Surge and destroy: the role of auxin in plant embryogenesis

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Development 132, 3577-3585
Published by The Company of Biologists 2005
doi:10.1242/dev.01952

Summary

For nearly a century, the plant hormone auxin has been recognized for its effects on post-embryonic plant growth. Now recent insights into the molecular mechanism of auxin transport and signaling are uncovering fundamental roles for auxin in the earliest stages of plant development, such as in the development of the apical-basal (shoot-root) axis in the embryo, as well as in the formation of the root and shoot apical meristems and the cotyledons. Localized surges in auxin within the embryo occur through a sophisticated transcellular transport pathway causing the proteolytic destruction of key transcriptional repressors. As we discuss here, the resulting downstream gene activation, together with other, less well-understood regulatory pathways, establish much of the basic body plan of the angiosperm embryo.

Introduction

The plant hormone auxin was identified originally from the effects it had on modulating plant growth (see Went, 1974). Among the classically studied auxin effects are plant tropic responses (growth toward or away from environmental signals) and apical dominance (repression of branch outgrowth by cells at the shoot tip). Plant growth in response to gravity and light requires asymmetrically distributed auxin across the stem or root. This causes one side to grow more than the other. Similarly, the production of auxin by the 'apically dominant' shoot tip, followed by its transport down through the stem, represses the outgrowth of lateral buds.

It has taken longer to recognize and understand the role auxin plays in the development of cell or organ types in the embryo. This is partly because the plant embryo is safely, and for many experimental purposes, inaccessibly, tucked away inside the ovule. It is also because we have had, until recently, a very limited understanding of the molecular mechanisms that underlie auxin action. Despite these difficulties, the profound effects of auxin on in vitro-cultured plant tissues have suggested that auxin had the power not just to regulate growth but also to dictate the fate of a cell. Skoog and Miller's famous experiments showed that the ratio of auxin to cytokinin in the growth medium determined whether roots or shoots developed from cultured cell clusters (Skoog and Miller, 1957). The concentration of auxin in the medium could also be manipulated to cause cultured Zinnia mesophyll cells to develop into xylem cells (Fukuda and Komamine, 1980).

In 2000, Geldner and colleagues (Geldner et al., 2000) wrote a short review entitled, 'Is there a role for auxin in early embryogenesis?' Around that time, researchers were finding that molecules involved in early embryonic development bore similarities to molecules involved in auxin signal transduction. These were the first hints of a molecular mechanism for auxin action in embryonic development. Five years later, the answer to Geldner et al.'s question is an emphatic 'yes'. In this review, we discuss some of the more recent experiments linking auxin to important events in the development of the early embryo. These include the establishment of the root apical meristem at the basal end of the embryo and the partitioning of the apical portion of the embryo into cotyledons (seed leaves) and shoot apical meristem. We begin with brief summaries of the mechanism of auxin action and of wild-type embryogenesis.

Auxin activity and transport

Auxin activates transcription by destroying transcriptional repressors

Auxin refers to any of a group of chemically related small molecules, which includes indole acetic acid and indole butyric acid. Our knowledge of auxin action comes chiefly from experiments in which exogenous auxin has been applied to cells, and from the study of mutants unable to respond normally to exogenously applied auxin. Auxin application causes rapid changes in transcription (Abel and Theologis, 1996; Hagen and Guilfoyle, 2002) through the destruction of AUX/IAA transcriptional repressors (Gray et al., 2001; Zenser et al., 2001) (Fig. 1). Auxin does this by binding to and activating the TIR1 F-box component of the SCF^{TIR1} E3 ubiquitin ligase, which then ubiquitinates AUX/IAA proteins, targeting them for destruction by the proteosome (Dharmasiri et al., 2005; Kepinski and Leyser, 2005) (see also Moon et al., 2004). AUX/IAA proteins contain potent transcriptional repression domains but do not bind DNA on their own. Instead, AUX/IAA proteins dimerize with AUXIN RESPONSE FACTOR (ARF) DNA-binding transcriptional activators and prevent them from activating transcription (Tiwari et al., 2001; Tiwari et al., 2004). Upon auxin-mediated degradation of an AUX/IAA factor, the ARF protein is free to activate transcription from its target promoter.

Microarray analysis of auxin-treated tissues shows that there are several hundred genes that are regulated at the transcriptional level by auxin (Zhao et al., 2003; Goda et al., 2004; Nemhauser et al., 2004). Among the wide variety of gene products regulated by auxin are those involved in cell

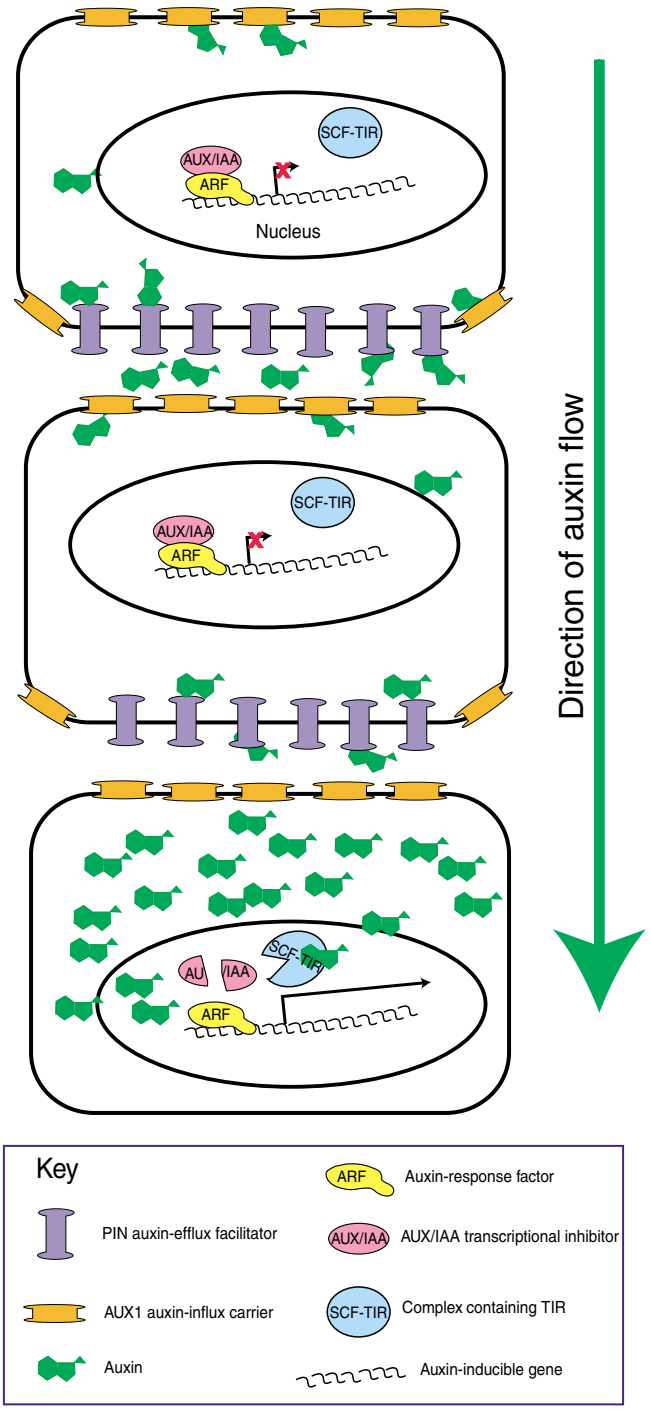

Fig. 1. Model for auxin transport and action in the plant cell. Auxin is moved into cells through influx carriers encoded by the *AUX1* gene. It is expelled from the cell by localized auxin-efflux carriers associated with PIN auxin facilitators. Auxin efflux carriers are asymmetrically targeted through a mechanism that involves the presence/absence of the PINOID kinase. Auxin-efflux carriers cycle to and from the membrane through the endosomal compartment. At some threshold concentration, auxin triggers the activity of the SCF^{TIR} ubiquitin E3 ligase which marks the AUX/IAA transcriptional represssor (IAA) for destruction via the proteosome. The auxin-response factor is then free to act as a transcriptional activator.

elongation, consistent with the known role of auxin on growth. Induced genes also include many of the AUX/IAA genes, indicating the potential for a negative-feedback loop in which auxin action is self-limiting.

As predicted by the model for auxin action described above, the levels of most auxin-regulated transcripts go up, but a sizable fraction of auxin-regulated transcripts (about 1 in 5) were found to be downregulated (Nemhauser et al., 2004). This could reflect that the AUX/IAA and ARF transcriptional regulatory network is more complex than is currently understood. Indeed, AUX/IAA and ARF interactions are likely to be complex, as there are many members of both gene families (29 AUX/IAA family members and 22 ARF family members) (Liscum and Reed, 2002; Guilfoyle and Hagen, 2001). All AUX/IAA gene products seem to be transcriptional repressors (Tiwari et al., 2001). The ARF proteins are more variable; some have transcriptional activation domains whereas others have transcriptional repression domains (Tiwari et al., 2003). Moreover, systematic analysis of loss-of-function mutations in members of the ARF family has shown that most do not result in mutant phenotypes (Okushima et al., 2005). Thus, there is likely to be a high degree of functional redundancy among members of this family. Alternatively, the negatively regulated transcripts could reflect a more direct effect of auxin on transcript stability, through, as yet, unknown mechanisms.

ARFs bind to TGTCTC auxin response elements (auxREs), which lie upstream of auxin-activated genes. A synthetic reporter, DR5, consisting of multiple tandem repeats of this sequence upstream of either β-glucuronidase (GUS) or green fluorescent protein (GFP), is frequently used to detect auxin activity in plant cells (Ulmasov et al., 1997a; Ulmasov et al., 1997b). The DR5 reporter is strongly activated in protoplasts (dissociated plant cells from which the cell wall has been removed) treated with auxin in the presence of ARFs with transcriptional activation domains (Tiwari et al., 2003).

The extent of DR5 reporter activity is often used as an indicator of the active auxin present in a given cell. Two observations suggest that DR5 activity alone is not a sufficient way to measure intracellular auxin activity. First, in the presence of ARFs with transcriptional repression domains, the DR5 reporter is repressed in protoplasts (Tiwari et al., 2003). High levels of such ARFs in a cell could theoretically compete with activating ARFs for the auxREs. Second, the DR5 reporter requires the plant growth regulator brassinolide for full activity (Nemhauser et al., 2004). Moreover, high levels of brassinolide can activate DR5 in the apparent absence of changes in auxin levels (Nakamura et al., 2003). Thus, high or low levels of brassinolide within a cell could potentially confound the readout of the DR5 reporter. These findings show the need to use additional experimental methods to confirm findings based on DR5 activity. In some cases, a combination of microdissection and mass spectrometry has been used to measure auxin levels (e.g. Friml et al., 2002a; Friml et al., 2004). Although such measurements have limited spatial resolution, they tend to support the results obtained with the DR5 reporter. Immunolocalization following crosslinking of IAA to protein has also been developed as a technique to directly visualize the distribution of auxin (Moctezuma, 1999; Friml et al., 2003; Benkova et al., 2003).

PIN proteins regulate the asymmetric transcellular transport of auxin

Auxin is transported from its site of synthesis to its site of action by a transcellular mechanism (Fig. 1). Directional auxin transport is mediated by the combined action of auxin influx carriers and auxin efflux carriers. The auxin expelled from one cell is taken up by the next cell in line, which expels it from its opposite end, and so forth.

Extracellular auxin is thought to be transported across the membrane and into the cell by the AUX1 protein, a member of the auxin amino acid permease (AAAP) family of proton-driven transporters (Bennet et al., 1996; Swarup et al., 2001). The AUX1 influx carrier may be asymmetrically localized. In some cells, AUX1 is present on the cell surface opposite to the efflux carrier (Swarup et al., 2001) (Fig. 1). In other cases, it is lateral to and on the same surface as the efflux carrier (Reinhardt et al., 2003). The latter configuration could allow the auxin flow to be 'focussed' to a single column of cells by promoting the reuptake of auxin effluxed laterally, thus preventing auxin from affecting neighboring cell columns. *aux1* mutants, although resistant to the effects of exogenous auxin, have not been reported to have embryonic pattern defects. If, indeed, auxin influx is required for embryonic development, it occurs via a second, redundant permease family member or an alternative mechanism for importing auxin into the cell.

Members of the PIN family of membrane proteins are thought to play an important role in auxin efflux from the cell (Gälweiler et al., 1998; Chen et al., 1998; Friml et al., 2002a; Friml et al., 2002b; Müller et al., 1998; Luschnig et al., 1998; Friml et al., 2003). *Arabidopsis* plants homozygous for mutations in several of the *PIN* genes show phenotypes consistent with auxin transport defects. *Arabidopsis pin1* mutants fail to produce flowers, as is seen in wild-type plants treated with the auxin transport inhibitor naphthylphthalamic acid (NPA) (Okada et al., 1991; Gälweiler et al., 1998). *pin2* and *pin3* mutants display abnormal tropisms (Chen et al., 1998; Müller et al., 1998; Luschnig et al., 1998; Friml et al., 2002b). *pin* mutants show reduced auxin transport, as determined by direct measurement or as inferred by the expanded pattern of auxin accumulation in them. PIN proteins are polarly localized, typically localizing to one or two of the six faces of the nearly brick-shaped plant cell. Their location correlates well with the expected direction of auxin transport. PIN proteins are continuously recycled to and from the cell surface via an endosomal compartment in a process that requires the GNOM membrane-associated ARF-GEF (guanine nucleotide-exchange factor on ADP-ribosylation factor G) protein (Steinmann et al., 1999; Geldner et al., 2003). This recycling could allow the rapid redistribution of PIN proteins in response to changing environmental stimuli, such as changes in light or gravity.

It is not known whether PIN proteins themselves export auxin from the cell or whether they promote efflux by interacting with an auxin efflux transporter. The *Arabidopsis* multidrug resistance (MDR)-like ABC transporter proteins MDR and PGP are candidates for auxin efflux transporters. They are required for auxin transport and for normal tropisms in *Arabidopsis* seedlings (Noh et al., 2001), and also bind directly to the auxin transport inhibitor NPA.

The MDR transporters also affect PIN localization, as these are mislocalized in *mdr* mutants (Noh et al., 2003). Additional factors influencing PIN localization are flavonoids, a family of secondary metabolites (Peer et al., 2004), and the PINOID kinase (Friml et al., 2004). PINOID kinase activity appears to direct the PIN proteins to the apical end of the cell, while lack of PINOID kinase function directs the PIN proteins to the basal end of the cell. The target for the PINOID kinase is as yet unknown.

Arabidopsis embryogenesis

All parts of the *Arabidopsis* seedling – cotyledons, shoot apical meristem (SAM), hypocotyl, vascular cylinder, root apical meristem and root cap – are present in primordial form in the heart-stage *Arabidopsis* embryo (Fig. 2) (for a review, see Laux, 2004). Thus, the most dramatic steps in pattern

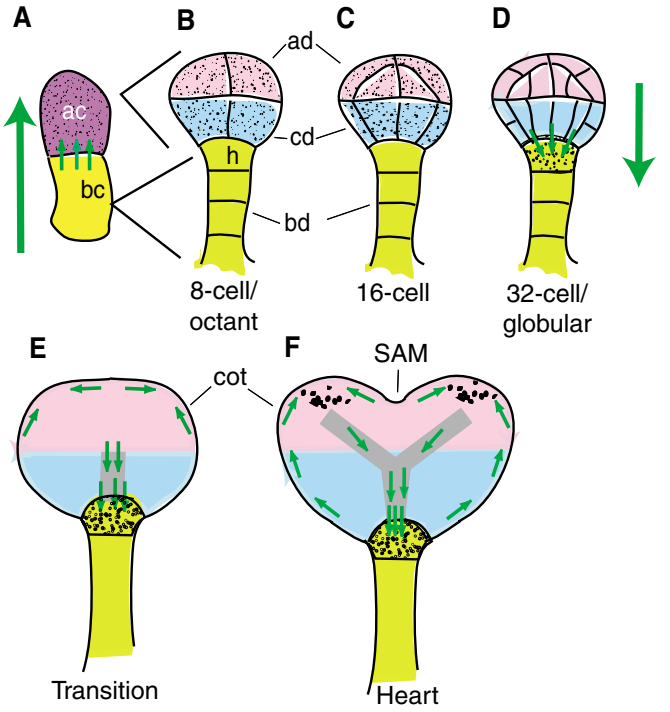

Fig. 2. Auxin transport relative to early events in *Arabidopsis* embryo patterning. (A) An early *Arabidopsis* embryo, consisting of an apical cell (ac) and a basal cell (bc). Green arrows indicate the direction of auxin transport; stippling indicates regions with high auxin levels. (B) Eight-cell/octant-stage embryo. (Cell numbers used to stage embryos reflect the number of cells in the apical cell lineage.) The apical domain (pink) and central domain (blue) both derive from the apical cell and each consists of four cells. The basal domain (yellow) derives from the basal cell. (C) A 16-cell stage, early globular embryo. (D) In a 32-cell stage globular embryo, auxin transport has shifted direction (green arrows), and auxin now accumulates in the hypophyseal lineage. The hypophyseal lineage is derived from the hypophysis (h) – the suspensor cell closest to the embryo proper. This lineage gives rise to a portion of the root meristem, specifically the quiescent center and the central columella with associated stem cells. (E) A transition stage (transitioning between globular and heart stage) embryo. Auxin transport in the apical domain is directed toward the center of the cotyledon primordia (cot). (F) An early heart-stage embryo, showing the emergence of cotyledons and a cleft where the shoot apical meristem (SAM) will form. Gray indicates regions of vascular development.

formation occur prior to this, as the embryo develops from zygote to transition stage.

The polarly organized zygote houses a large vacuole at the basal end of the cell and its nucleus at the apical end. The first cell division is asymmetric, generating a larger vacuolated basal cell and a smaller, more densely cytoplasmic, apical cell. The basal cell divides transversely to form a filament of cells. Most of this filament is the suspensor, an extraembryonic structure that tethers the embryo to the inside of the ovule and may function in the transport of nutrients from the maternal tissues. The exception is the filament cell closest to the apical cell. This cell, the hypophysis, becomes part of the embryo itself. Its descendants make up the quiescent center of the root, as well as the central root cap and its associated stem cells.

The vast majority of the embryo derives from the apical cell. The first three rounds of cell division in this lineage, the first two longitudinal and the third transverse, result in an octant-stage embryo that is divided equally into top (apical domain) and bottom (central domain) halves (Fig. 2B). The spatially restricted expression patterns of the *WOX* homeobox-containing genes clearly distinguish these domains and precede their overt differentiation (Haecker et al., 2004). *WOX9* and *WOX2* mRNAs are expressed in the apical and central domains, respectively, of the octant embryo (apical cell lineage), whereas *WOX8* mRNA is expressed in the hypophysis and suspensor (basal cell lineage). The apical domain of the octant-stage embryo gives rise to cotyledons and the SAM, while the central domain gives rise to the hypocotyl and most of the root and root meristem.

The root meristem contains stem cells (also called initials) for the root cortical and endodermal layers, for the root epidermis and lateral root cap, for the vascular cylinder, and for the central root cap (columella). These stem cells are arranged around a small group of very slowly dividing cells called the quiescent center. The quiescent center and the central root cap with its associated stem cells derive from the hypophyseal lineage – all other parts of the root meristem derive from the central domain.

Approximately four more rounds of division generate a larger late globular embryo. This subsequently transitions from a globular-shaped (radially symmetrical) to a heart-shaped (bilaterally symmetrical) embryo, with the outgrowth of the two cotyledon primordia flanking the SAM.

Surges of auxin in the early embryo

Four members of the PIN family of auxin efflux facilitators are expressed in the embryo – *PIN1*, *PIN3*, *PIN4* and *PIN7* (Friml et al., 2002a; Friml et al., 2003). They are expressed in different places and at different times during embryonic development. The temporally and spatially controlled sequence of expression of the PIN proteins appears to be responsible for the dynamic and shifting pattern of auxin accumulation observed in the embryo, as principally determined using the DR5 reporter. In the early embryo, auxin flows upward, away from the extraembryonic suspensor and into the embryo proper. By the heart stage, auxin has reversed course and flows to the developing root. Experiments in which these flows are disrupted show that auxin may play important roles in the development of the apical/basal axis.

PIN7 and *PIN1* are the earliest *PIN* genes expressed in the embryo (Friml et al., 2003). PIN7 protein is found at the apical membrane of the basal cell in the two-cell embryo and at the apical membrane in suspensor cells until the 32 cell stage. This localization pattern suggests a role for PIN7 in transporting auxin from the basal cell to the apical cell. In support of this, the DR5 reporter is expressed in the apical cell in wild-type embryos. When auxin transport inhibitors were applied to embryos, or to a *pin7* mutant, the DR5 reporter was instead expressed in the basal cell (Friml et al., 2003).

By contrast, PIN1 protein is localized in a non-polar fashion throughout the embryo proper, from the one-cell stage until the 32-cell stage, when it becomes polarly localized to the basal ends of the provascular cells (Steinmann et al., 1999; Friml et al., 2003). At the same time, PIN7 protein switches to the basal ends of suspensor cells, PIN4 protein begins to accumulate in the hypophyseal lineage and in vascular initials, and auxin accumulation, as detected by DR5 expression, shifts downward into the hypophysis and suspensor (Friml et al., 2002a; Friml et al., 2003). In the absence of either *pin1* or *pin4*, DR5 remains expressed in the globular embryo proper. Thus, the combined action of *PIN1*, *PIN4* and, probably, *PIN7* appears to reverse auxin flow in the globular-stage embryo, causing it to be exported from the embryo proper and to accumulate at the basal pole.

PIN3 mRNA expression is detected at the root pole of the heart-stage embryo (Friml et al., 2003). Given the late appearance of *PIN3* mRNA in the heart-stage embryo after all pattern elements are present, it seems unlikely that *PIN3* plays a role in early apical basal pattern formation in the wild-type embryo.

What role do these auxin surges play in embryogenesis and, more specifically, in apical-basal pattern formation? Mutants lacking *PIN1*, *PIN4* or *PIN7* show abnormal cell division patterns in the cells to which the auxin is expected to be targeted by the respective PIN protein: the apical cell and the hypophysis in *pin7* mutants (Friml et al., 2003); the hypophysis in *pin1* mutants (Friml et al., 2003); and the columella initials and hypophysis in *pin4* mutants (Friml et al., 2002a). Changes in the expression of cell-specific reporters consistent with cell fate changes in the embryo have been seen in *pin7* mutants (Friml et al., 2003). However, in all cases, the reporters used were related to auxin metabolism, so it is not possible to determine whether these changes represented changes in developmental fates or in auxin homeostasis. It should now be possible to repeat these experiments using the *WOX* genes as markers for embryonic cell fates.

The defects in *pin* single mutants are rather subtle and the embryos ultimately recover to become near normal embryos. Only when the *pin* mutations are combined in double, triple and quadruple mutant combinations are more severe defects seen. The most severely affected multiple mutants develop as ball-shaped embryos without any clear evidence of apical-basal polarity. Thus, the PIN proteins appear to act redundantly. Consistent with this, severe *gnom* mutants are defective in the endosomal recycling of PIN proteins and also develop into ball-shaped seedlings. The recycling defect in *gnom* mutants presumably affects all members of the family of PIN proteins.

Because the different *PIN* proteins are expressed at different times and in different locations, the substitution of one *PIN* family member by another is not simple. Indeed, auxin accumulation in the predicted sink cells appears to be abolished in *pin1* and *pin7* mutants (Friml et al., 2003). Nevertheless, the

embryo appears to be able to re-establish the correct pattern, perhaps by re-establishing the auxin gradient after the globular stage. Support for such reorganization is found in the recent observations by Blilou et al. (Blilou et al., 2005). These authors found that, in embryos mutant for a subset of *PIN* family members, the remaining functional PIN proteins altered their distribution.

The role of ARF and AUX/IAA proteins in embryogenesis

The auxin surges that occur during embryogenesis are predicted to result in the local degradation of AUX/IAA proteins, with the consequent activation of ARF-regulated genes, resulting in local gene activation in the embryo. Studies of *Arabidopsis* mutants in this pathway have led to an understanding of the role auxin plays in the developing embryo. Indeed, gain-of-function, degradation-resistant mutant alleles of the *BODENLOS/IAA12* (*BDL*) gene, and loss-of-function mutant alleles of the *MONOPTEROS/ARF5* (*MP*) gene cause embryos to develop without roots and with much of their hypocotyls missing (Hardtke et al., 1998; Hamann et al., 2002). Several lines of evidence support the hypothesis that these particular factors function as an interacting pair in the embryo. For example, *BDL* and *MP* are co-expressed in the early globular embryo and can interact to form heterodimers, and the overexpression of *MP* can suppress the gain-of-function *bodenlos* phenotype (Hardtke et al., 2004).

MP is highly related to the *NONPHOTOTROPIC HYPOCOTYL/ARF7* (*NPH4*) gene (Harper et al., 2000; Hardtke et al., 2004). Both ARFs have transcription activation domains and are expressed in overlapping expression domains. Although the single *mp* mutant lacks basal structures and has reduced vasculature, the single *nph4* mutant shows no morphological abnormalities but instead shows defects in auxin-dependent hypocotyl cell expansion. Double mutants homozygous for both *mp* and *nph4* show a more severe embryonic defect than *mp* single mutants, indicating that *nph4* plays a role in embryo development that is normally masked by wild-type *MP* function. Conversely, when 'weak' mutants of *mp* were generated by RNAi, these had hypocotyls that showed defects in auxin-dependent cell expansion. Thus, *MP* plays a role in tropic responses to environmental stimuli that involve auxin.

These observations fit with a model in which *PIN7*-directed auxin flow into the embryo proper causes the degradation of BODENLOS. This allows MP and NPH4 proteins to activate, as yet, unknown target genes in the embryo proper. Because the early basal defects seen in *mp* mutants primarily affect the hypophysis where *MP*, *NPH4* and *BDL* are not expressed, changes in target gene expression in the apical and central domains are predicted to have a cell non-autonomous effect on the hypophyseal lineage. Alternatively, because protein localization data are not available for these gene products, it is possible that the MP, NPH4 and BDL proteins are transported into the hypophyseal cell, where the entire pathway could act to directly influence the development of this lineage.

Arabidopsis plants mutant for other components of auxin-mediated signaling also have defects in the hypophyseal lineage. These include plants with mutations in the *CUL1/AXR6* gene, which encodes the CULLIN subunit of the auxin-regulated SCF^{TIR1} ubiquitin E3 ligase (Hobbie et al., 2000; Hellman et al., 2003).

The role of auxin in establishing and maintaining the root stem cell niche

The root apical meristem is fully organized and functional by the end of embryogenesis and is a site of high auxin accumulation that is important for stem cell function. The AP2 domain-containing transcription factor genes *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) act redundantly to promote the development of the root stem cell niche (Aida et al., 2004; Blilou et al., 2005). In the absence of *PLT1* and *PLT2* function, the hypophyseal lineage divides abnormally and fails to express markers for the quiescent center. The quiescent center is a small group of slowly dividing cells that is located adjacent to the root initials (the root stem cells) and is required for the activity of these stem cells. *PLT1* and *PLT2* are expressed in the basal end of the embryo, and their expression is dependent upon *MP* and *NPH4* function. Moreover, *PLT1* and *PLT2* transcription is induced following auxin treatment, and the ectopic expression of *PLT1* and *PLT2* promotes root formation in the absence of auxin. Taken together, these data suggest a pathway in which prolonged auxin signaling, acting through MP and NPH4 ARFs, promotes the formation and development of root stem cells by activating the *PLETHORA* genes. The substantial delay between auxin treatment and *PLT* activation suggests that the action of auxin on *PLT* transcription is not direct, but rather proceeds through intermediate steps.

The *PLT* genes in turn are required for the normal accumulation of PIN proteins (Blilou et al., 2005). Thus, the auxin signaling and PLT accumulation are connected in a positive-feedback loop. This loop is thought to stabilize the size and position of the root apical meristem.

The importance of auxin for the survival of the root meristem can be seen in situations where the direction of auxin transport is altered. In mutants overexpressing the *PINOID* kinase gene, PIN proteins are localized apically instead of basally (Friml et al., 2004). In these mutants, auxin flow is inappropriately directed up and away from the root meristem. The result of this is the cessation of stem cell activity in the root meristem, which can be prevented if the PINOID-overexpressing plants are grown in the presence of auxin transport inhibitors.

Subdivision of the apical domain is affected by auxin

So far, this review has described the role of auxin in developmental events that occur in the basal portion of the embryo. Several lines of evidence show that auxin plays a role in the apical domain as well.

The two cotyledons and the SAM develop from the apical half of the globular embryo (Fig. 3). At the late globular stage, the two cotyledon primordia grow rapidly relative to the slow-growing cleft region between them, giving the embryo its characteristic heart shape. The cleft region consists of the (central) SAM primordium and the (lateral) intercotyledonary zones, which will form the 'separation' between the two cotyledons. The outgrowth of the intercotyledonary zones is suppressed by the redundantly acting *CUP-SHAPED COTYLEDON1* and *2* (*CUC1* and *CUC2)* gene products, which belong to the larger family of plant-specific NAC domain

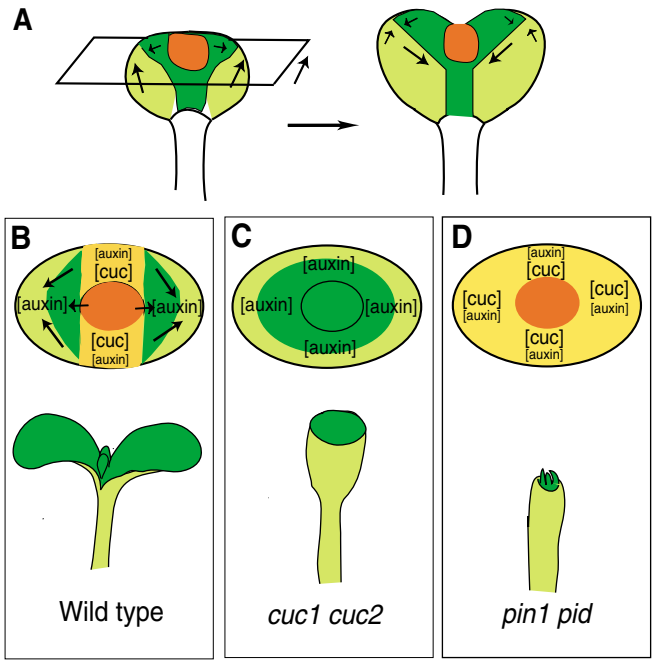

Fig. 3. Partitioning of the apical domain into shoot apical meristem and cotyledons. (A) Transition-stage and early heart-stage *Arabidopsis* embryos showing the direction of auxin transport (arrows) and the partitioning of the embryo into cotyledonary (green) and meristem (orange) domains. (B) Cross section (as shown in A) through the apical domain of a wild-type embryo, showing the region that will develop into the shoot apical meristem (dark orange), the intercotyledonary zones (light orange), the adaxial domain (top) of the cotyledon (dark green) and the abaxial domain (bottom) of the cotyledon (light green). The meristem and intercotyledonary zones have low auxin levels and high CUC levels, whereas the opposite holds for the cotyledon primordia. (C) Cross section of the apical domain from a cup-shaped *cuc1 cuc2* cotyledon mutant. No separation is made between the cotyledons and no shoot apical meristem is made in the central region of the embryo. (D) Cross section of the apical domain from a *pin pid* embryo. These embryos lack the PIN1 auxin transporter. They also lack the PID kinase that appears to be responsible for positioning PIN1 on the apical side of the cell. Lack of high auxin levels in the cotyledon primordia in these embryos would allow CUC accumulation throughout the apical domain, thus preventing cotyledon outgrowth.

transcription factors and probably mediate their action through the transcriptional control of downstream genes (Aida et al., 1997; Aida et al., 1999; Takada et al., 2001). When both *CUC1* and *CUC2* are mutant, embryos develop a single cup-shaped cotyledon that encircles the apical pole of the embryo.

CUC mRNA is normally expressed in the intercotyledonary regions, where growth is suppressed and is excluded from the developing cotyledons (Aida et al., 1997; Takada et al., 2001). In mutants expected to be highly deficient in normal auxin transport, i.e. *pin1 pinoid* double mutants, ectopic *CUC* mRNA accumulates not only in the intercotyledonary regions, but also in the cotyledon primordia themselves (Furutani et al., 2004). In these double mutants, the cotyledons fail to grow out and the result is a radially symmetrical seedling lacking cotyledons. Cotyledon outgrowth can be restored in *pin1 pinoid* double mutants by eliminating *CUC* gene function in them. Embryos doubly mutant for both the MP and NPH4 ARF transcriptional activators entirely lack cotyledon outgrowth, similar to the *pin pid* double mutants (Hardtke et al., 2004).

These observations suggest a model in which auxin is transported away from the intercotyledonary regions and towards the cotyledonary domains, where it directs degradation of an AUX/IAA repressor, which then allows MP/NPH4 ARF activity to repress *CUC* gene transcription (Fig. 3). The repression of *CUC* RNA accumulation by MP/NPH4 need not be direct. This model for early leaf/cotyledon development is supported by observations that DR5 activity accumulates at the site of future cotyledons, and by experiments in which auxin applied to the shoot apical meristem causes the outgrowth of leaf primordia at the site of application, as well as by the analysis of PIN protein localization and auxin transport in wild-type leaf primordia (Benkova et al., 2003; Reinhardt et al., 2003).

Other lines of evidence indicate a role for auxin in patterning the cotyledonary and intercotyledonary regions of the embryo apex. Embryos treated with exogenous auxin or auxin transport inhibitors develop cup-shaped cotyledons (Liu et al., 1993; Hadfi et al., 1998; Aida et al., 2004), as do triple and quadruple *pin* mutants (Friml et al., 2003), and single mutants of the *gnom* ARF-GEF required for PIN protein localization.

Combinatorial effects of auxin action

This review has described evidence that supports a role for auxin in key embryonic developmental events. Auxin, of course, does not act alone but rather in the environment of other regulators of plant growth and development. The complexities of these interactions will probably be important for a more complete understanding of the role of auxin in embryo development.

Recent work has highlighted several ways in which auxin action interacts with other plant hormones, such as brassinosteroids, gibberellins or ethylene, tissue-specific transcription factors and small regulatory RNAs (microRNAs).

Brassinosteroids are important for cell expansion. Exogenously added brassinosteroids were found to induce expression of the auxin responsive DR5:GUS reporter (Bao et al., 2004). Brassinosteroids appear to have this effect by positively regulating auxin transport. Nemhauser et al. found that auxin-responsive promoters and brassinosteroid-responsive promoters share a common element that requires brassinosteroid synthesis for its expression (Nemhauser et al., 2004).

Gibberellins regulate many aspects of plant growth, including the elongation of the stem and root. The response of root growth to gibberellins was found to be influenced by auxin (Fu and Harberd, 2003). Gibberellin normally acts by destabilizing nuclear DELLA proteins. DELLA proteins (named for the presence of a conserved amino acid sequence) are thought to be transcriptional repressors that repress gibberellic acid (GA)-inducible genes. Auxin derived from the shoot and transported to the root was found to enhance the gibberellin-induced destabilization of DELLA proteins.

The ability of ethylene, another hormone involved in seedling elongation, to inhibit auxin activity has recently been explained by its ability to destabilize the ARF2 proteins (Li et al., 2004). Finally, auxin appears to rapidly repress cytokinin biosynthesis (Nordström et al., 2004).

Transcription factors also can have different effects, depending on the presence of auxin in the cellular environment. The WUSCHEL homeobox protein, when ectopically expressed in roots, can induce the formation of embryos, but only if exogenous auxin is added to the growth medium (Gallois et al., 2004).

Small RNAs are also likely to influence the auxin pathway of signaling, as many of the genes involved in auxin control are also controlled by microRNAs. These include members of the TIR, ARF and CUC gene families (Reinhart et al., 2002; Mallory et al., 2004). Thus, small RNAs have the potential to either counteract (TIF, ARF) or reinforce (CUC) the auxin signal by altering the stability of particular mRNAs in the auxin pathway. ARF17 has also been recently confirmed to be a microRNA-targeted mRNA. Plants expressing an *ARF17* cDNA that encodes a wild-type ARF17 protein but carries silent mutations preventing it from being recognized by microRNA 160 (mir160) accumulate higher levels of ARF17 protein and show alterations in the levels of auxin-regulated transcripts (Mallory et al., 2005). Some embryos carrying this transgene developed extra or split cotyledons, indicating a role for mir160 in partitioning the apical domain of the embryo. Guo et al. (Guo et al., 2005) found evidence that mir164, a microRNA that targets member of the NAC domain-containing family of genes, is regulated by auxin. The *CUC* genes described above belong to this family. This finding suggests that auxin could act by targeting RNA destruction, as well as bringing about protein destruction. However, Mallory et al. found no evidence of auxin-induced changes in the levels of mir160 or mir164 (Mallory et al., 2005), casting some doubt on this result.

Is auxin a morphogen?

A morphogen is generally thought of as a chemical whose concentration varies in space, and for which varying threshold concentrations direct qualitatively different cellular responses or fates. In many ways, auxin appears to be in a good position to dictate cell fates in the embryo in a concentration-dependent manner. Through regulated transport, it can accumulate in a spatially asymmetric concentration gradient. It acts as a transcriptional regulator. Varying concentrations of auxin could result in different degrees of IAA/AUX degradation, thus releasing variable amounts of ARF protein that could then activate downstream targets in an ARF concentration-dependent manner. [For a more complete discussion of auxin as a possible morphogen, see the reviews by Bhalerao and Bennett (Bhalerao and Bennett, 2003), and by Friml (Friml, 2003).] However, a direct correspondence between the cellular auxin concentration gradient and the development of discrete cell types or regions in the embryo has yet to be proved. We cannot rule out that auxin, although required for the execution of key developmental decisions, does not on its own specify distinct cell fates but rather provides a permissive environment in which other factors act as the sought after morphogens. In part, this is because it is technically very difficult to accurately measure auxin levels in situ and in real time. The development of in vivo auxin sensors will be important in helping to solve this problem, as will a better understanding of which target genes are directly regulated by auxin in the embryo. If the next five years see as many advances in this field as the past five years have, the debate about whether auxin acts as a morphogen will have been resolved, as will, no doubt, other, as-yet unimagined, questions.

P.D.J. was a DOE-Energy Biosciences Fellow of The Life Sciences Research Foundation.

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