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Vascular Patterning

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INTRODUCTION

The vascular system of the plant is a network of cells that interconnects all major plant organs. A vast body of literature describes both the patterns of vascular development and a variety of experimental approaches to study factors controlling vascular development (for example (Shininger, 1979; Sachs, 1981; Aloni, 1987), however the use of molecular genetic approaches using Arabidopsis is a relatively recent entry. The vast majority of Arabidopsis references cited in this review were published within the last five years, and the significance of this work is reflected by the publication of several recent reviews on vascular development that prominently feature work on Arabidopsis (Nelson and Dengler, 1997; Berleth and Mattsson, 2000; Berleth et al., 2000; Dengler and Kang, 2001; Ye, 2002).

In this review, we focus on two major aspect of vein patterning: the apical/basal pattern (found in stems and roots); and the reticulate patterns (found in all broad flat organs) (Figure 1). The vascular system is composed of two tissues, xylem and phloem, which function to transport water and solutes, or sugars, respectively. Each of these tissues is composed of multiple specialized cell types. In comparison to information gained from the zinnia *in vitro* system for tracheary element differentiation from leaf mesophyll cells (see Fukuda, 1997; Roberts and McCann, 2000) for review), work to date using Arabidopsis has not contributed greatly to our knowledge of vascular cell type differentiation. Consequently, only a brief description of vascular cell types is included (below) It is considered that the molecular genetic analysis of vascular cell type differentiation is likely to be a productive area of future work involving Arabidopsis.

It is important to point out that alterations in vascular patterning can also occur as a secondary consequence of an otherwise unrelated defect. Examples include the *leafy cotyledon1* (*lec1*), *abscissic acid insensitive3* (*abi3*) and *fusca3* mutants, which all exhibit altered seed development and premature vascular development (Holdsworth et al., 1999). It is unclear what information such mutants will contribute to our understanding of vascular patterning per se, consequently they are not considered further in this review.

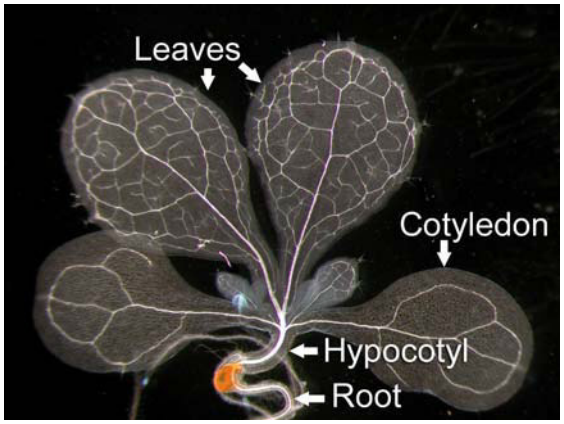

Figure 1. Vein Pattern in an Arabidopsis Seedling. A cleared wild type (L.*er*) 11 day old seedling, photographed using dark-field optics. The tracheary elements refract light, and show up as bright white lines. Note the reticulated patterns of veins in the cotyledons and leaves, and the linear pattern in the hypocotyl and roots.

Vascular tissue and differentiation

The first visible indication of vascular specification is the appearance of procambial cells, which are identified by their narrow elongated cell shape (Figure 2) (Jurgens, 1994; Scheres et al., 1995; Busse and Evert, 1999a). Procambial cells are the progenitors of all primary vascular cells and can be detected very early in organ development (e.g. in leaf primordia less than 40µm long; (Mattsson et al., 1999; Sieburth, 1999). The stage of development just prior to procambium is termed provascular tissue (Esau, 1965; Xia and Steeves, 1999, 2000). Provascular cells are hard to visualize and follow through development, so they have been the focus of few investigations. However, as a wider selection of marker genes are described, research on provascular cell specification and function should become more tractable,

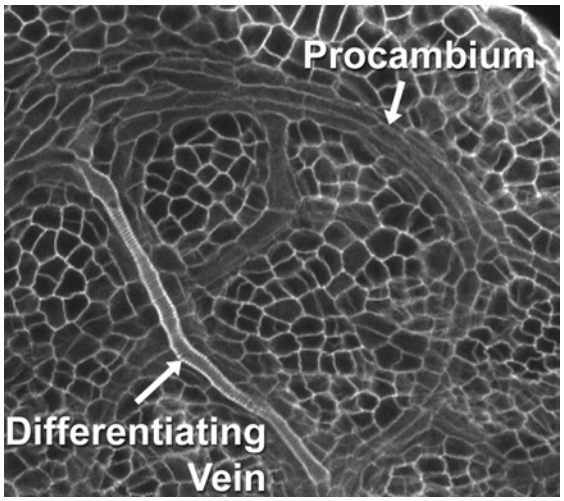

Figure 2. Vascular Differentiation in a Developing Leaf. A confocal image of a differentiating leaf. In the differentiating vein, striations corresponding to tracheary element secondary cell wall are clearly visible. Adjacent to and connected with the differentiating vein, the elongated procambial cells are also clearly visible.

and this area is likely to be of critical importance for understanding vein patterning.

Primary xylem is composed of two cell types: the tracheary elements and the xylem parenchyma. Tracheary elements (TEs) are elongated cells connected end to end and form the water conducting vessels. In common with most flowering plants, TE differentiation in Arabidopsis entails patterned cell wall deposition, followed by programmed cell death and the formation of a perforation plate between adjacent members of a vessel (Figure 3) (Mittler and Lam, 1995; Turner and Hall, 2000). The first xylem cells to differentiate, the protoxylem, are characterized by annular or spiral thickening, whilst later forming metaxylem elements have more complex reticulate or patterned areas of secondary cell wall deposition (Dharmawardhana et al., 1992; Busse and Evert, 1999a; Mahonen et al., 2000). Xylem parenchyma cells are relatively short cells with uniform secondary cell walls. These cells do not undergo cell death, but are located within the xylem (Figure 3).

Phloem structure and its implication for sugar transport in the veins of mature leaves has been carefully described (Haritatos et al., 2000). Phloem is composed of companion cells, sieve tube elements, and phloem parenchyma cells. The primary conducting cells is the sieve tube element. In Arabidopsis these cell types are typical in that they contain few organelles and are linked to one another by a sieve plate. The sieve tube elements are found with adjacent companions cells, which make up the sieve element – companion cell complex (SE-CCC). The phloem parenchyma cells, when in contact with companion cells, frequently form cell wall ingrowths characteristic of transfer cells specialized for sugar

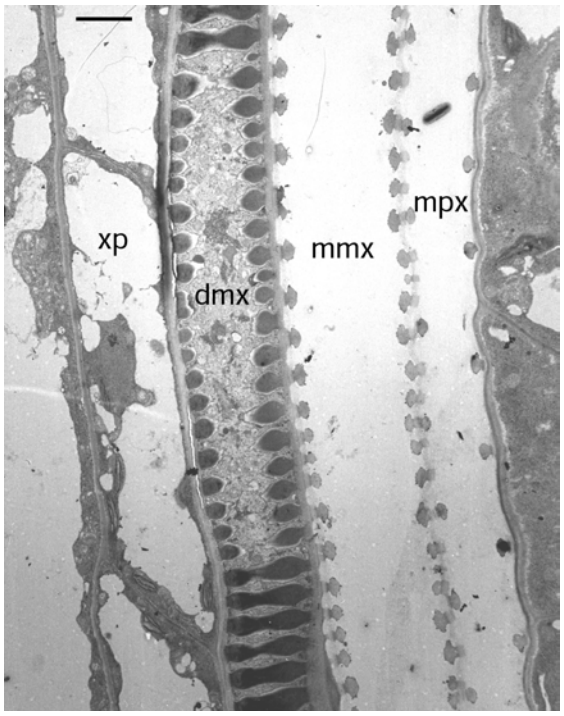

Figure 3. Xylem development in a developing inflorescence stem. Transmission electron micrograph showing developing xylem in a vascular bundle from an Arabidopsis inflorescence stem. Mature protoxylem (mpx), mature metaxylem (mmx), developing metaxylem (dmx) and xylem parenchyma (xp) are indicated. Size bar = 7μm.

loading (Dolan et al., 1993; Dolan and Roberts, 1995; Busse and Evert, 1999a; Haritatos et al., 2000).

Tools for following Vascular Development

Because vascular tissues are relatively inaccessible and because the identification of vascular cell types can be difficult, cell-specific and tissue-specific reporter gene lines provide important tools. Two of the most useful reporter constructs are derived from the Athb-8 and TED3 genes. These reporter genes confer GUS expression in procambium and early differentiating tracheary elements, respectively (Demura and Fukuda, 1994; Baima et al., 1995; Igarashi et al., 1998). These constructs are useful for identification of early stages of vascular development (for example see (Koizumi et al., 2000), and are likely to be especially useful for distinguishing the similarly-shaped procambial cells at early stages of TE differentiation. An enhancer trap line, VPP, confers GUS staining at early stages of leaf development. GUS staining is first observed in isodiametric cells, and then at slightly later stages in procambial cells; suggesting that this reporter

gene may identify provascular cells (Holding and Springer, 2002).

Vascular tissue reporter genes related to vascular function are also available. A GAS::GUS (melon Galactinol Synthase) reporter gene confers GUS staining in sucrose-loading (minor) leaf veins, and timing of expression corresponds to the organ's transition from a sink to a source (Haritatos et al., 2000). Also, a GFP fusion to the sucrose symporter promoter (*AtSUC2::GFP*) is transported through the phloem, and unloaded from the phloem in a manner consistent with the presumed photoassimilate path (Imlau et al., 1999). Together, these constructs provide a broad range tools for studies ranging from vein pattern establishment to vascular function.

In addition to reporter genes, histochemical stains are also useful for identification of vascular cell types. The lignified walls of mature xylem cells are readily stained with a variety of stains such as phloroglucinol (Clifford, 1974) and Toluidine blue (see Figure 7 for an example) that identify lignin. While these stains only indicate a lignified cell wall, other stains such as Maules reagent discriminate between xylem and interfascicular cells based on their lignin composition (Chapple et al., 1992). In addition, staining with Aniline blue causes the phloem to fluoresce yellow under UV due to the Aniline blue binding to callose deposited on the sieve plates. Together, these constructs and stains provide a broad range tools for studies ranging from vein pattern establishment to vascular function.

Models and Molecules

A large number of studies indicate roles for the plant hormone auxin in vascular tissue development. In the major sections of this review, influences of auxin will be discussed where contributions have been made. A synthesis of these auxin results, discussed in terms of vein pattern models, will be presented in the discussion.

APICAL BASAL PATTERNING

Apical/basal patterning, in its simplest form, is the pattern that connects the shoot apical meristem with the root apical meristem. In this section, we consider establishment of the apical/basal vascular pattern during embryogenesis, then consider post-embryonic apical/basal vascular patterning in the root and shoot separately.

Apical/Basal Patterning in the developing embryo

Mature embryos contain procambial cells that will differentiate into xylem and phloem following germination. Consequently during the course of embryogenesis, the vascular pattern is established, but the differentiation of vascular cell types arrest at the procambial stage. Several excellent descriptions of embryogenesis in Arabidopsis are available (Mansfield and Briarty, 1991; Jurgens, 1994). In addition, a description of embryogenesis occurs elsewhere in this volume and we recommend readers refer to that chapter for embryo staging information.

The first indication of procambial cell formation is during the late globular stage of embryogenesis, when the isodiametric cells of the lower tier of the embryo elongate in the apical-basal axis. These elongated cells are a distinct feature of the heart-stage, and are present in the centre of the developing embryo. Further elaboration of the procambium occurs as the embryo completes differentiation, with the fully differentiated embryo having its apical/basal axis defined by a hypocotyl that contains a variable number of procambium cell files bounded by a single layer of pericycle (Busse and Evert, 1999a).

In most non-embryonic tissue, vascular development is a continuous process in which procambium development is followed by differentiation of the xylem and phloem. In embryos however, further differentiation of the procambium is halted during embryo maturation and only proceeds when growth resumes following seed germination. How procambium differentiation arrests during embryogenesis remains unclear, but it is tightly linked to the same processes that regulate seed maturation. This point is emphasized by observations on a series of mutants, such as *abscissic acid insensitive* (*abi*), that develop seedling characteristics within the seed (Holdsworth et al., 1999). For example *abi3* mutants exhibit premature activation of the apical meristem and also exhibit differentiation of the xylem (Holdsworth et al., 1999). There are currently no characterised mutants that separate the development of the vascular tissue within the embryos from the development of the embryo as a whole.

Development of the root and hypocotyl vascular tissue following germination

Vascular pattern in the root and hypocotyl exhibit many similarities, however their developmental origins differ: hypocotyl vascular tissue is formed from pre-existing procambial cells (see above) whereas root vascular tissues are derived from activity of the root apical meristem. Within the hypocotyl the first sign of vascular differentiation is the formation of two files of protophloem elements, a process that occurs almost synchronously throughout the embryo approximately 2-3 days after germination (Busse and Evert, 1999a). The two protophloem cell files are located on opposite sides of the vascular cylinder, adjacent to the pericyle. Although the

precise procambial cells that will give rise to the protophloem are not distinguishable by any morphological feature in the embryo, they can be identified from their relationship to other cell types, and from the known location of the phloem at later stages of development (Busse and Evert, 1999a). Xylem differentiation is observed at a similar stage, but generally lags behind protophloem development. Two files of protoxylem elements form first; these are located on opposite sides of the cylinder from one another, and alternate with the protophloem (Figure 4). Xylem development proceeds from the two poleson outside of the cylinder towards the centre until the xylem forms a continuous plate. This plate bisects the vascular cylinder and separates the developing phloem into two regions (Figure 4). This centripetal pattern of xylem development, known as exarch, is typical of angiosperms. The organisation of the vascular cylinder is normally classified by the number of xylem poles and consequently the organisation of the hypocotyl in Arabidopsis is described as diarch. The organisation of primary vascular tissue in the root is similar to the hypocotyl and also exhibits a diarch pattern of organisation (Dolan et al., 1993). Root vascular tissue development occurs acropetally and consequently the earliest stages of root vascular development are always closest to the root tip. Vascular cell lineages have been determined in the developing root using serial sections (Mahonen et al., 2000). The xylem cell files form an axis of 4 or 5 cells that derive from initials very close to the quiescent centre. These cell files form the xylem cell plate that separates the rest of the vascular cylinder into two domains in a manner similar to the hypocotyl (see above). Each of these domains derive from 2-5 initials and are composed of both the phloem and the undifferentiated procambial cell lineages (Figure 5). The exact number and pattern of cell divisions that occur in the procambial cells appears to vary between different seedlings. Consequently, no strict cell lineage is apparent in contrast to the invariant pattern of cell lineage seen in the endodermis and outer layers (Mahonen et al., 2000).

It remains unclear how the diarch pattern of vascular tissue development (seen in both the hypocotyl and the root) is controlled. In the mature embryo no morphological features distinguish the cells destine to become the first protophloem elements (Busse and Evert, 1999a); that they arise in a predictable position following germination leaves the possibility that their cell fate may be determined during embryogenesis. That the root pattern is so similar to that of the hypocotyl also suggests that patterning information may be transmitted from the hypocotyl to the developing root, similar to the transmission of other root patterning signals (Vandenberg et al., 1995).

In addition, the relatively simple diarch organisation of root and hypocotyl primary vascular tissue is probably a reflection of the narrow vascular cylinder in Arabidopsis. Several lines of evidence have linked the number of xylem poles with root diameter. For example, pea roots normally exhibit a triarch pattern. When grown in culture however, root tips occasionally showed a reduced root diameter and a reduction to a diarch or monarch pattern within the vascular cylinder. Similarly, pea vascular cylinders regenerated from decapitated pea roots and cultured in the presence of auxin exhibited an increase in diameter and an increase to a hexarch pattern of vascular tissue. In all cases the organisation of the vascular tissue was related to the diameter of the apical meristem (Torrey, 1955, 1957). What these experiments fail to distinguish, however, is whether the controlling feature is the vascular cylinder diameter itself, or proximity of xylem poles. It is clear, however, that whatever the number of xylem poles they are always organized in a symmetrical pattern that maintains the maximum possible spacing between xylem poles (Barlow, 1984).

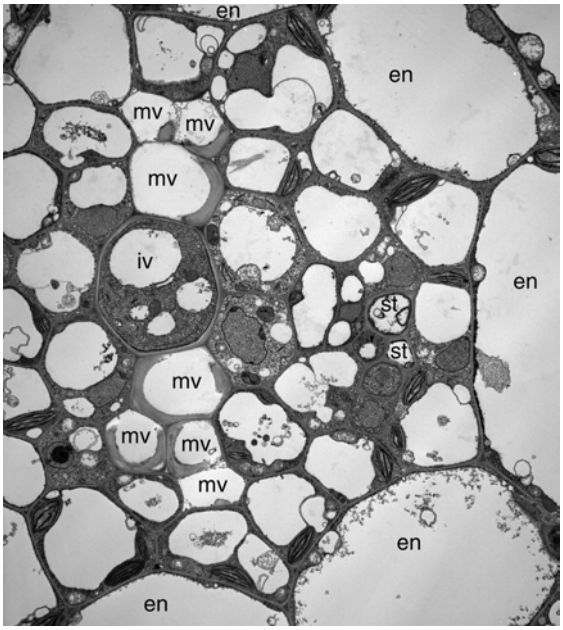

Figure 4. Organisation of the vascular tissue within the vascular cylinder of the developing hypocotyl. Transmission electron micrograph showing that primary vascular development is almost complete; maturation of the immature xylem vessel (iv) will link the mature xylem vessels (mv) to form a plate that spans the vascular tissue. The phloem tissue indicated by the presence of sieve tubes (st) and procambial cells are located in two domains either side of the xylem plate. The entire vascular cylinder is surrounded by the endodermis (en).

Genetic insight into root vein pattern comes from analysis of the *woodenleg* (*wol*) mutant. In the primary root and lower hypocotyl, the vascular cylinder of *wol* plants has fewer cells files than wild type. In addition the vascular cylinder lacks phloem and is composed solely of protoxylem (Scheres et al., 1995; Cano-Delgado et al., 2000). This phenotype has been traced back to a defect

in periclinal divisions in the embryonic procambium that results in a reduced number of cell files in the vascular cylinder. This defective division pattern is maintained in the root apical meristem during early root formation, however lateral roots that form from the hypocotyl of *wol* plants have an intermediate vascular phenotype (Scheres et al., 1995). In addition, double mutants with *fass* have been examined. The *fass* mutation causes an increase in number of cells in the vascular cylinder (Torresruiz and Jurgens, 1994) and *wol:fass* double mutants can form both xylem and phloem (Scheres et al., 1995). Furthermore in these double mutants the xylem is composed of both protoxylem and metaxylem (Mahonen et al., 2000). These results demonstrate that WOL is not essential for either phloem or metaxylem development, but that the absence of these cell types in the primary root of *wol* mutants is a consequence of the altered division pattern (Mahonen et al., 2000).

The *WOL* gene has recently been cloned and shown to encode a novel two component regulator that functions as a cytokinin receptor (Mahonen et al., 2000; Inoue et al., 2001). *WOL* gene expression is detected early in the globular embryo, within a small group of cells destined to become the vascular cylinder, and is maintained within the vascular cylinder during the course of embryogenesis (Mahonen et al., 2000).

Taken together, these results suggest that cytokinin signaling is required for asymmetric divisions within the developing procambium of the root. Some supportive evidence for the role of cytokinin in controlling cell divisions within the vascular tissue has come from studies of the *schizoid* (*shz*) mutant. *shz* plants exhibit a pleiotrophic phenotype that includes an large number of adventitious meristems and a proliferation of tissue within the vascular cylinder (Parsons et al., 2000). Preliminary analysis has suggested that *shz* may be involved in the regulation of active cytokinin levels.

It is unclear why the resulting reduction in cell number within the vascular cylinder of *wol* plants leads to all the cells becoming specified as xylem. One suggestion is that specification of xylem cells temporally precedes phloem specification, even though phloem differentiation precedes that of the xylem (Scheres et al., 1995). Understanding why in *wol* plants, with fewer procambial cells, only xylem forms should give some insight into how these two tissue types are specified.

A number of additional mutants, identified on the basis of abnormal leaf venation pattern (see section below), also exhibit root/hypocotyl vascular tissue defects. Six *vascular network* (*van*) mutants with altered leaf vein patterns described by Koizuma and co-workers (Koizumi et al., 2000) all have defects in the organisation of

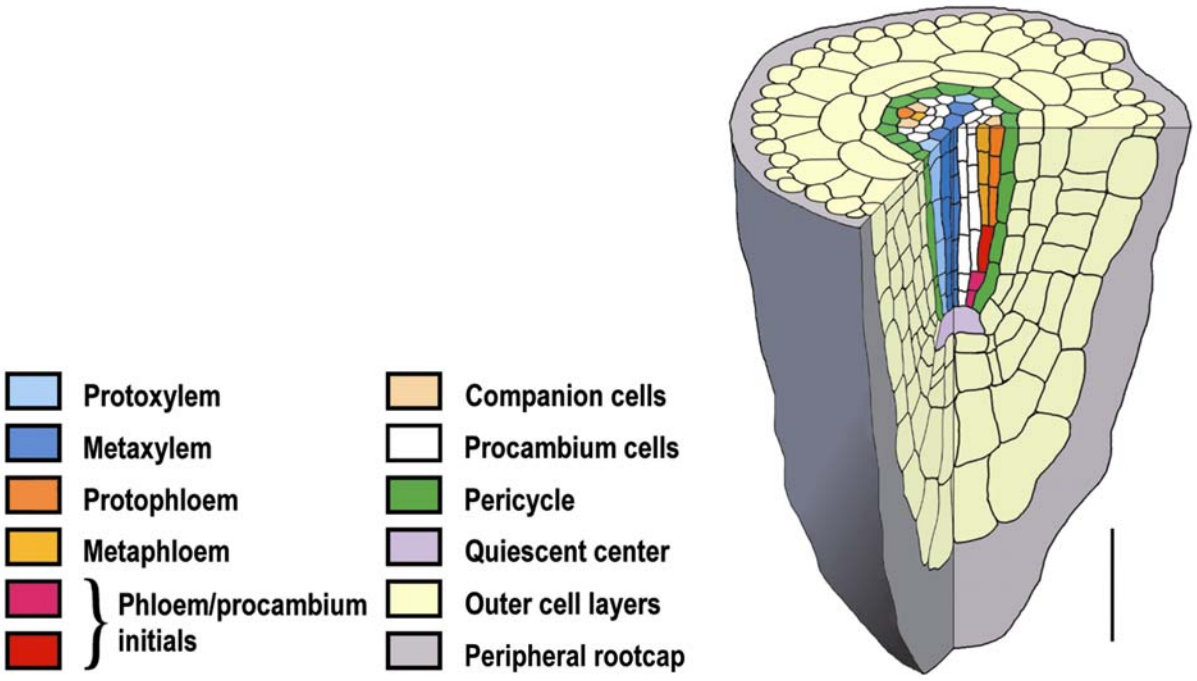

Figure 5. Diagram to illustrate the development of the vascular tissue within the primary root. Reprinted with permission from (Helariutta et al., 2000)

vascular tissue in the hypocotyl. In addition, at least one of these mutant (*van1*) had abnormal xylem development in roots, with the vessels improperly aligned with one another (Koizumi et al., 2000). The clearly defined and relatively easily visualized pattern of vascular development within the growing root is likely to provide additional mutants in the future and form the basis of a productive system for studying vascular tissue development.

Hypocotyl/Root Secondary Growth

Secondary growth is the increase in vascular cylinder diameter that results in a larger organ diameter. In Arabidopsis, secondary growth of the hypocotyl and root can be initiated as soon as 6 days after germination (Busse and Evert, 1999b). Initiation of secondary growth occurs when groups of procambial cells located between the primary xylem and the primary phloem poles begin to divide periclinally (Dolan et al., 1993; Dolan and Roberts, 1995; Busse and Evert, 1999b). These cell divisions eventually lead to a continuous ring of narrow thin walled cells (the cambium). Divisions within the cambium generate the cells that differentiate into secondary xylem (toward the inside of the organ) and secondary phloem (toward the outside of the organ) (Figure 6). Secondary growth in the hypocotyl occurs in two phases. Initially, only vessels differentiate within the secondary xylem, however subsequent growth results in the formation of both vessels and fibers (Chaffey et al., 2002).
The switch to secondary growth is accompanied by changes in cell surface epitopes that may act as useful markers for cell differentiation (Dolan and Roberts, 1995). Other markers for cell differentiation may derive from the construction of cDNA libraries specifically from developing secondary xylem and bark (Zhao et al., 2000). In tree species, careful measurements of auxin concentration have demonstrated a steep concentration gradient of auxin around the cambium zone with the highest concentration of auxin located within the cambium. Furthermore, there is a strong correlation between the radial width of the concentration gradient and cambial growth rates (Uggla et al., 1996; Uggla et al., 1998). These results indicate a role for IAA in controlling growth and/or differentiation in the cambial zone. Furthermore a detailed analysis of expression pattern of nearly 3000 poplar ESTs using microarrays has been performed during xylem formation from the cambium (Hertzberg et al., 2001). Due to the size limitations, it is unlikely that these kinds of experiments will be easily repeated in Arabidopsis, however, the poplar EST analysis reveals a list of genes for which Arabidopsis homologs are attractive candidates for reverse genetics. Consequently, it is likely that secondary growth in the Arabidopsis root and hypocotyl will be a productive model, helping to elucidate the pathways that control cambial growth and differentiation of secondary vascular tissue.

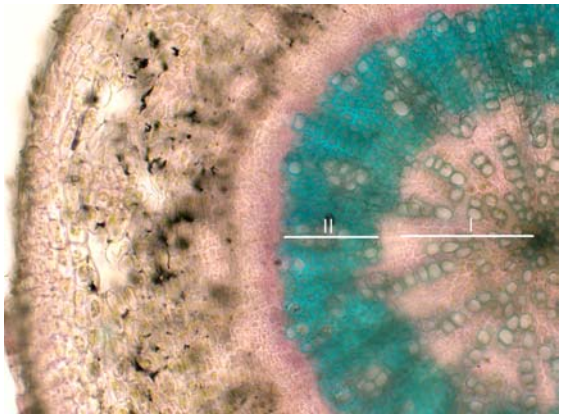

Figure 6. Secondary growth in the Arabidopsis hypocotyls . Toluidine blue stained, freehand sections differentiate between the lignified cell walls (blue) and the primary walls (red). Two distinct stages of development are marked (I and II). In the first phase only vessels are formed, whereas in stage II both vessels and fibers are formed.

Vascular patterning in the shoot

Vascular tissue in the shoot (rosette and inflorescence) is present as a ring of separate veins. This pattern of stem veins serves two essential functions: it provides an apical/basal axis for material transport and it connects with the veins of lateral organs. This pattern contrasts with that of the hypocotyl and root, and the region at the apex of the hypocotyl where vascular interconnections between the root and shoot are formed is called the

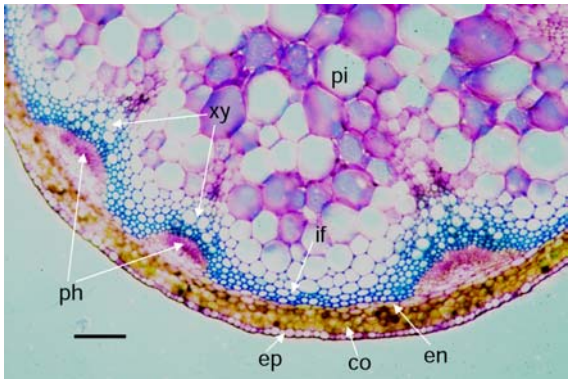

Figure 7. Organization of wild type Arabidopsis inflorescence stems following primary vascular tissue development.Position of the phloem (ph), xylem (xy), pith (pi), interfascicular cells (if), endodermis (en), cortex (co) and epidermis (ep) are marked. Size bar = 100nm.

transition zone. A detailed study of this transition is available elsewhere (Busse and Evert, 1999b).

Following primary vascular tissue development, the stem contains 5-8 vascular bundles arranged in a ring; between the vascular bundles are interfascicular cells (Figure 7). Although the interfascicular cells are lignified, they are not normally considered to be part of the vascular tissue. The interfascicular cells are composed solely of fibers with pointed ends that form following tip growth (Burk et al., 2001; Zhong et al., 2001). Differentiation of the fibers appears to be regulated, at least to some extent, independently of the xylem (Zhong et al., 1997; Zhong and Ye, 1999). The entire cylinder of cells comprising the vascular tissue and the interfascicular cells is surrounded by a single layer of cells variously described as an endodermis or a starchy sheath (Figure 7). Whilst this cell layer lacks a casparian strip, a characteristic of endodermis, it shares many characteristic with the endodermis of the hypocotyl/root (Wysocka-Diller et al., 2000).

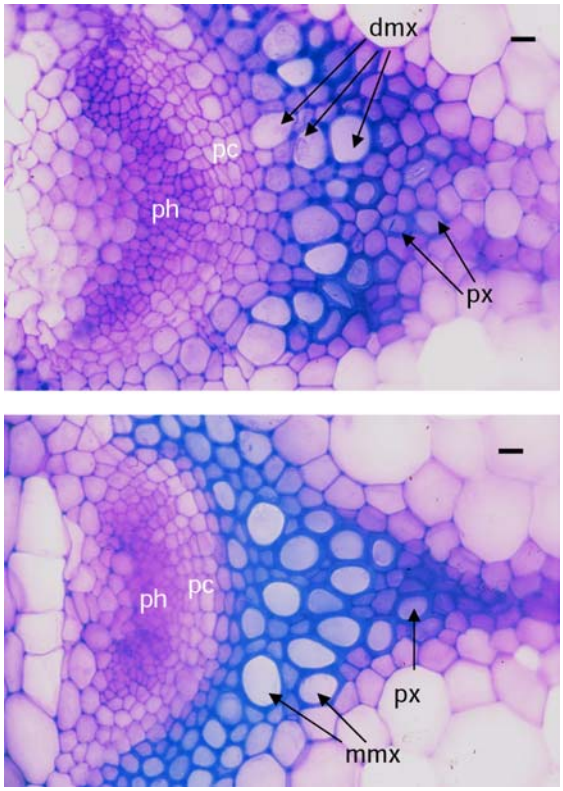

Figure 8. Differentiation of vascular tissue within the inflorescence stems. Sections showing vascular bundles close to the apical meristem (top) and base (bottom) of a single inflorescence stem. Phloem (ph), procambium (pc), protoxylem (px), mature metaxylem (mmx) and developing metaxylem (dmx) are indicated. Size bar = 12.5nm

The differentiation of vascular cell types within stem vein procambium has also been studied. The first protoxylem, with characteristic helical or annular patterns of cell wall thickening, differentiate on the side of the vascular bundle closest to the centre of the stem, and proceeds centrifugally towards the outside of the stem (Figure 8) (Turner and Somerville, 1997). This pattern of xylem differentiation is described as endarch, and contrasts with exarch pattern of xylem differentiation observed in the root in which xylem differentiation starts at the outside, and proceeds toward the center. Phloem differentiation is difficult to visualize, but appears to precede xylem development. The mature phloem occupies the outer part of the vascular bundle. Upon completion of primary vascular development only one or two layers of undifferentiated procambium remain that separate the xylem and phloem (Figure 8).

A major issue in stem vein patterning is how the vascular tissue of lateral branches and organs connects with the veins of the stem. The compressed nature of the Arabidopsis rosette makes this a difficult region in which to study this problem, but the connection between the first rosette leaf veins and the stem has been described (Busse and Evert, 1999b).

Mutants with altered stem vascular tissue organization

Few mutants are available that radically alter patterns of vascular tissue in the stem while maintaining a relatively normal apical meristem. The *cvp1* mutant, isolated on the basis of its abnormal cotyledon vein pattern, occasionally has shorter inflorescence internodes and increased xylem formation (Carland et al., 1999). The most striking mutant, however, is the *amphivasal vascular bundle* (*avb*) mutant (Zhong et al., 1999). *avb* mutants exhibit two patterning defects. Firstly, stems of *avb* plants have increased numbers of veins. Many of these are abnormally localized within the pith. These pith-localized veins arise from inappropriate branching of normally-positioned veins within the stem. Secondly, avb mutants also exhibit an altered organization of vascular cell types within these veins. Instead of the collateral pattern (xylem toward the inside, phloem toward the outside), *avb* vascular bundles showed amphivasal arrangement with the phloem surrounded by a ring of xylem (Figure 9). This amphivasal vascular cell arrangement also extends to leaf veins. The linking of vein branching and radial organization of vascular cell types in the *avb* mutant suggests that similar positional signals may be used in both processes. The small number of mutants with severe shoot vascular defects might indicate that many of the genes controlling shoot vein pattern also have essential functions early in development, preventing the later shoot phenotype from being observed.

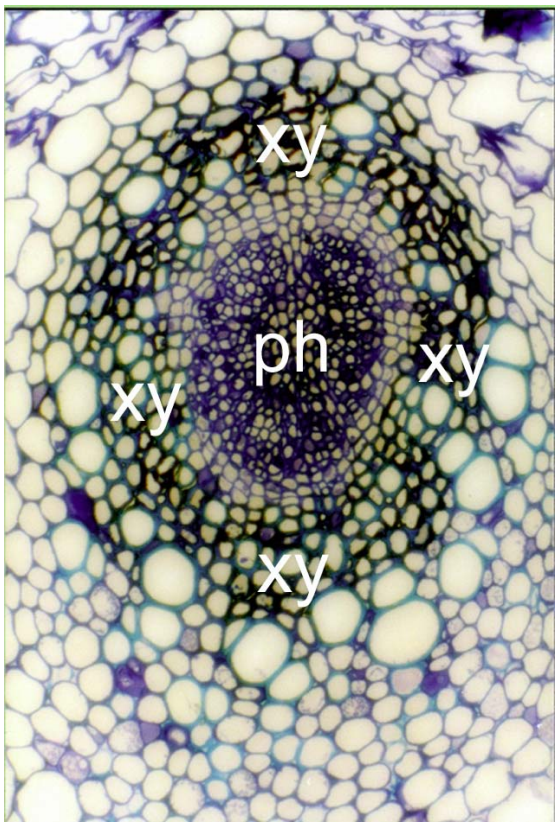

Figure 9. Organisation of vascular tissue within a vascular bundle from the stem of an *avb* mutant plant. Xylem (xy) and phloem (ph) are indicated. Reproduced with permission from (Zhong et al., 1999).

Relationship between the shoot apical meristem and vascular development

Formation of the vascular tissue within the shoot is intimately linked with the activity of the apical meristem. This point is illustrated by mutants such as *clavata* (*clv*), which exhibit an enlarged apical meristem that initiates an increased number of lateral organs. Characterization of *clv1, clv2* and *clv3* has demonstrated that these mutants exhibit an increased number of initials within the apical meristem (Brand et al., 2000; Weigel and Jurgens, 2002). The *clv* mutants also exhibit enlarged, flattened stems that contain increased numbers of stem veins (Figure 10). Nevertheless the spacing and organization of vascular bundles in *clv1* stems are strikingly similar to wild type. Whether the alteration in the number of vascular bundles is related to the increase in primordia initiation or whether it is related to the alteration in stem morphology is unclear at present.

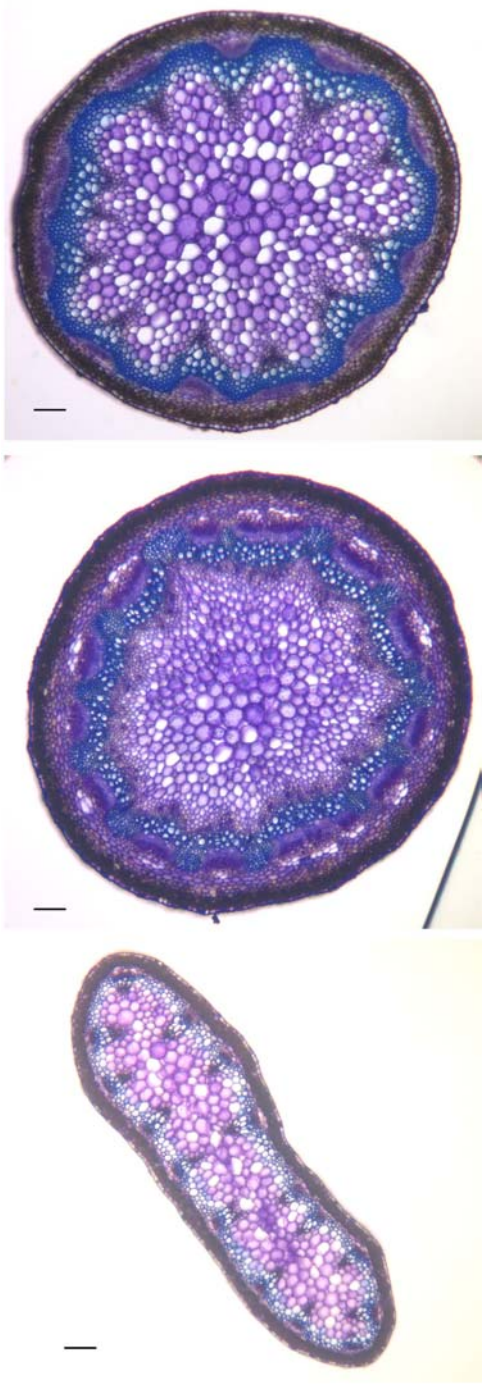

Figure 10. Organisation of vascular tissue in the Arabidopsis inflorescence stem. Sections from the base of mature stems of wild type (top), pin1-1 (middle) and clavata1-8 (bottom) are shown. Size bar = 100nm

The role of vascular tissue in meristem activity has been debated for many years. It has often been suggested that the growing primordia are a source of auxin that induces vascular development (see Shininger, 1979 for review). Other workers, however, recognize a provascular stage that precedes the initiation of primordia. This provascular stage is considered to be under the control of the apical meristem, while further differentiation of the vascular tissue is dependent upon the formation of leaf primordia (Xia and Steeves, 1999, 2000). Evidence that organization of vascular bundles within the stem is independent of organ initiation comes from studies of the *pin* (Okada et al., 1991) and *mp* (Przemeck et al., 1996) mutants. Both *pin1* and *mp* mutants fail to develop lateral organs on their inflorescence stems, and instead make only a limited number of "pin-like" structures. Nevertheless, the inflorescence stem of *mp* plants does form vascular tissue. The organization of these tissues is very similar to the wild type, albeit the xylem tracheary elements are not properly aligned (Przemeck et al., 1996). Similarly, *pin1* mutants also produce vascular tissues organized into distinct vascular bundles in their stems, although there is a slight increase in the size of vascular bundles (Okada et al., 1991) (Figure 10), These observations provide strong evidence that the initiation and development of vascular bundles within the stem are independent of the formation of lateral organs.

Further studies on the *pin1* apical meristem have demonstrated that initiation of flowers may be induced by the local application of auxin. Similar experiments have been performed on a "pin-like" structure induced by the culturing vegetative tomato apical meristems in the presence of the auxin transport inhibitor NPA (NPA-pins) (Reinhardt et al., 2000). When NPA is removed leaf development is resumed but the initial pattern of leaf initiation exhibits a virtually random phylotaxis (Reinhardt et al., 2000). These experiments demonstrate that preexisting leaves are not required for leaf initiation per se, but that they are required for correct positioning of new leaves. It is suggested that the influence of pre-existing leaves on the site of new leaf initiation is mediated by their vascular tissue, possibly by defining the sites of auxin transport to the meristem. The authors go on to suggest that the vascular pre-pattern within the apex defines the site of new leaf initiation (Reinhardt et al., 2000).

Vernoux et al. (2000) studied meristems from *pin1* plants using a variety of markers for organ identity. Based upon these studies the authors suggest a model in which organ formation is initiated by high concentration of auxin while auxin transport is also required for the "lateral inhibition" that prevents new primordia formation nearby (Vernoux et al., 2000). A similar model has been proposed to control the spacing of vascular bundles (Sachs, 1981, 1991) (see below). Further evidence supporting the idea that vascular development may regulate the pattern of phylotaxis has come from the studies of the *pinhead/zwille* (*pnh/zll*) gene. *PNH* is required for leaf development (see below), but is also required for normal development of the shoot apical meristem and is highly expressed in vascular tissue (Lynn et al., 1999). Studies on *PNH* in the apical meristem reveal that *PNH* expression marks the site of vascular development below the site of future primordia development. The appearance of *PNH* expression precedes alteration in the expression of other marker genes such as *shoot meristemless* that normally mark the site of the next primordia formation (Lynn et al., 1999). This result suggests that the site of vein development that connects new primordia to the existing vascular structure is defined prior to any signs of primordia formation within the shoot apical meristem.

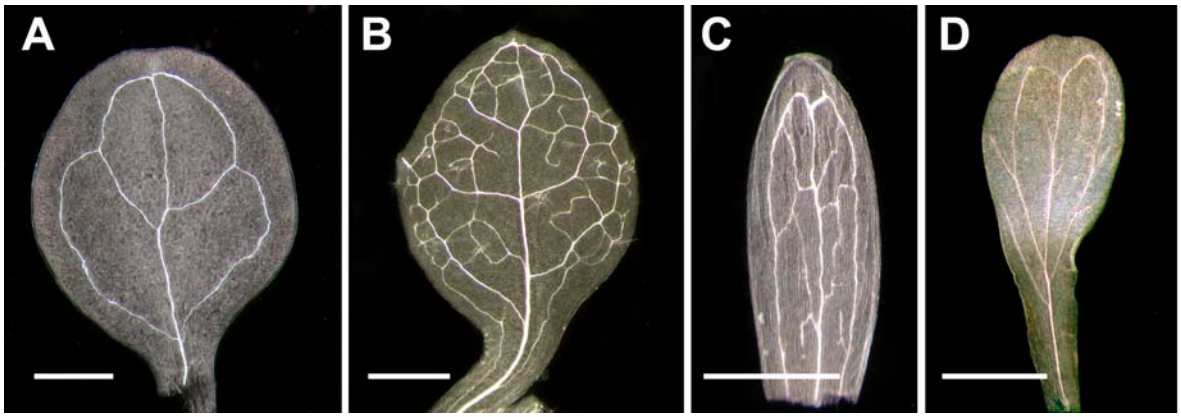

Figure 11. Reticulate Vein Patterns in Organs of Arabidopsis Plants. All tissue was fixed, cleared in chloral hydrate, and illuminated using dark field optics. (A) Cotyledon; (B) Leaf; (C) Sepal; (D) Petal. Xylem (x) and phloem (p) are indicated. Size bars = 1 mm

Secondary growth in the stem

The transition to secondary growth in the stem is less clear than that in the root hypocotyl axis. A distinct phase of primary growth – prior to onset of secondary growth – is distinguished by a ring of vascular bundles linked by a ring of interfascicular cells (Chapple et al., 1992; Przemeck et al., 1996; Turner and Somerville, 1997; Mattsson et al., 1999). Secondary growth initiates both within the bundles (between the xylem and phloem) and in the interfascicular region from division in the starchy sheath (Talbert et al., 1995; Altamura et al., 2001; Baima et al., 2001). The exact timing of when initiation occurs is likely to vary depending on the growth conditions, the proximity to a leaf trace and the genotype studied (Altamura et al., 2001). Secondary growth within the vascular bundle cambium gives rise to additional cell files of phloem and xylem. In contrast, secondary growth within the interfascicular region gives rise to secondary phloem on the outside of the stem and additional lignified, but flat-ended cells toward the center of the stem (Altamura et al., 2001). The absence of vessels or cell types unambiguously recognizable as xylem suggests that secondary growth in the interfascicular region is distinctly different from the secondary growth associated with wood formation and that of the hypocotyl/root axis (described above). Some mutants, however, such as acaulis5 (acl5), exhibit enormously decreased internodal elongation accompanied by a proliferation of vascular tissue that resembles increased secondary growth (Hanzawa et al., 1997). The ACL5 gene encodes a spermidine synthase (Hanzawa et al., 2000). The gene contains a putative auxin response element and is upregulated by auxin. One possibility is that spermidine is a signalling molecule that acts downstream of auxin. This analysis is complicated by the fact that acl5 mutants affect the expression of genes regulated by gibberellin (Hanzawa et al., 1997). Consequently, an alternative explanation is that spermidine is involved in regulating the interactions between different hormone pathways (Hanzawa et al., 2000). Further analysis of these kinds of mutants is likely to be productive.

RETICULATE PATTERNING

Broad flattened organs, such as leaves and petals, have veins that are distributed across these planar organs in a well-dispersed and interconnected network. Representative images of these vein patterns are shown in Figure 11. Within these veins, vascular tissues are generally arranged collaterally, with the xylem is located on the upper (adaxial) side of the leaf (Figure 12). In this section, we consider together the patterning of veins in cotyledons, leaves, sepals, and petals. With the exception of petals, these broad flattened organs are photosynthetic, and have an acute physiological need for a well-dispersed vein pattern. Efficient photosynthesis requires gas exchange, which is accompanied by water loss. The lost water must be resupplied via the xylem. In addition, the end-product of photosynthesis (sugar) is supplied to the growing tissues via the phloem. Thus, underlying physiological requirements necessitate all cells having proximity to a vein.

Patterns of leaf veins have been described extensively, and a leaf vein pattern classification system categorizes patterns based on the numbers and relative positions of different vein orders (Hickey, 1973; Hickey, 1988). The vein orders are a hierarchical classification of veins based on their size (diameter) and relative positions. The primary vein is located centrally and has the thickest vein diameter (Figure 13). Secondary veins branch from the primary vein,

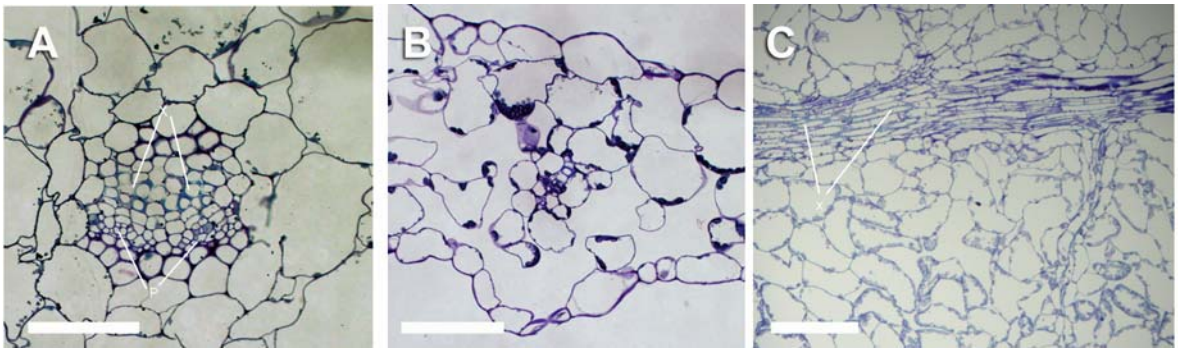

Figure 12. Vascular Cell Type Organization in the leaf. (A) Leaf primary vein, from a transverse section taken approximately half way down the leaf. (B) leaf minor vein. (C) Paradermal section of the primary and a secondary vein. The xylem (xy) and phloem (ph) are indicated. Size bars: A, B=50 µm, C=100 µm.

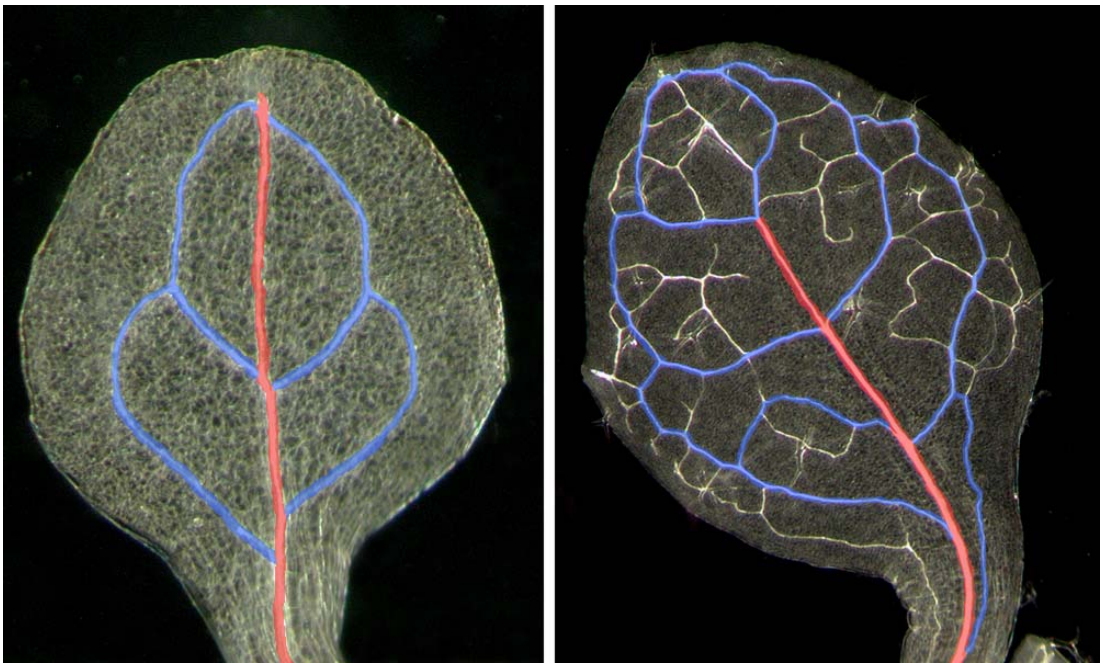

Figure 13. Vein Order Designation in Arabidopsis Cotyledons and Leaves. These dark field images of cleared cotyledons (A) and leaves (B) have been colorized so that the primary vein shows in red and the secondary veins are blue. Tertiary and quaternary veins have not been colored. Size bar =1 mm

and at the branch site are narrower than the primary vein. Similarly, tertiary veins branch from secondary veins, quaternary veins branch from the tertiary veins, and each is narrower than the vein from which it branched. This convenient vein order nomenclature, however, is not without problems. Within a single vein, proximal positions (closest to the leaf base) are wider in diameter than more distal positions (closest to the leaf apex) (shown clearly in Candela et al. 1999). Furthermore, a typical vein branches multiple times and at branch point the vein becomes narrower. As a result, at positions near to the leaf margins, vein order assignment is problematic. In addition, small (tertiary sized) veins are sometimes seen branching from the primary vein. Finally, this system of vein order classification is not applicable for mutants with disrupted vein patterns such as veins that lack connections with other veins, have abnormal diameters, or have abnormal positions.

Despite the problems with vein order terminology, the normal leaf vein pattern in Arabidopsis is easily recognized (Figures 11 and 13). A single primary vein extends toward the leaf apex, and 6 to 10 secondary veins branch from the primary. The secondary veins extend toward margin, bend, and then run parallel to the margin. At the adjacent secondary vein, the two secondary veins connect, resulting in an overall pattern of closed loops (with the space defined by a closed loop being called an areole). The tertiary and quaternary veins either subdivide the areoles defined by the 1˚ and 2˚ veins, define areoles between the secondary vein and the margin, or form free endings within an areole (these free-ending veins are also called veinlets). Candela et al., (1999) analyzed leaf vein patterns of 266 Arabidopsis accessions, only two of which exhibited marked differences in vein pattern. This well-conserved and relatively easily visualized pattern of vein development in Arabidopsis has facilitated the identification of a number of mutants.

Cotyledon vein pattern is simpler than that of the leaf (Figure 11A). In Arabidopsis, cotyledons contain a single primary vein that extends though the center of the organ. In addition, there are generally four secondary veins that form four closed loops (areoles). However, the number of fully differentiated cotyledon secondary veins varies. In approximately half the cotyledons, one or both of the proximal secondary veins fails to connect with the primary vein at its proximal end. Petal vein pattern (Figure 11D) is similar to that of the cotyledon in that there is a central primary vein and a small number of secondary veins. However, positions of petal secondary veins are somewhat variable. Sepal vein pattern (Figure 11C) differs from the other broad flat organs in that there is no single large central vein. Instead, typically three veins run the length of the organ, uniting adjacent to the organ's apex, and the space defined by these veins is further subdivided by additional veins. However, clear distinction between primary or secondary sepal veins is not obvious as all the veins have similar diameters.

Reticulate Vein Pattern Development

The establishment of reticulate vein patterning has been studied most extensively for leaves, and both quantitative and developmental approaches have been taken. Measurements of leaf area, branch point number, vein lengths, and vascular cell volume as a function of organ age or size have shown that the relative abundance of vascular tissue is highest early in development (Pyke et al., 1991; Candela et al., 1999). Qualitative and developmental analyses have described the sequence of events during leaf vascular tissue development (Telfer and Poethig, 1994; Nelson and Dengler, 1997; Kinsman and Pyke, 1998; Mattsson et al., 1999; Sieburth, 1999). Elongated procambial cells in the center of the developing leaf are the first visible sign of leaf vascular tissue. Primary vein formation initiates below the leaf primordium and proceeds toward the leaf apex (acropetal), followed rapidly by acropetal differentiation of vascular cell types. Secondary veins first become apparent as the primary vein differentiates; pairs of these veins typically develop sequentially starting at the leaf apex. Procambium for tertiary and quaternary veins is first observed at the leaf apex, at a time when approximately half the secondary veins have formed. Further development of the tertiary and quaternary veins proceeds basipetally. Thus, leaf vein development occurs in three waves, and in two different directions, suggesting that at least some elements of the signalling pathways may be distinct.

Vascular tissue development in other flattened organs is less well characterized. Cotyledon vein patterning (the appearance of procambium) occurs during embryogenesis, whereas differentiation of cotyledon vascular cell types takes place after germination. The precise timing of cotyledon procambium differentiation has not been fully characterized, however cotyledon procambium is evident in torpedo-staged embryos (West and Harada, 1993). Developmental analyses of sepal and petal vein patterning are also lacking. Although each of these organs shows a distinctive pattern of veins, it is likely that at least some aspects of their vein patterning pathways are shared, as mutations in several genes affect vein patterning in multiple different organ types (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000; Semiarti et al., 2001).

Auxin Transport Inhibitor Studies Of Leaf Vein Patterning

Auxin has been implicated in several aspects of vascular tissue development, including vein patterning (Aloni, 1995). The influence of polar auxin transport on vein patterning in Arabidopsis has been explored using polar auxin transport inhibitors (Aloni, 1995; Mattsson et al., 1999; Sieburth, 1999) (Figure 14). These inhibitors have a profound effect on vein pattern, including the elimination of the acropetally-differentiating primary vein and the ability to independently disrupt secondary and tertiary vein differentiation when the inhibitors are applied after leaf initiation. However, it is possible that vein pattern changes were not direct effects of the presumed auxin transport block, as a recent study has shown that the dynamics of many membrane proteins are affected by polar auxin transport inhibitors (Geldner et al., 2001). Nevertheless, vein pattern in the *pin1-1* mutant, which has a defect in one of the auxin efflux carrier genes, is similar to that of the inhibitor-grown plants (Mattsson et al., 1999). These studies highlighted the critical importance of developing methods for analyzing auxin transport paths in developing tissues.

A major step toward this goal was recently achieved by the development of an immunocytochemical approach for directly assaying the level of free IAA (Avsian-Kretchmer et al., 2002). Using both this technique and auxin-inducible GUS reporter lines, auxin levels in the meristem and developing leaves were assessed in both control and polar auxin transport-inhibited plants. The results indicated that auxin is transported to both the shoot apical meristem and the young leaf primordia, and suggested that the cotyledons might be the auxin source. Older leaf primordia synthesized their own auxin. The pattern of auxin accumulation and presumed movement match the pattern of vascular development in both wild type and auxin transport-inhibitor grown plants. These data are consistent with auxin's presumed role as a positive regulator of vein patterning.

Genetic approaches to Understanding Reticulate Vein Patterning

To identify genes that function in vein patterning, mutants

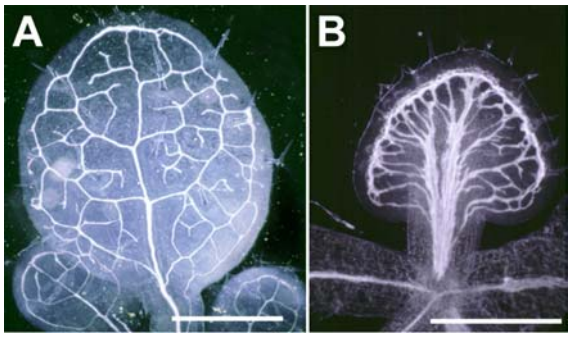

Figure 14. Polar Auxin Transport Inhibitors Affect Leaf Vein Patterning. Dark field images of cleared leaves showing the leaf vein patterns. (A) Leaf from a plant grown in normal growth medium; (B) Leaf from a plant grown in the presence of 100 µm NPA, a polar auxin transport inhibitor. Size bars = 1 mm.

with vein patterning defects have been identified; these derive both from directed screens and from phenotypic analyses of mutants obtained in different morphological screens (mutants are listed in Table 1). The vein pattern phenotypes are highly varied (Figure 15), suggesting that vein patterning pathways may be complex. In addition, molecular identity has been reported for only a few of these genes. Molecular characterization and defining relationships between the genes using double mutant analyses are promising areas for futures study.

Cotyledon Vein Patterning and Seedling Apical/Basal Patterns Require Auxin

Plants mutant for *MP*, *BDL*, or *AXR6* have severely reduced cotyledon vein pattern, lack primary roots and have reduced hypocotyls (Przemeck et al., 1996; Hamann et al., 1999; Hobbie et al., 2000). The vein pattern of *axr6-3* is shown in Figure15C-15D. All three of these mutants show similar cotyledon vein patterns and seedling defects,

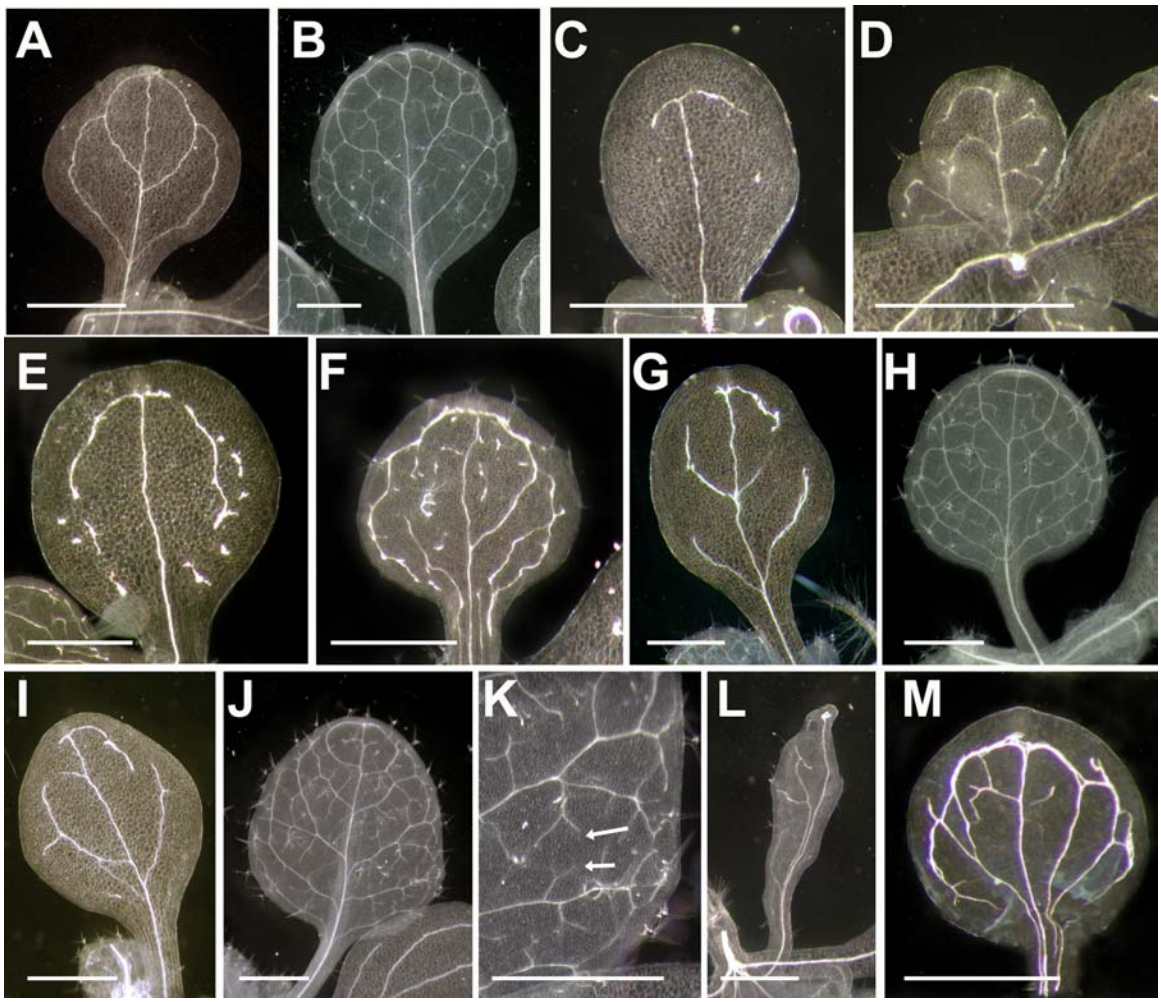

Figure 15. Reticulate Vein Patterns Mutant Phenotypes. Dark field images of cleared cotyledons and leaves from selected Arabidopsis mutants. (A) Wild type (Landsberg *erecta*) cotyledon vein pattern. (B) Wild type leaf vein pattern. (C-D) Vein patterns of *axr6-3* cotyledon and leaf respectively. (E-F) Cotyledon and leaf vein patterns of *sfc* mutant. (G-H) Cotyledon and leaf vein pattern of a *cvp1* mutant. (I-K) Vein pattern of the *cvp2* mutant, (I) shows the cotyledon vein pattern, J shows the over-all leaf vein pattern, and K shows a higher magnification image from the lower right and portion of the same leaf as shown in J. (L) Leaf vein pattern of an *as1-1*mutant. (M) Leaf vein pattern of an *as2-1* mutant. (N) Leaf vein pattern of a *lop1* mutant. (O) Leaf vein pattern of *pin1-1* mutant. Size bars = 1 mm.

suggesting that these genes might function in the same developmental pathway. The *AXR6* gene identity has not been reported, but the mutant's resistance to auxin strongly suggests that it encodes an auxin-related molecule. The molecular identities of both MP and BDL have been reported. *MP* encodes ARF5, an auxin response factor gene that is believed to mediate auxin-induced gene expression (Hardtke and Berleth, 1998; Ulmasov et al., 1999), and *BDL* encodes IAA12 (Hamann et al., 2002). ARF and IAA proteins have been shown to interact; ARF proteins can bind DNA and activate gene expression while IAA proteins bind to ARFs, repressing their ability to activate downstream genes (Tiwari et al., 2001). That *mp* and *bdl* have very similar phenotypes and encode potentially interacting proteins suggested that these two proteins may interact *in vivo*. Indeed, both genes are co-expressed during embryogenesis (from a generalized proembryo expression to concentrated expression in vascular precursor cells) and they interact *in vitro* (Hamann et al., 2002). Identification of the genes being regulated by this complex, and determining how these downstream genes function in vein patterning is likely to be productive area.

Formation of Continuous Veins

A major feature of vein pattern is that at least one end of each vein is connected to another vein, and this connectivity is critical for vascular function. Mutants with vein segments that fail to interconnect (called vascular islands) have been identified; these include *cotyledon vein pattern 1 (cvp1)*, *cotyledon vein pattern 2 (cvp2*), *scarface* (*sfc*), *van3*, and *van6* (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000), see Figure 15E to 15K. The *sfc* and *van3* mutants are likely to be allelic, as they share similar phenotypes and map positions, and so will be considered together (Deyholos et al., 2000; Koizumi et al., 2000). Vascular islands could be consequences of defects in establishing the pattern for the vein (i.e. the pattern for the procambium), or defects in procambial cell differentiation. These possibilities have been assessed for *cvp1*, *cvp2*, and *sfc/van3*. Using morphological (Carland et al., 1999; Deyholos et al., 2000) or reporter gene expression studies (Koizumi et al., 2000), defects in all of these mutants are manifest in their procambium. These observations indicate roles for the products of these genes in events leading to patterning of the procambium rather than the differentiation of vascular cell types.

Phenotypes of these vascular island-containing mutants vary. On the seedling level, *cvp1* and *cvp2* mutants appear wild type, whereas *sfc/van3* mutants are reduced in size, have epinastic cotyledons and leaves, and do not produce fertile flowers. In terms of vein patterning, *cvp1* mutants have defects that are restricted to cotyledon vein pattern, whereas *cvp2*, and *sfc/van3* mutants have vein pattern defects in all broad flat organs. In *cvp1* and *cvp2* mutants, the cotyledon vascular defect is mostly restricted to secondary vein regions adjacent to the margin (Figure 15G-15H). Leaves of *cvp2* mutants contain apparently normal primary and secondary veins, but the higher order veins are either free-ending veinlets or vascular islands (Carland et al., 1999)(Figure 15I-15K). In contrast, *sfc/van3* mutants, the primary veins are intact, but all other veins are largely replaced by vascular islands (Figure 15E to 15F).

In general, the vascular islands in these mutants appear in positions appropriate for a vein. Thus, the overall pattern of vascular islands suggests that mutants may have defects in transducing a positive patterning signal along the path for a vein. Thus, affected genes could function in production, perception, or transduction of a putative positive signal. A strong candidate for this positive signal is auxin, however all tests for auxin responses in *cvp1* and *cvp2* showed the mutants to be identical to the wild type (Carland et al., 1999). In contrast, the *sfc* mutant shows exaggerated auxin responses, suggesting it may function as a negative regulator of auxin responses (Deyholos et al., 2000). Recently, the molecular identity of *CVP1* has been determined (Carland et al., 2002). *CVP1* encodes sterol methyltransferase 2, an enzyme in the sterol biosynthetic pathway. Consistent with this defect, *cvp1* mutants contain elevated levels of brassinosteroid precursors and decreased levels of sterols. However, how these changes cause the vein pattern defects is unknown. One possibility is that the defect in *CVP1* may directly affect production of a lipid based signalling molecule. Alternatively, the defect could affect membrane properties such that membrane protein function (such as the putative auxin efflux carrier PIN proteins) is compromised.

Decreased Vein Complexity Mutants

In an analysis of vein patterns in Arabidopsis ecotypes, two were identified as having reduced vein pattern complexity (ecotypes a-1 and Ei-5) (Candela et al., 1999). In addition, two mutants with modest leaf shape defects, but otherwise are similar to the wild type, also have altered vein patterns. These mutants are *asymmetric leaf 1* (*as1*) and *asymmetric leaf 2* (*as2*). The striking feature of both these mutants is their irregular lobed leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). *AS1* encodes a myb domain protein that is related to *PHAN* and *RS2* of Antirrhinum and maize, respectively (see chapter on leaf development, this volume), but the identity of *AS2* has not been reported. These mutants' altered leaf shape was reminiscent of plants overexpressing the

homeodomain gene *KNAT1* (Lincoln et al., 1994; Chuck et al., 1996), and several *KNAT* genes were found to be ectopically expressed in small regions of the mutants' abnormal leaves. In addition to the altered leaf shape, both *as1-1* and *as2-1* also have vein pattern defects. The vein pattern of *as1-1* is shown in Figure 15L. The leaves showed a modest decrease in the number of veins, and a modest decrease in vein connectivity. The vein pattern defects have been well characterized for *as2* mutants. Their leaves contain multiple veins of similar diameter. This superficial similarity suggested that they could all be secondary veins, however a developmental time course showed that these veins arose in a temporal sequence that is similar to normal primary and secondary veins. In addition, *as2* mutants have vein patterning defects in cotyledons, sepals, and petals.

Increased vein pattern mutants

In the vein pattern mutant screen reported by Koizumi (2000), a mutant with an excessive number of leaf veins (and disrupted cotyledon veins) was identified as an allele of *gnom/emb30*. GNOM/EMB30 encodes an ARF GEF (Shevell et al., 1994; Busch et al., 1996). Altered vesicle transport in *gnom/emb30* plants result in mislocalization of PIN1, an auxin efflux carrier (Steinmann et al., 1999). Thus *gnom/emb30* defects are likely to result in ectopic auxin delivery and the increased leaf vein number observed in *gnom/emb30* mutants is consistent with a positive role for auxin in specification of leaf veins (see below).

Pleiotropic mutants affecting both leaf and leaf vein pattern

Many mutants with pleiotropic defects that include altered vein patterns have been described. For example, *lop1/trn1* mutants have leaves with irregular blade development, a forked primary vein, and a variable number of additional aberrantly positioned veins (Figure 15N). In addition, *lop1/trn1* mutants also have short twisted roots with aberrant specification of cell identities, and reduced apical dominance (Carland and McHale, 1996; Cnops et al., 2000). As with the other mutants in this category, the pleiotropic phenotype suggests that the affected gene is required for multiple developmental processes. Auxin transport assays have shown that *lop1* mutants have severely decreased polar auxin transport (Carland and McHale, 1996), however *pin* mutants have similarly decreased polar auxin transport and a very different vein pattern (Figure 15O). These observations hint that polar auxin transport and its relationship to vein patterning is complex. Additional pleiotropic mutants with leaf vein pattern defects include *lem7*, *van1*, *van2, van4,* and *van5* (Meisel et al., 1996; Koizumi et al., 2000). That many leaf vein pattern mutants also have defects in other leaf pattern attributes suggests also that the affected gene(s) may play primary roles in early leaf primordium development, and that the vein pattern defects are secondary consequences of the leaf defects.

Relationship of Vein Pattern to Abaxial/Adaxial Patterning

Recently, insight into leaf shape and development has come from genetic studies with Arabidopsis, and for details we refer readers to the chapter on leaf development in this volume. An emerging theme is the identification of genes with functions in either abaxial or adaxial patterning pathways. That these genes can provide positional cues with functions that extend beyond the cell identity of each leaf surface is suggested from studies of the *phantastica* mutant of Antirrhinum. These studies led to a model in which leaf blade expansion requires the juxtaposition of cells with adaxial and abaxial fates (Waites and Hudson, 1995).

In Arabidopsis, the most severe abaxial/adaxial patterning defect is shown by the semi-dominant *phabulosa* (*phb*) and *phavoluta* mutants (McConnell and Barton, 1998; McConnell et al., 2001). These mutants produce radial needle-like or trumpet shaped leaves. In the *phb* radialized leaves, vascular tissue varies between being absent, to veins with aberrantly arranged xylem and phloem (xylem surrounding the entire vein). These data suggest that positional cues either underlying abaxial/adaxial patterning, or derived from normal abaxial/adaxial pattern provide positional cues to the procambium for the radial patterning of vascular cell types within a vein.

Additional support linking abaxial/adaxial patterning to vascular tissue formation comes from work on the *KANADI* (*KAN*) gene (Kerstetter et al., 2001). *kan* mutants have defects in abaxial patterning, but whether this defect alone affects leaf or cotyledon vein patterning has not been addressed. However, over-expression of *KAN* using the 35S promoter produces plant with a small number of radialized leaves that largely lack vascular tissue. Vascular tissue loss also often extends to the hypocotyl of these transgenic plants (Figure 16) (Kerstetter et al., 2001). Although ectopic expression experiments can be misleading, the simplest interpretation of these results is that in the transgenic plants, internal tissues are mis-specified as ones with more peripheral identities, thus precluding specification of that innermost of tissues, the vascular tissues.

While its not surprising that gross alterations of leaf organization and cell identity specification is accompanied by defects in vascular tissue, a more relevant question for

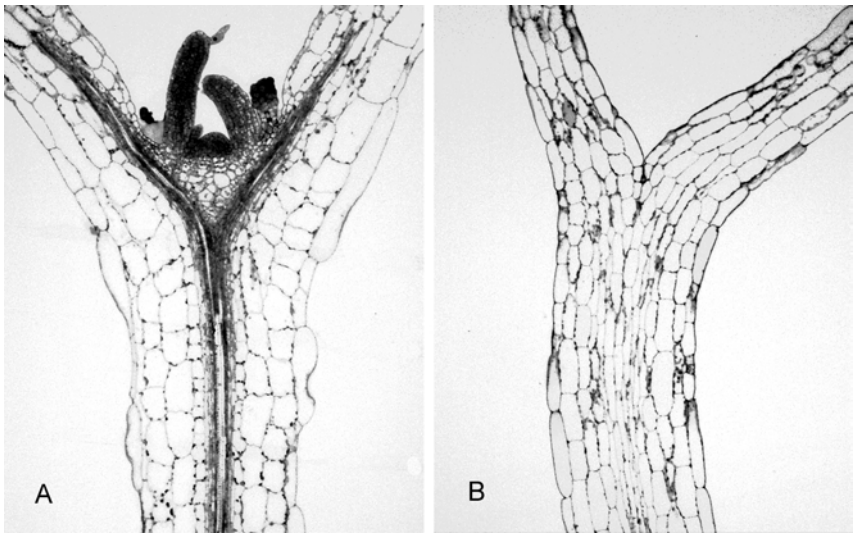

Figure 16. Seedling of plant ectopically expressing *KAN*. Section through the hypocotyl and cotyledons of wild type (A) and plants transformed with a 35S::*KAN* construct (B). Note the absence of both a vascular cylinder and the apical meristem in plants overexpressing *KAN*. Reprinted by permission from Nature (411, 706-709) copyright (2001), Macmillan Publishers Ltd.

this review is whether the normal patterning of veins uses positional cues related to abaxial/adaxial cell fates. This possibility is strongly suggested by the phenotype of a *filamentous flower-5 yabby3-1* double mutant, in which two abaxial-specifying gene activities are removed (Siegfried et al., 1999). This double mutant produces abnormal leaves with apparently disorganized secondary veins and no tertiary or quaternary veins.

Another mutant with vein pattern defects, *argonaute1* (*ago1*), has also recently been implicated as functioning in abaxial/adaxial patterning. Plants homozygous for either *ago1-1* or (pnh/*zll*), and heterozygous for the other mutation produce radialized organs, suggesting that these two genes function in the same pathway (Lynn et al., 1999). *ago1* single mutants have vein pattern defects in both their cotyledons and their leaves (Bohmert et al., 1998), further strengthening the link between organ polarity and vein patterning. *AGO* and *PNH/ZLL* encode similar genes of unknown function. *AGO1* RNA is detected throughout developing leaves, suggesting a role for *AGO1* in leaf development itself (Lynn et al., 1999), whereas *PNH/ZLL* expression is on the abaxial sides of cotyledons and leaves, and the highest concentration is in vascular tissues. Note that *pnh/zll* is also discussed in the section on apical/basal patterning.

Genetic approaches to vein patterning are still in the early phase where the diversity of mutants with interesting phenotypes exceeds our ability to explain the basic mechanisms underlying the developmental process. However, the near future promises to provide many gene identities that may provide us with the tools to construct testable models.

AUXIN AND MODELS FOR VASCULAR TISSUE DEVELOPMENT

Since Jacobs work on vascular tissue formation showing that IAA could replace the inductive effects of developing leaves on vascular tissue formation (Jacobs, 1952), auxin has featured prominently in research into the control of vascular tissue development. Jacobs was able to demonstrate that basipetal regeneration of both xylem and phloem could be quantitatively induced by increasing concentrations of auxin. Evidence that it is the transport of auxin that is crucial for patterning vascular tissue regeneration came from observations that regeneration could be reduced by the application of polar transport inhibitors such as TIBA (Thompson and Jacobs, 1966). A large body of experimental evidence now clearly indicates that auxin plays inductive roles in vascular tissue regeneration (reviewed in Sachs, 1981)

In addition to exploring the inductive effects of auxin, Sachs also demonstrated that auxin could have an inhibitory effect on vascular development (Figure 17) (Sachs, 1966). A newly induced strand of vascular tissue will not join to a pre-existing vascular strand if that strand is already transporting high concentrations of auxin. Sachs rationalized these many observations of auxin's influence on vascular tissue development in a model described as canalization (Sachs, 1981, 1991). According to this model, cells with slightly higher concentrations of auxin become specialized for polar transport of auxin. One consequence of auxin polar transport is that auxin is delivered to underlying cells, inducing their specialization

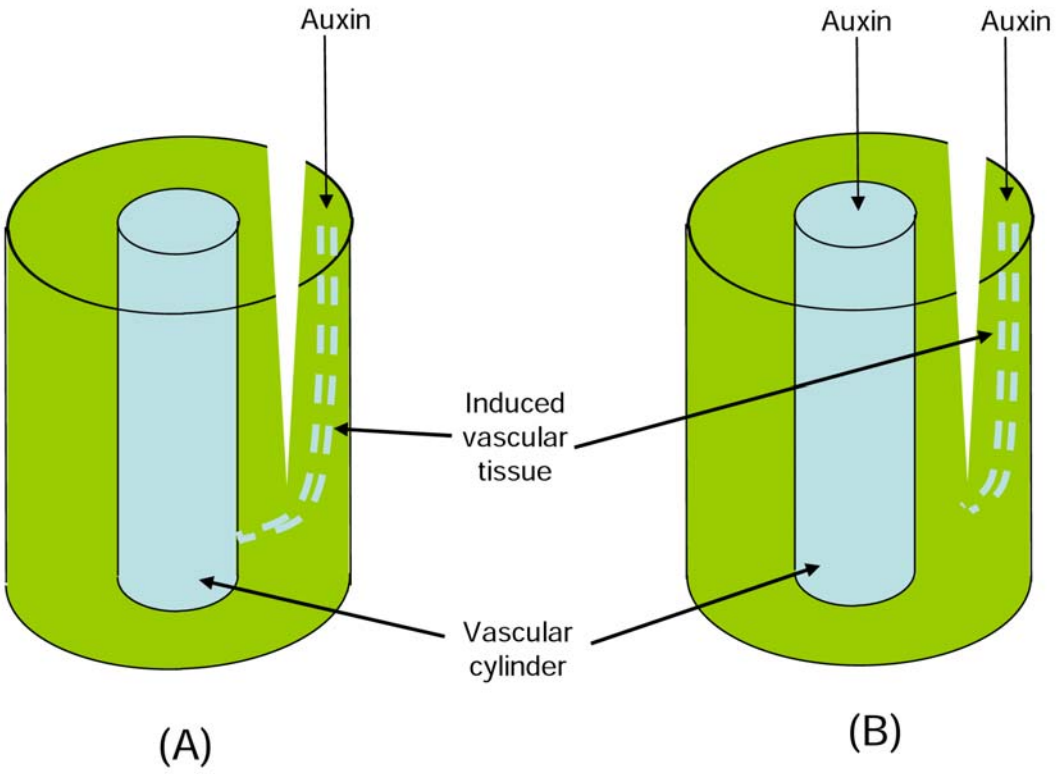

Figure 17. Inhibition of vascular bundle formation by auxin. Adapted from (Sachs 1966). (A) Following application of auxin to a partially separated section of a pea epicotyl, a new vascular bundle is induced that joins up with the main vascular cylinder. (B) If the experiment is repeated but with auxin also applied to the main vascular cylinder the newly formed vascular strand no longer joins to the existing vascular tissue.

for auxin transport, resulting in files of specialized auxin transporting cells. A second consequence is that that these cell files drain away the auxin from surrounding tissue. Finally, the canalization hypothesis goes on to suggest that these auxin transporting cell files eventually differentiate into the vascular strand (Figure 18). Consequently, the formation of the new vascular tissue is an autocatalytic process in that increased transport leads to increased concentration of auxin and vascular differentiation, which in turn leads to better auxin transport.

A mathematically based model for reticulated vein patterns has also been proposed (Meinhardt, 1976; Meinhardt, 1984). This model is based on two components: an activator, which is autocatalytic and capable of moving to adjacent sites; and a fast-acting long-range inhibitor that restricts the lateral spread of the activator. Using these two parameters, Meinhardt demonstrated that is possible to generate stable patterns that strongly resemble normal leaf vein patterns (Meinhardt, 1984). This model does not specify specific molecules for activator and repressor and it is not incompatible with the canalisation hypothesis. It is possible that auxin is the activator and that repression results from veins with high auxin concentrations being a poor “auxin sink” and therefore preventing vascular development. Two precedents for local activation-repression mechanisms being used in Arabidopsis development have been described. These are trichome spacing and stomatal development. In both of these processes, a differentiated cell apparently prevents adjacent cells from becoming differentiated through a local repression mechanism (reviewed in Scheres, 2000).

In recent years, genes whose products are believed to encode components used in polar auxin transport have been identified. One of these genes, *PIN1*, is believed to encode an auxin efflux carrier (Galweiler et al., 1998), and its molecular identification led to the realization that a family of related genes are encoded in the Arabidopsis genome. The identification of this gene, as well as identification of additional molecules that function in the transport pathway (reviewed in Muday and Murphy, 2002) provides tools for identification of cells that are specializing for auxin transport.

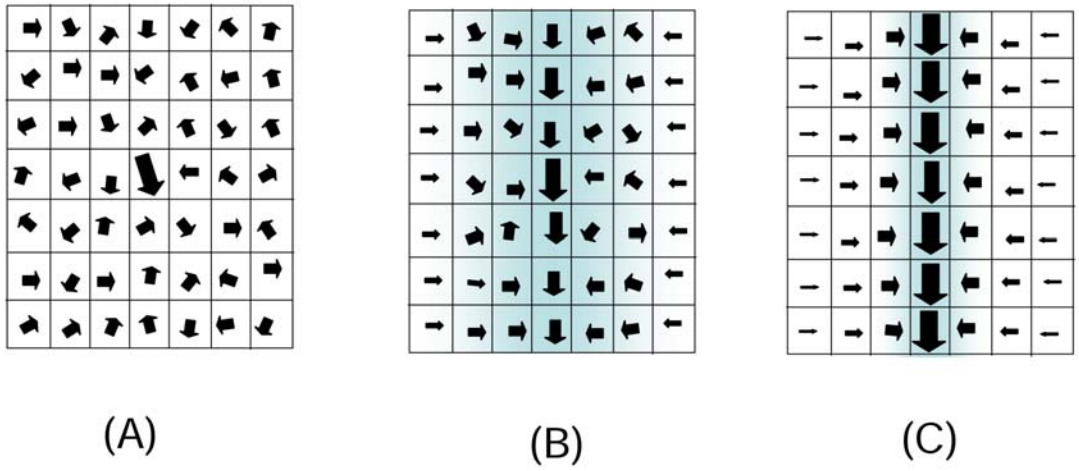

Figure 18. Formation of vascular bundles by canalization.The arrows indicate the direction of auxin flow and the width of the arrow is proportionate to the capacity of a cell to transport auxin. (A) Most cells have a similar capacity to transport auxin. (B) Increased auxin flow in some cells leads to an increase in their capacity to transport auxin. (C) Specialization of cells to transport auxin drains auxin from the surrounding tissues and leads to vascular bundle formation.

The PIN1 protein is initially distributed throughout the globular embryo, but in heart-staged embryos it becomes localised in the elongated procambial cells (Galweiler et al., 1998). This expression pattern suggests a self-reinforced accumulation; that the accumulation occurs in cells that will go on to differentiate into vascular tissues is consistent with canalization. In *pin1* mutants, the stem accumulates an excess of vascular tissues, especially in regions just below cauline leaves (Okada et al., 1991; Galweiler et al., 1998). These observations might be explained by the canalisation hypothesis if the loss of PIN1 impedes auxin drainage down the stem while not affecting the leaf's ability to deliver auxin to the stem. These conditions should lead to a local accumulation of auxin just below the cauline leaf, in precisely the place the excess vascular tissue differentiation is observed. However, if the *pin1* defect does affect auxin movement out of the cauline leaf, then the localized accumulation of stem vascular tissues is more difficult to explain. Mattsson et al (1999) showed that *pin1* mutant rosette leaves have modest vein pattern defects reminiscent of leaves grown in low levels of polar auxin transport inhibitors, suggesting at least a partial block to polar auxin transport. The extent to which auxin transport in the *pin1* leaf is reduced, and whether redundancy of *PIN* gene family members or diffusion still allows some auxin to reach the stem awaits either characterization of additional *PIN* genes or development of methods to measure auxin transport through leaves.

Further evidence for canalization has come from studies on PIN1 localization in the developing embryos. The transition from non-oriented distribution of PIN protein found in the early embryo to the polar localization of PIN along the apical basal axis suggests a positive feedback loop with auxin consistent with the canalization hypothesis (Steinmann et al., 1999). In *gnom/emb30* mutants, PIN1 does not become localized to basal membranes, but instead it remains evenly distributed around the cell (Steinmann et al., 1999). Canalization might predict that mislocalized PIN1 could lead to auxin delivery to inappropriate cell types, resulting ultimately in too many veins. Indeed, *gnom/emb30* alleles have been identified in screens for leaf vein pattern mutants as a mutant with an excess number of leaf veins (Koizumi et al., 2000; Sieburth, unpublished data).

In contrast to the above results, some vein patterning mutants, such as *cvp1*, *cvp2*, and *sfc/van3*, are difficult to reconcile with canalization (Carland et al., 1999; Deyholos et al., 2000; Koizumi et al., 2000). These three mutants are all characterized by having discontinuous veins (vascular islands). On the one hand, the lack of procambial strands between the vascular islands could be explained by canalization if the mutants had impaired auxin responses. However, both *cvp1* and *cvp2* have normal auxin responses, and *sfc/van3* has heightened auxin responses. Alternatively, these mutant phenotypes may be more interpretable in terms of the mathematical diffusion/reaction prepattern hypothesis. If the defect affected the autocatalyic component, it is possible that the result might be vascular islands. However, other aspects of this model, especially evidence for a repressor component, currently lack experimental evidence.

One problem with finding a model that covers all vascular development is highlighted by the difference in the development of major and minor veins within the leaf. In Arabidopsis the formation of the midveins is clearly polar and proceeds in an acropetal direction up the developing leaf (Carland et al., 1999; Sieburth, 1999). The formation

of such veins can easily be accounted for by canalization. Minor veins in leaves, however, lack obvious polarity since they form a bridge between higher order veins and they appear to differentiate almost simultaneously along their entire length. Although modifications to the canalization hypothesis have been proposed to explain these observations, to date there is little experimental evidence to support them (Sachs, 2000). Also highlighting the difference between leaf major and minor veins is the observation that minor vein density is dependent on light intensity (Haritatos et al., 2000). High light intensity gives high minor vein density, which suggests that minor vein density may be linked to the photosynthetic capacity or some other physiological function of the leaf (Haritatos et al., 2000).

CONCLUSIONS

Research into vascular development in Arabidopsis is a comparatively new field, which is reflected by the fact that many of the genes identified in genetic screens specifically for vascular defect mutants remain to be cloned. While determining identities for vein patterning genes will certainly help to define pathways used in vascular development, it is also clear that many aspects of vascular development are intimately tied to development of the organ as a whole. This is evident both in the pleiotropy of most vein pattern mutants and in the gross vascular defects identified in plants with leaf polarity defects (see above) (Kerstetter et al., 2001). Defining which aspects of vein patterning depend on organ development specific signals and how organ specific vascular development integrates with the vascular system as a whole remains an important question for future research.

An equally compelling complication is that, in some instances, it is clear that organ development requires signals originating within the vascular tissue. For example the *SHOOTROOT* (*SHR*) gene is required for divisions in the cortex of the root, even though the *SHR* gene is expressed exclusively within the vascular tissue (Helariutta et al., 2000). Similarly, a number of important developmental genes either exhibit high levels of expression within the vascular tissue (e.g. *PHD*) or in cells immediately adjacent to the vascular cylinder (*WUSHEL*). Interestingly, plants overexpressing the *KAN* gene lack a vascular cylinder in the hypocotyl and also lack an apical meristem (Figure 16) (Kerstetter et al., 2001), suggesting that positional signal from within the vascular tissue may be required for meristem development. The fact that vascular mutants typically have pleiotropic phenotypes may be a result of secondary defects in positional signals generated in the vascular tissue that are essential for other aspects of plant development.

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