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Regulation of Vascular Development

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ABSTRACT

The regulation of vascular development is one of the major unresolved issues of plant developmental biology. This overview provides a framework for the following four papers by describing key events of vascular development and highlighting recent advances in understanding their regulation. Vascular development includes: (1) formation of the longitudinal pattern of primary vascular strands (2) formation of the radial pattern of xylem and phloem within vascular strands (3) differentiation of specialized cell types from xylem and phloem precursors; and (4) cell proliferation and cell differentiation within the vascular cambium. Integration of information from diverse sub-disciplines, including comparative anatomy of primary vascular patterns, manipulative experiments testing the role of hormones in pattern formation and cell differentiation, analysis of mutual phenotypes, detailed characterization of cellular events, and use of molecular tools to identify and determine gene function, will be essential for further progress in understanding the regulation of these processes.

Key words: Cell differentiation; Meristem; Phloem; Procambium; Sieve tube element; Tracheary element; Vascular cambium; Vascular pattern formation; Xylem

INTRODUCTION

Plant vascular systems provide conduits for the movement of water and solutes over long distances, more than 100 meters in the largest trees and vines. Water and mineral nutrients travel from the roots to the sites of evapotranspiration in the shoot system via the conducting cells of the xylem. Dissolved carbohydrates move from tissues that are net producers of photoassimilate to tissues that are net users, through the conducting cells of the phloem. Features that are prerequisite for these transport functions in both xylem and phloem are: (1) presence of elongate strands that link root and shoot systems, (2) enlargement and differentiation of specialized conducting cells in a pattern that aligns cells end to end, and (3) in woody species, a pattern of cell proliferation and differentiation within the cambial zone that provides continuous channels for transport throughout development. How this overall vascular architecture is formed and how the appropriate cell types differentiate with that pattern are major unresolved issues of plant developmental biology.

During the primary stage of plant growth, long continuous strands of vascular tissue connect every part of the shoot system, including stems, leaves, and flowers, with the root system. Within each strand, the appropriate proportions of xylem and phloem are formed, and within each tissue, conducting, parenchyma, and sclerenchyma cells must

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differentiate in appropriate numbers and spatial relationships that give a functional whole. Developing vascular tissues must be able to carry out their functions in expanding organs; this is particularly challenging in grasses and other groups of plants where leaves and stems grow from the base, and vascular continuity must be maintained across this growth zone. The primary vascular system also retains a capacity for modification. For instance, fully expanded leaves may develop additional vascular connections in the stem, and older stems can still form new vascular bridges to circumvent a wound. In woody plants, primary vascular strand function is replaced by the products of vascular cambium activity; this transition must occur without disruption so that shoot and root systems have a continuous supply of water and solutes.

How is the development of these complex structures and functions regulated? This brief overview cannot be comprehensive, but will point to some major themes and sources in the literature. I discuss four specific aspects of the regulation of vascular development: (1) formation of the longitudinal pattern of primary vascular strands; (2) formation of the radial pattern of xylem and phloem within vascular strands; (3) differentiation of specialized cell types from xylem and phloem precursors; and (4) cell proliferation and cell differentiation within the vascular cambium. Substantial progress has recently been made in each of these areas, and, in many ways, we are on the brink of a more complete understanding of vascular development. Most significantly, a central role for polar auxin transport continues to receive strong support from both physiological and molecular studies. At the same time, it is clear that multiple additional factors act in concert with this important directional signal to provide an integrated developmental and functional whole. The interplay of these factors provides a vascular system that is reproducible, yet flexible, and one that achieves an overall pattern and arrangement of cell types that works, yet is not identical from individual to individual (Mattsson and others 1999; Sachs 2000; Berleth 2001; Berleth and Sachs 2001; Savidge 2001).

The turn of the 21st century has been an exciting time for understanding the regulation of vascular development. We have an extensive knowledge base of the principles of primary vascular architecture that govern both the longitudinal and radial patterns of vascular tissues within plant shoots (Esau 1954, 1965a, b; Larson 1994). There is a strong tradition of experimental manipulation that has demonstrated the regenerative capacities of the vascular systems of stems and leaves and the roles of phytohormones in both pattern formation and cell differentiation (for example, Jacobs 1952; Sachs 1975, 1981, 1989, 1991a, 1991b; Aloni 1987, 1995, 2001). Importantly, early experiments led to the development of a testable hypothesis, canalization of auxin flow. This idea has recently received compelling support from the localization of auxin efflux carrier molecules (Lomax and others 1995; Galweiler and others 1998), from studies of auxin transport inhibitors (for example, Mattsson and others 1999; Sieburth 1999), and from characterization of the vascular phenotype of mutants that are defective in auxin synthesis, perception, or polar transport (Przemeck and others 1996; Carland and McHale 1996; Deyholos and others 2000; Hobbie and others 2000). Development of the *Zinnia* cell culture system in which mesophyll cells transdifferentiate directly into tracheary elements (TE) in response to phytohormone application has greatly advanced our understanding of cell differentiation events, as well as their regulation (Fukuda and Komamine 1980a, b; Fukuda 1992, 1994, 1996, 1997a, b, 2000; Fukuda and others 1998; Kuriyama and Fukuda 2001). A combination of careful sectioning techniques and mass spectrophotometric detection of auxin led to the suggestion that auxin concentration gradients may provide the positional information required to coordinate cell division and differentiation across the cambial zone (Savidge and others 1982; Uggla and others 1996, 1998; Sundberg and others 2000).

Below I give an overview of developmental events and highlight recent advances in regulation of primary vascular pattern formation, vascular cell differentiation, and cambial activity. Readers are also referred to recent reviews by Aloni (1995), Aloni and others (2000), Northcote (1995), Savidge (1996, 2000), Nelson and Dengler (1997), Sjolund (1997), Sachs (2000), Berleth and Mattsson (2000), Berleth and others (2000), and Fukuda (1997a, b, 2000).

Development of Longitudinal Vascular Pattern

Embryo and Shoot-Root Axis

Formation of vascular pattern is an integral part of embryogenesis and reflects the expression of apical-basal polarity in the zygote. During the early stages of embryogenesis, organogenesis and histogenesis occur simultaneously at different levels of organization. Organogenesis gives rise to the distinctive shapes and positional relationships of root, stem, and cotyledon, whereas histogenesis gives rise to the precursors of the dermal, ground, and vascular tis-

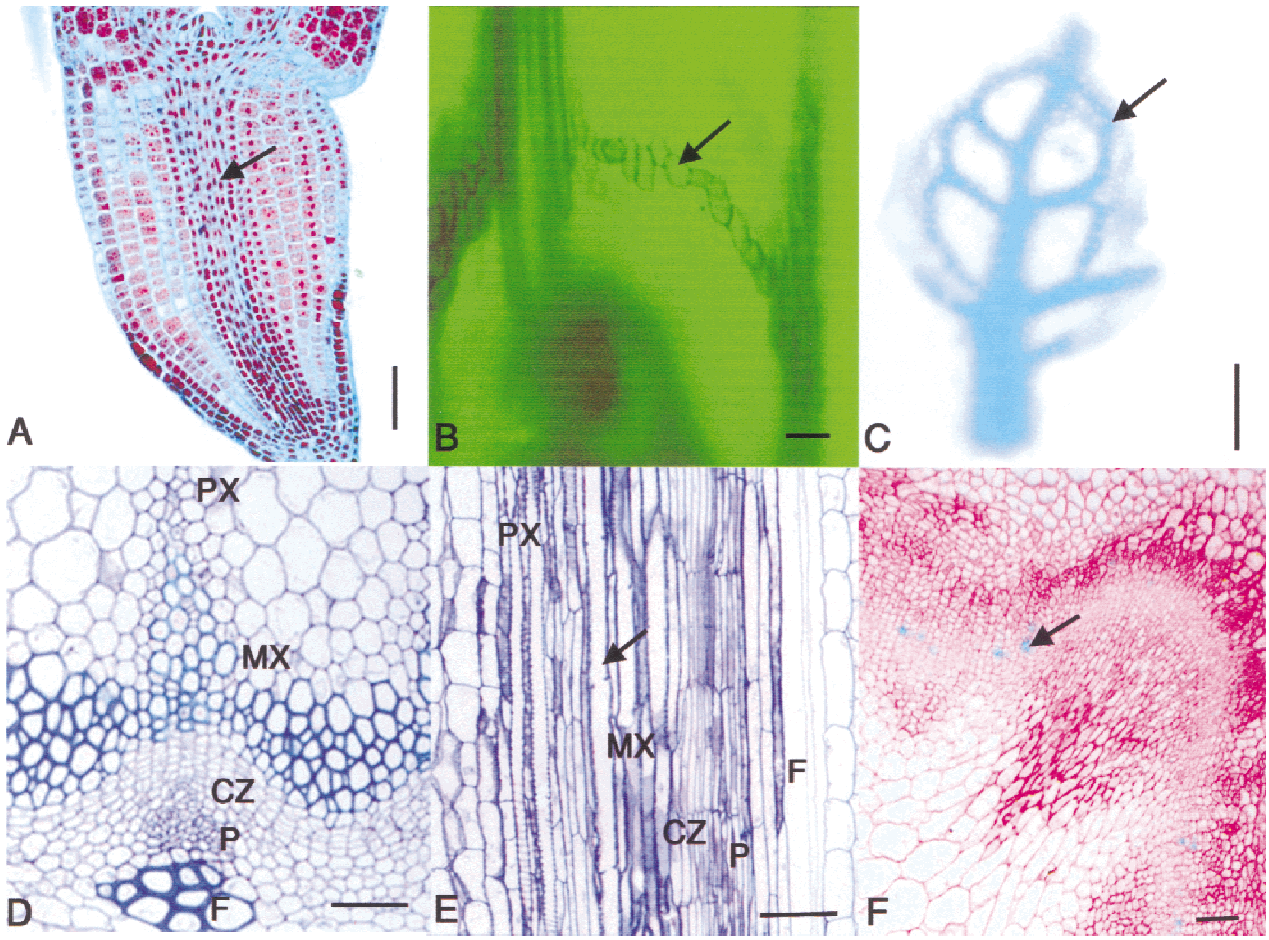

Figure 1. Examples of vascular development. **(A)** Mature embryo of *Clarkia rubicunda* showing delineation of procambium (arrow) from ground meristem (photo courtesy R. Dengler). **(B)** Regeneration of xylem TE (arrow) after wounding in internode of *Coleus blumei*. **(C)** Developing leaf of *Arabidopsis thaliana* expressing a *Athb8::GUS* construct. Arrow indicates secondary vein (construct courtesy G. Morelli). **(D)** Cross-section of inflorescence stem of *Arabidopsis* showing primary xylem and phloem and cambial zone (slide courtesy J. Tang). **(E)** Longitudinal section of inflorescence stem of *Arabidopsis* showing primary vascular tissues and cambial zone. Arrow points to aligned TE (slide courtesy B. Ibarguchi). **(F)** Cross-section of rosette stem of *Arabidopsis* expressing *cyc1At::GUS* construct (courtesy J. Celenza). Arrow points to cyclin-expressing cell in cambial zone. CZ, cambial zone; F, fibers; MX, metaxylem; P, phloem; PX, protoxylem. Scale bar = 50 µm.

sue systems (protoderm, ground meristem, and procambium, respectively). These tissue precursors generally become delineated by distinctive patterns of cell enlargement and cell division. Procambial cells are recognized on a histological basis by their elongated, narrow shape, and unvacuolated, darkly stained cytoplasm (Figure 1A) (Esau 1965a).

At present, there are only a few known molecular markers for the procambial stage of development. For instance, expression of the *Arabidopsis thaliana homeobox gene 8* (*Athb-8*) is clearly detectable in putative procambium of heart stage embryos in both *Arabidopsis* and tobacco (Baima and others 1995, 2000). The *Tracheary Element Differentiation 3* construct (*pTED3::GUS*) marks both early stages of tracheary element differentiation in *Zinnia* cell cultures (Demura and Fukuda 1993, 1994) and is expressed in embryo cotyledon procambium (Igarishi and others 1998; Koizumi and others 2000). At present, the significance of the expression of homeobox genes, particularly those belonging to the HD-Zip family, within developing vascular tissue is not fully understood. Recently, careful analysis of the expression patterns of the rice HD-ZIP transcription factor, *Oshox 1*, indicates that this gene may regulate cell commitment to a procambial cell fate (Scarpella and others 2000). Early embryo stages were not examined, however, so the relationship between *Oshox1* expression and the earliest anatomical indication of procambium is unknown.

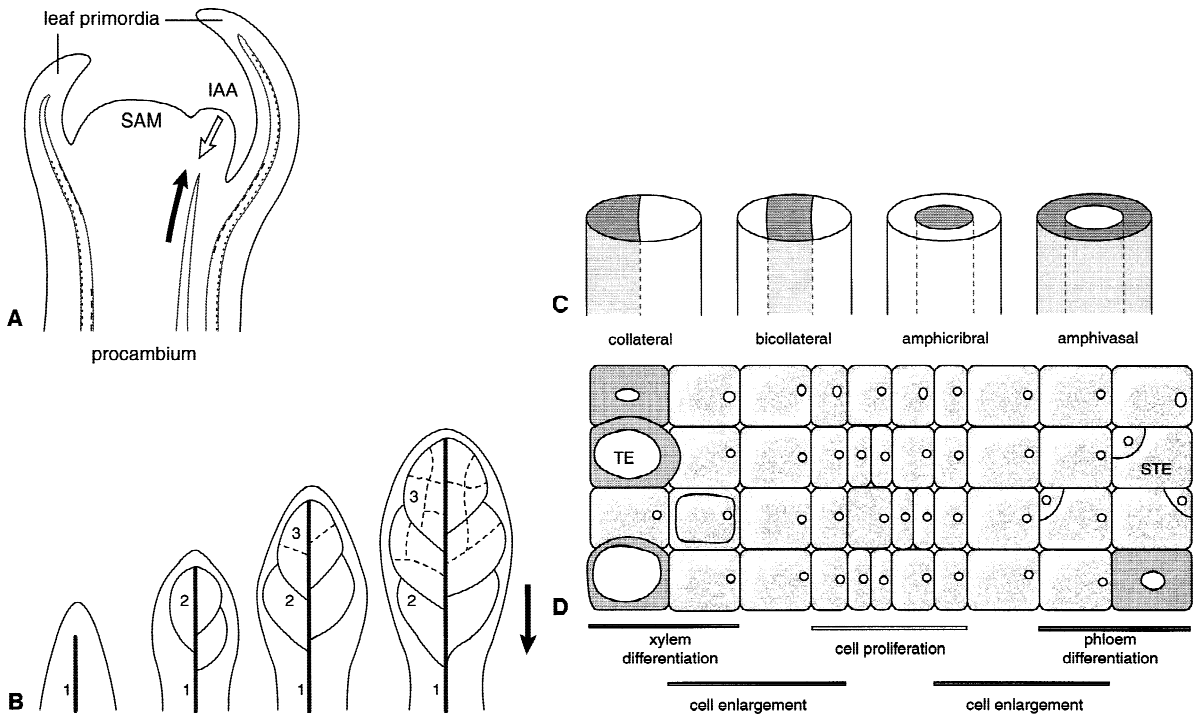

Figure 2. **(A)** Diagram showing longitudinal course of procambial strands within shoot apical meristem (SAM) region. Strands develop acropetally (solid arrow) into leaf primordia, in continuity with older strands within the stem. Leaf primordia are the source of a basipetally transported auxin (IAA) signal (open arrow). Phloem tissue (dotted line) differentiates in continuity with phloem in the older part of stem, and xylem tissue (dashed line) differentiates discontinuously. **(B)** Diagram of leaf vascular pattern development. Leaf veins are formed in hierarchical order, with primary (1) veins appearing before secondary (2) veins, and secondary veins generally appearing before tertiary (3) veins. The spatial pattern of secondary, tertiary, and higher order vein formation is basipetal (arrow). **(C)** Radial patterns of xylem (shaded) and phloem within vascular strands. **(D)** Diagram showing approximate regions of cell proliferation, cell enlargement, and differentiation and xylem and phloem within the cambial zone. Dark shading, thickened secondary walls of tracheary elements (TE); light shading, living cells with primary walls, including the sieve tube elements (STE).

Upon embryo germination, new leaves are generated by the shoot apical meristem, and each new leaf is connected with preexisting vasculature by one or more leaf traces. In all species that have been studied in detail, primary vascular strands develop in continuity with preexisting strands and extend acropetally into newly formed leaves and other shoot components (Figure 2A) (Esau 1954, 1965a, b). Procambial strand longitudinal growth keeps pace with shoot extension so that continuity is maintained. In *Arabidopsis thaliana*, the vegetative rosette is highly condensed, and new leaf traces have an almost horizontal orientation, making it difficult to determine whether leaf traces have a basipetal or acropetal pattern of development (Busse and Evert 1999).

How is the development of primary vascular pattern regulated at the physiological and molecular levels? Both observations of wildtype development and experimental manipulation of vascular development have provided support for the canalization of auxin flow hypothesis developed by Sachs (1981, 1989, 1991a, b). It has long been known that exogenous application of a point source of auxin can induce new vascular strands that connect the source of auxin with preexisting strands in the shoot (Figure 1B) (Jacobs 1952; Sachs 1981). Sachs (1981) hypothesized that these strands develop *de novo* from a uniform field of parenchyma cells that initially show random variation for the presence or absence of auxin transporters. Strand formation is self-reinforcing in the sense that, once a cell begins to transport auxin, it becomes a more effective transporter of auxin. A cell at the basal end of a transporting cell is induced to become a specialized transporter itself, whereas laterally adjacent cells are drained of their auxin and are inhibited from developing auxin transport capacity (Sachs 1981, 1991a, b; Aloni 1987, 2001; Berleth and Sachs 2001; Berleth 2001; see his Figure 1E). Thus, a narrow continuous strand develops basipetally from a source of

auxin toward an auxin sink. The identification of auxin efflux carrier molecules at the basal end of vascular parenchyma cells has provided important support for this hypothesis (Lomax and others 1995; Galweiler and others 1998). The apparent contradiction between the basipetal flow of auxin and the visible acropetal development of procambial strands needs to be resolved, however (Figure 2A). Molecular markers of early events in vascular development could contribute to understanding how these two processes are coupled.

The discovery of new genes through mutagenesis screens and the application of molecular tools have provided the strongest support thus far for the role of auxin in primary vascular strand formation. For instance, the phenotype of *MONOPTEROS* (*MP*) mutants of *Arabidopsis* display both a reduction in the basal end of the embryo and, in weaker alleles, discontinuities in leaf vascular pattern (Berleth and Jurgens 1993; Przemeck and others 1996). Mutant inflorescence stems are impaired in the polar transport of auxin (Przemeck and others 1996). Positional cloning has identified *MP* as a transcription factor that recognizes auxin response elements in the genome (Hardtke and Berleth 1998). *In situ* hybridization experiments also showed that *MP* is expressed throughout the subepidermal tissue of globular stage embryos, but gradually becomes restricted to the central procambial tissue of the embryonic axis (Hardtke and Berleth 1998). The *MP* mutant phenotype provides support for the idea that auxin polar transport and signaling are required for both embryo organogenesis (for example, formation of the shoot-root axis) and histogenesis (for example, formation of longitudinally arranged procambial strands).

Leaf Vascular Pattern

Is the development of leaf vascular pattern consistent with the canalization of auxin flow hypothesis? The leaves of flowering plants are characterized by elaborate vascular networks with veins arrayed both longituindally and transversely within the blade. In *Arabidopsis* and most other dicotyledons, the vascular system consists of several vein size classes, including a prominent midvein, divergent secondary veins, and a reticulum of smaller veins (Nelson and Dengler 1997; Candela and others 1999; Sieburth 1999). As a continuation of leaf trace procambium within the stem, the midvein develops acropetally into the leaf primordium. Secondary veins develop in continuity with the midvein and appear in a basipetal sequence (Figures 1C, 2B). A detailed analysis of cell wall patterns in leaves less than 0.3 mm in length indicated that procambium forms simultaneously, that is, the full course of the secondary vein appears at once, rather than progressively (Mattsson and others 1999). The canalization of auxin flow hypothesis predicts that cells would be added incrementally to the end of developing procambial strands, but these observations suggest that this step happens at a prepatterning stage and that morphological appearance of procambium is expressed only after the bridge from auxin source to sink is complete (Mattsson and others, 1999).

Screens for leaf vascular pattern mutants have yielded a handful of genes that appear to be required for vein pattern formation (Carland and McHale 1996; Candela and others 1999; Carland and others 1999; Deyholos and others 2000; Koizumi and others 2000). Mutant phenotypes typically display reduced continuity of leaf vasculature: in cotyledons and foliage leaves, the midvein and occasionally the marginal veins are intact, but the connecting secondary and higher order veins are disrupted, often resulting in vein islands. Vein discontinuity is strongly linked to abnormal cell elongation and alignment, and careful examination of the procambial stage in some cases revealed that the primary defect is in vein pattern, not cell differentiation (Carland and others 1999; Deyholos and others 2000; Koizumi and others 2000). The discontinuous leaf vein phenotype has been shown to be correlated with defective auxin transport in the *LOPPED1* (Carland and McHale 1996), *MONOPTEROS* (Przemeck and others 1996), *SCARFACE* (Deyholos and others 2000), and *AUXIN-RESISTANT6* (Hobbie and others 2000) mutants. Application of auxin transport inhibitors partially phenocopies these mutant phenotypes, lending support to the importance of auxin in establishing a continuous vein network (Mattsson and others 1999; Sieburth 1999). In contrast, auxin synthesis, transport, and perception appeared normal in the *COTYLEDON VASCULAR PATTERN* mutants (Carland and others 1999), indicating that additional factors are involved in regulation of leaf vascular pattern.

These mutant phenotypes, results from auxin transport inhibition experiments, and earlier vascular regeneration experiments (Sachs 1975, 1981, 1989) led Aloni to propose a comprehensive leaf venation hypothesis (Aloni 2001). This hypothesis postulates that shifting sites of auxin synthesis within the leaf could explain the acropetal development of the primary vein, as well as the basipetal course of secondary and higher order vein formation, and the different spatial and temporal patterns

of xylem and phloem differentiation from procambium. The combination of careful studies of vein pattern formation and cell differentiation (for example, Canadela and others 1999; Sieburth 1999; Carland and others 1999) coupled with molecular tools that can detect auxin transport or signaling (for example, Steinman and others 1999) make tests of this hypothesis feasible, despite the complexity of vein pattern formation.

Radial Pattern of Xylem and Phloem

Not only must developmental signals provide information about the longitudinal pattern of procambial strands, but they must also provide information about the radial pattern so that xylem and phloem tissues differentiate from procambial precursors in the appropriate locations and ratios (Figures 1D, 2C). Nowhere is regulation of this radial pattern of vascular tissues more apparent than in the transition zone of the seedling. Within the root and hypocotyl, xylem tissue is mostly interior to the phloem, but the first-formed xylem tracheary elements (protoxylem) differentiate toward the exterior of the seedling. Above the cotyledonary node, xylem is fully interior to the phloem, but now the protoxylem differentiates toward the interior of the shoot (for example, Esau 1965a, b; Busse and Evert 1999). The continuity of conducting cells must be maintained, despite this intricate diagonal course of the protoxylem and the global alteration of tissue pattern within the vascular strands.

Developmental regulation of the radial pattern of tissue differentiation within procambium within the transition zone of the seedling, as well as within roots, stems, and leaves is unknown. So far, only one gene that appears to regulate the relative positions of xylem and phloem within vascular strands has been identified, the *amphivasal vascular bundle* gene (Zhong and others 1999). Several other mutations indicate that events taking place at the organogenetic level, particularly those that affect organ symmetry, have a dramatic effect on the radial pattern of xylem and phloem within leaf veins (Dengler and Kang 2001). For instance, in the most extreme phenotypes of the *phabulosa-1d* mutation of *Arabidopsis* (McConnell and Barton 1998) and the *phantastica* mutation of *Antirrhinum* (Waites and Hudson 1996), leaves develop as cylindrical organs. Rather than displaying the typical collateral pattern, the single vein of *phantastica* leaves is amphicribral (Waites and Hudson 1996), and the single vein of *phabulosa-1d* leaves is amphivasal (McConnell and Barton 1998). Although the discontinuous leaf veins of the *VASCULAR NETWORK* mutants remain collateral, the volume of phloem in relation to xylem is enhanced (Koizumi and others 2000). These mutations point to the integration of developmental signals involved in longitudinal patterning, alignment of conducting cells, and the positional relationships of xylem and phloem within the veins. Thus, major factors, including the axial flow of auxin, would be expected to affect all of these developmental processes. Other major players need to be identified, however, and, at present, those influencing radial patterning represent a large gap in our knowledge of the regulation of vascular development.

Xylem and Phloem Differentiation

Xylem

In the *Zinnia* model system, leaf mesophyll cells are induced to transdifferentiate as tracheary elements (TE) when cultured in a medium containing auxin and cytokinin (Fukuda and Komamine 1980a, b; Fukuda 1996; McCann 1997). Up to 60% of the cultured cells differentiate synchronously, allowing a precise characterization of cytological, biochemical, and molecular events (Fukuda 1992, 1996, 1997a, b, 2000; Kuriyama and Fukuda 2001). Such a system has been invaluable in understanding the temporal course of cell differentiation. In intact plants, tracheary element differentiation follows a complex spatial and temporal pattern, so that adjacent cells undergo asynchronous differentiation (Esau 1965a). Thus, though it is possible to analyze cytological aspects of cell differentiation *in planta,* identification of gene expression patterns would be much more challenging. A number of genes with specific patterns of expression during TE differentiation have been identified through screening cDNA libraries from the *Zinnia* system, however (Demura and Fukuda 1993, 1994; Ye and Varner 1993, 1994; Ye and Droste 1998). These include *TED2,* with homology to an alcohol dehydrogenase, *TED3,* a hydrophobic protein of no known homologies, and *TED4,* a lipid transfer protein, in addition to other genes related to lignification and cell death (Fukuda 1997a, 2000; Ye and Droste 1996). Comparison of the timing and sequence of expression patterns for some of these genes are similar to those in cells differentiating from procambium in intact plants, as indicated by *in situ* hybridization analysis (Demura and Fukuda 1994). Thus, the *Zinnia* system provides a powerful tool for understanding the complexities of TE cell differentiation in intact plants.

The events of transdifferentiation of *Zinnia* mesophyll cells to mature tracheary elements have been summarized in detail (Fukuda 1996, 1997a, 2000; Groover and others 1997; Groover and Jones 1999; Kuriyama and Fukuda 2001; see their Fig. 1). In the *Zinnia* system, mesophyll cells must first dedifferentiate (Stage I). During Stage II, cells become committed to differentiating as TE, and this stage is thought to correspond to setting aside certain cells of the procambial strands as TE precursors in the intact plant. Interestingly, though auxin and cytokinin are required for cell commitment to TE differentiation during Stage II, they appear not to be required for differentiation *per se,* since cells can be transferred back to non-inducing medium at the end of Stage II and still complete differentiation (Stacey and others 1995). The final stage of cell differentiation (Stage III) involves alterations to the cytoskeleton, formation of secondary wall cellulose microfibrils and matrix polysaccharides, synthesis of several cell wall proteins, lignification of the secondary wall, and programmed cell death (Fukuda 1996, 1997a, b; McCann 1997; Fukuda and others 1998; Groover and Jones 1999). TE cell death is thought to be a vacuole-dependent type and to differ from programmed cell death observed in senescence or apoptosis-like events seen in response to pathogenesis (Fukuda 2000). One of the earliest events is the collapse of the vacuole preceded by changes in permeability of the vacuolar tonoplast to organic anions (Kuriyama 1999).

While the events of TE differentiation are now well-documented and functions of some of the genes expressed during this process are becoming better understood, the regulation of these events appears to be highly complex. This complexity is highlighted in the review by Kuriyama and Fukuda (2001): essentially, all plant hormones and a host of other factors are known to influence TE differentiation. What makes the matter more complicated is that in the plant, TE differentiation is not synchronous: xylem cells differentiate in a discontinuous fashion along a procambial strand (Esau 1965a; Busse and Evert 1999). In addition, the first TE to differentiate at any given location are protoxylem elements with annular or helical secondary walls; later TE differentiate with reticulate or pitted secondary walls. These cells must be able to interpret positional information to follow such predictable patterns. Xylem also is a complex tissue, comprised of TE, parenchyma cells, and sclerenchyma fibers and, though the placement of these cell types within the xylem tissue is not precise, they generally differentiate in predictable ratios (Esau 1965a), indicating that cell interactions play a role in determining cellular identity. Communication between like cells is suggested by the fact that TE are aligned longitudinally. The most common TE type in angiosperms, the vessel element, undergoes a specific degradation of the primary wall where cell ends in a file abut, forming the perforation that allows the bulk flow of water within each vessel (Figure 1E). In plants with long (10 m) vessels such as certain oaks, thousands of individual TE are aligned to form a single vessel. In the *Zinnia* system, Nakashima and others (2000) showed that two TE derived from the same mother cell formed small perforations in adjoining walls. *In planta,* both TE and fiber cells generally have matched pits on the lateral walls, indicating that inductive signals are communicated between adjacent cells. The nature of these signals and how adjacent cells within the xylem are induced to become strikingly different mature cell types is currently unknown.

Positional information and cell—cell communication must coordinate the differentiation of several cell types over a small spatial scale, but must also act to coordinate larger scale patterns of cell differentiation. One of the best-documented large scale patterns is the variation in TE size and number with position in large woody trees: vessel diameter generally increases from the leaves to the roots, so that vessel density decreases along the same gradient (Aloni and Zimmerman 1983; Aloni 1987, 1995, 2001; Aloni and Barnett 1996). Aloni hypothesizes that the increase in vessel size along the plant axis reflects a gradient of decreasing auxin concentration from leaves to roots and that auxin concentration is directly correlated with the rate of cell expansion and cell differentiation (Aloni 2001). Procambial or cambial cells located near the leaves are exposed to higher concentrations and thus differentiate more quickly, before substantial cell expansion takes place, whereas those located at a greater distance from a source of auxin differentiate more slowly, allowing for greater cell expansion. A similar hypothesis might explain the differences in TE diameter between early wood and late wood within a single growth ring, or between protoxylem and metaxylem within a primary vascular strand.

Phloem

Phloem cell differentiation parallels that of the xylem in that several distinct cell types differentiate from procambial or cambial precursors, including highly specialized conducting cells, the sieve tube elements (STE) (Figure 1D, E). As with the xylem,

the STE must be aligned end to end and develop a highly specialized end wall, the sieve plate, where adjacent cells abut. Unlike xylem differentiation, however, differentiation of STE appears to always be continuous and acropetal within the shoot, providing a conduit for the import of carbohydrates at the earliest stages of leaf development (Esau 1954, 1965a, b; Busse and Evert 1999). Like xylem, adjacent STE appear to have an inductive influence on each other, forming matching clusters of modified plasmodesmata (sieve areas) that functionally link the cells through their lateral walls.

The cytological details of STE differentiation have been well characterized from intact plants (for example, Thorsch and Esau 1981a, b, c; Northcote 1995; Eleftheriou 1996). A striking feature of STE differentiation is that cells undergo selective degradation of organelles, including the nuclei, vacuoles, rough endoplasmic reticulum, golgi, ribosomes, and microtubules. At the same time, cell membranes, plastids, mitochondria, smooth endoplasmic reticulum, and copious phloem protein are protected from degradation and survive in mature functioning cells. The basis of this differential organelle targeting remains an unsolved, yet fascinating, mystery (Sjolund 1997).

In addition to STE, phloem tissue contains fibers, parenchyma cells, and specialized companion cells. Adjacent companion cells and STE are typically derived from a common precursor, and companion cells are thought to perform two important roles in the functioning of enucleate STE (Sjolund 1997). Companion cells are the site of synthesis of both transcription and translation for all proteins required by mature STE (Thompson and Schultz 1999). For example, the filamentous phloem protein PP1 is found primarily within STE, and *PP1* mRNA is localized to the companion cells (Clark and others 1997). Many cell differentiation events are likely regulated by the STE themselves, however, since they take place when developing STE are still nucleate. In addition, short-lived protophloem STE may lack companion cells, whereas longer-lived metaphloem STE are always associated with companion cells (Eleftheriou and Tsekos 1982), suggesting that STE may direct their own differentiation events, but rely on companion cells for post-differentiation function. In addition to synthesis, companion cells are involved in the loading of photoassimilates into the STE prior to transport to other parts of the plant. The plasmodesmatal connections between STE and companion cells are highly modified (Thompson and Schulz 1999), reflecting both the volume of carbohydrate movement and the trafficking of macromolecules. Thus, in addition to having a unique pattern of cell differentiation themselves, STE require close coordination with companion cells, for both proper development and for mature functioning.

How are these differentiation events regulated at the hormonal level? Regeneration experiments indicate that low concentrations of auxin stimulate phloem differentiation, whereas higher levels induce xylem differentiation (Aloni 1987, 1995, 2001). Under tissue culture conditions, high auxin levels can result in differentiation of both phloem and xylem, but only phloem under lower concentrations (Aloni 1980). Other phytohormones, particularly cytokinins, have been shown to induce STE differentiation in wounded stems (Aloni and others 1990; Aloni 1993).

Although STE differentiation has been studied in intact plants, regenerating internodes, and in tissue culture, the lack of a reliable model system comparable to the *Zinnia* system for TE differentiation has been a major limitation to progress. Even under optimum conditions, only 5 to 10% of cells in tissue culture are induced to differentiate as STE, and differentiation is not synchronous (Sjolund 1997). Although the use of reporter constructs and other molecular tools will aid in understanding the regulation of phloem differentiation in intact plants, it will be essential to develop a tissue culture system that allows the large scale identification of genes involved in phloem development, as has been possible for xylem (Kuriyama and Fukuda 2001).

Vascular Cambium

The primary vascular system acts as a template for the development of the vascular cambium. In species lacking secondary growth, the procambial strands are said to be "closed" or determinate—the meristematic tissue is consumed by vascular cell differentiation. For species with secondary growth, procambial strands are "open" or indeterminate—the panel of meristematic tissue between the primary xylem and phloem does not differentiate and becomes mitotically active (Figure 1D, E, F) (Esau 1965a; Larson 1994). The zone of cell divisions spreads laterally from the vascular strands, so that interfasicular parenchyma cells transdifferentiate as meristematic cambium (Esau 1965a). The transition from procambial to cambial cells forms a continuum, but cambial cells are typically recognized by the regularity of division plane and the presence of two cell types: the fusiform initial cells (giving rise to the axial elongate cells of the secondary xylem and

phloem) and the cuboidal ray initials (giving rise to radially oriented parenchymatous rays). Not only is the initial formation of cambium a complex process, but cell numbers and dimensions change over time with the increase in circumference of stems and roots. In addition, most temperate-zone woody plants undergo cycles of cambium dormancy and reactivation that are cued by environmental signals.

At least in concept, the vascular cambium is comparable to an apical meristem. Centrally located cells are regarded as "initial" cells that have the stem cell-like property of replacing themselves with each division. Cells derived from the initial cells are "derivatives"; these cells might divide numerous times, but eventually all descendants differentiate. The cambium resembles the root apical meristem in that it is bifacial. Cambial initials produce derivatives toward the inside (secondary xylem) and toward the outside (secondary phloem) of the axis. Like an apical meristem, the cambium needs to maintain a fine balance between cell proliferation and cell differentiation (Figure 2D). It is clear from experimental manipulation that the patterns of cell divisions in the cambial zone are not genetically programmed (Savidge 1996, 2001; Chaffey 1999). Rather, it appears that cells perceive their local environments, including hormonal signals, physical factors, and intercellular messenger molecules, and act accordingly. In a parallel situation in the shoot apical meristem, the *WUSCHEL* and *CLAVATA* genes interact antagonistically. *WUSCHEL* promotes stem cell division behavior, and the *CLAVATA 1, 2,* and *3* genes repress mitotic activity and promote commitment to leaf primordium formation (Schoof and others 2000; Brand and others 2000). In addition, the three *CLAVATA* genes encode components of a receptor kinase signaling pathway, including a putative receptor kinase (Clark and others 1997). It is likely that as yet unidentified genes perform similar functions in the cambial zone.

The vascular cambium forms a continuous sleeve within the shoot-root axis. Individual cells within the cambial zone experience hormonal and other signals travelling both longitudinally and radially (Little and Pharis 1995; Uggla and others 1998; Savidge 2001). Although endogenous levels of almost all plant hormones have been shown to be present in the cambial zone, the polar transport of auxin from leaves and shoot tips toward the roots is thought to provide the major signal for cambial development (Little and Pharis 1995; Aloni and others 2000; Savidge 2001; Sundberg and others 2000). Physiological experiments support this idea: for instance, cambial activity is restricted by removal of natural sources of auxin and by application of auxin transport inhibitors, and auxin application enhances cambial activity and induces differentiation of secondary xylem and phloem (Savidge and Wareing 1981; Savidge 1983; Little and Savidge 1987; Leitch and Savidge 1995; Little and Pharis 1995; Savidge 2001). Recently, cryosectioning techniques in combination with gas chromatography—mass spectroscopy detection of auxin have demonstrated the occurrence of an auxin concentration gradient across the cambial zone (Uggla and others 1996, 1998; Sundberg and others 2000). Peak auxin concentrations coincide with the cambial intial zone and levels tail off within xylem and phloem derivatives. The observation that cells divide, enlarge, or differentiate at different concentrations across the gradient indicates that auxin could provide a positional signal that cues cell behavior and could potentially affect expression and function of signalling genes as in the shoot apical meristem.

Secondary xylem and secondary phloem contain the same cell types as the primary vascular tissues, but in different proportions and different spatial relationships. The regular arrangement of secondary tissues has facilitated characterization of some aspects of the cell biology of cell differentiation. For example, the positioning of cytoskeletal microtubules and microfilaments in relation to the formation of vessel element pits, perforations, and tertiary wall thickenings has been described with beautiful precision (Chaffey and others 1999; Chaffey 1999, 2000). Presumably lessons learned from the study of primary differentiation will also apply to cell differentiation within the cambial zone, but undoubtedly some regulatory factors will be unique to either system. For instance, physical pressure is required for normal cambial behavior and must be applied for induction of cambium in callus tissue or grafts (Savidge 1996). A radial gradient of auxin concentration may be necessary to maintain cell division activity, cell expansion, and cell differentiation, all in close juxtaposition within the cambial zone. Whether a similar gradient might be present within primary procambial strands and contribute positional information for the spatial arrangement of xylem and phloem is unknown at present.

The ability to isolate large quantities of cambium has made it possible to carry out biochemical and molecular analyses (Savidge 1996, 2000, 2001). To date, cDNA libraries have been constructed from the cambial zones of a pine (Allona and others 1998), a hybrid poplar (Sterky and others 1998; Hertzberg and Olsson 1998), a eucalypt (Bossinger and Leitsch 2000), and *Arabidopsis* (Zhao and others 2000). At least some of these cDNAs represent genes involved in cellular differentiation. For instance, expression

of the homeobox gene *VAHOX1* in tomato is restricted to secondary phloem and is excluded from the initial zone of the cambium, suggesting that it may be involved with the acquisition of phloem cell identity (Tornero and others 1996). Homeobox gene expression specific to the xylem maturation zone has been identified for hybrid poplar (Hertzberg and Olsson 1998). The challenge for the future will be to identify additional genes that are directly involved with the regulation of cell cycling, cell expansion, and cell differentiation within the cambium, as well as genes involved in the perception and translation of positional signals.

CONCLUSIONS

In the past decade there has seen dramatic progress in understanding the regulation of vascular development. Many of these new findings, as well as testable hypotheses and perspectives derived from them, are detailed in the four papers to follow (Berleth 2001; Aloni 2001; Kuriyama and Fukuda 2001; Savidge 2001). In this overview, I have attempted to highlight issues that I see as important ones for solution. For instance, the apparent paradox of basipetal auxin flow and the acropetal development of procambial strands within the shoot system needs to be resolved. It may be that molecular markers of earlier stages of procambial strand formation, including those involved with auxin perception and signalling, will help to resolve this discrepancy. Although auxin transport inhibitor studies and characterizations of mutant phenotypes have contributed much to understanding regulation of the complex vein network of dicot leaves, it is still not clear how this pattern is formed. Additionally, we do not know how the spatial pattern of xylem and phloem cell types within a procambial strand is achieved and how this might be influenced by organogenic events. The *Zinnia* model system has led to a detailed understanding of the events of xylem TE differentiation and the factors regulating these events, but comparable understanding of phloem STE differentiation is almost completely lacking. Finally, the factors regulating the maintenance of the balance between cell proliferation and commitment to differentiation in the vascular cambium, as well as the interaction between long distance signaling and local cell-to-cell communication are unknown.

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