

RESEARCH COMMUNICATION

WUSCHEL protein movement mediates stem cell homeostasis in the *Arabidopsis* shoot apex

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WUSCHEL (WUS) is a homeodomain transcription factor produced in cells of the niche/organizing center (OC) of shoot apical meristems. WUS specifies stem cell fate and also restricts its own levels by activating a negative regulator, *CLAVATA3* (*CLV3*), in adjacent cells of the central zone (CZ). Here we show that the WUS protein, after being synthesized in cells of the OC, migrates into the CZ, where it activates *CLV3* transcription by binding to its promoter elements. Using a computational model, we show that maintenance of the WUS gradient is essential to regulate stem cell number. Migration of a stem cell-inducing transcription factor into adjacent cells to activate a negative regulator, thereby restricting its own accumulation, is a theme that is unique to plant stem cell niches.

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Cell–cell communication between distinct cell types within stem cell niches is critical for stem cell maintenance in both plants and animals, although they differ in their niche architecture and cell behaviors (Spradling et al. 2008; Rieu and Laux 2009). In animal systems—for example, the *Drosophila* germline—stem cells make direct contact with the niche cells through adherens junctions and receive local signals that prevent their differentiation (Spradling et al. 2008). Oriented and asymmetric division of stem cells places one of the progeny at a distance by a cell diameter, which no longer can receive signals from the niche and differentiates; thus, precise local cell behaviors regulate stem cell number. Whereas in the shoot apical meristem (SAM) stem cell niche not all stem cells make contact with the niche, they also do not exhibit oriented and asymmetric cell divisions to regulate stem cell numbers. For example, the *Arabidopsis* SAM stem cell niche

is a collection of ~500 cells located at the growing tip of each shoot (Reddy 2008). The CZ of the SAM harbors stem cells. The stem cell progeny that are displaced into the adjacent peripheral zone (PZ) proliferate before differentiating (Fig. 1A; Reddy 2008). Visually, the SAM stem cell niche is a multilayered structure consisting of three clonally distinct layers of cells, and stem cells are found in each of these layers. The cells in the L1 and the L2 layers divide parallel to the surface to remain as monolayers. The cells in the L3 layer divide in random orientations to form a multilayered structure referred to as the rib meristem (RM) or the organizing center (OC), which provides stem cell-promoting cues (Rieu and Laux 2009).

Previous studies have shown that WUSCHEL (WUS), a homeodomain-containing transcription factor, is both necessary and sufficient for stem cell specification (Laux et al. 1996). WUS RNA is found in a few cells of the RM/OC located just beneath the CZ (Fig. 1A; Mayer et al. 1998). Restriction of WUS transcription to cells of the OC is critical for maintaining a constant number of stem cells, and this is mediated by the CLAVATA (CLV) signaling pathway (Mayer et al. 1998; Fletcher et al. 1999; Brand et al. 2000). *CLAVATA3* (*CLV3*), expressed in the CZ, encodes a small peptide that is secreted into the extracellular space and binds to CLAVATA1 (CLV1), a leucine-rich repeat receptor kinase predominantly expressed in cells of the RM (Clark et al. 1997; Fletcher et al. 1999; Kondo et al. 2006; Ogawa et al. 2008). Activation of CLV1 and related receptor kinases (Müller et al. 2008; Kinoshita et al. 2010) has been shown to mediate repression of WUS transcription through a signaling cascade that is not well understood. WUS, which is expressed in cells of the RM/OC, not only specifies stem cell fate in overlying cells of the CZ, but also activates its own negative regulator, CLV3, in a non-cell-autonomous manner. Thus, the WUS–CLV feedback system forms a self-correcting mechanism for maintaining a constant number of stem cells and the SAM size.

A live-imaging study has shown that the transient down-regulation of *CLV3* results in sequential expansion of *CLV3* promoter activity and of the CZ cells, suggesting that WUS could activate a diffusible stem cell-promoting signal (Reddy and Meyerowitz 2005). This apart, an earlier study has revealed that a transient increase in WUS levels not only results in expansion of the CZ, but also leads to an increase in cell division rates in the stem cell progenitors located in the PZ (Muller et al. 2006; Yadav et al. 2010). Thus, WUS, expressed in as few as 25–30 cells of the RM/OC, performs both the short- and the long-range functions that span across the CZ and the PZ. Gaining insights into the non-cell-autonomous functions of WUS requires identification of stem cell-promoting signals that emanate from the RM/OC, and also an understanding of the mechanism of communication between the RM/OC and rest of the SAM.

Results and Discussion*WUS protein migrates from OC/niche into adjacent cells*

Intercellular movement of transcription factors forms an important mode of cell–cell communication in several

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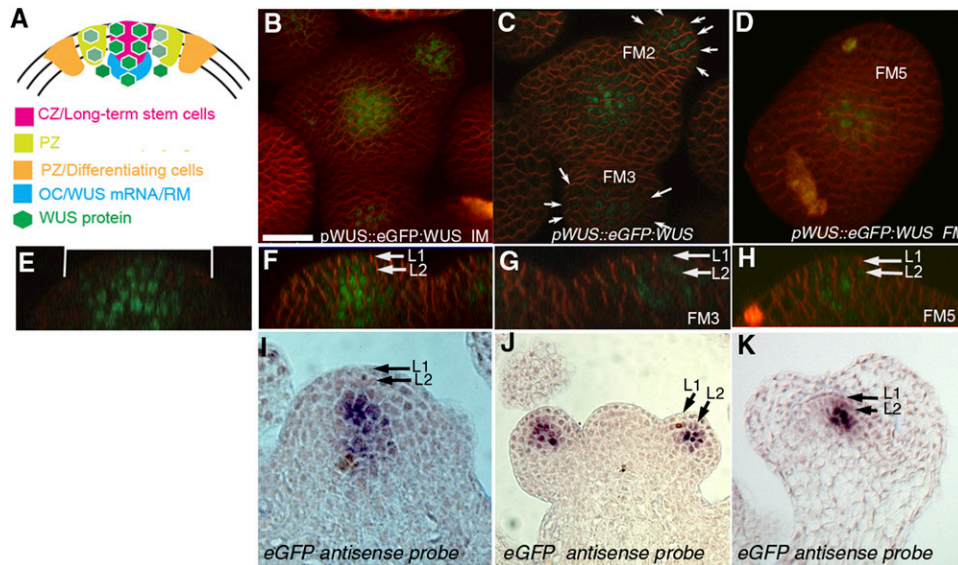


Figure 1. WUS protein movement. (A) A sketch of the *Arabidopsis* shoot apex showing various cell types compared with domains of WUS RNA and protein distribution. 3D reconstructed top view of a *wus-1* mutant SAM rescued by *pWUS::eGFP-WUS* (B) and FMs of stage 2 (C), stage 3 (C), and stage 5 (D) showing eGFP-WUS protein (in green); cell outlines were stained with FM4-64 (red). (E,F) Side views of SAMs showing eGFP:WUS protein extending into the PZ and into the L1 layer. (G,H) Side views of early and late stage FMs showing WUS protein localization. I, J, and K are side views of SAMs, early flowers, and late flowers, respectively, showing *eGFP* mRNA distribution in *pWUS::eGFP:WUS*-expressing plants. Bar, 20 μ M.

plant developmental processes (Gallagher and Benfey 2005). To test the possibility of cell-to-cell movement, we examined WUS protein localization. We generated a chimeric WUS protein by fusing *eGFP* (enhanced green fluorescent protein) protein-coding sequences with the N terminus of the *WUS* ORF. This fusion construct was expressed from the native *WUS* promoter, *pWUS::eGFP:WUS*. After transformation into the *wus-1* mutant background, this construct was able to rescue the mutant phenotype, confirming that it is functional (see the Materials and Methods; Supplemental Fig. S1A) and the eGFP:WUS fusion protein junction was not cleaved (Supplemental Fig. S1G,H). We observed a brighter fluorescence signal in cells of the RM/OC where WUS is expressed and a relatively weaker signal in adjacent cells that extended up to the L1 layer of SAMs and into the PZ (Fig. 1B,E,F). The *eGFP* mRNA distribution in tissue sections of *pWUS::eGFP:WUS*-expressing plants revealed that *eGFP* transcripts are localized to the L3 and deeper layers of the SAMs (Fig. 1I), very similar to the native *WUS* expression pattern described earlier (Mayer et al. 1998), excluding the possibility of *WUS* promoter misexpression from these constructs. A comparison of eGFP:WUS fluorescence with the *GFP* RNA in situ pattern in cells of the top-most L3 layer revealed that the WUS protein moves laterally by at least two cell layers (Supplemental Table S1). De novo stem cell specification occurs in early stages of floral meristem (FM) development, and the *CLV*-*WUS* feedback loop has been shown to mediate stem cell homeostasis in FMs. It has been shown that *WUS* transcripts are restricted to the L2 and deeper cell layers of FMs (Mayer et al. 1998). We observed a WUS protein gradient in FMs that extended up to the L1 layer of both early and late stage FMs (Fig. 1G,H). In early FMs, protein was detected in a much broader radial domain (Fig. 1C) than the *eGFP* mRNA distribution (Fig. 1I), whereas in late stage FMs, the radial spread of the WUS

protein was limited to fewer cells (Fig. 1D). Taken together, the above observations reveal that WUS protein synthesized in cells of the RM/OC of SAMs and FMs migrates to adjacent cells.

WUS protein migration is required for shoot meristem function

Next, we tested whether WUS protein movement is required for its function. It has been shown that efficient targeting of plant transcription factors to the nucleus restricts their movement between cells (Crawford and Zambryski 2000; Gallagher et al. 2004). We generated a chimeric WUS protein in which *eGFP* containing a nuclear localization tag was fused to the N terminus of *WUS* and expressed from a native *WUS* promoter, *pWUS::nls-GFP:WUS*. Nls-GFP:WUS fusion protein was found in the L2 layers of SAMs and in fewer cells in the radial domain (Fig. 2H; Supplemental Fig. 1I) in comparison with the eGFP:WUS protein distribution (Fig. 2B), showing that addition of a nuclear localization signal (nls) inhibits WUS protein movement. In early stage FMs, the fusion protein was detected in the L1 layer (Fig. 2K) and in a broader domain (Fig. 2J), similar to the eGFP:WUS distribution (Fig. 2D). However, in late stage FMs, nls-GFP:WUS was restricted to deeper cell layers and to fewer cell layers in the radial domain, revealing inhibition of protein movement (Fig. 2L). Plant transcription factors have been shown to move between cells through plasmodesmata, plasma membrane-lined channels (Crawford and Zambryski 2000). Plasmodesmata have been shown to have a size exclusion limit (SEL), allowing passage only to those molecules whose size falls below their SEL (Crawford and Zambryski 2000). To test this, we generated a chimeric WUS protein of higher molecular weight by fusing two tandem repeats of *eGFP* with the N terminus of *WUS*, *pWUS::2XeGFP:WUS*.

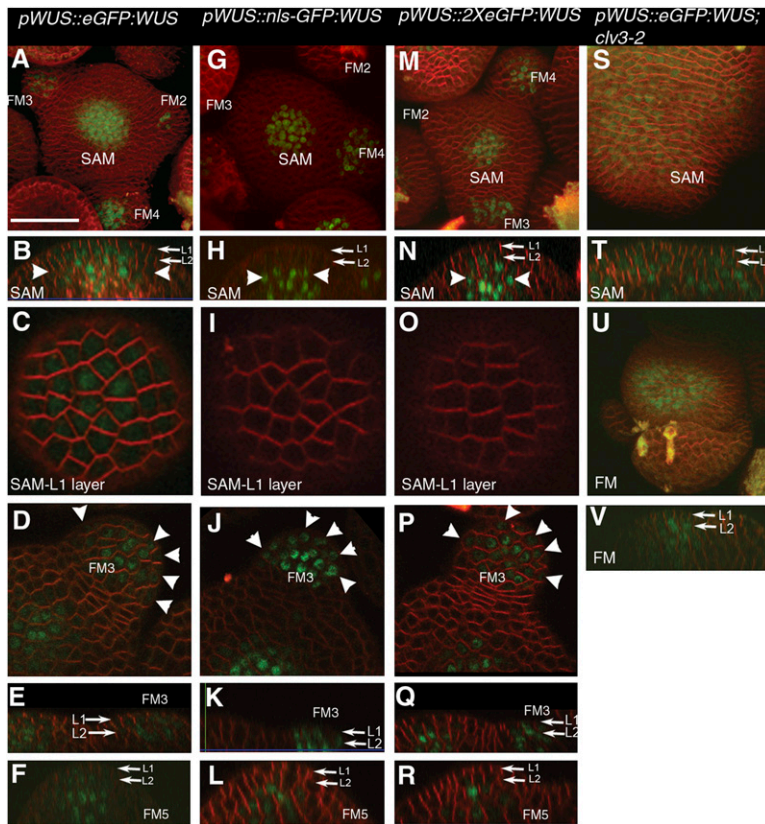


Figure 2. WUS protein movement is required for its function. WUS protein localizations of *pWUS::eGFP:WUS* (A–F), *pWUS::nls-GFP:WUS* (G–L), and *pWUS::2XeGFP:WUS* (M–R). (S–V) *pWUS::eGFP:WUS* in *clv3-2* mutants. (Green) WUS protein; (red) cell membranes. A, G, M, and S are 3D reconstructed top views of SAMs. C, I, and O are confocal cross-sections showing the L1 layer of SAMs. B, E, F, H, K, L, N, Q, R, T, and V are reconstructed side views of SAMs and FMs. (FM3) Stage 3 flower; (FM4) stage 4 flower; (FM5) stage 5 flower. Arrows point to the L1 layer (L1) and the L2 layer (L2). Opposing arrows in B, H, and N indicate the width of WUS protein domains. Arrowheads in D, J, and P indicate the radial domain of WUS expression in FM3. Bars: C, I, O, 10 μ M; all other panels, 35 μ M.

This fusion protein failed to move beyond the L2 layer of SAMs (Fig. 2N) and was detected in fewer cells in the radial domain (Fig. 2N). In early stage FMs, the protein was observed in the L1 layer (Fig. 2Q) and in a broader domain (Fig. 2P), similar to the eGFP:WUS and nls-GFP:WUS. However, in late stage FMs, the 2XeGFP:WUS expression was restricted to deeper layers, showing inhibition of WUS protein movement (Fig. 2R). Both *pWUS::nls-GFP:WUS* and *pWUS::2XeGFP:WUS* failed to rescue the SAM phenotype of *wus-1* mutants (Supplemental Fig. S1B,C). However, in a few cases, they developed axillary shoot meristems, which produced five to six flowers that contained a complete set of organs (see the Materials and Methods; Supplemental Fig. S1D,E). *wus-1* mutants never produce complete flowers, and the rescue of flower phenotype reveals that the fusion proteins retain their biological activity. These results are consistent with the inhibition of movement of nls-GFP and 2XeGFP in SAMs and the lack of inhibition observed in the early stages of FMs. Taken together, these results show that inhibition of WUS protein movement does not interfere with its activity but interferes with SAM function, demonstrating that WUS protein movement is critical for SAM function.

It has been shown that CLV-mediated signaling negatively regulates WUS transcription to restrict its expression to a few cells of the OC (Fletcher et al. 1999; Schoof et al. 2000). In *clv3* mutants, a massive expansion of the CZ and CLV3 promoter expression have been observed, along with the expansion of the WUS domain in underlying cell layers of SAMs and FMs (Fletcher et al. 1999; Schoof et al. 2000; Reddy and Meyerowitz 2005). To examine WUS protein distribution in these enlarged meristems, we introduced *pWUS::eGFP:WUS* into *clv3-2* mutants. WUS protein was detected in the L1 layer cells and also expanded radially in *clv3-2* mutant SAMs (Fig. 2S,T; Supplemental Fig. S1F) and FMs (Fig. 2U,V), suggesting that expansion of the CZ and the CLV3 promoter activity may be a consequence of the presence of the WUS protein in these cells.

WUS binds to CLV3 regulatory regions to activate its transcription

In light of WUS protein movement into superficial cell layers of the CZ, we next examined the possibility of WUS activating CLV3 transcription directly. To test whether WUS is capable of activating CLV3 expression, we used a dexamethasone (Dex)-inducible form of WUS, *35S::WUS-GR*. This construct was generated by fusing the WUS protein-coding region with the ligand-binding domain of the rat glucocorticoid receptor (GR) and was expressed from a ubiquitous promoter. Quantitative RT-PCR (qRT-PCR) analysis on RNA samples extracted from carefully dissected wild-type SAMs expressing *35S::WUS:GR* showed that CLV3 was activated within 4 h of Dex application in both the absence and presence of cycloheximide (Cyc), a protein synthesis inhibitor. This result suggests that CLV3 may be a direct transcriptional target of WUS (Fig. 3A).

To test whether WUS directly binds to the promoter regions of CLV3, we performed chromatin immunoprecipitation [ChIP] coupled to qPCR analysis (ChIP-qPCR) by using anti-WUS (peptide) antibodies (Supplemental Fig. S2A,B). After screening different promoter regions of CLV3, we obtained immunoprecipitated DNA that was enriched in sequences located upstream of and downstream from the ORF of the gene (Fig. 3B; Supplemental Fig. S3A). Fine mapping of WUS-binding sites within the two regions was carried out by testing a series of short oligonucleotides for their ability to bind to WUS using electrophoretic mobility shift assays (EMSA) (Fig. 3D). This analysis revealed the presence of one WUS-binding element in the upstream region and two elements in the downstream region (Fig. 3D). A comparison of DNA sequences of both the upstream and downstream binding elements revealed the presence of a conserved TAAT core in all three (Fig. 3E), which shares similarities with the WUS-binding element described for AGAMOUS (AG) (Lohmann et al. 2001). We confirmed the presence of WUS protein in the WUS-DNA complex by observing a “supershift” when we incubated CLV3 promoter oligonucleotides representing the three binding sequences with anti-WUS antibody (Fig. 3D; Supplemental Fig. S4G–I). Introduction of single

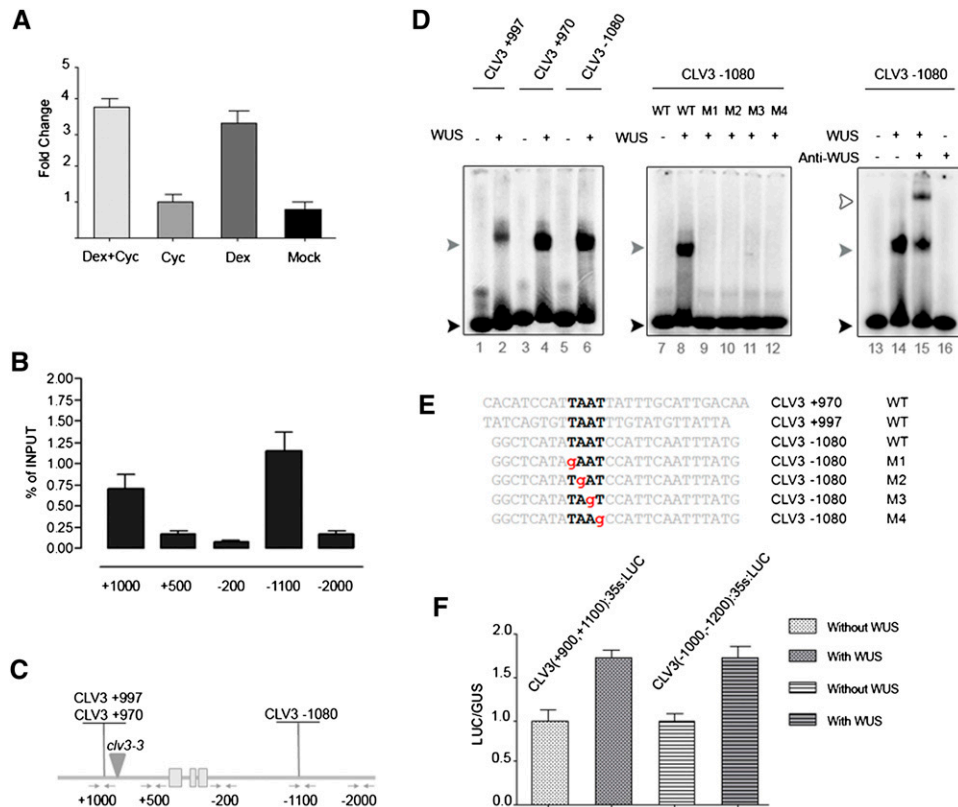


Figure 3. CLV3 is a direct transcriptional target of WUS. (A) qRT-PCR showing the increase in CLV3 transcripts upon activation of WUS by using a Dex-inducible system within 2 h of Dex application in the absence and presence of protein synthesis inhibitor Cyc. Error bars represent standard deviation. (B) ChIP-qPCR showing relative enrichment of regulatory regions of CLV3. Regions are mapped with respect to transcription start site (+1). (C) The *clv3-3* T-DNA allele is located 55 nt in front of the WUS-binding elements located downstream from the ORF. (D, left panel, lanes 1–6) EMSA showing recombinant WUS protein bound to radiolabeled CLV3 oligonucleotides from the +970 and +997 downstream regions and from the –1080 upstream region. (Middle panel, lanes 7–12) Absence of binding of WUS to mutant forms of CLV3 (–1080)-radiolabeled oligonucleotides in which each of the four bases (in red) within the TAAT core have been substituted. (Right panel, lanes 13–16) EMSA showing recombinant WUS protein bound to radiolabeled CLV3 oligonucleotides –1080 in the absence and presence of anti-WUS antibody. A black arrowhead indicates free probe, a gray arrowhead indicates band shift, and a white arrowhead indicates “supershift.” The sequences of wild-type and mutant form (M1–M4) oligonucleotides are indexed in E. (F) Transient transfection assay plots showing activation of LUC reporter when cloned downstream from a 35S minimal promoter region containing the WUS-binding element found in the CLV3 promoter [CLV3(–1000,–1200)::35s::LUC] and a region containing WUS-binding elements found in the CLV3 downstream region [CLV3(+900,+1100)::35s::LUC].

base pair mutation within the TAAT core of the WUS-binding site in the *CLV3* promoter completely abolished WUS binding (Fig. 3D). Competition experiments using increasing concentrations of specific wild-type cold (Supplemental Fig. S4A–C) and mutated cold oligos (Supplemental Fig. S4D–F) demonstrated the specificity of binding of WUS to all three binding sites. We next tested whether the WUS-binding element in the *CLV3* promoter is capable of activating transcription in a transient expression system by examining the LUCIFERASE (LUC) reporter expression levels in protoplasts isolated from mesophyll cells of leaves. These experiments revealed a WUS-dependent activation of *LUC* when the *CLV3* promoter contexts containing the WUS-binding elements were used to drive the *LUC* gene (Fig. 3F). The WUS-dependent activation of *LUC* was abolished when the *CLV3* promoter context carrying a mutated WUS-binding element was used (Supplemental Fig. S5). The loss-of-function *CLV3* allele *clv3-3* (a T-DNA insertion described in an earlier study) (Fletcher et al. 1999) is located ~55 nucleotides (nt) upstream of the 3' WUS-binding element,

suggesting that it may have disrupted WUS-mediated transcriptional regulation. Moreover *CLV3* promoter deletion covering the region containing downstream WUS-binding elements identified here has been shown to significantly reduce the promoter activity (Muller et al. 2006). Taken together, these results demonstrate that WUS binds to the genomic regions of *CLV3* to activate its transcription.

Computational model explains the importance of maintaining WUS protein gradient

Our results show that the expression of the WUS gene is restricted to a few cells of the RM/OC, while the WUS protein migrates into adjacent cells of the CZ to activate *CLV3* through a direct transcriptional control. To understand the importance of WUS protein migration into adjacent cells of the CZ to activate the *CLV3* promoter in the context of the known stem cell regulatory network, we implemented a computational model of a three-dimensional (3D) SAM tissue (Supplemental Fig. S6; see the Supplemental Material). The model includes the direct activation

of *CLV3* transcription from the *WUS* protein (Fig. 2B) and a negative regulation of *WUS* from a *CLV3* signal that emanates from the CZ (Brand et al. 2000; Schoof et al. 2000). In addition, *WUS* is activated by a localized cytokinin signal (Jönsson et al. 2005; Gordon et al. 2010; Hohm et al. 2010), and a hypothetical signal originating from cells of the L1 layer is used as a cofactor for *CLV3* activation (see the Supplemental Material; Jönsson et al. 2003). This minimal network was sufficient to reproduce the wild-type expression domains of *CLV3* and *WUS*, as well as to produce several perturbations (Supplemental Fig. S7). The perturbations include blocked migration of *WUS* protein (Fig. 4B), which results in meristem termination (Supplemental Fig. S1B,C), and also misexpression of *WUS* in cells of the CZ by using the *CLV3* promoter (Fig. 4B), which has been shown to result in expansion of the stem cell domain and *CLV3* expression (Yadav et al. 2010; Brand et al. 2002).

Taken together, our work shows the importance of maintaining the *WUS* protein gradient in regulating stem cell number. In the absence of cell behavioral mechanisms such as asymmetric and oriented cell division and cell migration to fine-tune stem cell numbers, the self-repression of stem cell inducer through a direct transcriptional mechanism may allow quick resetting of the *CLV*-*WUS* feedback loop. Therefore, it is essential to understand the regulation of *WUS* protein movement together with the *CLV3*-mediated transcriptional repression of *WUS*. This will require an understanding of the structural properties of plasmodesmata, the symplasmic connectivity patterns of cells, signals intrinsic to the *WUS* protein, and extrin-

sic signals such as plant hormone cytokinin, which has been implicated in stem cell homeostasis (Leibfried et al. 2005; Gordon et al. 2010). We observed that *WUS* protein migrates laterally into the differentiating progeny of stem cells. New studies are required to unravel *WUS* function in these cells, which may lead to better insights into the molecular mechanisms that regulate differentiation behavior and cell division patterns of stem cell progenitors, both of which are critical for stem cell maintenance.

Materials and methods

Plant growth and live imaging

Plant growth, live imaging, microscopic protocols, and phenotypic analysis have been performed as described earlier (Reddy et al. 2004; Reddy and Meyerowitz 2005; Yadav et al. 2009, 2010).

Plasmid constructs, selection of transgenic lines, and rescue analysis

The details of the *35S::WUS:GR* construct have been published (Yadav et al. 2010). To generate the *pWUS:eGFP:WUS* plasmid, *eGFP* was fused in-frame to the N terminus of the *WUS* ORF and introduced into the *WUS* promoter containing 5.6-kb upstream and 1.2-kb downstream regions between *AscI* and *StuI* in pCAMBIA2300 (Yadav et al. 2008). To generate *nls-GFP:WUS*, *nls-GFP* (kind gift from Dr. Ji Young-Lee) was fused in-frame with the N terminus of the *WUS* ORF and introduced into the *WUS* promoter. To generate *2xeGFP:WUS*, two tandem repeats of *eGFP* were fused in-frame with the N terminus of the *WUS* ORF and introduced into the *WUS* promoter. Transgenic lines were generated in the Landsberg *erecta* background.

To test the functionality of *pWUS:eGFP:WUS*, it was introduced into the *wus-1^{-/-}* background. We generated 55 T1 lines. Through PCR-based genotyping and sequencing, we identified only 16 T1 lines of the *wus-1^{-/-}* or *wus-1^{-/+}* genotypes, which were phenotypically similar to wild-type plants. Since it was not possible to distinguish between *wus-1^{-/-}* or *wus-1^{-/+}* genotypes because of the *eGFP:WUS* transgene, we followed five independent T1 lines in subsequent generations. The progeny of five T2 lines that could be either *wus-1^{-/-}* or *wus-1^{-/+}* were subjected to phenotypic analysis in the T3 generation. All progeny in the T3 generation that carried the transgene showed complete rescue and resembled wild-type plants (Supplemental Fig. S1A). Two independent lines were followed until the T4 generation to establish a homozygous *wus-1^{-/-}* line that is phenotypically similar to wild type. To test the functionality of *pWUS::nls-GFP:WUS*, we generated 47 T1 lines. We identified only seven lines that could be either *wus-1^{-/-}* or *wus-1^{-/+}*, out of which two lines showed *wus-1*-like phenotype. To evaluate the functionality of the *nls-GFP:WUS* protein further, we followed five T1 lines of the *wus-1^{-/+}* genotype in subsequent generations. The T2 progeny of three independent lines that carried the transgene segregated *wus*-like phenotype (Supplemental Fig. S1B). The T2 progeny of the remaining two lines occasionally produced secondary shoots that terminated after making four to six wild-type-like flowers that set seeds, suggesting that *nls-GFP:WUS* can partially rescue secondary shoots, whereas it can completely rescue FM phenotypes (Supplemental Fig. S1D). In the case of *pWUS::2xGFP:WUS*, we generated 27 independent T1 lines. We identified only seven T1 lines that could be either *wus-1^{-/-}* or *wus-1^{-/+}*, and five of these lines exhibited *wus*-like phenotype (Supplemental Fig. S1C). The T2 progeny of the remaining two lines that carried the transgene segregated *wus-1*-like phenotype or produced occasional secondary shoots with few wild-type flowers before terminating (Supplemental Fig. S1E).

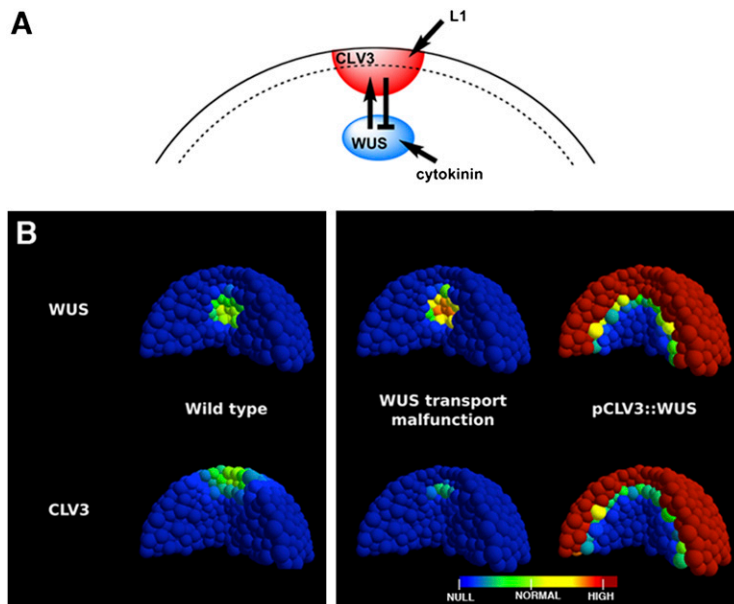


Figure 4. A computational model for 3D patterning of gene expressions in the SAM. (A) Illustration of the gene interactions in the model showing typical expression domains in the SAM. (B) Simulation results from a *CLV3*-*WUS* feedback model (see the Supplemental Material). The *top* row shows *WUS* expression, and the *bottom* row shows *CLV3* expression. The *left* panel shows the wild-type expression domains, the *middle* panel shows the results from a perturbed *WUS* diffusion (the disappearance of the *CLV3* expression can be interpreted as a terminated meristem), and the *right* panel shows the expanded stem cell domain/*CLV3* expression domain along the outer layers of cells upon misexpression of *WUS* from the *CLV3* promoter, as shown in earlier studies (Brand et al. 2002; Yadav et al. 2010). In the color bar, a linear scale is used and “normal” represents the template value used for optimization (1; i.e., concentration value 1 in arbitrary units).

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