Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem

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A central unanswered question in stem cell biology, both in plants and in animals, is how the spatial organization of stem cell niches are maintained as cells move through them. We address this question for the shoot apical meristem (SAM) which harbors pluripotent stem cells responsible for growth of above-ground tissues in flowering plants. We find that localized perception of the plant hormone cytokinin establishes a spatial domain in which cell fate is respecified through induction of the master regulator WUSCHEL as cells are displaced during growth. Cytokinin-induced WUSCHEL expression occurs through both CLAVATA-dependent and CLAVATA-independent pathways. Computational analysis shows that feedback between cytokinin response and genetic regulators predicts their relative patterning, which we confirm experimentally. Our results also may explain how increasing cytokinin concentration leads to the first steps in reestablishing the shoot stem cell niche in vitro.

Plants ranging from the small weed Arabidopsis to the giant sequoia tree, maintain growth of stems, leaves, flowers, and branches through the action of stem cells. In the model plant Arabidopsis, as in other flowering plants, stem cells which give rise to above-ground tissues reside in a structure termed the shoot apical meristem (SAM) (1, 2). The Arabidopsis SAM is composed of three functionally distinct zones. The central zone (CZ) at the tip of the SAM harbors pluripotent stem cells which are necessary for the indeterminate growth and development of the plant. As the plant grows, CZ cells become either multipotent peripheral zone (PZ) cells on the sides of the meristem, capable of differentiating to leaf and flower primordia, or multipotent rib meristem (RM) cells beneath, which can differentiate to the cell types of the stem (3). Positions of zones within the meristem are maintained even as individual cells are displaced from the CZ through the PZ and RM into differentiating tissues. Molecular mechanisms by which meristematic zones are maintained as cells comprising these domains change remains a fundamental question in plant biology (1, 4). One mechanism involves the transmembrane receptor kinase CLAVATA1 (CLV1), expressed in cells of the RM (5). Its ligand, the extracellular peptide product of the CLAVATA3 (CLV3) gene, is produced in the CZ (6), and when it signals the RM cells, they reduce the activity of the WUSCHEL (WUS) gene, which codes for a homeodomain transcription factor also expressed in the RM (7, 8). WUS activity is nonautonomously necessary for the maintenance of the CZ cells as pluripotent stem cells, and therefore for persistence of the SAM (9). Loss of CLV3 activity causes enlargement of the CZ by conversion of PZ cells on the PZ-CZ border to CZ cells within hours, followed by enlargement of the SAM through increased cell division or reduced differentiation, or both, over days (10).

Multiple lines of evidence show that the plant hormone cytokinin is involved in the CLV/WUS circuit, as well as SAM formation, maintenance and growth (1). Cytokinins stimulate the formation of new shoot apical meristems in culture (11). Cytokinin application rescues the SHOOTMERISTEMLESS (STM) mutant, which lacks the ability to maintain the SAM (12), and STM induces cytokinin biosynthetic genes (13, 14). Mutants for the LOG1 gene of rice, which encodes an enzyme that catalyzes the production of active cytokinins in the apical stem cell region of the SAM, have reduced shoot meristem size and prematurely terminate floral meristems (15, 16). Cytokinins act via receptors of the histidine kinase class (AHK2, 3, and 4), which when activated transfer phosphoryl groups to histidine phosphotransfer proteins (HPTs) and thence to two classes of Arabidopsis response regulators (ARRs) (17, 18). The Type-B ARRs activate transcription of cytokinin-induced target genes; Type-A ARRs negatively regulate cytokinin signaling (18–20). WUS has recently been shown to repress the genes for Type-A ARRs, thus likely increasing cytokinin signaling (21). Furthermore, overexpression of a Type-A ARR reduces WUS RNA levels, and can mimic the wus mutant phenotype (21) [SAM termination (22)]. Cytokinin treatment induces CLV loss-of-function phenotypes and causes increased WUS and decreased CLV1 expression (23, 24).

In this study, we reveal multiple feedback loops between cytokinin response and WUS which influences gene expression and patterning within the Arabidopsis SAM. We use live imaging and an array of reporters to show that cytokinin perception and response is localized within the SAM where it regulates the pattern of WUS, a key positive genetic regulator of stem cell fate. We demonstrate that cytokinin signaling activates WUS expression through both CLV-dependent and CLV-independent pathways. We develop a computational model of cytokinin signaling which shows that feedback between cytokinin response and key genetic regulators determines the probability that a cell will express WUS. Given that WUS-expressing cells promote stem cell number and recent evidence that stem cells are a source of active cytokinins (16), our results may support a positive feedback loop between stem cells and underlying RM cells that maintains the organization of the SAM as stem cells are displaced during growth.

Results

CLV-Dependent and CLV-Independent Regulation of WUS by Cytokinin Signaling. Prior studies have shown that WUS expression within the SAM is partly restricted spatially through negative feedback from the CLV pathway shown in Fig. 1A. Recent studies have shown that treatment of plants with high levels of cytokinin leads to CLV loss-of-function phenotypes and causes increased WUS expression (21, 23, 24). Our results here, detailed below, extend these observations and suggest an additional level of regulation by cytokinin.

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and decreased CLV1 expression (23, 24). These data lead to a qualitative model in which cytokinin treatment increases WUS expression through suppression of CLV-mediated negative feedback on WUS levels (Fig. 1B). To test this starting hypothesis, we quantified the effect of cytokinin treatment on CLV1 and ARR5 transcription, as measured by quantitative reverse transcriptase PCR (qRT-PCR). As previously reported (20, 23), 24 h of cytokinin treatment reduced CLV1 RNA levels and increased RNA for the Type-A ARR, ARR5 (Fig. 1 C and D). To test whether repression of the CLV pathway is the only mechanism of WUS induction by cytokinin (23), we performed cytokinin treatments in a clv1–11 loss-of-function mutant. At 4 and 24 h after cytokinin treatment, WUS RNA increased in both wild-type and mutant lines compared to mock-treated samples (Fig. 1E and F); by 24 h WUS transcript was increased approximately 40-fold in both genotypes. Pretreatment of the plants with the protein synthesis inhibitor cycloheximide did not prevent this induction (Fig. 1G), suggesting a direct effect. Cytokinin treatment also induced WUS transcript accumulation in a clv3–2 loss-of-function mutant background, suggesting that induction in the clv1–11 mutant is not due to redundant function of related CLV3-dependent kinases active in the SAM, such as BAM1, 2 and 3 (25), CLV2 (26), or CORYNE (27). We observed greater phenotypic enhancement of floral organ number (an indicator of increased floral meristem size and stem cell activity) by cytokinin in clv1 and clv3 mutants compared with wild type, suggesting a synergistic interaction between cytokinin and CLV loss of function (Fig. 1H, two-way ANOVA, F = 81, P < 0.0001). In contrast to wild type, cytokinin (benzylationpurine, BAP) treatment of clv mutants resulted in massive enlargement of the SAM and floral meristems (Fig. S1). Similar fold induction of WUS transcript in wild type and clv mutants after continuous cytokinin treatment reveals the existence of CLV-independent mechanisms of cytokinin-induced WUS expression (Fig. 1 E–G). However, greater phenotypic enhancement in CLV loss-of-function background indicates that the CLV pathway limits the effect of transient perturbations in cytokinin signaling and therefore indicates that there are also CLV-dependent effects (Fig. 1H; see Computational Modeling in SI Appendix).

Feedback Between Cytokinin Signaling and the WUS/CLV Circuit Influences Patterning of Gene Expression. After 24 h, CLV1 and ARR5 transcript levels were altered at low cytokinin concentrations. However, increase in WUS transcript occurred only at high concentration (Fig. 2A). Finer dilutions showed a steep rise in WUS transcript and corresponding decrease in ARR5 beginning at 400 μM and peaking near 600 μM (Fig. S1). We reasoned that the observed increase of WUS transcript after cytokinin perturbation could be indicative of a role for endogenous cytokinin response in influencing the pattern of WUS expression. Cytokinin response can be visualized at high resolution using a synthetic reporter, pTCS::GFP (28), which reports downstream activation of the cytokinin signaling pathway. Our data showing the sharper and higher threshold of cytokinin required for WUS induction as compared to ARR5 (Fig. 2A) indicates that WUS should closely overlap spatially with high levels of cytokinin response and drop off sharply in cells less responsive to cytokinin within the SAM. Consistent with this hypothesis, the pTCS::GFP reporter for cytokinin response was activated in a similar domain to WUS (Fig. 2 B–D), pTCS::GFP expression mirrored temporal dynamics of WUS reporter expression during floral meristem development and SAM regeneration in culture (Fig. S2), consistent with a model where WUS is spatially regulated by cytokinin signaling during development (29).

Previous studies have shown that WUS directly suppresses the transcription of a subset of Type-A ARRs involved in negative feedback on the cytokinin signaling pathway. Thus WUS likely increases cytokinin signaling (21). From the perspective of a gene regulatory network, regulation of WUS levels by cytokinin signaling, either by CLV-dependent or independent pathways, completes a positive feedback loop between cytokinin signaling and WUS. Cytokinin-induced increase in WUS levels leads to greater suppression of Type-A ARRs which leads higher cytokinin signaling and thus higher WUS levels. To better understand the nature of these potential positive feedback loops we used computational modeling (see Computational Modeling in SI Appendix for details) to plot predicted steady state values of WUS as a function of cytokinin signaling (Fig. 2 F–J). We first considered the three hypothetical networks shown in Fig. 2E. In the circuit displayed in Fig. 2E (1), cytokinin signaling regulates WUS through suppression of CLV1 alone. Alternatively, in the network displayed in Fig. 2E (2) WUS transcription is activated...
WUS can be induced an order of magnitude greater than in the first two cases, similar to the experimentally observed an approximate 40-fold increase. Suppression of CLV1 transcription by cytokinin signaling allows CLV-independent induction of WUS to occur with less suppression from the CLV pathway. The effect of functional CLV negative feedback is shown in Fig. 2G. Plots in Fig. 2G show that in the absence of CLV negative feedback (3*), WUS is induced at lower levels of signaling than in circuits in which the CLV pathway is present (3). Negative feedback on cytokinin signaling through Type-A ARRs also contributes to the high threshold required for WUS induction.

We used our computational model (see Computational Modeling in SI Appendix) to predict the pattern of components within the circuit displayed in Fig. 2E (3), given our data showing a central peak of cytokinin signaling within the SAM (Fig. 2B–D). Plots of predicted steady state values of activated B-Type ARR, WUS, and the Type-A ARR, ARR5 (known to be suppressed by WUS) are shown in Fig. 2H–J. These plots show that WUS is predicted to closely overlap with cytokinin signaling as we observe experimentally. In contrast, ARR5 is predicted to be suppressed where cytokinin signaling is highest and expressed strongly in a peripheral ring-shaped domain.

**Distribution of Cytokinin Receptor, Cytokinin Response, and WUS Correlate in Individual Cells Where ARR5 Is Suppressed.** To test the predictions of our model, we experimentally determined the relative spatial expression of WUS and the Type-A ARR, ARR5. We observed that a transcriptional reporter for ARR5 was suppressed in the WUS domain but expressed strongly in adjacent cells forming a ring-like expression pattern (Fig. 3A–C), consistent with the predictions of our computational model.

Activation of WUS expression by cytokinin perturbation and overlap of WUS expression with the reporter of downstream cytokinin response, pTC5::GFP, suggested that endogenous cytokinin response might act as a positional cue for patterning WUS transcription.

Upstream of WUS function, cytokinin response is governed by cytokinin receptor availability and the local concentration of cytokinin. Therefore, localized cytokinin response within the center of the SAM could be indicative of either a higher local concentration of cytokinin or increased perception of cytokinin in these cells through localized receptor expression. To investigate the latter possibility we determined the distribution of cytokinin receptor expression within the SAM. Indeed, fluorescent reporters for the cytokinin receptor AHK4 (30), and WUS transcription were expressed in overlapping domains within the SAM and were correlated in individual cells (Fig. 3D–H). AHK4 and WUS reporters were similarly regulated during floral meristem development, expanded similarly in the clv3–2 mutant and were both altered in super-enlarged cytokinin-treated clv3–2 SAMs (Fig. 3I–L and Fig. S1). AHK4 and WUS reporters also overlapped during SAM regeneration in culture. AHK4 reporter was induced in cultured cells during pretreatment on auxin-rich medium known to promote regeneration (Fig. S2). Transfer to cytokinin-rich medium resulted in WUS induction (29) in cells marked by the AHK4 reporter in developing SAMs (Fig. S2).

**Cytokinin Regulates Domain of Cytokinin Signaling Output, WUS and CLV3 Expression.** The above results suggested that cytokinin receptor distribution initiates a gradient of cytokinin signaling peaking within the center of the SAM and which patterns WUS expression in multiple contexts. This model predicts that treatment of plants with exogenous cytokinin would extend sufficient signaling to cells farther from the center of the SAM which have lower levels of receptor, causing WUS activation in an expanded domain. Indeed, live imaging before and after 12 h of cytokinin treatment showed expansion of the WUS reporter expression domain (Fig. 4A–D and Fig. S3). We observed respecification of
cells that previously did not express WUS, indicating that WUS domain expansion was not solely due to increased cell division in the RM (Fig. 4 A–D). Therefore, similarly to recruitment of surrounding cells into CZ cells after loss of CLV3 activity (10), cytokinin increase leads to recruitment of surrounding cells into WUS expressing cells. After 24 h of cytokinin treatment we observed expanded pTCS::GFP and pCLV3::GFP-ER reporter expression within inflorescence and floral meristems which was not observed in mock treated samples (Fig. 4 E–H). Cytokinin treatment was also sufficient to induce ectopic WUS expression, but only in cells which express high levels of cytokinin receptor (Fig. S3) (30).

Cytokinin-Induced Increase of WUS Transcript and Related Meristem Phenotypes Requires a CLV-Independent Pathway Through an AHK2/AHK4-Dependent Mechanism. Our results suggest that cytokinin receptor distribution controls the distribution of cytokinin response and thereby influences the pattern of WUS expression within the SAM. To determine whether the induction of WUS by cytokinin perturbation requires functional cytokinin receptors we quantified WUS levels in cytokinin receptor loss-of-function backgrounds (Fig. 5 A). Of the three characterized cytokinin

receptors only AHK2 and AHK4 mutants showed significantly lower relative WUS transcript levels after cytokinin treatment as compared to wild type (one-way Anova, F = 42, P < 0.05, Fig.
expressing cells in the RM promote stem cell fate in overlying cells. Such positive reinforcement between these two domains could maintain their juxtaposition as the apical stem cells are displaced during post embryonic growth.

During in vitro reestablishment of the shoot stem cell niche in tissue culture, high cytokinin signaling triggers induction of ectopic WUS expression leading to stem cell fate in surrounding cells (29). Ectopic WUS expression is sufficient for induction of shoot tissues and WUS is functionally required for de novo formation of the SAM in vitro (29, 31, 32). Therefore induction of WUS through cytokinin treatment may be a key link in triggering the formation of shoot tissues in culture. Cytokin-in is sufficient to induce WUS expression in the stele of root explants where AHK4 is expressed (Fig. S3). Auxin pretreatment of tissue explants is used to enhance the efficiency of regeneration in culture (29). Our results show that auxin treatment leads to callus formation associated with broad up-regulation of the AHK4 receptor (Fig. S2). Thus, it is possible that the ability of auxin pretreatment to enhance regeneration in culture is mediated through the up-regulation of cytokinin receptor expression. This enables a larger population of cells to be competent to respond to cytokinin and trigger high cytokinin signaling required for up-regulation of WUS when explants are subsequently induced with cytokinin. The ability of cytokinin signaling to alter cell fate through induction of WUS expression is a common thread that links in vitro regeneration of shoot tissues and normal shoot development.

Cytokinin receptors appear to be redundant in many contexts (18). One known example of cytokinin receptor specificity is in the control of leaf senescence in Arabidopsis that specifically requires the AHK3 receptor (33). We demonstrate that cytokinin-induced up-regulation of WUS transcript is mediated primarily through AHK2 and AHK4 dependent pathways and does not require the AHK3 receptor. Furthermore, cytokinin-induced clv mutant-like phenotypes associated with WUS misregulation are not observed in the ahk2–2 mutant. Published microarray data suggests that expression of the cytokinin receptors is not involved in a positive feedback loop with WUS, as transient overexpression of WUS leads to reduction of AHK4 levels and does not significantly alter AHK2 levels (21).

Previous computational models have addressed how WUS expression is confined to a small number of cells within the SAM (34), and recently how the CLV and WUS cell populations maintain each other through feedback between WUS and the CLV pathway (35). In the first study, several alternative hypotheses to maintain WUS spatial pattern were considered. A model that assumed a localized activation of WUS was best able to reproduce its experimentally observed pattern. In this study we propose that cytokinin is a potential candidate for an activator similar to the hypothetical activator described in the above study, which is locally perceived within the SAM and thus influences the WUS expression pattern. Our study represents an attempt to computationally model the cytokinin signaling pathway. We then integrate this model with components of the WUS/CLV feedback system. Hence, our model is similar to previous studies (34, 35) in how components of the WUS/CLV pathway interact to spatially maintain their regions within the SAM. However, unlike previous studies, the model reported here provides an understanding of hormonal feedback on gene expression, through a detailed study of the cytokinin perception network. This study (see Computational Modeling in SI Appendix) reveals the functionality of several nested feedback loops which suggest a threshold-dependent activation of WUS, as a function of cytokinin. The threshold for activation occurs because sufficient cytokinin must build up before: (i) negative feedback of the CLV pathway is suppressed; (ii) sufficient WUS is accumulated to repress Type-A ARRs and promote further increases in WUS transcription. The positive feedback inherent in this circuit has

**Discussion**

We propose that within the shoot meristem a standing gradient of cytokinin response, dictated in part by cytokinin receptor distribution, acts as spatial reference to inform cells of their position. As cells move into the RM, high cytokinin signaling triggers cell respecification through induction of WUS. Given recent evidence for localized production of active cytokinins in shoot stem cells (15), our results support a feedback principle for maintenance of stem cell niche organization during growth (Fig. 5D). Stem cells specify RM cell fate through production of active cytokinins which are locally perceived by underlying cells leading to induction of the master regulator WUS. In turn, WUS-

**Fig. 5.** Cytokinin regulates WUS expression through an AHK2/AHK4 dependent mechanism while CLV1 suppression has no requirement for individual receptors. (A) Relative WUS or (B) CLV1 transcript levels in wild-type and individual cytokinin receptor mutants after 24 h of mock treatment or cytokinin treatment. (C) Cytokinin-induced clv mutant-like carpel number phenotypes in wild type (COL), ahk2–2, and ahk3–3 mutants. (D) Hypothetical positive feedback between apical stem cells and RM cells. Apical stem cells produce active cytokinins (CKs) perceived by RM cells expressing sufficient cytokinin receptor to activate WUS expression, WUS, in turn, promotes stem cell fate in apical cells. Negative feedback from the CLV pathway is also shown.

5A). In contrast, relative WUS transcript was not significantly different between cytokinin treated AHK3 mutant and wild-type samples. Consistent with this observation, clv mutant-like phenotypes associated with WUS misregulation after cytokinin treatment were not observed in the ahk2–2 mutant but were observed in the ahk3–3 mutant similar to wild-type plants (Fig. 5C). In comparison to WUS, cytokinin-induced suppression of CLV1 transcript occurred at a similar magnitude in all backgrounds (Fig. 5B). These data indicate that the AHK2 and AHK4 receptors are required for cytokinin-induced up-regulation of WUS transcript levels and associated clv mutant-like phenotypes. In contrast, cytokinin-induced suppression of CLV1 transcript does not have specific requirements for individual receptors. The fact that CLV1 was suppressed in all backgrounds but WUS up-regulation and cytokinin-induced clv mutant-like phenotypes were blocked in the AHK2 mutant (ahk2–2), suggests that induction of clv mutant-like phenotypes by cytokinin treatment requires a CLV-independent pathway of WUS induction by cytokinin.

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that our experimental and computational modeling data suggests mechanisms. Given that expression through both CLV-dependent and CLV-independent required to extend the domain of cytokinin response and unperturbed pattern, as monitored by the pTCS::GFP reporter. The relatively sharp expression profile of the cytokinin receptors may also explain why strong cytokinin perturbations were required to extend the domain of cytokinin response and WUS cytokinin response within a given cell is the output of receptor concentration and cytokinin. Therefore cells with only low levels of receptor, such as cells farther away from the center of the SAM, require high levels of cytokinin to have significant cytokinin response.

This study shows that cytokinin response regulates WUS expression through both CLV-dependent and CLV-independent mechanisms. Given that WUS promotes cytokinin response (21), our experimental and computational modeling data suggests that WUS and cytokinin signaling interact through multiple positive-feedback loops which ultimately control stem cell number in the SAM. Future studies will show whether cytokinin acts as signaling cue to relay information between cells in different domains of the SAM as cells comprising different zones change during growth of the plant.

**Materials and Methods**

**Plant Materials and Reporter Constructs.** cvt–1, cvt–2, and cvt–2 alleles in Ler background have been previously described (36, 37). The pWUS::GFP line in Columbia (Col-0) background has been previously described (38), and it recapitulates expression patterns observed in the shoot and root via in situ hybridization (30). The pARR5::GFP line in WS ecotype has been previously described (13). The pWUS::GFP-ER and pCLV3::GFP-ER lines have also been previously described (10, 34) for details of other lines see SI Materials and Methods.

**Plant Growth and Cytokinin Treatment Conditions.** Plants were grown as previously described (29). Cytokinin treatments with N6-benzylaminopurine (BAP; Sigma–Aldrich Co.) were performed as described (23) except that shoots were sprayed with the respective solutions (for details see SI Materials and Methods).

**Quantitative Real-Time PCR (qRT-PCR).** Quantitative real-time PCR (qRT-PCR) was performed with Roche Universal Probe Library hydrolysis probes. Each sample represents tissue harvested from 50 two-week-old seedlings just transitioned to flowering (for details see SI Materials and Methods).

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