The control of the location and activity of stem cells depends on spatial regulation of gene activities in the stem cell niche. Using computational and experimental approaches, we have tested and found support for a hypothesis for gene interactions that specify the Arabidopsis apical stem cell population. The hypothesis explains how the WUSCHEL gene product, synthesized basally in the meristem, induces CLAVATA3-expressing stem cells in the meristem apex but, paradoxically, not in the basal domain where WUSCHEL itself is expressed. The answer involves the activity of the small family of HAIRY MERISTEM genes, which prevent the activation of CLAVATA3 and which are expressed basally in the shoot meristem.

Distinct cell types in multicellular organisms form specific patterns during development, and how the patterns are regulated is a critical question. In Arabidopsis shoot apical meristems (SAMs), stem cells reside at and near the apex, whereas the cells specifying the stem cells are located more basally (1). Along the apical-basal axis, the homeodomain transcription factor WUSCHEL (WUS) and the secreted peptide CLAVATA3 (CLV3) form a negative feedback loop mediating communication between the stem cells and the beneath-rib meristem cells (2–9). CLV3 is highly expressed in the apical stem cells (2–4), whereas the WUS transcript is confined to the center of the rib meristem (5, 6). WUS protein moves apically via plasmodesmata (cell-cell connections) into the stem cells (10–12) to activate CLV3 expression, reportedly through direct binding to the CLV3 promoter (10, 12). How the apical-basal patterns of CLV3 and WUS mRNAs are initiated and maintained is unknown. One central question is why CLV3 is activated only by WUS that has transited into the apical stem cells and is not maintained in the interior cells, where the WUS protein concentration is highest and where WUS is actively expressed. It has been proposed that either WUS requires an as-yet-unidentified signal from the epidermis (L1) of the meristem for CLV3 activation (13, 14) or WUS can convert itself from a repressor to an activator when its concentration is low (12). Here, we propose a different mechanism. We previously found that members of the HAIRY MERISTEM (HAM) family, GRAS-domain transcription factors, function as interacting partners of WUS to control the production of shoot stem cells (15). The HAM proteins are involved in meristem regulation and the CLV3-WUS pathway (15–18), and CLV3 is ectopically expressed in the rib meristem of a ham multiple mutant (16). Using computational and experimental approaches, we have shown that in the SAMs, WUS activates
CLV3 only in the absence of HAM, and in the initiating meristems, an apical-basal gradient of HAM defines the patterning of the CLV3 expression domain.

Imaging of fluorescent reporters for both HAM and CLV3 in the same living SAMs shows that the expression patterns of HAM and CLV3 mRNAs are nearly complementary, with opposite concentration gradients along the apical-basal axis (Fig. 1, fig. S1, and movies S1 to S3). HAM1 and HAM2 are highly expressed in the rib meristem and peripheral zone in the corpus (Fig. 1 and fig. S1), where CLV3 expression is reduced. In contrast, HAM1 and HAM2 expression is not detected in the L1 and L2 layers of the central zone, where CLV3 is highly expressed (Fig. 1 and fig. S1). In addition, we previously showed that HAM1, HAM2, and WUS are co-expressed in the same cells at the center of the corpus (15), and HAM protein was not detected in the central zone (15). This imaging (Fig. 1 and fig. S1) and previous reports (4, 7, 10–12, 15) have revealed the distinct and overlapping expression patterns of WUS, CLV3, and HAM in the SAM (fig. S2), which leads to the hypothesis that in the apical stem cells where HAM is absent, CLV3 mRNA production is activated by WUS; at the basal part of the SAM where HAM proteins are present, the ability of WUS to activate CLV3 mRNA production is suppressed.

To test this hypothesis, we first established a new three dimensions–plus–time (3D+t) computational model to simulate the patterns of CLV3 and WUS transcripts and the movement of CLV3 peptides and WUS proteins during meristem development (see methods). The model incorporated the current knowledge of the CLV-WUS feedback (2–8, 19); included an activator of WUS transcription, the organizing center signal at the center of the meristem corpus; and took the concentration gradient of HAM as an input. We modeled the movement of WUS protein as a passive diffusion-like transport, as previously reported (13, 14). Most notably, we defined WUS as the activator of CLV3 transcription when HAM is absent but not when HAM protein is present. We represented CLV3 peptide as a repressor of the WUS mRNA level and modeled the peptide’s rapid apoplastic movement between cells (8). This model was able to reproduce the specific patterns of the WUS transcript, WUS protein, and CLV3 transcript in a wild-type SAM, and these patterns were resistant to perturbations introduced by cell growth and divisions (movies S4 to S6, S8, and S9). In addition, a simplified 1D+t cell layer model was able to predict the patterns of WUS and CLV3 expression along the apical-basal axis (movie S7), suggesting that the apical-basal polarity of gene expression can be uncoupled from lateral cell proliferation. By contrast, if HAM was converted into an activator (either together with WUS or in addition to WUS) of CLV3 transcription, we were not able to reproduce the wild-type CLV3 mRNA pattern (fig. S3).

We further tested the hypothesis and validated the computational model by introducing a spatial perturbation of gene expression both in silico and in vivo. The absence of HAM protein at the center of the meristem L1 layer (Fig. 1, A to E, and fig. S1, A to E) prompted us to test the effect on CLV3 patterning of specifically expressing HAM in the L1 layer. Our model predicted that CLV3 mRNA in the L1 layer would be markedly reduced because of the absence of activation in the L1 and that the peak of CLV3 expression would shift into deeper cell layers (Fig. 2, A and B). We reproduced this perturbation by generating pATML1::HAM1m-GFP (LI-HAM) transgenic plants that express a HAM1m–green fluorescent protein (GFP) fusion in L1 (fig. S4 and movies S10 and S11) from an epidermis-specific promoter (20). We found that the activity of the CLV3 reporter in L1 of the LI-HAM SAM was reduced compared with either the level in cells below L2 from the same LI-HAM SAM or the level in L1 in a wild-type SAM (Fig. 2, C to H). In addition, the SAMs of LI-HAM plants were substantially enlarged compared with the wild type (Fig. 2, C and D, and fig. S5), demonstrating the functional implications of keeping HAM levels low and CLV3 high in the epidermis.

The model also predicted that partial repression of HAM (18, 21) is sufficient to alter the CLV3 pattern (fig. S6, A and B), which is consistent with the experimental results and quantitative analyses (fig. S6). This partial repression of HAM led to a substantial increase in CLV3 mRNA levels (fig. S6, C and D), more cells expressing CLV3 in the SAMs (fig. S6E), and a basal shift of the CLV3 expression peak (fig. S6F).

A series of genetic perturbations was introduced to further dissect the roles of HAM and WUS in CLV3 patterning along the apical-basal axis in the SAM. The model made the following predictions in parallel (Fig. 3, A to D): When HAM is absent in a SAM, CLV3 mRNA will be locally activated by WUS, with the concentration peak in the basal part of the SAM (Fig. 3, A and B). When HAM is present in a SAM but the transcriptional activity of WUS is reduced, the CLV3 mRNA will still be expressed in the apical part of the SAM, with the level substantially reduced (Fig. 3C). Furthermore, when HAM is absent and the transcriptional activity of WUS is reduced, CLV3 will be expressed in the basal part of the SAM at a reduced level (Fig. 3D). To
test these predictions, we examined the CLV3 mRNA in the SAMs of the wild type, ham1 ham2 ham3 triple loss-of-function mutants, wus-7 partial loss-of-function mutants (15, 22), and wus-7 ham1 ham2 ham3 quadruple mutants (Fig. 3E to H). As assessed by in situ hybridization experiments, the CLV3 expression domain in ham1 ham2 ham3 mutants shifted to the center of the rib meristem (Fig. 3F), as observed previously (16), whereas the CLV3 mRNA level was reduced locally in the central zone in a wus-7 SAM (Fig. 3G). In the wus-7 ham1 ham2 ham3 SAM, CLV3 mRNA expression shifted to the rib meristem, and its level was substantially reduced compared with that in the ham1 ham2 ham3 SAM (Fig. 3H).

In addition, the wus-7 ham1 ham2 ham3 mutant displayed a much reduced SAM size (Fig. 3H), consistent with the previous finding that HAM and WUS work together in control of stem cell homeostasis (15). Aside from the partial loss of function (Fig. 3C), the computational model predicted that when WUS activity is completely lost (Fig. 3, I to L), CLV3 mRNA will be absent because this major activator of CLV3 is absent, regardless of whether HAM is present (Fig. 3, K and L). These predictions were validated through the CLV3 mRNA in situ hybridization in the SAMs of the wild type, ham1 ham2 ham3 and wus-1 null mutants (5), and wus-1 ham1 ham2 ham3 quadruple mutants (Fig. 3, M to P, and fig. S7). We found that CLV3 mRNA was undetectable in both wus-1 and wus-1 ham1 ham2 ham3 plants, and wus-1 ham1 ham2 ham3 plants displayed a terminated meristem similar to that in wus-1 plants (Fig. 3, O and P). These results (Fig. 3) reveal distinct roles of HAM and WUS in determining the CLV3 mRNA pattern in an established SAM: WUS maintains the overall CLV3 level, whereas HAM defines the apical-basal positioning of the CLV3 expression domain.

We further examined whether the HAM-WUS-CLV3 regulatory loop defines the initiation of polarity during de novo meristem formation from leaf axils (Fig. 4, A to P, and figs. S8 and S9). Different from that in the already established apical meristem, expression of CLV3 in the initiating axillary meristem (AM) (stages S2 and S3) (23, 24) is first seen in the corpus (24). To seek the underlying mechanism, we first examined the expression pattern of HAM1 at early stages of AM initiation. At S2, HAM1 is evenly expressed in the initiating meristem, with no gradient from the epidermis to the interior cells (Fig. 4A). At S3, HAM1 is also expressed throughout the initiating meristem, lacking a clear gradient (Fig. 4E). Both patterns are distinct from the HAM1 pattern in the established SAMs (Fig. 1) (15). When these HAM1 patterns (Fig. 4, A and E) were used as inputs for AM simulation (Fig. 4, B and F), the model predicted that the CLV3 domains in the
wild type would be confined to the basal part of the initiating meristems (Fig. 4, C and G), suggesting that when a HAM concentration gradient does not exist or is very shallow, the CLV3 mRNA pattern is determined predominantly by the WUS concentration. These predictions are consistent with our experimental results (Fig. 4, D and H) and previous observations (24). The concentration gradient of HAM1 from the epidermis to interior cells at late stages of AM initiation (Fig. 4, I and M) has been established and is comparable to the pattern in the SAMs (Fig. 1). With this information as the input (Fig. 4, J and N), the model predicted that at these stages (Fig. 4, K and O), CLV3 would be expressed predominantly in the apical domain of the initiating AMs; this was experimentally validated (Fig. 4, L and P) (24). Thus, the dynamic gradient of HAM drives the CLV3 pattern dynamics during de novo formation of a new stem cell niche (movie S12).

We then simulated the patterns of CLV3 mRNA expression at different stages during AM initiation when HAM activity is absent (Fig. 4Q). The model predicted that CLV3 expression domains

Fig. 4. Patterning during the de novo formation of axillary stem cell niches in the wild type and ham1 ham2 ham3 mutants. (A, E, I, and M) In situ hybridization to HAM1 RNA in the wild type at early [(A) and (E)] and late [(I) and (M)] stages of AM initiation. (B, F, J, and N) Levels of HAM concentration in the wild type at early [(B) and (F)] and late [(J) and (N)] stages as the input. (C, G, K, and O) Simulated CLV3 mRNA levels at early [(C) and (G)] and late [(K) and (O)] stages in the wild type. (D, H, L, and P) Validation of the simulation through the in situ hybridization to CLV3 mRNA in a wild-type AM at early [(D) and (H)] and late [(L) and (P)] stages. (Q) The HAM concentration was set at zero in ham1 ham2 ham3 mutants at all stages as the input. (R) Simulated CLV3 mRNA levels at different developmental stages in ham1 ham2 ham3 mutants. (S and T) Validation of the simulation through RNA in situ hybridization of ham1 ham2 ham3 AMs. The initiation of AMs in ham1 ham2 ham3 plants was disturbed and did not follow the well-characterized developmental stages (23, 24). The early (S) and late (T) stages of AM initiation in ham1 ham2 ham3 mutants were defined on the basis of the distance of leaf axils from the same main SAM in longitudinal sections. In each individual cell, the relative HAM protein level (input) is indicated by color (blue, 0; red, maximal level of 1), and the relative CLV3 mRNA level (output) is also indicated by color (blue, 0; red, 1.23 a.u.). Arrows: HAM1-expressing cells [(A), (E), (I), and (M)] and CLV3-expressing cells [(D), (H), (L), (P), (S), and (T)] during new meristem initiation. Scale bars, 50 µm.
would be confined to deep cell layers at all times (Fig. 4R and movies S13 to S15), which is consistent with the experimental results (Fig. 4, S and T). The computational and experimental results together demonstrated that the lack of either a HAM gradient or HAM expression leads to similar CLV3 RNA patterns, suggesting that the HAM concentration gradient in a meristem acts in specifying the apical-basal polarity of the CLV3 mRNA pattern. Additionally, the defects in CLV3 patterning during meristem initiation (Fig. 4, S and T) could be the molecular basis of the shoot-branching phenotypes of the ham1 ham2 ham3 mutants (16–18).

In contrast with previous models (33, 14, 25), this work, complemented by a recent theoretical study (26), reveals a regulatory circuit involving three components—WUS, HAM, and CLV3—that sustains both the initiation and the maintenance of the apical-basal polarity of distinct cell types in the plant apical stem cell niche.

REFERENCES AND NOTES

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SUPPLEMENTARY MATERIALS
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Hairy Meristem with Wuschel confines CLAVATA3 expression to the outer apical meristem layers

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**Enough but not too many stem cells**

In the shoot apical meristem of plants, just the right number of stem cells generates a steady supply of cells with which to build differentiated tissues. Too few stem cells, and the plant cannot grow. Too many, and growth runs amok. Zhou *et al.* analyzed the controls on stem cell proliferation. They found that the HAIRY MERISTEM proteins define a domain within which WUSCHEL (WUS) cannot work, but beyond which WUS is left free to promote stem cell proliferation.

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