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Short communication

Sequential activation of two Dof transcription factor gene promoters during vascular development in *Arabidopsis thaliana*

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Abstract

Plant-specific Dof transcription factors play various roles in plants. We show the strictly regulated activity of two *Dof* gene promoters during procambium formation, an early process for vascular development, in *Arabidopsis thaliana* (L.) Heynh. The *AtDof2.4* promoter was active in procambial cells of leaf primordia, roots and embryos, whereas the *AtDof5.8* promoter activity was specifically detectable in the cells of prospective veins in leaf primordia of seedlings and cotyledons of developing embryos, and the vascular tissue of developing flower buds. The *AtDof5.8* promoter but not the *AtDof2.4* promoter showed strong activity in advance of perceptible procambium formation. AtDof2.4 and AtDof5.8 might function in the early but different processes for vascular development.

Keywords: *Arabidopsis thaliana*; Dof transcription factor; Procambium; Vasculature

1. Introduction

Dof transcription factors that are widely distributed in the plant kingdom regulate gene expression through the specific interaction of their plant-specific Dof DNA-binding domains with 5′-AAAG-3′ sequences in the plant gene promoters [25,26]. They have been suggested to play various roles in growth and development and in the responses to phytohormones and environmental signals in various plant species [25,26]. In the genome of *Arabidopsis thaliana* (L.) Heynh., 36 putative *Dof* genes have been identified [25]. Nine genes of them have been characterized and shown to be involved in the salicylic acid-response, seed germination, phytochrome signaling, flowering and glucosinolate biosynthesis [7,21,23,25,26], while others remain uncharacterized.

The vascular tissue of higher plants is a well-differentiated tissue for transport of water and nutrients including minerals and sugars. The vascular network of seedlings is primarily patterned by formation of the vascular precursor called procambium during embryogenesis and established by differentiation of the procambium into phloem and xylem after germination [4,22]. Formation of the vascular tissue in true leaves is also caused by differentiation of the procambium that arises from ground meristem cells during leaf primordium development. Vascular development involves many processes, including cell division, cell elongation and specification of cell types. Although strict regulation of the expression of genes involved in vascular development has been suggested, transcription factors associated with vascular development, especially procambium formation, have been poorly identified [2,11,16,18,20,22].

In this report, we show that two *Dof* gene promoters are sequentially activated during procambium formation in *Arabidopsis*, implying possible involvement of Dof transcription factors in vascular development of higher plants.

Abbreviations: GUS, β-glucuronidase; HD-ZIP III, Class III homeodomain-leucine zipper; RT-PCR, reverse transcription-PCR.

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2. Methods

2.1. Plasmid construction

The binary plasmids containing the *AtDof* promoters fused to the GUS gene were made by a series of modifications of the pCB302 vector [24]. First, pCB302-K4 was constructed from pCB302 by deletion of a HindIII site in the MCS2 region, and replacement of the EcoRI-SacI fragment of the MCS1 with a double-stranded linker that was made by annealing two oligonucleotides, 5′-AATTAAGCTTCTATCTGCAGAGCTC GACAGAATTCAGCT-3′ and 5′-GAATTCTGTCGAGCTCT GCAGATAGAAGCTT-3′. The pCB302-*35S*-Ω-*LUC* plasmid was constructed by inserting the HindIII-EcoRI fragment of pJD301 that contains a transcriptional unit, *35S*-Ω-luciferase-*nos* 3′ [13] into the new multicloning site of pCB302-K4. The luciferase gene in pCB302-*35S*-Ω-*LUC* was then replaced by *GUS* [8] using NcoI and SacI sites, to generate the pCB302-*35S*-Ω-*GUS* plasmid. Subsequently, to produce plasmids containing putative *AtDof* promoter sequences (pCB302-*AtDof2.4-GUS* and pCB302-*AtDof5.8-GUS*), the *35S*-Ω sequence in pCB302-*35S*-Ω-*GUS* was replaced by the approximately 1.5 kb DNA fragments that corresponded to the 5′ flanking regions and the 5′-untranslated regions of *AtDof2.4* and *AtDof5.8*, respectively. The putative promoter sequences used for construction were cloned by PCR using pairs of primers (5′-GAGAAGCTTGGAATATCTTTGGTCTCATTC GTC-3′ and 5′-ATGGAGGAGAAACCATGGTTATTCTC-3′ for *AtDof2.4* and 5′-GAGCTGCAGATACGGACCAGAAGG TAAATGACG-3′ and 5′-GAGCCATGGTGTTGAGAAGAG AGGTTATCTGAA AG-3′ for *AtDof5.8*). All constructs were verified by DNA sequencing.

2.2. Plant transformation

Transformation of the Columbia strain of *Arabidopsis thaliana* (L.) Heynh. was carried out with the floral dip method. Seedlings were grown under constant light at 22 °C on half-strength MS plates [14] and used in the GUS staining analysis. For the analysis of embryos, immature siliques were taken from plants grown on soil under constant light at 22 °C. In addition to two independent homozygous T_3 lines, more than eight independent lines of T_1 or T_2 generation were used in the GUS staining analysis for respective constructs.

2.3. Histochemical GUS staining and microscopy

Histochemical staining of GUS activity was performed basically as described in [10]. When diffuse staining patterns were observed, the concentration of potassium ferricyanide in the staining solution was increased to 3 mM to prevent diffusion of the signal [16]. Stained samples were observed either under a stereomicroscope (MZ16F, Leica Microsystems, Germany) and photographed with a DXM1200C digital camera (Nikon, Japan) or under a light microscope (CK40, Olympus, Japan) and photographed with a COOLPIX 990 digital camera (Nikon, Japan).

2.4. RNA preparation and RT-PCR analysis

Total RNA was extracted from samples frozen in liquid nitrogen with TRIzol reagent (Invitrogen, USA) and reverse-transcribed by SuperScript II (Invitrogen) with oligo$(dT)_{15}$. PCR reactions were carried out using Ex Taq DNA polymerase (Takara Bio, Japan) with 31 cycles (for *AtDof5.8*) and 25 cycles (for β-tubulin) of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min. We confirmed that there was no amplification from contaminating genomic DNA under these conditions. Primers for the detection of *AtDof5.8* are 5′-GATCTGTTGCGATTCCGACGGATC-3′ and 5′-CAATGTGGCTTGTAGTAGTTGCAGC-3′. Primers for a β-tubulin gene were described in [9].

3. Results

3.1. AtDof2.4 promoter activity specific to the vascular tissue

We were interested in the promoters of *AtDof2.4* and *AtDof5.8* from the results of our preliminary reverse transcription-PCR (RT-PCR) analysis (unpublished data). To investigate the organ- and tissue-specific activities of these promoters, we generated transgenic *Arabidopsis* plants carrying the β-glucuronidase (*GUS*) gene reporter under the control of the *AtDof2.4* or the *AtDof5.8* promoter. As shown in Fig. 1A, GUS activity directed by the *AtDof2.4* promoter was specifically detected in the vascular tissue of seedlings. In leaves, GUS activity was observed in the vascular network of the basal portions of the first pair of true leaves, including both the midvein and higher-order vascular bundles, and stronger one was observed in the vascular bundles of petioles (Fig. 1A). Considering that the differentiation of the leaf primordia proceeds from the apex to the base [4,22], the *AtDof2.4* promoter might be more active in the vasculature of the immature portions of the leaves. In fact, GUS activity was stronger in immature vasculature of the third and fourth leaf primordia (Fig. 1A–C). The expression of *AtDof2.4* appeared to precede the completion of xylem differentiation, because lignification of the xylem strands was not observed in the third and fourth leaf primordia (data not shown).

GUS expression was also observed in the vasculature of the hypocotyls and the upper portion of the roots adjacent to the hypocotyls (Fig. 1A,D). In the primary root tips, two bundles of GUS staining extended upward from the staining in the central cylinder near the apex, with a gradual reduction in intensity (Fig. 1E). GUS staining was also detectable from the cells of the future vasculature of the lateral roots (Fig. 1F,G). Consistent with our finding, a recent study reported that the *AtDof2.4* promoter directed the specific expression of the GFP reporter gene in phloem precursor cells in roots [12].

We investigated the linkage between the *AtDof2.4* promoter activity and vascular development in embryos (Fig. 1H–J).