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Expression of *DOF* genes identifies early stages of vascular development in *Arabidopsis* leaves

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ABSTRACT The sequence of events underlying the formation of vascular networks in the leaf has long fascinated developmental biologists. In *Arabidopsis* leaves, vascular-precursor procambial cells derive from the elongation of morphologically inconspicuous ground cells that selectively activate expression of the *HD-ZIP III* gene *ATHB8*. Inception of *ATHB8* expression operationally defines acquisition of a typically irreversible preprocambial cell state that preludes to vein formation. A view of the constellation of genes whose expression is activated at preprocambial stages would therefore be particularly desirable; however, very few preprocambial gene expression profiles have been identified. Here, we show that expression of three genes encoding members of the DOF family of plant-specific transcription factors is activated at stages overlapping onset of *ATHB8* expression. Expression of *DOF* genes is initiated in wide domains that become confined to sites of vein development. Congruence between *DOF* expression fields and zones of vein formation persists upon experimental manipulation of leaf vascular patterning, suggesting that *DOF* expression identifies consistently recurring steps in vein ontogeny. Our results contribute to defining preprocambial cell identity at the molecular level.

KEY WORDS: *Arabidopsis thaliana, leaf development, vein patterning, procambium, auxin transport*

Introduction

The vascular system of plants is composed of strands that extend and intersect throughout all organs (Esau, 1965). Vascular strands are responsible for long-distance transport of water and nutrients, and are source of signals that act locally, to direct the development of neighboring cells, and systemically, to coordinate the initiation of new shoot organs with that of new roots (Berleth and Sachs, 2001). Sites of vascular strand differentiation are defined during organ development by emergence of continuous lines of elongated vascular-precursor procambial cells (Esau, 1943).

Few natural phenomena have attracted more widespread attention than the patterned formation of vascular strands in the leaf. From a developmental standpoint, the process is particularly fascinating because it seems to be organized de novo in each leaf primordium. At early stages of leaf ontogeny, in fact, the cells located beneath the epidermis appear as a morphologically homogeneous population of tightly connected, polygonal, isodiametric cells ('ground cells') (Smith, 1934; Foster, 1936, 1952); during leaf development, complementary, anatomically inconspicuous subsets of ground cells will differentiate to generate procambial strands and the photosynthetic tissue of the leaf, the mesophyll.

The molecular details of the mechanism by which cells acquire procambial identity during organ development are not entirely clear, but transport and transduction of the plant signaling molecule auxin have long been implicated in defining paths of vascular differentiation (Berleth *et al.*, 2000; Sachs, 1981). In leaf development, ground cells are directed towards procambial fate through induction of wide domains of expression of the PIN-FORMED1 (PIN1) auxin exporter and of the auxin response

Abbreviations used in this paper: CFP, cyan fluorescent protein; DAG, days after germination; DOF, DNA-BINDING WITH ONE ZINC FINGER; GFP, green fluorescent protein; HD-ZIP III, class III HOMEODOMAIN-LEUCINE ZIPPER; LUT, look-up table; NPA, 1-N-naphthylphthalamic acid; YFP, yellow fluorescent protein.

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transcription factor MONOPEROS (MP) (Donner *et al.*, 2009; Hardtke and Berleth, 1998; Scarpella *et al.*, 2006; Wenzel *et al.*, 2007). Decay of PIN1 and MP expression and associated relapse to ground state occur in some of the cells, and domains of PIN1 and MP expression are eventually restricted to individual files of cells that will elongate into procambial cells (Donner *et al.*, 2009; Scarpella *et al.*, 2006; Wenzel *et al.*, 2007).

While onset of PIN1 and MP expression marks an unstable and reversible state in formation of leaf vascular strands (or 'veins'), lines of PIN1 and MP-expressing ground cells that are stabilized towards procambial fate activate expression of the class III *HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIP III*) gene *ATHB8* (Baima *et al.*, 1995; Donner *et al.*, 2009; Kang and Dengler, 2004; Sawchuk *et al.*, 2007; Scarpella *et al.*, 2004). Initiation of *ATHB8* expression is directly controlled by MP (Donner *et al.*, 2009), and identifies acquisition of a critical and typically irreversible 'preprocambial' cell state that accurately predicts sites of vascular differentiation (Alonso-Peral *et al.*, 2006; Candela *et al.*, 2001; Carland and Nelson, 2004; Cnops *et al.*, 2006; Donner *et al.*, 2009; Kang and Dengler, 2004; Koizumi *et al.*, 2000; Petricka and Nelson, 2007; Pineau *et al.*, 2005; Sawchuk *et al.*, 2007, 2008; Scarpella *et al.*, 2004, 2006). A view of the transcriptional landscape of cells at preprocambial stages would be particularly valuable as it might provide insight into the molecular pathways underlying vein formation. However, very few genes have been identified whose expression is initiated at stages prior to procambium formation [e.g., (Alonso-Peral *et al.*, 2006; Baima *et al.*, 1995; Carland and Nelson, 2009; Hardtke and Berleth, 1998; Kang and Dengler, 2004; Konishi and Yanagisawa, 2007; Scarpella *et al.*, 2004;, 2006; Steinmann *et al.*, 1999; Wenzel *et al.*, 2007)].

In this study, we searched for gene expression profiles associated with early stages of vascular development in *Arabidopsis* leaves. We reasoned that at least some of the genes expressed during vein formation may be expected to display similar vascular-biased expression in other organs. We extracted root vascular-specific gene expression profiles from available whole-genome transcript accumulation data sets (Birnbaum *et al.*, 2003), and converged on genes encoding transcription factors of the plant-specific DOF (DNA-BINDING WITH ONE ZINC FINGER) family (Lijavetzky *et al.*, 2003; Yanagisawa, 2002) because of the correlation between their molecular diversification and the functional differentiation of the plant body into physiologically distinct organs during evolution (Shigyo *et al.*, 2007). Our findings suggest that expression of three *DOF* genes can be assigned to stages of preprocambial development that overlap with acquisition of the *ATHB8* cell state. Under both undisturbed and pharmacologically perturbed leaf development, broad domains of *DOF* gene expression become restricted to sites of vein formation, suggesting that dynamics of *DOF* expression define reproducible cell states in preprocambial development. Our results assist in the molecular characterization of cell identity at morphologically indistinguishable stages of vein formation.

Results

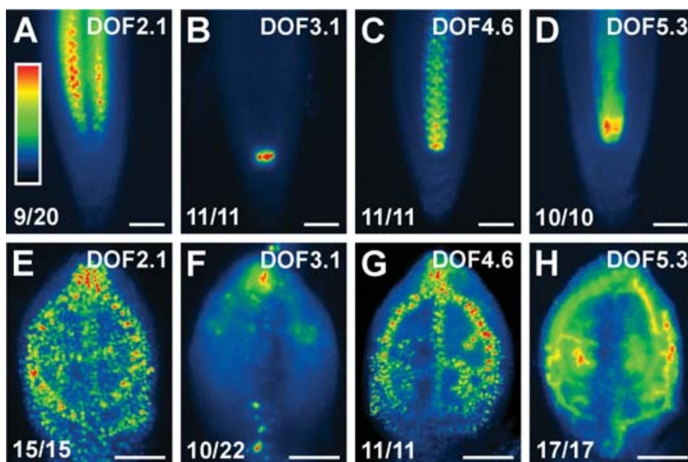
All genes whose expression has previously been assigned to early stages of vascular development in the leaf have also been reported to be expressed in root vascular cells [e.g., (Alonso-Peral *et al.*, 2006; Baima *et al.*, 1995; Carland and Nelson, 2009; Hardtke and Berleth, 1998; Kang and Dengler, 2004; Konishi and Yanagisawa, 2007; Scarpella *et al.*, 2004; Scarpella *et al.*, 2006; Steinmann *et al.*, 1999; Wenzel *et al.*, 2007)]. Therefore, to identify new preprocambial gene expression profiles, we interrogated an available global gene expression map of the *Arabidopsis* root (Birnbaum *et al.*, 2003) to extract root vascular-specific gene expression datasets. We focused on genes encoding members of the DOF family of plant-specific transcription factors because their evolutionary diversification is associated with the compartmentalization of the plant body into separate organs (Shigyo *et al.*, 2007). We found that nine of the 36 *DOF* genes in *Arabidopsis* (Lijavetzky *et al.*, 2003; Yanagisawa, 2002) displayed a strong expression bias for root vascular cells (Fig. 1). Detailed expression data for five of these nine *DOF* genes are already available and support their vascular-specific expression in roots and other organs (Gualberti *et al.*, 2002; Skirycz *et al.*, 2006; Ward *et al.*, 2005; Zhao *et al.*, 2005), suggesting that preselecting vascular gene expression profiles based on root expression patterns from whole-genome microarray datasets may be an effective criterion. Here we investigated leaf expression of the remaining four *DOF* genes *2.1*, *3.1*, *4.6* and *5.3*.

Expression of DOF *genes in seedling organs*

Because the upstream noncoding region is sufficient to recapitulate the endogenous transcript accumulation pattern in 80% of the cases for 44 *Arabidopsis* transcription factors (Lee *et al.*, 2006), to visualize *DOF* gene expression patterns at high resolution, we employed transcriptional reporter gene fusions with an

Gene	Locus	Root cap	Epidermis	Cortex	Endodermis	Stele
DOF1.1	At1g07640	16 ± 7.8	18 ± 8.9	33 ± 16.5	42 ± 20.8	114 ± 56.5
DOF2.1	At2g28510	22 ± 4.3	17 ± 3.4	69 ± 13.9	76 ± 15.2	123 ± 24.7
DOF2.2	At2g28810	28 ± 7.1	18 ± 4.6	35 ± 8.7	35 ± 8.9	104.3 ± 26.3
DOF2.5	At2g46590	11 ± 5.7	8 ± 4.4	29 ± 15.7	28 ± 14.8	60 ± 32.4
DOF3.1	At3g21270	35 ± 12.1	28 ± 9.7	39 ± 13.6	41 ± 14.4	121 ± 41.9
DOF3.6	At3g55370	2± 1.4	4 ± 3.3	9 ± 7.0	6 ± 4.3	36 ± 26.5
DOF3.7	At3g61850	26 ± 12.2	19 ± 8.6	74 ± 34.4	69 ± 32.2	289 ± 134.2
DOF4.6	At4g24060	15 ± 5.4	14 ± 4.9	78 ± 27.5	67 ± 23.8	170 ± 59.9
DOF5.3	At5g60200	3 ± 0.7	6 ± 1.5	26 ± 6.2	29 ± 7.1	168 ± 40.7

Fig. 1. Chart of *DOF* expression in root tissues. *Heat map showing levels of* DOF *expression in different tissues of the Arabidopsis root. Data compiled in (Birnbaum* et al.*, 2003) were interrogated with the AGI codes of the 36* DOF *genes in Arabidopsis (Lijavetzky* et al.*, 2003; Yanagisawa, 2002) through the Arabidopsis eFP browser tool (Winter* et al.*, 2007), and profiles of the nine genes with biased expression in vascular cells ('stele') are represented. Values represent mean ± SD of gene expression levels at development stages I-III [see (Birnbaum* et al.*, 2003) for more details]. To visualize changes in gene expression, a light-to-dark blue look-up table (LUT) with 25-percentile color steps was adopted, in which darker shades represent progressively stronger expression.*



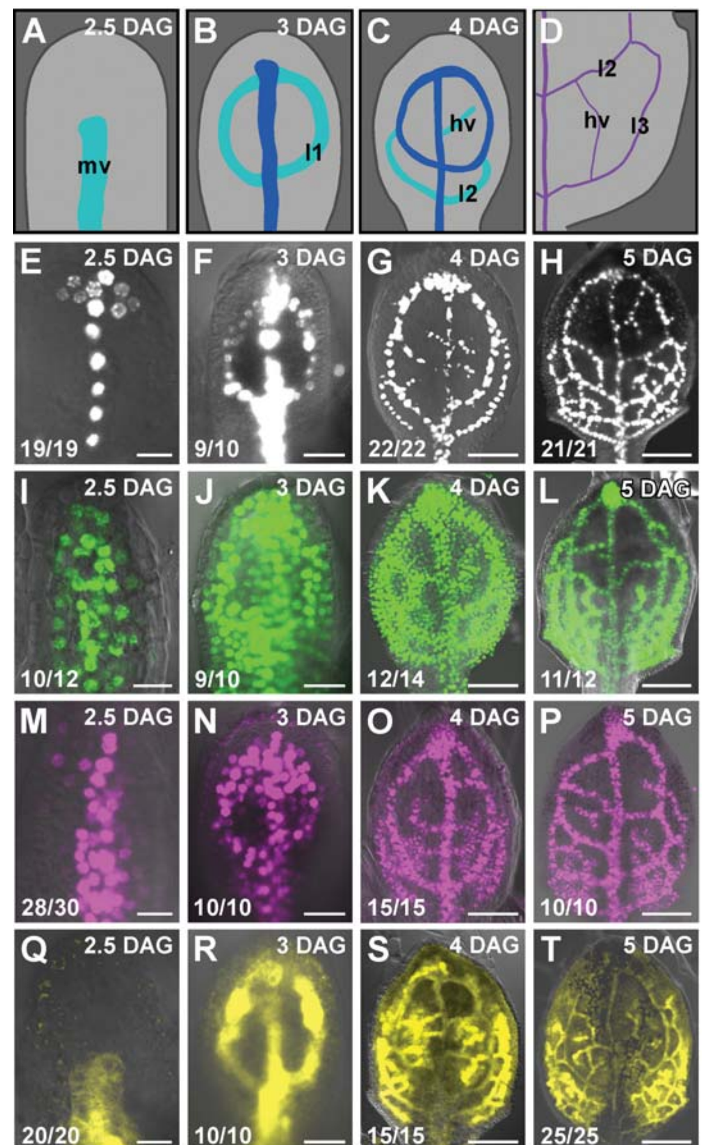


Fig. 2 (Left). *DOF* expression in seedling organs. *Top right, gene identity. Bottom left, fraction of samples showing the displayed features.* **(A-H)** *Wide-field epifluorescence microscopy. An LUT (displayed in A) in which black was used to encode global background, blue to encode local background, and cyan, green, yellow, orange, and red to encode increasing signal intensities (Sawchuk* et al.*, 2008) was applied to eight-bit grayscaled images to generate color-coded images.* **(A-D)** *Root tips.* **(E-H)** *Leaves (abaxial view).* **(A)** *See Supplementary Figure S1 for additional expression patterns and their frequencies. Scale bars, 50 μm.*

Fig. 3 (Right). *DOF* expression in first leaf development. *Top right, leaf primordium age in days after germination (DAG). Bottom left, fraction of samples showing the displayed features.* **(A,E,I,M,Q)** *Lateral view (adaxial side to the right).* **(B-D,F-H,J-L,N-P,R-T)** *Abaxial view.* **(A-D)** *Illustrations depicting the spatiotemporal course of vein formation in Arabidopsis first leaf development as inferred from published works (see text for references), and definition of terms used in this study; see also Materials and methods.* **(A-C)** *Whole leaves.* **(D)** *Detail of the lower-right region of a mature leaf. Cyan, preprocambial stages; blue, procambial stages; purple, mature veins; hv, higher-order vein; l1, l2 and l3, first, second and third loop, respectively; mv, midvein.* **(E-T)** *Overlay of confocal laser scanning and differential interference contrast microscopy images.* **(E-H)** *White, ATHB8$_{pro}$:HTA6:EYFP expression.* **(I-L)** *Green, DOF2.1$_{pro}$:HTA6:EYFP expression.* **(M-P)** *Magenta, DOF4.6$_{pro}$:HTA6:EYFP expression.* **(Q-T)** *Yellow, DOF5.3$_{pro}$:mGFP4er expression. Scale bars: E,I,M,Q, 10 μm; F,J,N,R, 20 μm; G,K,O,S, 50 μm; H,L,P,T, 75 μm.*

endoplasmic reticulum-targeted GFP (mGFP4er) (Haseloff *et al.*, 1997) or a nuclear localized YFP (HTA6:EYFP) (Zhang *et al.*, 2005).

We first asked whether *DOF* promoter activity could recapitulate the root vascular-specific expression suggested by transcript profiling (Birnbaum *et al.*, 2003). To address this question, we imaged expression of DOF5.3$_{pro}$:mGFP4er (Lee *et al.*, 2006), DOF2.1$_{pro}$:HTA6:EYFP, DOF3.1$_{pro}$:HTA6:EYFP and DOF4.6$_{pro}$:HTA6:EYFP in the root of seedlings 4 days after germination (DAG). While transcriptional fusions of *DOF2.1*, *DOF4.6* and *DOF5.3* were expressed in root vascular cells, DOF3.1$_{pro}$:HTA6:EYFP fluorescence was confined to the quiescent centre region (Fig. 2 A-D), suggesting that, at least in the root, patterns of *DOF3.1* promoter activity may not reflect the gene's transcriptional profile.

We next asked whether *DOF* gene expression was associated with sites of vascular strand formation in the leaf. To address this question, we visualized expression of DOF2.1$_{pro}$:HTA6:EYFP, DOF3.1$_{pro}$:HTA6:EYFP, DOF4.6$_{pro}$:HTA6:EYFP and DOF5.3$_{pro}$:mGFP4er in 4-DAG first leaves as their venation is predominantly preprocambial and procambial (Donner *et al.*, 2009; Sawchuk *et al.*, 2007). Expression of fusions of *DOF2.1*, *DOF4.6* and *DOF5.3* was activated in correlation to zones of vein emergence, while DOF3.1$_{pro}$:HTA6:EYFP signals were restricted to the leaf tip and the most basal portion of the central midvein (Fig. 2 E-H), suggesting that the *DOF3.1* transcriptional fusion is not appropriate for marking early stages of vein formation. Therefore, successive analyses were performed on DOF2.1$_{pro}$:HTA6:EYFP, DOF4.6$_{pro}$:HTA6:EYFP and DOF5.3$_{pro}$:mGFP4er lines.

DOF *expression during leaf development*

Expression of *DOF2.1*, *DOF4.6* and *DOF5.3* all seemed to label early stages of vein formation (Fig. 2 E,G,H). However, patterns of initiation, progression and termination, or persistence, of expression could be remarkably different, even for genes that are expressed similarly at any single stage of leaf development. Therefore, to visualize dynamics of *DOF* expression over time, we

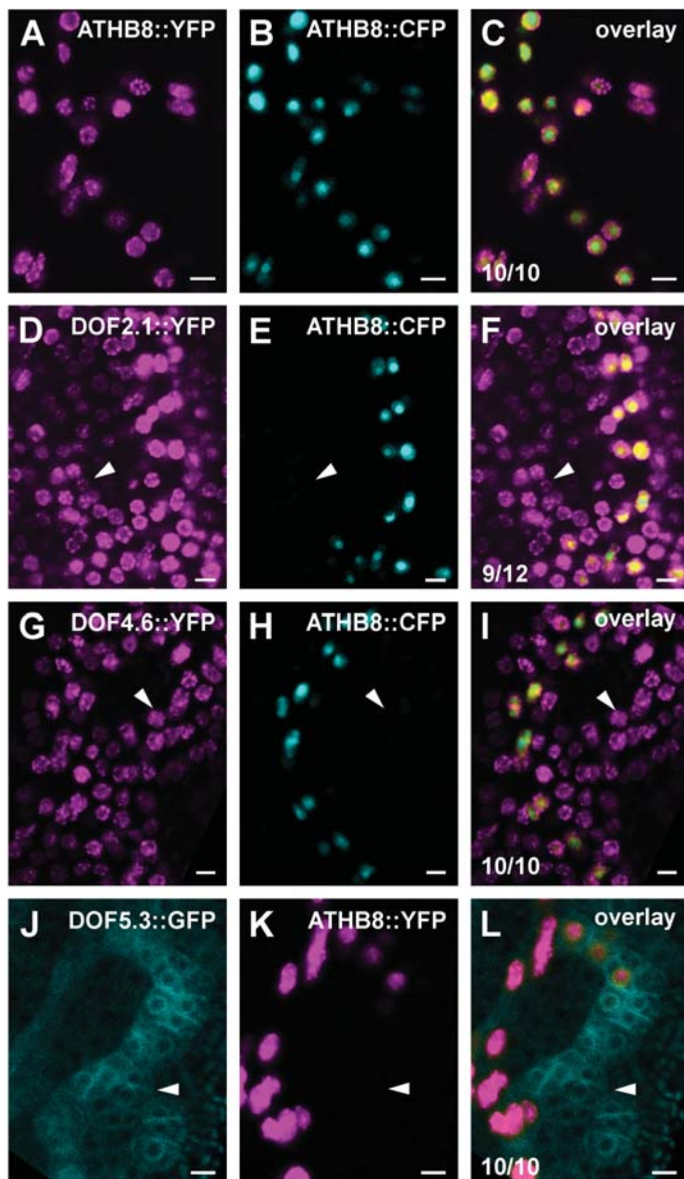


Fig. 4. Vein stage-specific *DOF* expression. *Top right, marker identifier. Bottom left, fraction of samples showing the displayed features.* **(A-L)** *4-DAG leaves, abaxial view. Confocal laser scanning microscopy.* **(A,C,K,L)** *Magenta, ATHB8$_{pro}$:HTA6:EYFP expression.* **(B,C,E,F,H,I)** *Cyan, ATHB8$_{pro}$:ECFP-Nuc expression.* **(D,F)** *Magenta, DOF2.1$_{pro}$:HTA6:EYFP expression.* **(G,I)** *Magenta, DOF4.6$_{pro}$:HTA6:EYFP expression.* **(J,L)** *Cyan, DOF5.3$_{pro}$:mGFP4er expression.* **(C,F,I,L)** *Images color-coded with a dual-channel LUT from cyan to magenta through green, yellow, and red (Demandolx and Davoust, 1997). Fluorescence in each detection channel was displayed in either cyan or magenta. Single-fluorophore images were then merged using a differential operator. As a result, preponderance of cyan signal over colocalized magenta signal is encoded in green, opposite in red, and colocalized cyan and magenta signals of equal intensity in yellow. Arrowheads indicate domains of DOF promoter activity lacking expression of ATHB8 fusions. Scale bars, 5 μm.*

monitored activity of DOF2.1$_{pro}$:HTA6:EYFP, DOF4.6$_{pro}$:HTA6:EYFP and DOF5.3$_{pro}$:mGFP4er, and of the reference preprocambial marker ATHB8$_{pro}$:HTA6:EYFP (Donner *et al.*, 2009; Sawchuk *et al.*, 2007) in first leaf primordia at 2.5, 3, 4, and 5 DAG. In *Arabidopsis*, veins of subsequent orders become recognizable progressively later in the same area of the developing leaf primordium, and veins of the same order appear in a tip-to-base sequence during leaf development (Candela *et al.*, 1999; Kang and Dengler, 2002 , 2004; Kinsman and Pyke, 1998; Mattsson *et al.*, 1999; Scarpella *et al.*, 2004; Sieburth, 1999; Steynen and Schultz, 2003; Telfer and Poethig, 1994). The illustrations in Figure 3 (Fig. 3 A-D) schematically depict the temporal sequence of vascular development events in *Arabidopsis* leaf primordia, and define the stages and terminology to which we refer throughout this study (for additional details, see Materials and methods).

At 2.5 DAG, expression of all transcriptional fusions was visible in the central region of the leaf primordium (Fig. 3 E,I,M,Q). While ATHB8$_{pro}$:HTA6:EYFP signals were confined to a single cell file, expression of *DOF* fusions occupied wider territories, which in DOF5.3$_{pro}$:mGFP4er comprised two adjacent columns of cells and in DOF2.1$_{pro}$:HTA6:EYFP encompassed nearly all subepidermal cells. Expression of fusions of *ATHB8*, *DOF2.1* and *DOF4.6* was additionally detected at the tip of the 2.5-DAG primordium. At 3 DAG, all fusions were strongly expressed at sites of midvein and first loop appearance; however, *DOF* promoters were active in broader domains than ATHB8$_{pro}$:HTA6:EYFP, with only slightly expanded fields of DOF5.3$_{pro}$:mGFP4er expression at the apical side of the developing first loops and weak DOF2.1$_{pro}$:HTA6:EYFP fluorescence in almost all subepidermal cells (Fig. 3 F,J,N,R). At 4 DAG, activity of all fusions marked zones of formation of midvein, first and second loops, and higher-order veins, even though levels of midvein-associated DOF5.3$_{pro}$:mGFP4er expression were considerably lower than those detectable in all other veins (Fig. 3 G,K,O,S). Further, while domains of *ATHB8* promoter activity were equally narrow in all developing veins, expression of *DOF* fusions in prospective second loops and higher-order veins pervaded larger fields of cells than in the emerging midvein and first loops. Finally, at 5 DAG, all promoters directed expression in developing midvein, first, second and third loops, and in higher-order veins (Fig. 3 H,L,P,T), but fields of *DOF* fusion activity were wider in veins emerging in basal areas of the leaf, and DOF5.3$_{pro}$:mGFP4er expression had subsided in midvein and first loops.

In summary, expression of all *DOF* genes seemed to be tightly associated with regions of vascular strand formation throughout leaf development.

Stage-specific DOF *expression in vein formation*

Comparison between *DOF* and *ATHB8* expression profiles during leaf development (Fig. 3) suggests that expression of *DOF* genes is initiated as early as that of *ATHB8*, and that therefore *DOF* expression could be assigned to preprocambial stages of vein formation. An unambiguous criterion to test such a hypothesis, however, would be to visualize expression of individual *DOF* genes and *ATHB8* within the same sample. We therefore tested the degree of colocalization between DOF2.1$_{pro}$:HTA6:EYFP and ATHB$_{pro}$:ECFP-Nuc (Sawchuk *et al.*, 2008), between DOF4.6$_{pro}$:HTA6:EYFP and ATHB$_{pro}$:ECFP-Nuc, and between DOF5.3$_{pro}$:mGFP4er and ATHB8$_{pro}$:HTA6:EYFP.

Covisualization of *DOF* transcriptional fusion signals and inception of *ATHB8* promoter activity showed overlapping expression of the fluorescent reporters (Fig. 4 D-L), suggesting that *DOF* expression is initiated at preprocambial stages. However, at onset of

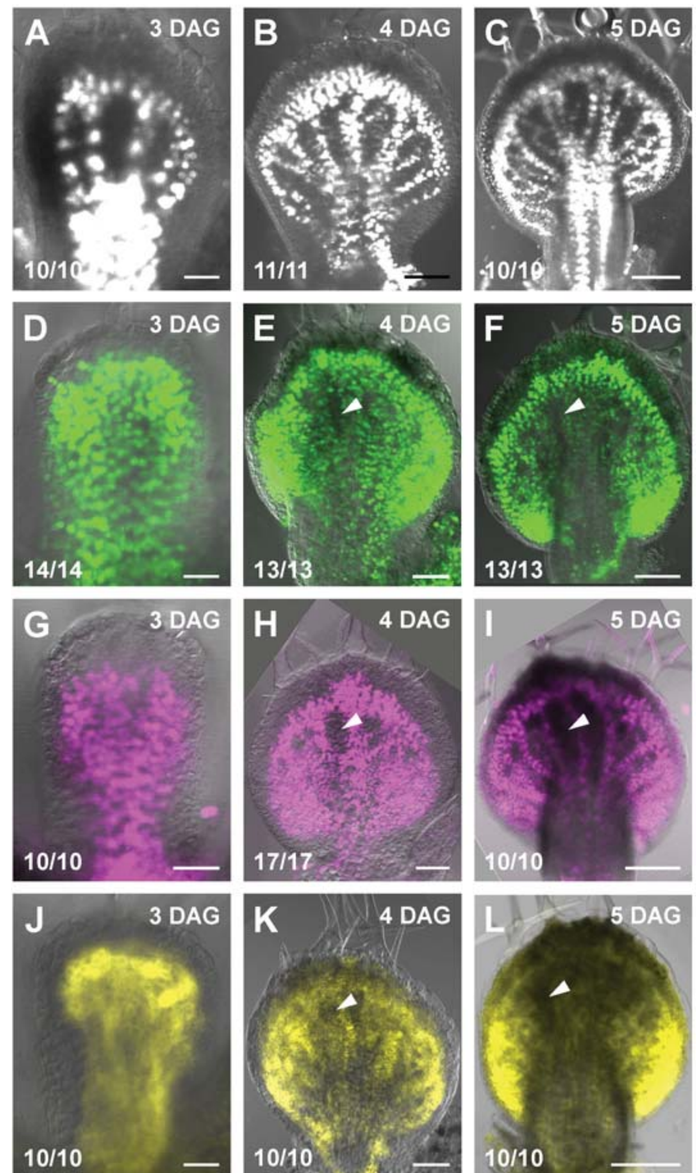


Fig. 5. *DOF* expression in auxin transport-inhibited first leaves. *Top right, leaf age in DAG. Bottom left, fraction of samples showing the displayed features.* **(A-L)** *Leaves (abaxial view) developing in the presence of 2.5 μM NPA. Overlay of confocal laser scanning and differential interference contrast microscopy images.* **(A-C)** *White, ATHB8$_{pro}$:HTA6:EYFP expression.* **(D-F)** *Green, DOF2.1$_{pro}$:HTA6:EYFP expression.* **(G-I)** *Magenta, DOF4.6$_{pro}$:HTA6:EYFP expression.* **(J-L)** *Yellow, DOF5.3$_{pro}$:mGFP4er expression. Note how the size of areas devoid of* DOF *expression (arrowheads) expands during leaf development, suggesting progressive restriction of* DOF *expression domains. Scale bars: A,D,G,J, 20 μm; B,E,H,K, 50 μm; C,F,I,L, 75 μm.*

ATHB8 promoter activity, expression domains of *DOF* fusions were always wider than those of *ATHB8*. Further, domains of *ATHB8* fusion expression were visible that did not extend throughout the full length of fields of *DOF* promoter activity (Fig. 4 D-F, J-L). Finally, we observed entire expression domains of *DOF* fusions that were totally devoid of *ATHB8* promoter activity (Fig. 4 G-I).

DOF *expression in auxin transport-inhibited leaves*

We finally asked whether expression of *DOF* genes remained associated with zones of leaf vascular strand formation upon experimental interference with vein patterning. Auxin transport has been shown to define sites of vein appearance in developing leaf primordia (Mattsson *et al.*, 1999; Scarpella *et al.*, 2006; Sieburth, 1999). Therefore, we grew seedlings harboring the *DOF* and *ATHB8* transcriptional fusions in the presence of the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) and imaged fluorescent protein expression in first leaves at 3, 4, and 5 DAG.

Leaves of plants germinated and grown in the presence of auxin transport inhibitors are characterized by a number of distinct anomalies in vascular organization; most conspicuously, great numbers of broad vein loops fuse along the entire margin of the leaf, to give rise to a wide zone of vascular differentiation, and extend parallel at the centre of the leaf, to give rise to a laterally expanded midvein (Mattsson *et al.*, 1999; Sieburth, 1999). As shown in Figure 5, domains of ATHB8$_{pro}$:HTA6:EYFP expression retained their characteristically slender shape throughout development of auxin transport-inhibited leaves (Fig. 5 A-C). Expression of *DOF* transcriptional fusions, on the other hand, appeared extremely expanded in leaves with reduced auxin transport, such that *DOF* promoters were active in nearly all subepidermal cells at 3 DAG (Fig. 5 D,G,J). Nevertheless, quasi-ubiquitous expression of *DOF* fusions became resolved into more discrete domains, which were already visible in the apical half of 4-DAG leaves (Fig. 5 E,H,K) and became further restricted in leaves at 5 DAG (Fig. 5 F,I,L).

In conclusion, the association between *DOF* expression domains and regions of vein formation observed under undisturbed conditions persisted in auxin transport-inhibited leaves, suggesting non-circumstantial correlation between zones of *DOF* expression and sites of vein emergence.

Discussion

The molecular details of the mechanisms controlling the recruitment of ground cells in the leaf towards procambium formation are largely unknown. Available evidence, however, suggests that the selection process terminates with emergence of files of ground cells that have activated expression of the *HD-ZIP III* gene *ATHB8* and that will successively elongate into procambial cells (Alonso-Peral *et al.*, 2006; Candela *et al.*, 2001; Carland and Nelson, 2004; Cnops *et al.*, 2006; Donner *et al.*, 2009; Kang and Dengler, 2004; Koizumi *et al.*, 2000; Petricka and Nelson, 2007; Sawchuk *et al.*, 2007, 2008; Scarpella *et al.*, 2004, 2006). Therefore, the events preceding acquisition of the *ATHB8* preprocambial cell state are crucial for vein formation.

In this study, we have sought gene expression profiles that were initiated at preprocambial stages. We have found that expression of *DOF2.1*, *DOF4.6* and *DOF5.3*, which encode members of the DOF family of plant-specific transcription factors (Lijavetzky *et al.*, 2003; Yanagisawa, 2002), reproducibly identifies morphologically inconspicuous cell states in the process that culminates into onset of *ATHB8* expression.

Cell state transitions in vein formation

Transcription of *DOF2.1*, *DOF4.6* and *DOF5.3* could be con-

trolled by regions other than the upstream noncoding sequences used here to monitor their expression, and abundance of transcripts of *DOF2.1*, *DOF4.6* and *DOF5.3* could be regulated at the post-transcriptional level. However, our results are in good agreement with expression profiles extracted from publicly accessible large-scale microarray data sets (Birnbaum *et al.*, 2003; Schmid *et al.*, 2005; Winter *et al.*, 2007), suggesting that expression patterns of *DOF2.1*, *DOF4.6* and *DOF5.3* can be accurately visualized by transcriptional fusions.

During leaf development, *DOF2.1*, *DOF4.6* and *DOF5.3* were expressed in seemingly overlapping subepidermal domains and with amazingly comparable dynamics. At early stages of leaf development, very low levels of *DOF2.1* expression embraced all ground cells; within these fields, however, broad domains of maximum expression of *DOF2.1* were distinguishable that became associated with sites of vein emergence, as identified by *ATHB8* expression. During subepidermal tissue ontogeny, weak *DOF2.1* expression became extinguished from subsets of ground cells, leaving only the intense vein-associated expression domains. Expression of *DOF4.6* and *DOF5.3* was initiated in wide domains that seemed to coincide with peaks of *DOF2.1* expression, but their expression never incorporated all surrounding ground cells. Expression of *DOF2.1* and *DOF4.6* was sustained at all stages of vein formation, while that of *DOF5.3* became terminated during procambium differentiation. Areas of *DOF* expression overlapped with sites of initiation of *ATHB8* expression, suggesting that *DOF2.1*, *DOF4.6* and *DOF5.3* are expressed at preprocambial stages. However, that discrete *DOF* expression domains were visible that were partially or completely free of *ATHB8* expression may suggest that expression of *DOF2.1*, *DOF4.6* and *DOF5.3* is initiated prior to acquisition of the *ATHB8* preprocambial cell state. Alternatively, or in addition, this observation may point to transient appearance of *DOF* expression in cells that will never activate expression of *ATHB8*.

If congruence between expression of *DOF2.1*, *DOF4.6* and *DOF5.3* and sites of vein formation is more than a coincidence, one would expect to observe such association even under conditions of manipulated leaf vascular patterning. Expression of *DOF* genes in leaves with reduced auxin transport, which dramatically changes the shape of vein networks (Mattsson *et al.*, 1999; Sieburth, 1999), retained dynamics comparable to those observed under undisturbed development. Furthermore, all aspects of *DOF* expression, including onset, intensity, decline, relation to *ATHB8* expression and association with vein-forming cells under all experimental conditions proved to be highly reproducible. We therefore suggest that expression of *DOF2.1*, *DOF4.6* and *DOF5.3* identifies regularly recurring steps in preprocambial development.

Unlike *ATHB8*, *DOF* expression was always initiated in wide domains, and *ATHB8* expression appeared within broad *DOF* expression domains. Furthermore, initially-wide fields of *DOF* expression became laterally confined over time, while *ATHB8* expression domains are always narrow at inception and progress longitudinally during vein formation. In this respect, expression of *DOF2.1*, *DOF4.6* and *DOF5.3* resembles that of genes that have been functionally implicated in selection of *ATHB*8-expressing preprocambial cells [e.g., (Alonso-Peral *et al.*, 2006; Candela *et al.*, 2001; Carland and Nelson, 2004; Donner *et al.*, 2009; Hardtke and Berleth, 1998; Petricka and Nelson, 2007; Sawa *et al.*, 2005; Sawchuk *et al.*, 2007; Scarpella *et al.*, 2006)]. However, *DOF2.1*, *DOF4.6* and *DOF5.3* are not expected to be directly involved in this process because preprocambial expression of *ATHB8* is under the immediate control of MP through a noncanonical auxin response element located in the *ATHB8* promoter (Donner *et al.*, 2009).

Vascular expression profiles of DOF *genes*

The *DOF* genes whose leaf expression was investigated here were selected because of their biased expression for root vascular cells in an *Arabidopsis* transcription map (Birnbaum *et al.*, 2003), and because their leaf expression had not previously been reported. Root vascular expression was recapitulated by patterns of promoter activity for three of the four *DOF* genes, and all of the three root vascular-specific promoters were also active at early stages of vascular strand formation in the leaf. Preselecting vein-associated gene expression profiles based on root expression patterns proved to be a valuable strategy, but this does not exclude the possibility that other *DOF* genes may be expressed in leaf vascular strands. Indeed, *DOF5.8*, which is negligibly expressed in root microarray datasets, displays prominent vein-associated expression (Konishi and Yanagisawa, 2007). Tissue-specific expression patterns are available for 13 of the 36 *Arabidopsis DOF* genes (Fornara *et al.*, 2009; Gardner *et al.*, 2009; Gualberti *et al.*, 2002; Imaizumi *et al.*, 2005; Konishi and Yanagisawa, 2007; Skirycz *et al.*, 2006, 2007, 2008; Ward *et al.*, 2005). These 13 genes sample the diversity of the *DOF* family, yet all of them appear to be expressed in vascular strands. While it will be interesting to understand the significance of the association between the expression of at least a large fraction of *DOF* genes and vascular cells, our study already contributes to the characterization of a largely unexplored class of plant-specific transcription factors.

Materials and Methods

Terminology

We apply the generic term ‘subepidermal’ to all positions of the leaf beneath the epidermis. We refer to ‘ground cells’ as polygonal, isodiametric, subepidermal cells of the leaf. We use the terms ‘procambial’ and ‘procambium’ to indicate morphologically identifiable vascular cell precursors. We designate as ‘preprocambial’ all stages of vein development prior to procambium formation.

Vector construction

To generate *DOF* transcriptional fusions, the genes’ entire noncoding regions were amplified from *Arabidopsis* (*Arabidopsis thaliana*) ecotype Col-0 genomic DNA using Finnzymes Phusion high-fidelity DNA polymerase (New England Biolabs Inc., Ipswich, MA, USA) and gene-specific primers (Supplementary Table S1), integrated into pDONR221 (Invitrogen, Carlsbad, CA, USA) with BP clonase II (Invitrogen), sequence-checked, and recombined into the Gateway-adapted pFYTAG binary vector, which contains a translational fusion between the coding region of histone 2A (HTA6; At5g59870) and that of the enhanced YFP (EYFP) (Zhang *et al.*, 2005), using LR clonase II (Invitrogen).

Plant material and growth conditions

The origins of the ATHB8$_{pro}$:HTA6:EYFP, DOF5.3$_{pro}$:mGFP4er and ATHB8$_{pro}$:ECFP-Nuc have been described (Lee *et al.*, 2006; Sawchuk *et al.*, 2007, 2008;). Seeds were sterilized and germinated, and seedlings and plants were grown, transformed and selected as described (Sawchuk *et al.*,

2007, 2008). For DOF2.1$_{pro}$:HTA6:EYFP, DOF3.1$_{pro}$:HTA6:EYFP and DOF4.6$_{pro}$:HTA6:EYFP, the progeny of 11 to 18 independent transgenic lines were inspected to identify the most representative expression pattern. Successive expression analysis was performed on the progeny of at least four lines per construct, which were selected because of strong YFP expression that was emblematic of the expression profile observed across the entire series of transgenic lines and that resulted from single insertion of the transgene. For DOF5.3$_{pro}$:mGFP4er, expression analysis was performed on the progeny of two lines per construct (JYB818.3, ABRC stock number: CS70640; JYB821.1, ABRC stock number: CS70641). In genetic crosses, the progeny of at least two independent lines per construct were examined. For auxin transport inhibition, seeds were germinated on growth medium supplemented with 2.5 μM NPA (Chem Service Inc., West Chester, USA). We define 'days after germination' (DAG) as days following exposure of imbibed seeds to light.

Microscopy and image processing

Dissected seedling organs were mounted and imaged as described (Donner *et al.*, 2009; Sawchuk *et al.*, 2007, 2008). Brightness and contrast were adjusted through linear stretching of the histogram in ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Signal levels and colocalization were visualized as described (Sawchuk *et al.*, 2008).

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