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REVIEW

Signaling Network for Stem Cell Maintenance and Functioning in *Arabidopsis* Shoot Apical Meristem

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The shoot apical meristem (SAM) domain, which contains a self-renewing population of undifferentiated pluripotent cells, produces all of a plant's post-embryonic aerial organs. Intra- and intercellular signaling networks are essential to the maintenance of SAM size and structure, and for coordinating the formation and patterning of new organs. A key regulatory system for meristem functioning consists of a non-cell-autonomous WUSCHEL (WUS)-CLAVATA (CLV) regulatory loop and class I KNOX-related signaling in the shoot apex. Meristem activity is also dependent on short- and long-distance signaling from the organizing center, organ boundaries/primordia, and distant organs. Here, we provide an overview of SAM organization and present our current knowledge about the signaling network for stem cell maintenance and functioning in the vegetative SAM in *Arabidopsis*. Transcription factors belonging to the WOX, KNOX, HD-ZIP, MYB, SAND, AP2, and NAC domain protein families, as well as microRNA, play central roles in this network, along with hormonal cross-talk. We also discuss regulation at different levels, such as for protein interactions, and transcriptional and epigenetic controls. Our intent is to show how various signals are integrated to maintain a stem cell niche in the SAM.

Keywords: CLAVATA, epigenetic regulation, plasmodesmata, shoot meristem signaling, SHOOT MERISTEMLESS, WUSCHEL

STEM CELL BIOLOGY IN PLANTS

A plant meristem cell can be defined by its ability to divide for an indeterminate period while simultaneously maintaining some stable form of identity. The plant body includes a variety of meristem types, e.g., apical meristems located at the apices of roots and shoots, lateral meristems (cambial and marginal meristems), intercalary meristems, and meristemoids. The post-embryonic aerial structure of a higher plant is produced dynamically throughout the life cycle via activity within the shoot apical meristem (SAM).

The SAM is a spatially grouped collection of cells that continuously divide to renew themselves and provide cells for new organs. Despite this on-going differentiation by their daughters, the number of stem cells remains constant. This indicates that a balance is critical between the recruitment of cells into new organs and the formation of new stem cells. Recent studies have shown that intercellular communication, rather than genetically predetermined cell-fate specification, controls the organization and activity of the SAM (Poethig, 1987). One of the key questions in plant biology is how different signals integrate to maintain stem cell niches and to coordinate proper meristem functioning.

SAM maintenance in *Arabidopsis* involves an initiation of stem cells by WOX homeodomain protein WUS-signaling, as well as the perpetuation of indeterminate cell populations by a KN1-related homeobox (KNOX) protein SHOOT MERISTEMLESS (STM). Here, we provide a general overview of SAM organization, meristematic functioning, and the regulation of WUS-CLV-signaling and KNOX-signaling. We specifically focus on how the activities of these proteins are integrated within the framework of pathways that control plant development.

STRUCTURE AND ORGANIZATION OF SAM

All plant SAMs share a similar fundamental architecture that is partitioned into radial domains comprising discrete cell layers and concentric zones (Steeves and Sussex, 1989). The SAM is divided into three zones according to histological phenotypes and patterns of division (Fig. 1). The apical central zone contains stem cells that divide very slowly – a phenomenon that perhaps protects the fidelity of the genome by reducing the frequency of mutations (Irish and Sussex, 1992). The lateral peripheral zone, which recruits daughter cells divided from the central zone, displays cell differentiation and organ initiation. Division rates in that peripheral zone are faster than in the central zone (Reddy et al., 2004). Finally, the central rib zone forms organizing-center and stalk cells. A typical dicot shoot meristem has three discrete layers (L1, L2, and L3; Satina et al., 1940), while monocot SAMs have two layers (L1 and L2). In the dicot *Arabidopsis*, the tunica cells in the two outermost layers divide anticlinally, i.e., perpendicular to the surface of the meristem. These anticlinal divisions maintain continuity during growth of the L1 and L2 layers that gives rise to the epidermis and subepidermis, respectively. The L3 layer of the SAM, called the corpus, contains multi-cell layers where divisions occur in both periclinal and anticlinal planes to form the internal tissues.

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Abbreviations: CLV, CLAVATA; CUC, CUP-SHAPED COTYLEDON; KN1, KNOTTED1; KNOX, KN1-related homeobox; PD, plasmodesmata; SAM, shoot apical meristem; STM, SHOOT MERISTEMLESS; WUS, WUSCHEL

Because meristem cells from different layers take part in new organ formation, tight coordination for cell growth (division and expansion) and cell-fate specification between layers are critical for maintaining the SAM as well as correcting the patterning of new organs in the shoot apex (Clark, 2001). This orchestration may require non-cell-autonomous intercellular signaling between different layers, or within separate zones in the meristem.

Two cell-to-cell communication pathways are typical: 1) apoplastic signaling, involving secreted ligands and their membrane-localized receptors; and 2) symplasmic signaling, using a direct symplasmic route without the secretion of signal molecules (Kim, 2005). Most plant cells are joined by intercellular nanotubes called plasmodesmata (PD), which connect cytoplasm, ER, plasma membrane, and cell wall. The PD allow not only the passage of small molecules, such as nutrients or small signaling molecules, via diffusion-based movement, but also the trafficking of macromolecules, such as proteins or mRNAs, through a selective-trafficking mechanism (Lucas et al., 1995; Kim et al., 2002; Zambryski, 2004).

Early-stage embryonic cells initially form a single symplast, but symplasmic subdomains develop along a major morphological region and include the shoot apex, cotyledons, hypocotyl, and roots (Kim et al., 2005). Microinjection experiments using the fluorescent dye LYCH have shown that the birch SAM is divided into 'symplasmic domains', compartments that limit the movement of molecules (Rinne and van der Schoot, 1998; Gisel et al., 1999). When injected into the L1 cells of the peripheral zone, the dye rapidly spreads throughout the same zone but not into the central zone nor into the leaf primordia. This indicates that the meristem-organ primordia boundary as well as the boundary between the central and peripheral zones acts as a barrier that restricts the diffusion of relatively small molecules.

Intercellular trafficking of transcription factors or RNAs through the PD appears to be an important communications tool. Class I KNOX family proteins, KNOTTED1 (KN1) and STM, are essential meristematic housekeepers that function non-cell-autonomously through direct cell-to-cell movement (Kim et al., 2003). For example, layer-specific expression of a GFP fusion protein to KN1 or STM in a strong *stm-11* mutant demonstrates the shoot rescue of this strong mutant allele through inter-layer protein movement that follows 'secondary PD', which are produced via *de novo* cell-wall drilling after cytokinesis (Fig. 1B). How this plasmodesmal communication network is regulated and how widespread SAM-signaling is remain mostly unknown.

SAM INITIATION AND MAINTENANCE BY THE WUSHEL-CLAVATA REGULATORY LOOP

WUS as a master regulator of SAM fate

WUS, in the WOX family of homeodomain transcription factors, is a master regulator of stem cell fate. WUS protein is required for the initiation and maintenance of stem cells in meristems (Laux et al., 1996; Mayer et al., 1998). Loss-of-function *wus* mutants cannot initiate or support self-maintaining stem cells. During embryogenesis, the apices of *wus* mutants are enlarged and flat compared with wild-type shoot meristems, which are convex and dome-shaped. *WUS* expression is first detected in the early embryo and continues in a small region of cells underneath the center of the SAM, i.e., the organizing center (Laux et al., 1996). These *WUS*-expressing organizing-center cells play a role similar to those in the quiescent center of the root apical meristem (Vernoux and Benfey, 2005). Ectopic WUS expression in roots is sufficient to induce ectopic stem cell fate, and it makes the root meristem cells responsive to signals that specify SAM cell identity, as shown by leaf development on roots (Schoof et al., 2000; Gallois et al., 2004).

WUS acts in a non-cell-autonomous fashion, either by moving cell-to-cell or by activating an intercellular signal (Mayer et al., 1998). According to the 'stem cell competency' theory, this signal is released to all surrounding cells, but only 'stem cell competent' cells are capable of perceiving it.

WUS-CLV feedback regulation for SAM maintenance

The WUS signals back to the organizing center via the CLV-signaling pathway. In experiments of ectopic *WUS* expression using a *CLV3* promoter, a large apical dome develops that induces the stem cell marker gene *CLV3* (Brand et al., 2002). This gene encodes a stem cell-specific protein that is secreted by the L1/L2 stem cells (Rojo et al., 2002; Lenhard and Laux, 2003). Application of matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) to *in situ Arabidopsis* tissues has determined the structure of a modified hydroxyl 12-amino acid peptide, which is derived from a conserved motif in the CLV3 sequence (Kondo et al., 2006). Furthermore, a family of CLV3/ENDOSPERM SURROUNDING REGION (ESR), consisting of 31 members in the *Arabidopsis* genome, is able to generate an extracellular peptide hormone to regulate cell division and differentiation (Fiers et al., 2007). The modified 12-amino acid peptide of CLV3 moves between cell layers and binds to the CLV1–CLV2 heterodimer complex in the underlying cells, including the organizing center. CLV1, a transmembrane receptor-like kinase with extracellular leucine-rich repeats (LRRs), possesses serine/threonine kinase activity (Clark et al., 1997). CLV2 lacks the cytoplasmic kinase domain that is required for stabilizing the CLV1 complex (Kayes and Clark, 1998; Jeong et al., 1999). Single or double mutations in *CLV1*, *CLV2*, or *CLV3* exhibit similar phenotypes that produce larger SAMs than those of the wild type due to an increased number of cells in the central zone (Clark et al., 1995, 1997). These mutants show an enlarged WUS-expression domain while activation of CLV-signaling limits the size of the WUS expression domain. This suggests that WUS is the target of CLV-signaling that suppresses stem cell proliferation (Clark et al., 1993).

A model (Fig. 2) for the regulation of stem cell fate in *Arabidopsis* proposes that the organizing center of the meristem expressing WUS acts to initially specify stem cells in an overlying region, and then activates CLV3 non-cell-autonomously through unknown signaling factors (Lenhard and

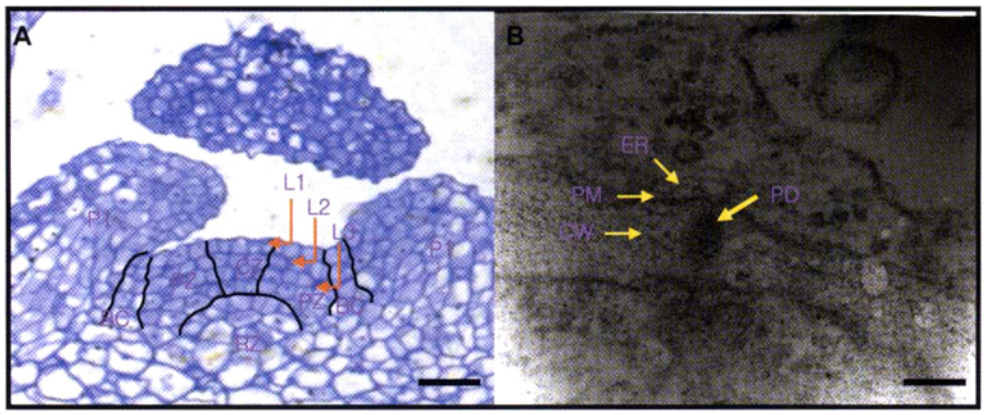

Figure 1. Structure of shoot apical meristem, and presence of secondary plasmodesmata in *Arabidopsis*. BC, boundary cells; CW, cell wall; CZ, central zone; ER, endoplasmic reticulum; P1, leaf primordia; PD, plasmodesmata; PM, plasma membrane; PZ, peripheral zone; RZ, rib zone. Scale bars: 20 μm (**A**), 100 nm (**B**).

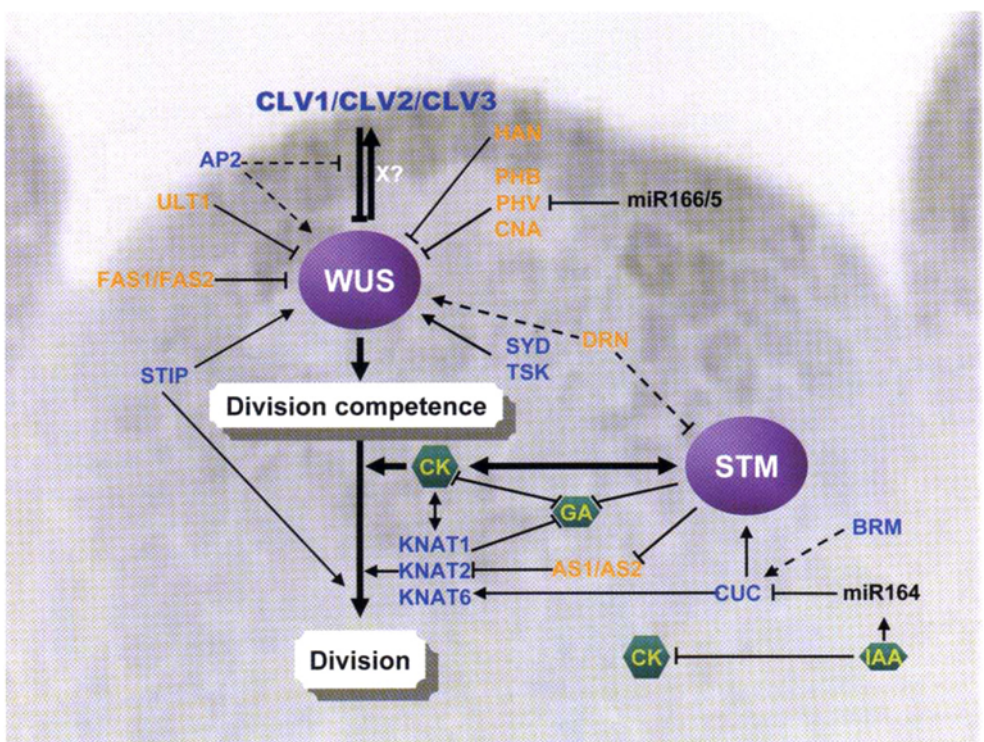

Figure 2. Molecular network to regulate stem cell fate in *Arabidopsis* SAM. WUS/CLV and STM are central regulators for meristematic functioning, including initiation and maintenance of division-competent cells. Stem cell population in outermost cell layers is maintained by unknown factor (X) generated in WUS expression cells of organizing center. CLV pathway limits size of organizing center. CLV-independent pathways involving FAS1/2, PHB/PHV/CAN, ULT1, and HAN restrict expansion of WUS-expression domain through CLV-independent pathways. Class III HD-ZIP proteins PHB, PHV, and CNA are subject to negative regulation by microRNA166/5. STIP, SYD, and AP2 are proteins that positively regulate WUS expression. STM is expressed throughout meristem, where it maintains division competency of stem cells by repressing differentiation. KNOX (KNAT1, KNAT2, KNAT6, and STM) and cytokinin in SAM promote mutual synthesis and antagonize GA synthesis. Activation of STM expression requires functions of CUC that control organ separation and are regulated by microRNA164 activated by auxin (IAA). Positive and negative regulators are shown in yellow and blue, respectively. Dotted lines indicate potential regulation. Thick lines indicate central regulatory pathway in SAM control. CK, cytokinin; GA, gibberellin; IAA, auxin.

Laux, 2003). CLV3 secreted from the L1 and L2 stem cells non-cell-autonomously counteracts WUS activity, comprising a negative feedback loop. Such a coordinated loop allows the SAM to maintain equilibrium between stem cell division in the central zone and cell loss due to differentiation in the peripheral zone (Schoof et al., 2000).

KNOX GENES IN SAM DEVELOPMENT

Role of class I KNOX family in SAM functioning

KNOX genes are found in species throughout the plant kingdom, and are part of a large super-class of home-

odomain proteins (Muller et al., 2001) that contain a three-amino acid loop extension (TALE) motif in the homeodomain (Burglin, 1997). Among the class I KNOX (STM, KNAT1/BP, KNAT2, KNAT6), STM plays an essential role in the formation and maintenance of the SAM (Long et al., 1996). Other members of the KNOX1 family-KNAT1 and KNAT6 – show some redundant functioning to maintain the SAM. Even though STM is naturally essential for SAM maintenance, *stm* knock-out mutants can be rescued by the loss-of-function of an MYB transcription factor, ASYMMETRIC LEAVES1 (AS1) (Byrne et al., 2002). This rescue is again lost in the triple mutant *stm/as1/kant1*. These results can be explained by KNAT1 activity that is de-repressed by the loss of AS1 functioning. KNAT6 also contributes redundantly with STM to the maintenance of the SAM and organ separation, as demonstrated by the lack of meristematic activity in *knat6/stm-2* double mutants. Expression of *KNAT6* in the SAM requires CUP-SHAPED COTYLEDON (CUC), and is located at the boundaries between the SAM and the cotyledons (Belles-Boix et al., 2006).

STM as a housekeeper of the SAM

stm mutations result in the loss of meristematic organization and eventual meristem arrest. Although *stm* mutants have a phenotype that is superficially similar to that of *wus* (Laux et al., 1996), their meristem-arrest phenotypes differ; stem cells are consumed into developing organs in *stm* mutants but are retained in a somewhat disorganized and non-meristematic state in *wus* (Laux et al., 1996; Mayer et al., 1998). When over-expressed, STM can promote cell proliferation in the absence of WUS, suggesting a WUS-independent STM pathway (Lenhard et al., 2002). Importantly, however, enhanced STM activity cannot compensate for the loss of WUS because the frequency of ectopic shoot meristem formation is reduced in the *wus* background. This further indicates synergism between STM and WUS functioning (Brand et al., 2002).

Two main pathways operate in shoot meristem development: 1) the STM-based KNOX pathway, which regulates meristem cell fate, preventing meristematic cells from adopting organ-specific cell fates and 2) the WUS pathway, which modulates initial specification of stem cell identity in the central zone. Thus, the combined synergistic activities of these pathways are required for proper meristem organization and functioning.

REGULATION OF PROTEIN NETWORKS IN SAM DEVELOPMENT

Transcriptional or post-transcriptional regulation

Until now, little has been known about how highly the expression of WUS is restricted, and the means by which STM is controlled. Analysis of the *WUS* promoter has indicated that a 57 b regulatory region (two adjacent short sequence motifs) located 529 b upstream of the start codon is sufficient to provide all the information required for *WUS* transcription in the SAM (Baurle and Laux, 2005). Several new studies have identified regulators that are involved in the spatial and/or temporal expression of *WUS*. These include a GATA-3-like transcription factor, HANABA TARANU (HAN); the SAND domain ULTRAPETALA 1 and 2 (ULT1 and ULT2); a WUS-related homeobox containing protein, STIMPY/WOX9 (STIP); a class III homeodomain-leucine zipper (HD-ZIPIII); APETALA (AP2); CORONA (CAN); PHABULOSA (PHB); PHABULOTA (PHV); and SPLAYED (SYD) (Williams and Fletcher, 2005). The WUS-signaling system seems to be associated with signals from different parts of the meristem, including those from the neighboring differentiating cells.

HAN is essential for meristem formation in a non-cell-autonomous manner (Zhao et al., 2004). Its expression in the SAM boundary from early embryogenesis suggests that it functions to synergistically control WUS expression through a pathway distinct from CLV. ULT1 and ULT2 have overlapping functions as negative regulators of WUS and STM expression, but act in separate genetic pathways (Carles et al., 2004, 2005). The SAM of *stip* mutants has a flat structure and lacks both CLV3 and WUS expression. Loss of STIP functioning completely suppresses the *clv3* phenotype whereas overexpression enhances it, consistent with reports that STIP is a positive regulator acting in the WUS pathway (Wu et al., 2005).

CNA, *PHB*, and *PHV* may regulate stem cell accumulation in a pathway parallel to that of the CLV loci (Green et al., 2005). The triple mutant *can/phb/phv* of the HD-ZIP III gene family effectively recreates the *clv* enlarged-SAM phenotype (Prigge et al., 2005). A gain-of-function *jabba-1D* (*jba-1D*) mutant (Williams et al., 2005) that reduces *CNA*, *PHB*, and *PHV* transcripts in the shoot meristem shows an enlarged and fasciated SAM, both features correlated with a dramatic increase in the level of WUS transcription. Plants of the *jba-1D* mutant over-express microRNA (miR166g), which dramatically reduces *PHB*, *PHV*, and *CNA* transcripts in the SAM. These researchers have concluded that PHB, PHV, and CNA restrict SAM activity by down-regulating *WUS* transcription in wild-type plants. Other aspects related to distal activity by the WUS may reflect the action of DORNROSCHEN (DRN), which encodes an AP2 class transcription factor (Kirch et al., 2003). AP2 has been proposed to function in stem cell maintenance by modifying the WUS-CLV3 feedback loop. Genetics studies using a dominant negative *l28* mutant have shown that the dominant inhibition of AP2 activity results in either CLV-dependent or -independent down-regulation of *WUS* (Wurschum et al., 2006).

STM is first activated at the late globular stage of embryogenesis, in the center of the apical domain that will give rise to the SAM (Long and Barton, 1998). Activation of *STM* expression requires the functioning of *CUC* genes, which encode NAC-domain transcriptional regulators that control organ separation and are regulated by microRNA during embryogenesis (Vroemen et al., 2003; Laufs et al., 2004). STM is subsequently responsible for restricting CUC expression to the peripheral boundary of the embryonic SAM, mimicking its expression at the boundaries between lateral organ primordia and the SAM during post-embryonic growth (Aida et al., 1999).

Epigenetic regulation by chromatin remodeling

Cellular chromatin reorganization is a means to control meristematic functioning and organogenesis. TONSOKU (TSK)/BRUSHY1 (BRU1), a protein involved in the post-replicative stabilization of chromatin structure, is associated with positive regulation of the *WUS* expression domain (Takeda et al., 2004). *WUS* is also a direct target of the chromatin remodeling factor SPLAYED (SYD), an SNF2 class ATPase (Kwon et al., 2005). *syd* plants have reduced levels of *WUS* and *CLV3* mRNAs, and undergo premature termination of the SAM. Chromatin immunoprecipitation experiments have demonstrated that SYD is specifically recruited to the promoter region of the *WUS* gene; SYD is a direct and specific, positive upstream regulator of *WUS*. AtBRAHMA (AtBRM), the closest homolog of SYD in *Arabidopsis*, up-regulates the transcription of *CUC1*, *CUC2*, and *CUC3* (Farrona et al., 2004; Kwon et al., 2006).

Mutations in the FASCIATA1 (FAS1) or FAS2 genes, which encode two subunits of the three components of CHROMATIN ASSEMBLY FACTOR-1 (CAF-1), cause ectopic expression of *WUS* in the SAM (Kaya et al., 2001). AtMSI1, the *Arabidopsis* counterpart of the third CAF-1 subunit, is required for normal shoot development, as indicated by transgenic *Arabidopsis* plants with reduced AtMSI1 expression and abnormal development (Hennig et al., 2003).

Characterization of the functioning of chromatin remodeling factors in SAM regulation will bring answers to how chromatin factors integrate developmental signals to regulate meristematic functioning.

Regulation by protein-protein interactions

Class I KNOX proteins interact with the BEL-like homeodomain protein, BELLRINGER/PENNYWISE (BLR/PNY), which is required in order for KNAT1 to functionally compensate for the loss of STM (Byrne et al., 2003; Smith and Hake, 2003). Genetics studies have demonstrated that these interactions are also essential for normal KNOX functioning in the SAM. Single or double mutants of *BLR/PNY* and the closely related *POUNDFOOLISH* genes exhibit several alterations to the SAM, including smaller meristems, aberrant STM expression, and an altered phyllotaxy (Smith et al., 2004).

Nuclear import of STM depends on hetero-dimerization with BELL1-like homeodomain proteins expressed in discrete sub-domains of the SAM in *Arabidopsis thaliana* (Cole et al., 2006). Protein interaction between the KNOX and BELL1-like proteins is used to achieve high DNA-binding affinity (Smith et al., 2002).

The WUS homeodomain protein may also function as a homodimer or heterodimer. OsWUS forms a homodimer and interacts with PRS carrying the WUS subfamily homeodomain. However, OsWUS cannot interact with a TALE subfamily homeodomain protein such as OSH15 (Nagasaki et al., 2005).

Cross-talk with hormonal signaling pathways

KNOX gene expression is mutually and positively correlated with hormonal signaling pathways. For example, transgenic plants with a high cytokinin content express KNOX genes at greater levels than do wild-type plants (Rupp et al., 1999; Frank et al., 2000), while KNOX-overexpressing plants accumulate more cytokinins through the increased expression of a cytokinin biosynthesis gene, *ISOPENTENYL TRANSFERASE 7* (*IPT7*) (Hewelt et al., 2000; Yanai et al., 2005). Artificial expression of *IPT7* in the SAM can partially suppress the *stm* mutant. Moreover, both the KNOX overexpressor and plants with elevated levels of cytokinins show delayed senescence (Ori et al., 2000). WUS functions as a negative regulator of cytokinin-signaling by direct binding to the *ARR7* promoter. The overexpressor of *ARR7* phenocopies wus mutants (Leibfried et al., 2005), suggesting that down-regulation of *ARR7* by WUS is biologically relevant.

STM negatively regulates the expression of a GA-20 oxidase required for gibberellin biosynthesis, and positively regulates the expression of a gene encoding a GA 2-oxidase, which deactivates bioactive GAs in the SAM (Sakamoto et al., 2001; Jasinski et al., 2005). In *Arabidopsis*, exogenous GA applications can partially alleviate the ectopic phenotype conferred by KNOX overexpression, while the constitutive GA-signaling *spy* mutation enhances the phenotypic severity of mild *stm* mutants (Hay et al., 2002). The combination of reduced cytokinin and increased gibberellin in the background of the *spy* mutant results in a range of phenotypes similar to those of strong *stm* alleles, including meristem abortion and fused cotyledons (Jasinski et al., 2005). This antagonistic relationship between KNOX genes and GA is in general accordance with the view that gibberellin promotes polar cell expansion/organ growth and is incompatible with meristematic homeostasis.

Auxin classically demonstrates antagonistic interactions with cytokinin. High relative auxin concentrations in the P_0 region down-regulate the expression of *CUC* and KNOX genes, allowing primordium initiation (Heisler et al., 2005). A proposed pathway to down-regulate *CUC* expression includes activating the expression of miR164 by auxin (Guo et al., 2005). Alternatively, some evidence suggests that KNOX proteins might also inhibit auxin transport (Tsiantis et al., 1999), indicating a possible feedback relationship between auxin and the KNOX proteins. Thus, KNOX-mediated meristem maintenance probably involves a delicate balance in the levels of several hormones.

CONCLUDING REMARKS

We have described how two central signaling pathways in the SAM operate and are integrated into a functioning SAM. Recent studies have provided exciting new data that enable us to better understand the molecular mechanisms of stem cell specification and meristem maintenance. Significant progress is also being made to elucidate the factors controlling *WUS* transcription. Identification of regulatory *cis* elements in the *WUS* promoter and a description of the first protein (SYD) shown to bind specifically to the *WUS* promoter now provide clues to the transcriptional regulation of *WUS* by chromatin remodeling. The identification of ULT1, HAN, AP2, CNA, and STIP presents new insight into the activities that set the boundaries of the *WUS* expression domain. Discovery of microRNA involvement and hormonal

cross-talks in the regulation of SAM-functioning add to a more complex signaling network.

Still many questions remained unanswered. What are the direct target genes regulated by *WUS* that are responsible for transcriptional repression of *WUS*? How do chromatin factors operate as integrated developmental signals to regulate meristem functioning? How do different hormonal and protein signals integrate to coordinate proper meristem functioning? What factors or mechanisms work non-cell-autonomously for SAM-functioning? When we combine traditional genetics and biochemical methods with modern, state-of-the-art technologies, such as bioinformatics, meristem imaging, microdissection, microarrays, proteomics, and chromatin immunoprecipitation, we obtain useful assets that help us disentangle the complex integrated signaling network for SAM development.

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