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A systems approach to understand shoot branching

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Shoots are ramifying systems with regular initiation of new growth axils called shoot branches. In seed plants, shoot buds initiate in leaf axils from axillary meristems (AMs) containing stem cells. The activities of AMs and buds play vital roles in plant architecture and crop yield. Whereas recent years have witnessed enormous progress the control of bud outgrowth, our knowledge of AM initiation remains rudimentary. Recently, systems biology approaches have been employed to study AM initiation, and have substantially expanded our understanding of the underlying gene regulatory network (GRN). Systems approaches uncovered transcriptional signatures, predicted cellular functions, and identified new regulators and regulatory relationships. Complementary molecular genetic studies support and extend findings from systems studies.

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1. Shoot branching is fundamental to plant architecture

1.1. Branching, plant body plan, and crop yield

Body plan evolution of plants has been distinct from animals since their divergence around 1 billion years ago. Branching is one of the inventions plants made [1]. Whereas branching has also been found in animals in rare cases [2], it is widely adopted by presumably all embryophytes (land plants), including liverworts, hornworts, mosses, pteridophytes (ferns), gymnosperms, and angiosperms [3]. Acquisition of branching is believed to enable plants' adaptation to the changing environment. Because plants maintain apical meristems to keep organogenesis capacity during postembryonic development, multiple apical meristems are favored for the survival of sessile plants. Multiple shoot apical meristems lead to shoot branching and new growth axils.

Different branching systems have been adopted by each group of embryophtes. Mosses, and likely liverworts and horneworts, use mainly terminal branching, in which one shoot meristem, often equally, separates into two [4]. Pteridophytes have most diversified branching systems, including terminal branching, adventive branching, and occasionally axillary branching [5]. Axillary branching, in which branches initiate laterally at some distance away from the shoot apical meristem (SAM), has become the dominant branching system in angiosperms and gymnosperms (Fig. 1A). Although it remains unclear why spermatophytes prefer axillary branching, the axillary position is likely an optimal location for branching. Axillary branches are associated with the phyllotaxis, i.e., the arrangement of leaves on a plant stem. The associated leaf may protect its axillary meristem (AM), which resides in the leaf axil. Molecular evidence suggests that the floral meristem is a type of specialized AM [6].

Shoot branching profoundly affects plant architecture, and is, not surprisingly, a key factor affecting crop yield. In cereal crops, especially grasses, shoot branches during the vegetative stage are termed tillers. The number of tillers determines the number of inflorescences. Each inflorescence further branches one or more times to increase its complexity and finally flower and seed numbers [7]. Thus shoot branching ability during reproductive stage is also critical to final crop yield. In fact, axillary bud activity has long been a target of breeding selection [8], because it significantly affects crop yield by affecting both tiller (and therefore inflorescence) number and inflorescence complexity.

Axillary branch formation can be divided into two steps, the initiation of an axillary bud, and the outgrowth of the bud into a branch [9]. Axillary bud outgrowth is under apical dormancy control, as the main stem shoot apex is dominant over axillary buds' growth. Extensive study has been carried out in recent years on axillary bud outgrowth, as summarized in an excellent recent review [10]. On the other hand, axillary bud formation, i.e., the initiation of an AM, is less studied. We will discuss in this review how systems approaches speed up our understanding of AM initiation.

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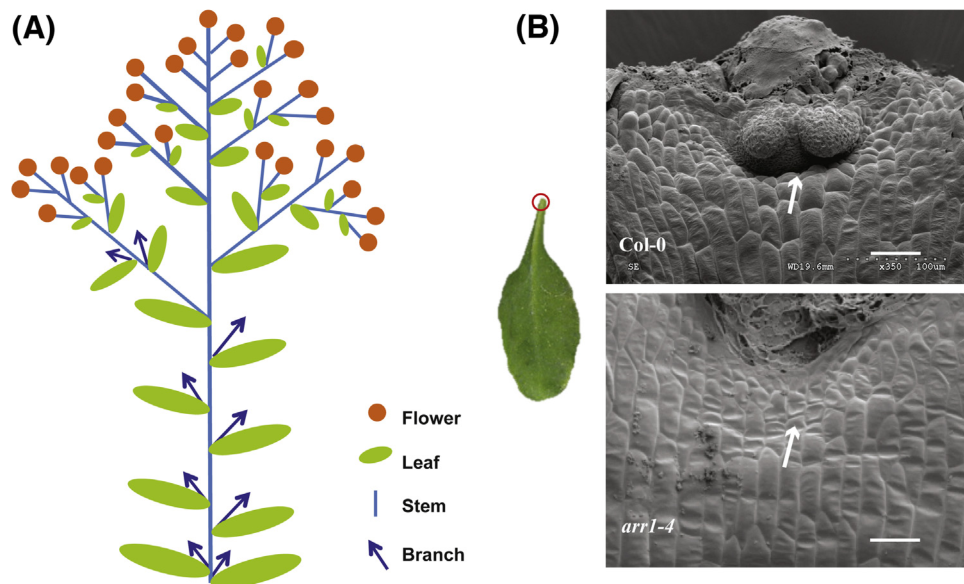

Fig. 1. Schematic representation (A) and morphology (B) of AM initiation. (A) Schematic representation of shoot and inflorescence branching patten in a typical seed plant, showing axillary branching at leaf axils. (B) Scanning electron micrograph of *Arabidopsis* leaf axil with (Col-0) or without (*arr1–4*) an axillary bud. Arrows indicate the position where AM initiates. Bars = 50 μM.

1.2. Genetic analysis of axillary meristem initiation

AM specification occurs during modular plant growth into phytomers, which consists of a leaf, an AM on the adaxial side of leaf base, and a stem segment. As its name suggests, AMs arise on the adaxial side (i.e., the upper side) of the subtending leaf base (Fig. 1B). AM initiation is closely associated with the adaxial leaf fate, because abnormal leaves with altered adaxial development has compromised AM initiation [11,12], and ectopic AMs arise on the abaxial side of fully adaxialized leaves [13]. Clonal analysis indicated that the AM and the subtending leaf share a common pool of ancestral cells [14,15].

Two models have been proposed to explain the origin of AMs. In the “detached meristem” model, a small number of stem cells detach from the SAM and associate with the leaf axil as the leaf differentiates from the SAM. In the “de novo induction” model, an AM initiates from leaf cells which have lost stem cell identity and have differentiated to be leaf cells. It remains to be tested which model better explains the AM initiation process [6].

Genetic studies have identified a small number of transcription factor encoding genes affecting AM initiation. *LATERAL SUPPRESSOR* (*Ls* or *LAS*) of tomato and *Arabidopsis*, and their homolog in rice *MONOCULM1* are the first identified AM initiation regulators [16–18]. *Arabidopsis las* mutants are specifically defective in AM initiation, and its orthologs in tomato and rice also affect flower development. *LAS* encodes a GRAS family transcription factor and its downstream targets are largely unknown. Another group of transcription factors specifically affecting AM initiation are the R2R3 MYB-family *Blind* gene of tomato and its homologs *REGULATORS OF AXILLARY MERISTEMS1–3* (*RAX1–3*) in *Arabidopsis* [19–21]. This *Blind*/*RAX* pathway affects vegetative AM initiation in *Arabidopsis* and vegetative and reproductive branching in tomato. It has also been found that a bHLH family transcription factor, LAX PANICLE1 (LAX1) in rice, barren stalk1 in maize, and REGULATOR OF AXILLARY MERISTEM FORMATION (ROX) in *Arabidopsis*, affects AM initiation [22–25]. Whereas severe vegetative and reproductive branching defects are found in rice and maize, the *Arabidopsis* ortholog only has marginal effect on vegetative stage branching. The *CUP-SHAPED COTYLEDON* (*CUC*) genes encode NAC domain transcription factors [26]. In addition to their roles in embryonic shoot meristem formation, phyllotaxis patterning, and lateral organ boundary specification, *CUC2* and *CUC3*, as well as *CUC1*, play partially redundant roles in AM initiation in *Arabidopsis* [27,28]. All these above mentioned transcription factor-encoding genes are specifically expressed in the axils of leaves from which new AMs initiate. On the other hand, more broadly expressed HD-ZIP III transcription factor *REVOLUTA* (*REV*) also affects AM initiation [29]. Consistent with its broad expression in leaves, stems and roots [30], development of all these tissues is compromised in *rev* mutants. The *PINHEAD/ZWILLE/AGONAUTE10* (*PNH*) gene also has pleiotropic effects on *Arabidopsis* development, including AM initiation [12]. Recent studies have shown that *PNH* encodes a member of the AGO proteins, which are involved in small RNA biogenesis, and that PNH specifically binds to and sequesters micro RNAs miR166/165, which promote *REV* and related HD-ZIP III transcripts degradation [31]. It is thus conceivable that reduced *REV* level in *pnh* is likely responsible for its AM initiation defects.

Identification of additional regulators of AM initiation by forward genetic approaches has been difficult, because strong apical dormancy in the model plant *Arabidopsis* significantly inhibits axillary bud outgrowth. In addition, using molecular genetic approaches to resolve the gene regulatory network (GRN) underlying AM initiation is labor-intensive and time consuming. Systems biology approaches allow high-throughput integrated and comprehensive research to resolve interactions of multiple components of biological systems, such as GRNs. We have recently combined two genome-wide study approaches to tackle the GRN underlying AM initiation and associated organ boundary formation. A first glimpse of the GRN not only identified new directions for further study, but has also successfully connected known regulators and identified novel regulators for AM initiation.

2. Gene expression pattern specified in organ boundary

2.1. Cell type-specific transcriptomes to identify expression patterns

With the rapid development of microarray and, more recently, next generation sequencing technologies, transcriptome analysis has been widely adopted in biological research. For the study of

Table 1
Technologies for cell type specific transcriptome analysis.

	FACS	LM	INTACT	TRAP
Cell type determination	Marker gene expression	Morphology and location	Marker gene expression	Marker gene expression
Specialized equipment required	Flow cytometer	Laser capture microdissection apparatus	No	No
Isolation procedure	Complicated	Complicated	Simple	Simple
Yield	Low/medium	Low	High	High

multicellular organisms, especially their development, cell type-specific transcriptome analysis has obvious advantages. AMs and their progenitor cells reside in the leaf axils and contribute to a very small proportion of leaves or whole shoots. Therefore, cell type-specific analysis of gene expression is highly valuable for the study of AM initiation.

There are at least four approaches for cellular level transcriptome analysis in plants (Table 1). Laser microdissection (LM) uses laser to isolate cells from fixed tissue sections according to cell morphology and position. Because LM does not require transgenic lines, it can be easily adopted to many cell types with high flexibility. For e.g., a rice transcriptome atlas project has used LM to isolate 40 cell types from rice shoot, root and germinating seeds [32]. Another approach to accomplish cell type transcriptome is fluorescence activated cell sorting (FACS), in which a fluorescent protein, such as green fluorescent protein (GFP), is expressed in specific cells. Protoplasts with fluorescent signals are then isolated by cytometry after wall digestion. High-resolution spatiotemporal maps of different cell types organized around the radial axis of *Arabidopsis* root were resolved and revealed dominant expression patterns [33,34]. Another methods, INTACT (isolation of nuclei tagged in specific cell types), which was developed by Deal and Henikoff, employs affinity-based nuclei isolation from specific individual cells [35,36]. To this end, total affinity-labeled nuclei can be isolated from transgenic plants with expression of a biotinylated nuclear envelope protein in target cell types. The isolated nuclei can be subsequently used for both gene expression and chromatin features profiling. The forth approach, Translating Ribosome Affinity Purification (TRAP), use a biochemical approach to isolate polysomes, which contain mRNAs and ribosomes, from labeled cells. TRAP introduces a tagged ribosomal protein under cell type-specific promoters to label cell types of interest and enable downstream immunopurification of polysomes from target cell types. TRAP has been applied in mammals [37], and also in plants [38,39]. It can efficiently obtain translating mRNAs with high yield, making it highly suitable to be combined with RNA-seq [39].

2.2. Organ boundary cell specific genes identified by cell-type specific transcriptomes

AMs initiate in leaf axils, from the boundary between SAM and leaf primordia. The boundary region is a narrow band with a small number of cells (Fig. 2). We have employed TRAP in combination with RNA-seq to acquire boundary- and leaf cell-specific gene expression at the genome scale [40]. Comparisons between the two cell types identified 466 genes as boundary-enriched and 868 as leaf-enriched (or boundary-depleted). Genes within several Gene Ontology molecular function groups, including cell cycle, epigenetic regulation, transcription, meristem functions, and phytohormone regulation, are significantly enriched in boundary cells (Fig. 2). These newly identified biochemical and physiological roles lead to new directions to understand AM initiation and boundary formation. Many of these observations from a genome-scale study are also in line with previous observations and the developmental context.

Whereas both meristem cells and early primordium cells maintain relatively active cell proliferation patterns, boundary cells are expected to maintain relatively low cell division rate [41]. In fact, a semi-vivo analysis of flower development has shown that boundary cells within a floral bud have very low cell proliferation, and are maintained in a quiescent state [42]. More recent live imaging analysis also shows that boundary cells exhibit a very low growth rate [43]. Boundary cells maintain meristematic activity, which is likely pivotal for AM initiation from leaf axils. As mentioned earlier, almost all AM initiation regulatory genes encode transcription factors, implying that a GRN is critical for the AM initiation process. In

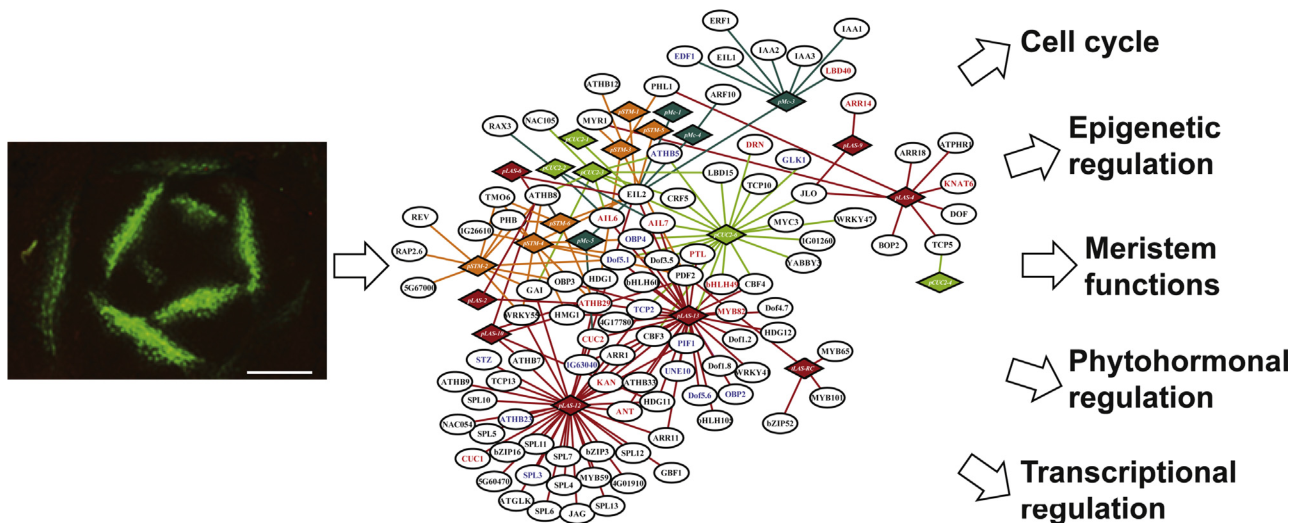

Fig. 2. An organ boundary-enriched gene regulatory network. Left panel, a transverse section of the leaf axil region highlighted by the expression pattern of *pLAS≫GFP-ER* (green). Scale bar = 50 μm. Middle panel, PDI (protein–DNA interaction) network modified from [40]. Circle, TF. Diamond, promoter fragment. Edge, PDI. Boundary-enriched TFs are shown in red, and boundary-depleted TFs are shown in blue. Diamonds of the same color represent promoter fragments of the same gene. Right panel, selected gene categories enriched in boundary specific transcriptomes reported in [40].

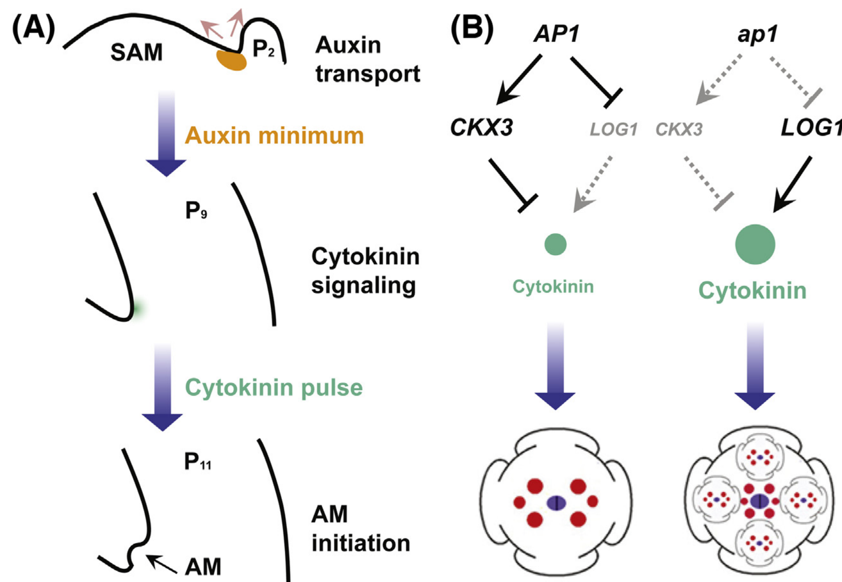

Fig. 3. Conceptual summary of hormonal regulation of AM initiation in leaf axils (A) and floral meristem formation in sepal axils (B). (A) During vegetative development, an auxin minimum (orange) is observed in the leaf axil, which depends on PIN1-mediated transport, and then a cytokinin signal pulse (cyan) appears prior to axillary buds formation in the second-youngest primordium (P_2). (B) During flower development, *AP1* suppresses sepal axil stem cell activity through direct activation of *CKX*3 and inhibition of *LOG1*. Elevated cytokinin levels can induce secondary flower formation, as seen in caulifower and brocoli. The models at the bottom show a flower structure, in which red circles represent stamens and the two purple semi-circles in the center represent carpels. Note secondary flowers form in sepal axils in *ap1* flowers.

some types of boundaries without bud formation, such as boundaries within a flower, meristematic activity is gradually lost and boundaries can differentiate in abscission zones [41]. In leaf axils, cell proliferation is later evoked to become new meristem cells [44]. The cell cycle machinery is likely reprogramed during the AM initiation process. Epigenetic regulation, including histone modification and DNA methylation, are widely involved in cell fate determination in both animals and plants [45]. It has been found that the expression of shoot meristem marker gene *SHOOTMERISTMLESS* (*STM*) together with other *KNOX1* genes is suppressed by histone H3K27-methylation through POLYCOMB REPRESSIVE COMPLEX1 (PRC1) and PRC2 complex in differentiated cells [46]. It is conceivable that more epigenetic regulation exists in AM initiation.

3. Hormone regulation

3.1. Genome-wide evidence for hormonal regulation of AM initiation

Phytohormones play central roles in plant development, including lateral organ formation and meristem functions. Auxin and cytokinin are two groups of classical phytohormones with profound effects on plant development [47]. Leaf and floral organogenesis from the SAM is triggered by the auxin efflux carrier PIN1-mediated accumulation of auxin [48,49]. Polar auxin transport and signaling is also involved in the early lateral root initiation process [50–53]. On the other hand, cytokinins inhibit lateral root initiation [54–56], and constrain lateral root development [57,58]. Both auxin and cytokinin are involved in meristem function and maintenance. Two distinct coordinated interaction networks between auxin and cytokinin in the SAM and the root meristem lead to maintenance and organogenesis of these two types of stem cells [59,60].

Genome-wide cell type-specific analysis has provided new evidence for and new insights into phytohormone regulation of plant development. Earlier cell type-specific transcriptome analysis has found hormone-action centers in *Arabidopsis* root and flower, and rice shoot and root [32–34,39]. By comparing boundary cell-enriched genes and leaf-enriched genes, we also found distinct enrichment or depletion of phytohormone responsive genes, including those associated with abscisic acid, auxin, brassinosteroid, cytokinin, ethylene, gibberellins, and jasmonic acid [40]. This finding implies positive or negative effects of these five phytohormones on boundary formation and AM initiation. Whereas auxin responsive genes are enriched in leaf cells but depleted in boundary cells, cytokinin responsive genes are enriched in boundary cells. Detailed analysis of auxin and cytokinin has elucidated molecular mechanisms underlying their regulation of AM initiation.

3.2. Low auxin by auxin polar transport in leaf axil before AM initiation

Consistent with genome-scale evidence that auxin signaling is depleted from the leaf boundary, recent molecular genetic analysis by us and others indeed demonstrate that auxin is depleted from the leaf boundary, which is critical for normal AM initiation [44,61]. By imaging the distribution of the recently developed auxin sensor DII-Venus [62], it has been shown that auxin minimum exists in the leaf axil. Furthermore, this minimum establishment relies on both auxin efflux and influx, as mutations of auxin efflux carrier PIN1, its regulator PINOID (PID), or influx carrier AUXIN RESISTANT1 (AUX1) and related LIKE-AUX1 (LAX1) and LAX2 all resulted in a reduction of the auxin minimum. On the other hand, inhibition of auxin efflux by *N*-1-naphthylphthalamic acid (NPA) treatment led to the activation of auxin-responsive DR5 promoter in tomato. Consistently, PIN1 localization in the epidermis around the leaf boundary indicates that auxin transport toward both sides of the leaf axil (Fig. 3A).

Further experiments show that this leaf axil auxin minmum is required for AM initiation. The above-mentioned *pin1*, *pid*, and *aux1 lax1 lax2* mutants all have compromised AM initiation. More direct evidence comes from the targeted-expression of the *Agrobacterium tumefaciens* auxin indole-3-acetic acid biosynthesis enzyme iaaM in the leaf boundary region in *Arabidopsis* and local application of auxin in tomato, both of which perturb AM initiation. At the molec-

ular level, the meristem marker *STM* expression is diminished from the boundary region in these plants with elevated auxin levels in the boundary region.

Auxin transport also affects AM initiation in an indirect way through leaf polarity establishment. As mentioned earlier, AMs can only initiate from the adaxial (upper) side of the leaf axil but not the abaxial (lower) side. A recent study shows that auxin transport, mediated by PIN1, between the SAM and emerging leaf primordia leads to reduced auxin concentration in the adaxial domain, which further promotes adaxial development [63]. Interfering with auxin transport (by NPA treatment or in *pin1* or *pid* mutants) leads to compromised adaxial development, which further leads to AM initiation defects. These results provide molecular level explanations to the classical Sussex experiment on leaf polarity.

3.3. Cytokinin pulse during AM initiation process

In contrast to auxin, a cytokinin signaling pulse has been found prior to vegetative AM and reproductive floral meristem initiation [44,64]. During vegetative AM initiation, the cytokinin signaling pulse appears later than the auxin minimum, and depends on the auxin minimum. Consistently, cytokinin biosynthesis genes and receptor genes are highly expressed in the leaf axil [40,44]. Like the auxin minimum, the leaf axil cytokinin signaling pulse is also critical for AM initiation. It has been shown that AM initiation is compromised in high-order cytokinin biosynthesis isopentenyltransferase (*IPT*) gene mutants, cytokinin receptor mutants, and B-ARR (transcription factors downstream of cytokinin signaling) mutants [44,65] (Fig. 1B).

Cytokinin is not only a promoter of vegetative AM initiation, but also a positive regulator of reproductive AM initiation, i.e., floral meristem initiation. Because of the complexity and redundancy of the cytokinin signaling pathway, as well as cytokinin regulation of SAM maintenance, it is difficult to dissect in wild-type inflorescence. On the other hand, it has been found that ectopic cytokinin treatment can lead to axillary floral meristem initiation in the otherwise empty sepal axil [66], which is also found in the classical *apetala1* (*ap1*) mutants [67]. Our recent study shows that *AP1*, which encodes a transcription factor, represses the expression of the cytokinin biosynthetic gene *LONELY GUY1* (*LOG1*), and activates the cytokinin degradation gene *CYTOKININ OXIDASE/DEHYDROGENASE3* (*CKX3*), both through direct binding [64] (Fig. 3B). In the *ap1* mutants, cytokinin levels and signaling are ectopically activated in floral buds, which then promote sepal axil floral buds formation. Recursive axillary floral meristem initiation converts a simple flower with determinate developmental potential into a ramifying inflorescence, as seen in cauliflower and broccoli [7,68].

4. Organ boundary-enriched gene regulatory network

4.1. Genetic regulatory relationship involved in AM initiation

GRNs, which are networks of regulatory transcription factors that bind to specific genomic regions, are broadly involved in development and other biological processes. Distinct spatiotemporal expression of transcription factors and spatiotemporal restriction of their regulation result in diversified cell function, tissue specificity, and different developmental stages. Genetic studies have been widely used to understand regulatory hierarchies, but usually cannot distinguish between direct and indirect interactions. Genetic analysis of the gene regulating AM initiation shows that *LAS* acts downstream of *CUC1*/*CUC2*, but not *CUC3* to activate *STM* expression and initiate stem cells [28]. On the other hand, *ROX* modulates AM initiation in concert with *RAX* and *LAS* [25]. The underlying molecular mechanisms of these regulations remain to be identified.

4.2. A systems approach to dissect transcriptional networks

Recent advances in genome-scale technologies are significantly speeding up our understanding of GRNs. Among others, yeast one-hybrid (Y1H) and chromatin immunoprecipitation together with sequencing (ChIP-seq) are two comprehensive approaches to identify protein–DNA interactions (PDIs) to resolve GRNs. Whereas ChIP-seq identifies genomic regions bound by a transcription factor, and can be considered as transcription factor-centered, Y1H defines the catalog of transcription factors bound to a DNA fragment of interest, and can be considered as gene-centered. Due to the low abundance of AM cells, regular ChIP is not suitable to analyze AM initiation, although ChIP using nuclei obtained by INTACT certainly provides an attractive alternative [35,36,69]. Combining Y1H and cell-type transcriptomes, we reconstructed a prototype GRN for AM initiation (Fig. 2). From the GRN, 180 PDIs between 103 TFs and 23 genomic regulatory regions were identified, in which expression phenotypes at the molecular level were identified for 73.3% PDIs tested. Combined with transcriptome data, we found that 13.6% of PDI-associated TFs were enriched in boundary and another 13.6% were boundary region-depleted, suggesting regenerative and degenerative regulation. We found morphological phenotypes for 31.8% of TFs tested which is much lower than expression phenotypes, and is likely due to genetic redundancy. Notably, this phenotype frequency is similar to those found in root by comparable approaches [70].

This GRN is able to link many known AM initiation regulators together. From this GRN, we now know that RAX1 directly activates *CUC2* expression, and CUC2 sequentially activates *LAS* expression. These PDIs explain and extend previous genetic results by providing direct molecular mechanisms [28]. Furthermore it was shown that REV binds to the regulatory regions of *STM*. The GRN also identifies new factors involved in this developmental process, such as *DORNR öSCHEN* (*DRN*) and *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE* (*SPLs*). We found AM initiation defects in the *drn-1* mutant and excessive AM initiation in the *spl9–4 spl15–1* mutant.

On a genome-wide level, these identified transcription factors involved in one or more PDIs are functionally enriched with meristem initiation, leaf development, and polarity specification, as shown by Gene Ontology analysis. Several transcription factor families, including HB, HD-ZIP, DOF and NAC families are enriched in PDI-associated transcription factors, and also in some boundary specific genes. These new factors provide new clues for further functional characterization of AM initiation and boundary formation.

4.3. Regulatory genomic region hubs

Biological networks are characterized by a scale-free connectivity distribution containing hubs with many connections and a large number of nodes with one or only a few connections. Transcription factor interactor hubs, where each hub is composed of several interacting transcription factor proteins, connect many genes expressed in different cell types, indicating the global role of the hub transcription factors [71–73]. However, no regulatory genomic region hubs, which are promoter regions bound by a large number of transcription factors, have been reported in previous studies. In the GRN underlying AM initiation, we identified a few regulatory genomic region hubs [40]. The existence of such regulatory genomic region hubs is also supported by a recent co-expression network analysis, which identified expression modules centered on common *cis*-motifs [74]. Our analysis of regulatory relationships within the GRN showed that both activation and repression functions by act-

ing on the identified genomic region hubs, implying competitive PDIs occurring on these regions. Although the number of genomic region hubs we identified is too small for statistical testing, it is speculated that such hub regions contain multiple *cis*-elements to facilitate binding by competing or promoting transcription factors, whose binding may be further modulated by and/or leads to epigenetic regulation. We expect further investigation of these putative genomic region hubs would uncover regulatory mechanisms to extend our understanding of AM initiation.

5. Perspective

The systems approaches emerging in biology promise to explain properties of biological systems based on genome-wide measurements of expression, interaction, regulation and metabolism [75,76]. To facilitate a systems approach, it is essential to first capture such components in a global manner and ideally at cellular resolution. Recent systems level studies has clearly accelerated our understanding of AM initiation, a fundamental developmental process with great implication in plant architecture and crop yield, yet a difficult target to study by reductionistic approaches. The first glimpse of the GRN underlying AM initiation and boundary formation has been fruitful by identifying new regulatory principles, regulators, and regulatory hierarchies. Still, our understanding of the gene regulatory network is far from comprehensive. The current GRN is gene centered, i.e., focusing on regulators of known key regulators. We still know little about genes downstream of these key regulators. Because AMs initiate from boundary cells that are very low in abundance, we would expect cell type-specific transcriptome analysis to enable the dissection of transcription factor-centered GRN. It is expected that epigenetic regulation plays important roles in meristems. Further exploration of epigenetic regulation at the genome-wide scale will add an additional layer of understanding of the GRN.

Systems approach can obtain a bird's-eyeview of GRNs and foster new hypotheses, and more focused studies will test these hypothesis to bring new understanding of AM initiation. We expect that systems approaches and reductionistic approaches will be combined to fully understand the GRN underlying AM initiation, which will provides a solid foundation for 'breeding by design' in crop improvement.

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