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Live-imaging stem-cell homeostasis in the *Arabidopsis* shoot apex

G Venugopala Reddy

A precise spatio-temporal regulation of growth and differentiation is crucial to maintain a stable population of stem cells in the shoot apical meristems (SAMs) of higher plants. The real-time and simultaneous observations of dynamics of cell identity transitions, growth patterns, and signaling machinery involved in cell–cell communication is crucial to gain a mechanistic view of stem-cell homeostasis. In this article, I review recent advances in understanding the regulatory dynamics of stem-cell maintenance in *Arabidopsis thaliana* and discuss future challenges involved in transforming the static maps of genetic interactions into a dynamic framework representing functional molecular and cellular interactions in living SAMs.

Addresses
2150 Batchelor Hall, Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, United States

Corresponding author: Reddy, G Venugopala (venug@ucr.edu)

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Introduction

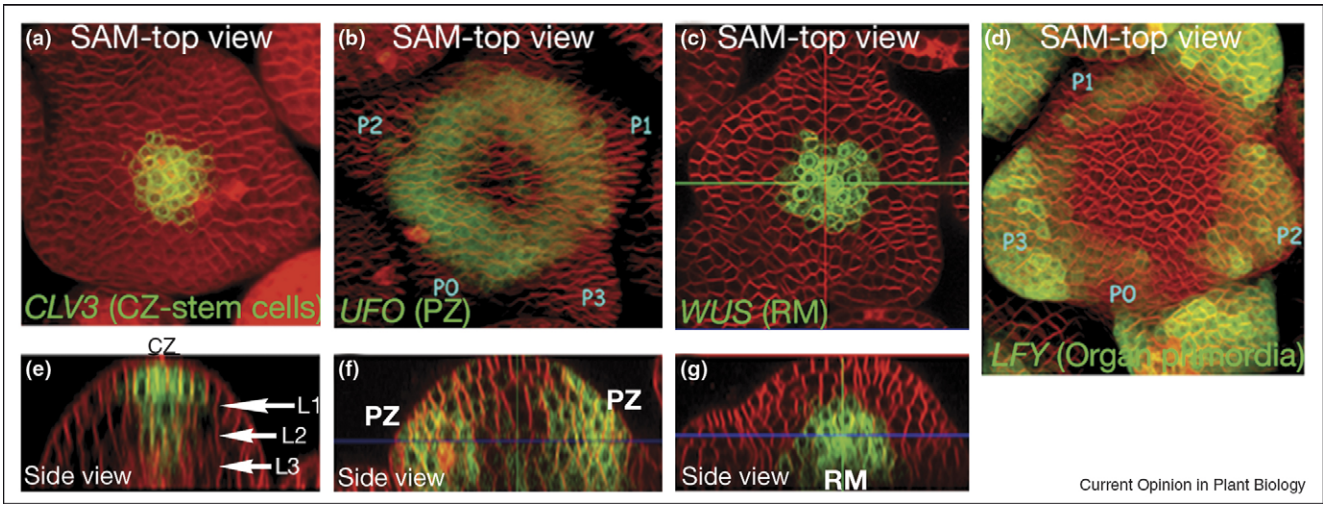
The sustained cell division activity of stem cells and timely transition of their progeny into differentiation states are crucial to ensure that a stable population of stem cells is maintained in the shoot apical meristems (SAMs) of higher plants. The SAM stem-cell niche is a dynamic developmental landscape of distinct types of cells displaying specific gene expression patterns and cellular behaviors [1,2,3•]. In *Arabidopsis*, the SAM is a multilayered structure consisting of approximately 500 cells, organized into three clonally distinct layers of cells. The cells in the outermost L1 layer and the subepidermal L2 layer divide in anticlinal orientation (perpendicular to the SAM surface), while the underlying corpus forms a multilayered structure where cells divide in random orientation. Within this framework, the SAM is divided into cytological and functional zones (Figure 1). The central zone (CZ) contains a set of stem cells and the progeny of stem cells differentiate within the surrounding peripheral zone (PZ). The CZ also supplies cells to the rib-meristem (RM) located beneath the CZ. Thus, cell fate specification within SAMs is a dynamic process intimately coupled to transient changes in gene activation/repression and changes in growth patterns. Therefore, dynamic imaging methods may provide new insights into the active interplay between growth and differentiation mediated by cell–cell communication. Genetic and molecular analysis carried out by several laboratories has resulted in a molecular framework that mediates cell–cell communication [4–6]. Recent studies have attempted to understand the dynamic spatio-temporal contours of cell–cell communication networks and that of patterns of gene expression and cell behaviors in living SAMs. Challenges, prospects, and the progress in live-imaging of plant tissues and image analysis have recently been reviewed in great depth [7•,8,9•]. In this article, I focus on studies aimed at deciphering the regulatory dynamics of stem-cell maintenance and SAM growth.

Spatio-temporal dynamics of cell–cell communication

CLAVATA–WUSCHEL feedback network

Earlier studies have revealed molecular and cellular interactions involved in stem-cell maintenance in the *Arabidopsis* SAMs. Mutations in *CLAVATA* class of genes result in enlarged stem-cell domain and bigger SAMs (reviewed in [4,5]). By contrast, mutation in the homeobox gene, *WUSCHEL* (*WUS*), results in premature termination of SAMs. The studies on gene expression patterns, genetic interactions, and biochemical characterization have revealed that stem cells secrete CLAVATA3 (CLV3), a predicted ligand that binds to and activates CLAVATA1 (CLV1)–CLAVATA2 (CLV2) receptor kinase complex within the RM cells. Once activated, CLV1–CLV2 receptor kinase complex functions to restrict the expression domain of WUS to few cells within the RM, also referred to as the stem-cell organizing center (OC) (reviewed in [6]). Spatial mis-expression of *WUS* results in the upregulation of *CLV3* expression in the overlying CZ and also promotes the expansion of stem-cell domain. Taken together, it has been proposed that a feedback loop links *CLV3*-expressing stem cells in the CZ to the *WUS*-expressing cells of the RM and thus forming a selfcorrecting mechanism that functions to ensure constant number of stem cells are maintained in the SAM. Better descriptions of expression patterns involving fluorescent chimeric constructs expressed from native promoters may be required to decipher the spatial and temporal dynamics of CLV–WUS feedback loop.

Figure 1



Functional subdomains of stem-cell niche highlighted by fluorescent reporter constructs. **(a)**, **(b)** and **(d)** are the 3D re-constructed views of shoot apical meristems (SAMs), highlighting the stem-cell domain (CZ) marked by *CLAVATA3* promoter **(a)**, the adjacent peripheral zone (PZ) is marked by *UNUSUAL FLORAL ORGANS* (*UFO*) promoter **(b)**, a section of the rib-meristem (RM) revealing *WUSCHEL* expression **(c)**, and the sites of differentiation are marked by *LEAFY* promoter **(d)**. **(e)–(g)** are the re-constructed side views of SAMs represented in (a)–(c), respectively, revealing three clonal layers of cells (e) and (f) and RM (g). Cell outlines are highlighted by FM4-64 dye (red).

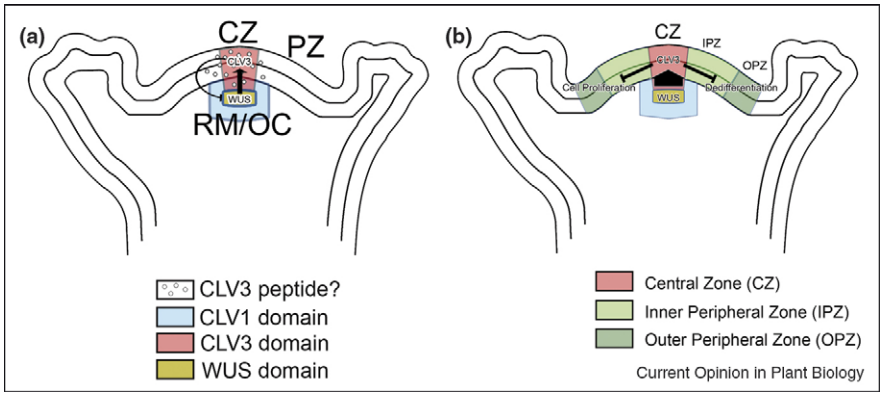
Efforts have been made to understand the regulatory dynamics of CLV–WUS feedback loop. For example, earlier studies have utilized CLV3:GFP protein chimera to follow the intracellular and spatial distribution of CLV3 protein within the SAMs [10,11]. When placed under the native CLV3 promoter, the CLV3:GFP fusion protein diffused laterally into the PZ cells. However, the CLV3:GFP fusion protein was excluded from the RM cells suggesting that the CLV3 protein was sequestered by CLV1 receptor kinase expressed in the RM [11]. Recent evidence suggests that CLV3 is processed to yield a biologically active 12-amino acid peptide [12–16]. In light of recent developments, the *in vivo* observations of CLV3 protein and the processed peptide may reveal spatial patterns of CLV3 diffusion (Figure 2A). The tagging of a small peptide with a relatively bulkier fluorescent protein may interfere with its function. A much smaller fluorescent tag, a tetracysteine peptide (Cys-Cys-Xaa-Xaa-Cys-Cys) which is capable of binding exogenously supplied fluorescent-bi-arsenic ligands (FIASHs-EDT2 and ReAsH-EDT2) with high affinity and thereby highlighting the protein chimera have been described [17]. Recently, the system has been used in *Arabidopsis* to visualize synthetic Serine-Proline [$(Ser\text{-}Pro)_7$ and $(Ser\text{-}Pro)_9$] glycomodule peptides of the arabinogalactan protein-motif [18•]. Generating a chimeric CLV3 protein consisting of an in-frame fusion with tracystein (TC) motif placed adjacent to the sequences corresponding to the CLE-peptide region of the protein might facilitate the *in vivo* detection of CLV3 peptide, thereby revealing the spatial parameters of CLV3 diffusion.

The terminal phenotypes of *clv* mutants have revealed an expanded *WUS* expression domain and a greatly disorganized RM [19]. However, a recent study implies that the story is not that simple. The analysis of *pWUS::GUS* reporter expression in *clv3* mutants showed that, intriguingly, the *WUS* promoter activity declined progressively as *clv3* plants develop. This indicates another type of CLV3-mediated temporal regulation of *WUS* expression and/or CLV3-mediated regulation of cell identity within the RM [20]. It is not possible to infer whether observed effects reflect the nature of CLV-mediated regulation of *WUS* expression and/or they reflect altered cell types in a disorganized RM because these studies analyzed terminal *clv3* SAMs, that is after the expansion of the *WUS* domain has occurred and RM has been disorganized. Development of new RM-specific fluorescent reporters, expressed independent of CLV signaling will be crucial to uncouple the effects of CLV signaling on *WUS* expression and cell fate specification within the RM. The dynamic and quantifiable spatio-temporal properties obtained by simultaneous monitoring of *WUS* expression, the expression of CZ and the RM-specific genes by live-imaging of both the wild-type SAMs and upon transient perturbations in CLV signaling can then be used to explicitly model the regulation and function of CLV–WUS feedback loop.

Quantification of *WUS* expression and computational modeling of spatial organization of *WUS* expression domain

Computational approaches have been employed to model CLV–WUS reaction network in an effort to recreate the

Figure 2



CLAVATA–WUSCHEL network: regulation and function. **(a)** Schematic representation of expression domains of CLV–WUS feedback network. CZ: central zone, PZ: peripheral zone, RM/OC: rib-meristem/organizing center. **(b)** Schematic representation of functional domains of SAM stem-cell niche and the dual function of CLV signaling in regulating stem-cell domain and SAM growth. CZ: central zone, IPZ: inner peripheral zone (region which retains competence to respond to the central zone promoting activity), OPZ: outer peripheral zone (region which fails to respond to the central zone promoting activity).

observed phenomena and to explore new hypotheses *in silico*. Computational modeling of biological phenomena requires quantitative determination of spatio-temporal parameters of network components. In a recent study, fluorescently labeled image data sets have been used to quantify *WUS* expression levels from individual cells of the RM [21••]. In order to quantify *WUS* expression levels, the SAM expressing an endoplasmic reticulum localized GFP under the influence of *WUS* promoter was counter labeled with FM4-64, a lipophilic dye, which can highlight cell boundaries/plasma membranes of all cells (Figure 1C). The FM4-64-labeled image was used as a template to extract individual cell boundaries by using watershed algorithm, which utilizes the pixel intensity gradient information to segment cells in a multicellular field. The computationally extracted individual cell compartments were then used to calculate average pixel intensity of GFP fluorescence within individual RM cells. The averaged GFP fluorescence intensity was taken as a direct measure of GFP protein concentration. The GFP protein concentration, in turn, was taken as a measure of *WUS* expression levels. The deduced *WUS* expression levels were taken as reference to compare with the output obtained from computational modeling of *WUS* expression domain in wild-type SAMs.

The model, based on reaction–diffusion (RD) system, invokes a hypothetical activator network which can induce *WUS* expression. Originally, the RD system was used to explain the behavior of a complex system in which two substances interact with each other and diffuse at different rates to generate regular spatio-temporal patterns in the field [22]. It was also noted, in the original study by Turing, that the spatial pattern generated by the RD system might provide positional information for a developing embryo. Though later studies have modified the RD system to fit various naturally observed events, however, all of them share the basic principle of the original model that the waves are generated as a result of an interaction between two chemical substances [23–25]. The RD system has been applied to simulate *WUS* expression pattern, on a two-dimensional static cellular template. The synthesis, degradation, and diffusion parameters of activator network are adjusted in such a way that a single peak appears within a given SAM. However, precise positioning of the peak within the SAM required another hypothetical inhibitory substance secreted from the L1 layer. Furthermore, this model could account for observed behavior of *WUS* expression both in *clv3* mutants and upon ablation of pre-existing *WUS* expression domain.

The components of the *WUS* activator network and the L1-derived repressive signals have not been identified. However, it has been suggested that CLV3 could provide the function of L1-derived repressive signal. In this context, analysis of the spatial diffusion patterns of CLV3 peptide ligand may provide clues to the spatial activation patterns of CLV1 and related receptor kinase network [26]. At present, the model may not be entirely predictive because of an incomplete network inference which is based on hypothetical factors. However, the highlight of this work is the utilization of imaging data as a reference to fine tune the parameters to achieve an expected output. Development of robust computational models, however, not only require quantifiable parameters of CLV–WUS network components, but it may also require a dynamic understanding of cell fate specification process.

Dynamic regulation of stem-cell pool and cellular memory system

Loss of function *clv3* or *clv1* mutants or ectopic expression of *WUS* has been shown to result in an enlarged CZ (stem-cell domain), and a much condensed PZ [19]. Two recent studies have attempted to address the mechanisms of enlargement of the CZ in *clv* mutants by employing transient perturbations in *CLV3* levels followed by the time-lapse observations of the stem-cell niche. Conditional over-expression of *CLV3* resulted in rapid loss of *WUS* expression which is followed by the loss of CZ identity [27•]. Analysis of dynamic cellular response to increased CLV3 signaling, as visualized by non-invasive replica method, revealed a gradual reduction in SAM size, with flower primordia forming closer to the CZ. These observations suggested that CLV signaling is required to maintain the boundary between the CZ and the PZ. A related study has employed conditional inactivation of *CLV3* and live-imaging to follow the reorganization patterns of the CZ and the SAM size [28•]. The conditional inactivation of *CLV3* gene resulted in a radial expansion of the CZ. Moreover, the expansion of the CZ was because of the re-specification/de-differentiation of pre-existing PZ cells into stem cells. This study indicates that a subset of the PZ cells located adjacent (inner peripheral zone, IPZ) to the CZ reverts back to the stem-cell fate. These results suggest that immediate progeny of stem cells retain their ability to respond to the stem-cell promoting factor. However, the ability to acquire stem-cell fate is lost as cells are displaced towards the outer edge (outer peripheral zone, OPZ) of the PZ ([28•]; Figure 2B). Thus, live-imaging analysis has not only provided clues to the function of CLV signaling in stem-cell maintenance, but also provided a better definition of the PZ. Further understanding of the PZ organization may require development of new cell-type-specific markers.

Understanding the molecular control of the cellular memory system in which cells can de-differentiate into stem cells, and how CLV signaling interfaces with this system may provide additional clues to the mechanisms of stem-cell homeostasis. An interesting possibility could be that cellular chromatin states play an important role in controlling the spatial limits of the cellular memory system. Studies on murine embryonic stem cells (ES) have identified bi-valent stretches of both H3 lysine 27 methylation (a chromatin mark which negatively regulates transcription) and H3 lysine 4 methylation (a chromatin mark which positively regulates transcription) within genes which encode developmentally important transcription factors, suggesting that the bi-valent domains are responsible for keeping differentiation genes silent in ES cells, while keeping them primed for activation [29,30]. Although the chromatin states of distinct cell types of the SAMs remains to be determined, however, earlier studies have shown that both the cellular chromatin organization and chromatin remodeling factors play an important role in stem-cell homeostasis and SAM development. Mutations in the *FASCIATA class of genes* (*FAS1 and FAS2*) result in over-proliferation of SAMs and an irregular expansion of the WUS domain [31]. *FAS1* and *FAS2* encode subunits of CHROMATIN ASSEMBLY FACTOR-1 (CAF-1) required for maintaining chromatin integrity. Mutations in *SPLAYED* (*SYD*) have been shown to result in premature SAM termination and *SYD* encodes a chromatin remodeling ATPase of the SNF2 class involved in transcriptional regulation [32]. A dynamic analysis of the function of FAS1, FAS2, and SYD involving their transient perturbations followed by live-imaging of gene expression and cell behavior within the stem-cell niche may provide clues to the function of chromatin in specifying the cellular memory system within the SAMs.

Another possible link between chromatin organization and stem-cell specification is the function of retinoblastoma-related (RBR) protein, a master regulator of G1–S cell-cycle transition. The emerging evidence from animal systems suggests that RB interacts with differentiation promoting factors and proteins involved in chromatin modification indicating that RB functions in epigenetic control of gene expression [33]. A recent study in the *Arabidopsis* root apical meristems (RAMs) has revealed that the local reduction in RBR protein results in expansion of columella stem-cell pool without altering their cell-cycle progression, indicating that RBR levels are crucial to turn off stem-cell program in daughters of columella initials [34•]. In addition, the local over-expression of RBR activity in tobacco SAMs has been shown to induce differentiation within the stem-cell domain [35]. Transient perturbation of RBR function followed by live-imaging of SAM stem-cell niche may reveal whether RBR function is required to turn-off stem-cell program in CZ daughters and whether the RBR levels/gradient determines the spatial limits of de-differentiation within the PZ. Taken together, the dynamic analysis of cell fate specification may result in a refined spatial map of different cell types and they may serve as ideal templates to model stem-cell homeostasis.

Conclusions

Live-imaging of cell–cell communication machinery, cell identity transitions and growth are beginning to yield new insights into the mechanisms underlying stem-cell maintenance in the SAMs. The information obtained from live-imaging has been used optimize parameters required to model the organization of the *WUS* expression domain. However, quantitative determination of the spatio-temporal parameters of signaling components and cell-autonomous factors of CLV–WUS network can be challenging considering the complex topology of SAM surface and limited intracellular-spatial resolution because of the smaller cell size. This can be overcome, to some extent, by employing photosensitive fluorescent proteins,

kinetic imaging methods, and image processing algorithms [8,36,37]. Additional experimental approaches are required to deduce new parameters and also to validate the parameters deduced from imagery. Protoplasts isolated from intact roots of *Arabidopsis* have been shown to retain their transcriptional states and this property has been used in generating selforganizing maps of cell-type specific expression patterns [38]. It remains to be tested whether isolated protoplasts, derived from a given cell type, can be used to model transcriptional changes, upon introducing transient gene manipulations. Such a system would not only provide a better temporal resolution in assaying for gene expression changes, but it can also be combined with proteomic approaches to quantify individual proteins of interest in the CLV–WUS reaction network.

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