VASCULAR-RELATED NAC-DOMAIN6 and VASCULAR-RELATED NAC-DOMAIN7 Effectively Induce Transdifferentiation into Xylem Vessel Elements under Control of an Induction System[^w]

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We previously showed that the VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 genes, which encode NAM/ATAF/CUC domain protein transcription factors, act as key regulators of xylem vessel differentiation. Here, we report a glucocorticoid-mediated posttranslational induction system of VND6 and VND7. In this system, VND6 or VND7 is expressed as a fused protein with the activation domain of the herpes virus VP16 protein and hormone-binding domain of the animal glucocorticoid receptor, and the protein’s activity is induced by treatment with dexamethasone (DEX), a glucocorticoid derivative. Upon DEX treatment, transgenic Arabidopsis (Arabidopsis thaliana) plants carrying the chimeric gene exhibited transdifferentiation of various types of cells into xylem vessel elements, and the plants died. Many genes involved in xylem vessel differentiation, such as secondary wall biosynthesis and programmed cell death, were up-regulated in these plants after DEX treatment. Chemical analysis showed that xylan, a major hemicellulose component of the dicot secondary cell wall, was increased in the transgenic plants after DEX treatment. This induction system worked in poplar (Populus tremula × tremuloides) trees and in suspension cultures of cells from Arabidopsis and tobacco (Nicotiana tabacum); more than 90% of the tobacco BY-2 cells expressing VND7-VP16-GR transdifferentiated into xylem vessel elements after DEX treatment. These data demonstrate that the induction systems controlling VND6 and VND7 activities can be used as powerful tools for understanding xylem cell differentiation.

[^w]: The online version of this article contains Web-only data.

[^1]: This work was supported by Grant-in-Aid for Scientific Research (grant nos. 20770041 [to M.Y.] and 21027031 and 22370020 [to T.D.]).

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[^1]: The online version of this article contains Web-only data.

Secondary cell wall formation is one of the characteristic features of plant cells. This structure is found in several types of plant cells, such as xylem vessels and fiber cells, as additional thick layers between the primary cell wall and plasma membrane. The major components of primary cell wall are cellulose, hemicellulose, and pectin. Secondary wall contains a wide range of additional compounds, e.g. a complex phenolic polymer, lignin, in xylem cells, which are believed to modify mechanical properties and permeability of walls.

Secondary wall formation has been intensively studied during xylem cell differentiation. Recently, several NAM/ATAF/CUC (NAC) domain protein transcription factors were shown to play crucial roles in specification into distinct xylem cells (Demura and Fukuda, 2007; Yamaguchi and Demura, 2010). The genes for VASCULAR-RELATED NAC-DOMAIN1 (VND1) through VND7 are preferentially expressed in differentiating xylem vessels (Kubo et al., 2005; Yamaguchi et al., 2008), and the overexpression of VND6 and VND7 can induce the ectopic differentiation of metaxylem-like vessels and protoxylem-like...
In vitro differentiation systems in which the transdifferentiation of non-xylem cells into xylem vessel elements is induced have been established; in particular, the zinnia (Zinnia elegans) system has been used as an effective means of studying the mechanism of tracheary element differentiation (Fukuda, 1997; Roberts and McCann, 2000). In addition to this system, the activation of key regulators in xylem cell differentiation is expected to provide new inducible differentiation systems. In this article, we describe the construction of a system with chimeric gene harboring VND6 and VND7 activates of key regulators in xylem cell differentiation. Recently, it has been reported that VND-INTERACTING2, isolated as an interacting factor with VND7 protein, negatively regulates xylem vessel differentiation (Yamaguchi et al., 2010). The NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST3/SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1 (SNDEL/ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN12 (ANAC012) are expressed in the interfascicular fibers and xylem fibers, and the simultaneous mutation of nst1 and nst3/snd1/anac012 severely inhibits secondary wall biosynthesis in fibers (Zhong et al., 2006, 2007b; Ko et al., 2007; Mitsuda et al., 2007).

We generated transgenic Arabidopsis plants expressing VND6-VP16-GR and VND7-VP16-GR. Under normal growth condition, there was no apparent difference in growth, development, and fertility among the transgenic plants expressing VND6-VP16-GR, VND7-VP16-GR, and VP16-GR (control vector). We selected T3 homozygous single insertion lines in which the transgenes were highly expressed (Fig. 1B). To check the effects of the induction of VND6-VP16-GR and VND7-VP16-GR, 7-d-old plants grown on agar medium were soaked in water in the absence or presence of DEX, a glucocorticoid derivative, for 4 d. Under the DEX-free conditions, seedlings expressing VND6-VP16-GR and VND7-VP16-GR did not exhibit apparent differences from the control plants, expressing VP16-GR (Fig. 1C). In the presence of DEX, VND6-VP16-GR and VND7-VP16-GR seedlings became pale in color and died (Fig. 1C). Microscopic observation of the seedlings revealed that patterns of the xylem formation were almost normal in the roots and leaves in the absence of DEX treatment, and those of the control plants were not altered in the presence of DEX treatment (Fig. 2, A–C). In contrast, DEX treatment induced transdifferentiation of nonvascular cells into xylem vessel elements in VND6-VP16-GR and VND7-VP16-GR seedlings (Fig. 2, D–F). Furthermore, secondary wall thickening was observed in differentiated cells, such as guard cells, columella cells, root hair cells, and trichome cells (Fig. 2, G–J).

RESULTS
Construction of a Postranslational Induction System for VND6 and VND7

We previously showed that constitutive overexpression of VND6 and VND7 induces transdifferentiation into xylem vessels (Kubo et al., 2005; Yamaguchi et al., 2008), but we could just obtain transgenic plants in which only some cells were transdifferentiated into xylem vessels. Xylem vessel differentiation is accompanied by cell death and secondary wall synthesis (Fukuda, 1997), suggesting the impossibility of obtaining transgenic plants in which VND6 or VND7 is strongly overexpressed under the control of a constitutive promoter, such as the CaMV 35S promoter. To efficiently obtain xylem vessel elements, we used a glucocorticoid-mediated postranslational induction system (Sablowski and Meyerowitz, 1998). Briefly, in the absence of glucocorticoid, GR is localized to the cytosol and forms an inactivated complex by binding heat shock proteins (Aoyama and Chua, 1997). When the glucocorticoid binds the GR, the receptor is released from the complex, translocates to the nucleus, and acts as a transcription factor. We constructed the binary vectors harboring the chimeric genes, which consisted of VND6 or VND7 fused to the activation domain of herpes virus VP16 protein and the hormone-binding domain of rat GR on the C-terminal region (VND6-VP16-GR or VND7-VP16-GR, respectively) under the control of the CaMV 35S promoter (Fig. 1A).
This difference between VND6-VP16-GR and VND7-VP16-GR seems to reflect metaxylem-like vessels and protoxylem-like vessels, respectively, similar to the transdifferentiated cells induced by overexpression of VND6 and VND7 (Kubo et al., 2005).

VND6-VP16-GR and VND7-VP16-GR Up-Regulate the Expression of Genes Involved in Xylem Vessel Element Differentiation after DEX Treatment

To investigate the expression levels of genes involved in the differentiation of xylem vessel elements in the VND6-VP16-GR and VND7-VP16-GR plants, we carried out reverse transcription (RT)-PCR analysis (Fig. 4). Fourteen-day-old plants were soaked in water in the absence or presence of DEX for 1 d and the total RNA extracted. VND6-VP16-GR and VND7-VP16-GR activated by DEX induced the expression of enzymes associated with developmental stages of xylogenesis, such as cellulose biosynthesis (CesA4/IRREGULAR XYLEM5 [IRX5], CesA7/IRX3, and CesA8/IRX1; Pear et al., 1996; Turner and Somerville, 1997; Taylor et al., 1999), hemicellulose biosynthesis (IRX8 and IRX10; Brown et al., 2005), lignin biosynthesis (CCoAOMT7 and IRX12/LAC4; Raes et al., 2003; Brown et al., 2005), and programmed cell death (XCP1; Zhao et al., 2000; Fig. 4A). Several MYB transcription factors were recently reported to regulate secondary wall biosynthesis downstream of VND6 and VND7 expression (Zhong et al., 2007a, 2008; Ko et al., 2009; McCarthy et al., 2009; Zhou et al., 2009). Therefore, we examined the expression of the MYB genes in VND6-VP16-GR and VND7-VP16-GR plants. Five MYB genes (MYB46, MYB63, MYB83, MYB85, and MYB103) were highly expressed in the transgenic plants treated with DEX (Fig. 4B). These data suggest that VND6-VP16-GR and VND7-VP16-GR strongly induce transdifferentiation into xylem vessels due to regulation of target gene expression dependent on DEX treatment.

Analysis of Cell Walls in VND6-VP16-GR and VND7-VP16-GR Transgenic Arabidopsis Plants

To investigate changes in cell wall composition during the differentiation of xylem vessel elements in the VND6-VP16-GR and VND7-VP16-GR plants, 14-d-old seedlings were soaked in water in the absence or presence of DEX for 1 d, and alcohol insoluble residue (AIR) was prepared. Cell wall sugars released from the acid-hydrolyzed AIR were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Fig. 5A). The proportion of Xyl increased in AIR from both VND6-VP16-GR and VND7-VP16-GR seedlings treated with DEX, consistent with an increase in secondary cell wall xylan during xylogenesis. There were no significant changes in any other cell wall monosaccharides (Fig. 5A). We analyzed the content and structure of the xylan in the cell walls using polysaccharide analysis by carbohydrate gel electrophoresis.
The quantity of glucuronoxyran was significantly elevated in both VND6-VP16-GR and VND7-VP16-GR seedlings treated with DEX. These data indicate that the transdifferentiation into xylem vessels induced by VND6-VP16-GR and VND7-VP16-GR leads to secondary cell wall xylan synthesis.

VND7-VP16-GR Induces Transdifferentiation into Xylem Vessel Elements in Cell Suspension and Trees

Suspension cell culture is easy to scale up for the production of large numbers of cells, collect samples and data, and control culture parameters. We applied the system to suspension cultures of the T87 line of Arabidopsis cells (Axelos et al., 1992). Treatment with DEX successfully induced transdifferentiation into xylem vessel elements in transgenic lines carrying VND7-VP16-GR (Supplemental Fig. S1A). We measured the percentage of differentiated cells in more than 100 independent transgenic lines 7 d after the addition of DEX and found a maximum of approximately 10% of cultured cells (Supplemental Fig. S1B). Because brassinosteroid has been shown to have a positive effect on the induction of xylem vessel element differentiation (Yamamoto et al., 1997), we attempted to increase the differentiation ratio by adding brassinolide. The addition of brassinolide increased the percentage of differentiated cells to more than 15%.
Supplemental Fig. S1B), suggesting that factors other than VND7 and brassinosteroid limit differentiation in T87 cells.

We previously reported that the inducible system for VND7 expression in which VND7 is expressed under the control of an estrogen receptor-based chemical-inducible system induced differentiation into xylem vessel elements in suspension cultures of the BY-2 line of tobacco cells at a low frequency (Yamaguchi et al., 2008). This finding suggested that tobacco BY-2 cells can respond to the up-regulation of VND7 activity. To examine whether our current system can also work in BY-2 cells, we transformed BY-2 cells with VND6-VP16-GR or VND7-VP16-GR constructs and obtained 37 and 50 independent colonies, respectively. The colonies originated from single cells harboring each construct. Interestingly, three transgenic lines expressing VND7-VP16-GR showed that more than 90% of cells were differentiated 7 d after the addition of DEX, whereas almost no differentiated vessel elements were observed in the absence of DEX (Fig. 6). In contrast to VND7-VP16-GR, the induction of transdifferentiation into xylem vessel elements was not observed in the transgenic BY-2 cells expressing VND6-VP16-GR.

In addition to the cells in suspension culture, we generated transgenic poplar plants. From the observations of 35S::VND6 and VND7 in poplar plants, overexpression of Arabidopsis VND6 or VND7 has been shown to induce the transdifferentiation of mesophyll or epidermal cells in the leaves into xylem vessel elements (Kubo et al., 2005). We obtained 11 and 20 transgenic poplar plants harboring VND6-VP16-GR and VND7-VP16-GR, respectively, which frequently showed the formation of ectopic xylem vessel elements in the leaves, especially in the regions around vasculature, by treatment with DEX (Fig. 7). These results indicate that this induction system also works effectively in trees.

DISCUSSION

In this article, we reported a glucocorticoid-mediated posttranslational induction system for
activating VND6 and VND7, key regulators of xylem vessel differentiation. In Arabidopsis cells transformed with these constructs, ectopic secondary wall thickening was observed in nonvascular cells after the activation of VND6 and VND7 by DEX application (Fig. 2). The expression levels of some genes involved in various stages of xylem vessel element differentiation, including secondary wall biosynthesis and program cell death, were up-regulated after the activation of VND6-VP16-GR and VND7-VP16-GR (Fig. 4). These data suggest that VND6-VP16-GR and VND7-VP16-GR induce xylem vessel transdifferentiation through control of the transcriptional activation of VND6 and VND7, depending on DEX treatment.

Measurement of the cell wall components demonstrated that xylan, a major component in secondary cell walls, was significantly increased in the transgenic Arabidopsis plants upon activation of VND6-VP16-GR and VND7-VP16-GR (Fig. 5). Like the glucuronoxylan of dicot secondary walls, the xylan synthesized by the induced transgenic plants is substituted by GlcA or MeGlcA. A hypothesis was recently proposed that IRX9, IRX10, and IRX14 form a protein complex to elongate the xylan chain (Brown et al., 2009; Wu et al., 2009). In our system, the expression of IRX10 was induced by the DEX application (Fig. 4), which is likely to be consistent with this hypothesis. Cellulose, a main component of the cell wall, is thought to be synthesized by the cellulose synthase (CesA) complex; however, the details of its components remain unclear (Mutwil et al., 2008). We previously identified membrane-bound factors, TRACHEARY ELEMENT DIFFERENTIATION6 (TED6) and TED7, specifically expressed in the differentiating xylem vessels of Arabidopsis roots (Endo et al., 2009). Down-regulation of TED6 and TED7 represses secondary cell wall formation, and TED6 interacts with IRX3/CesA7, a subunit of the secondary cell wall-related cellulose synthase complex (Endo et al., 2009). We showed that the transgenic plants expressing VND6-VP16-GR and VND7-VP16-GR induced expression of CesA after DEX treatment (Fig. 4). These suggested that our induction system can be used as material to purify the protein complexes, including IRX9/IRX10/IRX14 complex and CesA complex, responsible for secondary wall formation and to measure their activities.

In general, for further analysis of the molecular mechanisms underlying cell differentiation processes, in vitro inducible systems are fruitful because of its high-synchronous differentiation (Fukuda, 1997; Roberts and McCann, 2000; McCurdy et al., 2008; Pang et al., 2008). One well-investigated system for in vitro xylem vessel element differentiation is the induction system using isolated mesophyll cells from Zinnia elegans (Fukuda, 1997). This system shows highly synchronous tracheary element differentiation,
and the whole process of differentiation, including events upstream of VND6 and VND7, can be studied. However, two problems with significant variations in the yield of traecチャー elements and cell population heterogeneity exist. Arabidopsis orthologs of trachеary element differentiation inhibitory factor, which was isolated as an inhibitory factor of transdifferentiation in the zinnia system, are expressed in phloem cells but not xylem cells (Ito et al., 2006; Hirakawa et al., 2008), raising a possibility that the zinnia system induces transdifferentiation not only into xylem vessel elements but also other vascular cells. These facts mean that the transdifferentiated cells are a nonhomogenous cell population in the existing in vitro inducible system for zinnia. Although recent reports on the improvement of the zinnia system described an approximately 74% increase in the traecチャー element yield (Tuwumasi et al., 2009), our induction system has great advantages in regards to differentiation efficiency; it induced the transdifferentiation of more than 90% of tobacco BY-2 cells into vessel elements (Fig. 5). Our results indicate that we can make an almost homogeneous xylem vessel element population using this system. Because the tobacco BY-2 cell line has the additional advantages of rapid proliferation (Nagata et al., 1992) and easily extracting a huge amount of protein, the transgenic BY-2 cells differentiating into vessel elements can be good materials for purifying the protein complex located downstream of VND7 and to measure its activity.

Our induction system has another advantage of wide-range application over the existing system. We showed that the expression of Arabidopsis VND6 and VND7 under the control of the induction system induced the transdifferentiation of nonvascular cells into xylem vessel elements upon DEX application not only in Arabidopsis and tobacco BY-2 cells, but also in poplar trees (Fig. 7). Poplar has been accepted as a model tree for several reasons, including rapid growth, relatively small genome size (450–550 Mb), and the ease of genetic transformation (Taylor, 2002). The Populus trichocarpa genome was the first tree genome to be sequenced (Tuskan et al., 2006), and a number of full-length cDNA and EST clones are available (Nanjo et al., 2004, 2007; Sterky et al., 2004). Thus, our induction system can also be used to understand the molecular mechanisms underlying secondary wall formation in trees. In addition, our system would accelerate an integration of known information about secondary wall formation. In several in vitro cell differentiation systems, specific plant species are used as materials, giving some restriction of studies. For example, the inefficient transformation makes more extensive studies using zinnia system difficult. Although we recently established a method to introduce DNA/RNA into zinnia cells by electroporation-based transient transformation, the rate of transformation was still at a low level (Endo et al., 2008). In contrast, our inducible system is applicable to any known mutants and/or transgenic lines of plant species in which transformation has been established. We believe that our inducible system is the powerful tool to study xylem vessel element differentiation, which gives a lot of new knowledge and perspectives.

CONCLUSION

We showed that the up-regulation of VND6 or VND7 activities using the GR fusion system strongly induces most cells in Arabidopsis plants to transdifferentiate into vessel elements, and it can work in suspension cultures of Arabidopsis and tobacco cells and in poplar trees. These results indicate that this induction system provides powerful tools for understanding the molecular mechanism of xylem vessel differentiation. In addition to biochemical analysis as mentioned above, this inducible system is accessible for the transcriptome analysis of genes downstream of VND6 or VND7 and cytological analyses to monitor the sequence of events during xylem vessel differentiation (Oda et al., 2005; Wightman et al., 2009). The use of such an induction system is expected to generate new knowledge of xylem vessel differentiation including secondary cell wall formation.

MATERIALS AND METHODS

Plasmid Construction

The coding regions of VND6 and VND7 cDNA were amplified by PCR using primers containing the recognition sequence for XhoI and XbaI at the N-terminal end of VND6 and VND7, respectively, and XhoI at the C-terminal end (Supplemental Table S1). After double digestion, fragments were cloned into the XhoI–XbaI sites of the VP16-GR vector (gift from Dr. Nam-Hi Chua and Dr. Takashi Aoyama).

Generation of Transformants

The constructed plasmids were electroporated into Agrobacterium tumefaciens strain GV3101-pMP90. A simplified version of the floral-dip method was used for transformation of the Arabidopsis (Arabidopsis thaliana) plant (Columbia strain; Clough and Bent, 1998). Transgenic Arabidopsis seedlings were selected with growth medium containing Murashige and Skoog salt mixture (Wako Pure Chemical, http://www.wako-chem.co.jp/), 1% (w/v) Suc, 0.05% (v/v) MES-KOH (pH 5.8), B5 vitamins, and 1.5% (w/v) agar supplemented with 50 μg mL⁻¹ kanamycin. For the transformation of suspension cultures of Arabidopsis cells (T87; Axelos et al., 1992), we followed the methods of Forreiter et al. (1997). Tobacco (Nicotiana tabacum) suspension cells (cv BY-2) were cultured using the method described by Nagata et al. (1992). Agrobacteria culture (100 μL) was mixed with 4 mL of 4-d-old BY-2 cells and incubated at 28°C for 24 h. After washing with Linsmaier-Skoog (LS) medium (Linsmaier and Skoog, 1965), cells were suspended in 20 mL of LS medium. One milliliter of cells was spread on LS medium containing 0.8% (w/v) agar and 100 μg mL⁻¹ kanamycin and vancomycin. Transgenic poplars (Populus tremula × tremuloides; Nilsson et al., 1992) were generated using the method described by Eriksson et al. (2000).

DEX Treatment

To activate VND6-VP16-GR and VND7-VP16-GR, the whole Arabidopsis seedlings or poplar leaves were soaked in water containing 10 μM DEX. In the case of suspension cells, 10 μM DEX was added to the liquid medium.

Microscopic Observation

We carried out observations of the transgenic plants, including the differential interference contrast images and confocal laser-scanning microscopic...
images, according to the methods described by Kubo et al. (2005). BY-2 cells were fixed in fixative (3.7% [w/v] formalin, 25 mM PIPES, 2 mM Ecta, and 2 mM MgCl2) and washed with 50 mM phosphate buffer (pH 7.5) before staining with 0.1 mg mL−1 fluorescent brightener 28 (Calcifluor).

Cell Wall Purification
AIR was prepared according to Goubet et al. (2009).

Monosaccharide Analysis of AIR by HPAEC-PAD
The AIR (50 μg) was incubated in trifluoroacetic acid (2 μL, 200 μL) for 1 h at 121 °C, dried in vacuo, and resuspended in water. Chromatography was performed as described by Brown et al. (2009).

Enzymatic Hydrolysis of AIR and Analysis by PACE
The AIR (500 μg) was treated with NaOH (4 μL, 20 μL) for 1 h at 21 °C, and the pH adjusted to 5 to 6. The hydrolysis was performed as described by Brown et al. (2009) using Xylanase 11A from N. gracilis, and the reactions were stopped by boiling for 30 min, and the samples dried in vacuo. Oligosaccharides were labeled with 8-aminonaphthalene-1,3,6-trisulfonic acid and analyzed by PACE as described by Brown et al. (2009).

Expression Analysis
T3 homozygous single insertion lines were grown on the growth medium without any antibiotics, and total RNA was isolated using the RNeasy mini kit (Qiagen, http://www1.qiagen.com/). cDNA was synthesized using reverse transcriptase (SuperScript III; Invitrogen, http://www.invitrogen.com/) and oligo(dT) primer, and PCR was carried out using ExTaq DNA polymerase (Takara Bio, http://www.takara-bio.com/) and specific primers (Supplemental Table S1).

Sequence data from this study can be found in the GenBank/EMBL databases under the following accession numbers: At5g62380 (VND6), At1g71930 (VND7), At1g05320 (UBQ10), At3g18790 (ACT2), At4g35330 (XCPI), At5g4030 (Cesa4/IRX5), At5g17420 (Cesa7/IRX3), At4g18780 (Cesa8/IRX1), At5g54690 (IRX8), At4g27440 (IRX10), At4g26220 (CCoAOMT7), At2g38680 (IRX12/LAC4), At5g12870 (MYB46), At5g21910 (MYB63), At3g08500 (MYB83), At3g26280 (MYB85), and At5g56110 (MYB103).

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

Supplemental Figure S1. Xylem vessel element transdifferentiation in suspension cultures of Arabidopsis cells.

Supplemental Table S1. Oligonucleotides used in this study.

ACKNOWLEDGMENTS
We thank Dr. Nam-Hai Chua (Rockefeller University) and Dr. Takashi Aoyama (Kyoto University) for providing us with the VP16-GR vector. We also thank Ayumi Iihara, Mitsukata Araki, Sachiko Oyama, Tomoko Matsumoto, Ryoko Hiroyma, Hiromi Ogawa, Yoshie Kita, Akiko Sato, and Kayo Kitaura (RIKEN) and Zhinong Zhang (University of Cambridge) for excellent technical assistance.

Received January 29, 2010; accepted May 19, 2010; published May 20, 2010.

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