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Leaf Vascular Pattern Formation

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INTRODUCTION

The pattern and ontogeny of leaf venation appear to guide or limit many aspects of leaf cell differentiation and function. Photosynthetic, supportive, stomatal, and other specialized cell types differentiate in positions showing a spatial relationship to the vascular system. These spatial relationships are of obvious importance to leaf function, which relies on venation for the servicing of cells engaged in photosynthesis, gas exchange, and other leaf processes.

Although the need for coordinated organization of cell types around the vascular system is clear, the means by which this is achieved during development is not well understood. In the few systems in which it has been possible to follow the ontogeny of the venation along with the differentiation and function of surrounding cell types (e.g., in C_4 grasses), observations suggest that the developing vascular system may have a role in providing positional landmarks that guide the differentiation of other cell types. Another possible explanation is that an underlying pattern guides the differentiation of both venation and surrounding cells.

Whether the process of vascularization creates or reveals a pattern, studies to date are largely descriptive, and little is understood of the underlying mechanisms. These mechanisms must be highly regulated, as evidenced by the successful use of species-specific leaf vascular pattern as a taxonomic characteristic (e.g., Klucking, 1992) and by the predictable effect of certain mutations. In this review, we summarize the vascular patterns and their ontogenies in dicots and monocots, referring extensively but not exclusively to Arabidopsis and maize as examples. We also discuss a variety of models that seek to explain vascular pattern formation, and we provide a summary of molecular and genetic investigations of the process.

VASCULAR PATTERN IN MATURE LEAVES

Dicots

The relatively small simple leaves of Arabidopsis illustrate many of the characteristic features of leaf venation found in advanced dicots: a prominent midvein, several distinct vein size orders with smaller veins diverging from the larger, and a closed reticulum formed by several veins with the smallest veins forming so-called freely ending veinlets (Figures 1A and 1C; Troll, 1939; Esau, 1965a; Hickey, 1979; Gifford and Foster, 1988). The distinctive attribute of this overall pattern is the presence of discrete vein size orders that form a continuous branching system. The large primary vein or midvein is continuous with the stem vascular bundles and extends the length of the leaf. Secondary veins branch from the primary vein, and tertiary veins branch from secondary veins. In some plant groups, this pattern is reiterated through six or more additional size orders. Although determination of vein order may be unequivocal at the point of branching from a larger vein, veins typically diminish in size distally. Thus, a secondary vein may become indistinguishable from a tertiary vein more distant from its point of origin.

The largest vein orders are embedded in a ridge of parenchymatous and supporting tissues that projects from the abaxial surface of the leaf, forming a rib. These veins are visible macroscopically and are referred to as major veins. Smaller vein orders, which make up the minor venation of the leaf, are not associated with a rib but are embedded in the leaf mesophyll. The smallest polygonal area of mesophyll bounded by these veins is referred to as an areole (Figure 1C). Branched or simple freely ending veinlets may extend into the areole. Secondary veins may end blindly near the leaf margin, but in many species they bend and join with the adjacent secondary vein to form a closed loop. Often a series of closed loops forms a distinct intramarginal vein (Figure 1A).

Venation patterns of dicot species are exceptionally diverse, and detailed anatomical descriptions are available for numerous individual species (e.g., Avery, 1933; Pray, 1954; Merrill, 1978; Fisher and Evert, 1982; Russin and Evert, 1984; McCauley and Evert, 1988). This diversity may be phylogenetically informative at several levels of classification, from family to species (e.g., Klucking, 1989; Todzia and Keating, 1991; Dickinson and Weitzman, 1996; Eckenwalder, 1996).

The standard characterization of dicot leaf architecture, including venation pattern, has been made possible by the detailed terminology developed by Hickey (1973, 1979) on the basis of a scheme originally established by von Ettinghausen

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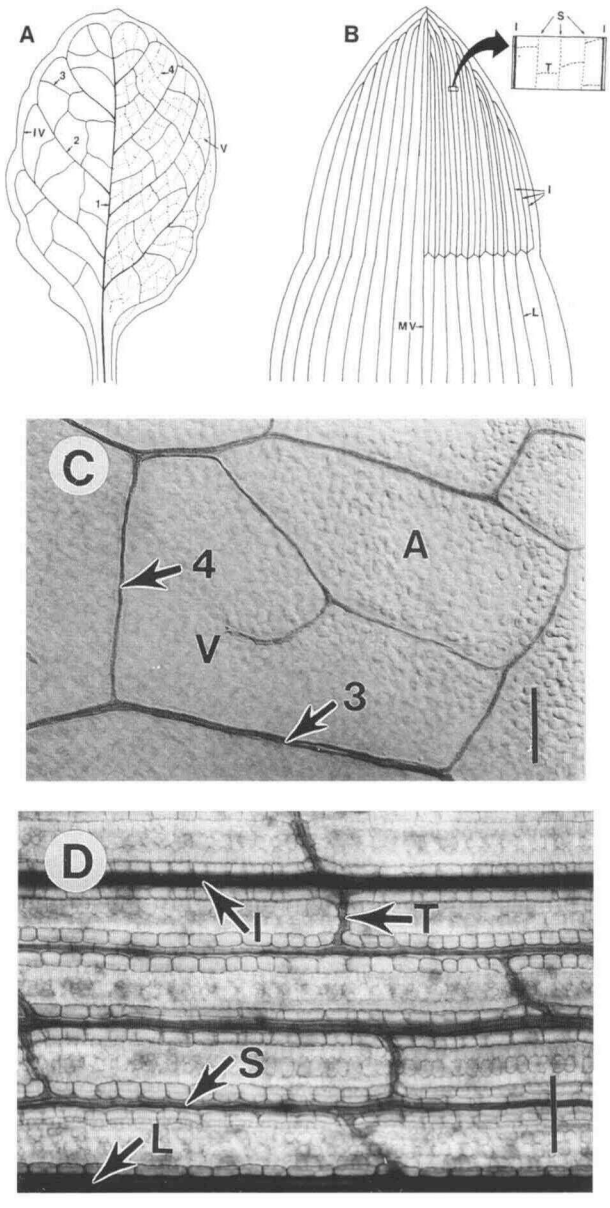

Figure 1. Comparison of Vascular Pattern in Mature Leaves of Arabidopsis (ecotype Columbia) and *Zea mays*.

(A) Diagram of vascular pattern in a rosette leaf of Arabidopsis. Note midvein (1), secondary veins (2), which are joined by an intramarginal vein (IV), tertiary (3), and quaternary (4) minor veins and freely ending veinlets (V).

(B) Diagram of vascular pattern in seedling leaf of maize. Note midvein (MV), large (L), intermediate (I), and small (S) longitudinal veins and transverse (T) veins.

(C) Photograph of cleared Arabidopsis leaf. The image shows tertiary (3) and quaternary (4) minor veins surrounding an areole (A), and freely ending veinlets (V). Bar = 100 μm.

(D) Photograph of cleared maize leaf blade. The image shows large (L), intermediate (I), and small (S) longitudinal veins and transverse (T) veins. Bar = 100 μm.

(1861). In this scheme, typology is based first on the pattern of major veins, which may be pinnate, with secondaries branching laterally from a single primary vein; actinodromous, with several primaries diverging from the leaf base; parallelodromous, with several primaries having a more or less parallel course; or campylodromous, with several primaries having an arcuate course (Hickey, 1979). This detailed scheme further provides for the description of arrangement, relative thickness, angles of divergence, and curvature of secondary and higher vein orders, for the shapes of areoles, and for special features of marginal venation. It was designed to facilitate the description of the amazing diversity of leaf vascular patterns. However, it should also assist in the identification of the developmental mechanisms that give rise to this diversity. The functional significance of this diversity remains almost unexplored, although there are indications that vein patterns may differ in the mechanical support provided to the leaf blade (Herbig and Kull, 1992) and in their efficiency as water conduits (Canny, 1990; Roth et al., 1995).

Monocots

Leaf venation patterns in monocot species are usually characterized as parallel; however, in most monocots, veins typically diverge at the base of the lamina and converge and fuse toward the apex, so that the term striate is more appropriate (Troll, 1939; Gifford and Foster, 1988). Leaf vascular pattern is better known for the grasses than for any other group of monocots (e.g., Sharman, 1942; Colbert and Evert, 1982; Russell and Evert, 1985; Dannenhoffer et al., 1990; Dengler et al., 1997). Maize leaves, like those of other grasses, have longitudinal veins of several classes that differ in size and features of component vascular and accessory tissues (Figure 1B; Russell and Evert, 1985). A median large vein is embedded in a thickened parenchymatous rib, forming a midrib. Several large lateral veins are present on either side of the midvein, intermediate longitudinal veins occur between laterals, and small longitudinal veins occur between adjacent intermediate and lateral veins (Figure 1B).

Although the longitudinal veins have a more or less parallel and equidistant course through the leaf blade, adjacent veins anastomose as the blade narrows distally; fusions occur between adjacent veins first near the margin and extend in a medial direction toward the blade apex. Small veins also fuse with adjacent veins near the base of the blade, so that only the midvein, large, and intermediate veins extend through the leaf sheath and connect with the stem vasculature.

Within the leaf blade, numerous transverse or commissural veins interconnect adjacent longitudinal veins so that, at the level of the smallest order of venation, the pattern is essentially reticulate, as it is for the dicots (Figure 1D). Veins of all classes diminish in size distally, so that a large lateral vein becomes intermediate and then small in anatomy near the apex of the leaf. The smallest longitudinal bundles as-

sume transverse vein anatomy where they bend to join an adjacent vein.

Relatively few detailed analyses of vein pattern exist for other groups of monocots. In the broad-leaved genus *Hosta*, unbranched independent primary veins diverge abruptly at the leaf base, forming a series of broad arcs that converge toward the leaf apex (Pray, 1955b). Commissural veins interconnect the primaries but form a much-branched reticulum with freely ending veinlets and a highly variable pattern of areoles. Although monocots typically have fewer vein size classes than do dicots (Esau, 1965a), some monocots have up to six orders of venation (Inamdar et al., 1983). Monocot midveins are usually regarded as composites of independent primary veins; however, a true midvein is present in several species (Inamdar et al., 1983).

Regional Identity and Symmetry

Vein patterns may vary among regions of the same leaf. For instance, the vein patterns of grass leaf blades and sheaths differ in the size orders of longitudinal veins that are represented and in the presence of transverse veins (Figure 1B); these differences are established early in development, before other indications of the identity of blade and sheath regions (Sharman, 1942; Dengler et al., 1997). Similarly, dicots often bear stipules, which are elaborations of the leaf base region. Stipule vascular pattern is usually simple with few vein orders, but in some species it resembles that of the blade or leaflets (Troll, 1939; Gifford and Foster, 1988).

Different parts of asymmetrical leaves may also differ in vascular pattern (Troll, 1939). In the lobed, asymmetrical leaves of *Begonia*, a major branch of the midvein supplies the large primary leaf lobe but not the smaller lobes, which are supplied by branches of the lateral leaf traces (Lieu and Sattler, 1976). Initial differences in vein pattern may be enhanced during unequal leaf expansion, as is the case for the asymmetrical leaves of *Tropaeolum* (Buis et al., 1995).

Leaflike Organs

Heteroblasty is expressed in venation pattern as well as in the different shapes and sizes of cotyledons, juvenile and adult foliage leaves, scale leaves, and floral organs such as sepals and petals (see Poethig, 1997, in this issue). The venation of cotyledons and bractlike leaves is generally similar to that of foliage leaves of the same species but is often simpler (Esau, 1965a). In Arabidopsis, cotyledons have a highly reduced vascular pattern, with the midvein and one or two secondary veins per lamina half forming a series of closed loops. Foliage leaf venation is qualitatively different, with well-developed minor venation and freely ending veinlets. The basic vascular pattern of juvenile leaves is elaborated in adult rosette and cauline leaves, such that numbers of secondary veins and orders of minor venation are greater. This distinction between cotyledon and foliage leaf vasculature contributed to the interpretation of the *leafy cotyledon* mutant of Arabidopsis as representing a homeotic shift of leaf characters to the cotyledonary node (Meinke, 1992).

Only a few detailed studies comparing scale and foliage leaf vascular pattern have been made, and these reinforce the generality that the venation of scale leaves in both monocots and dicots is essentially similar to that of foliage leaves but simpler (Foster, 1950; Pray, 1955b; Denne, 1960; Esau, 1965a). Often, the course of the large primary veins is similar, as are the patterns of the smallest veins, but intermediate vein classes may be absent in reduced scale leaves when compared with larger foliage leaves. For example, in a developmental comparison of vein pattern in juvenile and adult leaves in several species of the genus *Eucalyptus*, Carr et al. (1986) found that, although vein patterns were similar at maturity, the conspicuous marginal vein was formed differently in the two leaf types. This example reinforces the importance of studying developmental patterns directly to understand how intra- and interplant diversity in vein pattern may have been generated during evolution.

Floral organs possess distinctive patterns of vasculature, and there is an extensive classical literature devoted to floral vascular anatomy, particularly to the pattern of interconnections of vascular traces and its significance in floral evolution (e.g., Eames, 1931; Puri, 1951; Gifford and Foster, 1988). In Arabidopsis, both sepal and petal venation patterns are highly reduced, with three main veins in the sepals and one in the petals. In general, dicot sepal vasculature resembles that of foliage leaves, whereas the complexity of petal vasculature is correlated with petal size and longevity (e.g., Puri, 1951; Lee, 1986; Gustafsson, 1995).

Recently, sepal and petal venation patterns were used to evaluate the nature of the sepal-like petal of the *crinkled petal* mutant of *Clarkia tembloriensis* (Smith-Huerta, 1996). In this species, wild-type sepals have three primary veins and freely ending veinlets throughout the sepal, whereas petals have a single primary vein, secondaries that fan outward to the margins, and vein endings that are restricted to the margins. Based on mature and developing venation patterns, Smith-Huerta concluded that *crinkled petal* did indeed represent a homeotic transformation but an incomplete one, resulting in an organ that was a mosaic of sepal and petal characters (Smith-Huerta, 1996).

Comparisons among Leaf, Stem, and Root Vasculature

The primary vascular tissue forms a continuous system from the tips of roots to the vein endings of the leaves, flowers, and fruits, and distinctions between parts of the continuum are always arbitrary at some level (Esau, 1965b). The stem vascular system consists of a series of more or less distinct longitudinal strands that are organized in relation to the phyllotaxis of the shoot (Esau, 1965b). Associated with each node, one or more stem vascular bundles (or, more usually,

their branches) diverge into the base of each leaf. These divergent bundles are termed leaf traces, by contrast to the so-called sympodial bundles, which continue their course through the next internode.

Foliage leaves of dicots are typically connected to the stem vasculature by at least three leaf traces (one central and two lateral traces), each derived from a separate sympodial bundle. In dicot taxa with large leaves or with sheathing leaf bases, additional leaf traces are present, each derived from a different sympodial bundle (Larson, 1986). In grasses, leaf traces are more numerous (equivalent to the total number of large and intermediate veins) but connect in a similar way to independent stem vascular bundles (Sharman, 1942). In all cases, the continuity of the leaf vasculature with (usually) more than one stem bundle ensures a functionally important redundancy in the leaf vascular supply.

The most notable difference between the vascular organization of leaves, stems, and roots is related to organ symmetry. The vascular system of the root forms either a solid or pith-filled radially symmetrical cylinder, and the form of this simple vascular pattern is not altered by the formation of lateral organs. In the stem, the organization of sympodial bundles is radially symmetrical and organized in relation to shoot phyllotaxis. With few exceptions, bundles are collateral, with xylem toward the inside of the stem and phloem toward the outside. By contrast, the vascular pattern of typical dorsiventral leaves forms a two-dimensional array, and the relative positions of xylem (toward the adaxial side of the leaf) and phloem (toward the abaxial side) reflect the collateral arrangement of vascular tissue in the stem bundles. In nondorsiventral leaves, such as the unifacial leaves of certain dicots and monocots, veins form a radially symmetrical or compressed cylinder (Kaplan, 1975; Ruddall, 1995).

VASCULAR PATTERN ONTOGENY

Identification of Provascular Tissue

Vascular pattern is readily assessed once pattern elements such as the tracheary elements have begun differentiation from the provascular tissue, but identification at the provascular stage is less straightforward (see Fukuda, 1997; Sjölund, 1997, in this issue). Provascular tissue and ground meristem are derived from the uniformly meristematic tissue of the leaf primordium and become delimited through differential patterns of cell division, cell enlargement, and vacuolation (Figures 2A and 2B; Esau, 1965a; Meicenheimer and Leonard, 1990).

The earliest recognition of provascular cells is based on differential stain affinity; they become more densely staining, whereas their neighbors become less so, presumably through increased vacuolation (Esau, 1965a). Provascular cells are also elongate and narrow, with a long axis parallel to the axis of the provascular strand, by contrast to the more isodiametric ground meristem cells (Figures 2C and 2D). Because the axis of a provascular strand and its component cells may also parallel the direction of growth, provascular cells may break the normal "rules" for cell division (i.e., division plane normal to the growth direction) by dividing longitudinally in the growth direction (Lyndon, 1990). Thus, among the genes expressed early during provascular tissue formation may be those that control the plane of cell division.

The anatomical descriptions of vascular pattern ontogeny that follow are based on a qualitative assessment of cytological characteristics of putative provascular cells; these events may or may not coincide with the developmental determination of vascular pattern.

Dicot Ontogeny

The venation pattern of advanced dicots develops in three distinct phases during leaf morphogenesis and growth. First, the midvein provascular strand extends acropetally from the stem into the leaf primordium (Figure 3A). Second, the secondary vein provascular strands extend from the midvein toward the margin as the leaf lamina is formed (Figure 3B). Finally, the reticulum of tertiary and higher order veins, including the freely ending veinlets, is established during intercalary expansion growth (Figures 3C and 3D). These phases overlap to a greater or lesser degree depending on the duration of leaf growth and the numbers of discrete orders of venation. Formation of minor veins usually proceeds in a basipetal direction, so that the minor vein network is present in the apical region of the leaf while secondary veins are still forming near the leaf base (Figure 3D).

The general pattern found in derived dicots is characterized by the sequential appearance of each discrete order of minor venation. This is in contrast to the pattern found in putative ancestral dicots, in which the vein orders are poorly defined and form an irregularly ramifying pattern, and all vein orders appear more or less simultaneously (Foster, 1952; Doyle and Hickey, 1976). These contrasting patterns indicate that angiosperm evolution and diversification involved shifts in the mode of developmental regulation of leaf venation patterns.

In all dicot species that have been examined in detail, including Arabidopsis (Vaughan, 1955), the midvein provascular strand develops in continuity with stem provascular tissue and extends in an acropetal direction into the leaf primordium (Esau, 1965a; Larson, 1975). Midvein provascular tissue enters the leaf base when it is a simple axis and extends to the tip of the leaf primordium during the first plastochron (Lersten, 1965; Larson, 1975). As the leaf primordium extends in length through intercalary diffuse growth, so does the midvein provascular strand. The median axis of the leaf typically undergoes thickening growth early in development, by contrast to the thinner, laterally extending halves of the leaf blade (Esau, 1965a; Hagemann and Gleissberg, 1996). Continued thickening growth and, ultimately, the dif-

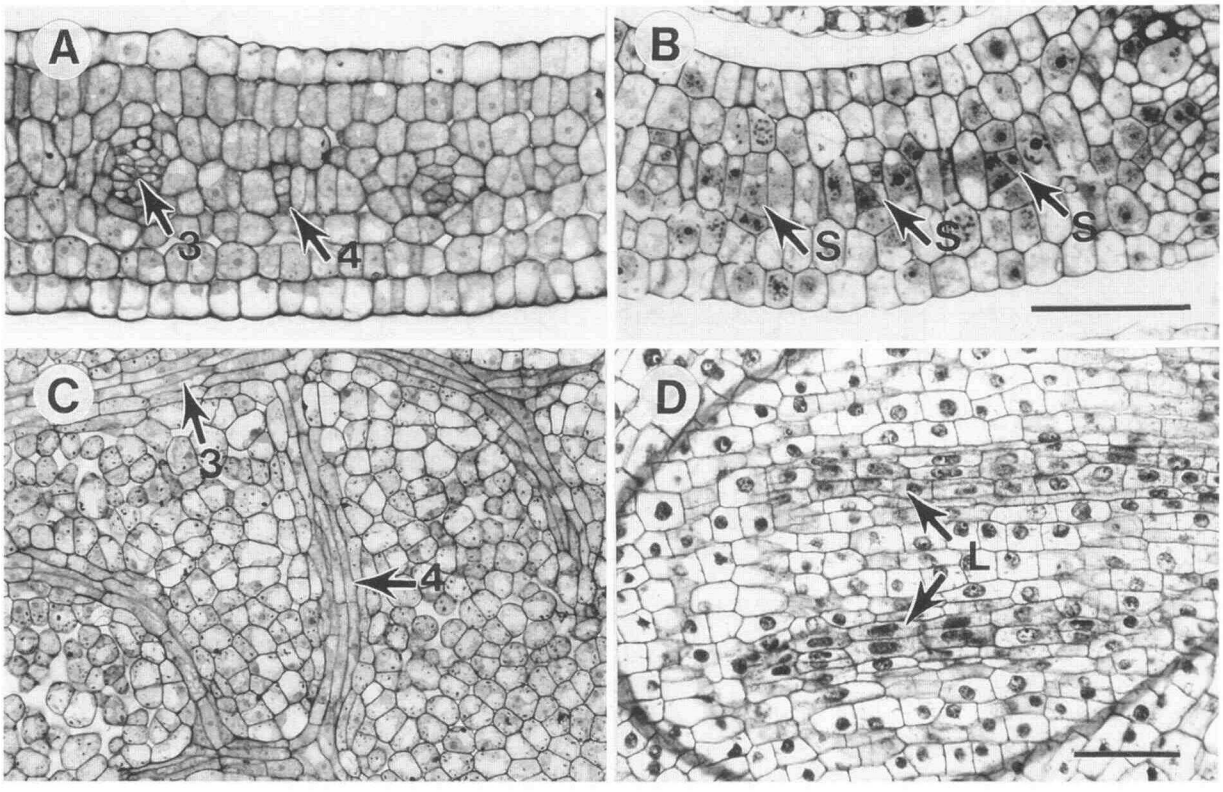

Figure 2. Provascular Tissue in Developing Leaves of Arabidopsis and Maize.

(A) and **(C)** Arabidopsis; **(B)** and **(D)** maize.
(A) Cross-section of provascular strands of tertiary (3) and quaternary (4) veins in a developing leaf of Arabidopsis.
(B) Cross-section of provascular strands of small longitudinal veins (S) in an extending leaf blade of maize. Bar = 50 μm for **(A)** and **(B)**.
(C) Paradermal section of tertiary (3) and quaternary (4) vein provascular strands in Arabidopsis.
(D) Paradermal section of provascular strands of large longitudinal veins (L) in maize leaf primordium. Bar = 50 μm for **(C)** and **(D)**.

ferentiation of supporting tissues along this axis result in the embedment of the midvein in a mechanically strong midrib and petiole.

In dicots with pinnate major venation, formation of secondary veins is coordinated with initiation and growth of the leaf lamina, and the sequence of vein formation reflects lamina morphogenesis (Foster, 1952; Esau, 1965a; Hagemann and Gleissberg, 1996). Secondary vein provascular strands are continuous with the midvein and extend from it toward the leaf margin (e.g., Pray, 1955a; Slade, 1957; Esau, 1965a; Lersten, 1965; Franck, 1979; Isebrands and Larson, 1980). Secondary vein formation is therefore described as progressive (although tips of the secondary provascular strands reach their mature distance from the leaf margin very early and almost all growth in length is intercalary).

Several patterns of secondary vein formation are observed. The sequence may be basipetal, as in Arabidopsis (Telfer and Poethig, 1994), in which the first-formed secondaries appear in the apical region of the leaf and later-formed secondaries appear sequentially toward the leaf base as the wave of intercalary lamina growth proceeds basipetally (Figures 3C and 3D; Lersten, 1965; Isebrands and Larson, 1980). In species with acropetal secondary vein formation, the earliest veins appear near the leaf base, and later ones form sequentially toward the apex (Slade, 1957; Herbst, 1972; Franck, 1979). In the divergent pattern, the earliest secondaries appear in the midregion of the incipient leaf blade, and new ones form both apically and basally (Pray, 1955a; Slade, 1957). In Arabidopsis and other species in which the secondaries form prominent marginal loops, these components appear early and follow the sequence of overall secondary vein pattern formation (Slade, 1957; Herbst, 1972; Telfer and Poethig, 1994).

Detailed analyses of the interconnections between leaf traces, midvein, and secondary veins have been made for only a handful of species (Larson, 1975, 1984, 1986; Isebrands and Larson, 1980). In these examples (all woody trees), the provascular strands of the lateral leaf traces extend into the leaf base one to two plastochrons after the median trace. Shortly thereafter, additional provascular strands, the subsidiary

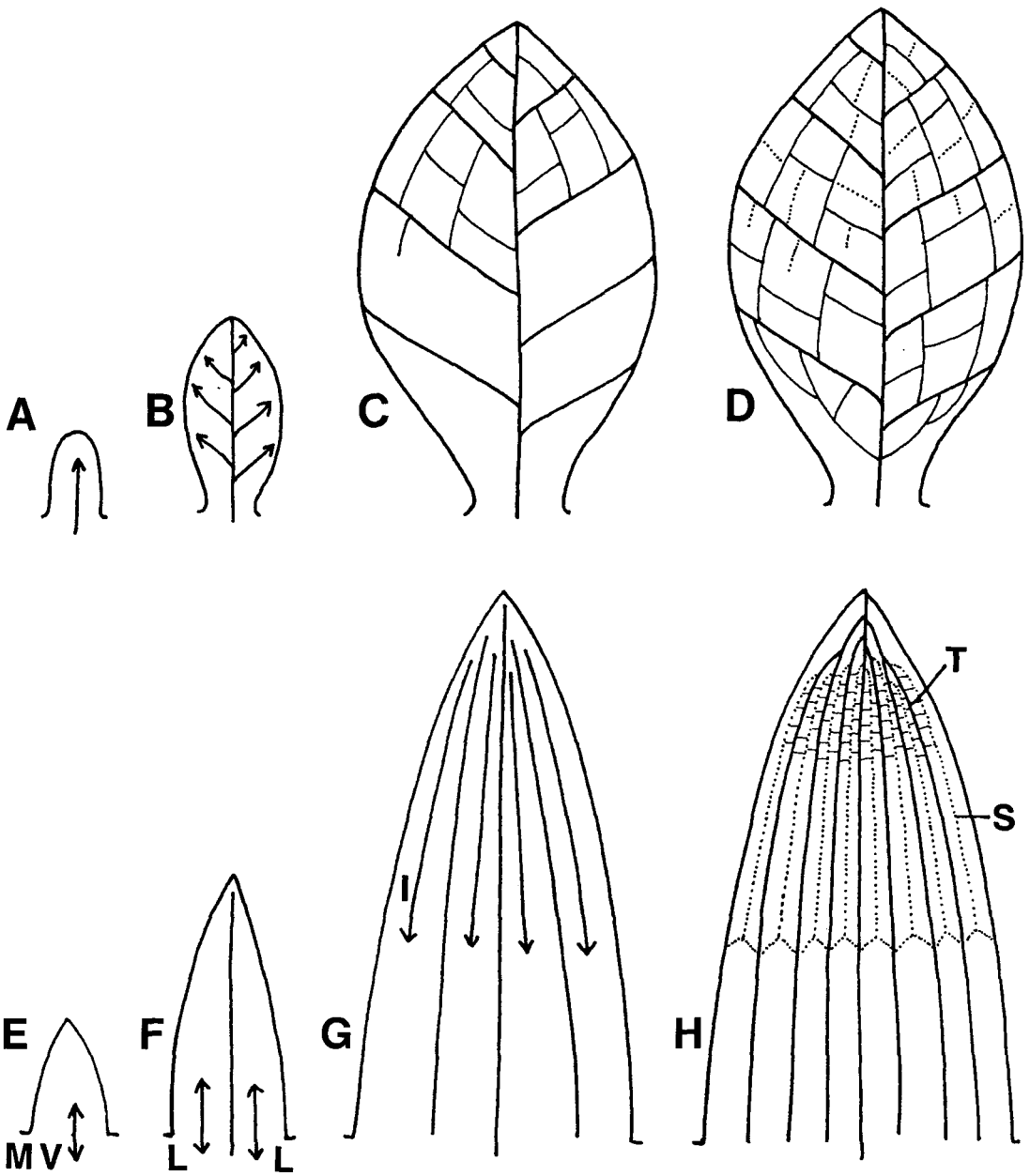

Figure 3. Vascular Pattern Ontogeny in Dicots and Monocots.

(A) to **(D)** Arabidopsis (a dicot); **(E)** to **(H)** maize (a monocot).

(A) Acropetal development of midvein provascular strand from stem vasculature (arrow).

(B) Progressive formation of secondary vein provascular strands (arrows).

(C) Simultaneous formation of tertiary vein network. Tertiary vein formation begins near the leaf apex and proceeds in a basipetal direction.

(D) Formation of quaternary veins and freely ending veinlets. The formation of minor-order veins (dashed lines) also proceeds in a basipetal direction from the apex of the leaf toward the petiole.

(E) Formation of midvein (MV) provascular strand in disk of insertion. The midvein extends acropetally into the leaf primordium and basipetally to connect to the stem vasculature (two-headed arrow).

(F) Formation of large lateral vein provascular strands (L) in disk of insertion. Large lateral veins develop acropetally into the leaf primordium and later basipetally to connect to the stem vasculature.

(G) Formation of intermediate longitudinal vein provascular strands (I) in distal portion of leaf. Only some of the intermediate longitudinal veins connect basipetally with the stem vasculature.

(H) Formation of small longitudinal (S) and transverse (T) veins in leaf blade region. Note the basipetal pattern of transverse vein formation.

bundles, appear at the leaf base and extend both acropetally into the leaf primordium and basipetally to connect with stem vasculature (Larson, 1975, 1984, 1986). In the simple leaves of *Populus*, midvein components remain discrete, so that distal secondary veins are derived from the central and lateral leaf traces, midsecondaries are derived from lateral and subsidiary trace elements, and basal secondaries are derived from the last-formed subsidiary traces (Isebrands and Larson, 1980). By contrast, in the compound leaves of *Gleditsia*, separate bundles fuse and branch so that the mature midvein is a composite of the three leaf traces and subsidiary bundles; each secondary vein combines elements of each leaf trace (Larson, 1984). As yet, this detailed analysis has not been extended to other species but points to both a complexity of primary vascular pattern and the secondary modification of the primary pattern (i.e., the formation of subsidiary traces and anastomoses between midvein components) during leaf development.

Minor vein pattern is formed during the intercalary extension growth of the leaf blade, usually in a basipetal sequence (Esau, 1965a). Tertiary vein provascular strands form in a simultaneous pattern, that is, a complete bridge between two secondary veins appears at once rather than developing progressively across the areole (Pray, 1955a; Hara, 1962; Lersten, 1965; Herbst, 1972; Merrill, 1979). As the leaf lamina expands, areoles formed by tertiary veins are subdivided by the next vein order, and this process is reiterated by higher order minor veins in a series of discrete steps, so that the initial coarse reticulum becomes finer and finer. This is a protracted process in species with numerous orders of minor venation but occurs quickly in species with only a few orders, such as Arabidopsis.

In the final round of minor vein formation, the provascular strands of the freely ending veinlets appear in the ultimate areoles. The pattern of formation of freely ending veinlets has been difficult to determine because they cannot be distinguished from the smallest order of minor veins in the reticulum at the provascular stage (Hara, 1962; Lersten, 1965; Herbst, 1972; Merrill, 1979). Apparently, a strand of cells with the potential to become provascular tissue bridges the areole, but cells at one end lose their provascular attributes and become indistinguishable from ground meristem, resulting in freely ending veinlets (Hara, 1962; Lersten, 1965). The hypothesis that free vein endings are formed because older provascular strands have lost their competence to form a vein juncture has not yet been tested experimentally and requires a better understanding of the nature of signals required for the coordinated formation of vascular pattern (Coleman and Greyson, 1976).

Monocot Ontogeny

The regular parallel (striate) venation of the leaves of most monocots also arises in an hierarchical sequence. First, the midvein provascular strand extends acropetally from its initiation at the disk of leaf insertion, which, in most species, occurs without initial attachment to stem vasculature (Figure 3E). Second, the major lateral provascular strands initiated at the disk of insertion extend acropetally (Figure 3F). Third, intermediate provascular strands initiated within the leaf primordium above the disk of insertion extend basipetally (Figure 3G), and fourth, small longitudinal and transverse vein provascular strands form, beginning near the tip and progressing in a basipetal direction to establish an interconnected vascular network within the leaf blade. Following the initial acropetal pattern of development, midvein and large lateral vein provascular strands extend basipetally to connect with stem vasculature; only some of the basipetally developing intermediate veins extend through the leaf sheath to connect with stem vascular bundles (Evert et al., 1996).

This ontogenetic sequence has been described in numerous monocot species, with particularly detailed descriptions available for the grasses maize (Sharman, 1942; Esau, 1943; Kumazawa, 1961; Bosabalidis et al., 1994), wheat (Sharman and Hitch, 1967; Blackman, 1971; Patrick, 1972), barley (Klauss, 1966; Dannenhoffer and Evert, 1994), rice (Kaufman, 1959; Inosaka, 1962), and *Arundinella* (Dengler et al., 1997). In leaves with close vein spacing, such as in those of the C_4 grasses, a second wave of basipetally differentiating provascular strands forms the small (minor) intermediate veins. In general, the pattern of major venation is well established within the first few plastochrons after leaf initiation, whereas the basipetal intermediate and minor veins continue to be initiated for an extended period.

A striking feature of the formation of most grass leaf venation patterns is the initiation of the midvein and major lateral veins in isolation from stem vasculature. This apparent isolation may simply be a limitation in our ability to detect the earliest stages of provascular activity. Cells committed to midvein initiation may, in fact, have an essential continuity with stem vasculature that will eventually be revealed by more sensitive molecular markers (see below). Regardless, the first cells that can be identified histologically as the precursors of the midvein and of the major lateral veins are generally found at the base of the primordium, with very evident gaps separating them from the nearest stem vasculature. The association with stem vasculature is established by extension of the provascular strand basipetally into the stem from the insertion site. The details of the connection of the midvein to stem vasculature have been described in few monocots. In maize, the midvein generally joins the stem trace of a major lateral vein of an opposite leaf several nodes below (Sharman, 1942).

The acropetal extension of the midvein into the primordium is associated with the differentiation of surrounding supportive tissues and with considerable (generally adaxial) thickening to form a midrib. In maize, the cell divisions producing this adaxial thickening follow midvein provascular divisions by approximately one plastochron (Sharman, 1942; J. Paxson and T. Nelson, unpublished observations). The differentiation of surrounding sclerenchymatous tissue is

concomitant with the differentiation of vascular tissues in that region. The ontogeny of the major lateral veins, initiated approximately one plastochron later than is the midvein, is similar to that of the midvein. These laterals are formed without initial connections to the midvein—a feature that suggests that the pattern of forming lateral provascular strands reveals a prepattern of positions (see below).

One or more waves of smaller basipetal veins are initiated during and after the period of leaf expansion. Like the lateral veins, these veins are initiated without initial connections to existing differentiated veins but instead occupy positions with fixed and parallel relationships to the existing vasculature. In the grasses, the large and small intermediate (basipetal) veins are formed without the extensive thickening and support tissues that accompany the major lateral veins. As differentiation progresses, the ranks of veins do join at leaf tips and margins, but at a time too late for older veins to have a significant role in guiding the differentation of newer veins. Rather, the ontogenetic patterns in monocot leaves suggest that existing veins serve as positional landmarks for the differentiation of later veins.

Development of the vascular pattern of the broad-leaved monocot *Hosta* resembles that of maize, but it is unclear whether the primary bundles are initially isolated from the stem vasculature (Pray, 1955c). The median primary bundle is present at the base of the leaf primordium early on, and lateral primaries form sequentially in a median to marginal direction. Formation of commissural and freely ending veinlets proceeds basipetally as the leaf expands. Appearance of commissural vein provascular strands (and most likely the freely ending veinlets) is continuous and simultaneous.

Vein Spacing

Spatial regularity is a striking feature of the vascular pattern of advanced dicots and monocots. This is most conspicuous in monocots and particularly in the grasses in which there is a constant relationship between blade width and longitudinal vein number (Russell and Evert, 1985; Dannenhoffer et al., 1990). However, regularity is also apparent in the reticulate venation pattern of dicots, despite the lack of uniformity in shape of the ultimate polygonal areoles (e.g., Pray, 1955a; Slade, 1957; Lersten, 1965; Herbst, 1972; Franck, 1979; Merrill, 1979). Where measurements have been made on mature leaves, the distance between branch points on the finest reticulum is remarkedly uniform regardless of whether veins branch from primary, secondary, tertiary, or higher order veins (Russin and Evert, 1984). Uniform spacing also appears to characterize the formation of all vein classes during leaf development, so that new provascular strands are intercalated between the old when a certain critical spacing is reached. Although there have been few quantitative studies of this phenomenon (Pray, 1955c; Dengler et al., 1997), many classic studies emphasized the regularity of vein spacing and of the number of cells between newly formed provascular strands (e.g., Foster, 1952; Pray, 1955a, 1955c; Lersten, 1965; Larson, 1984). These observations indicate a tight spatial control over vascular pattern formation throughout leaf development.

REGULATION OF VASCULAR PATTERN FORMATION

Pattern Formation Hypotheses

A universal theory of leaf vascular pattern formation would have to account for at least three quite different patterns: the continuous and acropetal formation of primary and secondary veins in dicot leaves (e.g., Figures 3A and 3B), the formation of parallel, isolated strands of provascular tissue in grass leaf primordia (Figures 3F and 3G), and the simultaneous formation of minor veins in dicots and monocots (Figures 3C, 3D, and 3H).

As yet, no single theory fully accounts for these disparate spatial and temporal patterns, but two general hypotheses, each using a different approach toward explaining the regulation of leaf vein pattern formation, have made a significant start. These are the canalization of signal flow hypothesis, which is based primarily on experimental observations of the inductive effects of auxin on vascular tissue formation (Sachs, 1981, 1989, 1991a, 1991b), and the diffusion–reaction prepattern hypothesis (Koch and Meinhardt, 1994; Meinhardt, 1995, 1996), which is based on computer modeling of interactions among hypothetical diffusible substances. Both hypotheses make use of the pioneering work of Turing (1952) on diffusion–reaction systems. Turing's models show that when small random deviations in an initially homogeneous morphogen field are reinforced by feedback, deviations from the initial concentration can form a stable pattern of peaks and troughs. Such a de novo formation of discrete pattern from a uniform field has analogies in many purely physical systems, such as the formation of a branched river system from a uniform drainage field (Meinhardt, 1996).

A third intriguing interpretation of the creation of venation patterns has been proposed recently (Kull and Herbig, 1995). This hypothesis relies on mathematical models for self-organization of two-dimensional space by using topological rules and fractality in combination with estimates of physiology and transport requirements in the leaf.

The canalization of signal flow hypothesis is derived from observations of the polar, unidirectional (i.e., progressive) differentiation of provascular strands under both experimental and nonmanipulated conditions, which suggests that a similar polar flow of a signal molecule may induce the formation of vascular strands (Sachs, 1981, 1989, 1991a, 1991b). Auxin transport is known to be polar (Lomax et al., 1995), and auxin induces the progressive differentiation of vascular strands in wounded tissues, indicating that this plant growth hormone may act as the signal for vascular pattern formation under natural conditions (Sachs, 1981, 1989, 1991a, 1991b).

Based on careful observations of regeneration of vascular tissue around mechanically induced wounds in stems and leaves, Sachs (1981, 1989, 1991a, 1991b) proposed that canalization of auxin flow occurred through a series of steps. Initially, all cells in a field adjacent to a severed vascular bundle are equivalent transporters of auxin (Figure 4A, left). Gradually, certain cells become better auxin transporters, and their capacity to transport auxin increases with auxin flux (Figure 4A, center). Eventually, the cells transporting auxin are induced to differentiate as provascular tissue, thus becoming determined as the preferred channels (Figure 4A, right. This process would induce cells at the terminus of a severed strand to become specialized as auxin transporters, thus canalizing the flow of inductive signal. Surrounding cells would be drained of auxin and therefore would be inhibited from forming vascular tissue. This hypothesis can readily account for the formation of an open, branching pattern such as that formed by the primary and secondary veins of a dicot leaf.

It is more difficult to account for the two other major patterns of vein pattern ontogeny (i.e., the simultaneous formation of minor vein networks and the sequential formation of parallel, isolated stripes) by using the canalization of signal flow hypothesis. To explain the formation of reticulate minor veins, both Sachs (1989) and Mitchison (1980) proposed that localized point sources of auxin induce the formation of bridging provascular strands either through a changing spatial pattern of such sources and/or through an alternation of the location of such sources between sides of a meristematic areole. Neither of these concepts is wholly satisfactory and requires the progressive, rather than simultaneous, formation of minor veins. Mitchison (1980) suggested that progressive formation occurs too quickly to observe; however, because detailed anatomical observations of vein ontogeny indicate that formation is simultaneous (Pray, 1955a; Hara, 1962; Lersten, 1965; Herbst, 1972; Merrill, 1979), other mechanistic explanations for the formation of reticulate vein

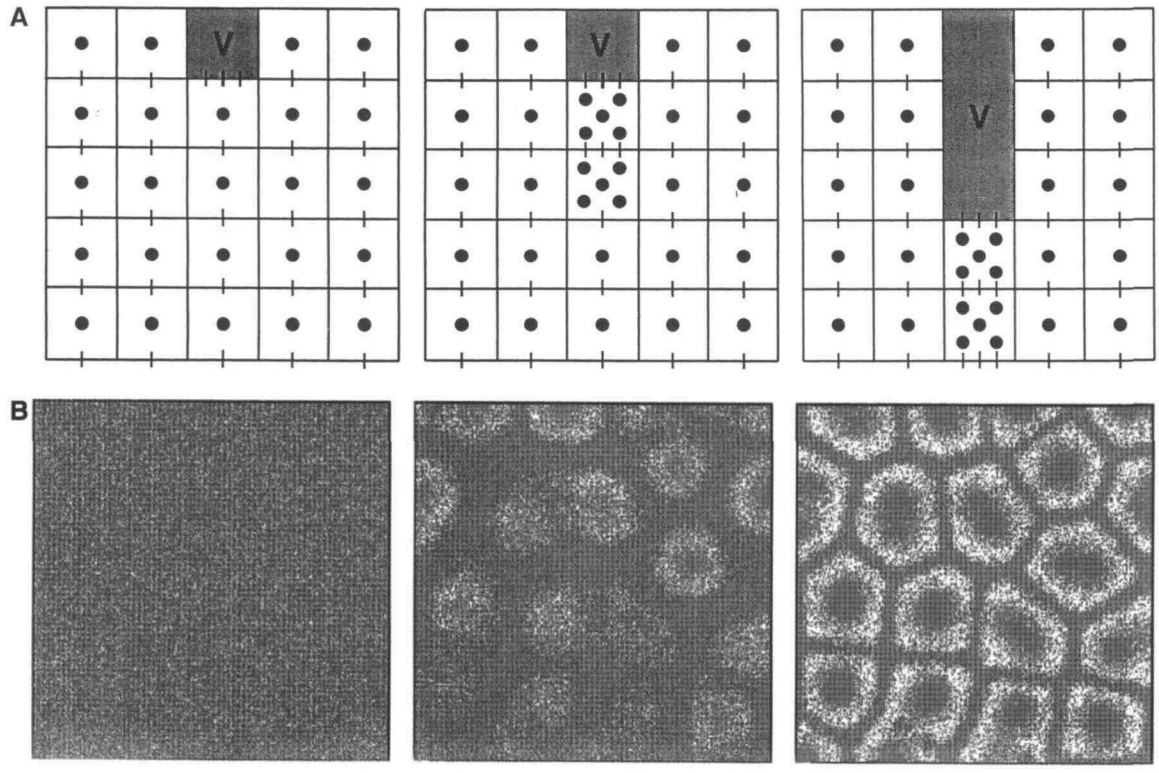

Figure 4. Hypotheses Explaining Vascular Pattern Formation.

(A) Canalization of signal flow hypothesis. Left, all cells in field adjacent to severed vein (V) are equivalent transporters of auxin (dots). Center, cells at the terminus of a severed vein are induced to become better auxin transporters (small vertical bars), with positive feedback so that their capacity to transport auxin increases with flux. Drainage of auxin from surrounding tissues inhibits expression of auxin transporters. Right, transporting cells differentiate as vascular tissue, thus becoming determined as preferred transport channels.

(B) Diffusion–reaction prepattern hypothesis. Computer simulation of a system forming patches combined with a system forming stripes. The patches specify where no stripes are allowed, and thus stripes appear at the largest possible distance from other stripes (see text for details; figure provided by H. Meinhardt, Max Planck Institut, Tübingen, Germany).

patterns should be sought. Canalization of auxin flow is also inadequate to explain the initial formation of grass leaf longitudinal veins but does provide a likely model for the secondary linkage of longitudinal veins with each other and with the stem vasculature.

The diffusion–reaction prepattern hypothesis has two essential components: (1) local self-enhancement or autocatalysis in which a small random increase in the concentration of an activator in an initially homogeneous field induces its further increase through positive feedback; and (2) long-range inhibition, in which a fast-diffusing antagonist prevents the spread of the self-enhancing reaction into neighboring tissue (Koch and Meinhardt, 1994; Meinhardt, 1995).

There are several attractive features to this model. If a morphogenetic field is growing isotropically, new peaks of activation emerge at positions in which the inhibitor concentration is low, thereby preserving the average spacing of the system. Moreover, depending on the kinetics of the interaction between activator and inhibitor molecules, different patterns may be formed de novo; for instance, if autocatalysis is not saturated, regularly spaced peaks are formed, but if autocatalysis saturates and production of the inhibitor substance is limited, a stripelike pattern results (Meinhardt, 1996).

Combining models of systems forming patches with systems forming stripes produces a closed reticulum, that is, patches where no stripes are allowed (Figure 4B; Koch and Meinhardt, 1994; H. Meinhardt, personal communication). The patterns produced by these modeling exercises mimic many common biological patterns, such as the spacing of leaf trichomes or stomata, insect segmentation patterns, coat patterns of zebras and giraffes, or fly wing venation patterns (Koch and Meinhardt, 1994; Meinhardt, 1995, 1996; Larkin et al., 1996, 1997, in this issue). Cellular response to gradual (but steep) morphogen gradients could produce sharp boundaries if autocatalytic feedback on gene expression and suppression of alternative genes occur simultaneously (Meinhardt, 1996). The autocatalytic nature of the expression of pattern formation genes, such as *engrailed, even-skipped,* and *fushi tarazu,* during Drosophila embryogenesis lends observational support to these ideas (Meinhardt, 1996 and references therein).

The diffusion–reaction prepattern hypothesis provides a useful perspective for understanding certain aspects of leaf vascular pattern ontogeny. For instance, the longitudinal veins of grass leaves essentially are formed in a homogeneous two-dimensional field (albeit rolled in a cone) and appear as a series of parallel stripes with new stripes intercalated between the preexisting ones as they grow apart (Dannenhoffer and Evert, 1994). New veins appear simultaneously not progressively, suggesting that cells in a uniform field are induced to develop as provascular tissue in response to prepatterns created by diffusing morphogens (Dengler et al., 1997).

The simultaneous formation of minor vein networks in dicot leaves and of commissural veins in monocot leaves could also be explained by the diffusion–reaction prepattern hypothesis. Moreover, alteration of the saturation coefficient of a hypothetical activator substance in computer simulations of pattern formation can result in a reticulate pattern that inserts new lines within an older reticulum so that the size of the enclosed domains remains the same, mimicking vein formation in insect wings and plant leaves (Koch and Meinhardt, 1994).

As yet, there is no direct observational evidence for morphogenetic substances that may act as activators or inhibitors of vein pattern formation in plants. Nevertheless, it is possible that auxin or other plant growth regulators may play a key role in a diffusion–reaction–induced prepattern; higher concentrations of auxin, brought about by efflux transporters that canalize the flow of auxin, may represent the activation component, whereas a low concentration of auxin in the surrounding field that has been "drained" of auxin may represent the inhibition component. Reconciling these points of view and furthering our understanding of the role of auxin and other unidentified factors during leaf development are crucial for answering unresolved questions about vascular pattern formation.

The role of auxins and other growth regulators as the possible agents of the pattern formation processes invoked in the models described above has not yet been subjected to rigorous experimental tests. Numerous wounding and auxin application experiments served as the basis for the canalization hypothesis (reviewed in Aloni, 1987; Sachs, 1991a, 1991b), yet none of these classic experiments unequivocally demonstrates that canalization of auxin flow is the mechanism that underlies provascular strand formation in normal development. It is possible, for example, that the directional flow of auxin is a means of guiding regeneration at wound sites but is not the primary mechanism in leaf development. In addition, it should be noted that these classic experiments were performed largely with the stems of dicots and that the ability of monocot tissues and leaves in general to regenerate vascular tissues at wounds or to respond to exogenous auxin is extremely limited (Aloni and Plotkin, 1985).

Genetic and molecular experiments to date have confirmed only that auxins and cytokinins can influence the degree and type of vascular differentiation. A number of mutants and transgenically modified plants have been described with auxin or cytokinin over- or underproduction, auxin transport defects, or auxin signaling defects (reviewed in Klee and Lanahan, 1995; see also Kende and Zeevaart, 1997, in this issue). Most of these have phenotypes that include vascular differentiation abnormalities. However, the cell-nonspecific nature of the alterations in these plants does not make it possible to evaluate the role of the corresponding genes, if any, in vascular pattern formation. For example, plants modified transgenically to have systematic alterations in auxin or cytokinin levels exhibit pleiotropic abnormalities in form and anatomy, such as excessive vascular differentiation, yet abnormalities in vascular pattern are impossible to distinguish (Medford et al., 1989; Romano et al.,

1991; Li et al., 1992; Ainley et al., 1993; Tuominen et al., 1995). Similarly, the in vitro tracheary element (TE) differentiation system (reviewed in Fukuda, 1992, 1997, in this issue; Church, 1993; Chasan, 1994), which permits the synchronized differentiation of TEs from *Zinnia elegans* leaf mesophyll protoplasts upon application of a hormone combination, serves to confirm that auxin and cytokinin are involved in vascularization but not necessarily in pattern formation. Other molecular genetic experiments, such as in situ hybridization measurements of the localization of mRNAs encoding the provascular markers *Arabidopsis thaliana homeobox-8 (Athb-8*; Baima et al., 1995) and *Tracheary Element Differentiation2* (*TED2*; Demura and Fukuda, 1993; see below), suggest that early steps in differentiation are similar in de novo and regenerating (wounded) vascular regions, but the distinction between differentiation and pattern formation is important.

Genetics of Vascular Pattern Formation

Remarkably few mutants with altered leaf vascular pattern in the context of otherwise normal vascular differentiation have been described. As a recent review of leaf cell differentiation observed, this could be due to redundancy of the genes responsible for leaf pattern, to the lethality of the loss of any component of such a system, or simply to the inadequacy of genetic screens to date in detecting such mutations (Hall and Langdale, 1996). Several potential pattern mutants were identified after chemical mutagenesis of the C_4 grass *Panicum maximum*, including variants in the spacing of veins, absence of minor veins, aberrant bundle sheaths, and absence of midrib (Fladung, 1994). The sterility of these variants and the heavy level of mutagenesis used to produce them suggest that the observed phenotypes are the consequence of combined mutations at more than one locus. Regardless, the study indicates that fundamental pattern alterations can be recovered in brute force screens.

A number of relevant mutations have been recovered from Arabidopsis, although all of them are in early stages of characterization, and their vascular defects could be secondary effects. In the *monopteros* mutant, leaf marginal veins are missing or interrupted, with little apparent effect on overall leaf morphology (Berleth and Jürgens, 1993; Przemeck et al., 1996). *monopteros* plants exhibit a reduced capacity for polar transport of auxin, but it is not clear whether this is the cause or a consequence of the vascular pattern abnormality. The *lopped1* mutant (and allelic *tornado*) also exhibits a defect in auxin transport, and its leaves are narrowed with a bifurcated and twisted midvein (Carland and McHale, 1996; Cnops et al., 1996).

Several narrow-leaf mutants have been described in maize, Antirrhinum, tobacco, and other species, in which leaves approach radial symmetry and the vascular pattern is reduced to a single midvein with zero to a few secondary veins (Miles, 1989; McHale, 1992, 1993; Waites and Hudson, 1995). Again, in these cases it is not possible to determine whether the vascular pattern defect is a cause or a consequence of the morphological phenotype.

Dominant mutant alleles of the maize homeodomain gene *Knotted* (which is normally downregulated at leaf initiation) cause the ectopic accumulation of the *Knotted* product along the lateral veins of leaves (Smith et al., 1992). In the blade, such lateral veins exhibit a differentiation pattern characteristic of the sheath, in which the bundle sheath is discontinuous and the immediately surrounding parenchyma is cleared (Sinha and Hake, 1994). Midribless mutants have been recovered from maize, barley, millet, and other grasses (Seip and Tsuchiya, 1979; Rao et al., 1988, 1989; Fladung et al., 1991; Fladung, 1994; J. Paxson and T. Nelson, unpublished observations). In grasses, the midrib region of the leaf blade normally exhibits a vascular pattern distinct from the adjacent laminae, with few basipetal veins between laterals, a pattern more typical of the sheath. In affected leaves of midribless mutants, adaxial thickenings are absent over the midvein, and the vascular pattern in the median region is the same as in the laminae, with the full complement of basipetal veins.

Molecular Markers for Provascular Cells

As should be evident from this review, a major limitation in the analysis of vascular ontogeny is the paucity of markers for provascular cells. Typically, provascular cells have been identified on the basis of their histological, or in some cases histochemical, properties. Provascular cells in many leaves can be recognized first by their dense staining patterns or by their characteristic tangential planes of cell division relative to surrounding ground cells. However, these properties are evidence of an earlier commitment to provascular behavior. It will be important to have earlier markers to evaluate critically the phenotypes of potential pattern mutants, because many if not all of the key events in pattern formation must occur before histological characteristics reveal the sites committed to vein formation.

As earlier provascular markers are found, the corresponding genes should be considered as candidates for roles in the provascular siting process. Some progress has been made in identifying such candidate markers. For example, expression of the *TED2* gene was identified as an early marker in the in vitro *Zinnia* TE differentiation system and was shown by in situ hybridization to be expressed in putative provascular cells of *Zinnia elegans* leaves and stems (Demura and Fukuda, 1993, 1994; Fukuda, 1997, in this issue). The *TED4* gene was recovered from the same system and is associated with a slightly later stage of vascular development (Demura and Fukuda, 1994; Fukuda, 1997, in this issue). The *Athb-8* gene, which was recovered from a screen for Arabidopsis homeodomain genes, is expressed in presumptive provascular cells of leaves, stem, and root (Baima

et al., 1995). The several enhancer- and gene-trap projects underway in Arabidopsis have recovered numerous lines with reporter traps of genes expressed in all or part of the vascular system. As these are analyzed in detail, additional provascular markers should become available.

PROSPECTS

Our knowledge of leaf vascular pattern and its ontogeny is largely descriptive, and so the models by which we can currently explain the observed patterns are limited. This review points out the need for additional genetic and molecular analysis of vascular pattern formation. Based on analyses of analogous processes in other organisms, such as wing venation and body segmentation in Drosophila, the intensive characterization of pattern-defective mutants should be a major experimental focus. In addition, we need a far better understanding of the provascular state, including the spatial signals that induce and maintain it, the earliest responses to those signals, and the stability of this state over time. Furthermore, we need to understand in more detail the role of hormonal signals in patterning and differentiation, their distribution in space, and local differences in their synthesis and reception. Finally, we need novel experimental approaches to the analysis of the patterned formation of provascular cells. The increased availability of vascular and provascular cell–specific promoters and enhancers, combined with strategies for genetic cell ablation, are examples of potentially useful novel tools, but certainly there is room for great creativity in this important area of vegetative development.

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