GENETIC ANALYSIS OF LEAF VASCULAR PATTERNING IN *Arabidopsis thaliana*

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Abstract

I have isolated and characterized a recessive mutation in the *FORKED* (*FKD*) gene that results in the abnormal initiation of vascular bundles in the foliar organs, such that the apices of the vascular bundles initiate freely. Once initiated, the development of Fkd vascular bundles is like wild type, generating an open vascular pattern of similar complexity to the closed venation pattern of wild type. Despite the significant alteration in the vascular pattern, Fkd plants are morphologically indistinct from wild type. *fkd* mutants do not show altered sensitivity to the effects of auxin and show additive phenotypes with auxin response mutants, suggesting that *FKD* is part of a pathway acting independently of auxin. The similarity of the open vascular pattern of Fkd plants to that of ancestral vascular plants suggests that acquisition of this pathway may have been critical in the evolution of the closed vascular pattern.

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Genes

AN	*ANGUSTIFOLIA*
AXR1	*AUXIN RESISTANT1*
AXR2	*AUXIN RESISTANT2*
AXR6	*AUXIN RESISTANT6*
CVP1	*COTYLEDON VASCULAR PATTERN1*
CVP2	*COTYLEDON VASCULAR PATTERN2*
FKD	*FORKED*
KNOX1	*KNOTTED1* like homeobox
LOP1	*LOPPED1*
PIN1	*PIN FORMED1*
ROT	*ROTUNDIFOLIA*
SFC	*SCARFACE*
VAN	*VASCULAR NETWORK*

Chemicals

2,4-D	2,4-dichlorophenoxyacetic acid
EMS	ethyl methnosulfonate
HFCA	2-chloro-9-hydroxyfluorene-9-carboxylic acid
IAA	indoleacetic acid
NPA	1-n-naphthylphtalamic acid
TIBA	2,3,5-triiodobenzoic acid

Terms

Col	Columbia ecotype
CCD	Charge-coupled device
DAG	Days after germination

FAA	Formalin
Ler	Landsberg ecotype
SAM	Shoot apical meristem
SSLPs	Simple sequence length polymorphisms
TE	Tris-EDTA
vis	Vascular islands

Genetic Nonmenclature

FORKED (*FKD*)	Wild type allele
forked (*fkd*)	Mutant allele
Forked (Fkd)	Mutant phenotype
FORKED (FKD)	Wild type protein product

Introduction

The development of the vascular system as a means of transporting water, minerals, and organic compounds throughout the plant body was integral to the migration of plants from an aqueous environment to land over 400 million years ago (Gifford and Foster, 1989). The development of specialized tissue types of the vascular system also eventually provided sufficient support for an upright growth habit giving plants an advantage in competition for sunlight and dispersal of spores.

The vascular system is composed of two main tissue types, xylem and phloem, arranged together in vascular bundles commonly referred to as veins (Esau, 1965; Gifford and Foster, 1989). The xylem is responsible for the movement of water and dissolved minerals from the roots to all aerial portions of the plant. The elongated cells that make up the xylem, the tracheary elements, are arranged end to end to form a continuous strand extending the entire length of the plant body. The tracheary elements are also characterized by lignified secondary cell walls, making them rigid and giving support to the plant body (Roth-Nebelsick et. al., 2001). The final step in the differentiation of tracheary elements is apoptosis (programmed cell death), leaving only a hollow cell with a thickened cell wall in the mature xylem. The phloem is responsible for the movement of photosynthates and other organic compounds as driven by a source-sink relationship. The cells making up the phloem, the sieve elements, are arranged in a manner similar to the tracheary elements in the xylem but do not have lignified secondary cell walls or undergo apoptosis. In addition to the xylem and phloem tissues there are other tissue types associated with the vascular bundles. Exchange of the material carried by the vascular

bundle with the surrounding tissues is partially regulated by the bundle sheath cells and sclerenchyma aids in support.

Leaf Development

The leaf is generally regarded as the evolutionary ground state of all other shoot derived organs (e.g. floral organs) (Tsiantis and Langdale, 1998). Thus, elucidating the mechanisms controlling leaf development, including vascularization, will be informative in understanding such mechanisms in overall plant development.

The leaf initials or primordia form at the flanks of the shoot apical meristem (SAM), a group of undifferentiated cells responsible for the post-embryonic growth of the shoot. During organogenesis approximately 100 – 200 founder cells (Poethig and Sussex, 1985) from all three cell layers (LI, LII, and LIII, Satina et. al., 1940) of the SAM show increased cell divisions. The cells of the outermost SAM layer (LI) divide exclusively anticlinally to give rise to the epidermal cell layer of the leaf while the other two internal cells layers (LII and LIII) undergo both anticlinal and periclinal divisions to form the internal tissues (Satina et. al., 1940; Satina and Blakeslee, 1941; Dermen, 1953; Lyndon, 1970 and 1972; Cunninghame and Lyndon, 1986). Although increased cell division rates are the first observable change, they are not necessary for primordia formation. Primordia formation can occur in plants with a significant decrease in cell division rate (Foard, 1971; Hemerly et. al., 1995) or with disorganized cell division planes (Traas et. al., 1995; Smith et. al., 1996), in some cases leading to a morphologically normal organ. In plants where cell division rates are reduced, a larger cell size compensates for the decreased cell number. The role of cell expansion in leaf

development has been further elucidated by two *Arabidopsis* mutants, *angustifolia* (*an*) and *rotundifolia* (*rot*), which are defective in polarized cell expansion (Tsuge et. al., 1996). The cells of An leaves are defective in lateral expansion leading to narrow leaves while *rot* mutants have short leaves because of a defect in proximal-distal expansion. Despite the abnormal shape, the mutant leaves have the same number of cells as wild type leaves, again suggesting that cell expansion is more important to proper leaf formation than cell division. In fact, a specific form of expansin, a protein that allows cell expansion, is expressed in the meristem at the site of the next incipient primordium (Reinhardt et. al., 1998). Expansin expression is one of the first indications of primordial fate and has been shown to be sufficient for primordia formation (Fleming et. al., 1997).

Expression of expansin coincides with loss of *KNOTTED1* like homeobox (*KNOX1*) gene expression (Jackson et. al., 1994; Long et. al., 1996; Fleming et. al., 1997). The *KNOX1* genes are responsible for maintaining the indeterminate identity of the meristem cells and loss of expression of these genes results in the cells acquiring a determinate fate (Smith et. al., 1992; Matsuoka et. al., 1993; Lincoln et. al., 1994). Shortly after the primordium begins to take shape, cells differentiate to form the specialized structures that allow the leaf to function as the photosynthetic factory of the plant. The process of cellular differentiation within the leaf occurs primarily basipetally so that the largest and most mature cells are found near the apex of the developing leaf (Sharman, 1942; Esau, 1965; Sylvester et. al., 1990).

Three major groupings of cells exist in the mature leaf, those of the epidermal layer, the internal photosynthetic layer, and the vascular tissue. The epidermal layer is derived from the LI of the shoot apical meristem. Two highly specialized types of cells

within the epidermis are the trichomes and those cells that make up the stomatal complexes. The trichomes are believed to reduce water loss and protect the plant from insects (Raven et. al., 1999). The stomatal complexes function in the exchange of gases and are composed of the pore, guard cells (the only photosynthetic cells of the epidermis), and subsidiary cells. The number and spatial arrangement of both of these specialized structures are species specific and can vary significantly with changes in environmental factors and age of the plant (Chin et. al., 1995; Willmer and Fricker, 1995; Yang and Sack, 1995; Steynen et. al., 2001). The internal photosynthetic cells of the leaf are derived from the LII and LIII of the SAM and are usually arranged into two distinct layers. The palisade layer is composed of tightly packed, elongated cells that are the primary photosynthetic cells of the leaf and are usually located on the adaxial side. The spongy mesophyll is made up of larger, loosely packed cells that are abaxial (Hall and Langdale, 1996). Embedded within the photosynthetic tissues of the leaf are the vascular tissues, derived from the LIII of the SAM.

Leaf Evolution

Leaves fall into two groups, microphylls and megaphylls, based upon their phylogenetic origin (not, as their names would suggest, based upon their size) (Esau, 1965; Gifford and Foster, 1989). Microphylls evolved as small outgrowths (enations) from the stems of lower vascular plants that eventually became vascularized by a single leaf trace (Figure 1). The resulting vascular pattern of microphylls is extremely simple, characterized by a single vein running the length of the leaf. Only a handful of exceptions to this vascular pattern exist and are found in some extinct arborescent lycopods (two

parallel veins running the length of the leaf) and two species of *Selaginella* (branched network of veins) (Wagner et. al., 1982; Gifford and Foster, 1989).

Megaphylls, as explained by the telome theory, evolved from the stems of isotomously branching primitive vascular plants where one branch eventually outgrew the other branch (Figure 2). Branches developing from the overtopped branch flattened out (planation) and tissue filled in the spaces between the branches to form a leaf. As in microphylls, the resulting vascular pattern of early megaphylls is open-ended but is usually more complex with an increased number of veins and vein branching. It is

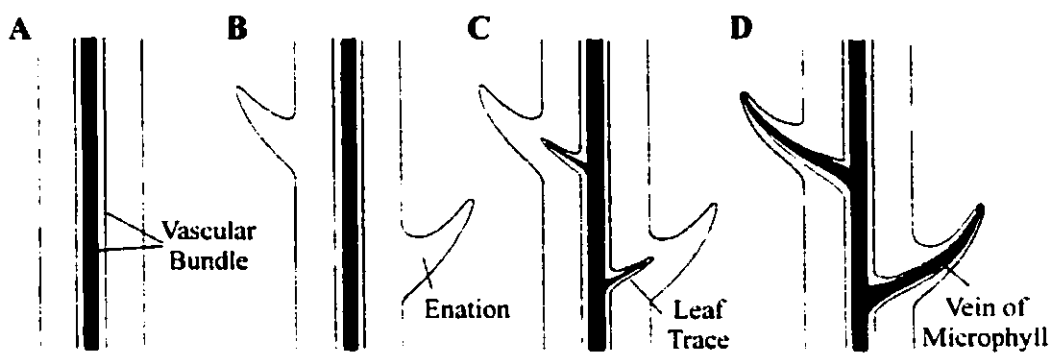

Figure 1 Schematic representation of the enation theory explaining the formation of microphylls. A small outgrowth or enation protrudes from the stem of them plant (**A**) and a vein diverges from the vasculature of the stem (**B**) to form a leaf trace (**C**). The enation is vascularized through extension of the leaf trace into the enation (**D**). (Gifford and Foster, 1989, used with permission)

believed that as atmospheric CO_2 levels began to decline approximately 400 million years ago plants bearing megaphylls were better able to cope than those bearing microphylls (Beerling et. al., 2001).

As primitive vascular plants evolved, the leaf vascular pattern became more complex, diverging from the simple dichotomously branching, open-ended vascular pattern. More advanced vascular plants (e.g. ferns and gymnosperms) exhibit a wide

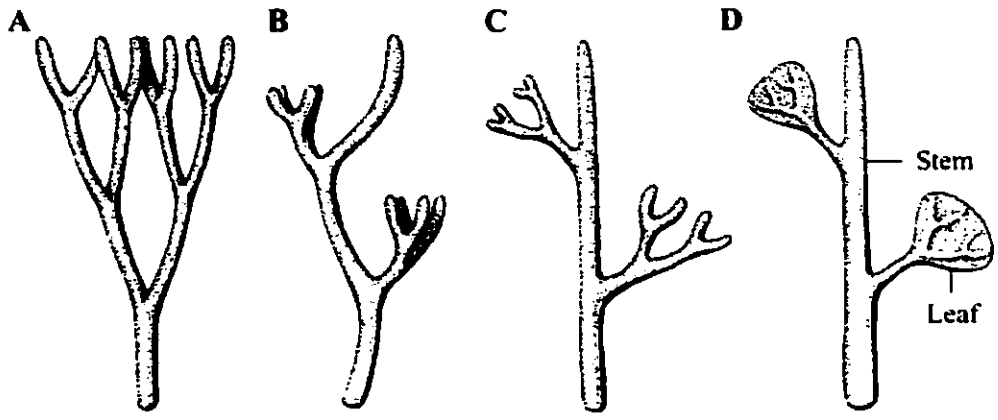

Figure 2 Schematic representation of the telome theory explaining the evolution of megaphylls. A primitive isotomously branching vascular plant (A) undergoes the process of overtopping in which one of the branches outgrows the other (B). The overtopped branches flatten out into a single plane (planation) (C) and the spaces between are filled in with laminar tissue to form a megaphylls (D). (Gifford and Foster, 1989, used with permission)

variety of leaf vascular patterns from open dichotomous branching to a complex closed pattern, similar to that associated with angiosperms (Figure 3). As in ferns, the open vascular pattern is most common in the gymnosperms, although several notable exceptions exist. In some cycads and *Gingko biloba*, the leaves have an open dichotomous venation pattern but the vascular bundles occasionally meet in an apparently random manner throughout the leaf blade and may represent a precursor to the evolution of a closed vascular pattern (Arnott, 1959) (Figure 3E&F). An extremely complex closed venation pattern that closely resembles that seen in angiosperms is found in the *Gnetum* of the Gnetophyta (Figure 3G). The pattern seen in *Gnetum* so closely resembles that seen in dicotyledonous angiosperms that, combined with other morphological data, some have considered the Gnetophyta the closest gymnosperm relative to the angiosperms (e.g. Doyle and Donoghue, 1987), suggesting that the closed venation pattern evolved only once. More recent data suggests the Gnetophyta are the closest relatives to the conifers

(Winter et. al., 1999; Bowe et. al., 2000), suggesting that the complex closed venation pattern has arisen at least twice during evolution. In either case the more complex closed venation pattern presumably provides a more efficient means to transport water and photosynthates throughout the plant body. Roth-Nebelsick (2001) have shown that a closed venation pattern provides redundancy which allows for alternate transportation routes to a specific area of the leaf in case of injury or blockage of a vascular bundle. The closed venation pattern also offers increased mechanical stability and support to the leaf (Roth-Nebelsick, 2001). The evolution of such a pattern would require a developmental mechanism enabling apical vein joining to close off the open vascular pattern of ancestral plants.

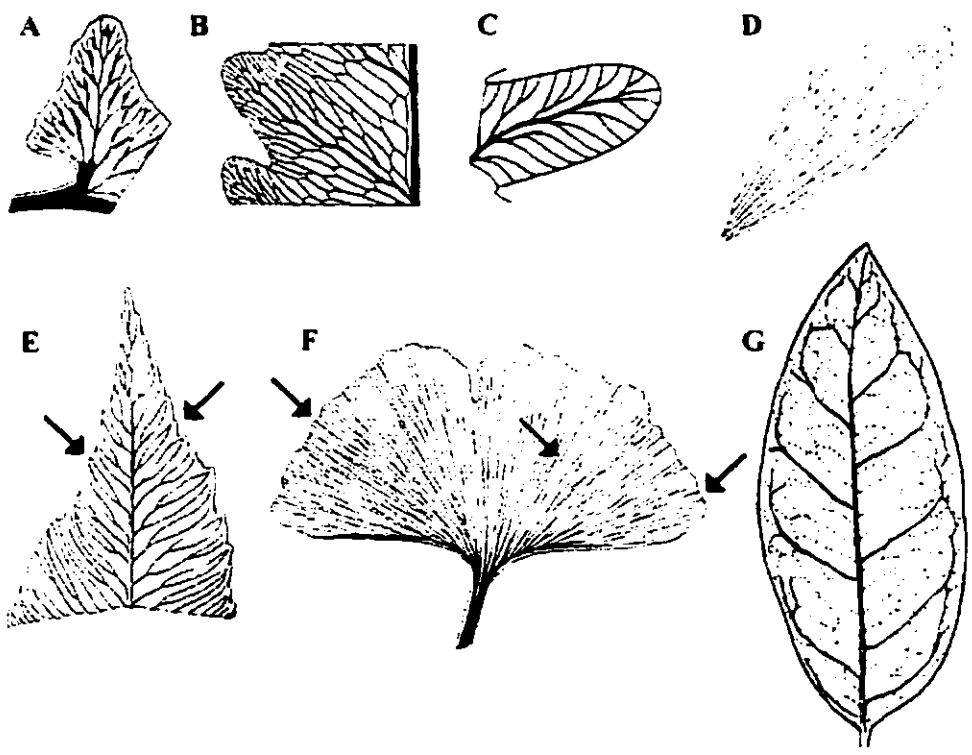

Figure 3 Examples of leaf venation patterns found in ferns (A&B) and gymnosperms (C-G). Note the open dichotomous venation pattern seen in the ferns (A), seed ferns (C), cycads (D&E) and *Gingko biloba* (F). Arrows in E and F show anastomoses (vein joining) that occur in *Gingko biloba* and in some cycads. The complex closed venation pattern of various species of Gnetales (G) closely resembles that observed in some angiosperms. (adapted from Eames, 1936 and Gifford and Foster, 1989)

Angiosperm Leaf Vascular Pattern

The angiosperms (Magnoliophyta) are subdivided into two classes, the Magnoliopsidas (dicotyledons) and the Liliopsidas (monocotyledons), in part due to the perceived differences in leaf vascular patterning, although both show a closed venation pattern. Monocotyledonous leaves have a closed vascular pattern that is commonly referred to as parallel (or striate) due to the appearance of the longitudinal veins (Figure 4A). Dicotyledonous plants (dicots) are characterized by a closed, netted leaf venation pattern (Figure 4B). Longitudinal veins in monocots are defined on the basis of size, with

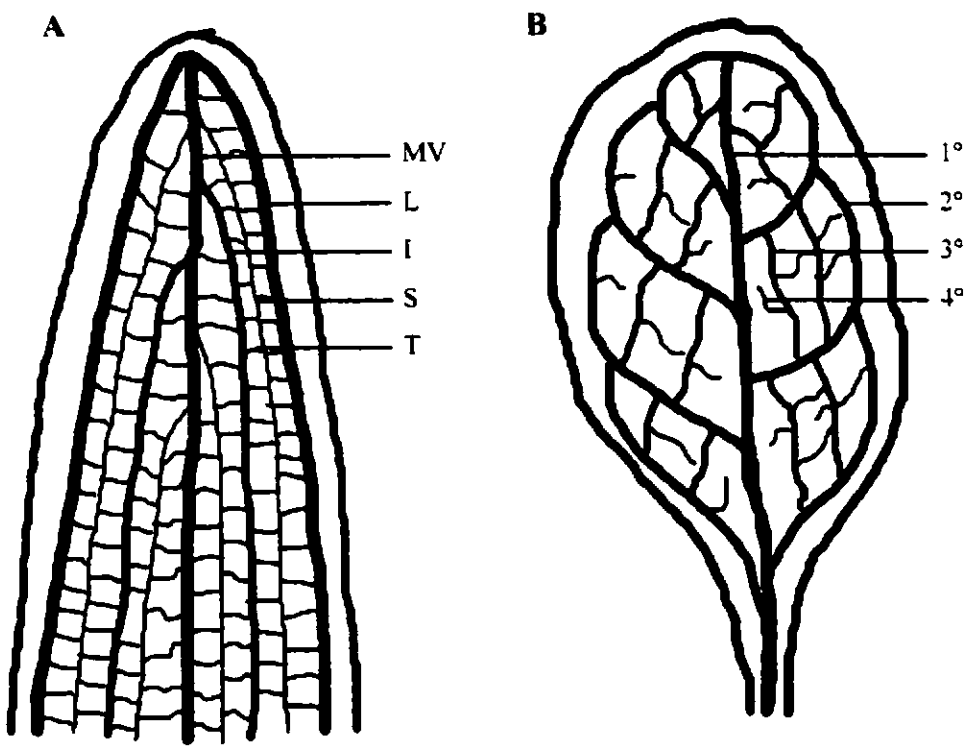

Figure 4 Typical leaf vascular pattern found in monocot (A) and dicot (B) leaves. (A) Shows the relative position and thickness of the midvein (MV), large longitudinal (L), intermediate longitudinal (I), small longitudinal (S), and transverse (T) veins and (B) shows the midvein (1°), secondary (2°), tertiary (3°), and quaternary (4°) veins.

large, intermediate, and small. Transverse veins are those that connect the longitudinal veins. In dicot leaves, vein orders are defined on the basis of connection. Secondary veins connect at least once to the midvein, tertiary veins to the secondary veins, and quaternary veins to the tertiary veins.

Another common feature of both monocot and dicot leaf venation patterns is their hierarchal development. The midvein always develops prior to the secondary or large longitudinal veins, which in turn develop prior to the tertiary or small longitudinal veins. This phenomenon can be seen later in development since early forming veins are generally thicker than later forming veins.

Mechanisms Regulating the Formation of the Vascular Pattern

Two theories have been put foward to explain vascular pattern formation in the leaves of angiosperms (Nelson and Dengler, 1997). The first is a reaction-diffusion hypothesis that has also been used to explain such patterning phenomena as zebra stripes, butterfly wings, and trichome spacing (Koch and Meindhart, 1994). In a reaction-diffusion system, small fluctuations in the concentration of a morphogen within an initially homogenous field are reinforced through a localized up-regulation event and a distant down-regulation event. The up-regulation event could take the form of a positive feedback loop resulting in the localized production of the morphogen. Alternatively, an initial fluctuation in morphogen concentration may be sufficient to start a signal cascade that has a positive feedback component. The end result of either mechanism would be to cause cells with high levels of morphogen to differentiate into a fate different from

neighbouring cells. The second requirement of the reaction-diffusion model is a diffusible, distantly acting substance, such as an inhibitor, that down regulates the process in the surrounding cells to prevent them from acquiring the same fate. Koch and Meindhart (1994) have shown through computer modeling that manipulating the variables of a reaction-diffusion system (e.g. the rate of diffusion of an inhibitor or the binding affinity of a signal molecule to its receptor) can lead to various patterns observed in nature such as regularly spaced points (as seen with trichome and stomata distribution) or broad, evenly spaced bands (such as zebra stripes).

The second model to explain vascular patterning was proposed based on experimental evidence by Sachs (1981, 1989, 1991). In these classic experiments, wounds were introduced to the stems and leaves such that vascular bundles were severed. Regeneration of the vascular bundles occurred progressively starting from the severed vascular bundle apical to the wound and developed basipetally until rejoining the vascular bundle basal to the wounding site. This is similar to the normal development of a vascular bundle in which differentiation of cells into vascular tissue adds to the length of the vascular bundle in a progressive and directional manner. These observations led to the canalization of signal flow hypothesis. This hypothesis requires that a signal molecule is moved in a directional manner, either through active transport or by diffusion, down a concentration gradient. Initially, the movement of the signal molecule through a field of cells is essentially equal, but slight discrepancies within individual cells in the field become positively reinforced as those cells are exposed to more of the signal molecule (i.e. a cell becomes more efficient at transport or more permeable to the signal molecule). Increased exposure to the signal molecule leads to the differentiation of that cell into a

vascular cell thereby further increasing its efficiency to transport the signal molecule. This increased ability to transport has two effects on the system: 1) cells immediately downstream of the vascular cell are exposed to an increased amount of the signal molecule which leads to them differentiating into vascular cells and 2) cells immediately adjacent to the vascular cell become drained of the signal molecule and are prevented from differentiating to the vascular fate. The pattern predicted from such a system is a series of discrete vascular bundles, regularly spaced, oriented parallel to the flow of the signal molecule.

There is mounting evidence that the canalization model more accurately describes the process of vascular tissue differentiation than the reaction diffusion model and that the plant hormone auxin may be the canalized signal molecule (Nelson and Dengler, 1997; Aloni, 2001). Auxin plays an important role in a wide variety of plant processes including apical/basal axis formation in embryos, establishment of the root quiescent centre, apical dominance, phototropism, gravitropism, root growth, cell differentiation, and cell elongation (Berleth and Sachs, 2001). Auxin is produced at the apex of the plant and in developing tissues, such as young leaves, and flows in a polar basipetal manner throughout the plant body (Wallroth-Marmonr and Harte, 1988; Lomax et. al., 1995). *In vitro* experiments have shown that auxin (in combination with cytokinin) can induce mesophyll cells from *Zinnia elegans* to differentiate into tracheary elements (Church, 1993). An important link between the polar flow of auxin and vascular patterning was established in the wounding experiments of Sachs (1981). When the apices and young leaves of the plants were removed, vascular tissue regeneration around the wounding site did not occur but was restored when auxin was applied to the site of apex removal.

More recent insights into the mechanisms of auxin transport have provided further support for its role in vascular patterning. The *Arabidopsis PIN-FORMED1* (*PIN1*) gene product has been shown to be an auxin efflux carrier located at the basal end of cells (Mattsson et. al., 1999; Steinmann et. al., 1999; Galweiler et.al. 1998; Muller et. al. 1998). In the early embryo this efflux carrier is found in most cells but as the embryo continues to mature and vascular tissue appears, PIN1 becomes localized within the vascular bundle. *Arabidopsis* plants mutant for *PIN1* exhibit a wide range of effects on growth and development including a leaf vascular pattern with increased vascular tissue along the margins of the leaf. Wild type *Arabidopsis* can be induced to phenocopy Pin1 plants when grown on media containing substances such as 2,3,5-triiodobenzoic acid (TIBA), 2-chloro-9-hydroxyfluorene-9-carboxylic acid (HFCA), or 1-n-naphthylphtalamic acid (NPA), all substances that inhibit the polar transport of auxin (Goto et. al., 1991; Okada et. al., 1991; Galweiler et. al., 1998). If the concentration of these substances is sufficiently high, vascularization of the leaf can be limited to only a broad band of vascular tissue along the margin of the leaf (Mattsson et. al., 1999, Sieberth 1999). The increased vascularization in either Pin1 mutants or plants treated with auxin transport inhibitors is presumed to result from the lack of auxin transport away from its site of synthesis, suggesting that the margin is a source of auxin.

A significant number of mutants defective in a variety of auxin pathways have been isolated. Analysis of their phenotypes and cloning of the genes identified by mutation have aided in the understanding of the critical role auxin plays in a wide variety of developmental processes. One might expect that mutants defective in components of the auxin pathway other than auxin transport such as those defective in auxin induced

targeting to an ubiquitin-like pathway (*auxin resistant1* [*axr1*], Carlos del Pozo and Estelle, 1999; Gray and Estelle, 2000), in an auxin inducible gene (*auxin resistant2* [*axr2*], Nagpal et. al., 2000), or in aspects of the pathway as yet unidentified at the molecular level (*auxin resistant6* [*axr6*], Hobbie et. al., 2000) might show vascular defects. In fact, the leaf vascular patterns of such auxin resistant mutants have not been thoroughly characterized. Although a number show aberrant leaf morphology, only Axr6 has been found to have some vascular abnormalities. Reexamination of additional mutants may uncover subtle vascular defects.

The canalization of signal flow hypothesis is now commonly referred to as the auxin canalization hypothesis because of the strong evidence that auxin is the signal molecule. This hypothesis for vascular pattern formation explains a number of important aspects of pattern development including: 1) the location of vascular tissue differentiation, 2) the direction of vascular tissue differentiation, 3) the confinement of the vascular tissue to a narrow region, and 4) the continuous nature of vascular tissue throughout the plant body (Palme and Galweiler, 1999). Despite these strengths there are a number of equally important aspects of vascular patterning that cannot be explained by the auxin canalization theory. For example, such a theory does not explain the formation of vascular bundles perpendicular to the flow of the signal molecule, a commonly observed event in both monocots (transverse veins) and dicots (tertiary and higher order vascular bundles). While the theory is consistent with the joining of basal ends of vascular bundles it is inconsistent with the joining of the apical portions of vascular bundles, which is commonly observed in leaf vascular patterning in higher vascular plants. Both apical vascular bundle joining and formation of vascular bundles in any

direction could be explained by the more theoretical reaction-diffusion hypothesis. However, this theory fails to explain the progressive or hierarchical development of vascular bundles.

While the two theories presented can explain certain aspects of vascular patterning, neither the reaction-diffusion nor the auxin canalization hypothesis accounts for the complexities of a complete vascular pattern as seen in either monocot or dicot leaves. Recently, a genetic approach has been utilized in an attempt to elucidate the mechanisms behind vascular patterning in plants. Several mutants have been reported after screening for cotyledon and leaf vascular patterning defects including *lopped* (*lop1*, Carland and McHale, 1996), *cotyledon vascular pattern* (*cvp1* and *cvp2*, Carland et. al., 1999), *vascular network* (*van1* thru to *van7*, Koizumi et. al., 2000), and *scarface* (*sfc*, Deyholos et. al, 2000), but the analysis of these mutants has not yet been well integrated into proposed models for vascular pattern formation. In fact, the occurrence of vascular bundles isolated from the rest of the vascular network (known as vascular islands or vis) in *van* and *sfc* mutants appears to contradict the auxin canalization hypothesis. Mutations in the *LOP1*, *SFC*, and *VAN* genes lead to significant aberrations in growth and development, suggesting that the vascular patterning defect may not be the primary defect but rather the indirect result of a more general developmental defect. The *cvp* mutants display otherwise normal development suggesting that the primary defect is an abnormality in vascular patterning. None of these genes have been cloned so the function and localization of their gene products remain unknown.

Objectives

The goal of this study is to elucidate, through genetic analysis, the mechanisms controlling leaf vascular patterning. Of particular interest is the mechanism controlling vein meeting because this character is nearly ubiquitous within the angiosperms and appears to have been important in their evolution. The model plant, *Arabidopsis thaliana*, offers an ideal molecular genetic system to study vascular pattern formation in leaves. Its small leaf size is well suited to large scale microscopic examination and screening, and the relative simplicity of its leaf vascular pattern makes phenotypic analysis of altered vascular patterns straightforward. Moreover, its short generation span and small, completely sequenced, diploid genome is ideal for molecular genetic analysis. To accomplish my goal, I have isolated and characterized a mutant of *A. thaliana* with an open leaf vascular pattern resembling that of an ancestral vascular plant. The characterization and future cloning of such a gene identified by mutation will be beneficial in elucidating the molecular mechanisms gained during evolution and will advance current theories for vascular pattern formation.

Materials and Methods

Plant Material

All seed material was obtained from the Arabidopsis Biological Resource Center (Columbus, Ohio) except for the EMS mutagenized seed (Columbia [Col]), which were purchased from Lehle Seeds (Round Rock, TX) and *pin1-1* seed generously provided by T. Berleth (University of Toronto, Toronto, ON).

Growth Conditions

Arabidopsis thaliana seed (Columbia) was sown on Metromix 200 (W.R. Grace Co., Marysville, OK) in 100 cm^2 pots or on *A. thaliana* growth medium (Ruegger et. al., 1997) in 78.5 cm^2 petri plates. All seed sown on *A. thaliana* growth medium were surface sterilized in 70% ethanol (30 seconds) followed by 10 minutes in 50% bleach. Pots or plates were incubated at 4°C in the dark for 4 – 5 days, and transferred to growth chambers (Percival Scientific, Perry, IA) set for 21°C, 60% relative humidity, and continuous light at an intensity of 130 µmol sec^{-1} m^{-2} provided by a combination of Sylvania Cool White, Gro Lux, and incandescent bulbs (Osram Sylvania Inc., Danvers, MA). For this study the time of transfer of the seed to growth chambers was considered to be the time of germination.

To assess if *fkd* mutants had altered sensitivity to an exogenous auxin source, vertically grown (such that the roots grew along the surface of the medium) five day old seedlings of wild type, *axr1-3*, and *forked* were transferred from *A. thaliana* growth medium to fresh medium containing 1.0 x 10^{-6} M 2,4-D. The root tips were positioned

along a line drawn across the bottom of the petri dish and grown vertically for another 4 days at which time root length was measured from the tip of the root to the previously drawn line on the petri dish.

Mutant Screening

Approximately 6000 EMS mutagenized M_2 seed from three families were sown at a density of 50 seeds per pot. A cotyledon and first leaf were taken two weeks after germination, mounted on slides in low viscosity Cytoseal (Stephen's Scientific, Kalamazoo, MI), and screened the following day for abnormalities in vascular patterning with the aid of a dissecting microscope (Stemi 2000, Carl Zeiss Inc., Thornwood, NY). M_3 seed of potential mutants was collected and sown for re-screening to ensure that the observed phenotypes were heritable. The mutant line of interest were backcrossed at least twice, using wild type as the female, before characterization of the phenotypes was performed. All crosses were carried out by hand on immature flowers (no petals visible) that had been emasculated the previous day.

Morphological and Anatomical Characterization

Wild type and *forked* plants (4 per pot) were scored for germination, total number of leaves, number of secondary stems, and time to flowering (days). A cotyledon was taken 14 days after germination (DAG) and the first leaf was taken 21 DAG and mounted as described previously. Images of the cotyledons and leaves were captured with a CCD camera (RS-170, Cohu Inc., Electronics Division, San Diego, CA) attached to a dissecting microscope and were analyzed for width, length, and area using NIH Image

(available for download from the United States National Institute of Health at http://rsb.info.nih.gov/nih-image/). The vascular pattern of the cotyledons and leaves were also scored by counting the number of branch points (two or more veins meeting), vein endings, and areoles (any area of the leaf blade completely bounded by veins) for whole cotyledons or half of the first leaf. Statistical differences were determined using Students' T test.

Fourteen-day-old cotyledons and 21-day-old first leaves of wild type and *fkd* were prepared for sectioning by vacuum infiltration in FAA, 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 prior to being viewed with a compound light microscope (Eclipse E600, Nikon, Mississauga, ON)

To examine development of both cotyledons and leaves, wild type and *forked* seeds were sown on *A. thaliana* growth medium (20 per plate) and plants were taken every 24 hours from 1 DAG. The plants were cleared in a solution of 3 ethanol : 1 acetic acid for 2 – 4 hours, 70% ethanol for 1 hour, 95% ethanol overnight, and finally 5% NaOH for 1 hour at 60°C . Whole plants were mounted on slides using a 50% aqueous glycerol solution and viewed with a compound light microscope (Eclipse E600, Nikon, Mississauga, ON).

Whole mature flowers were cleared by the same process as seedlings. Sepals and petals were dissected from flowers after clearing, mounted on slides using a 50% aqueous glycerol solution, and viewed with a compound light microscope (Eclipse E600).

Mapping of *forked*

Plants mutant for *FORKED* were crossed into the Landsberg erecta (Ler) background and DNA was extracted from F_2 plants exhibiting the Forked phenotype. Two leaves from each plant were ground for 15 seconds in 400 µL of extraction buffer (200 mM TrisHCl pH 5.0, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and centrifuged at 14,000 rpm for 5 min. Three hundred µL of the resulting supernatant was transferred to 300µL of isopropanol, mixed by inversion, and allowed to incubate for 2 minutes at room temperature before being centrifuged at 3000 rpm for 5 minutes. The supernatant was poured off and the pellet was washed once in 100 µL of 70% ethanol before being resuspended in 100 µL of TE buffer. Mapping was carried out as has been previously described by Bell and Ecker (1994) using primers obtained from Research Genetics Inc. (Huntsville, AL) and polymerase (Deep $Vent_R$) obtained from New England Biolabs (Mississauga, ON). PCR was carried out in a Personal Cycler (Biometra, Gottingen, Germany) under the cycling conditions of: 6 cycles of 94°C for 3 min, 55°C for 1 min, and 72°C for 3min followed by 31 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec except on the last cycle which had a 10 min extension time (72°C).

Generation of Double Mutants

Double mutants were generated by hand crossing pollen (as previously described) from *fkd* homozygotes onto plants homozygous for *axr1-3* and onto plants heterozygous for *axr2* and *pin1-1*. The F_1 progeny from each cross were all wild type and were allowed to self except for the progeny of the *axr2* x *fkd* which segregated 1:1 for Axr2. In this case, plants having the codominant Axr2 phenotype in the F_1 were allowed to self.

Segregation data from the F_2 populations were collected and analyzed using the χ^2 test. Selfed seed from Fkd F_2 plants were sown and the resulting F_3 progeny were screened for Axr1-3, Axr2, and Pin1-1 as appropriate. F_3 progeny from *axr2 fkd* and *pin1-1 fkd* double mutants and F_4 progeny from *axr1-3 fkd* were characterized.

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

I screened an M_2 population of EMS mutagenized plants for defects in leaf vascular patterning and chose for further characterization one mutant line (*forked* [*fkd*]) with vascular bundles that fail to meet in both the cotyledons and leaves (Figure 5) but with otherwise normal plant morphology.

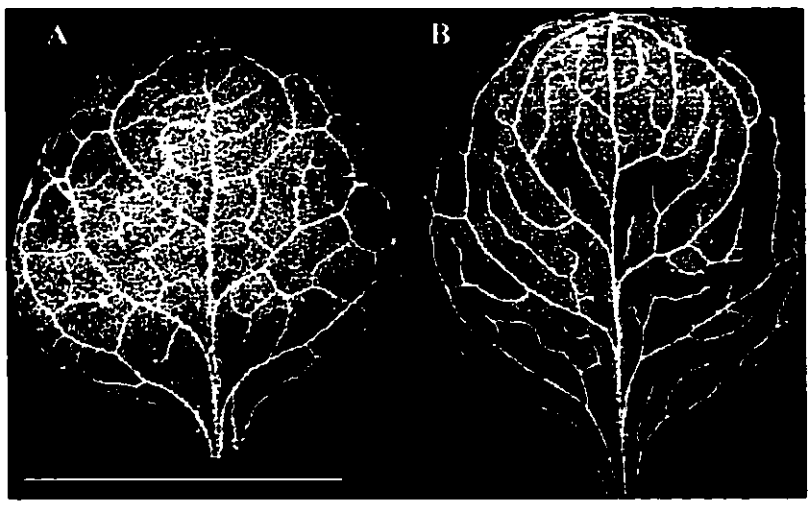

Figure 5 Vascular pattern of first leaves from wild type (**A**) and Fkd-1 (**B**) plants 21 days after germination. Bar, 5mm

Reciprocal crosses to wild type yielded a F_1 all of which had the wild type phenotype (n=36). In the F_2 the Fkd phenotype segregated from the wild type phenotype with a ratio of 3:1 (188 wild type: 53 Fkd-1, χ^2=1.037). I therefore concluded that the Fkd phenotype is the result of a single, nuclear, recessive mutation.

Fkd plants (Col background) were crossed into the Landsberg erecta (Ler) background to enable molecular based mapping of the *FKD* gene. DNA extracted from Fkd F_2 progeny was used to determine the map position of *FKD* by PCR based mapping using simple sequence length polymorphisms (SSLPs) between the Col and Ler backgrounds (Bell and Ecker, 1994). The *FKD* gene mapped to the south arm of

chromosome III at 89.48 cM based on recombination with nga112 (n=562 chromosomes) (Figure 6). No molecular marker south of this position was available so recombination with nga6 (n=210 chromosomes) was used to determine the position of *FKD* relative to nga112.

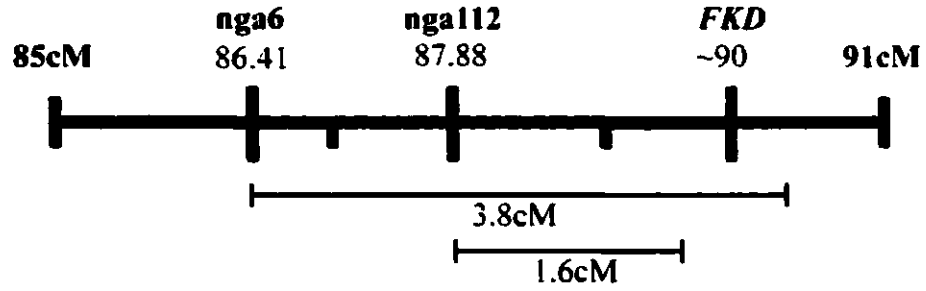

Figure 6 Schematic representation of the south arm of chromosome III from 85 to 91cM. *FKD* showed linkage to the markers nga6 (3.81% recombination, n=210) and nga112 (1.60% recombination, n=562) placing the *FKD* gene at approximately 90cM.

Cotyledon Vascular Pattern Development

In order to assess the differences between wild type and Fkd mature cotyledons, I first assessed the variability seen in wild type. The vascular pattern of the mature wild type cotyledon (Figure 7C) usually consists of a midvein and 4 secondary veins (72%, of n=29 cotyledons examined), although cotyledons with 3 secondary veins (21%) or 2 secondary veins (7%) were also observed as previously reported (Carland et. al., 1999; Sieburth, 1999). The vascular pattern was further quantified by assessing the number of vein branch points (meeting of any two veins), aeroles (area of the lamina completely bounded by vascular bundles), and freely ending veins (veins ending blindly in the lamina) in mature cotyledons (Table 1). The two apical-most secondary veins (apical secondary veins) are connected to the midvein at its apex (99.6%, n=827, 1 – 7 DAG)

and approximately at its midpoint while the two basal-most secondary veins (basal secondary veins) are connected to the apical secondary veins and to the midvein near the base of the cotyledon blade.

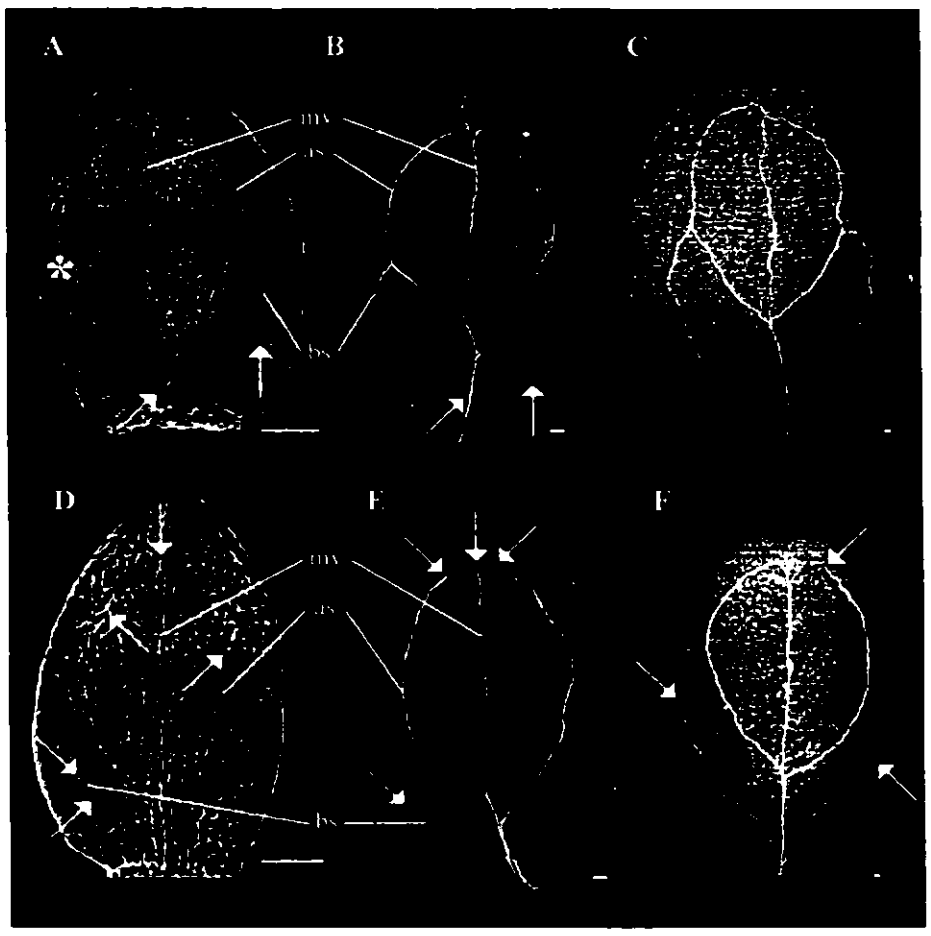

Figure 7 Vascular pattern development in wild type (A-C) and in Fkd cotyledons (D-F). Cleared cotyledons (1 DAG) of wild type (A) and Fkd (D) viewed with phase contrast optics, show the presence of provascular tissue of the midvein (mv), the apical secondary veins (as), and occasionally basal secondary veins (bs). By 3 DAG (B&E, DIC optics) maturation of the midvein and apical secondary veins is complete but is still progressing in the basal secondary veins. At 14 DAG, maturation of all vascular bundles is complete (C&F darkfield optics). Arrows indicate the ends of veins and * in A indicates the apical portion of the basal secondary vein initiating from the previously formed apical secondary vein. Scale, 50μm

I next determined the developmental events that give rise to the wild type cotyledon vascular pattern. The sequential differentiation of vascular tissue was followed by examining seedlings at 24-hour intervals following placement in a growth chamber (0 DAG). At 1 DAG, the provascular tissue for the midvein and the apical pair of secondary veins is complete for all cotyledons examined (Figure 7A). The provascular tissue of the apical secondary veins is connected to the midvein at its apex (Figure 8A&B) and also at a more basal point, approximately one-half to two-thirds down the length of the midvein (Figure 8I&J). The remaining 2 secondary veins are initiated from a basal and marginal proximal portion of the previously formed apical secondary vein (* in Figure 7A). The initiation of the third and fourth secondary veins is first evident as a single provascular cell oriented almost perpendicular to the existing secondary vein. Of the wild type cotyledons examined, 60% (n=28) have initiated a third secondary vein 1 DAG and by 2 DAG, 87% (n=15) have initiated a fourth secondary vein. The development of the new vascular bundle progresses with the addition of provascular cells in an end-to-end manner (Figure 8E). The basipetal differentiation follows the margins of the cotyledon blade before bending inwards to meet the midvein (Figure 7A&B and 8E&F). For some cotyledons, recruitment of cells to the vascular fate for the third and fourth secondary vein is never visible.

I defined the maturation of the provascular tissue as the time at which cell wall thickenings are visible in the xylem. Only 7% (n=28) of 1 DAG cotyledons show any signs of midvein maturation, but subsequent maturation occurs quickly and is complete 2 DAG for all cotyledons examined (n=15). The maturation of the apical secondary veins is not as rapid as the midvein. At 2 DAG, 40% (n=30) of these apical secondary veins are

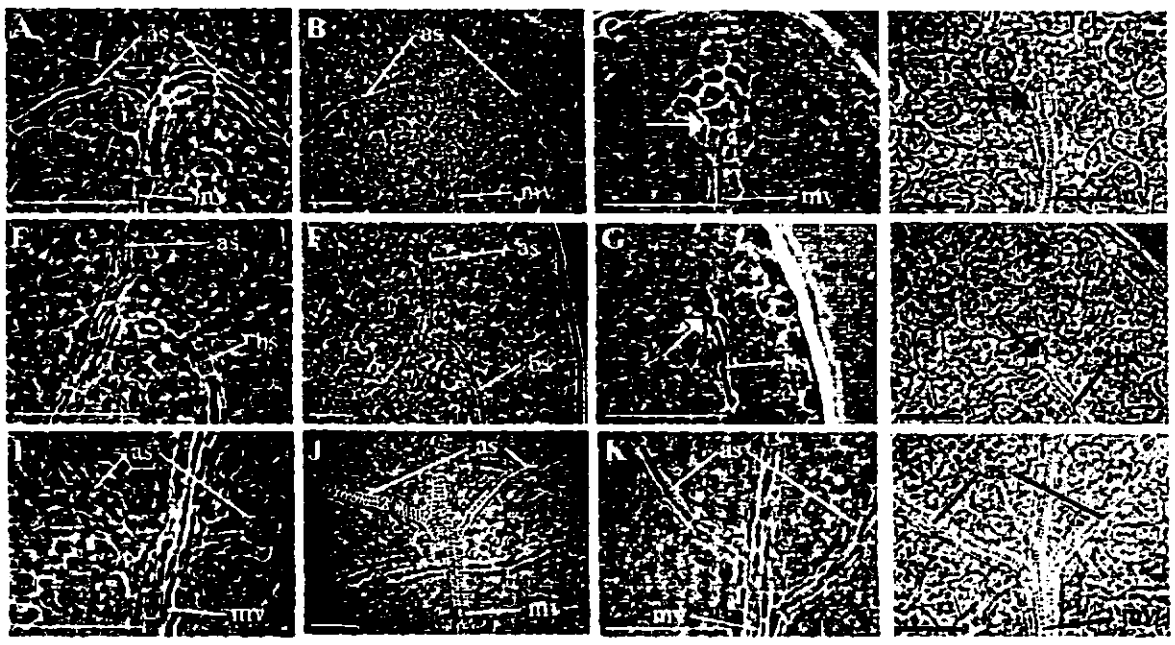

Figure 8 Apical and basal portions of vascular bundle junctions in wild type and Fkd cotyledons viewed with phase contrast optics. Provascular (A) and mature vascular tissue (B) of the wild type midvein (mv) and apical secondary veins (as) near the apex of the cotyledon. Provascular (C) and mature vascular tissue (D) of the Fkd midvein near the apex of the cotyledon. Provascular and mature vascular tissue of the apical portion of a basal secondary vein (bs) in wild type (E&F) and in Fkd cotyledons (G&H). Basal portions of the wild type (I&J) and Fkd (K&L) apical secondary veins showing their fusion with the midvein as provascular (I&K) and mature vascular tissue (J&L). Note that the apices of Fkd apical and basal secondary veins fail to connect with other veins (arrows) but that the basal portions of the same veins fuse with the midvein as in wild type. Scale, 50μm

mature throughout their entire length, 30% (n=30) are only partially mature, and 30% (n=30) have not yet begun to mature. Of the 30% (n=30) showing partial maturation, the majority (66%, n=30) showed mature xylem near the apex of the cotyledon, suggesting that maturation occurs primarily basipetally. The remaining apical secondary veins show mature vascular tissue at both the apical and basal ends, suggesting simultaneous bi-directional maturation. Maturation of all apical secondary veins is complete 3 DAG (n=42, Figure 7B).

The basal secondary veins mature more slowly than their apical counterparts, starting 3 DAG in 73% (n=42) of cotyledons examined (Figure 7B and 8F). As in the

apical secondary veins, subsequent maturation of these basal secondary veins occurs primarily in a basipetal manner (3 – 7 DAG, 95%, n=758), with rare bi-directional maturation (5%, n=758). Even at the point of cotyledon senescence (14 DAG), maturation (and recruitment of cells to the vascular fate) of 23% (n=46) of the basal secondary veins remains incomplete. Following their junction with the midvein, the basal secondary veins continue to mature basipetally, running parallel with the midvein and adding to its thickness (Figure 8I&J).

Mature Fkd cotyledons show approximately the same number and pattern of veins as their wild type counterparts but, in contrast to the apical connections seen between the apical loops and the midvein and between the basal and apical loops in wild type cotyledons, the apical connections between the veins are often not present in Fkd cotyledons. Approximately 78% (n=28) of Fkd cotyledons have 4 secondary veins, 18% have 3 secondary veins and 4% have 2 secondary veins, a variation in complexity similar to that in wild type cotyledons. However, the lack of apical vein meeting is evident by the significant decrease in the number of areoles and branch points and by the significant increase in the number of freely ending veins relative to wild type (Table 1).

The lack of apical vein meeting is evident at the earliest stages of cotyledon development. For many Fkd cotyledons 1 DAG, the provascular tissue of the apical secondary vein fails to join with the apex of the midvein for one (32%, n=28) or both veins (50%, n=28) (Figure 7D and 8C&D). In contrast to wild type cotyledons, where only a few apical secondary veins fail to join with the apex of the midvein (0.4%, n=827, 1 – 7 DAG). Maturation of the midvein and the apical secondary veins is indistinguishable in timing and direction from that in wild type cotyledons, such that

maturity is reached by the midvein 2 DAG (100%, n=12) and by the apical secondary veins 3 DAG (100%, n=47). Whereas the initial provascular cells of the basal secondary veins form perpendicular to the apical secondary vein in wild type cotyledons, these perpendicular cells are not observed in Fkd cotyledons. Instead, the newly forming secondary veins initiate at a point approximately halfway between the existing apical secondary vein and the cotyledon margin (Figure 8G). Moreover, the timing of initiation is delayed relative to wild type; whereas in wild type cotyledons, 60% have initiated the third secondary vein 1 DAG and 87% have initiated a fourth secondary vein by 3 DAG, only 7% (n=28) of Fkd cotyledons had initiated a third secondary vein 1 DAG and none (n=12) had a fourth secondary vein initiated 2 DAG. Once initiated, subsequent differentiation of the vascular tissue proceeds with similar timing and direction as wild type cotyledons (Figure 7E and Figure 8G,H,K,&L). Whereas in wild type, maturation of some basal secondary veins remains incomplete at maturation, in Fkd, all veins that are observed to initiate appear to connect basally with the midvein.

First Leaf Vascular Pattern Development

To compare the Fkd leaf venation pattern to that of wild type, I quantified the number of branch points, freely ending veins, and aeroles of first leaves 21 DAG (Table 1). The vascular pattern of mature, wild type first leaves consists of a midvein running along the apical-basal axis of the leaf blade with regularly spaced secondary veins extending from midvein to the leaf margin where they join with each other. Tertiary veins form connections between the secondary veins or occasionally end freely in the lamina and further subdivide the leaf lamina. Quaternary veins usually end blindly in the lamina.

Table 1 Cotyledon 14 DAG and first leaf 21 DAG vascular pattern characters. Values represent means with standard error for the entire cotyledon and half the first leaf as divided by the midvein. Values following genotypes in parentheses represent number of cotyledons or leaves examined.

Cotyledon	Free Ends	Areoles	Branch Points
Wild Type (31)	0.5±0.1	3.3±0.1	5.7±0.2
Fkd (31)	2.3±0.1[a]	1.6±0.1[a]	3.9±0.2[a]
Axr1-3 (31)	0.2±0.1	2.1±0.1[a]	3.5±0.2[a]
Axr1-3 Fkd (31)	2.0±0.0[a,c]	0.0±0.0[a,b,c]	2.0±0.0[a,b,c]
Axr2 (27)	0.3±0.1	3.3±0.2	6.0±0.2
Axr2 Fkd (27)	2.0±0.2[a,c]	1.5±0.1[a,c]	4.5±0.1[a,b,c]
Pin1-1 (15)	0.3±0.1	3.6±0.3	6.1±0.5
Pin1-1 Fkd (15)	0.7±0.2[b]	2.5±0.3[a,b]	4.6±0.5[a]
First Leaf			
Wild Type (31)	9.2±0.6	17.8±1.0	39.6±1.9
Fkd (31)	14.1±0.7[a]	4.0±0.3[a]	23.6±0.9[a]
Axr1-3 (31)	4.9±0.5[a]	4.2±0.2[a]	14.6±0.6[a]
Axr1-3 Fkd (31)	4.9±0.3[a,b]	0.8±0.1[a,b,c]	7.7±0.3[a,b,c]
Axr2 (27)	4.7±0.6[a]	9.7±0.5[a]	22.6±1.2[a]
Axr2 Fkd (27)	9.8±0.5[b,c]	2.3±0.3[a,b,c]	12.7±0.6[a,b,c]
Pin1-1 (15)	2.9±0.6[a]	10.5±0.9[a]	23.1±2.3[a]
Pin1-1 Fkd (15)	7.5±0.4[b,c]	5.1±0.8[a,c]	15.6±1.1[a,b]

[a] significantly different from wild type
[b] the double mutant is significantly different from Fkd
[c] the double mutant is significantly different from its corresponding single auxin mutant

Vascularization of the first leaf in wild type is first evident as acropetal recruitment and differentiation of provascular cells into the midvein. The midvein continues to mature until it reaches the apex of the developing leaf 6 DAG (87%, n=60,Figure 9A). Two secondary veins initiate from the apex of the midvein and recruitment of provascular cells and their subsequent maturation occurs basipetally following the margin of the young leaf blade before bending inwards to meet with the midvein 7 DAG (94%, n=50, Figure 9B). The majority of remaining secondary veins also recruit cells to the vascular fate in a basipetal manner, being initiated from the basal

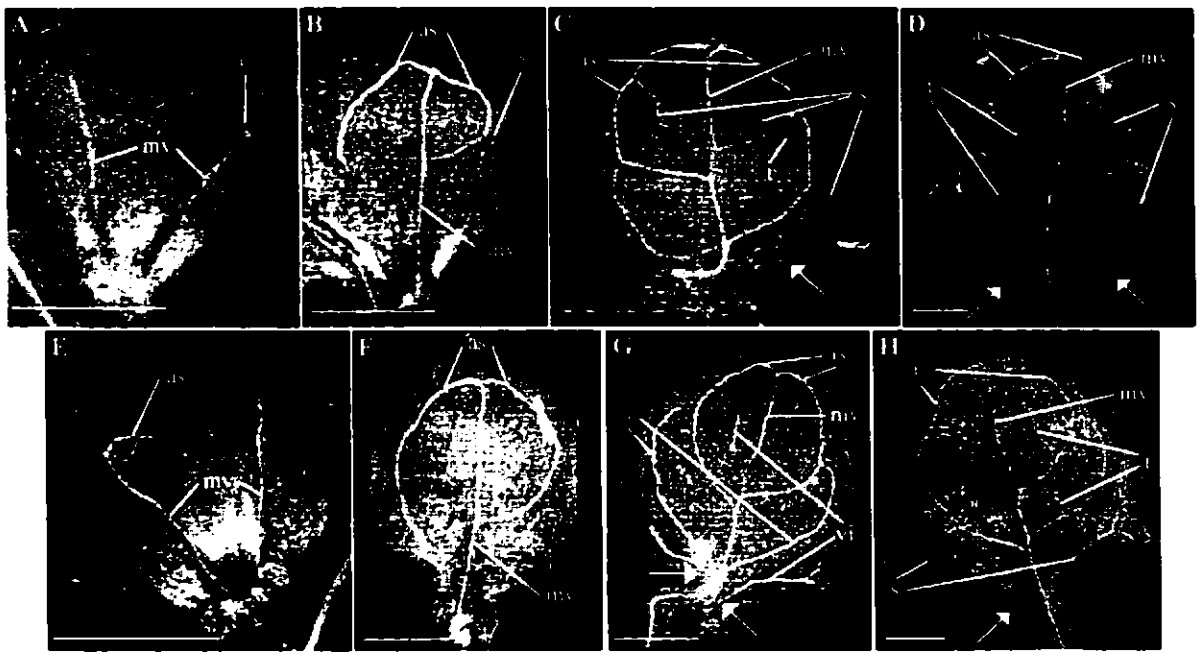

Figure 9 Vascular pattern development in the first leaf of wild type (A-D) and Fkd (E-H). Formation of the midvein (mv) and apical secondary veins (as) in wild type (A&B) is indistinguishable from Fkd (E&F). Subsequent secondary veins (s) initiate from existing vascular tissue in wild type (C) but initiate freely in Fkd (G), leading to the development of vascular islands (vi) in the immature leaf. Tertiary veins (t) fill in the developing vascular pattern being initiated in a similar manner as the secondary veins for their respective genotypes (D&H). Arrows indicate basal free vein ends. Scale, 250μm

portion of an apical secondary vein, following the margin of the leaf and bending inward to join with the midvein (Figure 9C). Additional secondary bundles commonly develop and mature within the area of the leaf blade bounded by the apical secondary vascular bundle, subdividing this area of the leaf blade. Recruitment and maturation of provascular cells of the secondary vascular bundles continues until leaf blade expansion is complete. Elaboration of the vascular pattern by tertiary and higher order veins occurs concurrently with the development of the secondary vascular bundles, making it difficult to initially distinguish between a secondary and a tertiary vein. While most secondary and tertiary veins join basally with the vascular network, the basal portion of a number of veins

(excluding quaternary vascular bundles) fails to rejoin with the vascular network 21 DAG (53% of leaves, n=38).

The vascular pattern of mature Fkd first leaves, as in wild type, consists of a midvein running along the apical-basal axis of the leaf blade with multiple secondary veins extending from the midvein. However, the apices of the majority of the secondary vascular bundles fail to join with one another and end freely near the leaf margin. Similarly, the apices of the tertiary and quaternary veins also end freely in the lamina (Figure 5B). Consistent with the failure of the vascular bundles to meet, Fkd first leaves have significantly fewer branch points and aeroles and significantly more veins ends than wild type (Table 1). Cross sections of wild type and Fkd leaves show no difference, suggesting that the tissue arrangement is unchanged in Fkd (Figure 10).

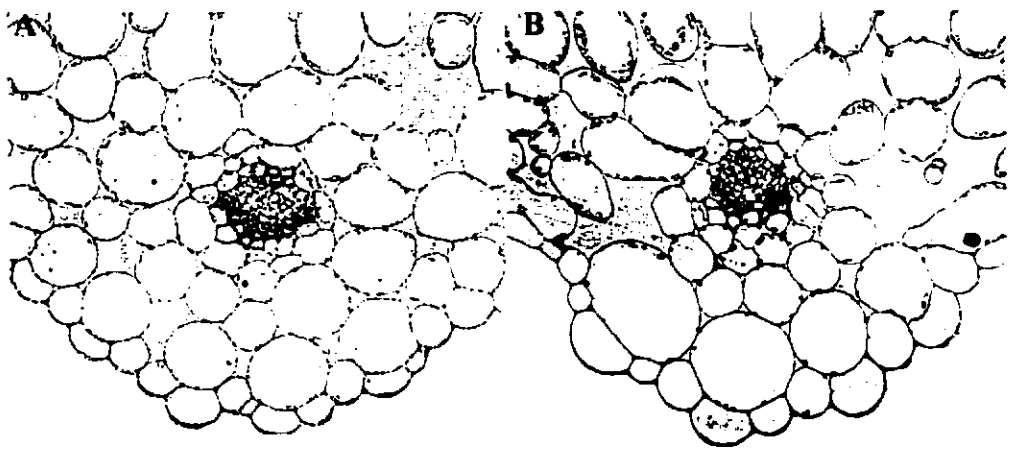

Figure 10 Cross sections through the mid-portion of wild type (A) and Forked (B) leaves taken 21 DAG.

The vasculature in Fkd leaves develops initially in a manner similar to wild type. The recruitment and maturation of provascular cells of the midvein, termination of the midvein (6 DAG, 95%, n=43), initiation of the apical secondary veins, and rejoining of the apical secondary veins with the midvein (7 DAG, 94%, n=31) is as observed in wild type (Figure 9E&F). The first noticeable defect in vasculature development of Fkd first

leaves occurs when the subsequent secondary veins initiate. Like the initiation of the secondary vascular bundles in Fkd cotyledons, the secondary veins of Fkd first leaves initiate at a point distant from the vascular tissue of the previously formed apical secondary veins (Figure 9G). Subsequent secondary and tertiary veins also initiate at a point distant from previously formed vascular tissue. Following their abnormal initiation, these secondary and tertiary veins develop in a normal fashion, maturing basipetally to join with the midvein or with secondary veins (Figure 9G&H). The abnormal initiation of the secondary and tertiary veins within the leaves results in the loss of the normal reticulate venation pattern observed in wild type *Arabidopsis*. Due to the manner of initiation of the vascular bundles, immature Fkd leaves are characterized by vascular islands (vis), vascular bundles that are disconnected from the remainder of the vascular network (Figure 9G). A number of leaves retained these vis 21 DAG (19%, n=43). The number of Fkd leaves with vis and veins failing to rejoin with the vascular network (43%, n=43) is similar to the number of wild type leaves in which the basal portion of vascular bundles fail to rejoin with the vascular network (53%, n=38), suggesting that they represent the apical non-meeting of Fkd with a normal level of basal non-meeting seen in wild type.

Vascular Pattern in Other Foliar Organs

The failure of the apical portion of the Fkd vascular bundle to initiate properly leads to a loss of the reticulate venation pattern observed in wild type *Arabidopsis* leaves and cotyledons (Figure 5 and 7C&F). Since leaves are considered the progenitors of the

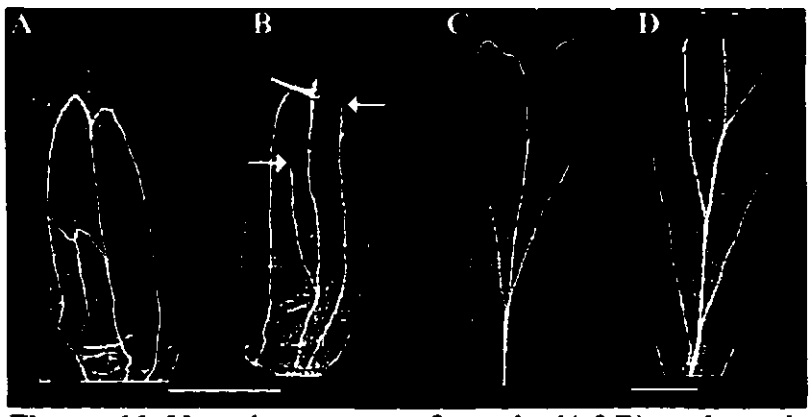

Figure 11 Vascular pattern of sepals (A&B) and petals (C&D) from wild type (A&C) and Fkd (B&D) plants. Arrows in B indicate freely ending veins in the Fkd sepal, an occurrence not normally observed in wild type sepals. Scale, 500mm

floral organs, one might expect that the loss of the reticulate venation pattern seen in Fkd leaves would also occur in the floral organs. Therefore, I examined the vascular pattern of sepals and petals. Wild type sepals (Figure 11A) usually have 3 main vascular bundles that meet in the apical region of the leaf blade to form 2 distinct loops (85%, n=81). In Fkd sepals (Figure 11B) the meeting of the 3 vascular bundles is rarely observed (11%, n=64). The petals of wild type (Figure 11C) are characterized by a single vascular trace that branches into a number of secondary veins, some of which meet at the margin (21%, n=73). In Fkd petals (Figure 11D) the vascular bundles never met in any of the petals examined (n=63).

Plant Morphology

Given the critical function of the vascular system, I expected that the loss of the reticulate venation pattern might result in an overall decrease in plant vigor. In an attempt to asses plant vigor, I compared seed germination, vegetative characteristics, flowering time, and seed production in Fkd and wild type. Germination of Fkd (98%, n=80) was

identical to that of wild type (98%, n=80). Moreover, the number of rosette and cauline leaves, the number of secondary stems, the time taken to flower, and seed production of Fkd plants was indistinguishable from wild type (Figure 12 and Table 2). Thus, despite the change from a closed to an open venation pattern, based on the parameters assessed Fkd shows no evidence of reduced vigor relative to wild type plants under the conditions tested. In order to assess the effect that the change in vascular pattern might have on leaf shape, I compared size and shape of Fkd cotyledons and leaves to wild type. Despite the observed differences in the vascular pattern, the size and shape of cotyledons and leaves are generally similar to wild type, although both are slightly longer. Additionally, Fkd cotyledons have increased surface area while first leaves have a more elongate shape (Table 3).

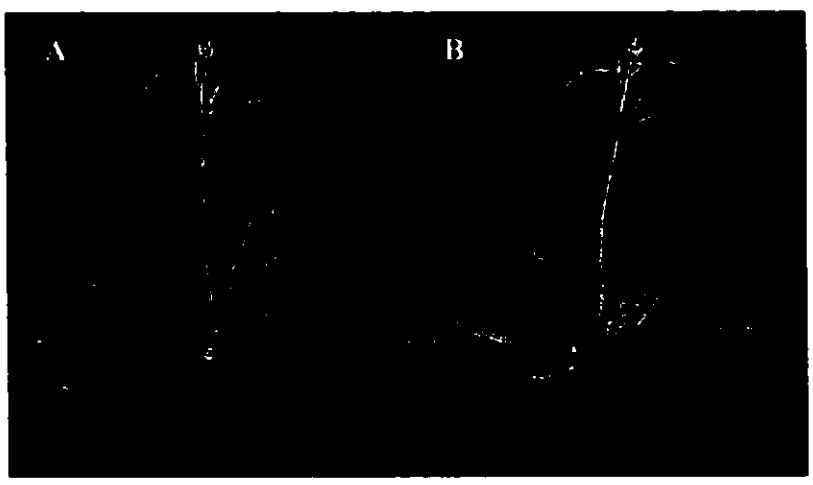

Figure 12 Wild type (A) and Fkd plants (B) 28 DAG.

Table 2 Morphological characters for wild type and Fkd plants. Values represent means ± standard error (n=31). No significant differences were observed for any of the examined characters.

	Wild Type	***Forked***
Total # of Leaves	11.3±0.3	10.8±0.3
# of Secondary Stems	3.4±0.1	3.9±0.3
Time to Flowering	21.6±0.3 days	21.2±0.3 days
Total Seed Weight	135±46 mg	147±45 mg

Table 3 Morphological characteristics of cotyledons 14 DAG and first leaves 21 DAG. Values represent means with standard error. Values following genotypes in parentheses represent number of cotyledons or leaves examined.

Cotyledon	Width (mm)	Length (mm)	Length/Width	Area (mm^2)
Wild Type (31)	2.24±0.07	2.70±0.08	1.22±0.02	4.77±0.25
Fkd (31)	2.38±0.06	2.97±0.09[a]	1.25±0.02	5.67±0.28[a]
Axr1-3 (31)	1.61±0.04[a]	1.77±0.04[a]	1.11±0.02[a]	2.25±0.1[a]
Axr1-3 Fkd (31)	2.08±0.07[b,c]	2.35±0.09[a,b,c]	1.13±0.02[a,b]	3.86±0.25[a,b,c]
Axr2 (27)	1.85±0.05[a]	1.88±0.06[a]	1.01±0.02[a]	2.71±0.17[a]
Axr2 Fkd (27)	1.90±0.04[a,b]	2.09±0.05[a,b,c]	1.10±0.02[a,b,c]	3.19±0.14[a,b]
Pin1-1 (15)	2.57±0.19[a]	2.36±0.10[a]	0.95±0.04[a]	4.63±0.56
Pin1-1 Fkd (15)	2.69±0.10[a,b]	2.88±0.09[c]	1.08±0.03[a,b,c]	5.81±0.33[a]
First Leaf				
Wild Type (31)	6.12±0.18	6.84±0.21	1.12±0.02	32.36±1.83
Fkd (31)	6.14±0.17	7.46±0.20[a]	1.22±0.02[a]	35.84±1.90
Axr1-3 (31)	3.83±0.11[a]	3.99±0.12[a]	1.05±0.02[a]	11.89±0.64[a]
Axr1-3 Fkd (31)	4.15±0.11[a,b,c]	4.54±0.11[a,b,c]	1.10±0.01[b,c]	14.29±0.74[a,b,c]
Axr2 (27)	3.89±0.10[a]	3.65±0.10[a]	0.94±0.01[a]	11.16±0.58[a]
Axr2 Fkd (27)	4.10±0.13[a,b]	3.78±0.13[a,b]	0.92±0.01[a,b]	12.29±0.78[a,b]
Pin1-1 (15)	5.33±0.377[a]	5.72±0.48[a]	1.07±0.04	24.20±3.57[a]
Pin1-1 Fkd (15)	5.44±0.43	6.42±0.42[b]	1.24±0.09	28.13±2.81[b]

[a] significantly different from wild type
[b] the double mutant is significantly different from Fkd
[c] the double mutant is significantly different from its corresponding single auxin mutant

Effect of Auxin on Fkd Plants

One explanation for the Fkd phenotype is that the mutation affects a component of auxin canalization. If correct, one might expect that Fkd plants would show altered sensitivity to changes in auxin levels, whether introduced exogenously or through double mutant combinations. Wild type plants grown on IAA or its synthetic equivalent, 2,4-D, exhibit inhibition of primary root growth and an increase in the induction of lateral roots. Therefore, a common technique to detect altered auxin response or transport is to grow plants on medium supplemented with 2,4-D and assess root response. To detect possible differences in Fkd responses to auxin, I compared root growth of Fkd to that of wild type

and to a known auxin resistant mutant, *axr1-3*. Both wild type and Fkd showed aninhibition of root growth relative to Axr1-3 but no significant difference in root growth inhibition was observed between wild type and Fkd plants (Figure 13).

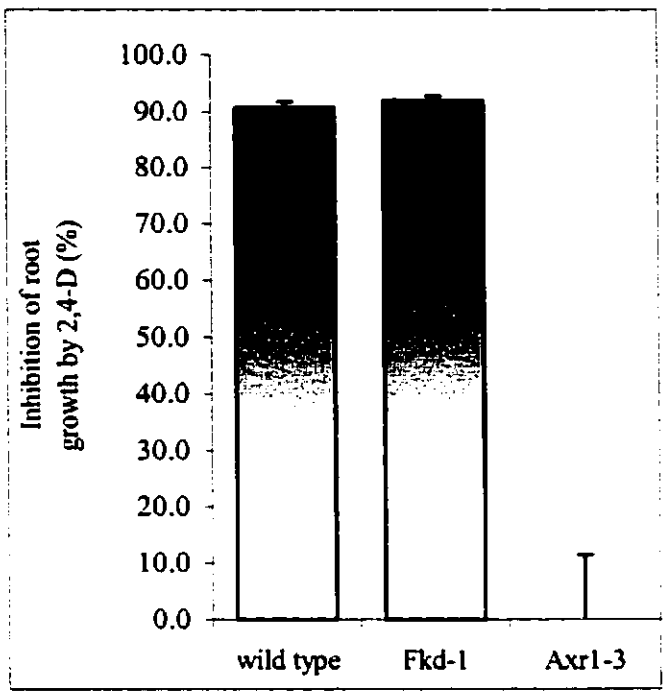

Figure 13 Mean root growth inhibition of 9-day-old wild type (n=42), Fkd (n=47), and Axr1-3 (n=57). Seedlings were transferred 5 DAG to medium containing $1.0x10^{-6}$M 2,4-D. Bars represent means with standard error.

While the auxin root assay is an effective method to screen for auxin resistance or sensitivity resulting from mutations to genes involved in the general auxin pathway, it is unlikely to detect changes resulting from mutations to genes involved in an auxin pathway specific to leaf vascular pattern formation. In an attempt to determine if *FKD* is part of a leaf specific auxin response, double mutants were generated between *fkd* and various auxin mutants known to have alterations in leaf morphology and in some cases leaf vascular patterning.

Axr1-3 Fkd

AXR1 acts in an ubiquitin-like pathway, that responds to the presence of auxin by targeting proteins for degradation (Carlos del Pozo and Estelle, 1999; Gray and Estelle, 2000). Plants mutant for *AXR1* are therefore resistant to auxin. The Axr1-3 phenotype is less severe than that of other *AXR1* mutants and includes a shortened plant height, root agravitropism, root failure to respond to auxin, decreased fertility, and smaller, wrinkled leaves (Lincoln et. al., 1990). My analysis indicates that Axr1-3 cotyledons and leaves are significantly smaller than their wild type counterparts (Table 3) and have a correspondingly simpler vascular pattern (Table 1). Axr1-3 leaves show frequent basal non-meeting (Figure 14) as indicated by a high number of freely ending veins considering the small size of the leaves (Table 1)

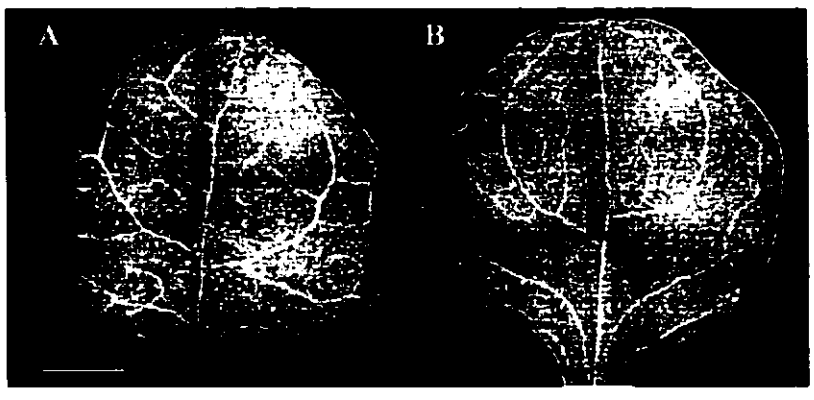

Figure 14 Vascular pattern of first leaves from Axr1-3 (A) and Axr1-3 Fkd (B) plants 21 DAG. * indicates basal non-meeting. Scale, 1mm

The F_2 progeny of the *axr1*-3 x *fkd* cross segregated 9 wild type: 3 Fkd: 3 Axr1-3: 1 novel phenotype (χ^2=0.159, n=163), consistent with the novel phenotype being the double recessive mutant. I allowed F_2 plants having the Fkd phenotype to self and two-thirds segregated the novel phenotype in their progeny, indicating that the novel phenotype is the *axr1-3 fkd* double mutant. Progeny of these plants were then analyzed.

Axr1-3 and Axr1-3 Fkd plants are similar in morphology and development (Figure 15). Both cotyledons and leaves of Axr1-3 Fkd are larger than those of Axr1-3

and first leaves are more elongate (Table 3). Relative to either single mutant, cotyledons and first leaves of Axr1-3 Fkd have significantly fewer branch points and areoles, consistent with the double mutant phenotype combining the apical non-meeting of Fkd with the basal non-meeting and the simplified vascular pattern of Axr1-3 (Table 1 and Figure 14). Relative to Axr1-3, Axr1-3 Fkd cotyledons show an increase in freely ending

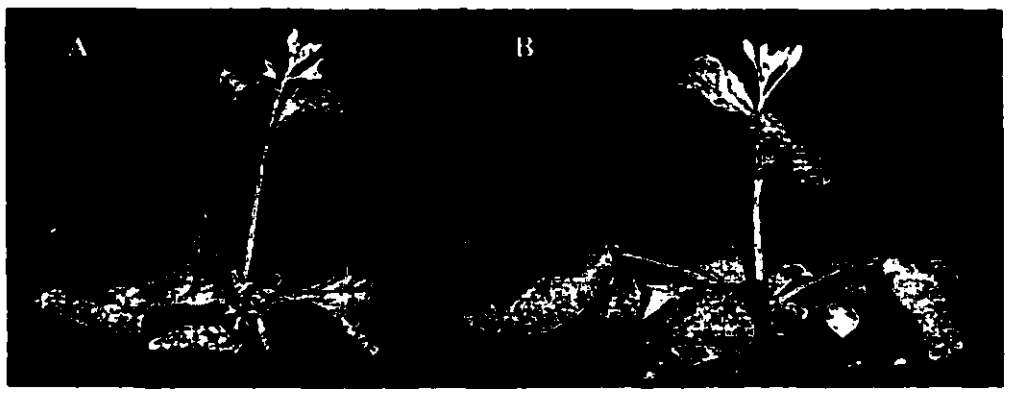

Figure 15 Axr1-3 (A) and Axr1-3 Fkd plants (B) 21 DAG.

vascular bundles consistent with the apical non-meeting in Fkd. Relative to Fkd, Axr1-3 Fkd cotyledons do not show a difference in freely ending vein number, reflecting the combined contribution of basal non-meeting and simplification typical of Axr1-3. In contrast, double mutant first leaves show a similar number of freely ending veins to Axr1-3 but significantly fewer than Fkd, consistent with the double mutant having the simpler vascular pattern associated with Axr1-3. Together, these results suggest that the double mutant phenotype is additive, combining the apical non-meeting of Fkd with the simple, basal non-meeting vascular pattern of Axr1-3.

Axr2 Fkd

AXR2 belongs to the IAA family of genes that are inducible by auxin. The dominant *axr2* mutation results in wide variety of developmental abnormalities such as

root agravitropism, shortened stem length, and failure of the root to respond to auxin (Nagpal et. al., 2000). My analysis indicates that Axr2 leaves are smaller and have a simpler vascular pattern (Tables 1 & 3, Figures 5A and 16A).

The F_2 progeny of an Axr2 x Fkd cross segregated 9 Axr2: 3 wild type: 3 novel phenotype: 1 Fkd (χ^2=4.22, n=166), consistent with the novel phenotype representing a double mutant between one recessive and one dominant mutation. I allowed F_2 plants showing the novel phenotype to self. Two-thirds segregated the Fkd single mutant phenotype alone and the remaining one-third were true-breeding for the novel phenotype. Therefore, I concluded that the novel phenotype was the *axr2 fkd* double mutant and selected a true-breeding F_3 population for analysis.

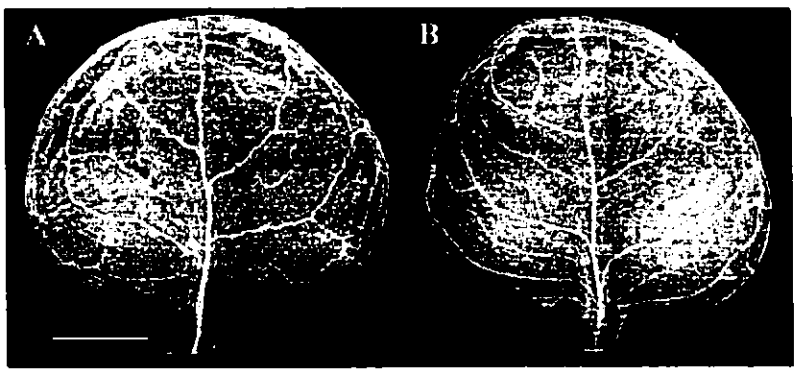

Figure 16 Vascular pattern of first leaves from Axr2 (A) and Axr2 Fkd (B) plants 21 DAG. Scale, 1mm.

The *axr2 fkd* double mutants are similar in morphology and development to Axr2 plants (Figure 17). The double mutant has cotyledons and leaves that combine the shape

Figure 17 Axr2 (A) and Axr2 Fkd plants (B) 21 DAG.

size and characteristics of both single mutants. As in Axr2, cotyledons and leaves of the double mutant are smaller and as in Fkd, the cotyledons are more elongate (Table 3). Similarly, the vascular pattern of cotyledons and leaves combines characteristics of both single mutants being simpler like Axr2 and showing reduced apical meeting like Fkd. Relative to Axr2, they show a reduced number of branch points and areoles, consistent with the apical non-meeting associated with Fkd. Relative to Fkd, they show a reduction in freely ending veins consistent with the simplification associated with Axr2 (Table 1).

Pin1-1 Fkd

PIN1 encodes an auxin transport efflux carrier and the loss of function allele, *pin1-1*, results in abnormal shoot development including sterility, occasional cotyledon and leaf fusion, and an increase in vascularization along the margins of the leaf (Mattsson et. al., 1999; Galweiler et.al. 1998). Compared to the Axr2 or Axr1-3 leaf phenotype, the Pin1-1 cotyledons and leaves are closer to wild type in size and shape, although the cotyledons are slightly rounder, the leaves are slightly smaller, and both sometimes show fusion. (Figure 18 and Table 3). The venation pattern of the cotyledons shows no difference from wild type while the leaf pattern is quite distinct, being simpler (fewer

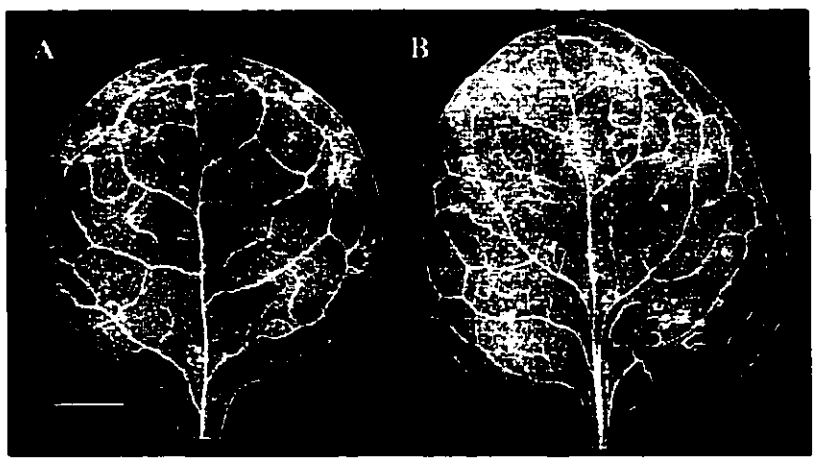

Figure 18 Vascular pattern of first leaves from Pin1-1 (A) and Pin1-1 Fkd (B) plants 21 DAG. Scale, 1mm.

areoles and branch points) and having fewer freely ending veins (Table 1). As well, the marginal vascular bundles occasionally have thickened areas indicating increased vascular tissue differentiation, as has been reported by Mattsson et. al. (1999).

The F_2 progeny of the *pin1-1* x *fkd* cross segregated 9 wild type: 3 Pin1-1: 3 Fkd: 1 novel phenotype (χ^2=5.30, n=234), consistent with the novel phenotype being the double recessive mutant. I allowed F_2 plants showing the Fkd phenotype to self, two-thirds of which segregated 3 Fkd: 1 novel phenotype indicating that the novel phenotype is Pin1-1 Fkd. I then selected an F_3 population segregating the novel phenotype for analysis.

The *pin1-1 fkd* double mutants are similar in morphology and development to Pin1-1 plants (Figure 19). As seen in other cases, the absence of FKD in a Pin1-1 background results in a more elongated cotyledon but otherwise similarly shaped and sized first leaves (Table 3). The Pin1-1 vascular pattern is epistatic to the Fkd vascular pattern in the double mutant cotyledons, such that the cotyledons show no significant difference from Pin1-1 cotyledons for any of the characters measured (Table 1). First leaves of Pin1-1 Fkd double mutants show characteristics of both single mutants. Relative to Pin1-1 they have more freely ending veins and fewer areoles, consistent with the apical non-meeting associated with Fkd. Relative to Fkd, they show a decrease in

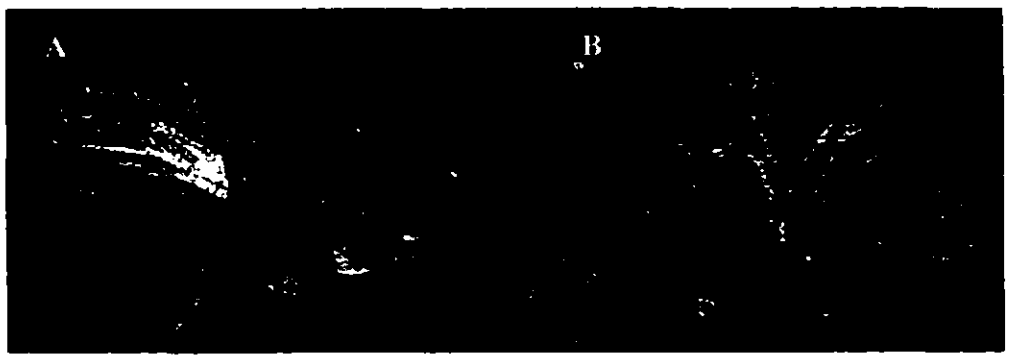

Figure 19 Pin1-1 (A) and Pin1-1 Fkd plants (B) 21 DAG.

freely ending veins, consistent with the increased vein meeting associated with the Pin1-1 phenotype. Specifically, as in Pin1-1, the increased meeting in double mutants occurs along the margin. Therefore, the vascular pattern in *pin1-1 fkd* double mutants may be described as combining the lack of apical non-meeting seen in Fkd in the internal regions of the leaf, with the increased vein meeting seen in Pin1-1 along the margins of the leaf. Interestingly, this results in a leaf that overall shows no significant difference from wild type with respect to freely ending veins.

Discussion

I have identified a mutant of *Arabidopsis*, *forked* (*fkd*), that is the result of a recessive allele of a novel gene *FORKED* (*FKD*) and results in aberrant vascular patterning within the foliar organs. Due to its recessive nature, I will assume that it represents a loss of function of the gene *FKD*. The primary defect is the initiation of newly developing vascular bundles such that they fail to join apically with previously formed veins. Due to the lack of provascular molecular markers, it is not possible to determine if the Fkd defect affects the specification of a cell to become a provascular cell or if the defect disrupts the differentiation of that cell once specified. The Fkd plants show no other significant alterations in morphology or anatomy except a slight increase in leaf and cotyledon length. A possible explanation for this increased length is that the interconnected vascular bundles of the wild type reticulate venation pattern constrain cellular expansion during leaf development, a constraint lacking in Fkd leaves. The tissues of the vascular system provide critical mechanical support to the leaf (Roth-Nebelsick et. al., 2001) and the Fkd defect may reveal a possible mechanism by which vascular patterning may influence leaf shape. Whereas *Arabidopsis* and other angiosperms normally have a closed venation pattern, the Fkd defect results in an open venation pattern reminiscent of that seen in lower vascular plants. This suggests that *FKD* may represent an important step in the evolution of the closed venation system seen in higher vascular plants.

One explanation of the Fkd phenotype is that the *FKD* gene is necessary for differentiation of vascular tissue in response to auxin. A second explanation is that *FKD*

acts independently of, but in conjunction with, auxin canalization to generate the complete vascular system. To distinguish between the two possibilities, I assessed the response of Fkd to synthetic auxin and in combination with auxin response or transport mutants. The root response of Fkd plants grown in 2,4-D is identical to that of wild type. While this suggests that *FKD* is not involved in a general auxin pathway, it does not eliminate the possibility that *FKD* action is related to auxin but leaf specific. However, all double mutants between *fkd* and the auxin response or transport mutants *axr1-3*, *axr2*, and *pin1-1* show an essentially additive phenotype suggesting that FKD acts independently of auxin. The *axr2 fkd* double mutant has an Axr2 phenotype for all aspects of plant and leaf morphology and a leaf vascular pattern that combines Fkd apical non-meeting with Axr2 simplification. The *axr1-3 fkd* double mutants have the plant and leaf morphology of Axr1-3 and a leaf vascular pattern that combines the apical non-meeting seen in Fkd with the simplified pattern and basal non-meeting seen in Axr1-3 to produce a simpler pattern with an increased number of vis. The *pin1-1 fkd* double mutants have all aspects of the Pin1-1 shoot phenotype, including cotyledon and leaf fusion. The Pin1-1 Fkd leaf vascular pattern combines the apical non-meeting typical of Fkd in the leaf interior with the increased vein meeting typical of Pin1-1 at the margin. This may be interpreted as epistasis of Pin1-1 at the leaf margin and an additive phenotype in the internal regions. Similarly, in Pin1-1 Fkd cotyledons where all the vascular bundles meet near the margin, Pin1-1 is epistatic to Fkd. This suggests that an altered distribution of auxin, such as that at the margin of Pin1-1, is able to compensate for the loss of FKD function. One explanation is that in the absence of *PIN1*, reduced basal auxin transport causes newly formed veins and the surrounding tissue to be

saturated with auxin through diffusion. Thus, cells apical to veins are exposed to auxin and may, in the absence of *FKD*, differentiate into vascular cells to create apical connections with the existing vascular bundle. A second explanation is that *FKD* results in vascular cell differentiation in response to auxin, and *fkd* is a partial loss of function allele. In the absence of *PIN*, the concentration of auxin along the margin is elevated sufficiently to induce the mutant gene product thereby restoring the wild type vascular pattern. While I cannot eliminate the second model, if *FKD* responds to auxin, I would expect to see evidence for this in the response of Fkd to exogenous auxin or to auxin response mutants. Instead, the double mutants with auxin response mutants are clearly additive and the root assay shows no evidence of decreased auxin sensitivity in Fkd plants. Therefore, I suggest that FKD is not directly involved in an auxin response and favour the first model.

My analysis suggests that *FKD* acts independently of auxin canalization in vascular pattern formation and therefore that at least two independent processes are coordinated to produce the final vascular pattern in *Arabidopsis* leaves. Consistent with the suggestion that auxin canalization cannot explain apical vein meeting, our results suggest that *FKD* represents a critical component of a mechanism that acts in conjunction with auxin canalization to establish a complete, closed vascular pattern. Thus, the Fkd phenotype may represent a leaf in which only auxin canalization is active and can provide further insight into how auxin canalization, acting alone, functions within the developing leaf.

The proposal that auxin, through canalization or polar flow, is the signal molecule determining the position of vascular bundles can explain some of the initial steps of leaf

vascular pattern formation in *Arabidopsis*. Aloni (2001) has proposed the leaf venation hypothesis based primarily on data of Mattsson et. al. (1999) and Sieburth (1999), who have shown that the leaf vasculature of wild type plants grown on media supplemented with substances inhibiting auxin transport are initially characterized by a band of vascular tissue parallel to the margin of the leaf near the apex. As the leaf develops this band lengthens following the margin of the leaf basipetally (Sieburth, 1999; Bolowkoski and Schultz, unpublished results). Not only is this evidence for the margin of the developing leaf being a source of auxin but it also suggests that the source is dynamic, starting early in leaf development at the leaf apex and moving along the margin to the leaf base progressing in the same basipetal direction as cellular differentiation (Mattsson et. al. 1999; Mattsson et. al., 1999; Aloni, 2001). A dynamic auxin source could explain the sequential development of the secondary vascular bundles within the leaves of Fkd. Initially, all cells of the leaf are similar in their capacity to transport auxin so the movement of auxin would proceed as a wave directed away from the margin (Figure 20A). The secondary veins initiate parallel to the proposed primary source of auxin, the leaf margin, due to the elevated auxin concentration in this region. Cells develop into vascular tissue distant from the margin suggesting that there is 1) a dynamic source of auxin such that a maximum is reached at a point distant from the margin and/or 2) prior competence to the vascular fate. Once the vascular tissue of the secondary vein begins to differentiate, its ability to transport auxin increases, providing a sink for auxin. This auxin sink drains auxin from the nearby leaf margin (Figure 20B), and results in a high concentration of auxin that is transported basipetally within the secondary vein. The flow of auxin would converge with the midvein near the base as the secondary vein converges,

guiding the basal portion of the developing veins to fuse together. This pattern would be reiterated at all basal meetings. The auxin resistant

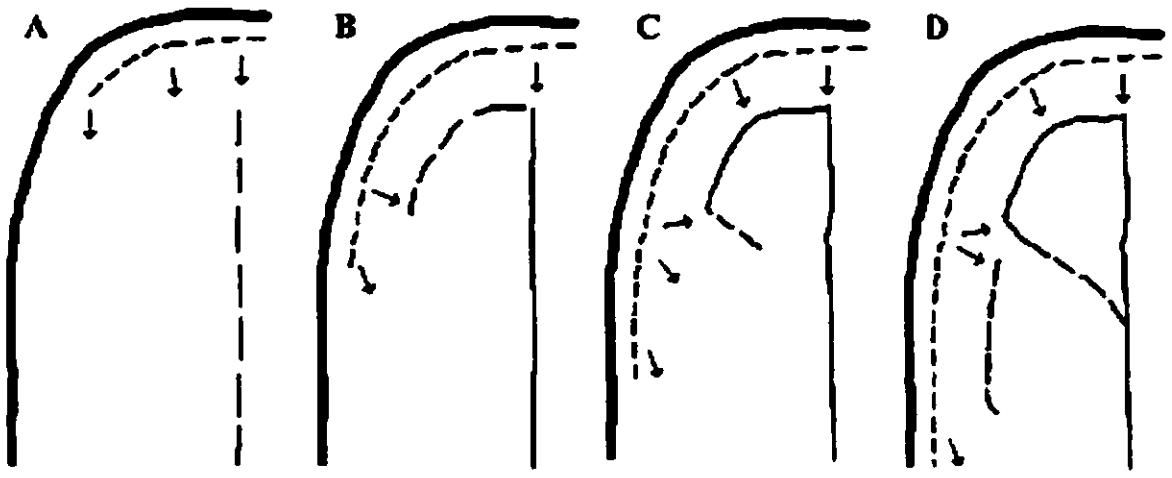

Figure 20 Vascular tissue differentiation as explained by the auxin canalization mechanism. Auxin is transported basally (arrows) from its marginal source (short dashed line) resulting in the determination of provascular tissue (long dashed line). As the provascular tissue matures (solid line) it acts as a sink for the auxin, transporting it basally out of the leaf.

mutant, *axr1-3*, shows reduced basal meeting suggesting that the concentration of auxin at the base of the vein is lower than that in the rest of the vein and is below a threshold level required for sufficient activity of the partial loss of function gene product. As thebasipetal development of the leaf progresses, a new marginal peak of auxin is created basal to the already developing secondary vein (Figure 20C). This initiates a new secondary vein parallel to the auxin source, and forms a new auxin sink (Figure 20D). As leaf expansion continues, the vascular bundles are separated and are insufficient to remove all the auxin being generated by the intervening margin. A new vein develops in this region of higher auxin concentration. While a dynamic marginal auxin source alone is sufficient to explain the vascular pattern in Fkd leaves, two aspects of the wild type phenotype require additional explanation: 1) apical meeting and 2) generation of

secondary veins that are separated from the marginal auxin source by a continuous loop of vascular tissue. The Fkd phenotype indicates that apical meeting occurs through a mechanism in which *FKD* is a major component. Generation of internal vascular bundles occurs even in the presence of apical loops and the absence of *FKD*, as demonstrated by the Pin1-1 Fkd phenotype. Therefore, there must be 1) an additional mechanism responsible for the development of veins internal to the marginal secondary vein or 2) an internal source of auxin that is insufficiently drained by existing vascular tissue as leaf expansion occurs, and induces the formation of tertiary and quaternary veins (Aloni, 2001). The reduced number of tertiary and quaternary veins seen in both Axr1-3 and Axr2 suggests that the internal development of these veins may also be in response to auxin. Moreover, the simplification suggests that the internal source of auxin may be 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.

If the Fkd leaf represents a leaf in which auxin canalization alone generates the vascular pattern, a pattern similar to that of lower vascular plants, it is plausible that the vascular pattern seen in lower plants is also the result of only the auxin canalization mechanism. Sztein et. al. (1995) have shown that primitive vascular plants are characterized by the presence of only simple IAA conjugates and as the these conjugates become more complex (as seen in higher vascular plants) there is a corresponding increase in the complexity of the 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.

The *FKD* gene may have contributed to the evolution of the closed venation pattern from the open vascular pattern (specified by auxin canalization) of more primitive plants. *FKD* enables apical meeting of the vascular bundles and functions along with auxin canalization mechanism to form a complete, closed vascular pattern. Once *FKD* is cloned, it will be interesting to determine if functional homologs exist in ancestral species suggesting that this second pathway has arisen multiple times in evolution to generate the closed venation pattern seen in some members of the gymnosperms and ferns.

Surprisingly, despite the alteration of the foliar vascular pattern of Fkd plants to a more ancestral state, the apparently normal morphology and development of this mutant suggests that overall plant metabolism and therefore the transport of water and photosynthates in Fkd is unaffected or that affects can be compensated by alterations to other mechanisms. A character that has arisen several times during evolution is assumed to confer some advantage to the plant, such as increased efficiency of water and photosynthate transportation (Roth-Nebelsick, 2001). Since growth conditions for all experimental procedures were ideal (i.e.; 21°C, 60% relative humidity) it is possible that

the benefits of a closed venation pattern are only apparent under conditions of stress. It will be interesting to determine if Fkd plants are more adversely affected by drought or increased temperature than wild type plants.

References

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

Arnott, H.J., (1959) Anastomoses in the venation of *Ginko biloba*. American Journal of Botany 46, 405-411.

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

Berleth, T. and Sachs, T., (2001) Plant morphogenesis: long distance coordination and local patterning. Current Opinion in Plant Biology 4, 57-62.

Bowe, M.L., Coat, G, and dePamphilis C.W., (2000) Phylogeny of seed plants based on all three genomic compartments: Extant gymnosperms are monophyletic and Gnetales' closest relatives are conifers. Proceedings of the National Academy of Sciences 97(8),4092-4097.

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

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

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

Chin, J., Wan, Y., Smith, J., and Croxdale J. (1995) Linear aggregations of stomata and epidermal cells in *Trandescantia* leaves: evidence for their group patterning as a function of the cell cycle. Developmental Biology 168, 39-46.

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

Cunninghame, M.E. and Lyndon, R.F. (1986) The relationship between the distibution of periclinal cell divisions in the shoot apex and leaf initiation. Annals of Botany 57, 737-746.

Dermen, H. (1953) Periclinal chimeras and origin of tissues in stem and leaf of peach. American Journal of Botany 40, 154-168.

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.

Doyle, J.A. and Donoghue. (1987) The importance of fossils in elucidating seed plant phylogeny and macroevolution. Review of Palaeobotany and Palynology 50, 63-95.

Eames, A.J. (1936) Morphology of vascular plants: lower groups (Psilophytales to Filicales). McGraw-Hill Book Company, Inc, New York and London.

Esau, K. (1965) Vascular differentiation in plants. Holt, Rinehart, and Winston Inc, New York.

Fleming, A.J., McQueen-Mason, S., Mandel, T., and Kuhlemeier C. (1997) Induction of leaf primordia by the cell wall protein expansin. Science 276,

Foard, D.E. (1971) The initial protrusion of a leaf primordium can form without concurrent periclinal cell divisions. Canadian Journal of Botany 49, 1601-1603.

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

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. W.H Freeman and Company, New York.

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

Gray, W.M. and Estelle M. (2000) Function of the ubiquitin-proteosome pathway in auxin response. Trends in Biochemical Sciences 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. American Journal of Botany 33, 532-543.

Hall, L.N. and Langdale, J.A. (1996) Molecular genetics of cellular differentiation in leaves. New Phytologist 132, 533-553.

Hemerly, A., de Almeida Engler, J., Bergounioux, C., Van Montagu, M., and Ferreira, P. (1995) Dominant negative mutants of the cdc2 kinase uncouple cell division from iterative plant development. European Molecular Biology Organization Journal 14, 3925-3936.

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

Jackson, D., Veit, B., and Hake, S. (1994) Expression of maize *Knotted1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. Development 120, 404-413.

Koch, A.J. and Meinhardt, H. (1994) Biological pattern formation: From basic mechanisms to complex structures. Review of Modern Physics 66, 1481-1507.

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

Lincoln, C., Britton, J.H., and Estelle, M. (1990) Growth and development of the *axr1* mutants of *Arabidopsis.* Plant Cell 11, 1071-1080.

Lincoln C., Long J., Yamaguchi, J., Serikawa, K., and Hake, S. (1994) A *knotted-1* like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell 6, 1859-1876.

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

Long, J.A., Moan, E.I., Medford, J.I., and Barton, M.K. (1996) A member of the knotted class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. Nature 379, 66-69.

Lyndon, R.F. (1970) Rates of cell division in the shoot apical meristem of *Pisum*. Annals of Botany 37, 1-17.

Lyndon, R.F. (1972) Leaf formation and growth of the shoot apical meristem. Physiologie Vegetale 10, 209-222.

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

Matsuoka, M., Ichikawa, H., Saito, A., Tada, Y., Fujimura, T., and Murakami, Y. (1993) Expression of a rice homeobox gene causes altered morphology of transgenic plants. Plant Cell 5, 1039-1048.

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 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 Physiology 123, 563-573.

Nelson, T. and Dengler, N. (1997) Leaf Vascular Pattern Formation. The Plant Cell 9, 1121-1135.

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

Palme, K. and Galweiler, L. (1999) PIN-pointing the molecular basis of auxin transport. Current Opinion in Plant Biology 2, 375-381.

Poethig, R.S. and Sussex, I.M. (1985) The cellular parameters of leaf development in tobacco: a clonal analsys. Planta 165, 170-184.

Raven, P.H., Evert, R.F., and Eichhorn, S.E. (1999) Biology of Plants. 6th ed. W.H. Freeman and Company, New York.

Reinhardt D., Wittwer, F., Mandel, T., and Kuhlemeier, C. (1998) Localized upregulation of a new expansin gene predicts the site of leaf formation in the tomato meristem. The Plant Cell 10, 1427-1437.

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

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J., and Estelle, M. (1997) the TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast GRR1p. Genes and Development 12, 198-207.

Sachs, T. (1981) The control of patterned differentiation of vascular tissues. Advances in Botanical Research 9, 151-262.

Sach, T. (1989) The development of vascular networks during leaf development. Current Topics in Plant Biochemistry and Phsiology 8, 168-183.

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

Satina, S., Blakeslee A.F., and Avery A.G. (1940) Demonstration of the three germ layers in the shoot apex of *Datura* by means of indiced polyploidy in periclinal chimeras. American Journal of Botany 27, 895-905.

Satina, S. and Blakeslee A.F. (1941) Periclinal chimeras in *Datura stramonium* in relation to development of leaf and flower. American Journal of Botany 28, 862-871.

Sharman, B.C. (1942) Developmental anatomy of the shoot of *Zea mays* L. Annals of Botany 6, 245-284.

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

Smith, L.G., Greene, B., Veit, B., and Hake, S. (1992) A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. Development 116, 21-30.

Smith, L.G., Hake, S., and Sylvester, A.W. (1996) The tangled mutation alters cell division orientation throughout maize leaf development without altering leaf shape. Development 122, 481-489.

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

Steynen, Q.J, Bolokowski, D.A, and Schultz, E.A. (2001) Alteration in flowering time causes accelerated or decelerated progression through *Arabidopsis* vegetative phases. Canadian Journal of Botany 79, 657-665

Steeves, T.A. and Sussex, I.M. (1989) Patterns in plant development 2nd ed. Cambridge University Press, New York.

Sylvester, A.W., Cande, W.Z., Freeling, M. (1990) Division and differentiation during normal and *liguleless-1* maize leaf development. Development 110, 985-1000.

Sztein, A.E., Cohen, J.D., Slovin, J.P., and Cooke, T.J. (1995) Auxin metabolism in representative land plants. American Journal of Botany 82, 1514-1521.

Trass, J., Bellini, C., Nacry, P. Kronenberger, J., Bouchez, D., and Caboche, M. (1995) Normal differentiation patterns in plants lacking microtubular preprophase bands. Nature 375, 676-677.

Tsiantis, M. and Langdale, J.A. (1998) The formation of leaves. Current Opinion in Plant Biology 1, 43-48.

Tsuge, T., Tsukaya, H., and Uchimiya, H. (1996) Two independent and polarized processes of cell elongation regulate leaf blade expansion in *Arabidopsis thaliana* (L) heynh. Development 122, 1589-1600.

Uggla, C., Moritz, T., Sandberg, G., and Sandberg, B. (1996) Auxin as a positional signal in pattern formation in plants. Proceedings of the National Academy of Sciences 93, 9282-9286.

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 Zentralblatt 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 Physiology 17, 715-721.

Willmer, C. and Fricker, M. (1995) Stomata. Chapman and Hall, London.

Winter, K.U., Becker, A., Munster, T., Kim, J.T., Saedler, H., and Theissen, G., (1999) MADS-box genes reveal that gnetophytes are more closely related to conifers than to flowering plants. Proceedings of the National Academy of Scienes 96, 7342-7347.

Yang, M. and Sack, F. (1995) The *too many mouths* and *four lips* mutations affect stomatal production in *Arabidopsis*. The Plant Cell 7, 2227-2239.