Stage-specific markers define early steps of procambium development in *Arabidopsis* leaves and correlate termination of vein formation with mesophyll differentiation

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Accepted 18 March 2004

Development 131, 3445-3455
Published by The Company of Biologists 2004
doi:10.1242/dev.01182

Summary

During leaf development, ground meristem cells along continuous lines undergo coordinated oriented cell divisions and differentiate to form procambial cells, the precursors of all vascular cells. The molecular genetic dissection of early procambial development suffers from the lack of easily identifiable markers, especially of cell states preceding procambium formation. In this study, we have identified and characterized three reporter gene expression markers that reflect three distinct preprocambial stages, as well as one marker whose expression seems to be perfectly congruent with the appearance of procambial cells. All four markers are invariably expressed in continuous domains connected to pre-existing vasculature and their expression profiles reveal a common spatiotemporal pattern of early vein formation. We observed progressive extension of vascular strands at the preprocambial stage, suggesting that veins are initiated as freely ending preprocambial domains and that network formation occurs through subsequent fusion of these domains. Consistent with this interpretation, we demonstrate that veins are generally not programmed to become freely ending or interconnected network elements. Instead, we found that the progressive extension of preprocambial domains can be interrupted experimentally and that this leads to less complex vein patterns consisting of fewer vein orders, in which even lower-order veins become freely ending. Mesophyll differentiation turned out to be strictly correlated with the termination of preprocambial domain extension. These findings suggest that *Arabidopsis* vein pattern is not inherently determinate, but arises through reiterative initiation of new preprocambial branches until this process becomes terminated by the differentiation of mesophyll.

Supplemental data available online

Key words: *Arabidopsis thaliana*, Procambium, Leaf development, Vascular patterning

Introduction

The vascular system of plants is composed of a continuous network of vascular bundles (Esau, 1965). These structures extend through all organs and throughout the entire plant, functionally connecting all parts of the shoot with the root system. Vascular bundles comprise two types of conducting tissues, phloem and xylem, each composed of a number of specialized cell types. The phloem provides the path for the translocation of dissolved photoassimilates, while the xylem is the conduit for water and minerals.

In leaves, all vascular cells differentiate from a vascular meristematic tissue, the procambium. Procambial cells become apparent as narrow, cytoplasm-dense cells emerging from the subepidermal tissue of the leaf primordium [ground meristem; GM (Foster, 1952; Esau, 1965)]. Procambial cells are characteristically arranged in continuous strands and acquire their narrow shape through coordinated, oriented divisions, parallel to the axis of the emerging strand. The mechanisms by which procambial cells are selected from the GM cell population in the young leaf primordium is not known, but the high degree of plasticity of the patterning process in most species suggests that positional cues influenced by growth conditions are of critical importance. The molecular details of positional specification of procambial cell fate are particularly intriguing since the underlying mechanism must reconcile the plasticity of vein patterns with the stringent requirement to keep all parts of the vascular system interconnected (Berleth et al., 2000).

Alternative models to explain vascular tissue patterning have been derived from the variability in natural vascular patterning, responses of vascular patterns to experimental interference and from the phenotypes of vascular mutants (reviewed by Nelson and Dengler, 1997; Turner and Sieburth, 2002). These mutants have provided evidence for the involvement of sterols (Szekeres et al., 1996; Choe et al., 1999; Jang et al., 2000; Carland et al., 2002; Souter et al., 2002; Willelmsen et al., 2003), small peptides (Casson et al., 2002), cytokinin (Mähönen et al., 2000; Inoue et al., 2001) and auxin (Hardtke and Berleth, 1998; Hamann et al., 2002; Hellmann et al., 2003) in vascular patterning, but the number of vascular pattern mutants is surprisingly small, which may in part be due to the scarcity of criteria by which abnormalities in vascular mutants can be discerned.

Mutant screens and the molecular genetic analysis of cell patterning processes are highly dependent upon the swift and precise recognition of cell types and differentiation stages, referred to as cell states. While this is usually unproblematic for anatomically conspicuous cells in exposed positions, genetic dissection of cell identity acquisition in internal tissues may only become feasible if distinct cell states are specifically visualized through gene expression markers. Marking individual cell states along vascular differentiation pathways should in principle be an easy task, as gene expression profiles associated with specific differentiation states have been identified (Ye, 2000; Turner and Sieburth, 2002). However, most of these gene expression profiles are associated with the terminal differentiation of various vascular cell types and very few are available to mark early stages prior to procambium formation.

In this study we have searched for reporter gene expression profiles suitable for high-resolution analyses of preprocambial patterning and procambium formation in young leaf primordia. In particular, we were interested in assigning these markers to specific preprocambial and procambial stages, and in visualizing very early stages of vein formation in different vein orders. Four marker gene expression profiles indicate striking similarities in the ontogeny of veins of different orders. When visualized at an early preprocambial stage, all veins seem to be generated as parts of a common reiterative branching pattern, which becomes terminated by negative interference with mesophyll differentiation.

Materials and methods

Terminology

Because the first pair of rosette leaves appears simultaneously, we use the term 'first leaf primordium' to refer to one of those two primordia. We refer to 'days after germination' (DAG) as days after exposure of imbibed seeds to light. Because leaf initiation is delayed under one of our growth conditions, we introduced the term 'days after primordium initiation' (DAI) as days after the emergence of the leaf primordium as a semispherical bulge.

Because of the different meanings associated with the term 'provascular' in the literature (Esau, 1965; Steeves and Sussex, 1989; Clay and Nelson, 2002; Holding and Springer, 2002), we have decided to exclusively use the terms 'procambial' and 'procambium' in this manuscript to refer to anatomically identifiable precursor cells of vascular tissues (illustrated in Fig. 2A-H). 'Ground meristem' (GM) refers to all immature subepidermal tissue in the leaf (Foster, 1952).

Plant material and growth conditions

The origins of all marker lines and their previous use are listed in Table S1, http://dev.biologists.org/supplemental/. We have not observed developmental abnormalities that could be attributed to the insertion of the reporter gene in any of the lines. In all experiments, seeds of *Arabidopsis thaliana* were surface sterilized in 15% (v/v) commercial bleach, 0.01% (v/v) Triton X-100 (VWR, West Chester, PA) for 20 minutes with shaking. Seeds were subsequently treated with 70% (v/v) ethanol for 1 minute and washed 10 times in sterile water. Seeds were germinated on medium composed of half-strength Murashige and Skoog salts (Sigma, St Louis, MO, USA), 0.5 mg/l morpholino ethane sulfonic acid (MES, Sigma, St Louis, MO, USA), 0.8% (w/v) agar (BioShop Canada Inc., Burlington, ON, USA), pH 5.7 at the approximate density of 1 seed/cm^2. Sealed plates were incubated in growth chambers with fluorescent light (100 μE/m^2/s^2) under three conditions: A, 8 hours dark cycle, 20°C; B, continuous light, 25°C; C, continuous light, 30°C. Plants grown under condition C had shorter roots and longer hypocotyls, confirming earlier reports of elevated auxin responses at high temperature (Gray et al., 1998). For studying the development of the first leaf, seed germination was synchronized through a modification of the procedure by Petrov and Vizir (Petrov and Vizir, 1982). Sterilized seeds were left to imbibed in sterile water at 25°C for 8 hours and then stratified on plates at 4°C for 5 days. For studying the development of later leaves, 1-week-old seedlings were transferred to Promix BX growing medium (Premiere Horticulture, Ref Hill, PA, USA) in 7×7×8 cm pots at the approximate density of 0.1 seedling/cm^2 and grown under fluorescent light (100 μE/m^2/s^2) in an 8-hour dark cycle, 20°C.

Microtechniques and microscopy

For histochemical detection of β-glucuronidase (GUS) activity, whole seedlings or detached leaves were permeabilized in cold (–20°C) 90% (v/v) acetone for 1 hour at –20°C (Hemerly et al., 1993), washed twice for 5 minutes with 100 mM phosphate buffer pH 7.7, and incubated in the dark at 37°C in freshly prepared 100 mM sodium phosphate buffer pH 7.7, 10 mM sodium EDTA, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc; BioShop Canada Inc., Burlington, ON, Canada). To facilitate the uptake of the X-gluc substrate, acetone treatment is indispensable, but should be limited to 1 hour at –20°C to reduce structural damage.

Best preservation of anatomical structures is achieved by limiting the GUS staining reaction to less than 2 hours. The incubation buffer was supplemented with either 1 (GT5211, ET1335 and 553-643) or 5 (Athb8-GUS) mM of both potassium ferrocyanide and potassium ferricyanide. Higher concentrations accelerate the oxidative dimerization of the reaction intermediate into the blue insoluble product and thereby reduce diffusion of the intermediate at the expense of sensitivity (Lojda, 1970). The indicated molarities account for the differential expression levels of the reporter genes and ensured that each was easily detectable after 2 hours of incubation.

Under these conditions, we have not observed diffusion of the intermediate, which typically results in a gradient of blue staining with a maximum towards a neighboring strongly stained cell, as opposed to uniform staining at all intensity levels resulting from genuine GUS expression.

The enzymatic reaction was terminated by fixing in ethanol:acetic acid (3:1, v/v) for 1-16 hours at room temperature, depending on the age of the seedlings. Samples were stored in 70% (v/v) ethanol at 4°C. For visualization of GUS activity in leaves older than 10 DAI, the enzymatic reaction was extended to 16 hours and the concentrations of both ferrocyanide and ferricyanide salts were increased to 10 mM (Athb8-GUS), 5 mM (553-643) or 2 mM (ET1335 or GT5211). For microscopy, rehydrated samples were dissected under water, mounted abaxial side up in chloral hydrate:glycerol:water (8:3:1, w/v/v) and viewed under differential interference contrast (DIC) optics or dark-field illumination (Olympus AX70 microscope, Olympus Optical Co., Tokyo, Japan). Images were acquired with a Fujifilm FinePix S1 Pro digital camera (Fuji Photo Film Co., Tokyo, Japan).

For confocal laser scanning (CLS) microscopy, LTI6b::YFP seedlings [line 29-1 (Cutler et al., 2000)] were mounted in water and observed with a Zeiss Axiovert 100M microscope (Carl Zeiss, Oberkochen, Germany) equipped with a Zeiss LCM510 laser module confocal unit (Carl Zeiss, Oberkochen, Germany). LTI6b::YFP signal was visualized using the 488 nm line of an argon laser and a BP 505-530 filter at the photomultiplier. Chloroplast autofluorescence was detected with the 543 nm line of a helium-neon laser and a LP 585 filter at the photomultiplier. Optical slices (<1.6 μm) were obtained with a pinhole diameter of 129 μm (2.00 Airy units).

Images were assembled using Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA, USA), and figures were labeled using Canvas 8 (ACD Systems Ltd, Saanichton, BC, Canada).

Results

In this study, we explored cellular events associated with specification of vascular cells in the leaf ground meristem. Stages and terminology used to describe the expression patterns are schematically depicted in Fig. 1. A total of 12 reporter gene markers (Table S1, http://dev.biologists.org/supplemental/) with potential expression in early vascular tissues were analyzed in the first rosette leaf of *Arabidopsis* (Figs 2-4; Figs S1-S3, http://dev.biologists.org/supplemental/). Four markers were found to be best suited for labeling cell stages preceding or associated with procambium formation and were analyzed extensively in the first rosette and other leaves (Figs 2-4; Figs S1, S2, http://dev.biologists.org/supplemental/; data not shown). To account for potential vascular tissue variability and to evaluate marker reproducibility, at least 50 first rosette leaf primordia were inspected for each stage and marker line (Figs 2-4; Figs S1, S2, http://dev.biologists.org/supplemental/).

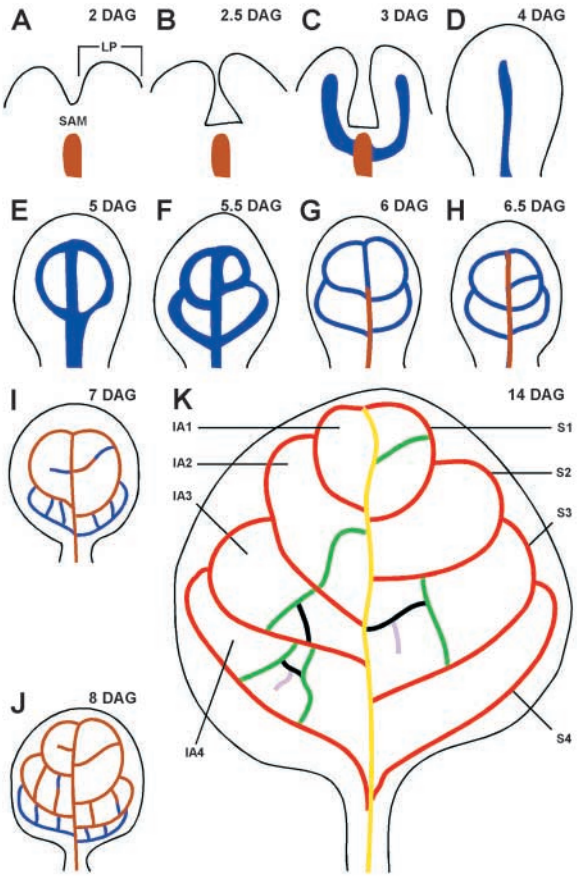

Fig. 1. Vascular development in the *Arabidopsis* first leaf. (A-C) Lateral view. (D-K) Abaxial view. Colors in A-J: procambium, blue, xylem, brown. Colors in K illustrate our application of the classification of vein orders by Hickey (Hickey 1973): 1st, yellow; 2nd, red; 3rd, green; 4th, black; 5th, purple. Veins of the 3rd and higher order can be formally classified as either branching from a next lower-order vein or connecting only lower-order veins. Only examples of these veins are shown in H-K. Numbering of 2nd order vein loops and of the enclosed intercostal areas (IAs) from distal to proximal. lp, leaf primordium, SAM, shoot apical meristem. DAG, days after germination.

In order to reliably recognize association of reporter gene expressions with stages of procambium development, GUS activity and the entire course of procambial strands had to be visualized in hundreds of synchronized primordia. Techniques to synchronize *Arabidopsis* seed germination and conditions optimizing the often problematic detection of GUS expression in early shoot organs are summarized in Materials and methods.

First leaf development and ET1335-GUS expression

The development of *Arabidopsis* leaves has previously been described (Telfer and Poethig, 1994; Kinsman and Pyke, 1998; Candela et al., 1999; Donnelly et al., 1999; Mattsson et al., 1999; Kang and Dengler, 2002; Mattsson et al., 2003). The schemes in Fig. 1 summarize the temporal sequence of vascular development events in first rosette leaf primordia to which we refer in Figs 2-4 and in the Figs S1 to S3, http://dev.biologists.org/supplemental/. The often oblique course of procambial strands was followed through various focal planes under DIC optics. Only one focal plane can be shown in Figs 2-4 and in Figs S1-S3, so that not all cellular features present are visible within a given marker gene expression domain. At all stages, the onset of ET1335-GUS expression coincided precisely with the onset of procambium formation (Fig. 2), except that ET1335-GUS was also transiently expressed in a larger domain at the tip of 3.5-5.5 DAG primordia (Fig. 3D-G). Because a gene expression marker that accurately monitored procambium formation would be very helpful, we rigorously reassessed the congruence of ET1335-GUS expression with procambium initiation at various stages and found it confirmed (Fig. 2). The following description therefore refers to the emergence of both patterns.

At 2 DAG, the first two vegetative leaf primordia become recognizable as semispherical protrusions flanking the shoot apical meristem (Fig. 1A, Fig. 2A). At 2.5 DAG, leaf primordia begin to elongate along the proximodistal axis (Fig. 1B, Fig. 2B). Elongated cells of the 1st order procambial strand and expression of ET1335-GUS become visible in the further elongated primordia at 3 DAG (Fig. 1C, Fig. 2A,B, Fig. 3C). At 4 DAG, lamina formation is initiated (Fig. 1D, Fig. 2E), followed by the formation of the first loops of 2nd order procambial strands at 5 DAG (Fig. 1E, Fig. 2C,D, Fig. 3F). At 5.5 DAG (Fig. 1F, Fig. 2E,F, Fig. 3G) the second loops and at 8 DAG (Fig. 1J, Fig. 2J) the third loops of 2nd order procambial strands appear. At 6.5, 7 and 8 DAG, the 3rd order procambial strands appear in the first, second and third intercostal area (IA), respectively (Fig. 1H-J, Fig. 2G,H, Fig. 3H-J). Differentiation of xylem reaches completion in the 1st order vein at 6.5 DAG (Fig. 1H) and in the first, second and third loops of 2nd order veins at 7 (Fig. 1I), 8 (Fig. 1J, Fig. 2K) and 10 DAG (data not shown), respectively. Xylem in 3rd order veins is initiated in the first IA at 7 DAG and then progresses towards higher-order veins as well as in more proximal IAs (Fig. 1I,J) until at 14 DAG xylem is continuous in veins of all orders (Fig. 1K). ET1335-GUS expression was initiated precisely with overt procambium formation but remained strong in differentiating vascular strands and was still visible even in the fully extended leaf at 14 DAG (Fig. 3L).

Expression of Athb8-GUS

Extremely weak Athb8-GUS expression was detected in the

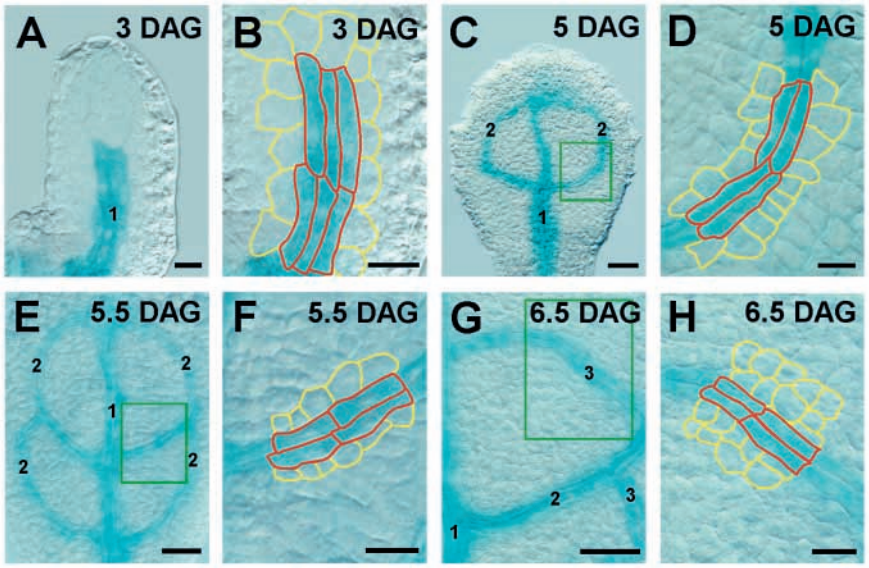

Fig. 2. Expression of ET1335-GUS in correlation to procambium formation. DIC micrographs of (A,B) lateral views, (C-H) abaxial views. (A,C,E,G) Congruence of ET1335-GUS expression and anatomically recognizable procambium in leaves of various ages as indicated. (B,D,F,H) Enlargements of A,C,E,G, respectively (boxed in C,E,G). Outlined cell boundaries illustrate our recognition criterion for procambium: procambial cells (red) are narrow and approximately twice the length of surrounding GM cells (yellow). Numbers refer to vein orders. Upper right: primordium age in days after germination (DAG). Scale bars: (A,B,D,F,H) 10 μm; (C,E,G) 25 μm.

innermost GM cells of the 2 DAG primordium (Fig. 4A). At 2.5 DAG, Athb8-GUS expression was considerably stronger and now visible in a central file of GM cells (Fig. 4B,C). At 3 DAG, Athb8-GUS was strongly expressed in the 1st order procambial strand and weakly in GM cells at the tip of the primordium (Fig. 4D). In slightly older but still cylindrical primordia (3.25 DAG), Athb8-GUS expression was reproducibly detected in single cells at the side of the 1st order procambial strand (arrow in Fig. 4E). With the beginning of lamina growth at 3.5 DAG, Athb8-GUS marked reproducibly a looped 2nd order preprocambial domain extending from proximal to distal (Fig. 4F). Expression of Athb8-GUS in the two loops of the first pair of 2nd order veins was consecutive rather than simultaneous (Fig. 4G), but both loops were complete by 4.25 DAG (Fig. 4H). Simultaneously with the completion of the first loop expression domain, Athb8-GUS expression marking the incipient second loops of 2nd order procambial strands became recognizable. As for the first loops, expression was initiated proximally in a domain extending from the 1st order procambial strand (Fig. 4I). From there, expression progressed distally to form a loop that eventually joined the first loop of 2nd order procambial strands in 4.75 and 5 DAG primordia (Fig. 4J-L). Athb8-GUS expression in the third loops of 2nd order vein loops followed the same temporal pattern as described for the first and second loops (not shown). The earliest 3rd order Athb8-GUS expression domains became visible in the first IAs in a significant portion (23/50) of 5 DAG primordia and were visible in this position in all primordia by 6 DAG (Fig. 4M,N). Formation of 3rd order Athb8-GUS expression domains in the second IAs followed about 1 day later (Fig. 4O,P). Immediately after their initiation, nearly all higher-order Athb8-GUS expression domains ended blindly (Fig. 4M-P), suggesting a polar progression

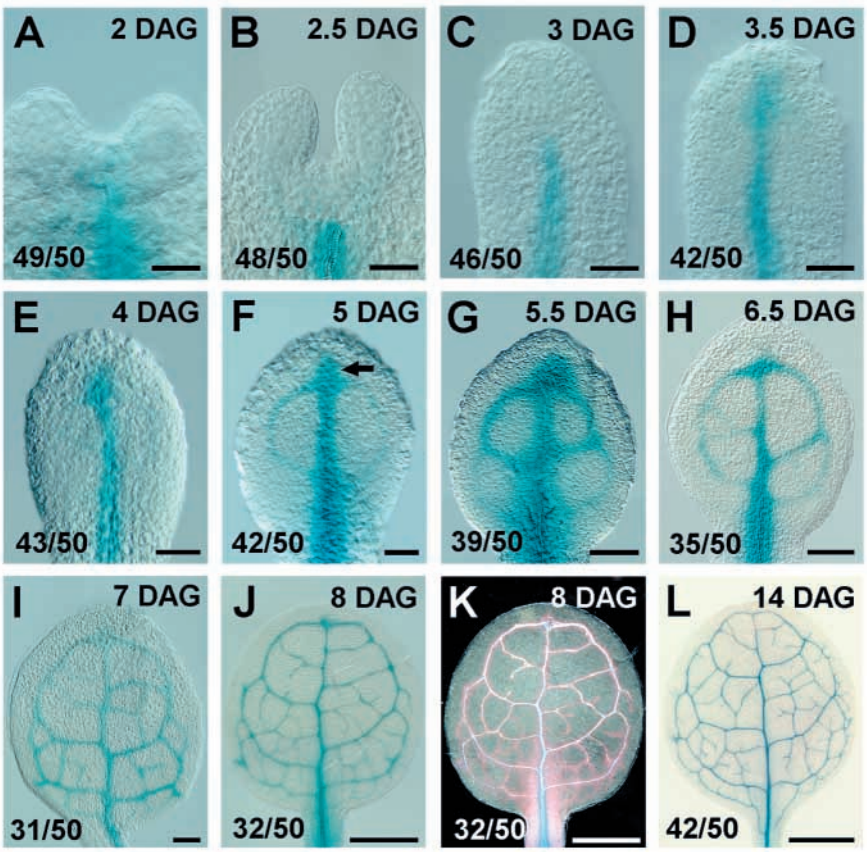

Fig. 3. Expression of ET1335-GUS during development of the first leaf. (A,B) Lateral view, (C-L) abaxial view; (A-J,L) DIC microscopy, (K) dark-field optics. Arrow in F indicates transient expression in GM cells at the tip of 3.5-5.5 DAG primordia. Upper right: primordium age in days after germination (DAG). Lower left: fraction of primordia showing nearly identical features. Scale bars: (A-F) 25 μm; (G) 50 μm; (H,I) 100 μm; (J,K) 500 μm; (L) 1 mm.

of Athb8-GUS expression domains also in the formation of higher-order veins (see below).

In summary, Athb8-GUS presages procambium formation with remarkable accuracy, except for a domain of transient expression at the tips of 3-5 DAG primordia. Expression of Athb8-GUS marks earlier preprocambial stages than any of the other markers in this study. Most conspicuously, Athb8-GUS expression progresses in a polar fashion from initiation points connected to pre-existing vasculature (see below and Discussion). Consistent with the auxin inducibility of the *Athb8* gene (Baima et al., 1995; Mattsson et al., 2003), Athb8-GUS expression domains are spatially correlated with the expression domains of the DR5-GUS auxin response marker in early leaf primordia (Fig. S3O-R, http://dev.biologists.org/supplemental/). In contrast to Athb8-GUS, however, the temporal expression profile of DR5-GUS is somewhat variable and the expression has been shown to be heterogeneous along individual veins (Mattsson et al., 2003). This marker is therefore unlikely to be correlated with a specific preprocambial cell state.

Reporter gene expressions in line GT5211 and in line 553-643

Two more of the surveyed twelve lines displayed remarkably invariant reporter gene expression patterns prior to procambium formation. As summarized in Fig. 6A, the onset of expression of both markers, gene-trap marker GT5211 and enhancer-trap marker 553-643, was delayed relative to Athb8-GUS expression, but clearly preceded the appearance of procambium. Expression of marker GT5211 strongly increased as cells adopted procambial shape. A detailed description of both expression profiles is provided in the supplementary material (see Figs S1, S2 at http://dev.biologists.org/supplemental/).

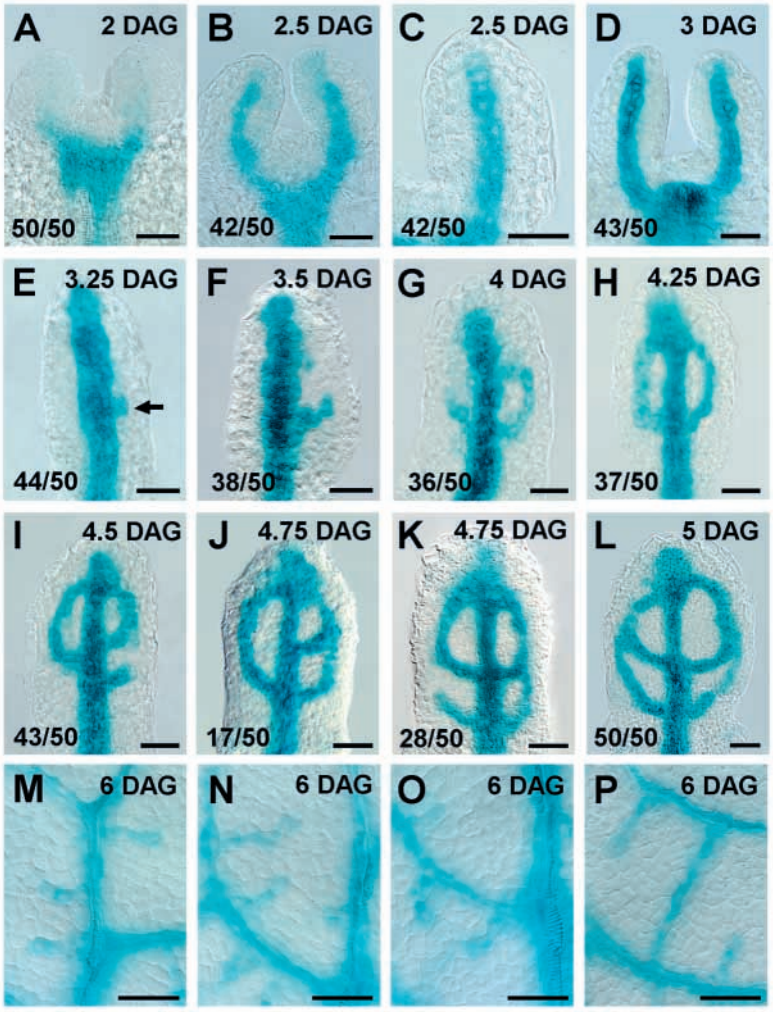

Fig. 4. Expression of Athb8-GUS during development of the first leaf. (A-D) Lateral view. (E-P) Abaxial view. (A-P) DIC microscopy. Arrow in E points to a single Athb8-GUS expressing cell at the onset of 2nd order loop formation. Upper right: primordium age in days after germination (DAG). Lower left: fraction of primordia showing nearly identical features. Scale bars: 25 μm.

Temporal patterns of early vascular differentiation

Arabidopsis rosette leaves have a closed venation pattern (Roth-Nebelsick et al., 2001; Steynen and Schultz, 2003) in which most veins connect with other veins (hereafter called 'connecting veins') and only a subset of veins ends blindly in the ground tissue ('freely ending' veins) (Fig. 1). Some vein orders produce only one type of veins. For example, under our standard growth condition (condition A), 2nd order veins are always connecting, while 5th order veins are invariably freely ending (Table 1). These regularities suggest that also in the relatively simple *Arabidopsis* vein pattern, veins of different hierarchical orders or vein types ('connecting' versus 'freely ending') may be specified as distinct elements in a genetic network-generating program. Alternatively, all veins may be produced through a reiterative branching mechanism and initiated as freely ending veins, as suggested by Athb8-GUS expression. Connecting veins would then be produced through the fusion of elongating preprocambial domains. We used our set of early markers to obtain more insight into the ontogeny of veins of different orders.

We first addressed the question of whether Athb8-GUS expression progresses polarly in the formation of higher-order veins as it does in primary and secondary veins (Fig. 4A-L). Since it is impossible to predict whether an individual higher-order vein will end up as a freely ending or as a connecting vein, we used a statistical approach, comparing the numbers of freely ending Athb8-GUS expression domains with the numbers of mature freely ending veins within defined IAs. We found that at the preprocambial stage the proportion of freely ending veins among all veins in a given vein order was consistently higher than in mature veins (Table 2), suggesting that at least some initially freely ending veins become connected as they are progressively extended and eventually fuse with other veins. We conclude that veins are not predisposed to become either freely ending or connected.

We next asked whether procambium (or ET1335-GUS expression) appears in the same temporal pattern in all vein orders. Procambium in the 1st order vein appeared as a short, always continuous stretch of elongated, ET1335-GUS-

Table 1. Manipulation of vein orders

Venation complexity*	Condition A High	Condition B Medium	Condition C Low
1st IA			
4th order veins	22 (9, 41%)	14 (14, 100%)	7 (7, 100%)
3rd order veins	–	69 (31, 45%)	37 (25, 68%)
2nd order veins	–	140 (0, 0%)	140 (21, 15%)
2nd IA			
5th order veins	28 (28, 100%)	not formed	–
4th order veins	389 (178, 46%)	106 (69, 63%)	–

*Overall degree of venation complexity developed under each growth condition reflected in total number of veins for each order.

Values in parentheses refer to the number of freely ending veins and their percentage of total number of veins.

Growth under condition B, and even more under condition C, is associated with a reduction in the complexity of the venation pattern compared to growth under condition A: growth under condition B results in the loss of 5th order veins, which are present under condition A, as well as in a reduction in number of 4th order veins and in an increased percentage of freely ending veins among them. Relative to leaves grown under condition B, growth under condition C results in a reduction in number of 4th order veins and in the appearance of freely ending 2nd order veins. Total number of veins and number of freely ending veins of the 2nd, 3rd, 4th or 5th order in the different intercostal areas (IAs) were determined in 70 first leaves of 14 DAG seedlings grown under condition A (8-hour day at 20°C), B (continuous light at 25°C) or C (continuous light at 30°C) (see Materials and methods and Fig. 1 for a more detailed description of growth conditions and terminology). Leaves were fixed and cleared as described in Materials and methods and inspected under dark-field illumination to visualize differentiated xylem.

expressing cells in primordia of stage 3 DAG (Fig. 2A,B). The distal end of the 1st order procambial strand remains separated from the primordium tip by 5-6 cells (at 3 DAG, 5.29±0.20, n=35; at 6 DAG: 5.78±0.15, n=41) throughout subsequent stages. Thus, the 1st order vein procambium is initiated simultaneously along the entire length of the strand and then is extended by interacalation as the primordium grows.

As shown in Fig. 2C-F and Fig. 3F,G,J, our results confirm earlier reports (Mattsson et al., 1999) that 2nd order vein procambium emerges simultaneously along the entire length of each loop. In higher-order veins, it is impossible to conclude from the expression pattern of a single marker whether the cells in an incipient vein acquire procambial identity simultaneously or progressively, since the final extension of a vein cannot be predicted. To determine the temporal pattern of procambium formation in higher-order veins, we took advantage of the marker Athb8-GUS, which marks the emerging vein at both the preprocambial and the procambial stage. We reasoned that if procambium is formed progressively, we should observe Athb8-GUS expression domains composed of stretches of procambial and GM cells. By contrast, if procambium is formed simultaneously throughout the full length of an Atbh8-GUS expression domain, procambial cells should be either visible along the entire domain or not at all. Among 314 Athb8-GUS domains, we did not find a single domain of composite appearance. We found five ambiguous domains, in which few cells showed aligned division axes, but these cells were still of the same maximum extension as surrounding GM cells.

In summary, the temporal expression patterns of early vascular gene expression markers and of procambium formation suggest a common pattern of differentiation in all vein orders, in which cells in a given vein acquire a preprocambial identity (marked by Athb8-GUS) progressively, while they acquire procambial identity simultaneously or extremely rapidly. The formal similarity of all vein orders and the extension of each vein from a single branch point is reminiscent of a typical reiterative branching pattern as opposed to a closed network pattern that could arise from dispersed differentiation zones.

Manipulation of vein orders

If veins of all orders are initiated as freely ending veins in a reiterative branching process and connecting veins are the

Table 2. Freely-ending vein and mesophyll development

	3 DAI		4 DAI		5 DAI		6 DAI		12 DAI	
	V*	M*	V	M	V	M	V	M	V	M
5th Vein order										
2nd IA (condition A)	–	–	–	–	28 (28)	0	**35 (35)**	**35*** **	**43 (43)**	**43**
4th Vein order										
1st IA (condition B)	–	–	**12 (12)**	**11****	**11 (11)**	**11*** **	–	–	**14 (14)**	**14**
1st IA (condition A)	–	–	11 (17)	0	**9 (21)**	**9****	–	–	**9 (22)**	**9**
2nd IA (condition B)	–	–	58 (70)	3	**70 (109)**	**66*** **	–	–	**69 (106)**	**69**
2nd Vein order										
1st IA (condition C)	**26 (140)**	**22*** **	**23 (140)**	**23*** **	–	–	–	–	**21 (140)**	**21**
1st IA (condition B)	0 (140)	na	**0 (140)**	**na**	–	–	–	–	**0 (140)**	**na**
1st IA (condition A)	0 (140)	na	0 (140)	na	–	–	–	–	**0 (140)**	**na**

*V, number of freely-ending Athb8-GUS expression domains (3-6 days after primordium initiation, DAI) or xylem differentiation (12 DAI) domains. Numbers in parentheses refer to the total number of Athb8-GUS expression or xylem differentiation domains; M, number of freely-ending Athb8-GUS expression (3-6 DAI) or xylem differentiation (12 DAI) domains surrounded by mesophyll. Bold indicates the presence of mesophyll in at least part of the respective IAs (intercostal areas). Note that in these cases, nearly all Athb8-GUS expression domains in developing leaves (3-6 DAI) were surrounded by mesophyll cells and that under these circumstances both the total number of veins as well as the proportion of freely-ending veins remains essentially unchanged.

Statistical evaluation: each IA at any given developmental stage and at any individual growth condition was considered as a distinct sample population. Immature leaf populations in which mesophyll had differentiated in the IAs in question were tested for significance of difference between frequency of freely-ending veins of the 2nd, 4th or 5th order surrounded by differentiated mesophyll cells and frequency of freely-ending veins of the respective order not surrounded by differentiated mesophyll cells by using Pearson's χ^2 test for proportions in a one-way table (null hypothesis: frequency of freely-ending veins of each order in any given IA at any individual growth condition that are surrounded by differentiated mesophyll cells equals frequency of freely-ending veins of the respective order, IA and growth condition, that are not surrounded by differentiated mesophyll cells). na, not applicable. **$0.001 \leq P < 0.01$, ***$P < 0.001$.

products of later vein fusions, it might be possible to convert connecting into freely ending veins and vice versa by manipulating the duration of the reiterative branching process. Interruption of this reiterative process would result in reduced numbers or even disappearance of higher-order veins (‘venation complexity’ below) and may simultaneously be associated with an increased proportion of freely ending veins in the remaining orders. We therefore assessed whether we could change the fate of defined orders of veins.

Differentiation in a leaf primordium proceeds in a distal-to-proximal direction and therefore all processes potentially terminating vein formation are probably not synchronized across the whole leaf. Therefore in order to look at a nearly homogenous group of veins, in each assay we monitored higher-order veins within a particular IA only (Table 1). In leaf primordia grown under our standard condition (condition A), less than half of all 4th order veins in the first IA were freely ending (Table 1). By contrast, in leaf primordia grown at a higher temperature (25°C) and under continuous illumination (condition B), 4th order veins in the first IA were less abundant and invariably ended freely (Table 1). Therefore, an incipient 4th order vein is not predisposed to become a freely ending vein, as it might appear in leaves grown under condition B. In the more complex higher-order venation pattern of the second IA, a change of growth condition from A to B is associated with a similar reduction in venation complexity. Veins of the 5th order, which are produced under condition A, are absent under condition B and the propensity of 4th order veins to become connected is significantly reduced (Table 1). Therefore, in both IAs, veins of the 4th order are becoming connected, if reiterative branching is allowed to proceed as under condition A.

The closed lobes of 2nd order veins are hallmarks of the brochidodromous *Arabidopsis* venation pattern. Each 2nd order vein connects precisely defined pre-existing veins with each other and this regularity lends strong support to a genetic specification of network patterns. We therefore explored the formation of the first loop of 2nd order veins in leaf primordia grown under various conditions. Remarkably, when leaf primordia were grown at 30°C and under continuous light (condition C), a significant portion of 2nd order vein loops were not connected at the distal end and fewer higher-order veins were produced (Table 1). Except for the complexity of their venation patterns, leaves grown under all three conditions were morphologically and developmentally similar (Table S2, http://dev.biologists.org/supplemental/).

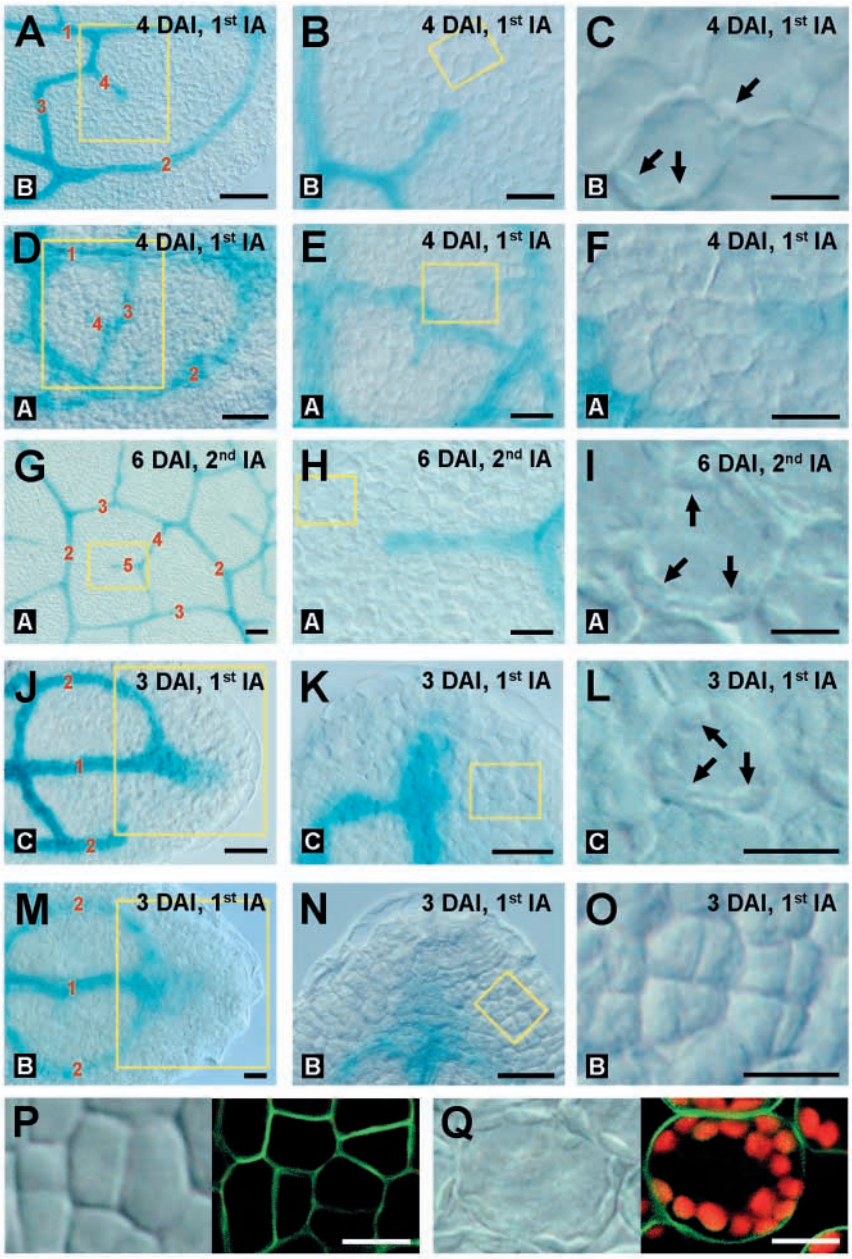

Fig. 5. Experimental manipulation of vein formation and mesophyll development. (A-O) Athb8-GUS expression, abaxial view, DIC microscopy. (B,E,H,K,N) Enlargements of boxed areas in A,D,G,J,M, respectively. (C,F,I,L,O) Enlargements of boxed areas in B,E,H,K,N, respectively. Upper right: primordium age in DAI (days after primordium initiation) and position in IA (intercostal area). Lower left: growth condition (A, B or C, as outlined in Materials and methods). Red numbers indicate vein order. Arrows point to examples of chloroplasts in mesophyll cells. (P,Q) Polygonal cells classified as GM (P) and mesophyll cells (Q) from leaves expressing the LTI6b::YFP plasma membrane marker. Left panel: cleared leaf, abaxial view, DIC microscopy; right panel: LTI6b::YFP expression (green) and chloroplast autofluorescence (red), abaxial view, CLS microscopy. CLS micrographs in P and Q were taken with identical microscope settings. Scale bars: (A,D,G) 50 μm; (B,E,H,J,K,M,N) 25 μm; (C,F,I,L,O,P,Q) 10 μm.

We conclude that even in vein orders that normally comprise only one vein type (freely ending or connecting), vein types can be changed if the duration of the reiterative branching

process is altered. This plasticity of vascular patterning is consistent with the initiation of all veins as freely ending veins, but inconsistent with a rigid specification of network properties.

Mesophyll differentiation and freely ending vein formation

We considered that if freely ending veins become connected through the fusion of elongating preprocambial domains, it might be possible to identify cellular events at the preprocambial stage that promote or prevent the formation of connecting veins. We began our analysis with higher-order preprocambial domains that predictably give rise to freely ending veins. For example, 5th order veins in the second IA grown under condition A will invariably be freely ending (Table 2). We analyzed the anatomy of the respective IA prior to procambium formation (6 DAI) and noticed that all Athb8-GUS expression domains were surrounded by mesophyll cells (Table 2), which were readily recognizable by their round shape, their separation by intercellular spaces and by the appearance of chloroplasts along the cell surface (Fig. 5G-I). The simultaneous appearance of all three features characteristic of mesophyll cells was confirmed in confocal images of GM and mesophyll cells in a line carrying the plasma membrane marker LTI6b::YFP (line 29-1) (Cutler et al., 2000). Round cells separated by intercellular spaces invariably contained large numbers of strongly autofluorescent plastids along the plasma membrane (Fig. 5Q), while polygonal cells were tightly connected and had no or few very weakly autofluorescent plastids, undetectable in whole-mount preparations (Fig. 5P). Under condition B, 4th order veins in the first IA are also invariably freely ending (Table 2), and again we observed that all corresponding preprocambial Athb8-GUS expression domains were surrounded by mesophyll cells (Table 2; Fig. 5A-C). The 4th order veins in the first IA grown under condition A and those in the second IA grown under condition B are not always freely ending (Table 2). We found that the corresponding preprocambial Athb8-GUS expression domains were not always embedded in mesophyll but could instead be surrounded by polygonal GM cells (Fig. 5D-F). Most strikingly, the percentage of Athb8-GUS domains surrounded by mesophyll cells nearly perfectly matched the percentage of freely ending 4th order veins in mature leaves under the same conditions (Table 2), strongly suggesting that initiation of mesophyll differentiation interferes with extension of preprocambial strands.

Finally, we tested whether the appearance of freely ending 2nd order veins might be correlated with mesophyll differentiation at the tip of the leaf. The 1st loops of 2nd order Athb8-GUS expression domains are formed between 2 and 3 DAI under all three conditions and are surrounded by GM cells under conditions A and B (Table 2; Fig. 5M-O). Under conditions C, mesophyll is still not differentiating in most leaves (compare Table S2, http://dev.biologists.org.supplemental/). However, in a minority of leaves (21/70, compare Table S2, http://dev.biologists.org.supplemental/), mesophyll differentiation has been initiated in the distal part of the leaf and enclosed 22/26 freely ending Athb8-GUS expression domains at this stage (Table 2; Fig. 5J-L). Because of the asynchronous formation of the two loops of one pair, often only one of the two 2nd order Athb8-GUS expression domains ended freely. As for higher-order veins, there was close correlation between the numbers of early Athb8-GUS expression domains enclosed by mesophyll and the proportion of freely ending veins in mature leaves (Table 2).

In summary, extremely high correlation between observations at the preprocambial and the mature state of vein formation indicates that preprocambial strand formation becomes terminated by the differentiation of the surrounding GM into mesophyll and that the prevalence of connecting versus freely ending veins in a given vein order depends on the relative timing of preprocambium specification (marked by Athb8-GUS expression) versus mesophyll differentiation. It should be emphasized that it is possible that mesophyll and procambium development influence each other mutually, but the design of our experiments can only trace an influence of mesophyll differentiation on the progression of preprocambial domains: in young leaves (3-6 DAI) the initiation of these domains is invariant and their progression becomes arrested only when they are surrounded by mesophyll cells.

Continuity of vascular expression domains

The above observations indicate that preprocambial strands, which are initially connected to pre-existing vasculature only at one end, can nevertheless give rise to veins that are eventually connected to vasculature at both ends. If fusions of vascular strands occur frequently, procambium might also be initiated as an island, entirely disconnected from pre-existing vasculature, to become fused at one or both ends at a later stage. In our extremely large test sample (more than 700 primordia were investigated for each of the four gene expression markers), the expression domains of all four markers were always in continuity with pre-existing vasculature. We conclude that the propensity of GM cells to adopt preprocambial and procambial cell fate is very strongly promoted by, if not strictly dependent upon, a direct connection to pre-existing vasculature (see Discussion).

Discussion

In this study we have monitored leaf procambium formation at high spatial and temporal resolution and provide a set of gene expression markers that identify distinct cell states among anatomically indistinguishable GM cells. The use of these markers made it possible to reveal stringent temporal correlations of differentiation events. Early development in all vein orders displayed remarkable similarities, and the relative timing of preprocambial domain growth and surrounding mesophyll differentiation defines the fate of a vein as either connecting or freely ending. Our findings suggest that the interpretation of vein patterns in wild-type and mutant leaves has to take the pattern of mesophyll differentiation into consideration and suggests a common developmental mechanism underlying the formation of all vein orders in the *Arabidopsis* leaf.

Early markers of vascular strand formation

Given the cell type complexity of mature vascular tissues and their high degree of functional specialization, it is not surprising that vascular tissue-specific gene expression profiles are common and readily observed in enhancer-trap and gene-trap expression pattern surveys (Sundaresan et al., 1995;

Geisler et al., 2002). However, many of these profiles do not include the earliest stages of vascular cell fate specification, and even the few markers that are expressed at early stages, among them Athb8-GUS (Baima et al., 1995; Kang and Dengler, 2002) and 553-643 (van den Berg et al., 1995), have not been characterized relative to each other or relative to anatomical features of early leaf development. The four markers presented here were selected from a larger collection (Table S1, http://dev.biologists.org/supplemental/) as it became apparent that they could identify consecutive stages preceding or accompanying procambium formation. In the development of *Arabidopsis* leaves, expression of these markers is invariably initiated in the temporal sequence: Athb8-GUS, 553-643/GT5211 (vein-associated weak), GT5211 (strong)/ET1335 (Fig. 6A), the latter two markers being expressed simultaneously with procambial cell features. In large numbers of leaf primordia, all aspects of marker gene expression, including onset, intensity and decline proved to be highly reproducible. We therefore suggest that the expression of these markers can be used to visualize distinct stages of procambial cell fate specification that cannot be identified by anatomical features.

Each of the four markers has an additional expression domain outside the incipient procambial domain. Three markers, Athb8-GUS, 553-643 and ET1335, are visible in a broad region near the tip of the leaf, where not all marker-expressing cells will become procambial. Marker GT5211 is initially weakly expressed in all GM cells, in addition to its strong procambial expression. Although these features show that the expression of none of these markers is absolutely restricted to vascular cells, this does not diminish their usefulness as indicators of specific vascular cell states. While it will be interesting to understand the molecular basis of the additional expression domains, the markers can already assist in the spatial and temporal dissection of early vascular development.

Polarities in *Arabidopsis* leaf vein ontogeny

Polarity in vein differentiation has been deduced from the directional differentiation of xylem (Telfer and Poethig, 1994; Kinsman and Pyke, 1998; Candela et al., 1999; Sieburth, 1999). In this study, markers of preprocambial and procambial cell states revealed that that the progression of vein differentiation at earlier stages can be strikingly different. For example, xylem differentiation in 2nd order vein loops is initiated at the leaf tip, while procambium formation occurs simultaneously along the entire vein and the still earlier cell fate, marked by Athb8-GUS, progresses from proximal to distal. This result is important because the polarity of xylem differentiation has fueled discussions about the position of signaling sources (Nelson and Dengler, 1997). However, if the polar progression of xylem differentiation is not correlated with that of earlier markers, it is unlikely that this polarity can provide clues as to the position of signaling sources defining the routes of vascular strands.

Expression of Athb8-GUS is the earliest marker in our study and, most interestingly, when judged by this marker, veins of all orders seem to have a common ontogeny. In all vein orders, expression of Athb8-GUS is initiated next to pre-existing vasculature and then progresses polarly away from this point of origin. More importantly, the expression of this marker identifies a critical stage in preprocambial cell specification. Expression of Athb8-GUS and the emergence of mesophyll cells seem to mark two mutually exclusive and typically irreversible stages, one leading to vascular and the other to mature mesophyll formation. Mutual exclusivity is evidenced by the fact that Athb8-GUS was never found expressed in cells of mesophyll appearance. Irreversibility of mesophyll differentiation is indicated by the observation that Athb8-GUS domains enclosed by mesophyll cells would not become elongated to form connecting veins. Finally, irreversibility of Athb8-GUS expression is indicated by the fact that islands of Athb8-GUS expression are not observed at any stage. If mesophyll cells could differentiate within Athb8-GUS expression domains, one would expect multiple interruptions of those domains.

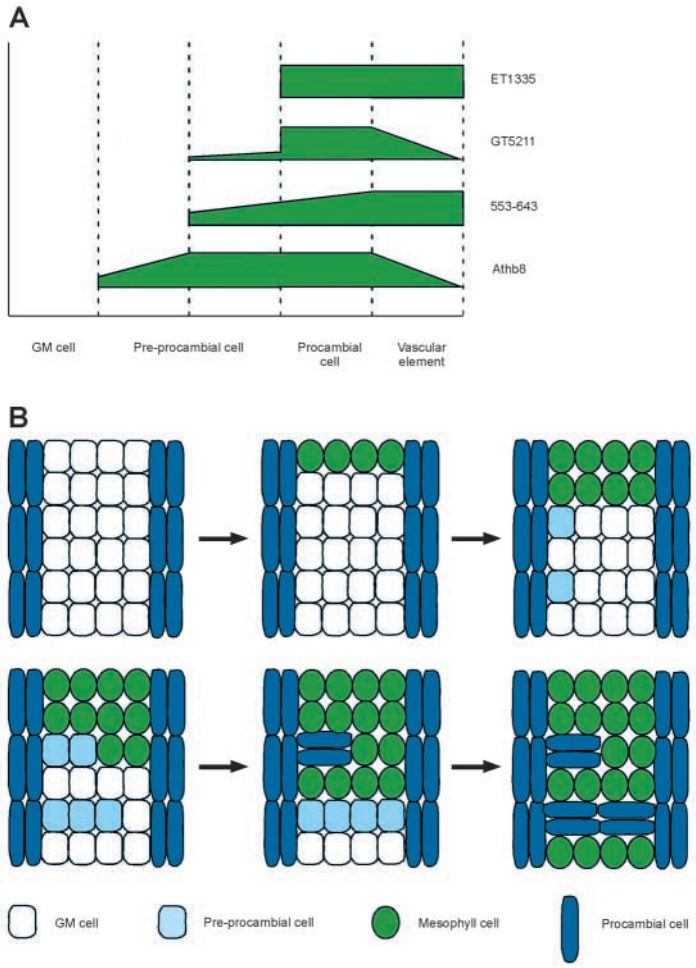

Fig. 6. (A) Schematic summary of vein-associated temporal expression profiles. Green bars indicate the duration of maximum expression of each marker, ramped termini indicate gradual onset or decline of marker gene expression. (B) Termination of vein formation by differentiating mesophyll (distal, top; proximal, bottom; arrows connect consecutive stages of leaf development). Mesophyll differentiation (green) progressing from distal to proximal interferes with polar acquisition of preprocambial cell fates (light blue) at different stages of vein ontogeny, resulting in the formation of a freely ending vein distally and a connecting vein proximally.

A common ontogeny of veins of different orders is not only suggested by Athb8-GUS expression, but also by procambium formation (or ET1335-GUS expression). Continued cell division is a defining characteristic of procambium. Cell cycling frequencies are initially similar in veins of different orders and differences between vein orders have been attributed to differential durations of cell cycling in vein development (Donnelly et al., 1999; Kang and Dengler, 2002). Furthermore, our data suggest that in all veins, procambium becomes recognizable along the entire length of a strand very rapidly, once the Athb8-GUS expression domain has reached its maximum extension, and is then extended by intercalary growth. Our half-day observation intervals do not exclude minor deviations from simultaneity, but the dynamics of procambium formation is in striking contrast to the clearly progressive expansion of the Athb8-GUS expression domain. The polar progression in the acquisition of a preprocambial cell identity is the first vascular patterning event we can monitor and it suggests that the vein network is formed through the fusion of initially free branches.

Formation of closed vascular networks

Vascular strands have existed since the Silurian period (some 400 million years ago), but were initially arranged in rather simple parallel patterns along the main axis of the plant (Stein, 1993). With the evolution of leaves in the Devonian and early Carboniferous, reiterative bifurcation leading to 'open' venation patterns was present in almost all plants with fern-like (macrophylls) leaves (ferns, progymnosperms and gymnosperms) (Roth-Nebelsick et al., 2001). According to the fossil record, simple network patterns, involving connected veins ('closed' networks) (Roth-Nebelsick et al., 2001) appeared during the Upper Carboniferous (Kull, 1999). These network patterns evolved repeatedly from open venation patterns in different lineages (e.g. Wagner, 1979) and were initially less complex than angiosperm venation patterns (Wagner, 1979; Kull, 1999). In angiosperms, these networks further evolved to produce veins of distinct hierarchical orders. Evolutionary history therefore suggests that closed venation patterns involving connecting veins evolved repeatedly from open venation patterns.

How vascular networks can be generated has been subject to some discussion. Given the high degree of complexity and reproducibility of vein patterns in many plant species, the existence of detailed genetic programs specifying the precise organization of individual vein orders seems plausible. However, at least for the brochidodromous venation pattern of the *Arabidopsis* leaf, several of our observations provide support for a reiterative, largely self-organizing mechanism underlying the formation of veins of all orders. First, in all vein orders, vein formation is initiated by the polar progression of a preprocambial cell state. Second, veins in all orders are initially freely ending and (except for the 1st order vein) will go on towards becoming connecting veins if network elaboration in higher vein orders is allowed to continue. Third, the number of vein orders in the *Arabidopsis* leaf is not genetically defined. Fourth, the developmental event terminating reiterative vein initiation and preprocambial vein elongation, the onset of mesophyll differentiation, occurs outside of the vascular system and is apparently not an inherent part of a vascular patterning program (Table 2; Fig. 6B). We therefore postulate that veins of all orders in the *Arabidopsis* leaf are initiated through the reiteration of a common branching program rather than through a genetically specified network plan.

Two alternative self-organizing mechanisms are often discussed (see Nelson and Dengler, 1997). One involves an apical-basal signal flow that becomes gradually restricted to vascular differentiation domains (Sachs, 1991; Sachs, 1981). The other postulates that vascular strand patterns are specified locally, for example through autocatalytic, local-activation-long-range-inhibition mechanisms (Meinhardt, 1982; Meinhardt and Gierer, http://www.eb.tuebingen.mpg.de/dept4/meinhardt/theory.html). Both concepts postulate a single self-organizing principle underlying the formation of all vein orders and are therefore compatible with a reiterative mode of vein initiation.

One crucial formal distinction between the two self-organizing models is that apical-basal signal flow involves signaling throughout the entire plant and thereby postulates that all vascular strands are connected from early on, while other types of self-organizing processes could generate transiently or permanently isolated vascular strands. Mutants in support of either of these models have been identified (see Turner and Sieburth, 2002). One group of mutants establishes the connection to apical-basal auxin signaling both phenotypically and biochemically (Hardtke and Berleth, 1998; Hamann et al., 2002; Hellmann et al., 2003). Another group highlights the formation of isolated procambial islands, thereby questioning the role of continuous apical-basal signaling in procambium formation (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). While the above mutant phenotypes help to explore the question of whether signaling through a procambial strand is critical in vascular patterning, our results suggest a common mechanism underlying the formation of all vein orders without specifically supporting one of the alternative models. Invariant continuity of expression domains of all four markers in extremely large numbers of leaf primordia can be considered as circumstantial support for signaling through emerging veins, but also this observation does not exclude the existence of isolated procambial strands in mutant backgrounds, which have been described in *Arabidopsis* (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000).

The observed interference between mesophyll and vascular cell specification introduces an additional patterning influence and therefore has important implications for the interpretation of venation patterns in accessions or mutants of *Arabidopsis* or in other species. For example a surprising portion of mutant venation patterns in *Arabidopsis* is characterized by failure of secondary veins to form closed loops at the leaf tip (Przemeck et al., 1996; Carland et al., 1999; Koizumi et al., 2000; Steynen and Schultz, 2003). Our results suggest that because of early mesophyll differentiation in this position, any delay of vascular differentiation and/or premature mesophyll differentiation is likely to result in this phenotype, which may explain its frequent occurrence.

We thank Simona Baima, Giorgio Morelli, Sean Cutler, Bernd Müller-Röber, Jane Murfett, Tom Guilfoyle and the Cold Spring Harbor Laboratory for seeds, and Wenzislava Ckurshumova, Nancy

Dengler, Julie Kang, Naden Krogan and Jaideep Mathur for helpful comments on the manuscript. This work was supported by an NSERC discovery grant to T.B.

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