The FORKED genes are essential for distal vein meeting in *Arabidopsis*

Quintin J. Steynen and Elizabeth A. Schultz

Department of Biological Sciences, University of Lethbridge, Lethbridge, AB, TIK 3M4, Canada
*Author for correspondence (e-mail: schultz@uleth.ca)

Accepted 3 June 2003

Development 130, 4695-4708

doi:10.1242/dev.00689

Summary

As in most dicotyledonous plants, the leaves and cotyledons of *Arabidopsis* have a closed, reticulate venation pattern. This pattern is proposed to be generated through canalization of the hormone auxin. We have identified two genes, *FORKED 1 (FKD1)* and *FORKED 2 (FKD2)*, that are necessary for the closed venation pattern: mutations in either gene result in an open venation pattern that lacks distal meeting. In *fkd1* leaves and cotyledons, the defect is first evident in the provascular tissue, such that the distal end of the newly forming vein does not connect to the previously formed, more distal vein. Plants doubly mutant for both genes have widespread defects in leaf venation, suggesting that the genes function in an overlapping manner at the distal junctions, but act redundantly throughout leaf veins. Expression of an auxin responsive reporter gene is reduced in *fkd1* leaves, suggesting that *FKD1* is necessary for the auxin reponse that directs vascular tissue development. The reduction in reporter gene expression and the *fkd1* phenotype are relieved in the presence of auxin transport inhibition. The restoration of vein junctions in situations where auxin concentrations are increased indicates that distal vein junctions are sites of low auxin concentration and are particularly sensitive to reduced *FKD1* and *FKD2* activity.

Key words: *Arabidopsis thaliana*, *forked1*, *forked2*, *mp*, *axr6*, *pin1*, Vascular patterning, Leaf venation, Auxin

Introduction

The spatial arrangement of the vascular bundles within plant leaves is crucial to plant function as it enables efficient transport of water, minerals and photosynthates and provides mechanical support to the leaf. Two major events are thought to have been fundamental in the evolution of the closed reticulate system present in all higher plant leaves: (1) the evolution of a branching system and (2) the formation of a closed pattern. The most primitive leaf vascular pattern, found in microphylls, consists of only a single vein or a pair of parallel veins running the length of the leaf (Wagner et al., 1982; Gifford and Foster, 1989). Primitive vascular plants bearing megaphylls evolved a more complex, open, dichotomously branching vascular pattern that is proposed to have conferred an advantage in dropping atmospheric CO_2 levels (Beerling et al., 2001). In more advanced vascular plants, including some members of the ferns and gymnosperms and all angiosperms, a closed venation pattern evolved through joining of distal branches (Trivett and Pigg, 1996). Distal meeting is proposed to be advantageous because it provides both redundancy in transportation routes in case of injury or blockage of a vascular bundle and increased mechanical stability and support to the leaf, particularly along the leaf margin (Roth-Nebelsick, 2001).

Both classes of angiosperms, the monocots and dicots, have a complex, branched and closed leaf venation pattern. In monocots the pattern is called striate with a series of longitudinal veins running approximately parallel to each other, meeting at the apex of the leaf and interconnecting through a multitude of smaller transverse veins. In dicots, the pattern is reticulate with a basic pattern of secondary veins branching from the midvein and connecting to one another or to higher order (tertiary and quaternary) veins at their distal ends near the leaf margin.

The primary model to explain vascular patterning, the auxin canalization model, is supported by evidence indicating a central role for auxin in vascular differentiation within the stem (Sachs, 1981; Sachs, 1989; Sachs, 1991), in isolated mesophyll cells (Church, 1993; Berleth and Sachs, 2001) and in leaves (Mattson et al., 1999; Sieburth, 1999; Aloni, 2001). According to the auxin canalization model, an initially homogenous field emanates from auxin sources, but random fluctuations expose some cells to increased auxin leading to vascular cell differentiation and increased efficiency in auxin transport. The increased transport results in two classes of cells: (1) those to which auxin is transported form vascular tissue and (2) those from which auxin is drained form non-vascular tissue.

Auxin is thought to be transported in a primarily basipetal manner throughout the plant body from primary sources of synthesis (Wallroth-Marmonr and Harte, 1988; Lomax et al., 1995), although recent studies indicate that the shoot apical meristem and very young leaf primordia are auxin sinks to which auxin is acropetally transported (Reinhardt et al., 2000; Avsian-Kretchmer et al., 2002). Basipetal transport from young developing leaves is proposed to direct stem vasculature since

removal of leaves eliminates vascular differentiation in the stem below and can be compensated by exogenous auxin (Sachs, 1981). Leaf venation is proposed to be directed by basipetal transport of auxin from the leaf margin (Mattson et al., 1999; Sieburth, 1999; Aloni, 2001; Avsian-Kretchmer et al., 2002), a mechanism consistent with auxin response patterns in regions that will become procambium (Mattsson et al., 2003). Leaves in which polar auxin transport is inhibited either chemically (Goto et al., 1991; Okada et al., 1991; Gälweiler et al., 1998; Sieburth, 1999), or genetically, through eliminating PIN-FORMED 1 (PIN1), a component of the auxin efflux carrier (Gälweiler et al., 1998; Müller et al., 1998; Mattsson et al., 1999; Steinmann et al., 1999), develop increased vascularization adjacent to the leaf margin.

Consistent with a role for auxin in vascular patterning, a number of *Arabidopsis* mutants known to affect aspects of auxin transport or response have defects in vascular development. Mutations in *MONOPTEROS (MP)*, *BODENLOS (BDL)* or *AUXIN RESISTANT 6 (AXR6)* show early defects in embryonic apical basal patterning followed by a reduction in cotyledon venation (Berleth and Jurgens, 1993; Hamann et al., 1999; Hobbie et al., 2000). *MP* encodes ARF5, an auxin response factor that activates auxin response targets, while *BDL* encodes IAA12, a protein that has been shown to bind to ARF5 and prevent its activity (Hamann et al., 2002). *AXR6* encodes the protein cullin, a subunit of the ubiquitin ligase complex SCF (Hobbie et al., 2002). Mutations in *SCARFACE* (*SFC*) result in plants that are more sensitive to auxin and show reduction and discontinuity of venation within foliar organs (Deyholos et al., 2000). Plants mutant for *PINOID*, which encodes a serine-threonine kinase believed to affect either auxin signaling or auxin transport, show altered venation within floral organs, while mutants in *LOPPED* (*LOP*) are defective in basipetal auxin transport and alter leaf venation (Carland and McHale, 1996; Christensen et al., 2000; Benjamins et al., 2001). Alleles of *GNOM* (*EMB30*), such as *van7* (*vascular network 7*) (Koizumi et al., 2000), result in discontinuous venation in cotyledons and increased marginal venation in leaves, a leaf phenotype similar to *pin1* and consistent with the proposed role of GNOM in PIN1 localization (Steinmann et al., 1999). While the interactions amongst these genetic factors are not yet well understood, the frequent association of defective auxin signaling or transport with defective vascular patterning clearly points to a primary role for auxin in establishing vascular pattern within shoots.

We report the isolation and characterization of mutants in two novel *Arabidopsis thaliana* genes, *FORKED1 (FKD1)* and *FORKED2 (FKD2)*, crucial to the formation of the closed leaf vascular pattern characteristic of dicot leaves. Recessive mutations in either *FKD1* or *FKD2* result in a failure of distal portions of the vascular bundles to form connections with the remaining leaf vascular network, resulting in an open leaf venation pattern reminiscent of primitive vascular plants. Our analysis suggests that *FKD1* responds to a particular auxin threshold and allows vascular development, and that this action is redundant to that of *FKD2* except at the lowest auxin levels. The function of these genes is of particular interest as the closed leaf vascular pattern is ubiquitous within the angiosperms and appears to have been important in their evolution.

Materials and methods

Plant material

All seed material was from the *Arabidopsis* Biological Resource Center (Columbus, Ohio) except the ethyl methane sulfonate (EMS) mutagenized seed (Columbia; Col), from Lehle Seeds (Round Rock, TX), *pin1-1* and *mp*^{G92} seed provided by T. Berleth (University of Toronto, Toronto, ON) and *DR5::GUS* seed provided by J. Murfett (University of Missouri, Columbia, Missouri).

Growth conditions and analysis

Seed were sown on Metromix 200 (W. R. Grace Co., Marysville, OK) in 100 cm^2 pots or on *A. thaliana* (AT) growth medium (Wilson et al., 1990) with or without 30 μM naphthylphthalamic acid (NPA; Sigma Chemical Company) in 78.5 cm^2 Petri plates. Following 4-5 days stratification, seeds were transferred (considered as the day of germination: 0 **D**ay **A**fter **G**ermination; DAG) to growth chambers (Percival Scientific, Perry, IA) set for 21°C, 60% relative humidity, and continuous light (130 mol $second^{-1}$ m^{-2}) provided by a combination of Sylvania Cool White, Gro Lux, and incandescent bulbs (Osram Sylvania Inc., Danvers, MA). To assess root growth, 5-day old seedlings vertically grown on plates were transferred to medium containing either 1.0×10^{-6}, 1.0×10^{-7} or 1.0×10^{-8} M 2,4-dichlorophenoxyacetic acid (2,4-D). Root growth was measured from the position of the root tip at transfer to the position 4 days later.

For isotopic analysis, wild-type and *fkd1* plants were greenhouse grown (16-hour days) on soil (as described above) and leaves were harvested when the shoot was 2 cm long. A 1-2 mg subsample of ground leaf material was sealed in a tin capsule and loaded into the elemental analyzer (NC2500, CE Instruments, ThermoQuest Italia, Milan, Italy) and the diatomic nitrogen and carbon dioxide gases generated therein were separated in a gas chromatographic column and passed directly, using a helium stream, to the inlet of the mass spectrometer (Delta Plus, Finnigan Mat, San Jose, CA, USA) for quantification and measurement of stable isotope ratios. Carbon stable isotope ratios ($^{13}C/^{12}C$) were expressed in delta notation (δ values presented in parts per thousand) where the international standard is CO_2 from Pee Dee Belemnite (PDB) limestone (Farquhar et al., 1989).

Mutant screening

Approximately 6000 EMS-mutagenized M_2 seed were sown at a density of 50 seed per pot. A cotyledon and first leaf was taken 14 DAG, mounted in low viscosity Cytoseal (Stephen's Scientific, Kalamazoo, MI) and screened for abnormalities in vascular patterning using a dissecting microscope (Stemi 2000, Carl Zeiss Inc., Thornwood, NY). M_3 seed of potential mutants was collected and re-screened. Lines with heritable phenotypes were backcrossed, using wild type as the female, at least twice before characterization.

Morphological and anatomical characterization

Wild-type and *fkd1* seeds (20 per plate) were sown on AT medium with or without NPA and plants taken every 24 hours from 1 DAG. Histochemical localization of GUS activity and subsequent clearing was performed as described previously (Kang and Dengler, 2002). For photography, mature cotyledons (14 DAG) and first leaves (21 DAG) of all genotypes, and fully open *fkd1*, *fkd2* and *fkd2 fkd1* flowers were removed and cleared in a solution of 3:1 ethanol:acetic acid for 2-4 hours, 70% ethanol for 1 hour, 95% ethanol overnight and 5% NaOH for 1 hour at 60°C. Dissections were performed in a 50% aqueous glycerol solution and samples viewed with a compound light microscope (Eclipse E600, Nikon, Mississauga, ON).

Cotyledons (14 DAG) and first leaves (21 DAG) of all genotypes were mounted in Cytoseal, images captured with a CCD camera (RS-170, Cohu Inc., Electronics Division, San Diego, CA) attached to a dissecting microscope (Stemi 2000, Carl Zeiss Inc., Thornwood, NY), and assessed using NIH Image (http://rsb.info.nih.gov/nih-image/).

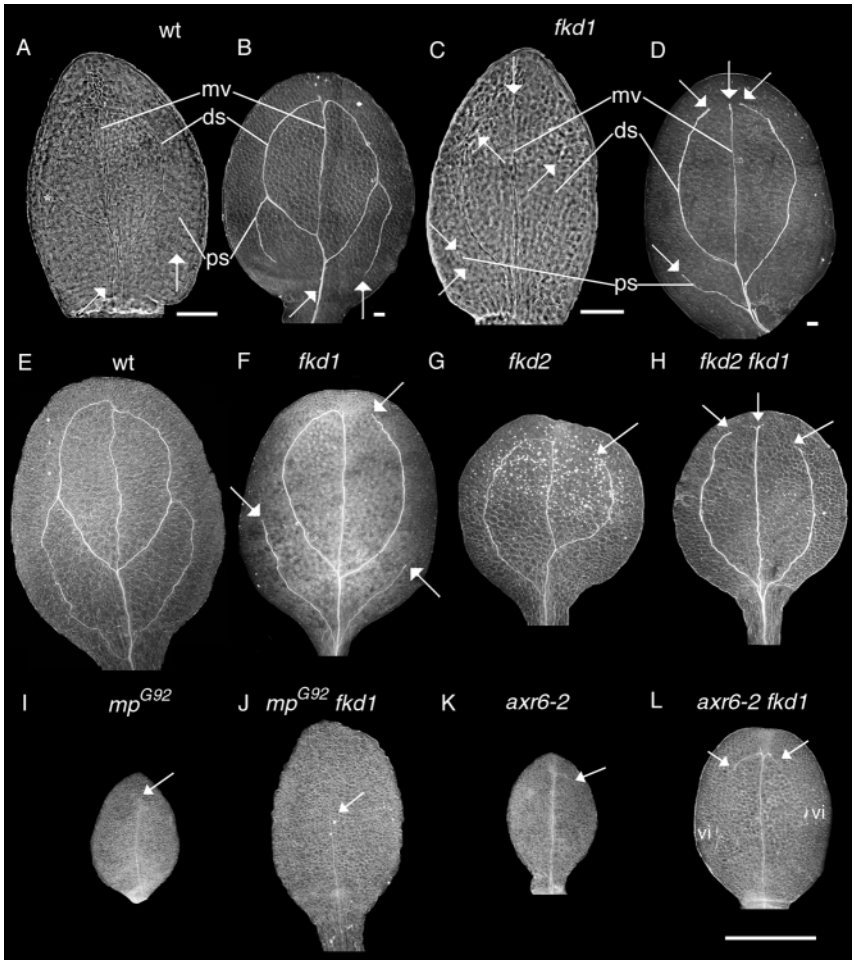

Fig. 1. Vascular pattern development in wild-type (A,B) and in *fkd1* (C,D) cotyledons and mature cotyledons of wild type (E), *fkd1* (F), *fkd2* (G), *fkd2 fkd1* (H), *mp*^{G92} (I), *mp*^{G92} *fkd1* (J), *axr6-2* (K) and *axr6-2 fkd1* (L), viewed with phase contrast optics (A,C), DIC optics (B,D) and dark-field illumination (E-L). Cleared cotyledons (1 DAG) of wild type (A) and *fkd1* (C), show the presence of provascular tissue of the midvein (mv), the distal secondary veins (ds), and occasionally proximal secondary veins (ps) that show partial maturation by 3 DAG (B,D) and complete maturation by 14 DAG (E,F). Arrows indicate freely ending veins and * in A indicates the initiation of a proximal secondary vein adjacent to the distal secondary vein. Scale bar: 50 μm.

Whole cotyledons were scored for numbers of secondaries (veins attached at least at one point to the midvein) and cotyledons and half leaves were scored for number of branch points (two or more veins meeting), vein endings and areoles (any area of the leaf blade completely bounded by veins). Cleared cotyledons and leaves were scored for the percentage showing vascular islands (VIs). Wild-type, *fkd1* and *fkd2* plants (4 per pot) were scored for germination, total number of leaves, number of secondary stems and time to flowering (days). Statistical differences were determined using Student's *t*-test.

Cotyledons (14 DAG) and first leaves (21 DAG) of wild type and *fkd1* were prepared for sectioning by vacuum infiltration in FAA (18:1:1 70% ethanol:formalin:glacial acetic acid), stored at 4°C overnight, dehydrated through an ethanol series, and embedded in Spurr's resin. Five μm sections were cut using a glass knife and stained with Toluidine Blue before being viewed with a compound light microscope (Eclipse E600, Nikon, Mississauga, ON).

Mapping of *FKD1* and *FKD2*

Plants mutant for *FKD1* and *FKD2* were crossed to Landsberg *erecta* (L*er*) ecotype and DNA was extracted from F_2 plants exhibiting the *fkd1* or *fkd2* phenotype (Edwards et al., 1991). Mapping using simple sequence length polymorphisms (SSLPs) between the Col and L*er* backgrounds was done using standard PCR conditions (Bell and Ecker,1994) and primers (Research Genetics Inc., Huntsville, AL).

Generation of double mutants

Double mutants were generated as described in Table 1. F_3 progeny from *axr2 fkd1*, *aux1-7 fkd1* and *fkd*2 *fkd*1 double mutants, F_3 progeny segregating for *pin1-1 fkd-1*, *mp fkd1* or *axr6-2 fkd1* double mutants and F_4 progeny from *axr1-3 fkd1* were characterized. To generate the *fkd1 DR5::GUS* line, *fkd1* plants were crossed to plants homozygous for *DR5::GUS*. F_2 progeny were screened on plates with 10 μg/ml kanamycin, surviving plants showing the *fkd1* phenotype were allowed to self, and three F_3 families entirely resistant to kanamycin were characterized.

Table 1. Generation of double mutants between homozygous *fkd1*(male parent) and various other mutant lines

Female parent	F_1 phenotype	F_2 phenotypic ratio†	F_3 phenotypic ratio§
fkd2 fkd2	Wild type	9:3:3:1, n=214, χ^2=0.621, P>0.75	3:1
pin1-1 PIN1	Wild type	9:3:3:1, n=234, χ^2=5.30, P>0.1‡	3:1
axr1-3 axr1-3	Wild type	9:3:3:1, n=163, χ^2=0.159, P>0.975	3:1
axr2 AXR2	1 *axr2:* 1 wild type*	9:3:3:1, n=166, χ^2=4.22, P>0.1	3:1
aux1-7 aux1-7	Wild type	9:3:3:1, n=155, χ^2=5.59, P>0.1	3:1
mp^{G92} *MP*	Wild type	9:3:3:1, n=126, χ^2=4.99, P>0.1‡	3:1
axr6-2 AXR6	1 *axr6-2:* 1 wild type*	9:3:3:1, n=220, χ^2=11.6, P>0.005	3:1

*Only F_1 plants with the codominant phenotypes were allowed to generate F_2 seed.

†As analysed by χ^2 test, all F_2 progeny segregated in an approximate 9 wild type: 3 *fkd-1*: 3 single mutant: 1 novel phenotype, except the *axr2* × *fkd1* F_2, which segregated 9 *axr2:* 3 wild type: 3 novel phenotype: 1 Fkd1. These data are consistent with the novel phenotype in each case representing the double mutant.

‡F_2 seed from single F_1 plants were screened; only those populations segregating *pin1-1* or *mp*^{G92} were considered further.

§In each case, selfed seed from several *fkd1* F_2 plants were sown, and in all cases, approximately two-thirds had F_3 progeny that segregated 3 *fkd1:* 1 novel phenotype indicating that the novel phenotype is the double mutant. In the case of *axr2* × *fkd1*, F_2 plants having the novel phenotype were allowed to self, and two-thirds segregated 3 novel phenotype: 1 *fkd1*, consistent with the novel phenotype being the double mutant.

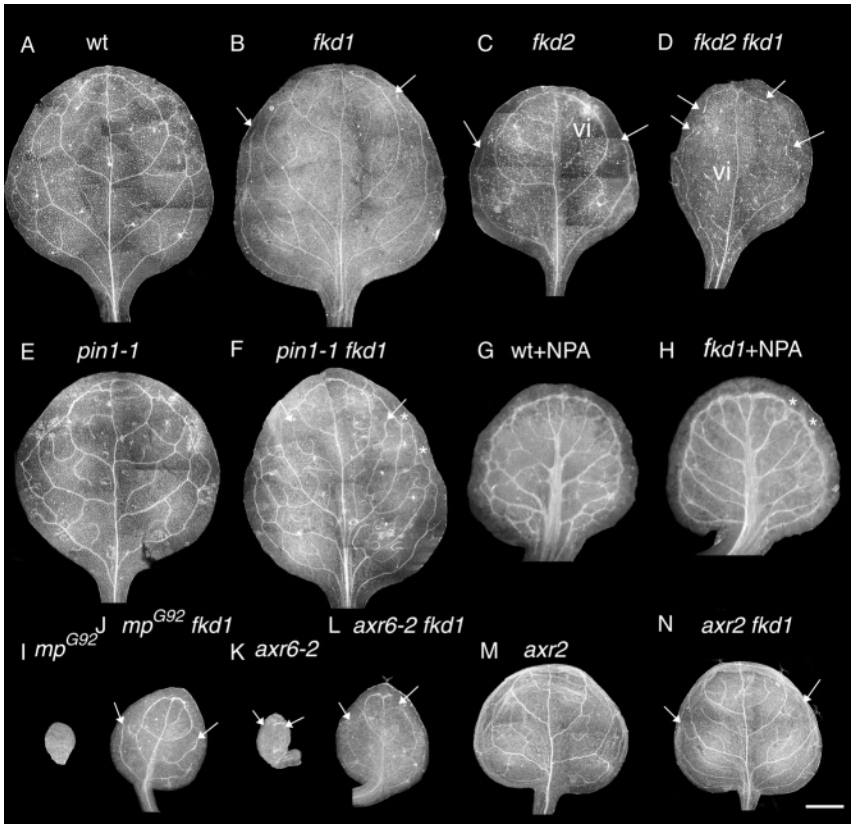

Fig. 2. Vascular pattern of cleared first leaves from wild type (A), *fkd1* (B), *fkd2* (C), *fkd2 fkd1* (D), *pin1-1* (E), *pin1-1 fkd1* (F), wild-type grown on 30 μM NPA (G), *fkd1* grown on 30 μM NPA (H), mp^{G92} (I), mp^{G92} *fkd1* (J), *axr6-2* (K), *axr6-2 fkd1* (L), *axr2* (M), *axr2 fkd1* (N) viewed under dark-field illumination. All leaves were removed 21 DAG, except I-L, which were removed 14 DAG. Arrows indicate freely ending veins, asterisks indicate distal meeting, and vi indicates vascular islands. Scale bar: 1 mm for A-F, L and M and 500 μm for G-K.

Photography and digital imaging

All images were captured using a digital camera (Coolpix 990, Nikon, Mississauga, ON) and were prepared for publication using Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, CA).

Results

Mutant isolation and genetic analysis

We screened an M_2 population (*gl1-1*, Col background) of EMS mutagenized plants for defects in leaf vascular patterning and chose, for further characterization, two mutant lines [*forked1* (*fkd1*) and *forked2* (*fkd2*)] with vascular bundles that fail to meet distally in both the cotyledons (Fig. 1F,G) and leaves (Fig. 2B,C).

Complementation tests between *fkd1* and *fkd2* yielded all wild-type plants (n=106). Crosses of either *fkd1* or *fkd2* to wild type yielded an F_1 of wild-type phenotype (n=36 and n=35, respectively). In the F_2 the *fkd1* and *fkd2* phenotypes segregated from the wild-type phenotype with a ratio of 3:1 (188 wild type: 53 *fkd1*, χ^2=1.037, P>0.25; 178 wild type: 57 *fkd2*, χ^2=0.070, P>0.75). We therefore concluded that the *fkd1* and *fkd2* phenotypes are each the result of a single, nuclear, recessive mutation.

For mapping, *fkd1* and *fkd2* plants were crossed into the L*er* background (Bell and Ecker, 1994). The *FKD1* gene mapped to 89.48 cM on chromosome III based on recombination with nga112 (1.6%, n=562 chromosomes) and nga6 (3.8%, n=210 chromosomes). *FKD2* mapped to 30.2 cM on chromosome V based on recombination with ciw8 (11.8%, n=152 chromosomes) and nga106 (14.0% n=50 chromosomes). This places *FKD2* near a previously described gene involved in vascular patterning, *SFC* (Deyholos et al., 2000). Plants mutant for *SFC* have severely altered morphology and leaves with highly disrupted vascular patterning, including VIs. Unfortunately, *sfc* seed was not available so we were unable to determine through complementation if *fkd2* is a weak allele of *sfc* or a mutation in a novel gene. For this reason, detailed developmental and double mutant characterization was performed only on *fkd1* plants.

Cotyledon vascular pattern development

In order to assess the differences between wild-type, *fkd1* and *fkd2* mature cotyledons, we first quantified wild-type numbers of vein branch points, areoles, freely ending veins and secondary veins (Table 2). Most commonly, the vascular pattern of the mature wild-type cotyledon consists of a midvein and 4 secondary veins (two distal and two proximal) that meet with the midvein and one another to generate four closed loops (Fig. 1E).

To determine the sequence of wild-type cotyledon vascular pattern development, we examined seedlings at 24-hour intervals and assessed the timing of developmental landmarks (Table 3, Fig. 1). By 1 DAG, provascular tissue of midvein and distal secondary veins is complete (Fig. 1A), the secondary veins connecting to the midvein at distal (Table 3, Fig. 3A,B) and proximal points (Fig. 3I,J). Midvein maturation (appearance of cell wall thickenings) begins at 1 DAG and is complete by 2 DAG, while distal secondary vein maturation is complete by 3 DAG. Both proximal secondary veins are initiated perpendicular to the distal secondary vein by 2 DAG, and maturation is initiated by 3 DAG (Fig. 1B, Fig. 3F). Completion is variable; at cotyledon maturity (14 DAG), some (23%, n=46) proximal secondary veins never join the midvein. Secondary veins develop either basipetally (66%, n=30 distal veins; 95%, n=758 proximal veins), or bidirectionally.

In mature (14 DAG) *fkd1* and *fkd2* cotyledons the distal connections between the secondary veins and the midvein, and between the proximal and distal veins often do not form (Fig. 1F,G), resulting in fewer areoles and branch points (Table 2). In *fkd1* cotyledons, the complexity of the venation pattern is unaffected relative to wild type, whereas *fkd2* cotyledons have a simpler pattern (Table 2).

The lack of distal vein meeting is evident early in *fkd1* cotyledon development. At 1 DAG, the provascular tissue of

Table 2. Cotyledon 14 DAG and first leaf 21 DAG vascular pattern characters for various genotypes

	Free ends	Areoles	Branch points	Secondary veins	% VIs
Cotyledon					
Wild Type (31)	0.5±0.1	3.3±0.1	5.7±0.2	3.7±0.11	0% (9)
fkd1 (31)	2.3±0.1*	1.6±0.1*	3.9±0.2*	3.8±0.1	0% (10)
fkd2 (31)	0.7±0.2	1.8±0.1*	3.4±0.1*	2.4±0.1*	25% (16)
fkd2 fkd1 (40)	2.1±0.1*,§	0.3±0.1*,†,§	2.4±0.1*,†,§	2.1±0.1*,†,§	62% (24)
axr1-3 (31)	0.2±0.1	2.1±0.1*	3.5±0.2*	2.2±0.1*	0% (12)
axr1-3 fkd1 (31)	2.0±0.0*,‡	0.0±0.0*,†,‡	2.0±0.0*,†,‡	2.0±0.0*,†,‡	0% (12)
axr2 (27)	0.3±0.1	3.3±0.2	6.0±0.2	3.5±0.2	17% (6)
axr2 fkd1 (27)	2.0±0.2*,‡	1.5±0.1*,‡	4.5±0.1*,†,‡	3.2±0.2*,†	0% (7)
pin1-1 (15)	0.3±0.1	3.6±0.3	6.1±0.5	3.2±0.2*,†	0% (9)
pin1-1 fkd1 (15)	0.7±0.2†	2.5±0.3*,†	4.6±0.5*	2.7±0.2*,†	0% (3)
aux1-7 (34)	0.5±0.1	3.6±0.6	6.7±0.1*	3.7±0.1	ND
aux1-7 fkd1 (37)	2.8±0.1*,†,‡	0.9±0.8*,†,‡	4.4±0.1*,†,‡	3.4±0.1†,‡	ND
mp ^{G92} (30)	1.4±0.1*	0.0±0.0*	0.8±0.1*	1.2±0.2*	0% (12)
mp ^{G92} *fkd1* (39)	1.1±0.0*,†,‡	0.0±0.0*,†	0.1±0.1*,†,‡	0.2±0.1*,†,‡	0% (35)
axr6-2 (41)	1.3±0.1*	0.0±0.0*	0.8±0.1*	1.0±0.1*	22% (23)
axr6-2 fkd1 (34)	1.6±0.1*,†	0.0±0.0*,†	1.0±0.1*,†,‡	1.6±0.1*,†,‡	68% (40)
First Leaf					
Wild type (31)	9.2±0.6	17.8±1.0	39.6±1.9	ND	0% (16)
fkd1 (31)	14.1±0.7*	4.0±0.3*	23.6±0.9*	ND	19% (43)
fkd2 (31)	11.1±0.6*	3.1±0.2*	16.5±0.8*	ND	78% (37)
fkd2 fkd1 (30)	41.1±3.1*,†,§	0.4±0.1*,†,§	13.8±1.1*,†	ND	100% (12)
axr1- 3 (31)	4.9±0.5*	4.2±0.2*	14.6±0.6*	ND	ND
axr1-3 fkd1 (31)	4.9±0.3*,†	0.8±0.1*,†,‡	7.7±0.3*,†,‡	ND	ND
axr2 (27)	4.7±0.6*	9.7±0.5*	22.6±1.2*	ND	ND
axr2 fkd1 (27)	9.8±0.5†,‡	2.3±0.3*,†,‡	12.7±0.6*,†,‡	ND	ND
pin1-1 (15)	2.9±0.6*	10.5±0.9*	23.1±2.3*	ND	ND
pin1-1 fkd1 (15)	7.5±0.4†,‡	5.1±0.8*,‡	15.6±1.1*,†	ND	ND
aux1-7 (40)	11.9±0.9	19.9±1.1†	44.6±2.3†	ND	ND
aux1-7 fkd1 (38)	15.2±0.9*,‡	4.1±1.2*,‡	24.4±2.9*,‡	ND	ND

Number in brackets represents number of organs scored. Values represent mean±s.e.m. for the entire cotyledon and half the first leaf as divided by the midvein, except for VIs, where values represent percentage of leaves having VIs. ND indicates that the value was not determined for this characteristic.

*Significantly different from wild type.

†The double mutant is significantly different from *fkd1*.

‡The double mutant is significantly different from its corresponding single auxin mutant.

§*fkd2 fkd1* is significantly different from *fkd2*.

Table 3. Landmarks of cotyledon and first leaf development

	Wild type	*fkd1*
Cotyledon		
Midvein		
Provascular complete	1 DAG 100% (*n*=15)	1 DAG 100% (*n*=44)
Maturation visible	1 DAG 7% (*n*=28)	1 DAG 0% (*n*=44)
Maturation complete	2 DAG 100% (*n*=15)	2 DAG 94% (*n*=48)
Distal secondaries		
Initiation	1 DAG 100% (*n*=15)	1 DAG 93% (*n*=44)
Connection of one vein to midvein	1 DAG 0% (*n*=15)	1 DAG 32% (*n*=28)
Connection of both veins to midvein	1 DAG 100% (*n*=15)	1 DAG 18% (*n*=28)
Maturation complete	3 DAG 100% (*n*=42)	3 DAG 97% (*n*=34)
Proximal secondaries		
First proximal initiated	1 DAG 60% (*n*=28)	1 DAG 7% (*n*=28)
Second proximal initiated	2 DAG 87% (*n*=15)	3 DAG 23% (*n*=47)
Maturation visible	3 DAG 73% (*n*=42)	3 DAG 50% (*n*=28)
First leaf		
Midvein		
Differentiation complete	5 DAG 54% (*n*=24)	5 DAG 21% (*n*=28)
Distal secondaries		
Vein initiated	5 DAG 42% (*n*=24)	5 DAG 14% (*n*=28)
Joined to midvein proximally	6 DAG 63% (*n*=41)	6 DAG 32% (*n*=43)

Values represent percentage of organs showing the developmental event on the day scored. Number in brackets represents number of organs scored.

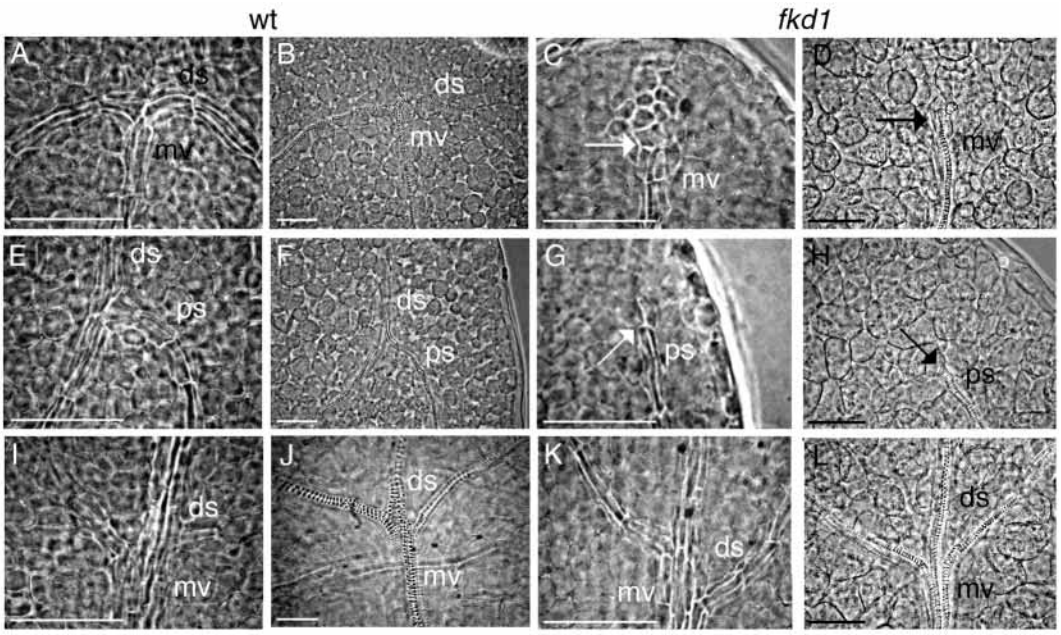

Fig. 3. Proximal and distal vein junctions in wild-type (A,B,E,F,I,J) and *fkd1* (C,D,G,H,K,L) cotyledons at provascular (A,C,E,G,I,K) and mature vascular (B,D,F,H,J,L) tissue stages viewed with phase contrast optics. (A-D) Distal vein junction between midvein (mv) and distal secondary veins (ds) near the apex of the cotyledon. (E-H) Distal vein junction of proximal (ps) and distal secondary veins. (I-L) Proximal vein junction between distal secondary veins and midvein. Arrows indicate the failure of distal junctions in *fkd1*. Scale bar: 50 μm.

one or both of the distal secondary veins fails to join the midvein (Table 3, Fig. 1C, Fig. 3C,D). Furthermore, *fkd1* proximal secondary veins initiate at a point distant from the existing distal secondary vein (Fig. 3G) and initiation is delayed relative to wild type (Table 3). Other aspects of *fkd1* vein development are similar to wild type, except that all secondary veins connect proximally with the midvein.

First leaf vascular pattern development

The vascular pattern of wild-type first leaves consists of a midvein from which regularly spaced secondary veins extend to the leaf margin where they join one another (Fig. 2A). Tertiary veins form connections between the secondary veins or occasionally end freely in the lamina, while quaternary veins usually end freely in the lamina. To compare the *fkd1* and *fkd2* leaf venation pattern to that of wild type, we quantified the number of branch points, freely ending veins and areoles of first leaves 21 DAG (Table 2).

The midvein of the wild-type leaf differentiates acropetally until it reaches the distal tip of the developing leaf 5 DAG where the distal secondary veins are initiated (Table 3, Fig. 4A). These develop basipetally, meeting the midvein between 6 and 7 DAG (Table 3, Fig. 4B). Most remaining secondary and tertiary veins initiate from previously formed, more distal veins and develop basipetally to join the vascular network at their proximal end (Fig. 4C). At least one vein (excluding quaternary) in 53% (n=38) of leaves at 21 DAG fails to rejoin the vascular network proximally.

In *fkd1* and *fkd2* first leaves the distal ends of most veins fail to join previously formed veins and end freely in the lamina (Fig. 2B,C), resulting in an open venation pattern with fewer branch points and areoles and more vein ends than wild type (Table 2). Proximal non-meeting of veins occurs in *fkd1* with a similar frequency (43%, n=43) as in wild type, resulting in VIs in mature leaves (Table 2). VIs occur more frequently in *fkd2* first leaves and are distributed throughout the leaf blade (Fig. 2C). Cross sections of *fkd1* leaves are indistinguishable from wild type (data not shown).

In *fkd1* leaves, development of the midvein, initiation of distal secondary veins and their proximal

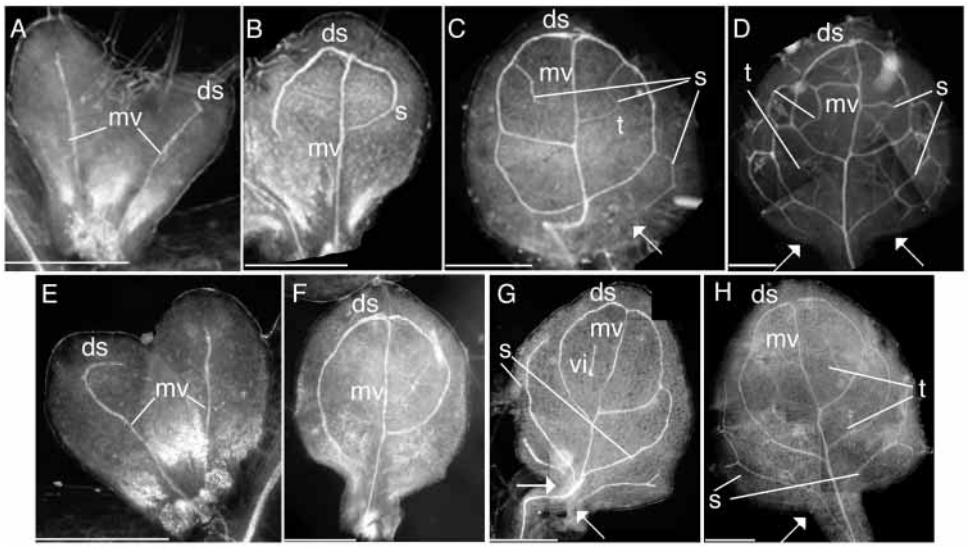

Fig. 4. Vascular pattern development in the first leaf of wild type (A-D) and *fkd1* (E-H). Formation of the midvein (mv) and distal secondary veins (ds) in wild type (A,B) is indistinguishable from *fkd1* (E,F). Subsequent secondary veins (s) initiate from existing vascular tissue in wild type (C) but initiate freely in *fkd1* (G), leading to the development of vascular islands (vi) in the immature leaf. Tertiary veins (t) fill in the developing vascular pattern (D,H). Arrows indicate basal free vein ends. Scale bar: 250 μm.

Table 4. Morphological characters for wild type, *fkd1* and *fkd2* plants

	Wild type	*fkd1*	*fkd2*
% Germination (n=40)	98%	98%	98%
Leaf total nitrogen (% of dry weight, n=8)	5.63±0.17%	5.62±0.29%	Not determined
$\delta^{13}C$ (n=8)	–28.69±0.05%	–29.26±0.07%	Not determined
Total no. of leaves (n=31)	11.3±0.3	10.8±0.3	9.4±0.2*,†
No. of secondary stems (n=31)	3.4±0.1	3.9±0.3	3.2±0.1†
Time to flowering (n=31)	21.6±0.3 days	21.2±0.3 days	22.9±0.2 days*,†
Total seed weight (n=31)	135±46 mg	147±45 mg	75±33 mg*,†

*Significantly different from wild type.
†Significantly different from *fkd1*.
Values for last six characters represent mean±s.e.m.

Table 5. Morphological characteristics of cotyledons 14 DAG and first leaves 21 DAG

	Width (mm)	Length (mm)	Length/width	Area (mm^2)
Cotyledon				
Wild type (31)	2.24±0.07	2.70±0.08	1.22±0.02	4.77±0.25
fkd1 (31)	2.38±0.06	2.97±0.09*	1.25±0.02	5.67±0.28*
fkd2 (31)	1.99±0.07*	2.55±0.09	1.29±0.02*	3.91±0.26*
fkd2 fkd1 (29)	1.76±0.05*,†,§	2.16±0.06*,†,§	1.25±0.04	2.92±0.13*,†,§
axr1-3 (31)	1.61±0.04*	1.77±0.04*	1.11±0.02*	2.25±0.1*
axr1-3 fkd1 (31)	2.08±0.07†,‡	2.35±0.09*,†,‡	1.13±0.02*,†	3.86±0.25*,†,‡
axr2 (27)	1.85±0.05*	1.88±0.06*	1.01±0.02*	2.71±0.17*
axr2 fkd1 (27)	1.90±0.04*,†	2.09±0.05*,†,‡	1.10±0.02*,†,‡	3.19±0.14*,†
pin1-1 (15)	2.57±0.19*	2.36±0.10*	0.95±0.04*	4.63±0.56
pin1-1 fkd1 (15)	2.69±0.10*,†	2.88±0.09‡	1.08±0.03*,†,‡	5.81±0.33*
aux1-7 (38)	2.47±0.05*	3.07±0.08*	1.24±0.02	5.96±0.27*
aux1-7 fkd1 (37)	2.41±0.06*	3.08±0.08*	1.28±0.01*	5.91±0.28*
First leaf				
Wild type (31)	6.12±0.18	6.84±0.21	1.12±0.02	32.36±1.83
fkd1 (31)	6.14±0.17	7.46±0.20*	1.22±0.02*	35.84±1.90
fkd2 (31)	5.93±0.19	7.05±0.19	1.21±0.02*	31.94±1.72
fkd2 fkd1 (33)	2.77±0.07*,†,§	3.63±0.09*,†,§	1.31±0.02*,†,§	7.20±0.32*,†,§
axr1-3 (31)	3.83±0.11*	3.99±0.12*	1.05±0.02*	11.89±0.64*
axr1-3 fkd1 (31)	4.15±0.11*,†,‡	4.54±0.11*,†,‡	1.10±0.01†,‡	14.29±0.74*,†,‡
axr2 (27)	3.89±0.10*	3.65±0.10*	0.94±0.01*	11.16±0.58*
axr2 fkd1 (27)	4.10±0.13*,†	3.78±0.13*,†	0.92±0.01*,†	12.29±0.78*,†
pin1-1 (15)	5.33±0.38*	5.72±0.48*	1.07±0.04	24.20±3.57*
pin1-1 fkd1 (15)	5.44±0.43	6.42±0.42†	1.24±0.09	28.13±2.81†
aux1-7 (38)	6.40±0.16*	7.91±0.21*	1.24±0.02*	38.54±1.64*
aux1-7 fkd1 (37)	6.93±0.15*,†,‡	7.94±0.16*	1.15±0.02†,‡	42.72±1.62*,†

Number in brackets represents number of organs scored. Values represent mean±s.e.m.
*Significantly different from wild type.
†The double mutant is significantly different from *fkd1*.
‡The double mutant is significantly different from its corresponding single auxin mutant.
§*fkd2 fkd1* is significantly different from *fkd2*.

joining with the midvein is slightly delayed relative to wild type (Table 3). Initiation of proximal secondary veins and subsequent secondary and tertiary veins occurs at a point distant from the previously formed distal secondary veins (Fig. 4G,H), but further development of all veins is normal (Fig. 4G,H).

Plant morphology

Given the crucial function of the vascular system, we expected that the loss of the reticulate venation pattern might result in a decreased growth rate or photosynthetic capacity. All characters analyzed were indistinguishable between wild type and *fkd1*. In contrast, *fkd2* plants produced fewer rosette leaves, flowered later, and produced less seed (Table 4). The similar photosynthetic capacities and growth rate in *fkd1* and wild-type plants suggests either that the altered vascular pattern has no effect or that the *fkd1* plants are compensating for their non-meeting venation by altering another component of the transpiration mechanism. Leaf carbon isotope composition (expressed using δ notation with units of parts per thousand [$\delta^{13}C$,‰]) provides information about the ratio of photosynthetic capacity to stomatal conductance: higher $\delta^{13}C$ values (less negative) mean a lower stomatal conductance in relation to photosynthetic capacity (Farquhar et al., 1989). The significantly lower $\delta^{13}C$ in *fkd1* than in wild type suggests that the *fkd1* vascular pattern provides less efficient water delivery that is compensated by increased stomatal conductance.

To assess any effect altered vascular pattern might have on leaf shape, we compared shape and size of *fkd1* and *fkd2*

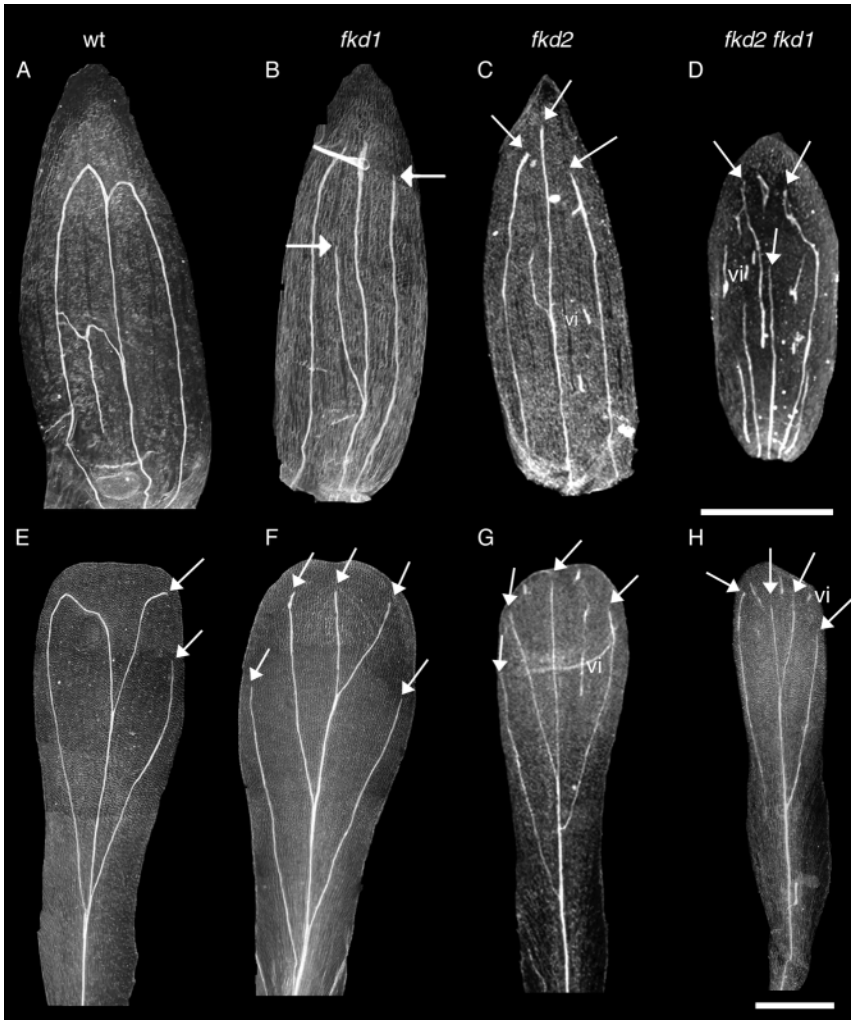

Fig. 5. Vascular pattern of sepals (A-D) and petals (E-H) from wild-type (A,E), *fkd1* (B,F), *fkd2* (C,G) and *fkd2 fkd1* (D,H) plants. Arrows indicate freely ending veins and vi indicates vascular islands. Scale bars: 500 μm.

cotyledons and leaves to wild type (Table 5). *fkd2* cotyledons are elongated compared to wild type and are somewhat smaller, while *fkd1* cotyledons are slightly larger. First leaves of both *fkd1* and *fkd2* are elongated relative to wild type. The increased length suggests that distal vein connections in wild type may constrain cellular expansion during leaf development, a constraint lacking in *fkd1* and *fkd2* leaves.

Since leaves are considered the progenitors of floral organs, one might expect a similar loss of the wild-type reticulate venation pattern to occur in *fkd1* and *fkd2* floral organs. In *fkd1* and *fkd2* sepals, distal meeting is reduced and VIs are increased compared to wild type (Fig. 5, Table 6), while in petals, distal meeting is reduced.

Vascular pattern in *fkd-2 fkd-1* double mutants

To determine if *FKD1* and FKD2 act in the same or different pathways, we generated plants doubly mutant for *fkd1* and *fkd2*. First leaves of the F_2 progeny of the *fkd1* × *fkd2* cross were scored as wild type if their leaves showed no distal non-meeting, as *fkd1* if they showed distal non-meeting and VIs concentrated to the proximal portion of the leaf and as *fkd2* if they showed distal non-meeting and VIs throughout the leaf lamina. We identified plants with a vascular phenotype more extreme than either single mutant (Fig. 2D), whose frequency was consistent with the plants being doubly homozygous for both *fkd1* and *fkd2* (Table 1). Relative to either single mutant, cotyledons of the double mutant were considerably smaller (Table 5) and had a simplified vascular pattern with more VIs. As in both single mutants, distal meeting of secondary veins was rarely seen. These trends result in a significant decrease in both the number of areoles and branch points relative to the single mutants (Table 2). The vascular pattern of double mutant first leaves consisted almost entirely of VIs, resulting in fewer areoles and more free ends than either single mutant (Table 2). Moreover, leaves were smaller and more elongated (Table 5). The increased severity of the venation phenotype was also seen in floral organs (Fig. 5D,E), with reduced distal meeting and increased frequency of VIs (Table 6). The increased severity in double mutant phenotype compared to either single mutant suggests that *FKD1* and *FKD2* have overlapping and partially redundant functions.

Effect of exogenous auxin on *Fkd1* roots

One explanation for the *fkd1* phenotype is that the mutation affects a component of auxin canalization and compromises distal vein meeting. If correct, one might expect that *fkd1* plants would show altered sensitivity to changes in auxin levels, whether introduced exogenously or through double mutant combinations. Since altered sensitivity of root growth to 2,4-D indicates altered auxin response or transport (Wilson et al., 1990), we compared root growth of *fkd1* to that of wild type at four 2,4-D concentrations. No significant difference was observed at any concentration (data not shown), suggesting that *fkd1* plants do not show a global change in auxin sensitivity.

While the auxin root assay effectively detects defects in the general auxin pathway, it may not detect defects specific to leaf vascular pattern formation. We therefore grew plants on auxin transport inhibitors and assessed alterations in leaf vascular pattern. As well, we generated double mutants between *fkd1* and various auxin mutants known to have alterations in leaf morphology and/or leaf vascular patterning.

Effect of an auxin transport Inhibitor on *Fkd1* leaf vascular patterning

The proliferation of veins adjacent to the margin in wild-type leaves treated with auxin transport inhibitors suggests that the leaf margin is a major source of auxin within the developing leaf (Mattsson et al., 1999; Sieburth, 1999). To assess whether, in the presence of an auxin transport inhibitor, *fkd1* alters the

Table 6. Vascular pattern of mature sepals and petals in wild-type, *fkd1*, *fkd2* and *fkd2 fkd1* flowers

	Wild type	*fkd1*	*fkd2*	*fkd2 fkd1*
Sepals, no. of distal joints	1.75±0.51 (120)	0.09±0.32*,‡ (103)	0.50±0.64*,† (118)	0.00±0.00*,†,‡ (120)
Sepals, no. of VIs	0.00±0.00	0.44±0.76*,‡	1.80±1.15*,†	5.00±1.89*,†, ‡
Petals, no. of distal joints	0.62±0.84 (112)	0.00±0.00* (99)	0.00±0.13* (118)	0.00±0.00* (120)
Petals, no. of VIs	0.05±0.32	0.04±0.00‡	0.25±0.49*,†	2.78±1.26*,†,‡

Number in brackets represents number of organs scored. Values represent means ± s.e.m.
*Significantly different from wild type.
†Significantly different from *fkd1*.
‡Significantly different from *fkd2*.

ability of marginal veins to meet, we grew *fkd1* and wild-type seedlings on 30 μM NPA. Mature (14 DAG) *fkd1* cotyledons had the same vascular pattern when grown with or without NPA (data not shown). Extensive marginal venation at the distal leaf tip was evident in both wild-type and *fkd1* leaves by 5 DAG, and vascular strands began to develop basipetally from the marginal region to the proximal leaf blade at 7 DAG. However, by 8 DAG, the interior of the wild-type leaf blade showed extensive vein branching, whereas in *fkd1* no branching was evident in the leaf interior. The marginal regions of *fkd1* and wild-type 21 DAG leaves were indistinguishable, however, significantly fewer secondary veins extended from the marginal region to the proximal leaf blade in *fkd1* (14.74±0.88) than in wild type (20.5±0.78) and tertiary veins were rare (Fig. 2G,H). Furthermore, 20% (n=23) of *fkd1* leaves had secondary veins that did not connect proximally, whereas all veins in all wild type leaves were connected (n=25). The ability of veins to meet distally in marginal areas of *fkd1* leaves where auxin is increased owing to reduced transport suggests that *FKD1* either acts in response to auxin to direct vascular tissue or is necessary for the auxin response that directs vascular tissue.

Effect of auxin mutants on the *fkd1* phenotype

pin1-1 fkd1

Like chemical inhibition of auxin transport, the loss-of-function allele, *pin1-1*, in the auxin efflux carrier results in a proliferation of marginal venation (Mattsson et al., 1999; Galweiler et al., 1998). *pin1-1* cotyledons and leaves are similar to wild type in size and shape, although the cotyledons are slightly rounder, the leaves are slightly smaller, and are sometimes fused (Fig. 2E and Table 5). The venation pattern of the cotyledons shows no difference from wild type while the leaf pattern is quite distinct, being simpler (fewer areoles and branch points) and having fewer freely ending veins (Table 2).

pin1-1 fkd1 cotyledons show no significant difference from *pin1-1* cotyledons for any of the characters measured (Table 2). Relative to *pin1-1*, the first leaves of *pin1-1 fkd1* have more freely ending veins and fewer areoles, consistent with the increased distal non-meeting of *fkd1*. Relative to *fkd1*, they show a decrease in freely ending veins, consistent with the increased vein meeting of *pin1-1*. Specifically, the increased meeting in double mutants occurs along the margin while the distal non-meeting occurs in the leaf blade (Fig. 2F). Therefore, like treatment of *fkd1* leaves with NPA, loss of *PIN1* compensates for lack of *FKD1* at the leaf margin, but not in the internal regions of the leaf.

axr1-3 fkd1; *axr2 fkd1*; *aux1-7 fkd1*

Plants mutant for any of *AXR1*, *AXR2* or *AUX1* are auxin resistant, with various defects in morphology. *AXR1* acts in an ubiquitin-like pathway that responds to the presence of auxin by targeting proteins for degradation (del Pozo and Estelle, 1999; Gray and Estelle, 2000), *AXR2* belongs to the IAA family of genes that are inducible by auxin (Nagpal et al., 2000) and *AUX1* encodes a membrane protein that is believed to be a component of the auxin influx carrier (Bennett et al., 1996; Marchant et al., 1999; Swarup et al., 2000). We found that both *axr1-3* and *axr2* cotyledons and leaves are smaller than wild type (Table 5) with a simpler vascular pattern (Table 2, Fig. 2M) that in *axr1-3* is combined with frequent proximal non-meeting (Table 2). In contrast, *aux1-7* cotyledons and leaves are significantly larger but maintain the wild-type vascular pattern, and *aux1-7* first leaves are more elongate (Table 5).

In double mutant combinations between these auxin resistant mutants and *fkd1*, both leaf morphology and leaf vascular patterning show essentially additive phenotypes (Tables 2, 5). Relative to the respective single mutants, cotyledons and first leaves of *axr1-3 fkd1*, *axr2 fkd1* and *aux1-7 fkd1* have significantly fewer branch points and areoles, consistent with the double mutant phenotypes combining the distal non-meeting of *fkd1* with the simplified vascular pattern of the auxin mutants (Table 2 and Fig. 2N).

mp fkd1 and *axr6-2 fkd1*

Plants with loss-of-function *MP* alleles or gain-of function *AXR6* alleles do not produce the basal embryonic structures, hypocotyl and root, but produce normal apical embryonic structures, including shoot apical meristem and cotyledons (Berleth and Jurgens, 1993; Przemeck et al., 1996; Hobbie et al., 2000). *AXR6* mutants rarely form a small number of rosette leaves; *mp* seedlings do not normally form leaves, but seedlings carrying weak *MP* alleles, such as mp^{G92}, can be induced by wounding to form roots, and the rooted seedlings go on to form leaves and inflorescences (Przemeck et al., 1996). Within these structures, defects occur in the leaf venation, including simplification of pattern and discontinuities in vascular strands. We found that mp^{G92} and *axr6-2* cotyledons have few distal secondaries arising from the midvein, and that these do not connect proximally with the midvein (Fig. 1I).

Many of the shoot defects seen in mp^{G92} and *axr6-2* plants are suppressed in the mp^{G92} *fkd1* or *axr6-2 fkd1* double mutants, while the morphology and development of double mutant basal structures is indistinguishable from mp^{G92} or *axr6-2* respectively. The vascular pattern of mp^{G92} *fkd1*

cotyledons is simpler than that of *mp*^{G92}, while the vascular pattern of *axr6-2 fkd1* cotyledons is slightly more extensive than *axr6-2* (Fig. 1J,L, Table 2) with increased frequency of VIs.

The remainder of the double mutant shoot structure diverges markedly from that of *mp*^{G92} or *axr6-2*: while *mp*^{G92} or *axr6-2* plants grown under the same conditions only rarely (*mp*^{G92} – 16.2%, *n*=37; *axr6-2* – 10%, *n*=19) develop a very small first leaf with highly reduced venation (Fig. 2I,K), 64.8% (*n*=54) of *mp*^{G92} *fkd1* double mutants and 39% (*n*=33) of *axr6-2 fkd1* double mutants show one to four small but well developed leaves, with extensive venation (Fig. 2J,L). Because *mp*^{G92} *fkd1* and *axr6-2 fkd1* double mutants rarely survived to 21 DAG, at which point we consider first leaves to be fully expanded, we could not make a quantitative comparison of their vascular pattern to either wild type or *fkd1*. However, examination of 20 *mp*^{G92} *fkd1* and 12 *axr6-2 fkd1* first leaves indicate that they show a simplified vascular pattern with reduced numbers of tertiary and quaternary veins (Fig. 2J,L), and that, as in *fkd1* leaves, the distal ends of veins often do not meet. Moreover, if *mp*^{G92} *fkd1* plants survive on medium for 21 days, 70% (*n*=10) form inflorescences, usually consisting of a single terminal flower, often reduced to a pistil.

Auxin response in *fkd1* mutants

To test the hypothesis that the *fkd1* phenotype is the result of reduced ability to respond to auxin, we introduced the synthetic AuxRE reporter gene construct (*DR5::GUS*) into *fkd1* plants. The *DR5::GUS* line contains a composite promoter with seven tandem repeats of the AuxRE TGTCTC fused to a minimal cauliflower mosaic virus 35S promoter-GUS reporter gene construct (Ulmasov et al., 1997). Initial reporter gene expression in the subapical cells of the 3 DAG *fkd1* leaves was indistinguishable from wild type (not shown). However, reporter gene expression in *fkd1* cells destined to be secondary veins was reduced in both intensity and duration relative to wild type (Fig. 6). Moreover, whereas in wild type, reporter gene expression at the distal junctions of secondary veins was somewhat reduced or delayed relative to the rest of the vein (see arrows, Fig. 6A,C) (Mattsson et al., 2003), in *fkd1*, such cells never expressed the reporter gene. In support of the hypothesis that auxin transport inhibition alleviates the *fkd1* phenotype by increasing auxin levels and allowing increased auxin response, both wild-type and *fkd1* leaves treated with NPA showed a broad band of intense reporter gene expression (Fig. 6I,J) that preceded the formation of vascular tissue (Fig. 6K,L).

Discussion

We have identified two mutants of *Arabidopsis*, *forked1* (*fkd1*) and *forked2*, that form an open vascular pattern in foliar organs because newly initiating veins do not join with previously formed veins. Owing to their recessive nature, we will assume both alleles represent loss of function of the respective genes *FORKED1* (*FKD1*) and *FORKED2* (*FKD2*). While both genes are necessary at distal vein junctions, they act redundantly throughout the leaf veins. Auxin responsive reporter gene expression (*DR5::GUS*) is reduced in *fkd1* plants. *DR5::GUS* expression and vein junctions are restored in areas of *fkd1* leaves where auxin concentrations are increased. We therefore propose that *FKD1* and *FKD2* are necessary for the auxin response that directs vascular development, and that distal vein junctions, being sites of low auxin concentration, are particularly sensitive to their reduced function.

FKD1 directs vascular differentiation in response to an auxin threshold

Several models for the role of *FKD1* in vascular pattern formation are consistent with the *fkd1* phenotype: (1) *FKD1* is a component of the auxin synthesis or transport pathway that controls distribution of auxin; (2) *FKD1* is necessary for differentiation of vascular tissue in response to auxin; (3) *FKD1* is necessary for the auxin response that results in vascular differentiation; or (4) *FKD1* acts independently of, but in conjunction with, auxin canalization to induce vascular tissue in areas where auxin alone is insufficient. To distinguish amongst the possibilities, we assessed auxin responsive reporter gene expression (*DR5::GUS*) in *fkd1*, the response of *fkd1* to synthetic auxin and

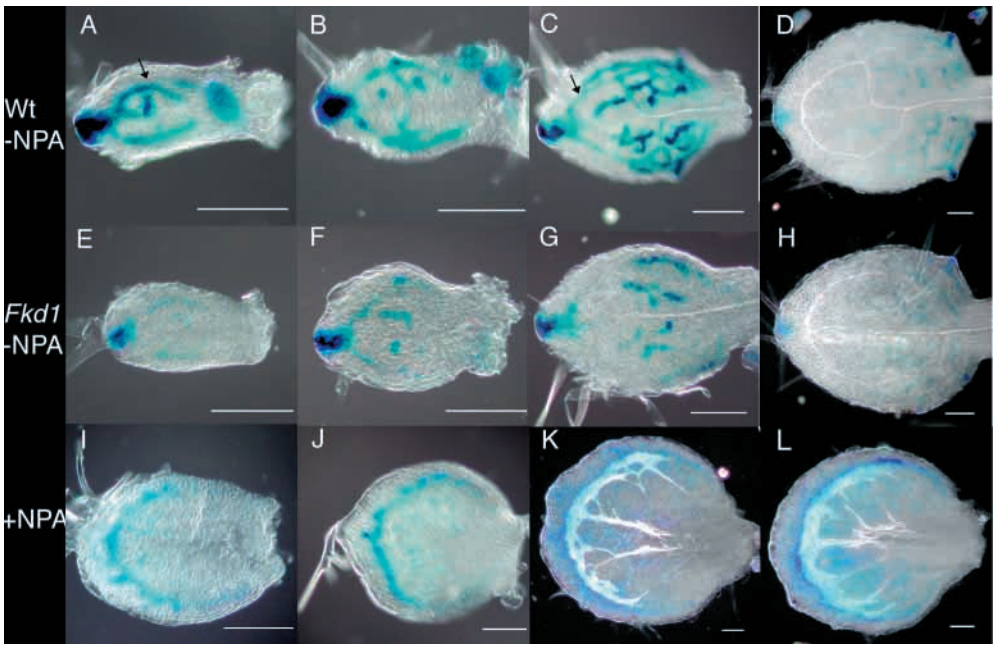

Fig. 6. Pattern of *DR5::GUS* expression in developing leaves of wild type (A-D,I,K) and *fkd1* (E-F,J,L) upon no exposure (A-H) or exposure (I-L) to auxin transport inhibition. Leaves were taken at 4 DAG (A,B,E,F), 5 DAG (C,G,I,J) and 6 DAG (D,H,K,L). Arrows indicate reduced reporter gene expression at distal joints of wild-type secondary veins. Scale bar: 100 μm.

auxin transport inhibitors and the response of *fkd1* to mutant backgrounds that affect auxin response or transport.

Our data provide the most support for Model 3. The expression of *DR5::GUS* is reduced in duration and extent throughout *fkd1* leaves, suggesting that *FKD1* is required for the auxin response. The reduced auxin response is sufficient to direct vascular development throughout most of the leaf, although it results in a slight developmental delay. At distal junctions, the level of auxin is sufficiently low that no auxin response occurs in the absence of *FKD1* and distal junctions do not form. When *fkd1* seedlings are grown on medium containing an auxin transport inhibitor or when double mutants are made between *fkd1* and the auxin transport mutant *pin1-1*, distal meeting of marginal veins in either cotyledons or leaves is more similar to wild type. Moreover, when auxin transport is inhibited, *DR5::GUS* expression in *fkd1* leaves is indistinguishable to that in wild type. Thus, the increased auxin at the margin following auxin transport inhibition reestablishes auxin response even in the absence of *FKD1* function. Our data suggest that *FKD1* activity is restricted to the leaves and is not present globally; however, the additive phenotypes of double mutants between *fkd1* and the auxin response mutants *axr1-3*, *axr2* and *aux1-7*, as well as the root response of *fkd1* plants grown in 2,4-D, may be the result of redundant activities in other tissues.

The hypothesis that *FKD1* is necessary for the auxin response is further supported by the effect of *fkd1* on both *mp*^{G92} and *axr6-2* phenotypes. Plants homozygous for *mp*^{G92} or *axr6*-2 show a similar, seedling lethal phenotype, forming no root, an abnormal, stubby hypocotyl, cotyledons with reduced venation and rarely a first leaf. Double mutants with *fkd1* show no change in the root or hypocotyl phenotype, but like *mp sfc* double mutants, *mp*^{G92} *fkd1* show enhanced vascular defects in the cotyledons. More surprisingly, *axr6-2 fkd1* or *mp*^{G92} *fkd1* double mutants exhibit suppression of the *mp*^{G92} or *axr6-2* leaf phenotypes, producing several well-expanded leaves and in the case of *mp*^{G92} *fkd1*, an inflorescence. *MP* encodes the auxin response protein ARF5 (Hardtke and Berleth, 1998), while *AXR6* encodes the protein cullin, a subunit of the ubiquitin ligase complex SCF (Hobbie et al., 2002), The similarities in *mp*, *axr6* and *bdl* phenotypes suggest that AXR6 may degrade IAA12 (product of *BDL)* and allow activity of ARF5. The enhancement of the *mp*^{G92} cotyledon phenotype by *fkd1* suggests that in cotyledons, *FKD1* acts redundantly with *MP*. While it is difficult to find a simple explanation for the suppression of *mp*^{G92} and *axr6-2* leaf phenotypes in a *fkd1* background, the most likely interpretation is that, like *MP* and *AXR6*, *FKD1* is involved in the auxin response. While it is tempting to speculate that FKD1, MP and AXR6 act as a complex required for auxin response, the function of which is restored if pairs of genes are mutated, the proposed activities of *MP* and *AXR6* products and the fact that *mp*^{G92} introduces a nonsense codon indicate that this is unlikely. A second possibility is that the suppression is due to indirect changes in auxin distribution caused by altered source/sink relationships resulting from *mp*^{G92} and *axr6-2* morphological defects. In wild-type seedlings, the initial source of auxin is probably the cotyledons (Ljung et al., 2001; Bhalerao et al., 2002; Marchant et al., 2002), while the region below the quiescent centre (QC) acts as a sink for auxin (Sabatini et al., 1999) that controls a gradient of auxin distribution (Casimiro et al., 2001; Marchant et al., 2002) and auxin-dependent patterning (Friml et al., 2002). Disrupting either auxin source or sink alters auxin distribution. In *stm1* seedlings, disrupting the auxin source alters auxin gradients within the root (Casimiro et al., 2001). One might predict a corresponding alteration in seedlings lacking an auxin sink, such as the rootless *mp*^{G92} and *axr6-2* seedlings. The lack of a sink could cause higher than normal auxin gradients within the shoot that would disrupt formation of vascular tissue and leaf development. Such a disruption would provide a possible explanation for the suppression of the *mp*^{G92} and *axr6-2* phenotypes in a *fkd1* background: while auxin levels are too high to allow the function of wild-type *FKD1*, they allow the product of *fkd1* to function and enable differentiation of vascular tissue in developing leaves.

Our analysis suggests that *FKD1* is necessary to induce an auxin response at low auxin levels and enable differentiation of vascular tissue. The product of *fkd1* cannot initiate a response to low auxin concentrations but can to higher levels as demonstrated by increased marginal vein meeting and *DR5::GUS* expression when auxin transport from the leaf margin is prevented. In contrast, one explanation consistent with the defective venation in *mp*^{G92} and *axr6-2* plants and suppression of this defect in a *fkd1* background is that the product of *FKD1* cannot function if auxin concentrations are too high.

Vascular pattern is driven by sequential dynamic auxin sources

If *FKD1* is necessary for responses to auxin concentrations below a particular threshold and direct vascular cell differentiation, then the *fkd1* phenotype may represent a leaf in which only higher auxin concentrations are driving vascular formation and provide insight into how auxin is distributed within the developing leaf. Based on mutant phenotypes, we suggest that three different sources of auxin are sequentially required for vascular pattern development within the leaf: (1) acropetally directed auxin directs midvein development; (2) a dynamic marginal source directs secondary vein development; (3) internal auxin sources direct tertiary and quaternary vein development.

Auxin from the first source is transported acropetally into the meristem and young leaf primordia (Reinhardt et al., 2000; Avsian-Kretchmer et al., 2002) where it directs differentiation of the midvein. The control of midvein differentiation by a different mechanism to that of subsequent veins is supported by its unique, acropetal developmental pattern and by the observation that all vascular pattern mutants described thus far affect subsequent veins but do not affect midvein development (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). Moreover, exposure to auxin transport inhibitors eliminates acropetal midvein development (Mattsson et al., 1999; Seiburth, 1999; Avsian-Kretchmer et al., 2002).

The second source arises at the margin, beginning at the distal tip and proceeding proximally with marginal cell differentiation, and directs differentiation of secondary veins. This idea is supported by this study and a number of previous studies. If auxin transport from the marginal source is inhibited, auxin accumulates at the distal leaf tip (Avsian-Kretchmer et al., 2002) and vascular development begins with a band of vascular tissue parallel to the margin of the leaf near

the distal tip which lengthens along the margin as the leaf develops (Mattsson et al., 1999; Sieburth, 1999). Moreover, high levels of auxin have been found to move basipetally during leaf development (Avsian-Kretchmer et al., 2002), coincident with highest rate of cell division and onset of vascular differentiation (Ljung et al., 2001). The sequential development of distally unconnected secondary vascular bundles originating at the margin of *fkd1* leaves also supports a dynamic auxin source. Moreover, the *fkd1* phenotype combined with reduced *DR5:GUS* expression at distal junctions in wild-type leaves suggest that auxin depletion of neighbouring cells by secondary veins results in low auxin concentrations at the point of distal vein connection. Interestingly, despite changes to marginal venation in *pin1*, *fkd1* and *fkd2* mutants, marginal vascular tissue always forms at a point distant from the margin, suggesting (1) that a maximum is reached at a point distant from the margin and/or (2) that cells at this point have prior competence for the vascular fate.

The third source of auxin is within the leaf blade, and directs formation of tertiary and quaternary veins (Aloni, 2001; Aloni et al., 2003). In *fkd1* and *fkd2* leaves the marginal auxin source alone is sufficient to explain the generation of these veins, since the open venation pattern would allow auxin from the margin to reach the interior leaf blade. However, the wild-type leaf blade interior is separated from the marginal auxin source by a continuous loop of vascular tissue, prompting the proposal that an internal source of auxin directs formation of tertiary and quaternary veins (Aloni, 2001; Aloni et al., 2003). The reduction in these veins in both *axr1-3* and *axr2* suggests that the internal source of auxin is lower than the marginal source so that the partial loss-of-function gene products can respond to the marginal source but not the internal source.

FKD1 acts redundantly with *FKD2* to allow vascular cell formation

We have identified a second gene, *FKD2*, whose mutant phenotype is very similar to *fkd1*, being distinguished only by a higher frequency of VIs in the distal portion of the leaf blade. *FKD2* maps very close to a previously characterized gene, *SFC* (Deyholos et al., 2000). Plants carrying mutations in *SFC* show severely disrupted vascular pattern with a very high frequency of VIs, and correspondingly severe defects in morphology, a phenotype consistent with *fkd2* being a weak allele of *SFC*. Furthermore, double mutants between *fkd1* and *fkd2* exhibit a phenotype that is very similar to that reported for *SFC* mutants. The double mutant phenotype suggests that both *FKD1* and *FKD2* are essential for distal vein meeting, presumably because auxin concentrations are extremely low in these areas. Within other regions of developing veins where auxin levels are higher, the two genes act redundantly such that partial loss of function of either gene is insufficient to alter vein morphology, but partial loss of function of both genes results in severe disruption to the vascular integrity. We suggest that both *FKD1* and *FKD2* are required to respond to auxin and allow vascular differentiation. In the partial absence of one gene product, only the lowest thresholds are not recognized, disrupting vascular differentiation at vein junctions. In the absence of both gene products, response to a range of thresholds is faulty, and vascular discontinuities occur throughout the leaf veins.

FKD1 and *FKD2* may provide an evolutionary link between open and closed venation patterns

If the *fkd1* and *fkd2* leaf pattern, which is similar to that of lower vascular plants, represents a leaf in which only high levels of auxin are able to initiate vascular differentiation, it is plausible that the vascular pattern seen in lower plants results from auxin canalization but that these plants have decreased sensitivity to auxin thresholds. Sztein et al. (Sztein et al., 1995) have shown that primitive vascular plants have only simple IAA conjugates and the increasing conjugate complexity occurring in higher vascular plants is associated with increasingly complex vascular tissue. It has been well established that leaves of conifers, *Gingko* and ferns are sources of auxin and that this auxin is at least partly responsible for the vascular pattern found within the stem of these plants (Gunckel and Wetmore, 1946; Steeves and Sussex, 1989; Wang et al., 1997). Additionally, lycopod leaves have been shown to have an effect upon the stem vasculature but it has not been conclusively demonstrated that auxin is responsible for this phenomenon (Freeberg and Wetmore, 1967). In conifers, it has also been shown that a concentration gradient of auxin is required for proper differentiation and patterning of xylem and phloem from the cambium (Nix and Wodzicki, 1974; Uggla et al., 1996). Given that auxin in primitive plants has a similar, if not identical, role to that in more advanced plants, it is likely that an auxin canalization mechanism is responsible for leaf vascular patterning in these groups, but that higher plants have evolved mechanisms to respond to a wider range of auxin thresholds. We suggest that *FKD1* and *FKD2* enable distal meeting of the vascular bundles in areas of low auxin concentration to form a complete, closed vascular pattern.

We thank members of the Flanagan lab for performing isotopic analysis, Beverly Burton, Larry Flanagan, Pat Kubik and James Meservy for technical assistance and John Bain, Beverly Burton, Jasmine Garrett, George Haughn and James Meservy for critical reading of the manuscript. This work was funded by an Operating Grant (E.S.) and a Postgraduate Scholarship (Q.S.) both from the Natural Science and Engineering Research Council.

References

Aloni, R. (2001). Foliar and axial aspects of vascular differentiation: Hypotheses and evidence. *J. Plant Growth Reg.* **20**, 22-34.

Aloni, R., Schwalm, K., Langhans, M. and Ullrich, C. I. (2003). Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in *Arabidopsis*. *Planta* **216**, 841-853.

Avsian-Kretchmer, O., Cheng, J.-C., Chen, L., Moctezuma, E. and Sung, Z. R. (2002). Indole acetic acid distribution coincides with vascular differentiation pattern during Arabidopsis leaf ontogeny. *Plant Physiol.* **130**, 199-209.

Beerling, D. J., Osborne, C. P. and Chaloner, W. G. (2001). Evolution of leaf-form in land plants linked to atmospheric CO_2 decline in the Late Palaeozoic era. *Nature* **410**, 352-354.

Bell, C. J. and Ecker, J. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. *Genomics* **19**, 137-144.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P. and Offrings, R. (2001). The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. *Development* **128**, 4057-4067.

Bennett, M. J., Marchant, A., Green, H. G., May, S. T., Ward, S. P., Millner, P. A., Walker, A. R., Schultz, B., and Feldman, K. A. (1996). Arabidopsis AUX1 gene: A permease-like regulator of rooth gravitropism. *Science* **273**, 948-950.

Berleth, T. and Jurgens, G. (1993). The role of the *monopteros* gene in

organising the basal body region of the *Arabidopsis* embryo. *Development* **118**, 575-587.

Berleth, T. and Sachs, T. (2001). Plant morphogenesis: long distance coordination and local patterning. *Curr. Opin. Plant Biol.* **4**, 57-62.

Bhalerao, R. P., Eklof, J., Ljung, K., Marchant, A., Bennett, M. and Sandberg, G. (2002). Shoot derived auxin is essential for early lateral root emergence in *Arabidopsis* seedlings. *Plant J.* **29**, 325-332.

Carland, F. M., Berg, B. L., FitzGerald, J. N., Jinamornphongs, S., Nelson, T. and Keith, B. (1999). Genetic regulation of vascular patterning in Arabidopsis. *The Plant Cell* **11**, 2123-2137.

Carland, F. M. and McHale, N. A. (1996). *LOP1*: a gene involved in auxin transport and vascular patterning in *Arabidopsis*. *Development* **122**, 1811-1819.

Casimiro, I., Marchant, A., Bhalerao, R. P., Beeckman, T., Dhooge, S., Swarup, R., Graham, N., Inze, D., Sandberg, G., Casero, P. J. and Bennett, M. (2001). Auxin transport promotes lateral root initiation. *Plant Cell* **13**, 843-852.

Christensen, S. K., Dagenais, N., Chory, J. and Weigel, D. (2000). Regulation of auxin response by the protein kinase PINOID. *Cell* **100**, 469-478.

Church, D. L. (1993). Tracheary element differentiation in *Zinnia* mesophyll cell cultures. *Plant Growth Reg.* **12**, 179-188.

del Pozo, C. J. and Estelle, M. (1999). Function of the ubiquitin-proteosome pathway in auxin response. *Trends Plant Sci.* **4**, 107-112.

Deyholos, M. K., Cordner, G., Beebe, D. and Sieburth, L. E. (2000). The *SCARFACE* gene is required for cotyledon and leaf vein patterning. *Development* **127**, 3205-3213.

Edwards, K., Johnstone, C. and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **19**,1349.

Farquhar, G. D., Ehleringer, J. R. and Hubick, K. T. (1989). Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 503-537.

Freeberg, J. A. and Wetmore, R. H. (1967). The Lycopsida – A study in development. *Phytomorphology* **17**, 78-91.

Friml, J., Benkova, E., Bililou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jurgens, G. and Palme, K. (2002). AtPIN4 mediates sink-driven auxin gradients and root patterning in *Arabidopsis*. *Cell* **108**, 661-673.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998). Regulation of polar auxin transport by *AtPIN1* in *Arabidopsis* vascular tissue. *Science* **282**, 2226-2230.

Gifford, E. M. and Foster, A. S. (1989). *Morphology and Evolution of Vascular Plants.* 3rd ed. New York: W. H. Freeman and Company.

Goto, N., Katoh, N. and Kranz, A. R. (1991). Morphogenesis of floral organs in *Arabidopsis*: predominant carpel formation of the *pin-formed* mutant. *Japanese J. Genetics* **66**, 551-567.

Gray, W. M. and Estelle, M. (2000). Function of the ubiquitin-proteosome pathway in auxin response. *Trends Biochem. Sci.* **25**, 133-138.

Gunckel, J. E. and Wetmore, R. H. (1946). Studies of development in long and short shoots of *Gingko biloba* L. II. Phyllotaxis and the organization of the primary vascular system; primary phloem and primary xylem. *Am. J. Bot.* **33**, 532-543.

Hamann, T., Benkova, E., Bäurle, I., Kientz, M. and Jürgens, G. (2002). The *Arabidopsis BODENLOS* gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes Dev.* **16**, 1610-1615.

Hamann, T., Mayer, U. and Jürgens, G. (1999). The auxin insensitive *bodenlos* mutation affects primary root formation and apical basal patterning in the *Arabidopsis* embryo. *Development* **126**, 1387-1395.

Hardtke, C. S. and Berleth, T. (1998). The *Arabidopsis* gene *MONOPTEROS* encodes a transcription factor mediating embryo axis formation and vascular development. *EMBO J.* **17**, 1405-1411.

Hobbie, L., McGovern, M., Hurwitz, L. R., Pierro, A., Liu, N. Y., Bandyopadhyay, A. and Estelle, M. (2000). The *axr6* mutants of *Arabidopsis thaliana* define a gene involved in auxin response and early development. *Development* **127**, 23-32.

Hobbie, L., Sherman, S., Adelphi Arabidopsis Mapping Team, Hellmann, H. and Estelle, M. (2002). Molecular characterization of the *AUXIN-RESISTANT6* gene. *13th International Conference on Arabidopsis Research, Seville, Spain.* **Abstract 4-75**.

Kang, J. and Dengler, N. (2002). Cell cycling freqency and expression of the homeobox gene ATHB-8 during leaf vein development in *Arabidopsis*. *Planta* **216**, 212-219.

Koizumi, K, Sugiyama, M. and Hiroo, F. (2000). A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. *Development* **127**, 3197-3204.

Ljung, K., Bhalerao, R. P. and Sandberg, G. (2001). Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant J.* **28**, 465-474.

Lomax, T. L., Muday, G. K. and Rubery, P. H. (1995). Auxin transport. In *Plant Hormones: Physiology, Biochemistry, and Molecular Biology*, 2nd edition (ed. P. J. Davies), pp. 509-530. The Netherlands: Kluwer Academic Publishers.

Marchant, A., Kargul, J., May, S. T., Muller, P., Delbarre, A., Perrot-Rechenmann, C. and Bennett, M. (1999). AUX1 regulates root gravistopism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO J.* **18**, 2066-2073.

Marchant, A., Bhalerao, R., Casimiro, I., Eklof, J., Casero, P. J., Bennett, M. and Sandberg, G. (2002). *AUX1* promotes lateral root formation by facilitating Indole-3-acetic acid distribution between sink and source tissues in the Arabidopsis seedling. *Plant Cell* **14**, 589-597.

Mattsson, J., Sung, Z. R. and Berleth, T. (1999). Responses of plant vascular systems to auxin transport inhibtion. *Development* **126**, 2979-2991.

Mattsson, J., Ckurshumova, W. and Berleth, T. (2003). Auxin signaling in Arabidopsis leaf vascular development. *Plant Physiol.* **131**, 1327-1329.

Müller, A., Guan, C., Gälweiler, L., Tänzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E. and Palme, K. (1998). *Atpin2* defines a locus of *Aribidopsis* for root gravitropism control. *EMBO J.* **17**, 6903-6911.

Nagpal, P., Walker, L. M., Young, J. C., Sonawala, A., Timple, C., Estelle, M. and Reed, J. W. (2000). *AXR2* encodes a member of the Aux/IAA protein family. *Plant Physiol.* **123**, 563-573.

Nix, L. E. and Wodzicki, T. J. (1974). The radial distribution of IAA-^{14}C in *Pinus echinata* stems in relation to wood formation. *Can. J. Bot.* **52**, 1349-1355.

Okada, K., Ueada, J., Komaki, M. K., Bell, C. J. and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* **3**, 677-684.

Przemeck, G. K. H., Mattsson, J., Hardtke, C. S., Sung, Z. R. and Berleth, T. (1996). Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta* **200**, 229-237.

Reinhardt, D., Mandel, T. and Kuhlemeier, C. (2000). Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* **12**, 507-518.

Roth-Nebelsick, A., Uhl, D., Mosbrugger, V. and Kerp, H. (2001). Evolution and function of leaf architecture: A review. *Annals Bot.* **87**, 553-566.

Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser, O., Bechtold, O., Weisbeek, P. and Scheres, B. (1999). An auxin-dependent distal organizer of pattern and polarity in the *Arabidopsis* root. *Cell* **99**, 463-472.

Sachs, T. (1981). The control of patterned differentiation of vascular tissues. *Adv. Bot. Res.* **9**, 151-262.

Sachs, T. (1989). The development of vascular networks during leaf development. *Curr. Topics Plant Biochem. Physiol.* **8**, 168-183.

Sachs, T. (1991). Cell polarity and tissue patterning in plants. *Development* **Supplement 1**, 83-93.

Sieburth, L. E. (1999). Auxin transport is required for leaf vein pattern in *Arabidopsis*. *Plant Physiol.* **121**, 1179-1190.

Steeves, T. A. and Sussex, I. M. (1989). *Patterns in Plant Development* 2nd edition. New York: Cambridge University Press.

Steinmann, T., Geldner, N., Grebe, M., Mangold, S., Jackson, C. L., Paris, S., Gälweiler, L., Palme, K. and Jürgens, G. (1999). Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* **286**, 316-318.

Swarup, R., Marchant, A. and Bennett, M. J. (2000). Auxin transport: Providing a sense of direction during plant development. *Biochem. Soc. Trans.* **28**, 481-485.

Sztein, A. E., Cohen, J. D., Slovin, J. P. and Cooke, T. J. (1995). Auxin metabolism in representative land plants. *Am. J. Bot.* **82**, 1514-1521.

Trivett, M. L. and Pigg, K. B. (1996). A survey of reticulate venation among fossil and living land pants. In *Flowering Plant Origin, Evolution and Phylogeny* (ed. D. W. Seldin and L. J. Hickey), pp. 8-31. New York: Chapman and Hall.

Uggla, C., Moritz, T., Sandberg, G. and Sandberg, B. (1996). Auxin as a positional signal in pattern formation in plants. *Proc. Natl. Acad. Sci. USA* **93**, 9282-9286.

Ulmasov, T., Murfett, J., Hagen, G. and Guilfoyle, T. J. (1997). Aux/IAA

proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**, 1963-1971.

Wagner, W. H., Jr, Reitel, J. M. and Wagner, F. S. (1982). Complex venation patterns in the leaves of *Selaginella*: megaphylls-like leaves in lycophytes. *Science* **218**, 793-794.

Wallroth-Marmonr, L. and Harte, C. (1988). IAA in the leaves of *Antirhinum majus* L. Sippe 50 and some mutants. *Biologisches Zentralbl.* **197**, 517-531.

Wang, Q., Little, C. H. A. and Oden, P. C. (1997). Control of longitudinal and cambial growth by gibberellins and indole-3-acetic acid in current year shoots of *Pinus sylvestris*. *Tree Physiol.* **17**, 715-721.

Wilson, A. K., Pickett, F. B., Turner, J. C. and Estelle, M. (1990). A dominant mutation in *Arabidopsis* confers resistance to auxin ethylene and abscisic acid. *Mol. Gen. Genet.* **222**, 377-383.

Zhong, R. and Ye, Z.-H. (2001). Alteration of auxin polar transport in the Arabidopsis *ifl1* mutants. *Plant Physiol.* **126**, 549-563.