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Invited Expert Review

Novel Insights from Live-imaging in Shoot Meristem Development

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Abstract

Microscopic imaging of fluorescent reporters for key meristem regulators in live tissues is emerging as a powerful technique, enabling researchers to observe dynamic spatial and temporal distribution of hormonal and developmental regulators in living cells. Aided by time-lapse microphotography, new types of imaging acquisition and analysis software, and computational modeling, we are gaining significant insights into shoot apical meristem (SAM) behavior and function. This review is focused on summarizing recent advances in the understanding of SAM organization, development, and behavior derived from live-imaging techniques. This includes the revelation of mechanical forces in microtubule-controlled anisotropic growth, the role of the *CLV-WUS* network in the specification of peripheral zone and central zone cells, the multiple feedback loops involving cytokinin in controlling *WUS* expression, auxin dynamics in determining the position of new primordia, and, finally, sequence of regulatory events leading to *de novo* assembly of shoots from callus in culture. Future studies toward formulating "digital SAM" that incorporates multi-dimensional data ranging from images of SAM morphogenesis to a genome-scale expression map of SAM will greatly enhance our ability to understand, predict, and manipulate SAM, containing the stem cells that give rise to all above ground parts of a plant.

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Introduction

Higher plants continuously produce new lateral organs throughout their lives owing to the stem cell activities at their growing tips. A long-standing question for plant developmental biologists has been how the spatial organizations of the shoot apical meristem (SAM) are maintained to ensure a constant number of pluripotent stem cells. Classical and anatomical studies showed that the SAM is a multilayered structure (**Figure 1A**) divided into the central zone (CZ), the periphery zone cells (PZ), and the rib zone (RZ) (Fletcher and Meyerowitz 2000; Clark 2001; Sablowski 2007). Superimposed upon this zonal organization are the three clonal layers (**Figure 1A**), the epidermal L1 layer, the subepidermal L2 layer, and the inner most L3 layer. While cells in L1 and L2 layers divide anticlinally with its division plane at right angle to the surface of the SAM, cells in the L3 layer divide in periclinal (parallel to the SAM surface) as well as anticlinal orientations (Steeves 1989).

However, underlying this stable organization of SAM are dynamic interactions among gene networks and hormonal signaling, which are being revealed owing to recent advances in live-imaging. This review is aimed at informing readers these recent findings, which have contributed significantly to our in-depth understanding of SAM organization, function, and

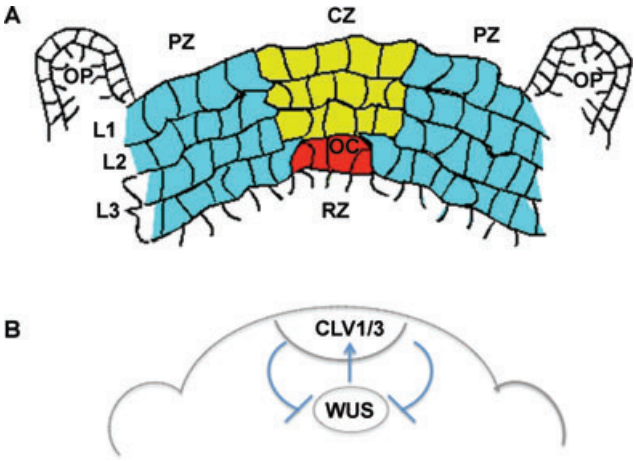

Figure 1. A diagram of the shoot apical meristem. L1, L2, and L3 cell layers are indicated.

The central zone (CZ) is marked yellow, the periphery zone (PZ) is in pale blue, and the rib zone (RZ) is below the CZ. The organizing center (OC), where *WUS* is expressed, is marked in red. Organ primordia (OP) arise from the PZ. (B) Feedback loop between *CLV* and *WUS* in SAM.

the underlying gene regulatory circuitries. For techniques and protocols of live-imaging, one should refer to several recent reviews (de Reuille et al. 2005; Reddy et al. 2007; Reddy 2008; Reddy and Roy-Chowdhury 2009).

The WUSCHEL (WUS)-CLAVATA (CLV) Feedback Circuitry Maintains SAM Size

The *WUSCHEL (WUS)-CLAVATA (CLV)* feedback circuitry was previously identified as the main genetic mechanism that maintains stem cell homeostasis in the SAM (Mayer et al. 1998; Schoof et al. 2000). While mutations in *WUS* result in premature termination of the meristems, overexpression of *WUS* is sufficient to convert cells of organ primordia back into pluripotent stem cells. Molecular studies revealed that WUS, a homeodomain protein, is expressed in only a few cells comprising the organizing center (OC) just above the RZ in SAM (**Figure 1A**). This OC-specific expression of *WUS* is controlled by members of the *CLAVATA (CLV)* family, which includes *CLV1*, *CLV2* and *CLV3* (Schoof et al. 2000). On the other hand, WUS activates, non-autonomously, *CLV3* expression in the stem cells of the CZ (Mayer et al. 1998), which then secret CLV3, a peptide ligand for the CLV1/CLV2 LRR-RLK receptors (Ogawa et al. 2008). The CLV1/CLV2 receptors, once activated by CLV3, negatively regulate *WUS*. This negative feedback loop between *WUS* and *CLV* ensures a constant number of stem cells in the SAM (**Figure 1B**).

SHOOTMERISTEMLESS (*STM*), a KNOX type homeobox protein, maintains SAM (Long et al. 1996) through a largely separate pathway from *WUS*/*CLV* (Endrizzi et al. 1996; Clark et al. 1996). STM prevents cells in the SAM from entering differentiation, allowing stem cell daughters to be amplified (Endrizzi et al. 1996). *STM* mRNA is expressed throughout the SAM but is excluded from incipient organ primordia to allow organ differentiation. Hence, while *WUS* specifies a subset of cells in the meristem center as stem cells, *STM* suppresses differentiation throughout the meristem dome. *STM* and *WUS* can function independently of each other, but co-expression of both genes produces a synergistic effect (Gallois et al. 2002; Lenhard et al. 2002). This suggests that the two pathways converge upon common target genes or processes.

Live-imaging and its Advantages

Live-imaging experiments are based on *in vivo* monitoring of spatial and temporal distribution of fluorescently labeled gene reporters in living tissues. Fluorescent reporter lines (**Table 1**) utilize either green fluorescent protein (GFP) and its derivatives, Yellow FP (YFP), Cyan FP (CFP), Red FP (RFP), or a more enhanced and faster maturated FP, such as eGFP, VENUS YFP, and DsRed. Some of the fluorescent lines, such as *p35S::YFP29–1*, label plasma membrane, while others, such as *p35S::H2B-mYFP* and *p35S::GFP-MBD,* label chromatin and microtubules, respectively. These fluorescent proteins that label chromatin or microtubules are ideal for observing dynamic cell division, expansion, and growth patterns (Reddy et al. 2004; Grandjean et al. 2004). Other fluorescent reporters, such as *pWUS::GFPer* and *pCLV3::GFPer,* allow one to observe gene expression patterns and identify and isolate stem cells (Jonsson et al. 2005; Reddy and Meyerowitz 2005; Gordon et al. 2007).

SAM is a dynamic structure, in which transient changes in gene expression, cell division and growth patterns, and cell-cell communications are difficult to study. In addition, SAM is very small and hidden, inaccessible for direct and real-time observation. The real-time live-imaging that combines the development of many fluorescent reporters (**Table 1**) with confocal laser microscopy has emerged as a powerful technique, which overcomes many of the limitations in the study of SAM (Reddy et al. 2007; Reddy 2008). First, researchers can now observe the immediate effect of transient perturbation of genes and gene networks. The transient perturbation could be genetic, chemical, or physical, such as using inducible RNAi, drug treatment, laser ablation, or chromophore-assisted light inactivation. Second, researchers can now monitor dynamic cell behavior and dissect complex cell signaling in real time. Third, one can easily observe cells buried several cell layers beneath the surface,

Table 1. List of fluorescent reporters for live-imaging of plant cells

Fluorescent marker	Description	Reference
pSTM::STM-VENUS	STM fused to a variant of YFP, VENUS, driven by a native promoter	Heisler et al. 2005; Gordon et al. 2007
pCLV3::GFPer	*CLV3* promoter driving GFP that localizes to endoplasmic reticulum	Reddy and Meyerowitz 2005; Gordon et al. 2007
pWUS::mGFPer	*WUS* promoter driving mutated, enhanced GFP that localizes to endoplasmic reticulum	Jonsson et al. 2005
pPIN1::PIN1-GFP	PIN1 protein fused to a GFP, driven by a native promoter	Heisler et al. 2005; Gordon et al. 2007
pPIN1::PIN1-CFP	PIN1 protein fused to a CFP, driven by a native promoter	Gordon et al. 2007
pDR5rev::3XVENUS-N7	*DR5*, an auxin responsive promoter, driving 3 VENUS proteins that localize to the nucleus	Heisler et al. 2005; Gordon et al. 2007
pCUC2::CUC2-VENUS	CUP-SHAPED COTYLEDON2 (CUC2) protein fused to VENUS, driven by a native promoter	Heisler et al. 2005; Gordon et al. 2007
pFIL::DsRed-N7	*FILAMENTOUS FLOWER (FIL)* promoter driving rapidly forming RFP variant that localizes to the nucleus	Heisler et al. 2005; Gordon et al. 2007
pARR5::GFP	*ARABIDOPSIS RESPONSE REGULATOR 5* promoter, responsive to cytokinin, driving GFP	Yanai et al. 2005; Gordon et al. 2007
pANT::GFPer	*AINTEGUMENTA* promoter driving GFP that localizes to endoplasmic reticulum	Grandjean et al. 2004
p35S::H2B-mYFP	HISTONE2B protein fused to mutated YFP, driven by a constitutive *35S* promoter that localizes to chromatin	Reddy et al. 2004
pCyCB1;1::CycB1;1-GFP	CYCLINB1;1 protein, G2-M cell cycle marker, fused to GFP, driven by the native promoter	Reddy et al. 2004
p35S::YFP29–1	YFP that localizes to the plasma membrane, driven by a *35S* promoter	Reddy et al. 2004
p35S::GFP-MBD	Microtubule-binding domain (MBD) of microtubule-associated protein 4 (MAP4) fused to GFP, driven by a *35S* promoter	Hamant et al. 2008
pTCS::GFP	A synthetic promoter, responsive to downstream cytokining signaling, driving GFP	Müller and Sheen 2008; Gordon et al. 2009
pAHK4::GFP	Cytokinin receptor promoter driving GFP	Gordon et al. 2009

leading to better mechanistic insights into cellular processes and development.

Live-imaging Provides a Comprehensive and Dynamic View of Cell Behavior in SAM

Using reporters that mark plasma membrane (*35S::YFP29–1*), nuclear (*35S::H2B:mYFP*), and cell cycle G2-M phases (*CyclinB1;1:GFP*), Reddy et al. (2004) monitored nuclear divisions and cell cycle length in live SAMs of *Arabidopsis*. The time-lapse imaging data were used to reconstruct events in time series to visualize morphogenesis in relation to cellular behavior. This led to several important observations. First, cell division rates vary across the SAM surface and cell division rates in the CZ are relatively heterogeneous when compared with those of PZ cells. Second, oscillations in division activities are uncoupled from events of circadian clock or primordial initiation. Third, oriented cell divisions in primordial progenitors and in cells located proximal to them are somewhat coordinated. Initial primordial outgrowth consists of oriented cell division, followed by a rapid and coordinated burst of cell expansion and cell division around flower primordium 2 (primordium 0 stage being the youngest primordium) to transform this extension into a three-dimensional flower bud.

In a similar study, mitotic drug oryzalin and DNA synthesis drugs were applied to meristems to achieve transient

perturbation of cell division, expansion and differentiation at the shoot apex (Grandjean et al. 2004). The drug effects were evaluated by visualizing live meristem cells stained with membrane-specific FM4–64 dye. While the mitotic drug stops cell division, DNA replication, cell expansion, and cell differentiation proceeded in the treated meristems, indicating that cell growth patterns can be uncoupled from the cell cycle. However, when inhibitors of DNA synthesis, aphidicolin and hydroxyurea, were applied, cell growth and, later, cell differentiation were inhibited, suggesting a coupling between DNA synthesis and cell growth patterns. This study indicated that DNA synthesis plays an important role in regulating growth behavior of meristem cells.

Mechanical-based Signaling also Contributes to SAM Morphogenesis

Living organisms are also subject to mechanical laws and thus their development may also have a physical basis in addition to biochemical ones (Uyttewaal et al. 2009). Hamant et al. (2008) used live-imaging, transient perturbation with laser beams and drugs, and cell-based computer modeling to determine the causal relationship between mechanical stress and morphogenesis in the SAM. Laser ablation or physical constrain were used to alter the stress of cells in SAM, which resulted in re-positioning of cortical microtubules, which align parallel to the maximal stress directions. This "parallel to stress direction" cortical microtubule alignment likely serves to deliver and guide cellulose synthesis complexes in order to modify the orientation of newly deposited cellulose microfibrils, thereby regulating morphogenesis. Interestingly, modeling based on a number of parameters allowed the researchers to calculate theoretical patterns of stresses in the SAM and predict patterns of microtubule alignment. The predicted microtubule distribution pattern was similar to what was observed with live-imaging (Hamant et al. 2008). While microtubule depolymerization only affected limited morphogenetic event such as the crease formation between the SAM and organ primordia, combined treatment of microtubule depolymerization drug with auxin transport inhibition resulted in spherical shoot tips. Therefore, microtubule-controlled anisotropy operates in parallel with auxin-driven growth rate patterns to regulate morphogenesis in SAM (Hamant et al. 2008; Uyttewaal et al. 2009).

Live-imaging Provides Novel Insights Into the CLV-WUS Network

A conditional inactivation of *CLV3* followed by a real-time live-imaging of SAM was used to further dissect *WUS-CLV* regulatory network (Reddy and Meyerowitz 2005). Upon induction of *CLV3-RNAi*, one can image in real time the expansion of CZ and determine which of the following mechanisms contributes to the CZ expansion. First, cell division rate in the CZ could increase upon *CLV3* inactivation. Second, CZ cells could delay their transition into PZ cells. Third, PZ cells could be re-specified into CZ cells. Upon induction of *CLV3* RNAi, investigators were able to follow cell division in specific cells of PZ and CZ using the plasma membrane marker (*p35S::YFP29–1*) and the CZ marker *pCLV3::GFPer*. They observed that some preexisting PZ cells began to express *pCLV3::GFP* and that cell division rate was increased in CZ but at a distance from the meristem center. Taking together, the investigators concluded that the expansion of the CZ in *CLV3* loss-of-function mutants was achieved by re-specification of PZ cells to take on the CZ fate as well as by a separate, long-range effect on cell division rate in CZ.

Cytokinin Promotes WUS via both CLV-Dependent and CLV-independent Pathways

Apart from the *CLV3* repressive signal, an unknown activator molecule responsible for inducing *WUS* expression in the OC was also proposed to originate from the stem cells (Jonsson et al. 2005). Plant hormone cytokinin is known to be involved in SAM formation, maintenance, and growth (Sablowski 2007) and thus a candidate for the proposed activator molecule. Cytokinin signaling is mediated via histidine kinase class receptors and Arabidopsis Response Regulators (ARRs). Two types of ARRs play opposite roles in cytokinin signaling (Kakimoto 2003; Müller and Sheen 2007; To and Kieber 2008). While Type-B ARRs positively regulates transcription of cytokinin-induced genes, Type-A ARRs negatively regulates cytokinin signaling. *WUS* directly suppresses the transcription of a Type-A ARR, *ARR5*, thereby positively regulating cytokinin signaling (Leibfried et al. 2005). Furthermore, high levels of cytokinin induce *WUS* by negatively regulating *CLV3*, releasing the repression of *WUS* by *CLV* (Lindsay et al. 2006).

Using live-imaging, the localized perception of cytokinin signaling within the CZ was recently shown to activate *WUS* expression through both CLV-dependent and a previously unknown CLV-independent pathway (**Figure 2A**) (Gordon et al. 2009). These regulatory relationships between *WUS* and cytokinin, either by *CLV*-dependent or independent pathways, constitute a positive feedback loop. Taking into account all these regulatory relations, computational modeling was used to predict steady state levels of activated type-B ARR, *WUS*, and type-A ARR, which were presented as peaks and donut shaped-rings at shoot apex (**Figure 2B-D**). *WUS* expression is predicted to closely overlap with cytokinin signaling and thus similar to the activated type-B ARR, whose activity peaks at the center of the SAM. In contrast, the expression level of *ARR5,*

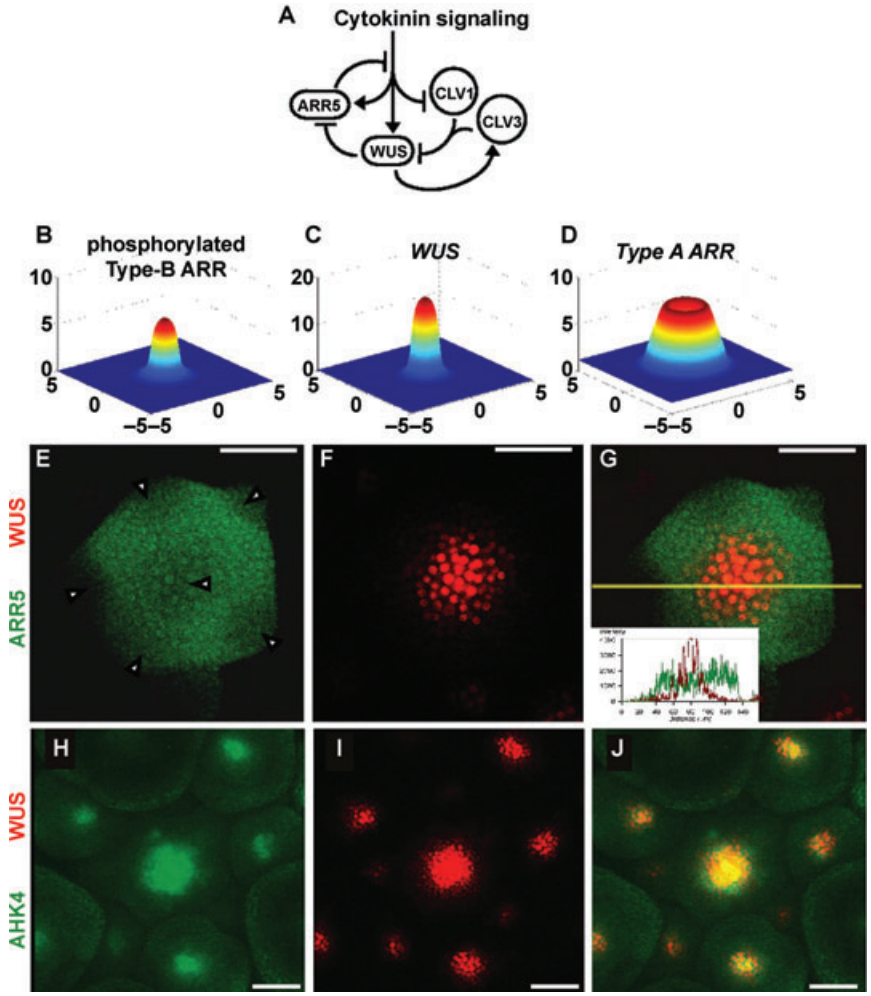

Figure 2. Computational modeling and live-imaging of the cytokinin and *WUS* regulatory circuitry.

Images are reproduced from Gordon et al. (2009) with permission from PNAS. (A) Cytokinin activates *WUS* through both suppression of *CLV1* and a *CLV*-independent pathway. (B-D) spatial distribution of phosphorylated B-type ARR (B), *WUS* (C), or *Type-A ARR* (D) predicted by modeling based on network proposed in (A). (E-G) *ARR5* (green) reporter is down regulated within the *WUS* domain (red) and organ primordial. Inset in (G) plots *ARR5* and *WUS* intensity (yellow line indicates profile). (H-J) Cytokinin receptor (*AHK4*, green) and *WUS* reporter (red) overlap within the SAM (center) or floral meristems (peripheral).

the type-A ARR, is predicted to be low in the SAM center, but high at the SAM peripheral, forming a donut-shaped ring at the shoot apex (**Figure 2D**). The predictions were experimentally tested and confirmed using live-imaging of *Arabidopsis* SAM. The spatial distribution of reporters for the cytokinin receptor *AHK4*, *WUS*, and *ARR5* in real time (**Figure 2E-J**) matches remarkably well with the computational predictions (**Figure 2B-D**). These results, combined with recent evidence that the stem cells produce active cytokinins (Kurakawa et al. 2007), indicate that cytokinin signaling is responsible for patterning *WUS* expression and ultimately controls the number of stem cells in the SAM.

Auxin Dynamics Controls Primordial Initiation and Growth

In previous studies, plant hormone auxin has been shown to play a central role in initiation and development of organ primordia within SAM (Reinhardt et al. 2000; Reinhardt et al. 2003; Benkova et al. 2003). This is primarily achieved by establishing a polarized auxin transport towards the SAM epidermis that results in auxin concentration being highest at the site of incipient organ primordia. Developing primordia would then act as auxin sinks and transport auxin into provascular tissue cells (Reinhardt et al. 2003). The distribution of the auxin efflux

carrier PIN1 regulates the direction of auxin polar transport and auxin concentration in cells. In turn, auxin has been recently shown to positively regulate *PIN1* expression (Heisler et al. 2005).

Using *pPIN1::PIN1-GFP* and *pDR5rev::3XVENUS-N7* (**Table 1**), Heisler et al. (2005) observed the dynamic polar auxin transport and auxin responses in living SAM. In incipient organ primordia, PIN1-GFP localization is directed toward them from adjacent sites, pumping auxin to these sites. As the primordium starts to grow and enlarge, PIN1-GFP in epidermal cells, especially cells adaxial to the primordium, reverses polarity from being directed toward the primordium to being directed away from the primordium and toward the meristem and adjacent new incipient primordia. The observed PIN1 polarity changes are correlated with auxin distribution changes revealed by the *pDR5rev::3XVENUS-N7*. The live-imaging data nicely supports earlier proposal that auxin is depleted from primordial regions after the primordium is formed and provides new insights into how auxin acts to position organ primordia within the SAM (Heisler et al. 2005).

De Novo Assembly of SAM From Callus Tissue

Unlike animal cells, plant cells are remarkable in their ability to regenerate. The ratio between plant hormone cytokinin and auxin determines the identity of the regenerated tissue. High auxin to cytokinin ratio induces roots, while high cytokinin to auxin induces new shoots. Live-imaging was recently used to analyze molecular patterning events during the *de novo* organization of shoot meristems from callus in culture (Gordon et al. 2007) using fluorescent reporters of genes with role in hormone transport, response, meristem specification and maintenance (**Table 1**). *pCUC2::3XVENUS-N7* was initially uniformly expressed in proliferating callus cells in auxin-rich Callus Inducing Medium (CIM). Upon the transfer of the callus to cytokinin-rich Shoot Inducing Medium (SIM), *CUC2* expression domain becomes complementary to that of *WUS*. This partitioning of *CUC2* and *WUS* expressing cells reveals one of the early steps in the self-organization of shoot meristem and is likely mediated by non-homogeneous distributions of auxin and cytokinin. Subsequently, *CUC2* and *WUS* expression may feedback on hormone synthesis, transport, and perception to enhance gradients of hormone signaling. Progressive refinement of hormone gradient and domains of meristem regulator expression ultimately lead to the *de novo* organization of shoot meristems.

Based on live-imaging data, Gordon et al. (Gordon et al. 2007) proposed four distinct steps in *de novo* shoot meristem initiation: callus induction, cytokinin-induced partition of cell identity within the callus, radial patterning within shoot progenitors, and meristem morphogenesis. The data also suggest that differences between shoot meristem initiation in planta and shoot meristem induction in culture may reside in the initial distribution of auxin and cytokinin (Gordon et al. 2007). In planta, auxin and cytokinin distribution is tightly controlled. In culture, their distribution is initially disrupted and requires subsequent reorganization.

Future Perspective

Live-imaging that consists of observing fluorescent markers with time-lapse imaging is emerging as a powerful technique in developmental biology. When live-imaging is combined with transient gene perturbation, multi-color labeling, and computational modeling of gene networks, researchers can integrate and superimpose gene regulatory pathways with growth patterns and morphogenesis in precise spatial domains and temporal sequence. Efforts are underway to develop “digital shoot apex” that incorporates multi-dimensional data from images of SAM morphogenesis to a genome-scale expression map of SAM (Yadav et al. 2009). Such progress requires the development of further computational and imaging tools and collaborations between biologists and computer scientists. Together, the field is moving closer than ever toward achieving a systemic view of SAM, enabling researchers to predict meristem behavior and gene expression patterns under any conditions.

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