Patterning and Polarity in Seed Plant Shoots

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Annu. Rev. Plant Biol. 2008. 59:67–88

The *Annual Review of Plant Biology* is online at plant.annualreviews.org

This article's doi: 10.1146/annurev.arplant.57.032905.105356

1543-5008/08/0602-0067$20.00

Key Words

embryogenesis, auxin, shoot apical meristem, hypocotyl, cotyledon, leaf

Abstract

Leaves and stems are ultimately derived from the shoot apical meristem (SAM); leaves arise from the peripheral zone of the SAM and stem tissue is derived from both the peripheral and central zones of the SAM. Both the peripheral and central regions of the SAM are formed during embryogenesis when the basic body plan of the plant is established. Interplay between points of maximal concentration of auxin and specific patterns of transcription of both auxin-responsive transcription factors and other patterning genes subdivide the embryo along both the apical-basal and central-peripheral axes. Differential gene expression along these axes leads to the differentiation of tissues, lateral organs, meristems, and boundary regions, each with varying responsiveness to auxin. Subsequent shoot growth and development is a reiteration of basic patterning processes established during embryogenesis.

Contents

THE DEVELOPMENT AND EVOLUTIONARY CONTEXT FOR ANALYSIS OF PATTERNING OF THE SHOOT SYSTEM

Seed plants have been the dominant component of land plant floras since the Middle Triassic (approximately 230 Mya) (72) and their foliage defines and colors our landscapes, marking the seasons in many regions of the world. The living seed plants include cycads, *Ginkgo*, conifers, the Gnetales, and flowering plants. Living seed plants range from small herbaceous annuals to succulent cacti to massive long-lived giant sequoia. The leaves of seed plants also vary tremendously, from needle-like to frond-like and everything in between. Despite this enormous variability in form, the basic building blocks of which the shoot systems of the 300,000 or so seed plant species are constructed, radial stems and dorsiventral leaves, are similar due to common ancestry. All living seed plants have a complex shoot apical meristem (SAM) that is unique among vascular plants. The seed plant SAM consists of distinct populations of cells, including one or more layers of initial cells below which are a group of relatively quiescent central mother cells that are surrounded by actively dividing cells of the peripheral zone (**Figure 1**). Cells in the central zone are larger in diameter and vacuolated compared with the small, densely cytoplasmic cells of the peripheral zone. Leaves arise from the peripheral zone of the SAM whereas stem tissue is derived from both the peripheral and central zones of the SAM. The reiterative production of leaves in a specific pattern, termed phyllotaxy, results in modular shoots composed of lateral leaves spaced along a central radial stem. Nearly all angiosperm leaves and some gymnosperm leaves have an associated axillary meristem from which additional shoot systems (i.e., branches) are generated. The phyllotaxy, rate of leaf production, and stem elongation, combined with the size and shape of leaves produced, give a species its characteristic morphology.

There has been tremendous interest in recent years in understanding the underlying processes that control the functioning of the SAM in seed plants, including the maintenance of stem cells, initiation and polarity

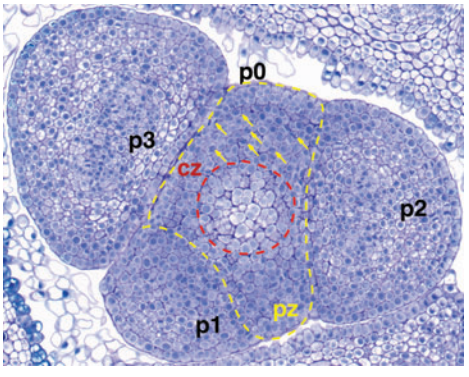

Figure 1

Shoot apex of *Ginkgo biloba*. The shoot apical meristem includes the central zone (cz) of larger vacuolate cells and the peripheral zone (pz) of smaller, less vacuolate cells. Leaves are derived from the peripheral zone whereas both the peripheral and central zone contribute to the stem. Leaf primordia are labeled youngest (p0) to oldest (p3). Note the many mitotic figures (*yellow arrows*) in the peripheral zone associated with p0.

of leaf primordia from the peripheral zone, and control of phyllotaxy. This work has focused on the model system *Arabidopsis*, which is amenable to genetic analysis and manipulation. The sporophyte (diploid) generation of seed plants (like all vascular plants) is capable of continuous development through the activities of shoot and root meristems. The stage of development where the basic body plan of the seed plant sporophyte is established is often referred to as embryogenesis, although the reiterative nature of plant development renders the demarcation between embryogenesis and postembryonic development nebulous (16, 56). The developmental patterning that is established during embryogenesis is repeated throughout the life of the plant. Thus, analyses focusing on the initial patterning events may be informed by findings from the study of the SAM and conversely, the study of embryogenesis contributes to our understanding of the SAM (56). For the purposes of this review we will use the term embryogenesis to encompass the developmental period in which the basic apical-basal, radial, and proximo-distal axes of the plant and its first lateral organs, the cotyledons, are established. For many species, this corresponds to the time between the formation of the zygote by fertilization of the female gametophyte and seed dormancy, when seeds are shed from the parent plant and dispersed. It must be noted that this is not always the case because some species do not undergo a 'dormant stage', and the seeds of many species contain 'embryos' with several leaves in addition to the cotyledons. Nonetheless, it is often convenient to think of seed development as embryogenesis.

Here we attempt to survey a limited number of recent findings that contribute toward understanding the development of the shoot system, and highlight a few key events about which little is presently known. We then attempt to extrapolate as to how generally applicable the developmental processes discovered in *Arabidopsis* are likely to be for other flowering plants and seed plants. Finally we discuss the significance of the emerging developmental genetic data for understanding the nature of some of the concepts in plant development, such as cytohistological zonation in the SAM, symmetry in the embryo, and the establishment of differential growth. Other excellent reviews highlight additional aspects of body plan establishment in *Arabidopsis* (12, 25, 54) and aspects of leaf development (7, 22, 95) and the ideas and experimental data in this review need to be considered in light of these other recent reviews on related topics.

ORIGIN OF PATTERN FORMATION IN *ARABIDOPSIS* SPOROPHYTES

In *Arabidopsis*, all the major pattern elements of the sporophyte body plan are established during the time between fertilization of the female gametophyte by the male gametophyte and the onset of seed dormancy. The basic patterns set up during this developmental window, including the apical-basal axis, the radial axis, and the programs necessary for the generation of lateral organs, are propagated during the remainder of the sporophytic phase of the life cycle. Thus, an understanding of the underlying molecular processes of these early stages can, to a large degree, be extrapolated to the rest of the growth and development of the sporophyte.

In general terms, development is the process by which different cells of a multicellular organism attain different fates. Although cell lineages are often visually conspicuous in plants, as in the files of cells readily apparent in the epidermises of stems, leaves, and roots, it is clear that position largely determines cell fate in most contexts, implicating a role for signaling molecules. Unlike animals, where many small, secreted, peptide signaling molecules have been identified as critical for developmental patterning, few such molecules have been identified in plants. Although this may merely be due to our present ignorance of the molecular mechanisms of plant development, it is also possible that molecules other than peptides may be more important. One

such molecule that has a pervasive role in plant development is auxin (12, 13, 25, 53, 60, 98). Indeed, three major patterning events in angiosperm embryogenesis, establishment of the apical-basal axis, establishent of the central-peripheral axes, and initation of lateral organs (cotyledons), are correlated with dynamic patterns of auxin concentration and flux. Thus, auxin may act in an instructive manner, with the potential for concentration gradients to pattern developmental events. Determining how these gradients are established and how they are modulated is critical to understanding pattern formation in plants.

The Flow of Auxin

In *Arabidopsis* apical-basal polarity of the sporophyte correlates with a polarity already established in the female gametophyte (**Figure 2**). Following fertilization, the

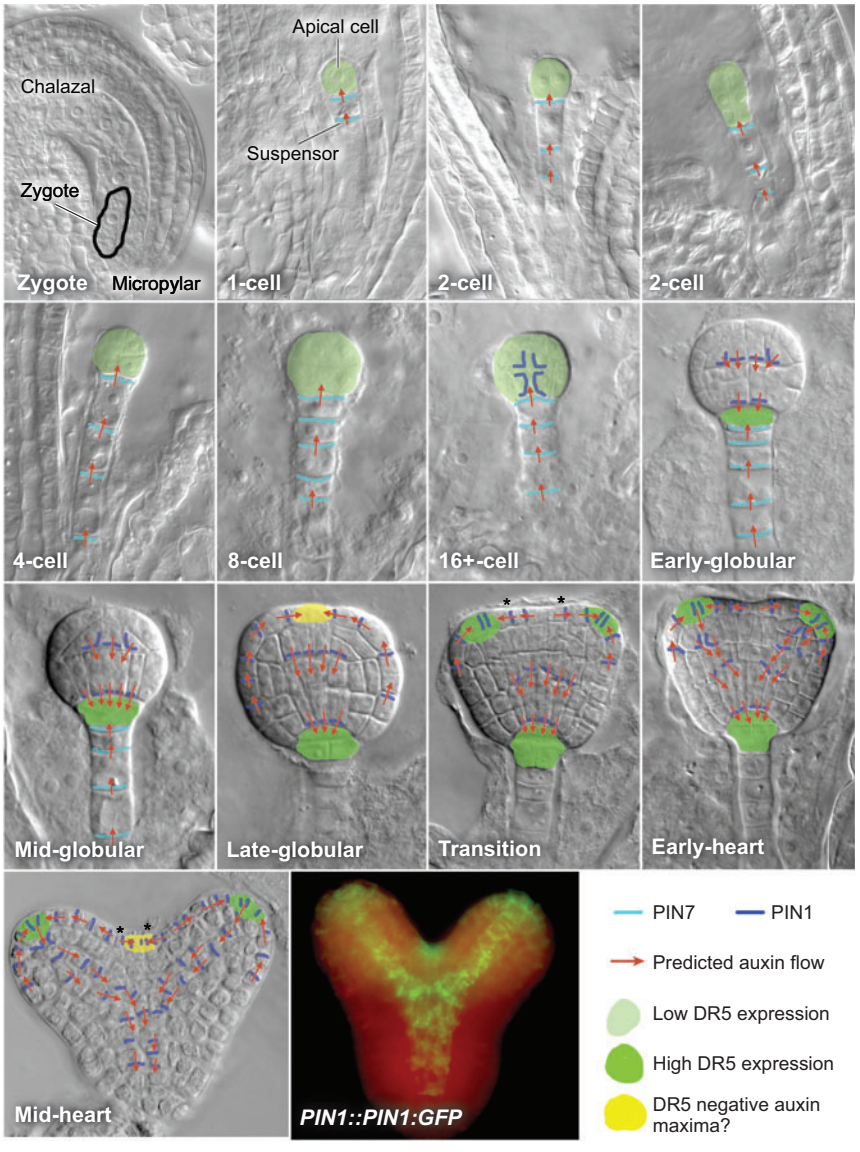

Figure 2

PIN FORMED (PIN) expression, auxin maxima, and predicted auxin flow during *Arabidopsis* embryogenesis. *PIN1::PIN1:GFP* in a late heart stage wild-type embryo. Reversals in PIN1 polarity marking the establishment of the cotyledons, and subsequently, auxin flow back toward the meristem following cotyledon formation are demarcated with asterisks.

zygote elongates and divides asymmetrically to produce a smaller apical cell, which will give rise to most of the embryo proper, and a larger micropylar basal cell that gives rise to the extraembryonic suspensor and the root meristem of the embryo. Thus, the polarity established at the first zygotic division of the embryo corresponds with the polarity of the embryo sac. Apical-basal axis formation is also marked by an asymmetric distribution of members of the PIN FORMED (PIN) family of proteins that mediate cellular auxin efflux from cells (39, 76, 102). Dynamic changes in auxin flux and maxima are mediated by both transcriptional control of PIN genes and the regulation of polar localization of PIN proteins (15, 99). The basal cell of the two-celled zygote expresses PIN7 protein at its apical end, presumably facilitating auxin flow into the apical cell (36). Although auxin has been difficult to detect directly, experiments using reporter genes, such as DR5::GFP, that respond positively to auxin suggest that auxin accumulates in the apical cell (reviewed in Reference 42). Although loss of *PIN7* activity has only minor effects on axis formation, this is likely due to extensive compensatory redundancy among PIN genes, in which the expression patterns of other family members change as a response to loss-of-function PIN alleles. Severe embryonic phenotypes, including embryos lacking apical-basal polarity and consisting of a file of suspensor-like cells, are observed in *pin1 pin3 pin4 pin7* quadruple mutants, suggesting that the early auxin asymmetry observed at the two-cell stage may be instructive with respect to apical cell fate (15, 36, 99).

The correspondence between embryo sac polarity and maternal sporophyte polarity brings up the question of how much instruction the maternal sporophyte provides with regard to embryonic polarity. Two observations suggest that embryonic patterning may be largely autonomous. Firstly, development of a second embryo, derived from cells normally fated to be extraembryonic suspensor cells, with an opposite polarity as compared with that derived from the apical cell, suggest that embryonic polarity can be established independently of maternal inputs (96). Secondly, the production of somatic embryos, free from the influence of any maternal sporophyte, indicates that embryonic polarity can be established autonomously. It is tempting to speculate that an asymmetric distribution of auxin between two daughter cells whose chromatin status reflects an embryonic cell fate may be sufficient to result in a progression into an embryonic developmental program. Embryo polarity could be established in the egg cell prior to fertilization because *Arabidopsis* egg cells are distinctly polar with a large vacuole at the micropylar end and the nucleus positioned at the chalazal end (66). These two poles correspond to the asymmetric first division of the zygote that results in a smaller apical cell and a larger basal cell that receives the egg vacuole (**Figure 2**).

Dynamic changes in auxin flow correlate with subsequent major events in embryonic development as described in **Figure 2** (36). First, auxin is thought to be drained from the embryo during the globular stage owing to the basa1 expression of PIN1. Second, an establishment of upward flow of auxin in the protodermal layer and thence downwards through the center contributes to axial elongation of the future procambium in the late globular embryo. This pattern presages much of what follows and highlights the need for understanding the establishment of the role of the epidermis in general patterning processes. Third, the establishment of bilateral symmetry and the SAM is correlated with a reversal in PIN polarity at the apex of the globular embryo. Finally, there is evidence that auxin is shunted back toward the meristem following the formation of lateral organ primordia (24, 50). That the dynamic flow of auxin and the creation of maxima is, if not instructive, at least necessary for proper embryo development is demonstrated by mutations in genes regulating PIN protein localization that result in abnormal auxin flow and embryonic patterning. GNOM (GN), a

membrane-associated guanine nucleotide exchange factor, regulates vesicle budding, an important process for recycling of PIN proteins between endosomal compartments and the plasma membrane (90). The *gn* mutant resembles the severe embryo phenotype of *pin1 pin3 pin4 pin7*, suggesting that dynamic recycling of PIN proteins is important for embryo development (15, 17, 67, 86, 99). However, the random changes in PIN1 distribution observed in *gn* mutants imply that GN does not determine the polarity of PIN localization (40, 90).

The creation of auxin maxima can be determined either by changing the direction of auxin flow such that opposing flows result in an accumulation of auxin, or alternatively, by localized synthesis of auxin. What little is known about the control of reversals in the polar localization of PIN proteins revolves around the activity of the *PINOID* (*PID*) gene. *PID* encodes a serine-threonine protein kinase, and may directly regulate PIN1 polarity because loss of PID activity leads to basal PIN1 localization and gain of PID function leads to apical PIN1 localization in cells of cotyledon primordia (23, 37). Phosphorylation of PIN proteins by PID is counteracted by the activity of PROTEIN PHOSPHATASE 2A (PP2A), such that phosphorylation of PIN1 promotes its apical cellular localization, whereas reduction in phosphorylation of PIN1 results in a basal cellular localization (70). Auxin maxima directing the initiation of organs are altered in plants lacking PID function; *pid* mutants often possess three cotyledons and fail to initiate floral organs (8, 10, 23). Furthermore, *pid pin1* and *pid enhancer of pinoid* (*enp*) double mutants lack cotyledons (38, 94). In these double mutant combinations, a transition stage embryo appears to develop, but the polar localization of PIN1 protein is not oriented normally and the cotyledons fail to develop appropriately. Thus, PID controls some aspects of polar PIN1 localization, but much of embryonic pattern formation occurs normally in *pid* mutants, indicating the existence of other controls. Furthermore, the loss of cotyledons in *pid pin1* mutants can be suppressed by mutations in CUP-SHAPED COTYLEDON (CUC) genes (38).

What is the origin of embryonic auxin? This is a question that has been largely neglected thus far, primarily owing to the incomplete knowledge of auxin biosynthesis and its sequestration via conjugation. Localized synthesis of auxin within the developing embryo could also play a role in creating auxin maxima and influencing auxin flows. This is especially relevant because *PIN1* expression is responsive to auxin concentration, suggesting the dynamic changes of auxin flux will include feedback regulation (50, 74, 84, 85, 99). At least two biochemical pathways can lead to auxin synthesis, one based on the products of the YUCCA genes (20, 104) and another via nitrilase (reviewed in Reference 73), and auxin can be inactivated via conjugation pathways (reviewed in Reference 103). Although increases in auxin concentration through exogenous application or expression of bacterial biosynthetic genes can often be accommodated, presumably through conjugative processes and increasing auxin flow, ectopic expression of YUCCA genes has severe morphological effects, suggesting that the control of auxin synthesis needs to be considered (104). At least four YUCCA genes are expressed during embryogenesis (21). *YUC1* and *YUC4* expression is detected as early as the transition stage in the apical regions of the embryo, with later expression primarily at the tips of the cotyledons, the SAM, and incipient leaf primordia (21). *YUC10* and *YUC11* are expressed in similar patterns during the heart stage. The expression of YUCCA genes in the presumptive SAM supports the hypothesis that elevated levels of auxin are present in the the SAM (**Figure 2**). *YUC4* is positively regulated by *STYLISH1* (*STY1*), and and multiple loss of function mutations in *STYLISH*-related genes result in similar carpel and leaf phenotypes as *YUCCA* loss-of-function mutant combinations (20, 58, 59, 88). On the basis of the coincident expression

patterns of *STY1* and YUCCA genes during embryogenesis, STYLISH genes are likely involved in embryonic YUCCA gene regulation. At present, the relative contributions of auxin synthesis and auxin flux in generating auxin maxima are not clear, but it is likely that a combination of localized auxin synthesis and directional auxin flow is required to generate and maintain auxin maxima.

One consequence of auxin maxima created by convergent auxin flow and localized auxin synthesis is the activation of AUXIN RESPONSE FACTORS (ARFs), transcription factors that activate and/or repress target genes (reviewed in References 61 and 82). ARF proteins are negatively regulated by AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins, which are targeted for degradation in response to auxin via a transport inhibitor resistant (TIR)-mediated targeting of the AUX/IAA protein to the proteasome (reviewed in References 26, 60, 82, 93, and 103). The release of the ARF proteins from their AUX/IAA partner allows their binding to auxin response elements (AREs) and the regulation (positive or negative) of target genes. Two members of the ARF gene family, *MONOPTEROS* (*MP/ARF5*) and *NONPHOTOTROPIC HYPOCOTYL 4* (*NPH4/ARF7*) have demonstrated roles in embryo axis patterning, with double mutants lacking both apical and basal embryonic development (11, 46, 47, 101). Specifically, the double mutants lack both the axial elongation of central cells and cotyledon initiation, suggesting that MP/NPH4 are required to interpret auxin maxima directing these two developmental processes. Because these processes, the initiation of lateral organ primordia and the establishment of procambial precursors, require different gene expression patterns, MP/NPH4 must interact with other cell type–specific factors to regulate morphogenesis. Dominant gain-of-function alleles in an AUX/IAA partner, *BODENLOS* (*BDL*), result in a similar loss of embryonic apical-basal polarity, suggesting that MP/NPH4 and BDL are the primary ARF-AUX/IAA proteins involved in early embryonic development (44, 45, 101). However, this does not preclude the participation of other family members in other aspects of embryonic pattern formation; *ETTIN*, *ARF2*, and *ARF4* are likely candidates for contributing to cotyledon patterning (4, 75).

Several significant morphological transitions during embryogenesis remain enigmatic. First, the establishment of the protoderm by periclinal divisions at the eight-cell stage sets the stage for signaling events between cell layers. The behavior of auxin flow differs significantly between the epidermal and internal cell layers. The establishment of auxin maxima that mark the cotyledons, and subsequently leaf primordia, occurs through changes in auxin flow within the epidermal layer and likely localized synthesis of auxin. Subsequent flow into the internal cells from sites of auxin maxima establishes the path of provascular tissue. How is the protodermal layer initated and differentated? Second, changes in auxin flow, from initially pumping auxin into the embryo, to a state where auxin is drained from the embryo at the early globular stage, imply that embryonic pattern formation requires both dynamic changes in PIN protein expression patterns and subcellular localization. During the transition from the early to late globular stages auxin is thought to flow upward in the epidermis and then downward through the central region instead of being drained from the embryo. The control and initation of this process is also unknown, although it is likely related to the origin of the protodermal layer. Third, the mechanism of reversal of *PIN1* polarity in the epidermis at the apex of the embryo that establishes the positions of cotyledon primordia is largely unknown. Fourth, how is auxin flow shifted from being primarily epidermal to an internal flow into subepidermal layers at sites of PIN1 convergence? Such changes result in a draining of auxin from the site of the epidermal maxima and mark the initial establishment of routes of vascular patterning. Finally, concomitant with the initiation of cotyledons

is the formation of the SAM, which completes the establishment of all the major pattern elements of the apical portion of the embryo. How are these dynamic changes achieved?

Interplay

What is known about the transcriptional control of the auxin synthesis, flux, and response machinery? Unfortunately, not much. Although the direct transcriptional control of PIN, PID, and ARF genes is a largely unknown territory, the expression patterns of several classes of transcription factors have been correlated with aspects of auxin biology. Here we describe a few, not to be comprehensive, but to highlight how little is known about their precise roles, even of those genes that have been subjected to significant analyses.

The activity of the CUC class of NAC [no apical meristem (NAM), *Arabidopsis* transcription activation factor (ATAF), and CUC] transcription factors causes a repression of growth between organs that creates boundaries, and their expression is correlated with low auxin levels (38). CUC genes are initially expressed in a domain extending across the upper region of the globular embryo, dividing the embryo into two halves (2, 89, 92, 100) (**Figure 3**). Once *SHOOT MERISTEMLESS* (*STM*) is activated in the center of the embryo (65) to mark the future SAM, CUC expression becomes limited to the marginal regions, effectively forming a boundary between the cotyledons (92). Loss of CUC

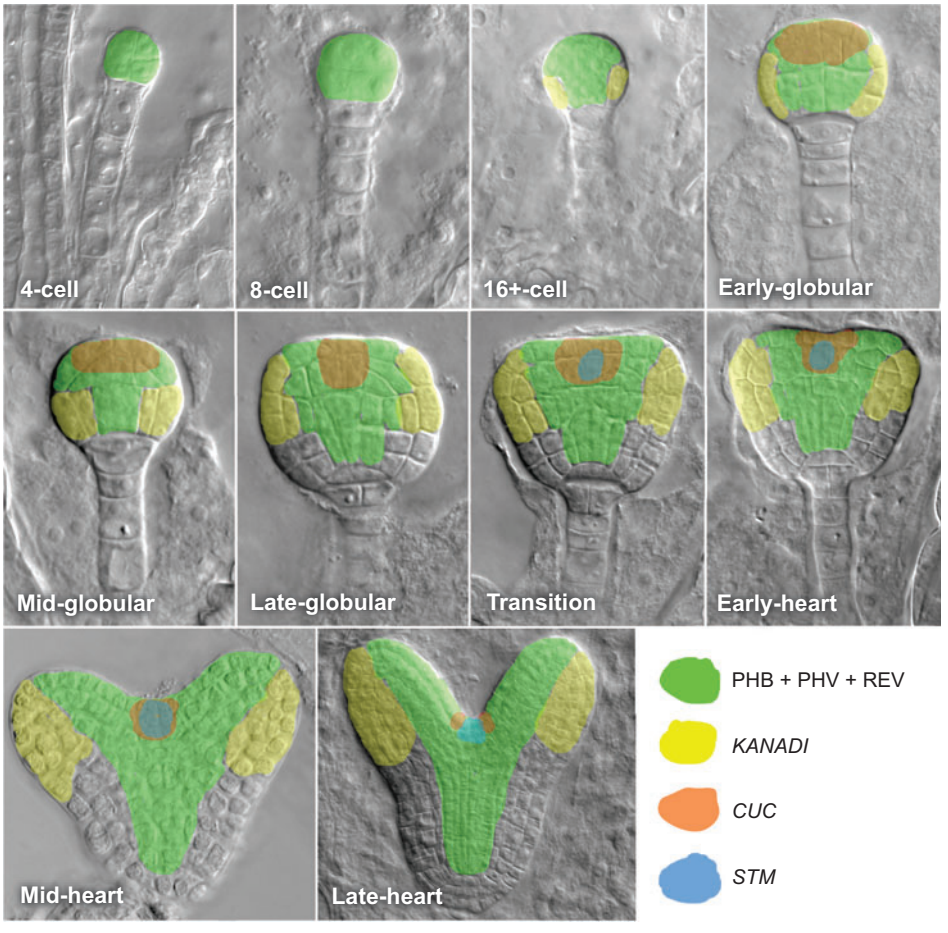

Figure 3

Expression patterns of four classes of transcription factors [*PHABULOSA* (*PHB*) + *PHAVOLUTA* (*PHV*) + *REVOLUTA* (*REV*) in green, *KANADI* in yellow, *CUP-SHAPED COTYLEDON* (*CUC*) in red, *SHOOT MERISTEMLESS* (*STM*) in blue] during early embryogenesis. Note that both the spatial and temporal patterns are approximate because the observations of expression for each gene were done independently, without multiple labeling of individual embryos.

activity results in a loss of separation between the cotyledons, such that the entire periphery of the embryo develops as a single cup-shaped cotyledon, a phenotype that can be phenocopied by application of auxin transport inhibitors to developing embryos (1, 62). In addition, *STM* is not activated appropriately in *cuc1 cuc2* embryos, such that a SAM is not formed (1). Conversely, ectopic CUC expression is correlated with a repression of growth. For example, CUC genes are ectopically expressed throughout the apical periphery of *pin1 pid* embryos and cotyledon primordia fail to initiate appropriately, but removal of CUC activity in this background results in the restoration of cotyledon development (38). The observation that some of the CUC genes are initially activated in a border domain suggests that they are in some respects responding to a preexisting boundary, but what establishes this boundary remains unclear. Are CUC genes activated in response to low auxin levels (e.g., at sites of PIN polarity reversal where auxin appears to be drained from a point), or does the activation of CUC genes result in a subsequent reduction in the response to auxin? These scenarios do not have to be mutually exclusive, and perhaps the variability in the initial expression patterns of *CUC1* and *CUC2* reflects an interplay between CUC and PIN gene regulation.

Analyses of CUC genes highlight two significant questions with respect to pattern formation. First, CUC gene expression defines boundaries, which can provide a separation between organs so that they can subsequently develop autonomously (reviewed in Reference 3). In addition, boundaries may facilitate communication pathways between the two separated organs by providing a third cell type distinct from each of the entities that the boundary defines, and thus boundaries may be instructive during developmental processes. Second, the nebulous relationship between CUC gene expression and the regulation of auxin synthesis and flow highlights the paucity of our knowledge about the relative temporal relationships of change in gene expression during embryonic pattern formation. Observations in real time of living inflorescence apices have been pivotal in providing insight into these processes, as described below (50).

One consequence of CUC expression in the central apical region of the embryo is the activation of *STM*, a Class I knotted1-like homeobox (KNOX) gene (1) (**Figure 3**). Class I KNOX gene activity is hypothesized to suppress differentiation within the SAM and the formation of organ boundaries (6, 52, 64). Several lines of evidence indicate that Class I KNOX genes may act to regulate and be regulated by plant hormones to promote maintenance or formation of the SAM (43, 49). Hay and coworkers (49) propose a model in which Class I KNOX genes in the SAM [such as *STM* and *BREVIPEDICELLUS* (*BP*) in *Arabidopsis* and *KNOTTED 1* (*KN1*) in maize] promote cytokinin synthesis and downregulate gibberellic acid (GA) levels, promoting meristematic activity. Class I KNOX genes are excluded from the positions of auxin maxima associated with the site of leaf initiation within the SAM, and it has been suggested that Class I KNOX genes are downregulated by high levels of auxin and may also act to repress auxin transport (48, 49). *BP* is also expressed in the cortex of the hypocotyl and in young internodal regions that become cortex, and directly downregulates components of the lignin biosynthetic pathway (69). In *bp* mutant plants, premature and increased levels of lignification occur in cortical and interfascicular regions of the stem, whereas overexpression of *BP* leads to a reduction in lignification throughout the stem (69). The accumulated evidence suggests that Class I KNOX genes are expressed in regions of low auxin concentration and low PIN-mediated transport and act to inhibit processes of growth and differentiation that are associated with auxin action.

In contrast to the Class I KNOX genes, members of the Class III homeodomain-leucine zipper (HD-Zip) gene family have expression patterns that correlate with known pathways of auxin flow out of the apex toward

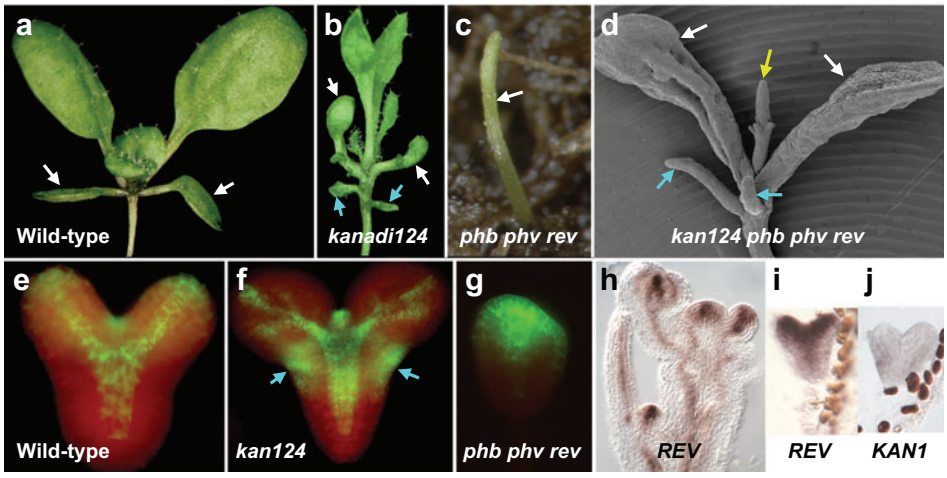

Figure 4

(*a–d*) Seedlings. White arrows denote cotyledons, blue arrows show 'hypocotyl leaves', and the yellow arrow shows the 'central' leaf. (*e–g*) *PIN1::PIN1:GFP*; blue arrows mark ectopic expression in *kan124* embryos. (*h–i*) *REV* expression in the inflorescence shoot and heart stage embryo. (*j*) *KAN1* expression in the heart stage embryo. Abbreviations: *phb*, *phabulosa*; *phv*, *phavulota*; *rev*, *revoluta*; *kan*, *kanadi*; *PIN*, *PIN FORMED*.

incipient leaf primordia and in the provasculature (27, 50, 78, 106) (**Figure 3**, **4*b–i***). Loss of Class III HD-Zip activity, as in *phabulosa* (*phb*) *phavoluta* (*phv*) *revoluta* (*rev*) mutants, results in a loss of central shoot identity (27, 78). The apical regions of mutant seedlings lack a SAM, and instead comprise a single radialized cotyledon, lacking adaxial cell types, with a small, radial vascular bundle (**Figure 4*c***). In this background, the reversals in PIN1 polarity that establish the divergent flow of auxin away from the center of the embryo and demarcate bilateral symmetry do not occur (51). Instead, the pattern of presumed auxin flow in the early globular embryo, upward through the epidermis and down centrally, continues such that an embryo with a single central auxin maximum is formed (**Figure 4*g***). Although the mechanistic relationship between presumptive pathways of auxin flow and Class III HD-Zip gene expression is unknown, that dynamic patterns of auxin flow appear to be established in a *phb phv rev* background in which KANADI activity (see below) is also removed suggests that that Class III HD-Zip activity may largely respond to, rather than direct, patterns of auxin flux. However, it has been reported that Class III HD-Zip genes influence polar auxin transport. Zhong & Ye (106) showed that auxin polar transport is reduced in *rev* plants and that the expression of two of the PIN genes is reduced. Class III HD-Zip genes may respond to auxin by promoting the differentiation of cell types (e.g., procambium) that further facilitate auxin polar transport away from the SAM and in the vasculature, and that by disrupting Class III HD-Zip activity reduced auxin flow occurs owing to a loss of proper conduits. Loss- and gain-of-function *REV* mutations lead to a reduction or expansion, respectively, of lignified tissues in the stem, which is consistent with a role for this gene in directing the normal flow of auxin within the shoot (27, 68, 105, 107).

Loss of *DORNRÖSCHEN* (*DRN*) and *DRN-LIKE* (*DRNL*) activity results in an embryonic mutant phenotype similar to that of loss of Class III HD-Zip gene activity (19). DRN and DRNL proteins physically interact with Class III HD-Zip proteins and the embryonic expression pattterns of *DRN* and *DRNL* overlap with those of Class III HD-Zip

genes, suggesting they may act in a common protein complex (19, 71).

Another class of gene that has been proposed to regulate auxin biology is the KANADI group of GARP transcription factors, named for maize Golden 2, ARR (*Arabidopsis* response regulators) and Psr1 (phosphorous stress response1 from *Chlamydomonas*). Loss- and gain-of-function phenotypes of KANADI activity are largely the converse of those of Class III HD-Zip genes (29, 30, 57). However, one phenotypic feature of loss of KANADI activity that is not observed in gain-of-function alleles of Class III HD-Zip genes is the formation of leaves from the hypocotyl of *kan1 kan2 kan4* plants (51) (**Figure 4*b***). The 'hypocotyl' leaves that develop during embryogenesis are associated with ectopic auxin maxima that form during the heart stage; PIN1 is already ectopically expressed during the globular stage. During the globular stage PIN1 normally becomes restricted to the apical portion of the embryo, but in KANADI mutants, PIN1 is expressed more basally around the entire circumference of the embryo (**Figure 4*j***). The ectopic auxin maxima are generated by ectopic reversals in PIN1 localization below the cotyledons as well as by the ectopic maintenance of PIN1 localization at the apical end of protodermal cells of the hypocotyl (**Figure 4*f***). One hypothesis for the origin of the ectopic reversal lies in what has been observed by live imaging of the dynamic changes in *PIN1* localization in the flowering shoot apices of *Arabidopsis* (50). Once a flower primordium is established, the orientation of PIN1 reverses such that auxin is transported away from the newly initiated flower primordium back toward the meristem (50). If this reversal is a general process, not limited to the establishment of flower meristems, one might expect to see a reversal of PIN1 from leaf/cotyledon primordia toward the SAM. Because *PIN1* is positively regulated by auxin (50, 85), such a reversal could account for the high expression levels of *PIN1* in the L1 layer of the meristem in late heart and torpedo stage embryos (51). In *kan1 kan2 kan4* embryos, if this reversal is not limited to the regions adaxial to the cotyledons, but also occurs below the cotyledons owing to the ectopic expression of PIN1 there, ectopic auxin maxima form in the hypocotyl.

Leaf primordia are initiated from the peripheral region when exogenous auxin is applied, but they are not initiated from the central zone even if auxin is applied to the center of the meristem (80). However, in *phb phv rev kan1 kan2 kan4* hextuple mutants, radial leaf-like organs also initiate from the hypocotyl and the shoot apex (**Figure 4*d***). These results suggest that auxin-mediated organ outgrowth can occur wherever opposing, PIN1-mediated auxin flow converges and appropriate auxin response factors are expressed. Normally this occurs only in the peripheral zone of the meristem. Following cotyledon formation, the flow of auxin back toward the center of the embryo in the hextuple *kan1 kan2 kan4 phv phb rev* mutant would create an auxin maximum in the position normally occupied by the SAM in the wild-type, but because *PHB*, *PHV*, and *REV* are required for normal SAM development (27, 78), the ectopic auxin maximum induces an ectopic leaf-like organ in the hextuple mutant.

The interactions between these four families of transcription factors and auxin biology highlight that the response to auxin maxima is context dependent, as was shown by the application of exogenous auxin to shoot apices (80). The lack of response in the central zone of the SAM to auxin requires the function of the Class III HD-Zip genes, perhaps acting through downstream genes, and the Class I KNOX genes likely play some role. Likewise, cells of the developing hypocotyl are initially competent to form and respond to auxin maxima, but this capability is restricted during the globular stages by the peripheral expression of KANADI. The idea that the meristem may also be a point of high auxin concentration (24, 50) also cautions against the use of using only DR5 to define auxin maxima, because this method depends on the distribution of ARFs that act as activators of transcription

(reviewed in Reference 42). DR5 is a synthetic promoter composed of four repeats of a composite auxin response element, whose regulation is thought to reflect ARF activity in cells. Because the distribution of ARF activation is dependent on auxin, patterning is a complex interplay between ARFs and other transcription factors within a cell, and these combinations feed back to further influence auxin biology as well as other transcriptional patterns.

The KANADI genes provide an example of a likely negative feedback loop involving ARF activity. Loss of *ARF2*, *ETTIN* (*ETT*; *ARF3*), and *ARF4* activity results in adaxialized leaves with outgrowths on their abaxial sides, a phenotype that resembles loss of KANADI activity (4, 75). Mutations in *ETT* also enhance the phenotypic effects of *kan1* mutations and suppress the effects of ectopic KANADI activity. Epistasis experiments suggest neither activity is upstream or downstream of the other, leading Pekker and colleagues (75) to speculate that KANADI and ARF proteins act together. The induction of ETT/ARF4 in response to auxin abaxially, in a domain in which KANADI activity is already present, could lead to the downregulation of auxin responses via their combined activity. Although the KANADI/ETT/ARF4 system exhibits a role only in the cotyledons and leaves, similar feedback modules could act in different cells on the basis of the different combinations of ARF proteins and other transcription factors expressed in particular cell types. In *Arabidopsis* the ARF gene family consists of 23 members and the AUX/IAA family consists of 29 members, a large fraction of which is expressed (based on microarray data) in the embryo or in the shoot tips (reviewed in References 61 and 82). A greater understanding of the expression patterns and loss-of-function phenotypes of ARF gene family members in addition to those of *MP* and *NPH4* is required to begin to understand the role of these genes in embryonic patterning.

The formation of auxin maxima exhibits hallmarks of a self-organizing system, both in the phyllotaxy of the shoot and in the development of leaf vascular patterns (9, 24, 55, 81, 85, 87). This could be extrapolated to the formation of auxin maxima in the embryo; auxin maxima form sequentially in the embryo proper, the root pole, the shoot pole, and finally at the sites of cotyledon primordia. Conceptually a self-organizing system could be established by the initation of auxin synthesis at sites of auxin maxima, perhaps at a certain threshold level of auxin; the increased auxin synthesis could then lead to a change in the flow of auxin away from the site of synthesis, perhaps at a second threshold level of auxin. Although some changes could occur in the absence of transcription, the action of ARFs in conjunction with other transcription factors is likely instrumental in coordinating these events. Again, the need for an understanding of the spatial and temporal patterns of auxin biosynthesis and ARF gene expression is highlighted, as well as a need for integrating these with the expression of other developmentally important genes.

Insights into Temporal Aspects of Auxin Biology and Gene Expression

A major limiting factor in our understanding of pattern formation is the lack of knowledge about the relative temporal relationships between the dynamic changes in auxin flow and gene expression of transcriptional regulators of patterning. Although in theory this could be accomplished by observing developing embryos, difficulties arise owing to their embedded position within the maternal sporophyte. However, recent technical advances in microscopy have allowed such observations in the *Arabidopsis* inflorescence meristem (41, 79) (**Figure 5**). The primary difference between the developmental dynamics of the inflorescence meristem and the establishment of embryonic patterning and subsequent functioning of the vegetative shoot meristem is that primordia formed on the periphery of the inflorescence meristem represent a reduced bract and its axillary meristem (the flower), whereas the primordia produced on the

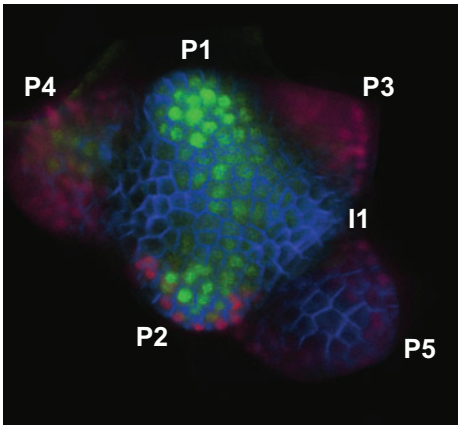

Figure 5

Arabidopsis inflorescence apex with PIN FORMED 1 (PIN1) (*blue*), REVOLUTA (REV) (*green*) and FILAMENTOUS FLOWER (FIL) (*magenta*) proteins labeled. Flower primordia (Pn) and incipient positions (In) where flower primordia will next arise are labeled.

periphery of the globular embryo or the vegetative meristem are leaf-like organs, with or without associated axillary meristems (63). Despite this caveat, studies on the dynamics of gene expression in the inflorescence and flower meristems obtained by live imaging (50) can likely be extrapolated to other stages of shoot development.

The dynamics of PIN1 protein expression observed with live imaging of inflorescence apices resembles that observed during embryogenesis (50). Expression of PIN1 marks the initiation of flower meristems in two respects. First, the polarity of PIN1 protein in the shoot apex is toward the position of the incipient flower meristem, suggesting that auxin is flowing from the meristem toward the position of the next flower primordium. On the basis of DR5 reporter gene expression, auxin maxima are formed in the periphery of the meristem at the positions predicted by the polarity of PIN1 within the meristem. Second, because PIN1 is positively regulated by increases in auxin concentration, the expression levels of PIN1 are highest at the sites of flower initiation (50, 74, 99). Following the formation of auxin maxima in the peripheral zone, the cells adaxial to the newly formed primordium exhibit a reversal in the polarity of PIN1 protein, suggesting the auxin adaxial to the primordium flows back toward the center of the meristem. In most respects this is similar to what has been observed during the globular to heart stages of embryogenesis, with the exception of the lack of an established shoot meristem in the embryo that could act as a source of auxin to be funneled to the sites of cotyledon initiation, further highlighting the need for information about the sites of auxin synthesis, both in the embryo and at later stages of development.

Concomitant with the formation of peripheral auxin maxima, both *CUC2* and *STM* are downregulated in regions predicted to have a high auxin concentration (50), suggesting both these genes may be regulated by ARF-mediated transcriptional responses. In addition, following the observed reversal of auxin flow back toward the meristem (presumably from where auxin is being drained), both genes are upregulated in a band of cells just adaxial to the region of downregulation; *CUC2* expression forms a boundary between the newly established flower primordium and the inflorescence meristem. *CUC2* and *STM* are downregulated in response to high auxin, whereas *REV* exhibits the converse expression pattern. *REV* is initially expressed in the central region of the shoot meristem, and its expression pattern expands within the epidermis in a ray from the center of the meristem toward the site of primordium initiation, as if following the flow of auxin predicted by the polar expression of *PIN1* protein. However, *REV* expression extends only to the abaxial edge of *PIN1* maximum expression and not all the way to the abaxial margin of the peripheral zone, suggesting that *PIN1* and *REV* expression interact to define a boundary, or that both *PIN1* and *REV* may be responding to a preexisting abaxial-adaxial boundary (50). At approximately the time when *CUC2* and *STM* are upregulated in a boundary region adaxial to the primordium, *REV* expression disappears from this region such that the *REV*

expression in the primordium is cut off from the *REV* expression in the meristem center. It is not clear at present what is cause and effect, but this event likely represents the beginning of autonomous development of the flower primoridum. Finally, *FILAMENTOUS FLOWER* (*FIL*) expression marks the abaxial region of the primordium, peripheral to the maximal region of *PIN1* expression; a slight overlap in the expression domains of *FIL* and *REV* is detected.

The significance of these results is that for the first time we have a glimpse into the temporal relationships between the establishment of auxin maxima and some gene expression patterns, allowing cause to be separated from effect with respect to some developmental events. The examination of additional genes, such as the YUCCA genes, *STY*, *MP*, and *KANADI*, and further multiple combinations of fluorescently labeled proteins will continue to provide additional insights into these developmental processes, and application of these techniques to embryo development and vegetative meristems will allow the identification of the common mechanisms of organ establishment and meristem function in *Arabidopsis*.

EXTRAPOLATING THE *ARABIDOPSIS* MODEL TO OTHER SEED PLANTS

What evidence exists for commonality in mechanisms of patterning and polarity among diverse seed plants? All seed plants exhibit a SAM with similar patterns of cytohistological zonation and spiral phyllotaxis. It seems reasonable to predict that PIN-mediated auxin maxima may be responsible for the initiation of organs from the peripheral zone. Although there is no direct evidence to date about expression and function of PIN genes in shoot apices of plants other than *Arabidopsis*, the PIN gene family seems to be ancient in land plants, with homologs identified in the lycophyte *Selaginella* and the moss *Physcomitrella* (32). Likewise, the Class III HD-Zip gene family is present in the genome of all land plants (33, 77, 83). Gymnosperms have a single ortholog of *PHB*, *PHV*, and *REV*, which is expressed in the peripheral zone, provascular tissue, and adaxially in leaves (31, 33). This suggests a conserved role for Class III HD-Zip function in establishing central shoot identity and adaxial leaf identity as well as promoting vascular development in seed plants. The KANADI gene family is also ancient, with homologs present in lycophytes and moss (32), although to date there is no evidence for the function of KANADI homologs in nonflowering plants. Class I KNOX genes are also ancient, and map to the common ancestor of mosses and vascular plants (18). The first to be identified, *KNOTTED-1* (*KN1*) in maize, is expressed in the SAM and is excluded from the sites of leaf initiation, as is *STM*, and loss of both genes leads to loss of SAM (reviewed in Reference 43). Class I KNOX genes are also expressed in the apices of gymnosperms, where they are excluded from the sites of leaf initiation (14, 91). Thus, the presumed role of Class I KNOX genes in suppressing growth and response to auxin in the *Arabidopsis* SAM may be conserved in seed plants. Although there are no published data, a search of the rice genome indicates that there is a single gene that is the likely ortholog of *CUC1* and *CUC2* (S.K. Floyd, unpublished data). It is not known if *CUC1/CUC2* orthologs exist in gymnosperms, but Axtell & Bartel (5) identified miR164, a microRNA that targets *CUC1* and *CUC2*, in a conifer. These data are consistent with the presence of at least one CUC1/CUC2 gene in all seed plants. Thus, patterning in the seed plant SAM likely involves conserved genes with conserved functions that evolved in a common ancestor.

Can we extend the model for patterning the *Arabidopsis* embryo to embryos of other flowering plants and seed plants and account for variability among them? One immediately obvious difference within flowering plants is the lack of bilateral symmetry of the monocot embryo, suggesting there is a single auxin

maximum during embryogenesis resulting in an asymmetrical embryo. In maize, leaf primordia are broader than in *Arabidopsis*, encircling the SAM, and *KN1* is excluded in an encircling zone on the SAM prior to the emergence of the leaf primordium (52). This suggests a broader auxin maximum throughout a larger arc of the peripheral zone in maize than in *Arabidopsis*. In conifers, a whorl of cotyledons is formed, suggesting numerous auxin maxima are formed in those embryos. Cycads and *Ginkgo* both have two cotyledons, but in cycads each cotyledon has a base that encircles the meristem much like a grass leaf base (35). Much emphasis is placed on the onset of bilateral symmetry in *Arabidopsis* embryogenesis. However, it is clear that seed plant embryos vary in terms of the number, position, and insertion of cotyledons so that cotyledon initiation leads to asymmetry (monocots, cycads), bilateral symmetry (dicotyledonous plants), or radial symmetry (conifers). The underlying commonality is that cotyledon initiation represents the onset of organogenesis from the shoot apex (56). Bilateral symmetry and heart stage are descriptive terms that apply to some seed plants and it is more broadly meaningful to focus on the developmental process of organogenesis (56). On the basis of the model for *Arabidopsis* we would predict that the differences in cotyledon number and arrangement among seed plants reflect spatial and temporal differences in PIN-mediated maxima and CUC-mediated boundaries. Expression and functional data are needed to confirm or reject these predictions.

Finally, embryogenesis in flowering plants begins with asymmetrical division of the zygote as in *Arabidopsis*. However, most gymnosperm embryos begin with a phase of free-nuclear division; the clearly bipolar embryos contain hundreds of nuclei (in cycads and *Ginkgo*). Most nuclei are aggregated at what will become the apical end and cellularization begins there as well. The shoot apex becomes organized even in the absence of complete cellularization of the basal end in cycads (35). PIN-mediated auxin flux occurs through polar localization of PIN proteins at the plasma membrane, which can happen only in multicellular tissue. Although auxin gradients may be involved, zygotic PIN-mediated polar transport cannot establish polarity within the free-nuclear embryo of gymnosperms (on the basis of our understanding of PIN function in *Arabidopsis*). The tightly controlled movement of auxin through particular cells and cell layers of the early embryo that occurs in *Arabidopsis* to create the shoot pole, create the root pole, and initiate the provasculature of the hypocotyl cannot occur in the gymnosperm embryo and may reflect a heterochronic shift to engage shoot patterning mechanisms at an earlier stage in development in the flowering plant lineage.

CONCLUDING REMARKS

A model is emerging in which auxin gradients and auxin transporters (PINs, PINOID) influence and are influenced by transcription factors, some of which are associated with high auxin concentration and flux (ARFs, Class III HD-Zips) and some of which are associated with low auxin concentrations (KNOX, CUC, KANADI). Together these transcription factors create patterns of differential growth in the shoot to maintain a meristem and produce lateral organs. As we mentioned at the beginning of this review, the SAM of seed plants is unique among land plants in that it exhibits cytohistological zonation that is associated with maintenance of stem cells (the central zone) and the production of lateral organs (the peripheral zone) (28, 34). The central zone includes the initial cells and their subadjacent derivatives, in which cell division is infrequent and the cells are larger and more vacuolate than in the peripheral zone (**Figure 1**). Auxin maxima form in the peripheral zone to determine where leaf primordia will emerge. Other genes that are expressed in the peripheral zone include *PHB*, *PHV*, and *REV* in the adaxial side of the entire peripheral zone,

KANADI in the abaxial side of the peripheral zone, *CUC1* and *CUC2* between adjacent primordia, and *STM* everywhere except the auxin maxima. The real-time analyses of Heisler and coworkers (50) suggest that the peripheral zone is already polarized with adaxial and abaxial domains when auxin maxima are formed and when elevated levels of *REV* expression occur. The fact that a ring of tissue with adaxial and abaxial characters develops in the *pin1* mutant inflorescence meristem and the *cuc1 cuc2* mutant embryo indicates that ad/abaxial polarity does not depend on the formation of auxin maxima or leaf primordia in the peripheral zone (1, 97). In the globular embryo, *PHB*, *PHV*, and *REV* are initially expressed throughout the embryo and then their expression becomes restricted to the apical and central regions (27, 68). At the same time, *KAN1* expression is evident around the periphery of the hypocotyl (57). The first auxin maxima forms at the boundary of *KANADI* and *PHB, PHV, REV* expression where there is no *STM* expression (50). Together this suggests that the peripheral zone is a region that represents the boundary of peripheral and central shoot expression domains in which auxin maxima form, with auxin flow converging from outer and inner domains. In both embryos and growing shoot apices organs form at the boundary of an abaxial/outer domain and an adaxial/inner domain of *PHB, PHV, REV* expression (50). This supports the view of Kaplan & Cooke (56) that the apical pole of the globular proembryo is the SAM and *STM* activity is required to maintain a population of stem cells, not establish them. However, cells at the center of the meristem do not acquire the larger, more vacuolate character that defines the central zone until after *STM* expression, which indicates that the peripheral zone is defined and functional prior to the establishment of the central zone and that in *stm* mutant plants the peripheral zone is established but the central zone is not. The data merging from detailed analyses of *Arabidopsis* are beginning to provide new insights into the developmental genetics of some longstanding questions, such as the developmental origin of the SAM in seed plants and the manner in which the SAM functions. There are still many gaps in the *Arabidopsis* model, such as the role of auxin biosynthesis and the causal relationship of auxin maxima, minima, and the transcription factors that are expressed in those regions. We also have still more to learn about how broadly the *Arabidopsis* model can be applied to understand seed plant shoot patterning in general. However, plant developmental genetics is beginning to address the fundamental issues of patterning in plants that we can apply to long-standing concepts from comparative anatomy to achieve a better understanding of seed plant development and evolution.

ACKNOWLEDGMENTS

We thank Gabriela Pagnussat for the images of embryo development in **Figures 2** and **3**, and Marcus Heisler for the image in **Figure 5**. We thank members of our lab, Marcus Heisler, and Yuval Eshed for helpful discussions over the years. We apologize for citing reviews in some cases rather than the primary literature owing to a lack of space. The authors' research is funded by the Australian Research Council (DP0771232, FF0561326), the United States National Science Foundation (IOB-0515435), and Monash University.

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Annual Review of
Plant Biology
Volume 59, 2008

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