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Auxin and cytokinin act during gynoecial patterning and the development of ovules from the meristematic medial domain

Authors:

Bhupinder Sehra and Robert G. Franks*

Authors declare no conflicts of interest

First author North Carolina State University, Interdepartmental Program in Genetics
Second author North Carolina State University, Department of Plant and Microbial Biology, rgfranks@ncsu.edu

Abstract

The gynoecium is the female reproductive structure of flowering plants, and is the site of ovule and seed development. The gynoecium is critical for reproductive competence and for agricultural productivity in many crop plants. In this review we focus on molecular aspects of the development of the *Arabidopsis thaliana* gynoecium. We briefly introduce gynoecium structure and development and then focus on important research advances published within the last year. We highlight what has been learned recently with respect to: 1) the role of auxin in the differential development of the medial and lateral domains of the Arabidopsis gynoecium; 2) the interaction between cytokinin and auxin during gynoecial development; 3) the role of auxin in the termination of the floral meristem and in the transition of floral meristem to gynoecium and 4) recent studies that suggest a degree of evolutionary conservation of auxin mechanisms during gynoecial development in other eudicots.

Introduction

Structure of the mature Arabidopsis gynoecium

The *Arabidopsis thaliana* gynoecium is made up of two fused carpels, generating a tube-like structure [1-6]. Along its apical to basal extent, the mature Arabidopsis gynoecium can be divided into four structurally and functionally different regions: the stigma, the style, the ovary and the gynophore (Figure 1A). The most apically located structure, the stigma, allows for pollen to be received and to germinate. The stigma and the style together comprise the upper-most portion of the transmitting tract. The transmitting tract is a specialized tissue that supports pollen tube growth and allows the pollen cell to reach the ovules located in the ovary. The gynophore, the most basally located portion of the gynoecium, attaches the gynoecium to the rest of the plant.

The ovary makes up the largest portion of the gynoecium. Within the ovary, about 50 ovules will form attached to a septum that separates the ovary into two locules (Figure 1B). Each ovule contains a female gametophyte, a multi-cellular haploid structure that includes both the egg cell and the central cell. After a double fertilization event, the egg cell will form into the embryo and the central cell into the endosperm. Thus the fertilized ovules mature into seeds containing embryo and the endosperm components.

Unlike the stigma, style and gynophore regions that are radially symmetric, the ovary is bilaterally symmetric to an imaginary plane that bisects the septum (Figure 1B). The ovary can be divided along this axis of symmetry into medial positions (those close to the axis of symmetry) and lateral positions (further from the axis)(Figure 1C). Within the mature gynoecium the structures located in the lateral positions are termed valves and make up the majority of the wall of the gynoecial tube. The ovules, septum and abaxial replum are located in medial positions. The septum contains the lower portions of the transmitting tract that are required for pollen tube growth and ovule fertilization. Late in development of the maturing fruit, cells of the valve margins, (where the valves meet the abaxial replum) differentiate into a dehiscence zone (Figure 1). Cells of the dehiscence zone undergo a set of specialized differentiation programs so that the valves can separate from the replum (dehisce) and allow seed dispersal [7].

Development of the Arabidopsis Gynoecium

The Arabidopsis gynoecium arises as a single, mound-shaped primordium in the center of the floral meristem, thus marking the beginning of stage 6 of floral development [6, 8]. Floral stages are according to Smyth et al. [8]. At this early stage of development a degree of positional identity has already been assigned that distinguishes the medial and lateral regions of the primordium [1]. Different positional identities are evidenced by the expression of several genes including *CRABSCLAW* (*CRC*) and *YABBY1* (*YAB1*) [9-11] in the lateral domains and *SHOOTMERISTEMLESS* (*STM*) and *SHATTERPROOF2* (*SHP2*) [1, 12, 13] in the medial domain (Figure 1E,F). These different positional domains will give rise to different functional structures. Although a careful clonal analysis of the developmental progression has not been published, based on patterns of cell division and gene expression, the lateral domains are thought to give rise to the valves while the medial domain gives rise to the ovules, the septum and the abaxial replum.

Early during floral stage 7, the mound shaped gynoecial primordium begins to morph into a tube-shaped structure that is still open at the apex [8]. Inside the tube, two meristematic ridges of tissue that span the apical-basal extent of the gynoecium form within the medial domain (Figure 1 C, D)[1]. These have been termed the medial ridges or alternatively the carpel margin meristems (CMMs). The meristematic nature of the medial ridge is indicated by the expression of *STM* [12] (Figure 1F), a marker of meristematic regions, as well as by the ability of the cells of the medial ridge to give rise to organ primordia (e.g. ovules). Ovule primordia arise from the medial ridge during late stage 8, as finger-like projections along the apical-basal extent of the ridge. These ovules continue to mature until stage 12 when the flower opens and the ovules are competent to be fertilized. Style and stigmatic tissues begin to differentiate in apical positions starting at late stage 9 [1, 6].

Evolutionary origin of the meristematic medial domain

The Arabidopsis gynoecium is typically represented as a composite structure derived from the congenital fusion of two component carpels (Figure 1B) [14]. In an evolutionary sense the two carpels are thought to be modified leaves [15, 16]. In this representation the margins of the two component carpels are seamlessly fused along the medial domain, and the medial domain is thought to represent the marginal regions of the component carpels. In this case the medial domain of the gynoecium would be evolutionarily derived from the leaf margin and the generation of meristematic fates in this position would likely reflect a developmental redeployment of the meristematic program along the organ margin.

An alternative possibility is that the ancestral Angiosperm carpel was ascidiate (cup-shaped) [17] and in Arabidopsis the meristematic medial ridge that sits between the two carpels would reflect tissue that is derived from a shoot or an axis. In this representation, the meristematic medial domain would arise directly from the terminating floral meristem or could arise in an axillary position relative to the two component carpels. In either of these cases, the meristematic identity of the medial domain would not reflect a re-deployment at the leaf margin, but would be more similar to an inflorescence branch meristems forming in the axil of a rosette leaf or a floral meristem forming in the axil of a subtending bract. Although the "modified leaf margin" representation is much favored in the current literature, the matter is not fully resolved. Future investigations of the molecular developmental mechanisms of female reproductive meristem development in basal angiosperms and gymnosperms may help to resolve this question.

Distinctive patterns of auxin transport and response differentiate lateral gynoecial domains from the meristematic medial domain

The auxin gradient model

A variety of experiments over the past several decades indicate that proper synthesis, transport and response to the plant hormone auxin are required within the developing gynoecium for proper development and female reproductive competence (See sidebar). See Larsson *et al.* and Dresselhaus and Schneitz for a recent reviews [18, 19]. One set of key experiments were those published by Nemhauser et al. [20] in which developing gynoecia were transiently treated with the auxin transport inhibitor 1-N-naphtylphthalamic acid (NPA)(See sidebar). An analysis of the resulting morphological defects demonstrated an important role for auxin transport in the patterning of the gynoecium and lead to a model that proposed that patterning along the apical/basal axis of the gynoecium required

the formation and action of an auxin gradient along this axis of the developing organ. In this model, high levels of auxin synthesis would be found at the apex of the gynoecium. Then through the action of basipetal (toward the base) transport of auxin, a gradient would form with high auxin concentrations at the apex and low concentrations at the base. The response of the cells to different threshold levels of auxin along this gradient would lead to the formation of gynophore, ovary, style and stigmatic tissues along the basal to apical extent of the gynoecium. Later efforts to visualize this auxin response gradient with the DR5-based auxin response reporters (see side bar) did reveal an auxin response maximum at the gynoecial apex, but no gradient of auxin response could be detected in more basal regions, thus calling into question tenets of this model [18, 21-24]

Early patterns of auxin response differ in medial and lateral gynoecial domains

In 2014, two papers were published that carefully examined the patterns of auxin transport, and auxin response in the developing gynoecia [21, 22]. Larsson et al. (2014) report the formation of two DR5 auxin response foci at the apex of the stage 5/6 gynoecium, one focus located within each lateral domain [21]. These DR5 foci likely represent the positions of the individual carpel primordia as they are forming. Two additional apical foci located in the medial domain can be detected, however these form later in development. These medial DR5 foci are weakly and inconsistently detected in stage 6 [21] and become established more strongly and consistently during stage 7 [21, 22]. Thus the formation of the medial DR5 foci is delayed by 24-48 hours relative to the lateral foci. During stage 8, the four apical foci of DR5 expression are transformed into a ring of apical expression that encompasses the entire apical tip of the gynoecium [18, 21-24].

***PINFORMED* (*PIN*) family of auxin transporters**

Based on the expression patterns and timing of expression of genes required for auxin synthesis (i.e. *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1* [*TAA1*]/ (*TRYPTOPHAN AMINOTRANSFERASE RELATED 2*)[*TAR2*] and *YUCCA*-family genes –See side box) relative to the timing of the appearance of the DR5 foci, it is unlikely that the apical DR5 response foci are formed entirely by local auxin synthesis [21, 22]. A variety of evidences suggest that the DR5 expression foci require directional transport of auxin. Both Larsson *et al.* and Moubayidin and Ostergaard report the expression patterns of PIN1, PIN3 and PIN7 auxin transporters during gynoecial development and highlight the functional importance of PIN1. At the earliest stage of gynoecium development, as the central portion of the floral meristem is transitioning into the gynoecial primordium (Stage 5 to 6), PIN1 protein is expressed in the epidermal cells and appears to transport auxin toward the apex and center of the developing gynoecial mound and toward the developing lateral DR5 foci [21]. The reduced expression of the DR5 lateral foci in the hypomorphic *pin1-5* allele [22] and the loss of valves observed in *pin1* mutant gynoecia [25] indicate that the PIN1 transporter is indeed important for the generation of the lateral DR5 foci, and subsequent valve growth.

As the gynoecial tube develops further, PIN1 transporters are expressed in the lateral domains in the L1 epidermal cell layer. Here the PIN1 transporter protein is polarly localized on the apical surface of the epidermal cells, orientated so as to transport auxin toward the apex of the gynoecium [21, 22, 26]. Within the medial domains, PIN 1 is not a strongly localized to just the apical surface of the cell, but rather is distributed evenly on all plasma membrane surfaces, suggesting a less polarized transport of auxin within the medial domain [21] . In addition, in the medial domain PIN1 is expressed throughout the internal cell layers (i.e. in the L2 and L3 mesophyll cell layers). This is in contrast to in

the lateral domains where PIN1 is expressed chiefly in the epidermis and in the developing vasculature. Thus PIN1 localization and DR5 reporter expression data suggest that the auxin transport and response in medial and lateral domains is different from an early developmental stage.

Taken together these data suggest that the flow of auxin in the epidermis of the lateral domains is acropetal (toward the tip of the gynoecium) [21, 22], then back down through the forming vasculature [21] similar to the reverse fountain model as proposed in the developing leaf [27] (Figure 2). In contrast, in the medial domains it appears that the net flow of auxin may be less strongly directed when compared to the flows in the lateral domains. Based on patterns of expression of auxin synthesis genes (e.g. *TAA1*/*TAR2*/*YUCCA*-family members), both Larsson *et al.* (2013) and Hawkins and Liu (2014) suggest that auxin synthesis in basal portions of the gynoecium or in the receptacle of the flower may be important for the proper formation of the gynoecium [18, 28]. Furthermore, Larsson *et al.* (2014) report a difference in the timing of the development of the vasculature in the medial and lateral domains using the *IAA2:GFP* reporter as a marker of early vasculature development; the lateral domain vasculature starts to develop by stage 5 while in the medial domain *IAA2:GFP* expression is not detected until stage 7-8 [21]. Thus, the patterns of auxin transport and the timing of vascular development differs between the medial and lateral gynoecial domains during early development.

Regulation of PIN1 subcellular polarity by PINOID kinase is required for proper medial domain development

PID (*PINOID*) encodes an AGC-3 type protein kinase that is required for apicial (polar) localization of the PIN transporter proteins within the apical/basal context of individual cells [29-31]. PID acts to phosphorylate serine residues in the PIN transporters, thus resulting in the apical (polar) localization of the PIN1 protein [32]. Mutation of two of the PIN1 serine residues (substitution of serine with alanine and thus preventing phosphorylation) results in a constitutive apolar localization of the PIN1 transporter [32] and in apical/basal patterning defects in the gynoecium that are similar to those observed in the hypomorphic *pin1-5* mutant or after NPA treatment [22]. Furthermore the serine-to-alanine substitutions in PIN1 condition the reduced expression of the DR5 reporter in the lateral apical foci.

Interestingly the DR5 medial foci and the DR5 apical ring are largely maintained in the *pin1-5* and *pid-8* mutants indicating that they are independent of polar PIN1 localization [22]. Moubayidin and Ostergaard propose that the medial foci are the result of the action of the transcription factors *SPATULA* (*SPT)* and *INDIHISCENT* (*IND)* that function to repress *PID* transcription in the medial domains [23, 26] thus resulting in the observed less polarized localization of PIN transporters in the medial domain.

Furthermore, the phosphorylated state of a serine residue can be mimicked by changing the serine residue to a glutamate residue, thus generating a constitutively pseudo-phosphorylated phospho-mimic [32]. When Moubayidin and Ostergaard expressed such a mutant PIN1 protein (*PIN1:GFP S1,2,3E*) from the PIN1 promoter, the phospho-mimic PIN1 protein was constitutively apically polarized, even in the medial domain, resulting in a split style phenotype similar to that observed in *spt-12* [22]. In the *PIN1:GFP S1,2,3E* expressing plants, the medial DR5 foci were not detected, while the lateral foci were detected as in wild type. Thus the medial DR5 foci do not require strongly apical

(polar) localization of PIN1, and in fact may require a greater degree of apolar PIN1 distribution. This greater degree of apolar PIN1 in the medial domains is important for DR5 foci in the medial domains and for the subsequent formation of the DR5 apical expression ring at stage 8 and the proper formation of style and stigmatic tissues. The Moubayidin and Ostergaard model further suggests that the medial DR5 foci promote the bilateral to radial transition at the apex of the gynoecium and the subsequent formation of the style, while the apical/basal gynoecial patterning defects are a result of a failure to form the lateral foci and the associated reduced growth of the lateral/valve domains.

Larsson et al. suggest that the drainage of auxin through the forming pre-vasculature may be required for carpel organ outgrowth in the lateral domains [21]. This is similar to what has been proposed to occur in developing leaves [33]. A disruption of auxin removal through the pre-vasculature (due to NPA treatment) would be expected to affect the lateral domains more severely than the medial domains, as the "reverse fountain" flow of auxin appears more predominant in the lateral domains. This may also contribute to the preferential growth of the medial domains over the lateral domain upon NPA treatment. This would provide a mechanistic explanation for the observation that the medial and lateral domains experience differential responses to the application of NPA [21].

Transient treatment with NPA results in ectopic expression of DR5 and expansion of medial domain markers

Larsson et al. tested the effects of a transient NPA treatment on development of the gynoecia and expression from the DR5 reporter [21]. In general, treatment of the floral buds with NPA resulted in a spreading or delocalization of the DR5 signal. More defined patterns of expression seen in the wild type such as lateral foci and pre-vascular strand specific expression patterns were broadened into more diffuse patterns that encompassed a greater proportion of the developing primordium. The ectopic DR5 response was often quite pervasive in the valve tissues suggesting that NPA treatment increased the level of auxin signaling generally throughout the valve. These gene expression changes were observed between 7 and 24 hours after treatment and preceded morphological changes.

NPA treatment also resulted in alterations of patterning along the medio-lateral extent of the gynoecium [21]. After NPA treatment, expression from medial-domain expressed reporters (i.e. *SHATTERPROOF2* [*SHP2*], *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1/BREVIPEDICELLUS* [*KNAT1/BP*], and *TAA1*) was expanded into lateral positions. These experiments suggest that blocking of polar auxin transport results in a partial reassignment of lateral fates into medial fates or in an expansion of the extent of the medial domain at the expense of the lateral domain.

SHOOT MERISTEMLESS (*STM*), like *KNAT1/BP*, encodes a Class I KNOX transcription factor and is expressed early within the developing medial domain of the gynoecium as well as within the shoot apical meristem [12]. *STM* has a key function in the formation, organization and maintenance of meristematic potential in the shoot apical meristem [12, 34-36]. *STM* maintains the pool of meristematic cells within the shoot apical meristem and more transiently at the center of the floral meristem, allowing carpel and placental development [37]. It would be interesting to look at the expression pattern of *STM* in response to NPA treatment. Based on the observed expansion of the medial domain markers (i.e. *SHP2*, *KNAT1/BP*, *TAA1*) upon NPA treatment, one might expect an expansion of *STM* expression upon NPA treatment, however to our knowledge this has not yet been investigated. Additionally, the recent report of a role for *STM* and *KNAT1/BP* during cell expansion

and differentiation of xylem in the cambium of Arabidopsis hypocotyls [38] suggests that, in addition to their role in maintenance of meristematic potential in the medial domain, *STM* or *KNAT1/BP* might function in the later differentiation of medial domain structures or vasculature.

PGP/ABCB-type auxin transporters are also important for proper auxin responses in the gynoecium

In addition to the PIN family transporters, the ATP-binding cassette subfamily B (ABCB)/MULTI-DRUG RESISTANT/ P-GLYCOPROTEIN (PGP) family proteins also function as auxin efflux transporters [39, 40]. These transporters are typically localized in an apolar fashion and are thought to alter polar auxin flux by regulating the intracellular auxin concentration that is available for polar transport by PIN proteins [41, 42]. *PGP1* and *PGP19* are expressed in the developing gynoecium and are differentially required in the medial and lateral domains. Larsson et al. reported that *pgp1/pgp19* double mutants displayed a reduced ability to generate the lateral DR5 apical foci while the medial DR5 foci were largely unaffected in this double mutant [21]. These results suggest a role for *PGP1* and *PGP19* in auxin transport in lateral domains. Larsson et al. suggest that when *PGP*-dependent functions are blocked by NPA treatment or in the *pgp1/19* double mutant, auxin becomes trapped in internal cell layers of the carpel and thus is not available for transport by PIN transporters in the epidermis. The broader expression domain of the PIN transporters in the medial domain (expression in epidermis as well as internal mesophyll layers) may in part explain why the medial domain is less sensitive to the application of NPA or the loss of *PGP1* and *PGP19* activity.

Interactions between cytokinin and auxin during gynoecial patterning, medial domain development and ovule initiation

Auxin and cytokinin regulate gynoecial patterning

The plant hormone cytokinin (see side bar) regulates the development of the shoot and root apical meristems [43, 44] and is required for proper development of key medial domain derived structures, including the ovules, as well as the valve margin (dehiscence zone)[45]. Reduced ovule formation is observed when cytokinin biosynthesis or perception is impaired [46-51]. Conversely, increasing cytokinin levels delay cell differentiation and enhance cell proliferation in the CMM-derived placental tissues and increase the number of ovules formed per gynoecium [52, 53]. The exogenous application of the cytokinin benzylaminopurine (BAP) as well as the analysis of transgenic cytokinin overexpression lines suggest that cytokinin plays at least three roles during gynoecial development: 1) an early developmental role in stimulating cell proliferation in the medial domain 2) a later role in the development of the valve margin [45], and a role in patterning along the apical-basal axis of the gynoecium [54].

Zuniga-Mayo et al. recently reported that the treatment of developing flowers with exogenous cytokinin, in addition to causing proliferation of medial domain structures, also alters apical-basal patterning within the gynoecium [54]. The observed apical-basal patterning defects were characterized by a reduction or loss of the valves and were similar to those caused by the blocking of auxin transport via NPA treatment. These results suggest interplay between auxin and cytokinin signaling pathways in the gynoecium. The similarity of the cytokinin and NPA treatment phenotypes suggests

that cytokinin reduces polar auxin transport in the gynoecium. Furthermore, the enhanced sensitivity of *auxin response factor19* (*arf19)* and *arf7* mutant gynoecia to the application of BAP suggests that these two *ARFS*, in addition to *ARF3*/*ETTIN* [3, 55], function in apical-basal patterning of the gynoecium [54].

A large degree of cross-talk between the cytokinin and auxin signaling pathways has been shown to influence the development of the shoot and root apical meristem [43, 44]. In peripheral portions of the SAM, auxin down regulates *STM* expression [56, 57] and reduces cytokinin biosynthesis [58]. Additionally auxin, acting through *MP/ARF5*, suppresses the expression of *ARABIDOPSIS RESPONSE REGULATOR7* (*ARR7)* and *ARR15,* two components of the cytokinin response pathway [59]. Given the meristematic nature of the gynoecial medial domain it seems likely that similar mechanisms of cross talk may shape medial domain development. Visualization of cytokinin and auxin responses in the gynoecium, using synthetic reporters (TCS-based and DR5-based, respectively), showed that auxin and cytokinin responses in the gynoecium tend to have complementary and mutually-exclusive expression patterns suggesting an antagonistic regulatory relationship between auxin and cytokinin [15, 45, 60].

The *NO TRANSMITTING TRACT* (*NTT*) zinc finger-containing transcription factor previously identified as a regulator of transmitting tract development [61] also plays a role in the development of the valve margin and the replum [62, 63]. *NTT* activates the expression of *KNAT/BP1* in the medial domain of the gynoecium, likely through the direct binding to the *KNAT1/BP* promoter sequences [63]. The KNAT1/BP and REPLUMLESS (RPL) transcriptional regulators form a protein complex that is important for replum development [64]. NTT physically interacts with itself as well as RPL, KNAT1/BP and STM and several other regulators of gynoecial development suggesting that it is a member of a multi component complex that regulates development within the medial domain [63]. *KNAT1/BP* and *STM* stimulate cytokinin synthesis in the SAM via the transcriptional upregulation of *AtIPT7* in the cytokinin biosynthesis pathway [65, 66]. Thus the stimulation of *KNAT1/BP* expression that is brought about by *NTT* action may support higher levels of cytokinin in the medial domain. This is consistent with the increase in the size of the replum that is observed upon exogenous cytokinin application as well as in response to *NTT* overexpression..

The splicing factor *CLUMSY VEIN (CUV)*, the Arabidopsis ortholog of the eukaryotic DEAH-box RNA-dependent ATPase Pre-mRNA-processing factor 16 (Prp16), affects the splicing and differential expression of key genes involved in auxin-mediated development [67]. *CUV* is required for efficient pre-mRNA splicing of several auxin biosynthesis genes including *TAA1, TAR2* and several *YUCCA* family members. The levels of properly spliced transcripts of a number of *PIN* genes, auxin receptor genes and auxin signaling genes were also reduced in *cuv* mutants. The expression and subcellular localization of the PIN1 protein was also altered in the *cuv* mutant roots. Consequently, *cuv* mutants exhibit a number of phenotypes that arise from altered auxin spatial distribution, as is corroborated by aberrant DR5-reporter expression. The *cuv* mutants exhibit embryonic defects, leaf vasculature defects, and ectopic vein formation in cotyledons, flowers and gynoecia. The apical-basal patterning of the gynoecium is also disrupted in *cuv* plants, with *cuv* mutants displaying shorter valves and longer gynophore and style domains.

Auxin and cytokinin regulate CMM development and ovule initiation from the meristematic medial domain

CUP-SHAPED COTYLEDON 1 (CUC1) and *CUC2* encode a pair of paralogous NAC-domain containing transcription factors that are expressed within adaxial portions of the gynoecial medial domain [68-71]. In order to study the functions of *CUC1* and *CUC2* during the development of the meristematic medial domain, Kamiuchi *et al*. examined gynoecium development in *cuc1 cuc2* double mutant plantlets that were regenerated from *cuc1 cuc2* calli [69]. The gynoecial phenotypes (i.e. reduced and misplaced CMMs) indicate a redundant role for *CUC1* and *CUC2* in the initiation and placement of the CMM within the medial domain. *STM* expression was greatly reduced in the *cuc1 cuc2* double mutant gynoecia, particularly in adaxial portions of the medial domain, indicating the *CUC1* and *CUC2* are required for the initiation of *STM* expression in the CMM. This is reminiscent of their role in the initiation of *STM* expression during the development of the embryonic shoot apical meristem [71, 72]. In addition to its role in shoot apical meristem development (see above), *STM* plays a role in the maintenance of undifferentiated cells in the floral meristem [34] as well as in the initiation of carpel development and in the formation or maintenance of the placental tissue of the medial domain [37]. Thus, much of the *cuc1 cuc2* double mutant gynoecial phenotype may be due to the failure to activate *STM* in the developing CMM [69]. The expansion of *FILAMENTOUS FLOWER (FIL)* expression into portions of the medial domain in the *cuc1 cuc2* double mutant suggests that *CUC1* and *CUC2* activities normally prevent valve differentiation in the medial domain, presumably through the activation of *STM* expression [69].

Kamiuchi *et al*. also generated microRNA-resistant versions of *CUC1* and *CUC2* that were expressed at higher levels and in expanded domains within the gynoecium relative to the expression of the wild type genes. Plants expressing these microRNA-resistant *CUC* constructs displayed supernumerary CMMs in adaxial portions of the medial domain and generated filamentous structures from ectopic CMM-like structures in abaxial portions of the medial domain [69]. Correspondingly, *STM* expression is expanded. The formation of filamentous structures from abaxial replum is similar to, albeit less severe than, the phenotype of BAP-treated gynoecia [45], again suggesting a role of cytokinin downstream of *STM* that is important for proliferation of cells of the CMM [45, 52].

In order to look at the function of *CUC1*, *CUC2* and *ANT* during ovule initiation Galbiati et al. generated an RNAi construct to reduce the activity of *CUC1* specifically in the developing ovules [73]. As *cuc1 cuc2* double mutants don't form a shoot apical meristem, this *CUC1_RNAi* construct allowed the ovule specific reduction of *CUC1* activity and thus enabled the analysis of *cuc1 cuc2* double and *cuc1 cuc2 ant* triple mutant ovules. The analysis of the *pPIN1:PIN1::GFP* reporter in a *cuc1_RNAi cuc2* double mutant revealed that *CUC1* and *CUC2* are redundantly required for the expression and proper localization of PIN1 in the developing ovules. In wild type plants *PIN1* is detected in epidermal cells and displays a polarized subcellular localization on the plasma membrane surface orientated toward the apex of the growing ovule primordium [27]. In the *cuc1_RNAi cuc2* double mutants, PIN1 protein was detected weakly and throughout the ovule primordium and failed to efficiently localize to the plasma membrane in an polar manner, instead being detected in vacuoles and diffusely on plasma membranes in an apolar fashion [73]. Previous work had demonstrated that exogenous application of cytokinin could stimulate the expression of PIN1 [74], and Galbiati *et al.* demonstrated that ovule loss and the reduction of PIN1 expression that was observed in the *cuc1_RNAi cuc2* double mutant could be partially rescued by the exogenous application of cytokinin. Interestingly the loss of ovule primordia in the *ant* mutant was not rescued by the application of BAP suggesting that *ANT* functions in a *CUC1/CUC2*-independent pathway.

The expression of *CUC1*, *CUC2* and *ANT* are stimulated by the MP/ARF5 auxin response factor through the direct interaction of MP/ARF5 with the cis-regulatory regions of these genes [73, 75]. Galbiati *et al.* recently presented a model that proposes that auxin via the MP/ARF5 protein stimulates *ANT* expression, and thus promotes cell proliferation in the placenta and ovules during early gynoecial development [73](Figure 3). In this model *MP/ARF5* also directly stimulates the expression of *CUC1* and *CUC2* that redundantly regulate *PIN1* expression through a non-cell autonomous action, allowing the formation of auxin maxima at the growing tip of the new ovule primordium. Cytokinin response, downstream of the *CUC* genes, is likely in part responsible for the *CUC1/CUC2* promotion of *PIN1* expression. At later stages of ovule development the auxin maxima at the apex of the developing ovule may repress *CUC* gene expression, as has been demonstrated in leaves [76].

Transition from floral meristem to gynoecial primordium

Auxin regulates the termination of the floral meristem and progression into the gynoecium

The earliest steps in the specification of the medial and lateral gynoecial domains, and the degree to which the termination of the floral meristem and the formation of the gynoecium is mechanistically coordinated, remain unresolved. A recent paper by Liu et al. revealed a role for auxin and the auxin response factor ARF3 in floral meristem termination [77]. They show that ARF3 functions as a repressor of *WUS* transcription and this action is in part dependent on the transcription factor *AGAMOUS (AG)*. Chromatin IP data indicate that ARF3 associates with the *WUS* cis-regulatory regions and this interaction is promoted by *AG* function. These experiments also argued for a rather complex role for *ARF3* in floral termination with ARF3 likely playing a direct role in *WUS* repression in the cells of the organizing center, while ARF3 may additionally function in a non-cell autonomous fashion outside the organizing center, exerting an indirect repression on *WUS*. The experiments of Liu *et al*. when taken together with those of Larsson et al., suggest that auxin plays a role in both the termination of the floral meristem [77] as well as the early patterning of the medial and lateral domains of the gynoecium [21] thus potentially linking these two processes mechanistically. However, the observation that NPA treatments have not been shown to regulate floral meristem determinacy is puzzling in this regard.

Another link between floral meristem termination and medial domain development is suggested by the work of Wynn et al. who recently demonstrated a role for the transcriptional regulator *PERIANTHIA* (*PAN*) in medial domain development and the subsequent formation of ovules [78]. *PAN* activates *AG* at the transcriptional level in the center of the floral meristem and thus promotes the proper termination of the floral meristem [79, 80]. *AG* functions as a repressor of *WUS* both directly and indirectly, as well as in concert with ARF3 as described above [77, 81-83]. Wynn *et al*. demonstrate that in *seu pan* double mutants the floral meristem termination defects are enhanced and *WUS* expression is temporally extended [78]. Thus they propose that PAN and SEU act as activators of *AG* in central portions of the flower. The enhanced indeterminacy in the *pan seu* double mutants is correlated with an enhanced loss of ovules suggesting a possible mechanistic link between these two developmental events. This apparent connection between floral meristem termination and ovule/medial domain formation was first noted by Zuniga-Mayo *et al.* in their study of *jaiba crc* double mutants [84, 85]. *JAIBA* and *CRC* are involved in the proliferation of the medial tissues and *jaiba*

crc double mutants display both abnormal floral meristem determination phenotypes, as well as reduced formation of ovule primordia. A large number of other genes have been shown to function during floral meristem termination in Arabidopsis [see Ito and Bo (2015) for a recent review [86]] perhaps providing additional opportunities to explore the proposed mechanistic link between floral meristem termination and CMM/ovule formation.

Conservation and divergence of gynoecial developmental mechanisms within eudicots

NGATHA (*NGA)* gene function is conserved in diverse eudicots indicating aspects of auxin regulation during gynoecial development are evolutionarily conserved

The four *Arabidopsis thaliana NGATHA* (*NGA*) genes are a part of the RAV clade within the B3 family of transcription factors [87, 88]. The Arabidopsis *NGA* genes act in a redundant manner to specify stigma and style development. The *nga1,2,3,4* quadruple mutants (hereafter *nga* quad. mutants) display a severe loss of style and stigmatic tissue as well as apical splitting of gynoecia. The strong apical ring of DR5-based auxin response that is normally detected at the gynoecial apex, and that precedes the morphological development of the stigmatic and stylar tissues, is also not detected in the *nga* quad. mutants [89]. The comparison of transcriptomic profiles of gynoecia from wild type, *nga* quad. mutant and *NGA3*-overexpression lines has identified nearly 2,500 putative targets of *NGA* regulation within the gynoecium many of which function in auxin synthesis, transport or signaling [89]. The expression of the auxin biosynthesis genes *YUCCA2, 4, 8,* and *TAA1* as well as *AMIDASE1* (*AMI1*) are reduced or absent in the apical gynoecial tissues in *nga1,2,3,4* quad. mutants [88-90]. Polar auxin transport may also be regulated by NGA genes through the repression of *PID* expression and the activation of the expression of *WAG2* [89].

Studies in the basal eudicot *Eschscholzia californica* and core eudicot *Nicotiana benthamiana* revealed that *NGA* function is highly conserved across eudicots [90]. Using degenerate primers, one *NGA* gene was identified in *E. californica* (*EcNGA*) while BLAST searches of available draft genomic sequences identified two *NGA* genes in *Nicotiana benthamiana*, termed *NtNGAa* and *NtNGAb.* Expression patterns of *EcNGA*, *NtNGAa* and *NtNGAb* are similar in floral tissues in both species and resembles the spatial-temporal expression of *AtNGA* genes, including expression in placenta, ovules and apical portions of the gynoecium. Knockdown of *NGA* expression using viral induced gene silencing (VIGS) (see side bar) in *E. californica* and *N. benthamiana* produced reduction of style and stigmatic tissue and incomplete closure of the gynoecial tube. The similarity of these phenotypes with those observed in the *A. thaliana nga* quad. mutants [87, 88] indicates a conserved function for *NGA* genes across these eudicot species. This functional conservation also correlates with high level of putative structural and sequence similarity across *NGA* orthologs investigated [90]. BLAST searches identified a putative *N. benthamiana YUCCA* gene with high sequence similarity to *A. thaliana YUC2* and *YUC6* sequences. The expression of this putative *N. benthamiana YUCCA* gene was significantly reduced in styles and stigmas after VIGS-induced reduction of the expression of *NtNGA*. These data suggest that the regulatory relationship between *NGA* and *YUC* genes characterized in *A. thaliana* gynoecia may well be conserved in *N. benthamiana.*

The *miR156-SBP* regulatory module may function differently during gynoecial development in Arabidopsis and tomato

In *A. thaliana* the *microRNA156* (*miR156*) regulates expression of several members of the *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPL* or *SBP* box) transcription factor family [91]. This *miR156-SBP* regulatory module functions during gynoecial patterning and female fertility in Arabidopsis [92]. Overexpression of the *miRNA156* in an *spb8* mutant results in reduced ovule number, deformed septa and an absence of transmitting tract tissue, as well as a shortened style. These data indicate that the targets of *miR156* act redundantly with *SPB8* (that is not targeted by *miR156*) to affect gynoecial development. These *spb8; miR156* overexpression "double mutants" also displayed a hypersensitivity to NPA and a reduced expression of the *YUCCA4::GUS* reporter. In addition, *SPB8* and targets of *miR156* from the *SBP* family were shown to interact genetically with *ettin/arf3, spt* and *crc*, suggesting that the *miR156-SBP* regulatory module functions during gynoecial patterning by regulating auxin homeostasis.

Silva et al. (2014) recently demonstrated that the *miR156-SBP* regulatory module also functions during gynoecial development in tomato [93]. However their results suggest that *miR156-SBP* module regulates tomato gynoecial development through pathways that differ somewhat from those in dry fruited species like Arabidopsis. In tomato the overexpression of *AtmiR156* led to floral indeterminacy, the development of extra carpels, as well as to the development of fruits that contained ectopic meristematic structures. The *AtmiR156* overexpression lines displayed an up-regulation of *GOBLET* (a *NO APICAL MERISTEM/CUC-like* gene) and *TOMATO KNOTTED-LIKE2* (a class I KNOX gene) perhaps providing a mechanistic explanation for the presence of ectopic carpels and ectopic meristematic structures. Thus, although the *miR156-SBP* regulatory module appears to be important for gynoecial and fruit development in both tomato and Arabidopsis, the downstream targets of the module may be different in these two rather different fruit types.

Auxin and auxin signaling

Auxin - A class of plant hormones originally defined by their ability to regulate growth via control of cell division and cell elongation. Recent reviews: [18, 94, 95]. Indole-3-acetic acid (IAA) is the most common plant auxin. The directional transport of auxins between the cells of developing organs can generate local auxin maxima or minima and thus support patterning events and patterns of organ growth. Two main types of auxin efflux transporters have been shown to participate in auxin-dependent patterning events: 1) *PINFORMED* (*PIN*) family members that are typically polarly-localized within a given cell and support directionally-biased or orientated flows of auxin within the organ primordium [96]; and 2) ABCB/PGP efflux transporters that are generally apolar localized within a cell, but may function in generating auxin patterns by regulating intracellular auxin concentration available to the PIN transporters [41, 42].

1-N-naphtylphthalamic acid (NPA) – A polar auxin transport inhibitor. NPA binds to ABCB/PGP transporters, but not to PIN transporters [39-41]. However NPA inhibits, either directly or indirectly, the ability of both ABCB/PGP and PIN type reporters to transport auxin.

PINOID – a kinase that phosphorylates PIN family transporters and regulates their subcellular localization [97, 98]

DR5 reporter– a synthetic auxin response reporter in which a known auxin responsive element is fused to a reporter gene to allow monitoring of auxin response *in vivo* [99]. Note that this is a reporter of the transcriptional response to auxin, not strictly a reporter of intracellular auxin levels.

TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1 (*TAA1*)/ *TAA-RELATED 2 (TAR2*) and *YUCCA*-family genes - Genes that encode key enzymes in the auxin biosynthesis pathway [100, 101].

AMIDASE1 (*AMI1*) - Enzyme that converts indole-3-acetamide (IAM) to IAA in an alternative pathway to the *TAA1*/*YUC*CA route [102]

Cytokinin synthesis and signaling

Cytokinins: A class of adenine-derived plant hormones primarily associated with cell growth and differentiation during plant growth and development. Recent reviews: [44, 103-105]. Cytokinin response and signaling involves a set of two-component histidine kinases. Cytokinins bind to membrane bound cytokinin receptors, *ARABIDOPSIS HISTIDINE KINASES* (*AHKs*), triggering a phosphor-relay ultimately causing the phosphorylation and activation of *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*). Phosphorylated type B *ARRs* activate transcription of cytokinin-responsive genes, including the type A *ARRs*. The majority of type A *ARRs* act as inhibitors of cytokinin signaling. Cytokinin biosynthesis is catalyzed by enzymes encoded by members of the *ATP/ADP ISOPENTENYLTRANSFERASE* (*IPT*) gene family.

TCS (Two-Component-Output-Sensor) reporter: A synthetic cytokinin-response reporter that allows *in vivo* measurement of levels of cytokinin response in a given tissue or cell type. The reporter is

driven by a promoter comprised of a concatenation of specific transcriptional elements that are responsive to cytokinin during immediate to early cytokinin response [106].

Benzylaminopurine (BAP) – A synthetic cytokinin analogue. The exogenous application of BAP allows the determination of the effects of increased levels of cytokinin on development.

Viral induced gene silencing (VIGS) – using a plant viral vector to deliver an interfering RNA (RNAi) construct that can target a specific gene within the host plant and reduce the ability of a specific RNA transcript to make a functional product.

Conclusion

The medial and lateral gynoecial domains develop via different mechanistic relationships

One conclusion that is becoming increasingly clear is that the developmental programs within the medial domain and the lateral domains of the Arabidopsis gynoecium proceed by different trajectories and are likely under the control of different molecular mechanisms. Larsson *et al.* and Moubayidin and Ostergaard clearly demonstrate that the formation of the DR5 expression foci in the medial and lateral domains are mechanistically different [21, 22]. Also the relationship between growth and auxin signaling appears substantially different in these two domains as well. Growth within the lateral domain appears to follow the existing paradigm for auxin flow and organ growth in lateral aerial organs: a reverse fountain flow pattern with an early apical maximum generates proximal to distal growth. What then is responsible for the growth of the medial domain if there are no early DR5 foci and vascular development is delayed in this domain? One possibility is that growth within the medial domain is coupled to lateral domain growth. Although some degree of coordination is perhaps likely, genetic evidence suggests that this is not absolute, as the growth of the medial and lateral domain can be genetically separated: *tousled ettin (arf3)* double mutants display a proliferation of medial tissues while lateral-derived valve tissues are greatly reduced [107]; and a somewhat complementary phenotype is observed in the *leunig ant* and *seuss ant* double mutants in which the valves form relatively normally in the lateral domains while the tissues from the medial domain are missing or are very reduced [108, 109].

Relationship of auxin to medio/lateral, apical/basal and abaxial/adaxial patterning

Another unanswered question relates to the relationship between auxin and cytokinin signaling, domain specific growth and the assignment of positional identities. Evidence from Larsson *et al*. suggests that early patterning events along the medio-lateral axis are regulated by auxin transport [21]. Early NPA treatment flattens the DR5 response foci and engenders an overall higher, but more diffuse, auxin response. This is correlated with the expansion of medial fates and a reduction in the extent of lateral fates. At least two possible mechanisms are consistent with this observation. It may be that the overall higher levels of auxin response favor the medial fates. Alternatively, medial fates are favored, not because auxin responses are higher across the primordium, but rather because the tight auxin response foci are not formed in the lateral domains when treated with NPA, and these tight foci are required for lateral domain outgrowth. In this second model, the growth of the medial domain would be favored as it is presumed not to require a well-canalized auxin flow to maintain growth. Larsson *et al.* also suggest that these early patterning defects might impact the subsequent

elaboration of the apical-basal gynoecial structures. This is similar to previously proposed ideas that linked early abaxial-adaxial patterning or medio-lateral patterning events to the later elaboration of the apical-basal patterning of the gynoecium [6, 18, 28]. Hawkins and Liu (2014) recently proposed a model for auxin function during gynoecial development that invokes a role for auxin in patterning along the adaxial-abaxial extent of the early gynoecial primordium. They propose that the juxtaposition of adaxial and abaxial identities within the developing carpels is necessary for the upward growth of the valve domains [28]. This is based on similar models of growth of the leaf lamina [110].

Cross talk between cytokinin and auxin signaling pathways in the gynoecium

In a manner similar to that observed in the shoot apical meristem, cytokinins appear to promote cell proliferation within and prevent the differentiation of the meristematic adaxial portions of the medial domain. This action of cytokinin contributes to the ability of the meristematic tissues to generate the medial domain-derived structures, particularly the ovules. Many observations suggest that there is significant crosstalk between the auxin and cytokinin signaling pathways during gynoecial apical/basal patterning, medial domain development and ovule initiation. The mechanisms of cross talk in the gynoecium may be similar to those observed in other structures, however a clear definition of the molecular mechanisms of auxin and cytokinin cross talk during gynoecial development awaits further investigations. Given the importance of the cytokinin-responsive *ARR5* and *ARR17* regulators in mediating of auxin and cytokinin cross talk in the SAM [59] it will be interesting to further explore the role of these ARR proteins in medial domain and gynoecial development.

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Figure captions

Figure 1. Structural components along the apical/basal and medial/lateral axes of the Arabidopsis gynoecium.

A) A scanning electron microscopic image of a mature Arabidopsis gynoecium. The stigma (stg), style (sty), carpel valve (cv), abaxial replum (abr), gynophore (gn), ovary (ovy) and valve margin/dehiscence zone (dz) are indicated. B and C) False-colored confocal gynoecial cross sections B) A stage 11 cross section. A plane of symmetry (indicated with a dashed line) divides the gynoecium into two component carpels. Ovules (ov)and septum (s) are indicated. C) A stage 8 cross section. Medial and lateral domains are indicated. The carpel margin meristem/medial ridge (mr) is false colored pink. D) A scanning electron microscopic image of a developing gynoecium from a stage 7 flower. Dashed line indicates plane of symmetry that bisects the medial domain. E and F) Stage 7 floral cross sections from *in situ* hybridization experiments. E) *CRC* expression (brown product) is detected in the two lateral domains. F) *STM* expression is detected in the medial domain. Panels A, B and C are adapted from Azhakanandam et al. [109] with permission. Panel D is adapted from Wynn et al. [111] with permission.

Figure 2. Diagrammatic representation of proposed patterns of auxin transport in the lateral and medial domains of a stage 7 gynoecium.

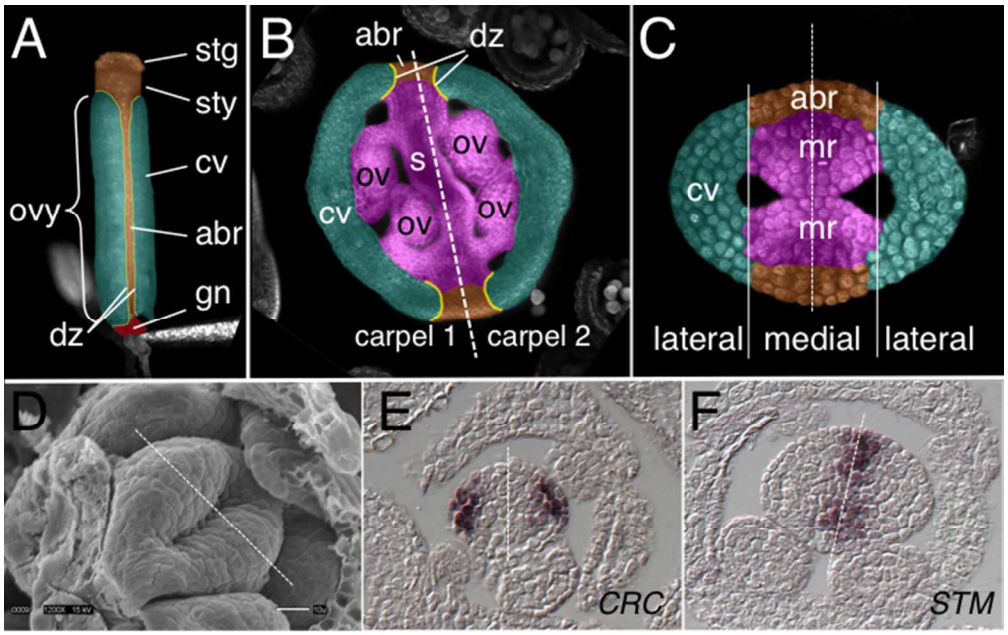
A) In the lateral domains auxin is transported predominantly via the PIN1 transporter and follows the "reverse fountain" model. Auxin flow in the abaxial and adaxial epidermal cell layers is toward the apex. Green circles indicate the lateral domain DR5 response foci. B) In the medial domain the flow of auxin in the abaxial epidermis is similarly toward the apex. However, in the adaxial epidermis and in subepidermal cells auxin transport is less polarized resulting in a less canalized (channelled) flow of auxin and in weaker and later-forming DR5 foci (smaller green dots). Note: the confocal longitudinal gynoecium images in A and B are for diagrammatic purposes and are both images of an oblique lateral plane section. C and D) A diagram of a cross-sectional view of a stage 7 gynoecium. The dotted line in A and B indicates the plane of section for which auxin flows are represented in panels A and B, respectively.

Figure 3 – A model of ovule formation from the meristematic medial domain as proposed by Galbiati et al.[73]

See text for details. Figure 3 is reproduced from Galbiati et al.[73] with permission.

Related Articles

Subtopic	Article title
e.g., Fertilization to Gastrulation	e.g., Maternal mRNA breakdown



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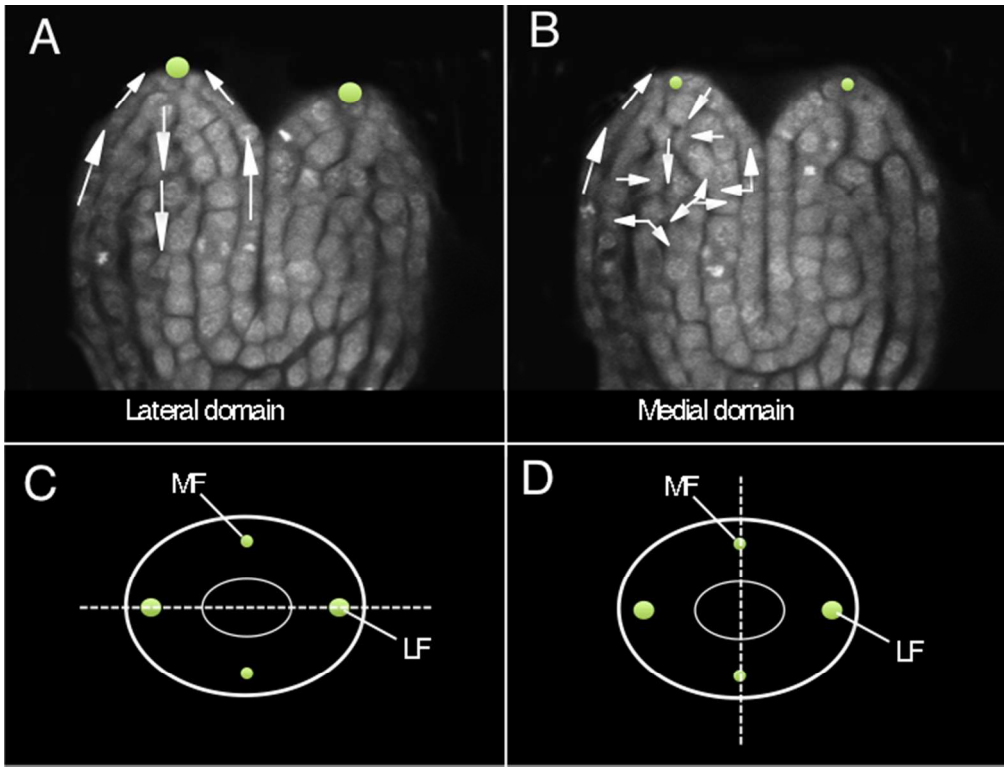

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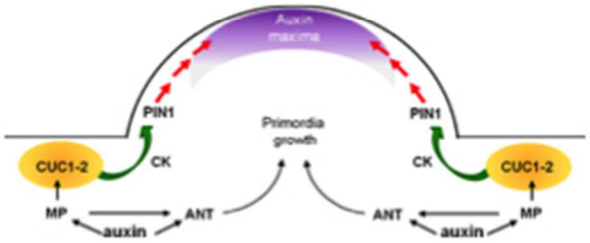

Figure 3 – A model of ovule formation from the meristematic medial domain as proposed by Galbiati et al. See text for details. Figure 3 is reproduced from Galbiati et al. with permission.

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