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Of Cells, Strands, and Networks: Auxin and the Patterned Formation of the Vascular System

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Throughout plant development, vascular cells continually form from within a population of seemingly equivalent cells. Vascular cells connect end to end to form continuous strands, and vascular strands connect at both or either end to form networks of exquisite complexity and mesmerizing beauty. Here we argue that experimental evidence gained over the past few decades implicates the plant hormone auxin—its production, transport, perception, and response—in all the steps that lead to the patterned formation of the plant vascular system, from the formation of vascular cells to their connection into vascular networks. We emphasize the organizing principles of the cell- and tissue-patterning process, rather than its molecular subtleties. In the picture that emerges, cells compete for an auxin-dependent, cell-polarizing signal; positive feedback between cell polarization and cell-to-cell movement of the polarizing signal leads to gradual selection of cell files; and selected cell files differentiate into vascular strands that drain the polarizing signal from the neighboring cells. Although the logic of the patterning process has become increasingly clear, the molecular details remain blurry; the future challenge will be to bring them into razor-sharp focus.

AUXIN AND THE PLANT VASCULAR SYSTEM

In most multicellular organisms, water, nutrients, and signals are transported by tissue networks. In animals, this essential transport function is distributed over separate tissue networks—for example, the nervous, circulatory, and respiratory systems. By contrast, in plants, all transport functions are performed by the only tissue network: the vascular system.

The plant vascular system is a network of vascular strands that connect the different parts of an organ and the different organs of a plant (Fig. 1; Esau 1965). Vascular strands are bundles of files of vascular cells that are elongated along the file and connected at their ends (Fig. 1). Vascular strands are named differently in different organs: veins in flat organs like leaves, petals, sepals, and cotyledons; vascular bundles in the stem; and vascular cylinder or stele in the root.

The relation between the plant hormone auxin and vascular development is old (e.g., Kraus et al. 1936) and varied (e.g., Snow 1935; Camus 1949; Jacobs 1952; Wangermann 1967). Here we discuss evidence that suggests that auxin controls the patterned formation of the vascular system at all the system's organization levels: the cells', the strands', and the network's.

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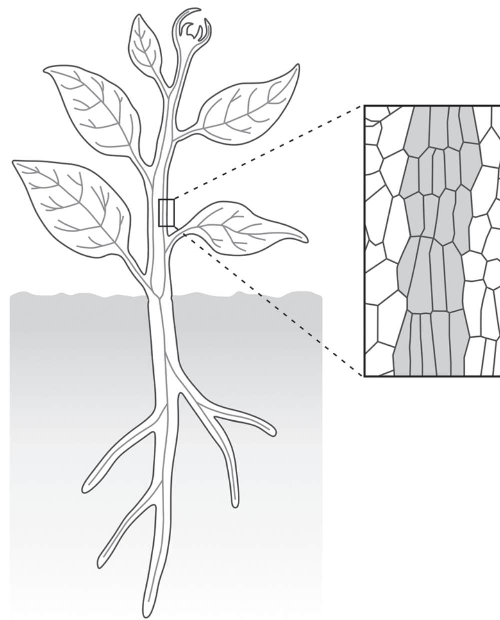

Figure 1. The plant vascular system. (*Left*) The plant vascular system is a network of vascular strands (gray lines) that connect the different parts of an organ and the different organs of a plant. (*Right*) Vascular strands are bundles of files of vascular cells (gray fill) that are elongated along the file and connected at their ends.

We will emphasize the "syntax" of such control, rather than its "semantics." This latter has been reviewed recently and comprehensively elsewhere (e.g., Jouannet et al. 2015; De Rybel et al. 2016; Etchells et al. 2016; Cho et al. 2017; Ramachandran et al. 2017; Ruonala et al. 2017; Anne and Hardtke 2018; Fischer et al. 2019; Fukuda and Ohashi-Ito 2019).

AUXIN SIGNALING AND THE FORMATION OF THE FIRST VASCULAR CELLS

Much of a seedling can be seen as a cylinder with a vascular strand in its center (Fig. 2A). The formation of this cylinder coincides with the formation of the first vascular cells in the *Arabidopsis* globular embryo (Fig. 2A; Scheres et al. 1994; De Rybel et al. 2014; Yoshida et al. 2014; Gooh et al. 2015). However, expression of vascular-specific markers suggests that the identity of these first vascular cells had been specified earlier, at the dermatogen stage (Fig. 2B; Smit et al. 2020).

The *Arabidopsis* dermatogen embryo is composed of 16 cells: eight outer cells, which are the precursors of the epidermis, and eight inner cells, which are the precursors of all the other tissue types (Fig. 2B; Mansfield and Briarty 1991; De Rybel et al. 2014; Yoshida et al. 2014; Gooh et al. 2015). These eight inner cells will divide periclinally and asymmetrically, and the resulting four larger, innermost cells in the basal half of the globular embryo will become the first vascular cells (Fig. 2B; Esau 1965).

Available evidence suggests that the formation of the seedling cylinder and the vascular strand in its center depend on auxin signaling. Indeed, dermatogen embryos of auxin signaling mutants express vascular-specific markers abnormally—if at all—and the eight inner cells in these mutant embryos fail to divide periclinally and asymmetrically and to form the first vascular cells in globular embryos (Fig. 2C; Berleth and Jurgens 1993; Hardtke and Berleth 1998; Hamann et al. 1999, 2002; Hobbie et al. 2000; Hellmann et al. 2003; Dharmasiri et al. 2005; Yoshida et al. 2014; Smit et al. 2020). Most of these mutant embryos develop into seedlings in which the vascularized cylinder is replaced by a vascularless cone (Fig. 2C). This defect characterizes mutants in auxin perception or response but also mutants in auxin production and embryos developed in the presence of auxin antagonists (Hadfi et al. 1998; Dharmasiri et al. 2003, 2007; Cheng et al. 2007; Stepanova et al. 2008; Thomas et al. 2009).

Among such mutants, defects are most severe in mutants lacking the function of the *MONOPTEROS/AUXIN RESPONSE FACTOR5* (*MP* hereafter) gene of *Arabidopsis*, which encodes a transcription factor that regulates auxin-responsive gene expression (Berleth and Jurgens 1993; Ulmasov et al. 1997, 1999; Hardtke and Berleth 1998; Mattsson et al. 2003), and in mutants with a stabilized variant of the otherwise short-lived MP-inhibitor INDOLE-3-ACETIC ACID12/BODENLOS (Hamann et al. 1999, 2002; Hardtke et al. 2004; Dharmasiri et al. 2005; Weijers et al. 2005, 2006; Schlereth et al.

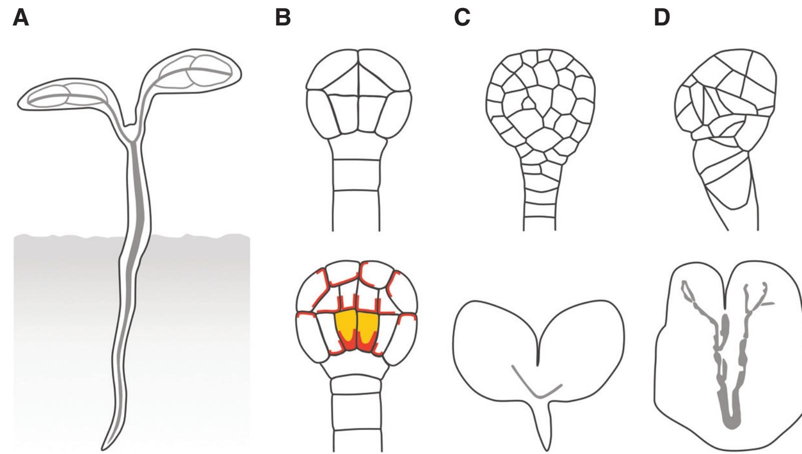

Figure 2. Auxin signaling and the formation of the first vascular cells. (*A*) Much of the seedling is a cylinder with a vascular strand (gray line) in its center. (*B*) The localization of PIN1 (red) is polarized in the first vascular cells (yellow fill) of the globular embryo (*bottom*), which originate from the periclinal, asymmetric division of the lower inner cells in the dermatogen embryo (*top*). (*C*) The lower inner cells in the dermatogen embryos of auxin signaling mutants fail to divide periclinally and asymmetrically, leading to vascularless globular embryos (*top*) and seedlings in which the vascularized cylinder is replaced by a vascularless cone (*bottom*). (*D*) Auxin-signaling-unrelated mutants whose cells divide along random planes (*top*) form vascular strands nonetheless (*bottom*).

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2010; Lau et al. 2011; Garrett et al. 2012; Krogan et al. 2012). Similar defects also characterize mutants in the *EMBRYO DEFECTIVE30/GNOM* (*GN* hereafter) gene of *Arabidopsis* (Mayer et al. 1993; Shevell et al. 1994; Busch et al. 1996; Yoshida et al. 2014), which encodes a regulator of membrane trafficking that controls auxin signaling through unknown pathways (Mayer et al. 1993; Wolters et al. 2011; Verna et al. 2019).

That these mutants fail to form the first vascular cells seems to result from the inability of the lower inner cells of the dermatogen embryo to divide periclinally and asymmetrically, suggesting that auxin signaling promotes such asymmetric cell division (Hamann et al. 1999; Yoshida et al. 2014). However, vascular cells—although abnormally shaped—still form in mutant embryos in which cells divide along random planes (Fig. 2D; Torres-Ruiz and Jurgens 1994; Yoshida et al. 2014)—random planes that presumably include those which in auxin signaling mutants are correlated with failure to form vascular cells. Furthermore, auxin application to various tissues induces the formation of vascular strands even in the absence of cell division (Fig. 3A; see, e.g., Sinnott and Bloch 1944, 1945; Sachs 1969). Therefore, the vascular-differentiation-promoting influence of auxin does not seem to necessarily depend on its cell-division-orienting activity.

AUXIN TRANSPORT AND VASCULAR STRAND FORMATION

The functional unit of the vascular system is the vascular strand, whose formation seems to depend on auxin transport. This conclusion is suggested by the result of experiments in which auxin is applied to mature plant tissues: Auxin application leads to the formation of continuous files of vascular cells that connect the applied auxin to the preexisting vascular strands basal to the auxin application site (Fig. 3A; Kraus et al. 1936; Jost 1942; Jacobs 1952; Sachs 1968; Linh et al. 2018), and this polar formation of vascular strands in response to auxin application is prevented by inhibitors of polar auxin transport (Roberts 1960; Thompson and Jacobs 1966), suggesting that it depends

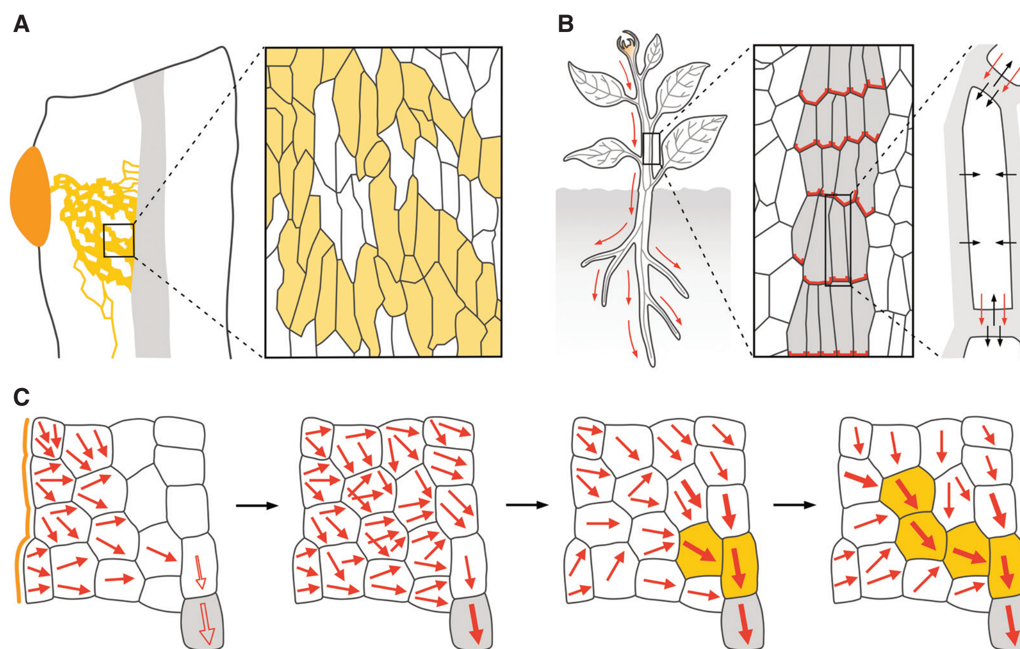

Figure 3. Auxin transport and vascular strand formation. (*A*) (*Left*) The application of auxin (orange) to mature plant tissues induces the differentiation of vascular cells in continuous files to form vascular strands (yellow lines) that connect the applied auxin to the preexisting vascular strands (gray fill) basal to the auxin application site. (*Right*) In the vascular strands formed in response to auxin application, vascular cells (yellow fill) are not aligned along the file like in Figure 1, but along the axis of the tissue. (*B*) (*Left*) Auxin (orange) is mainly produced in immature shoot organs and transported (red arrows) toward the roots through vascular strands (gray lines). (*Middle*) Apicobasal, polar auxin transport results from the localization of auxin efflux carriers of the PIN family (red) at the basal plasma membrane of vascular cells (gray fill). (*Right*) Auxin efflux carriers are required for auxin to leave the cell (red arrows) because auxin is mostly negatively charged inside the cell; by contrast, in the extracellular space, auxin is to a larger extent uncharged and can thus diffuse into the cell (black arrows). (*C*) The auxin canalization hypothesis assumes that auxin diffuses from the auxin application site (orange line) toward the preexisting vascular strands in the organ (gray fill). The positive feedback between auxin movement (red arrows) and localization of auxin efflux carriers to the site where auxin leaves the cell would gradually polarize auxin transport (thicker arrows). This would occur first in the cells connected to the preexisting vasculature (gray fill), which are still polarized along the original, apicobasal polarity of the tissue (empty red arrows) and thus orient auxin movement toward themselves. Increased auxin transport polarity, capacity, or velocity in the selected cells would lead to vascular differentiation (yellow fill) and drain auxin from neighboring cells, thus inhibiting their differentiation. The process would continue until a vascular strand formed that connected the applied auxin to the preexisting vascular strands basal to the auxin application site. Black arrows connect successive stages of auxin-induced vascular-strand formation.

on the ability of the responding tissue to transport auxin polarly.

Indeed, although auxin is mainly produced in immature shoot organs such as leaf and flower primordia (Thimann and Skoog 1934; Avery 1935), it is transported toward the roots through vascular strands (Fig. 3B; Went 1928; Wangermann 1974). This apicobasal, polar auxin transport is thought to result from the localization of auxin efflux carriers at the basal end of auxin-transporting cells (Fig. 3B; Rubery and Sheldrake 1974; Raven 1975). Indeed, the weak acid auxin is mostly negatively charged inside the cell and can thus efficiently leave the cell only through plasma-membrane-localized auxin efflux carriers (Fig. 3B). Although the mechanism of action is still unclear (for review, see Barbosa et al. 2018), available evidence suggests

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that these auxin efflux carriers are encoded by *PIN-FORMED* (*PIN*) genes (for review, see Adamowski and Friml 2015). Computational simulations of this model suggest that it can account for both polar auxin transport (Mitchison 1980b) and the polar formation of vascular strands in response to auxin application, provided that auxin movement through a cell positively feeds back on the localization of auxin efflux carriers to the site where auxin leaves the cell, as proposed by the auxin canalization hypothesis (Sachs 1981, 1991, 2000).

The auxin canalization hypothesis assumes that the applied auxin—or an auxin-dependent signal that is experimentally indistinguishable from auxin—initially diffuses from the auxin application site toward the preexisting vascular strands in the organ (Fig. 3C; Sachs 1981, 1991, 2000). In the cells between the application site and the preexisting vascular strands, auxin efflux carriers would initially be localized to all the different sections of the plasma membrane (i.e., nonpolarly). By contrast, in the preexisting vascular strands, auxin efflux carriers would be localized along the original apicobasal, auxin transport polarity of the tissue. Therefore, the preexisting vascular strands would efficiently remove any auxin that reached them by diffusion from the auxin application site, thereby acting as auxin sinks that orient toward themselves auxin movement in the neighboring cells and polarize the localization of auxin efflux carriers in those same cells (Fig. 3C). The resulting induction of polar auxin transport in the cells neighboring the preexisting vascular strands would be gradually enhanced by the positive feedback between auxin movement and localization of auxin efflux carriers proposed by the auxin canalization hypothesis. By draining auxin increasingly more efficiently and polarly, the cells neighboring the preexisting vascular strands would in turn induce polar localization of auxin efflux carriers and polar auxin transport in the cells above them and inhibit the same processes in the lateral neighbors (Fig. 3C). The repetition of these steps would eventually lead to preferential auxin transport through single cell files—the "canals" the hypothesis refers to—that would connect the applied auxin to the preexisting vascular strands basal to the auxin application site and that would differentiate into vascular strands (Fig. 3C). During this process, any random polarization in the localization of auxin efflux carriers would be stabilized by the positive feedback between auxin movement and localization of auxin efflux carriers proposed by the auxin canalization hypothesis and lead to random deviations in the course of the selected cell files and from the shortest route for auxin transport.

The plasma membrane localization of PIN1 auxin efflux carriers of *Arabidopsis* marks the presumed auxin efflux side of cells (Petrasek et al. 2006; Wisniewska et al. 2006). Therefore, the polarity of auxin transport can be inferred from the localization of PIN1 to the plasma membrane. Auxin application to mature plant tissues induces the formation of broad expression domains of nonpolarly localized PIN1 that connect the applied auxin to the preexisting vascular strands (Sauer et al. 2006; Mazur et al. 2016). Over time, the broad PIN1-expression domains (PEDs hereafter) become restricted to sites of auxin-induced vascular strand formation, and PIN1 localization becomes polarized: In the cells along the PEDs' midline, PIN1 localization becomes polarized toward the preexisting vascular strands basal to the site of auxin application; in the cells flanking the PEDs' midline, PIN1 localization becomes polarized toward the domains' midline. Eventually, narrow PEDs mark the sites of auxin-induced vascular strand formation, and polar PIN1 localization in the vascular strands formed in response to auxin application suggests auxin transport away from the applied auxin and toward the preexisting vascular strands basal to the auxin application site. Both the restriction of broad PEDs and the polarization of PIN1 localization during auxin-induced vascular strand formation initiate and proceed away from the preexisting vascular strands and are consistent with predictions of the auxin canalization hypothesis.

Consistent with predictions of the auxin canalization hypothesis is also the observation that auxin application fails to induce vascular strand formation in *pin1* mutants or plants treated with auxin transport inhibitors (Mazur et al. 2020). This observation is, however, unex-

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pected because auxin application to shoot tips of *pin1* mutants or wild-type plants grown in the presence of auxin transport inhibitors leads to the formation of whole leaves, including their veins (Reinhardt et al. 2000, 2003). This finding is not the only apparent inconsistency between assumptions or predictions of the auxin canalization hypothesis and experimental observations (Bennett et al. 2014; Runions et al. 2014; Ravichandran et al. 2020). For example, the auxin canalization hypothesis assumes that auxin readily diffuses out of the cells (Sachs 1981), but auxin diffusion out of the cell is unfavored over diffusion into the cell by nearly two orders of magnitude (Runions et al. 2014).

The auxin canalization hypothesis also assumes that cells can measure net auxin transport across their plasma membranes (Sachs 1969). No biological mechanism is known that allows cells to measure net auxin transport directly (Mitchison 1980a, 1981; Kramer 2009; Cieslak et al. 2015). However, cells could use the concentration of auxin, of auxin-bound auxin carriers, or of substances produced or consumed during auxin transport to measure auxin influx and efflux separately, which would indirectly provide a measure of net auxin transport (Mitchison 1980a; Coen et al. 2004; Cieslak et al. 2015). Alternatively, cells could measure intracellular auxin gradients, which depend on auxin transport: Auxin concentration would be higher where auxin enters the cell and lower near auxin efflux carriers, where auxin leaves the cell (Mitchison 1981; Kramer 2009). Even though these mechanisms await experimental testing, they are both plausible; instead, that auxin controls polarization of PIN1 localization by inhibiting PIN1 deallocation from the section of the plasma membrane from which auxin leaves the cell can be ruled out (Paponov et al. 2019).

Finally, the auxin canalization hypothesis assumes that developing vascular strands effortlessly connect to preexisting vascular strands, an assumption that seems to be justified by experimental observations (Sachs 1968) but that cannot be easily reproduced by computational simulations (Bayer et al. 2009; Smith and Bayer 2009). This limitation is overcome in plants that have two PIN1 proteins: one that broadly polarizes auxin transport toward preexisting vascular strands, and the other that restricts the broad auxin transport domains to narrow sites of vascular strand formation (O'Connor et al. 2014, 2017). In plants like *Arabidopsis* that have only one PIN1 protein, instead, a hypothetical substance has been proposed to diffuse from preexisting vascular strands and polarize PIN1 localization in developing vascular strands toward preexisting vascular strands (Bayer et al. 2009; Smith and Bayer 2009).

Despite the apparent limitations, vascular strand formation in response to auxin application seems to be an expression of auxin transport whose essence is captured by the auxin canalization hypothesis; can we say the same of the vascular strands that form during normal development?

AUXIN TRANSPORT AND VEIN FORMATION

Just like auxin application to mature plant tissues, auxin application to developing leaves leads to the formation of continuous files of vascular cells that connect the applied auxin to the preexisting veins basal to the auxin application site (Scarpella et al. 2006; Sawchuk et al. 2007; Verna et al. 2019). During both auxin-induced and normally occurring vein formation, PIN1 expression is initiated in broad domains of leaf inner cells connected to preexisting veins (Fig. 4A; Scarpella et al. 2006; Wenzel et al. 2007; Marcos and Berleth 2014). In the cells of those broad PEDs, PIN1 is initially nonpolarly localized (Carraro et al. 2006; Scarpella et al. 2006; Wenzel et al. 2007; Lee et al. 2009; Marcos and Berleth 2014; Johnston et al. 2015). Over time, however, the broad PEDs become restricted to sites of vein formation, and PIN1 localization becomes polarized: In the cells along the PEDs' midline, PIN1 localization becomes polarized toward preexisting veins; in the cells flanking the PEDs' midline, PIN1 localization becomes polarized toward the domains' midline (Fig. 4A). Both the restriction of broad PEDs and the polarization of PIN1 localization initiate and proceed away from preexisting veins and are delayed by auxin transport inhibition. And both auxin transport inhibition and higher aux-

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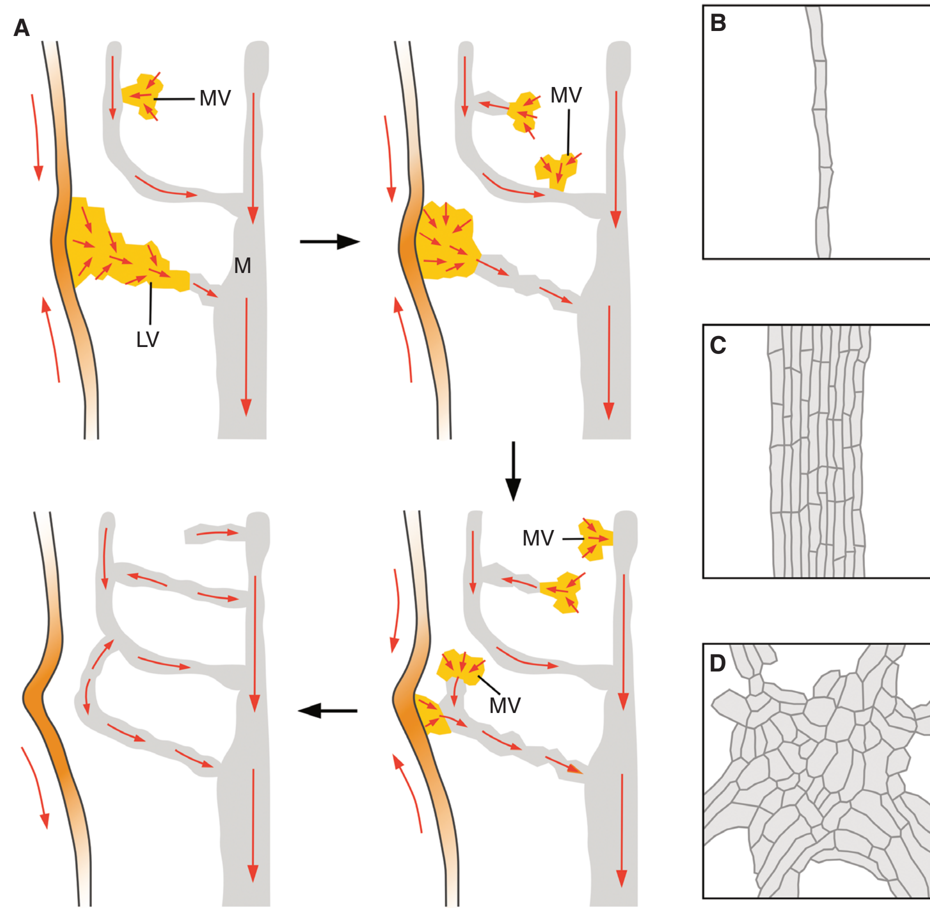

Figure 4. Auxin and vein formation. (*A*) In rounded leaves, PIN1 localization in epidermal cells of the leaf margin (red arrows) becomes polarized toward sites of leaf lateral growth (orange). Epidermal convergence points of PIN1 polarity are associated with sites of leaf lateral growth and broad PEDs in the inner tissue that become restricted to sites of lateral vein (LV) formation (yellow). Over time, PIN1 localization becomes polarized (red arrows) in the cells of these broad domains: In the cells along the PEDs' midline, PIN1 localization becomes polarized toward preexisting veins (gray); in the cells flanking PEDs' midline, PIN1 localization becomes polarized toward the domains' midline. Both the restriction of broad PEDs and the polarization of PIN1 localization initiate and proceed away from preexisting veins (gray), in which PIN1 localization is polarized (red arrows). Broad PEDs in the inner tissue that become restricted to sites of minor vein (MV) formation (yellow) branch from preexisting veins (gray). Over time, PIN1 localization becomes polarized (red arrows) in the cells of these broad domains: In the cells along the PEDs' midline, PIN1 localization becomes polarized toward preexisting veins (gray); in the cells flanking PEDs' midline, PIN1 localization becomes polarized toward the domains' midline. Both the restriction of broad PEDs and the polarization of PIN1 localization initiate and proceed away from preexisting veins, in which PIN1 localization is polarized (red arrows). Broad MV-associated PEDs can gradually disappear instead of becoming restricted and polarized. Initially, MV-associated PEDs connect to preexisting veins at one end only ("open" PEDs), but they can extend to connect to preexisting veins at both ends ("closed" PEDs). Open PEDs have a single polarity; closed PEDs have two opposite polarities, which are connected by a "bipolar" cell: a cell in which PIN1 localization is polarized at both ends (see also Fig. 6F). Each vein loop forms from the fusion of LV-associated PEDs and MV-associated closed PEDs. Black arrows connect successive stages of leaf development. (*B–D*) In wild-type (*B*) and auxin-transport-inhibited (*C*) leaves, vascular cells are connected end-to-end and aligned along the vein. By contrast, in leaves in which both auxin transport and auxin signaling are inhibited (*D*), veins are replaced by clusters of vascular cells that are randomly oriented.

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in levels lead to the formation of broader PEDs, but given time even these broader PEDs eventually become restricted to sites of vein formation (Aloni et al. 2003; Mattsson et al. 2003; Hay et al. 2006; Scarpella et al. 2006; Wenzel et al. 2007).

Many of these observations can be accounted for by the positive feedback between auxin movement and localization of auxin efflux carriers proposed by the auxin canalization hypothesis (Mitchison 1980a, 1981; Sachs 1981, 1991, 2000; Rolland-Lagan and Prusinkiewicz 2005). But if the auxin canalization hypothesis were truly to account for vein formation in response to auxin, leaves of *pin* mutant plants or wild-type plants grown in the presence of auxin transport inhibitors or lacking PIN proteins should form no veins (Rolland-Lagan and Prusinkiewicz 2005). However, this prediction seems to be unsupported by experimental evidence: Leaves of wild-type plants grown in the presence of auxin transport inhibitors do form veins; and as in normally grown plants, the veins of auxin-transport-inhibited plants are oriented along the apicobasal axis of the leaf, and their vascular cells are elongated along the vein and connected at their ends (Fig. 4B,C; Sieburth 1999; Verna et al. 2019). Veins with these same features also form in leaves of *Arabidopsis* plants lacking all the PIN proteins with vascular function (Verna et al. 2019). Therefore, that auxin-induced vascular-strand formation depends on auxin transport (i.e., the core prediction of the auxin canalization hypothesis) seems to be unsupported by experimental evidence. Moreover, unlike auxin application to mature plant tissues —but like auxin application to shoot apices— *pin* mutant leaves can still respond to auxin application by forming veins that connect to preexisting veins basal to the auxin application site (Verna et al. 2019). This observation seems to suggest that vein formation in *pin* mutants still depends on auxin, but how?

AUXIN SIGNALING AND VEIN FORMATION

The residual vein-formation activity in auxin-transport-inhibited leaves turns out to depend, at least in part, on auxin signaling. That auxin signaling controls vein formation has long been known. Indeed, in auxin-signaling-inhibited leaves, fewer veins form, and veins are often incompletely differentiated; yet in those veins, vascular cells are still elongated along the vein and connected at their ends (Przemeck et al. 1996; Hardtke and Berleth 1998; Candela et al. 1999; Alonso-Peral et al. 2006; Strader et al. 2008; Esteve-Bruna et al. 2013; Verna et al. 2019; Mazur et al. 2020). Instead, inhibition of auxin signaling, either because of growth in the presence of auxin signaling inhibitors or because of mutation in auxin receptors or their regulators, leads to entirely new vein defects in auxin-transport-inhibited leaves (Verna et al. 2019).

In the leaves of plants in which both auxin transport and signaling are inhibited, the end-to-end alignment of vascular cells oriented along the vein is replaced by the formation of clusters of randomly oriented vascular cells (Fig. 4D; Verna et al. 2019). Although hypotheses have been proposed (for a recent review, see Ravichandran et al. 2020), it is currently unclear how auxin signaling, which takes place in the nucleus and is thus inherently nonpolar (for review, see Leyser 2018), would control, in the absence of polar auxin transport, the polar propagation of the auxin signal.

AUXIN AND THE FORMATION OF CONTINUOUS VASCULAR STRANDS

If vascular strand formation depended on the cell-to-cell transport of an auxin-dependent signal, vascular strands would always be "continuous" (i.e., they would connect to other vascular strands at least at one of their two ends). Yet vascular strands that fail to satisfy this requirement—vascular "fragments"—have been observed in leaves of wild types and mutants (e.g., Pray 1955a,b; Lersten 1965; Herbst 1971; Berleth and Jurgens 1993; Carland et al. 1999; Deyholos et al. 2000; Koizumi et al. 2000; Steynen and Schultz 2003; Sawa et al. 2005). Such vascular fragments are of two types.

Vascular fragments of the first type, including those observed in auxin signaling mutants, are composed of files of mature vascular cells that are connected by files of immature vascular cells (Fig. 5A; Pray 1955a,b; Lersten 1965; Herbst

1972; Przemeck et al. 1996; Hardtke and Berleth 1998; Mähönen et al. 2006; Scacchi et al. 2010; Truernit et al. 2012; Ruiz Sola et al. 2017). Because the identification of immature vascular cells can be problematic (Esau 1943), vascular strands of this type have often been interpreted as fragmented when they are instead incompletely differentiated.

Vascular fragments of the second type are composed of files of vascular cells that are separated by nonvascular cells (Fig. 5B; Herbst 1972; Carland et al. 1999; Deyholos et al. 2000; Koizumi et al. 2000). Vascular fragments of this type originate from continuous PEDs within which some cells terminate PIN1 expression and differentiate into nonvascular cells (Scarpella et al. 2006; Naramoto et al. 2009). Therefore, both types of vascular fragments are continuous, at least at formative stages, and thus compatible with a vein-formation mechanism that depends on the cell-to-cell transport of an auxin-dependent signal.

Vascular fragments of the first type have been observed in the leaf and the seedling cylinder (Pray 1955a,b; Lersten 1965; Herbst 1972; Przemeck et al. 1996; Mähönen et al. 2006; Scacchi et al. 2010; Truernit et al. 2012; Rodriguez-Villalon et al. 2014, 2015; Ruiz Sola et al. 2017; Marhava et al. 2018). However, no vascular fragments of the second type have ever been observed in the seedling cylinder, even in those mutants with such vascular fragments in their leaves. This observation suggests that the function of the genes that control the formation of continuous veins in the leaf is not required for the continuity of the vascular strand in the seedling cylinder. But how could that be?

PIN1 localization is already polarized in the first vascular cells that form in the globular embryo (Steinmann et al. 1999). These first vascular cells are stem cells, and as such they continually divide into cells with unequal fates: One cell will maintain the stem cell population; the other will extend the vascular strand in the cylinder of the embryo during embryogenesis and of the seedling root upon germination (Scheres et al. 1994; van den Berg et al. 1997; Aida et al. 2004; Yoshida et al. 2014). Unlike in the embryo, in the leaf PIN1 localization is initially nonpolar, and PIN1-expressing cells do not behave like vascular stem cells. In developing leaves of both wild type and mutants with vascular fragments of the second type, PEDs are initially continuous. In wild type, over time, PIN1 localization becomes polarized (Scarpella et al. 2006; Wenzel et al. 2007; Marcos and Berleth 2014). By contrast, in mutants with vascular fragments of the second type, PIN1 expression is terminated in some of the cells in a PED before PIN1 localization becomes polarized in any of the cells in the domain (Scarpella et al. 2006; Naramoto et al. 2009; Verna et al. 2019). There-

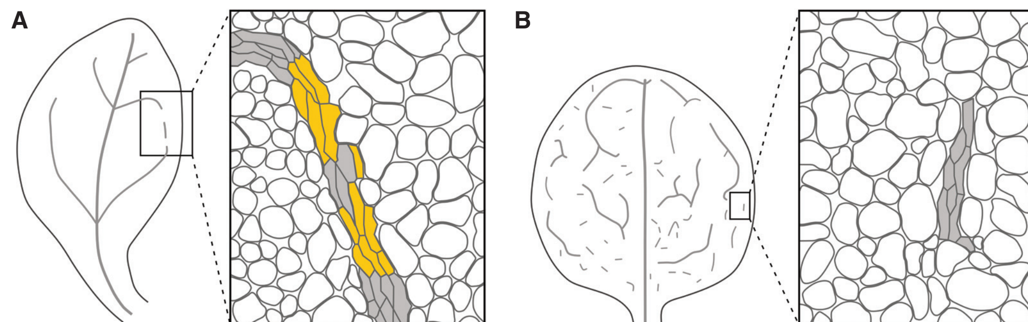

Figure 5. Auxin and the formation of continuous veins. (*A*) Vein fragments of the first type are composed of files of mature vascular cells (gray fill) that are connected by files of immature vascular cells (yellow fill). (*B*) Vein fragments of the second type are composed of files of vascular cells (gray fill) that are separated by nonvascular cells (white fill). Vein fragments of this type originate from continuous PEDs within which some cells terminate PIN1 expression and differentiate into nonvascular cells.

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fore, it is possible that the function of the genes that control the formation of continuous veins in the leaf is required for the polarization of PIN1 localization, and that it is the lack of such polarization that leads to PED fragmentation. If so, the function of the genes that control the continuity of the veins in the leaf would not be required during the extension of the vascular strand in the embryo and seedling cylinder because polar PIN1 localization would need to be maintained and propagated during such extension and not established like in leaf vein formation. In support of this interpretation, available evidence suggests that distinct mechanisms control the polarization of PIN1 localization and the maintenance of such polar localization (Kleine-Vehn et al. 2011; Łangowski et al. 2016).

AUXIN AND VASCULAR NETWORK FORMATION

Leaf Vein Networks

Just like a seedling can be seen as a vascularized cylinder, early, leafless plants can be seen as two systems of branched vascularized cylinders: one above ground and one below ground (Fig. 6A; Fairon-Demaret and Li 1993). And even flat organs such as leaves can be seen, at least at early stages of their development, as vascularized cylinders (Mattsson et al. 1999; Kang and Dengler 2004; Scarpella et al. 2004). However, during their development, flat-organ primordia soon lose their cylindrical shape by expanding laterally to acquire their distinctive flattened shape, a process that coincides with the formation of branched systems of veins that largely mirror the shape of the leaf (von Ettinghausen 1861; Ash et al. 1999; Dengler and Kang 2001). These vein networks are said to be "open" if all the veins connect to other veins at only one end or "closed" if at least some veins connect to other veins at both ends (Fig. 6B; Roth-Nebelsick et al. 2001; Verna et al. 2015).

In rounded leaves like those of *Arabidopsis*, lateral veins branch from a central midvein and connect to distal veins to form vein loops; minor veins branch from midvein and loops, and either end freely or connect to other veins to form a mesh; and loops and minor veins bend near the leaf edge to give the vein network a scalloped outline (Fig. 6C; Troll 1937; Gifford and Foster 1989; Nelson and Dengler 1997). In elongated leaves of grasses like maize, vein loops are compressed laterally and stretched along the leaf, such that midvein and lateral veins seem to run parallel to one another (Fig. 6D; Troll 1937; Gifford and Foster 1989; Nelson and Dengler 1997).

Auxin and the Formation of Open Vein Networks

In both rounded and elongated leaves, localization of PIN1 proteins at the plasma membrane of epidermal cells at the shoot tip becomes polarized toward sites of leaf primordium formation (Fig. 6E; Benková et al. 2003; Reinhardt et al. 2003; Carraro et al. 2006; Scarpella et al. 2006; Bayer et al. 2009; Lee et al. 2009; Johnston et al. 2015). The resulting epidermal "convergence points" of PIN1 polarity are associated with the appearance of broad PEDs in the inner tissue that will become restricted to sites of midvein formation. Likewise, epidermal convergence points of PIN1 polarity at the leaf edge are associated with both sites of leaf lateral growth and positions of broad PEDs in the inner tissue that become restricted to sites of lateral vein formation (Fig. 4A; Hay et al. 2006; Scarpella et al. 2006; Wenzel et al. 2007). Both auxin transport inhibition or higher auxin levels reduce the distance between successive epidermal convergence points of PIN1 polarity and leaf primordia and between midvein and lateral veins (Okada et al. 1991; Bennett et al. 1995; Mattsson et al. 1999; Sieburth 1999; Reinhardt et al. 2000; Scarpella et al. 2006; Guenot et al. 2012; Sawchuk et al. 2013; Verna et al. 2019). However, the relation between convergence points of epidermal PIN1 polarity and positioning of midvein and lateral veins seems to be correlative, rather than causal. Indeed, mutants that lack such convergence points form normal vein networks (Bilsborough et al. 2011), and epidermal expression of PIN1 is neither required nor sufficient for auxin-transport-dependent vein positioning (Govindaraju et

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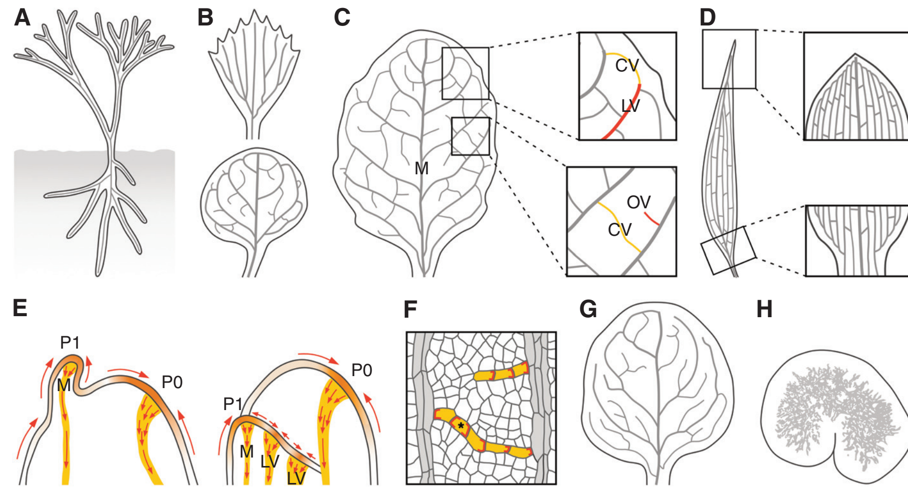

Figure 6. Auxin and vein network formation. (*A*) Early, leafless plants are systems of branched cylinders with a vascular strand (gray line) in their center. (*B*) Leaves of extant plants have "open" (*top*) or "closed" (*bottom*) vein networks. (*C*) In rounded leaves, lateral veins (LVs) branch from a single midvein (M); minor veins branch from M and LVs, and either end freely ("open" veins [OVs]; red) or connect to other veins ("closed" veins [CVs]; yellow); and vein loops form from the fusion of LVs (red) and closed minor veins (yellow) (*E*). (*D*) In elongated leaves, vein loops are compressed laterally and stretched along the leaf, such that M and LVs seem to run parallel to one another; and M and LVs are connected laterally by minor veins. (*E*) In epidermal cells at the shoot tip of plants with either rounded (*left*) or elongated (*right*) leaves, PIN1 localization becomes polarized (red arrows) toward sites of leaf primordium formation (orange). These epidermal convergence points of PIN1 polarity are associated with broad PEDs in the inner tissue that become restricted to sites of M formation (yellow). Over time, PIN1 localization becomes polarized (red arrows) in the cells of these broad domains: In the cells along the PEDs' midline, PIN1 localization becomes polarized toward preexisting veins; in the cells flanking PEDs' midline, PIN1 localization becomes polarized toward the domains' midline. Both the restriction of broad PEDs and the polarization of PIN1 localization initiate and proceed away from preexisting veins, in which PIN1 localization is polarized. P0 and P1 are successive stages of leaf primordium development. (*F*) The localization of PIN1 (red) in files of vascular cells (yellow) is polarized toward preexisting veins (gray; for simplicity, PIN1 expression in preexisting veins is not shown). In OVs, a single PIN1 localization polarity exists; in CVs, the two opposite PIN1 localization polarities are connected by a bipolar cell (asterisk): a cell with PIN1 at both ends. (*G*,*H*) Progressive reduction in the ability to polarize PIN1 localization during vein network formation leads to mutant vein networks with very few CVs (*G*); mutant vein networks in which vein fragments form along paths defined by initially continuous PEDs (Fig. 5B); or clusters of randomly oriented vascular cells, as in *gn* mutant leaves (*H*).

al. 2020). These observations suggest that the mechanism that controls the positioning of midvein and lateral veins depends on auxin levels and transport but is independent of epidermal convergence points of PIN1 polarity.

Auxin and the Formation of Closed Vein Networks

Unlike the broad PEDs that become restricted to sites of formation of midvein and lateral veins, those that become restricted to sites of minor vein formation in rounded leaves are not associated with epidermal convergence points of PIN1 polarity; instead, the broad PEDs that become restricted to sites of minor vein formation branch from preexisting veins (Fig. 4A; Scarpella et al. 2006; Wenzel et al. 2007; Marcos and Berleth 2014). Initially, these broad PEDs connect to preexisting veins at only one of their two ends ("open" PEDs), but they can extend to connect to other veins at both their ends ("closed" PEDs) (Fig. 4A; Scarpella et al. 2006; Wenzel et al. 2007; Marcos and Berleth 2014). The formation of such closed PEDs is promoted by auxin transport inhibition (Mattsson et al. 1999; Sieburth

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1999; Verna et al. 2015). Because auxin transport inhibition delays restriction of broad PEDs and polarization of PIN1 localization, only PEDs that have yet to differentiate into highly efficient auxin transport paths can connect to preexisting veins at both ends.

But not all broad PEDs become restricted and polarized: Some gradually disappear (Fig. 4A; Marcos and Berleth 2014), suggesting that PEDs compete for a limiting amount of auxin (Sachs 2003). Therefore, how many PEDs will form at any given stage of leaf tissue development, where exactly they will form, how broad they will be, what shape they will have, and which ones will become restricted and polarized and which ones will instead gradually disappear will not only depend on positive feedback between polarization of PIN1 localization and polar auxin transport but on random variations in the local production of auxin and on the number, shape, size, position, and polarization of preexisting PEDs.

Within narrow PEDs—whether open or closed—PIN1 localization is polarized toward preexisting veins (Scarpella et al. 2006; Wenzel et al. 2007; Marcos and Berleth 2014). Therefore, open PEDs have a single PIN1 localization polarity (Fig. 6F). By contrast, closed PEDs are composed of two segments, each with a single PIN1 localization polarity, opposite to that of the other (Fig. 6F). The two opposite polarities are connected by a "bipolar" cell, a cell in which PIN1 localization is polarized at both ends (Fig. 6F).

In rounded leaves, each loop forms from the fusion of a lateral-vein-associated PED and a minor-vein-associated closed PED (Fig. 4A; Scarpella et al. 2006; Wenzel et al. 2007). Whether in elongated leaves PIN1 is expressed and localized during the formation of minor veins and loops as it is in rounded leaves is currently unknown; however, computational simulations suggest that the same vein-formation mechanism embedded in different leaf growth patterns can account for the different vein networks of elongated and rounded leaves (Runions et al. 2005; Fujita and Mochizuki 2006b).

Therefore, available evidence suggests that like vascular strand formation in response to auxin application and vein formation during normal leaf development, vein network formation is an expression of auxin transport, many aspects of which are consistent with the auxin canalization hypothesis (Mitchison 1980a, 1981; Sachs 1991, 2000; Feugier et al. 2005; Rolland-Lagan and Prusinkiewicz 2005; Feugier and Iwasa 2006; Fujita and Mochizuki 2006a; Stoma et al. 2008; Bayer et al. 2009; Smith and Bayer 2009; Alim and Frey 2010; Wabnik et al. 2010; Walker et al. 2013; Lee et al. 2014; Cieslak et al. 2015; Abley et al. 2016). Nevertheless, the auxin canalization hypothesis predicts the formation of networks of "open" veins (i.e., veins that connect to veins at only one of their two ends) (Sachs 1975; Mitchison 1980a, 1981; Rolland-Lagan and Prusinkiewicz 2005). The formation of "closed" veins (i.e., veins that connect to other veins at both their ends) has thus been proposed to result from the diffusion of a hypothetical substance from preexisting veins that allows approaching veins to connect to preexisting veins (Feugier and Iwasa 2006). This hypothesis predicts that in closed PEDs, PIN1 is polarized *away* from the preexisting veins to which the closed PEDs connect, when in fact in closed PEDs, PIN1 is polarized *toward* the preexisting veins to which the closed PEDs connect (Fig. 6F; Scarpella et al. 2006; Wenzel et al. 2007; Marcos and Berleth 2014). Therefore, this hypothesis seems to be unsupported by experimental evidence.

Alternatively, closed veins could form from veins meeting at points of peak auxin levels (Dimitrov and Zucker 2006) or from localized auxin production at precisely defined times and places (Sachs 1975, 1989; Mitchison 1980a; Aloni et al. 2003; Rolland-Lagan and Prusinkiewicz 2005; Runions et al. 2005). Both hypotheses are consistent with the observation of bipolar cells and predict peak auxin levels in these cells; however, this prediction remains to be tested experimentally.

The formation of bipolar cells seems to be very sensitive to even the partial loss of function of the auxin-signaling- and auxin-transport-dependent pathway that controls the formation of continuous veins: Mutants partially lacking the function of this pathway often fail to form

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bipolar cells and thus to polarize PIN1 localization along closed PEDs (Naramoto et al. 2009; Hou et al. 2010). This reduced ability to polarize PIN1 localization along closed PEDs often leads to their "opening" and thus to vein networks with very few closed veins (Fig. 6G; Steynen and Schultz 2003; Naramoto et al. 2009). Nevertheless, these mutants are still able to polarize PIN1 localization along open PEDs (Naramoto et al. 2009; Hou et al. 2010).

More severe loss of function of the pathway that controls the formation of continuous veins leads to the inability to polarize PIN1 localization even along open PEDs (Carland et al. 1999; Deyholos et al. 2000; Koizumi et al. 2000, 2005; Willemsen et al. 2003; Carland and Nelson 2004, 2009; Scarpella et al. 2006; Sieburth et al. 2006; Naramoto et al. 2009). The inability of these mutants to polarize PIN1 localization along PEDs leads to termination of PIN1 expression in some of the cells in a PED before PIN1 localization becomes polarized in any of the cells in the domain (Scarpella et al. 2006; Naramoto et al. 2009). The cells that terminate PIN1 expression differentiate into nonvascular cells, while the remaining PED fragments differentiate into vascular fragments of the second type (Fig. 5B; Carland et al. 1999; Deyholos et al. 2000; Koizumi et al. 2000, 2005; Willemsen et al. 2003; Carland and Nelson 2004, 2009; Scarpella et al. 2006; Sieburth et al. 2006; Naramoto et al. 2009; Tan et al. 2020; Xiao and Offringa 2020). Nevertheless, these vascular fragments still form along the paths where continuous veins would form in wild type, a vestige that these fragments were once connected.

Defects in vein network formation are most severe in *gn* mutants. In *gn* cotyledons and leaves, individual cells can still localize PIN1 polarly—although to a lesser extent—but they seem to have almost entirely lost the ability to coordinate between them the polarity of PIN1 localization (Steinmann et al. 1999; Verna et al. 2019). The result of this inability is the formation of clusters of randomly oriented vascular cells (Fig. 6H; Mayer et al. 1993; Steinmann et al. 1999; Geldner et al. 2004; Verna et al. 2019; Amalraj et al. 2020).

Based on their biochemical function and cellular localization, proteins in the pathway that controls the formation of continuous veins have been proposed to localize PIN1 to or retain it in the plasma membrane, polarize PIN1 localization, or maintain such polar localization (e.g., Geldner et al. 2004; Koizumi et al. 2005; Sieburth et al. 2006; Kleine-Vehn et al. 2008; Carland and Nelson 2009; Naramoto et al. 2009, 2010; Prabhakaran Mariyamma et al. 2018). However, the function of these proteins seems to entail more than the control of auxin transport and to include at least the control of auxin signaling. Indeed, defects in mutants in the pathway that controls the formation of continuous veins cannot be phenocopied by mutation in *PIN* genes or growth in the presence of auxin transport inhibitors; instead, those defects are reproduced in plants in which both auxin transport and signaling are compromised (Verna et al. 2019). Although it is currently unclear how proteins in the pathway that control the formation of continuous veins control auxin signaling, the most parsimonious account is that such proteins control the polar localization of proteins with vein formation function that are produced in response to auxin signaling.

CONCLUDING REMARKS

The past 20 years have witnessed unprecedented advances in our understanding of the role of auxin in the patterned formation of the vascular system; however, the very same research that has led to such advancement has also exposed unexpected gaps in our current knowledge. For example, a role for auxin signaling in vein positioning had been unsuspected because the fewer and incompletely differentiated veins of auxin signaling mutants still form in the same positions as they would in wild type. It now turns out that functions of auxin signaling in vein positioning had been obscured by nonhomologous redundancy with auxin transport. Furthermore, it also turns out that auxin signaling and auxin transport had been eclipsing each other's functions in the end-to-end alignment of vascular cells oriented along the vein. However, how precisely auxin transport and signaling control all those processes remains unclear. These and other questions will have to be addressed in future

research, and as past research has taught us, even more surprises are awaiting ahead.

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