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Genetics of Shoot Meristem and Shoot Regeneration

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Abstract

Plants exhibit remarkable lineage plasticity, allowing them to regenerate organs that differ from their respective origins. Such developmental plasticity is dependent on the activity of pluripotent founder cells or stem cells residing in meristems. At the shoot apical meristem (SAM), the constant flow of cells requires continuing cell specification governed by a complex genetic network, with the WUSCHEL transcription factor and phytohormone cytokinin at its core. In this review, I discuss some intriguing recent discoveries that expose new principles and mechanisms of patterning and cell specification acting both at the SAM and prior to meristem organogenesis during shoot regeneration. I also highlight unanswered questions and future challenges in the study of SAM and meristem regeneration. Finally, I put forward a model describing stochastic events mediated by epigenetic factors to explain how the gene regulatory network might be initiated at the onset of shoot regeneration.

1. INTRODUCTION

The fascinating phenomenon of salamanders regrowing their limbs after amputation, or starfish regrowing lost arms (10), continues to intrigue biologists (35, 48). What are the mechanisms that underlie this regenerative capacity, and why has it become limited or completely suppressed in many lineages? How is patterning reestablished, directing just a few cells to form a perfect complex structure? These are just a few of the challenging questions in the field of regeneration (122).

Many scientists view regeneration in terms of wounding and damage and therefore use the term only in the context of repair and restoration of missing tissues or organs (34, 108). Others include in this term those phenomena of relevance to invertebrates and plants that form an entirely new individual from small body parts (14, 37).

Plants exhibit a remarkable capacity to regenerate tissue, organs, or entirely new individuals after major damage; as a form of asexual reproduction; or under in vitro conditions (**Figure 1**). The capacity to regenerate is extensive, has been reported for numerous plant species, and therefore can even be considered a signature feature of plants. This raises questions of why and by what means plants possess this exceptional regenerative capacity.

Plants maintain indeterminate growth and continuously form new organs throughout their life. This mode of development endows plants with morphological and physiological plasticity, which enables them to constantly adapt their development in response to dynamic environments and hence to cope with their sessile lifestyle. To achieve this ongoing organogenesis and developmental

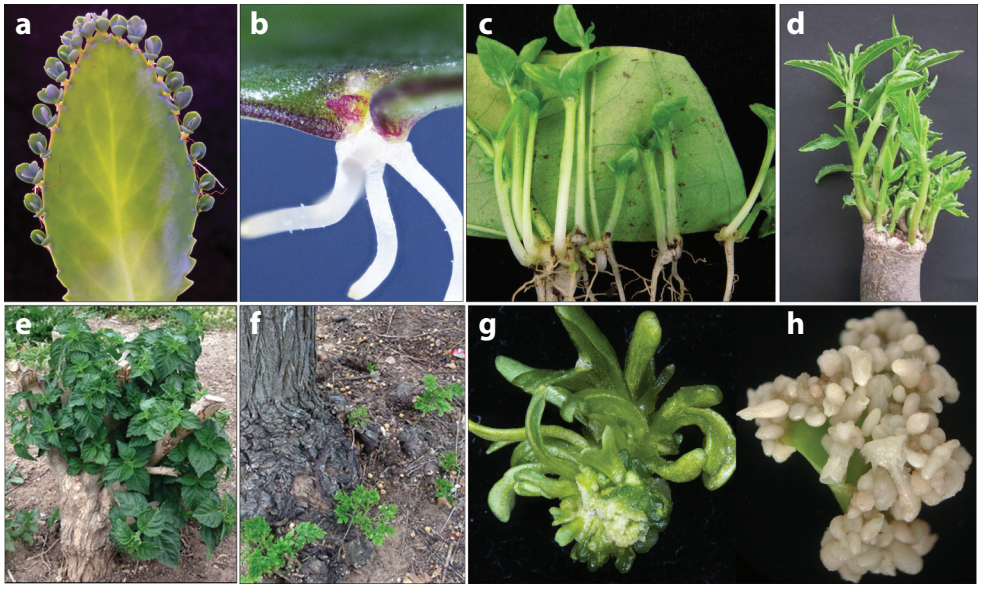

Figure 1

Different types of somatic embryogenesis and shoot regeneration. Somatic embryos form at specific positions on the leaf margin and develop into a shoot in species of the *Kalanchoe* genus as a form of asexual reproduction (*a*) while the leaf is still connected to the plant and (*b*) when triggered by leaf detachment. (*c*) Plantlets regenerate from a leaf of *Psychotria viridis* triggered by wounding only. (*d*) In vivo shoot regeneration from cut branches of *Ziziphus jujuba* Mill. is induced by wounding and growth regulators. Panel *d* is reproduced from Reference 110 with permission. (*e*) Multiple shoots regenerate from a mulberry tree stump. (*f*) Shoot suckers develop from unwounded lateral roots of a chinaberry tree. (*g*) Image of in vitro shoot regeneration from the callus of a *Nierembergia* plant. (*h*) Somatic embryos develop directly on leaf explants of *Coffea canephora*. Panel *h* is adapted from Reference 92 with permission and provided by Loyola-Vargas VM.

plasticity, plants maintain self-renewing populations of pluripotent cells, commonly termed stem cells (SCs), which reside in specialized tissues or structures called meristems.

Do these pluripotent cells, which are embedded within meristems and maintain the ability to divide and differentiate, grant exceptional capacities for regeneration? When considering this question, we must bear in mind that the presence of SCs embedded within organs is not unique to plants.

Multicellular organisms have developed two regenerative strategies. The first, which Thomas Morgan termed morphallaxis, relies on the transformation of preexisting tissues with little or no contribution from cell division (18, 85). A classic example from the animal kingdom is the regeneration of the *Hydra* head after decapitation, which depends on large stocks of adult SCs paused in G2 phase. Upon amputation, these proceed to mitosis and cytokinesis and further differentiate into apical structures even when the S phase is blocked (18). We can identify a few cases when plants employ this strategy as well. For example, when excised *Arabidopsis* roots are cultured on shoot-inducing media, lateral root primordia can be converted directly, via respecification of cell types, into a shoot apical meristem (SAM), which requires only a couple of cell division cycles (101, 123). Cell division–based regeneration is more common (96) and achieved through direct organogenesis or indirectly by first forming an intermediate mass of proliferating cells, the callus in plants (90) or the blastema in animals (26).

In plants, aside from specific cases of repair and restoration (5, 38, 53), regeneration commences with the formation of meristems (56). How do the proliferating cells of the callus, which are not organized in any particular structure, integrate intrinsic and/or extrinsic cues to instruct patterning and initiate cell fate reprogramming toward the regeneration of a complex structure like the meristem? Does meristem establishment during normal development and regeneration share the same principles? Does the same genetic network act in both?

In this review, I provide an overview of the current knowledge of plant regeneration, mainly in the model plant *Arabidopsis thaliana*, and I further focus on shoot regeneration. I begin by exploring the genetic networks and principles controlling the establishment of the SAM during normal development, while emphasizing aspects relevant to de novo meristem formation. Finally, I highlight the exciting new findings in the topic of lineage plasticity—a trait which allows the regeneration of organs with identities that differ from their source tissue. Within those contexts, I discuss emerging roles of epigenetic mechanisms in controlling plant regeneration.

2. MERISTEMS: THE KEY TO DEVELOPMENTAL PLASTICITY

Plants exhibit an indeterminate mode of development by maintaining populations of self-renewing SCs residing in specialized structures or tissues called meristems, which continuously provide cells for the development of new organs and tissues. Two primary meristems are established at opposite poles during early stages of embryogenesis: the SAM, which harbors the SC population from which all aboveground organs are derived, and the root apical meristem, which provides cells for the root system. A third lateral meristem, the (pro)cambium, gives rise to vascular tissues and later in development contributes cells for radial growth. Other meristems, such as lateral root and axillary meristems, will form de novo as the plant develops and will contribute to modeling of plant architecture (117).

The SAM is a dome-shaped structure located at the shoot apex, and although it is tiny (105), it is complex, organized in three clonally distinct cell layers, each generating separate lineages. The SAM can be divided into three functional zones (8, 32). The SC domain resides in the central zone at the tip of the meristem, across the three cell layers, such that each layer harbors a clonally independent pool of SCs. Just below this domain, and controlling it, is a molecularly distinct group

of cells constituting the organizing center. The SCs are characterized by low mitotic activity (32, 81, 141), producing cells with two alternative fates. Those daughter cells that remain at the same position in the center retain their SC identity and keep dividing infrequently (72). The others are pushed into the interior rib zone or radially toward the peripheral zone and start to divide at a faster rate (97). Their descendants will contribute to vascular tissue and stem structures at the rib zone or serve as founder cells for leaf or flower primordia at the peripheral zone (106, 117). Thus, cells across the meristem are passively displaced, and their lineage-based fate within each cell layer is further determined by their position (1, 65, 103).

Specification of shoot SC identity, as evinced by the expression of molecular markers, is initiated during embryogenesis at the future shoot apex, just before the heart stage (3, 146). Yet, new foci of SCs will also be established postembryonically, during de novo meristem formation from leaf axils, or during floral meristem initiation (4, 143). Injury of the SAM can also induce new SC foci. For example, laser ablation of the central zone in tomato SAM triggers the de novo establishment of an SC domain and an organizing center at the peripheral zone (98), demonstrating a remarkable flexibility of cell fate. Taken together, these examples show that the regulatory systems controlling the initiation of self-renewing SC populations can be initiated anew, a characteristic that contributes to the capacity for establishing new SAMs during shoot regeneration.

To ensure proper stable development, the relative ratio of cells in each functional domain at the meristem must be maintained. Precise coordination between cell proliferation and differentiation relies on a complex genetic network, involving multiple mechanisms of transcriptional regulation (102). At the core of this network is the WUSCHEL (WUS) transcription factor (TF), which specifies shoot SC fate and, as such, is required for meristem initiation and maintenance (73).

3. WUSCHEL: THE MASTER OF THE SHOOT APICAL MERISTEM

WUS was identified in a forward genetic screen by Thomas Laux and colleagues (73) in 1996 as it carried a mutation that disturbs shoot and floral meristem development in *Arabidopsis*. In situ hybridization analysis of *WUS* revealed its early expression at the 16-cell embryo stage prior to meristem formation. With the appearance of the fully developed SAM, *WUS* expression is confined to a small group of cells just below the SC domain (84). This finding led to the understanding that WUS acts in a non-cell-autonomous manner in the above cell layers, to specify SC fate. The small group of *WUS*-expressing cells functions as an organizing center, similarly to the quiescent center at the root meristem (131). Both centers could conform to the concept of animal SC niche, which is a specialized microenvironment controlling SC fate. Thus, these characteristics further justify the use of the term stem cells when referring to the group of pluripotent cells at the SAM: a population of self-renewing pluripotent cells controlled by a niche (86).

SC niches in animals utilize mostly secreted signaling factors to control cell division and prevent premature differentiation (93). In seeking a similar molecular mechanism in plants, many research groups have attempted to identify a secreted signal mediated by WUS (104). It was discovered, however, that the WUS protein itself moves from the organizing center to the overlying cells through intercellular plasmatic bridges called plasmodesmata (140), which provide cytoplasmic continuums with selective trafficking between neighboring cells (65).

WUS movement is essential for SC maintenance. Reducing the WUS intercellular trafficking by inducing plasmodesmata blockage resulted in a phenotype similar to the *wus* mutant. Likewise, preventing WUS trafficking by fusing WUS to nuclear localization signals, or by artificially increasing WUS molecular weight, failed to rescue the *wus* mutant (31, 140). The phenomenon of trafficking via plasmodesmata at the SAM was already shown for KNOX TFs (63). Yet the finding that WUS moves from the organizing center exposed another regulatory tier to WUS function,

namely the spatial regulation of protein mobility. This raises the question, By what mechanism does WUS specify the identity of SCs solely at their domain at the apex and not in the organizing center (where WUS itself is expressed) or in cells adjacent and subjacent to the organizing center?

3.1. Spatial Precision of WUS Function

Two cell subpopulations, the SCs and the meristematic cells, display divergent mitotic activity controlled by positional signaling via yet-unknown mechanisms (49). Ectopic activation of WUS function at the peripheral zone of the SAM or in the root was shown to suppress cell divisions (91, 141). It is therefore suggested that WUS movement solely to the upper cell layer provides the mechanism for setting up this sharp boundary of differential mitotic rates between the two subpopulations (91). In that scenario, WUS unidirectional upward movement might be mediated by spatial plasmodesmata distribution or permeability. However, confocal images of the SAM of *pWUS::WUS-green fluorescent protein* (*GFP*) transgenic plants clearly demonstrate that WUS moves from the organizing center in all directions and is present in a broader domain, which weakens the hypothesis of unidirectional movement. Nevertheless, the WUS-GFP protein signal is gradually diluted as WUS moves away from the organizing center (44). Thus, this analysis hints at a spatially dependent threshold mechanism of WUS function and further raises the question of how WUS specifies SC fate exclusively at the SC domain.

3.1.1. Non-cell-autonomous control of shoot apical meristem stem cells. The *WUS* level at the organizing center, which is crucial for maintaining the proper size of the SC population, relies primarily on a spatial regulatory loop between WUS and the CLAVATA (CLV) ligand-receptor system (29, 43). At the SC domain, WUS induces the expression of the SC-specific gene *CLV3*, which encodes for a secreted peptide (100). CLV3 then activates a signal transduction pathway to reduce *WUS* level at the organizing center, thereby limiting the extent of SC specification. WUS was shown to bind *cis* elements at the *CLV3* promoter in a concentration-dependent manner (94). Given that WUS can act as a transcriptional activator or repressor (54), researchers suggested that at the SC domain, where WUS is present at a low level, it switches to an activator that activates CLV3 (94, 99).

This proposed spatial functional switch provides a possible mechanistic framework for the WUS non-cell-autonomous activity of promoting transcription. However, the function of WUS as a repressor at a high level is arguable, since the ectopic expression of *WUS* at the SC domain, using the *CLV3::WUS* construct, results in seedlings having huge meristems (16). In those seedlings, high levels of *WUS* and *CLV3* messenger RNAs (mRNAs) were found in the same cells, which also indicates that the non-cell-autonomous mode of WUS is not obligatory for promoting *CLV3* expression. In addition, no expression of *CLV3* can be detected in cells positioned laterally and basally to the organizing center that display low levels of WUS as well (44). Therefore, new hypotheses and proposed mechanisms explaining how WUS acts specifically at the SC domain still remain to be explored.

3.1.2. Proposed mechanisms for spatial precision of WUS function. Directing WUS activity of SC specification exclusively to the SAM apex can be achieved through several plausible mechanisms. For example, does WUS activity, similar to that of KNOTTED1 (138), depend on chaperonin-mediated protein refolding after movement? Might this chaperonin be expressed specifically at the SC domain? Is WUS activity phosphor-regulated by plasmodesmata-localized kinases that preferentially accumulate in the SCs, such that WUS migration into the SC domain

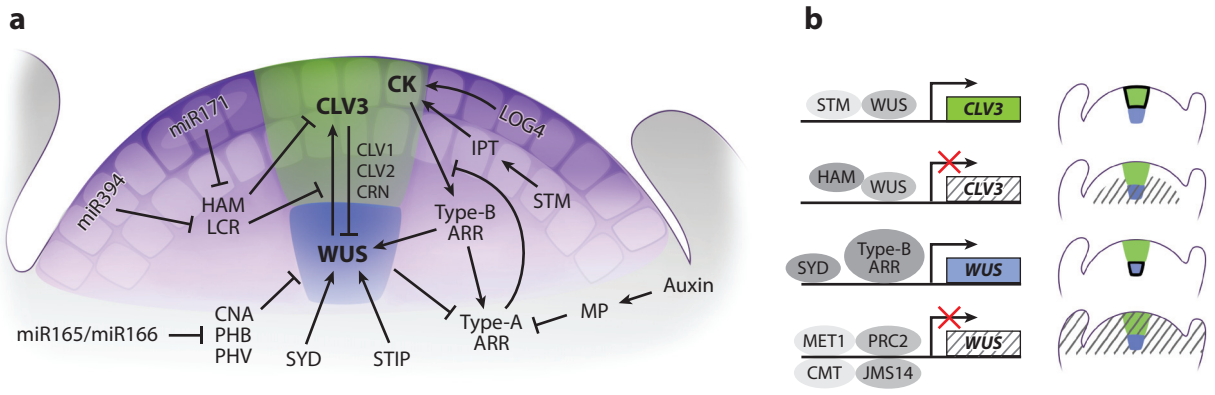

Figure 2

The control of *WUS* expression and WUS activities in the shoot apical meristem. (*a*) A model showing the action of CK, CLV3, and other factors in regulating *WUS* expression and activities. Activated CK and the microRNAs miR171 and miR394 are produced in the L1 layer, generating concentration gradients (*purple* indicates CK gradient). (For a review of the elements in the scheme, see 30, 51.) The SC domain is shown in green, and the OC is shown in blue. (*b*) At the SC domain (*green*) WUS and STM TFs bind *cis* elements in the *CLV3* promoter to activate its expression (119). Outside the SC domain (*diagonal lines*) the HAM TFs interact with WUS to suppress its ability to activate *CLV3*. At the OC (*blue*) the type-B ARRs bind to *cis* elements in the *WUS* promoter to activate its expression. The SNF2-class ATPase SYD is recruited to the *WUS* promoter to enhance its expression at the OC (68). Outside the meristem (*diagonal lines*) *WUS* is silenced by epigenetic repressors (reviewed in 137) (described in Section 6.1.4). Abbreviations: ARR, ARABIDOPSIS RESPONSE REGULATOR; CK, cytokinin; CLV, CLAVATA; HAM, HAIRY MERISTEM; IPT, ISOPENTENYL TRANSFERASE; LCR, LEAF CURLING RESPONSIVENESS; LOG, LONELY GUY; MET1, METHYLTRANSFERASE1; OC, organizing center; SC, stem cell; STM, SHOOT MERISTEMLESS; SYD, SPLAYED; TF, transcription factor; WUS, WUSCHEL.

activates the WUS transcriptional activity? Since many receptor-like kinases were shown to be localized to the plasmodesmata (40) and WUS was shown to contain putative phosphorylation sites (36), it is worth testing this hypothesis. The presence of WUS coactivators specifically at the SC domain or, conversely, of corepressors outside the domain might also contribute to the spatial precision of cell fate specification. One example is the HAIRY MERISTEM 1 (HAM1) and HAM2 TFs, which interact physically with WUS (107, 148). In the *ham1 ham2 ham3* triple mutant, the expression domain of *CLV3* is shifted inward (107, 149), suggesting that HAM TFs suppress the ability of WUS to activate *CLV3* outside the SC domain (149).

Another proposed mechanism for setting a sharp boundary for the SC domain is through threshold-based activity, laid out by opposite mobility gradients of microRNA (miRNA) and WUS (94, 114). *miR394* targets the *LEAF CURLING RESPONSIVENESS* (*LCR*) gene, which is ubiquitously expressed across the meristem and was shown to suppress the ability of cells to respond to WUS activity in an as yet unknown manner (66). *miR394* is produced in the L1 layer of the SC domain, moves inward to the two subtending cell layers, and generates a threshold-based gradient that outlines the boundaries for *LCR* degradation. The absence of *LCR* from the SC domain confers cellular competence to respond to WUS activity (**Figure 2**) (66).

In summary, assorted mechanisms that might operate in parallel have been proposed for directing WUS activity to a precise domain. Such mechanisms are based mainly on differential gene expression patterns across the meristem and on the principles of gradient- and threshold-dependent activity. Yet, imperative for proper WUS function is that its expression level be accurate and confined to the organizing center.

3.2. *WUS* Expression Is Regulated by Distinct Mechanisms

Cells at the SAM are continuously pushed by cell division in all directions, resulting in continual turnover of cells, while molecular subdomains are maintained. *WUS*-expressing cells, which perpetually occupy the organizing center domain at a fixed position relative to the shoot apex, must be constantly adjusted and thus are patterned by a positional cue mechanism. Given that WUS determines the size of the SC population, the location and boundaries of the *WUS* expression domain and the *WUS* transcript level per cell must all be under strict regulation. Deviations from this regulatory program might lead to an increase in the size of the SC domain and, as a result, to meristem enlargement or fasciation on one hand or to reduced meristem size or early meristem termination on the other (16, 73, 82, 132). When discussing the transcriptional regulation of *WUS*, we must distinguish between activation of *WUS* expression, fine tuning and patterning at the organizing center, and *WUS* silencing outside the meristem.

WUS is essential for de novo shoot organogenesis. Thus, release from silencing, which is mediated mainly by epigenetic mechanisms, and reactivation of *WUS* are some of the major obstacles to shoot regeneration (13). The epigenetic mechanisms regulating *WUS* outside of the SAM are discussed in Section 6.1.4. Temporal regulation of *WUS* expression at the floral meristem, which is crucial for the proper development of floral organs (69), is also mediated by epigenetic mechanisms (for recent reviews, see 19, 79, 120).

Following the discovery of the *WUS* and *CLV3* genes, systematic genetic screening identified multiple genes regulating *WUS* directly or indirectly by affecting the WUS-CLV3 regulatory loop. Those genes were isolated by their mutant-specific phenotypes displaying defects in meristem development or suppressing meristem phenotypes. Few such mutants turned out to be integral components of the CLV signaling cascade, such as receptors that interact with CLV1 to fine-tune the signaling activity (33, 89) or downstream genes (30, 116, 142). Other identified genes that encode for TFs, chromatin regulators, and small RNAs, as well as components of the cytokinin (CK) signaling, provided the basis to construct the gene network regulating *WUS* transcription at the SAM, and hence the regulatory scheme for SC control (thoroughly reviewed in 51, 116) (**Figure 2**).

For many years, in situ hybridization performed on meristem sections was the standard approach to study the spatial expression pattern of a gene at the SAM, thereby providing clues to the gene's function. Such analyses, while informative in capturing the position of mRNA accumulation, are limited in their ability to accurately measure mRNA levels and do not provide information on posttranscriptional regulation, timing, or dynamics.

Innovations in confocal microscopy, live-imaging technology, and computational modeling, together with the development of a repertoire of fluorescent reporters, have enabled researchers to expose additional mechanisms acting at multiple levels to regulate *WUS* and meristem homeostasis. It is now possible to discriminate between the site of gene expression and the site of protein accumulation, paving the way to the study of inter- and intracellular protein trafficking. After years of searching for non-cell-autonomous mechanisms of SC specification, it was exciting to discover that WUS itself moves to the SC domain. Novel imaging-based approaches have exposed additional gradients of protein concentration, with more likely to be revealed (52), as well as responses to hormones (28) and readouts of miRNA gradients across the meristem (52). These reinforce the notion of a threshold-based mechanism playing a role in *WUS* regulation and function. In addition, the powerful live-imaging technology made it possible to investigate several genes simultaneously (46), as well as to study the network dynamics, for example, to detect changes of gene expression patterns in living SAMs upon intervention, using inducible systems (136) or exogenous application of hormones (28) or other compounds (77).

Based on new discoveries, two models have emerged: the multiple CK feedback loops for patterning *WUS* at the SAM (46) and the L1 layer-driven mechanism for defining the domain competent for WUS activity. Both are relevant to shoot regeneration (50).

4. CYTOKININ AS THE GOVERNOR OF THE SHOOT APICAL MERISTEM

Two phytohormones, auxin and CK, play pivotal roles in SAM regulation, and both are essential for directing de novo shoot organogenesis (87, 118). Here I focus on CKs (but see also two recent excellent reviews on the role of auxins and their crosstalk with CK in References 74 and 126).

CKs are a class of small, adenine-derived organic molecules that act both over long distances and in the vicinity of their biosynthesis site. CKs regulate various aspects of plant development, physiology, and growth through transcriptional responses (151). At the meristem, CKs promote *WUS* transcription as reflected by the reduced meristem size in mutants altered at any step of CK signaling (70) and by the increase in *WUS* levels and meristem size upon exogenous CK application (28, 46, 128).

Recent studies provide new insights into the principles of CK signaling at the meristem, suggesting that CKs provide positional cues for *WUS* patterning via positive and possibly also negative spatial feedback loops. Those feedback loops are adjusted by multiple inputs, including from auxin (147), or are intertwined with other feedback loops, including the CLV-WUS signaling pathway (28, 46, 129). To understand how CKs contribute to *WUS* patterning, we first need to dissect the spatial organization of CK signaling across the meristem.

CKs are synthesized by three key enzymes encoded by multigene families: *ISOPENTENYL TRANSFERASE* (*IPT*), cytochrome P450 mono-oxygenase (*CYP735A*), and *LONELY GUY* (*LOG*), which convert the CK to its active form (39). CK molecules bind the ARABIDOPSIS HISTIDINE KINASE 2 (AHK2)–AHK4 receptors to initiate a multistep phosphorelay signaling cascade that ultimately leads to phosphorylation of the nuclear ARABIDOPSIS RESPONSE REGULATORS (ARRs) (88). Upon phosphorylation, activated type-B ARRs act as TFs to activate CK-responsive genes including the type-A ARRs that negatively regulate CK signaling, thereby establishing a negative feedback effect (124). (For in-depth coverage of CK signaling, see recent excellent reviews in References 62, 76 and 135.)

LOG4 is expressed in the L1 layer of the SAM (50) (**Figure 2**), suggesting that active CKs are produced in L1 and move basipetally into inner layers. This generates a concentration gradient that extends into the rib zone, thus defining a possible domain for CK responses in a threshold-dependent manner.

The expression of *AHK* receptors in inner tissues, excluding the L1 and L2 layers, defines the rib zone as a potential domain for CK signal perception (28, 46, 50). The overlap between cells having a threshold CK concentration and cells with competence for CK perception presumably occurs at a precisely fixed distance from the CK source (the L1 layer), leading to *WUS* activation and thereby defining the organizing center domain. How the spatial *AHK* expression is regulated is still unknown. The upregulation of *WUS* by CK application was indeed reported to be mediated primarily through AHK2- and AHK4-dependent pathways (46). Consistently, the downstream type-B ARR1 and ARR12 TFs bind the *WUS* promoter and activate its transcription upon CK treatment (136) (**Figure 2**). Type-B ARRs also activate the expression of *type-A ARR*s (125), whereas WUS directly represses the *type-A ARR* members, thus setting up a positive feedback effect that enhances the CK signaling (75). Plants overexpressing the *type-A ARR7* allele that mimics the active phosphorylated form exhibit the *wus* mutant phenotype (75).

It was therefore not surprising to discover that *type-A ARR5* and *WUS* exhibit complementary expression patterns, suggesting that outside of the organizing center, type-A ARRs reduce the responses to CKs to prevent *WUS* activation (46).

LOG4 expression is reduced in the *clv3* mutant, which exhibits high *WUS* levels (46). It is therefore suggested—although solid evidence is still missing—that WUS negatively regulates *LOG4* expression in the L1 layer, thereby exposing a plausible additional spatial feedback.

Adopting the model for CK patterning, we would expect to see significant expansion of the *WUS* domain upon exogenous application of CKs to the SAM and, consequently, an enlarged meristem. However, effects of added CKs were modest, except in meristems bearing mutations in *CLV3* or in the *ERECTA* (*ER*) receptor gene family. The remarkably increased responses to CK in *clv* or *er* family mutant backgrounds suggest that these pathways act to buffer the CK responsiveness at the SAM via yet-unknown mechanisms (46, 64a, 128, 129).

5. THE L1 LAYER AS A SOURCE FOR PATTERNING SIGNALS

The concept of positional specification by morphogen gradients was raised decades ago based on observations from animal development (20). In current models, morphogens, defined as molecules responsible for pattern formation (127), are proposed to be produced in specific regions. Depending on the morphogens' mobility, they generate a concentration gradient among surrounding cells. To interpret the gradient, the signal is read out at fixed concentration thresholds to specify gene profiles and cell fates in target tissues (134), thereby defining the boundaries for distinct domains (6). In recent years, this concept has been taking shape for use in modeling patterning at the meristem, as more morphogen gradients are discovered. Several molecules mentioned in previous sections fit into this model, patterning *WUS* and delineating the SC domain. The WUS protein is produced at the organizing center and forms a gradient along the apical–basal axis that acts in a concentration-dependent manner to specify SC fate (94). The establishment of negative feedback by CLV3, via an inverse route (apical to basal) to confine *WUS* expression, conveys robustness to SC homeostasis.

Within the meristem, a gradient can be formed on the radial axis, for example, by producing signals in lateral primordia. Recent discoveries, however, point to the L1 layer as the source for patterning signals. These include the production of active CK molecules (see above). Two other signals, in the form of miRNAs also produced at the L1 layer, generate a gradient that directs an inverse gradient of their targets through a threshold-based readout. The target genes gradient thereby defines the domain which will be competent for WUS activity. *miR394* degrades *LCR* mRNA and thus ensures LCR absence from the three apical cell layers at the central zone (see Section 3.1.2) (66). Overexpression of a form of *LCR* that is resistant to *miR394* causes shoot meristem termination (66), highlighting the substantial role of *miR394* in positioning and maintaining the SC domain.

Another signal, *miR171*, targets the *HAM* genes (see Section 3.1.2) (139). Confocal live-imaging analysis demonstrated that *HAM* and *CLV3* exhibit almost complementary expression patterns with opposite concentration gradients along the apical–basal axis (149), raising the question of how this pattern of expression is established. The recent in-depth analyses of reporters for the four *miR171* family members revealed their L1-restricted expression pattern (52, 121), consistent with the findings that *miR171* genes are direct targets of the epidermis-specific TFs ATML1 and PDF2 (52). The *HAM2* transcriptional reporter *pHAM2::H2B-GFP*, which is insensitive to *miR171*, is highly expressed across the meristem. However, the *HAM2* translational reporter *pHAM2::YFP-HAM2* clearly shows a concentration gradient across the apical–basal axis, from undetectable in L1 to an intense signal at the meristem interior (52, 149).

All of the evidence thus supports the model in which *miR171*, which is produced specifically at the L1 by ATML1, acts as a morphogen to pattern the *HAM*s expression, thereby defining the SC domain.

The CK, *miR394*, and *miR171*, and probably also other as-yet-unidentified signals, are epidermis-derived signals serving as positional cues to pattern *WUS* and the SC domain. This mechanism exposes another level of regulation at the SAM: the control of mobility (115). Cell-to-cell trafficking of miRNAs and proteins occurs through plasmodesmata. The number of plasmodesmata, their spatial distribution, the size of their aperture, and the ability to gate their channels are all tightly regulated and serve as mechanisms to control intercellular trafficking. The accumulating evidence on gradients forming across the SAM, in developing leaves, and in the root (115) makes plasmodesmata the new star in the study of plant development.

6. REGENERATION IN PLANTS: CONCEPT AND DEFINITION

In the animal kingdom, regeneration is defined as the repair or replacement of lost cells, tissues, organs, and entire body parts as well as the formation of a new individual from a small part of an organism (12, 85). Plants exhibit different modes of regeneration, and discussions on the precise use of the term plant regeneration can be traced to the nineteenth century (67). What should be included under this term? Unlike animals, plants can form new organs that are distinct in identity, number, and position from those that were lost. Such cases do not fall into the definition of repair or replacement but are clearly situations of bona fide regeneration (**Figure 1**), as manifested, for example, by multiple roots formed on stem cuttings. Should the term regeneration be restricted to those cases in which wounding triggers the formation of a new organ? And if so, how should we refer to the formation of shoots or entire individuals as a form of asexual reproduction that does not involve wounding? One example is root suckering, i.e., the formation of shoots originating from unwounded lateral roots in trees (2) (**Figure 1*f***). In tissue cultures, direct or indirect regeneration with intermediate callus formation can be triggered by wounding or by exogenous application of phytohormones. We might want to consider limiting usage of the word regeneration to events in which organs or entire individuals are formed under unexpected conditions or sites and not as part of the developmental program (67). Accordingly, somatic embryos that are formed in certain species of the *Kalanchoe* genus at specific positions on the leaf margin, whether triggered by leaf detachment or formed with the leaf still connected to the plant (**Figure 1*a*,*b***), should not be considered examples of true regeneration.

The regenerative potential of plants varies dramatically within and between species (71). A genome-wide association study (GWAS), seeking to associate genetic variations within 190 natural *Arabidopsis* accessions with shoot regeneration capacity, identified a few genes, including *WUS*, that act as master regulators of this capacity. A strong correlation between single nucleotide polymorphisms in the *WUS* promoter and shoot regeneration capabilities highlights the contribution of *WUS* transcriptional regulation to regeneration capacity (71). Another element affecting regeneration is the developmental stage of the plant. The competence to regenerate declines in most plants with the juvenile-to-adult transition mediated by *miR156* (144). Reduction of *miR156* expression in mature plants leads to an increase in the expression level of its target *SQUAMOSA PROMOTER BINDING-LIKE9* (*SPL9*). The SPL9 inhibits the transcription of *type-B ARR* and thereby the responsiveness to CK, which decreases the shoot regenerative capacity (144).

I now focus on the in vitro SAM regeneration from root explants in *Arabidopsis*, widely used as an experimental system for studying mechanisms of regeneration (25, 130). I refer to all cases of SAM formation as regeneration, whether or not they are triggered by wounding and whether they occur through meristem conversion or via callus formation.

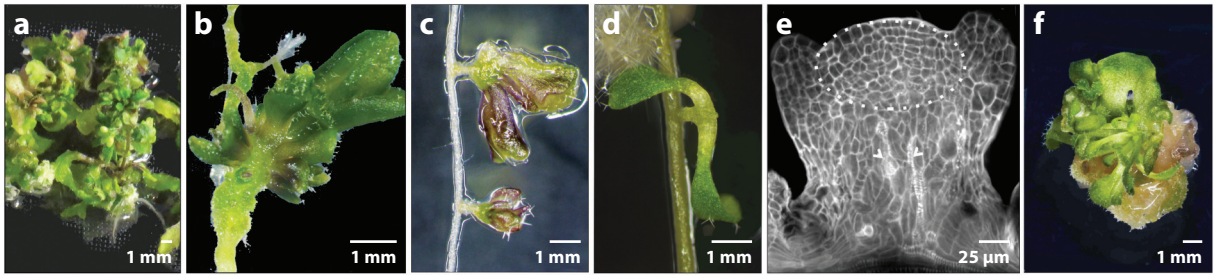

Figure 3

Shoot regeneration in *Arabidopsis*. (*a*) *Arabidopsis* shoot regeneration from excised roots that were incubated on callus-inducing media for 72 h, followed by incubation on shoot-inducing media (SIM). (*b*) Close-up of one shoot from panel *a*, demonstrating the regeneration via callus. (*c*) Direct shoot regeneration from an excised root explant of the *drm1 drm2 cmt3* triple mutant incubated directly on SIM. Panels *a–c* reproduced from Reference 109 with permission from *Plant Science*, Elsevier. (*d*) Direct shoot regeneration from a root explant of the transgenic plant *35S::WUS-GR* upon activation of WUS by Dex. Panel *d* reproduced from Reference 91. (*e*) Cytokinin induces the direct conversion of root primordium into shoot meristem at a specific stage of primordium development. Panel *e* reproduced from Reference 101 with permission from *Development* (**https://dev.biologists.org**). (*f*) Shoot regeneration from *Arabidopsis* callus incubated on SIM.

6.1. In Vitro Shoot Regeneration

In vitro shoot regeneration in *Arabidopsis* is usually achieved through two culturing steps. First, explants are cultured on callus-inducing media (CIM), containing a balanced auxin-to-CK ratio (113), to initiate cell division, followed by culturing on a CK-rich shoot-inducing medium (SIM) to induce SAM formation (42). Cell proliferation that is initiated on CIM and proceeds under the high-CK medium facilitates the acquisition of pluripotency, resulting in high capacities for regeneration (109). In most cases, direct incubation on SIM is not sufficient to induce SAM formation. Use of this two-step protocol for excised roots (130) provides an extensive system for studying the molecular characteristics and epigenetic regulation of pluripotency acquisition, pattern formation, and de novo organogenesis during shoot regeneration.

Five key steps take place successively during shoot regeneration from root explants and are also well suited, with minor adjustments, to describe other cases of regeneration. These steps are as follows: on CIM, (*a*) hormone and wounding signals are perceived, (*b*) cells start to proliferate, and (*c*) epigenetic reprogramming is taking place to acquire callus cell identity and the competency to regenerate shoot. Upon transfer to SIM, (*d*) patterning is achieved by spatial partition of auxin and CK responses within the callus, simultaneously with the erasure of repressive epigenetic marks to enable *WUS* expression, and (*e*) de novo organogenesis to establish a functional SAM (not discussed here).

The direct conversion of lateral root meristems into SAMs, without the formation of callus (**Figure 3*c–e***), occurs through reprograming and cell fate switch in the absence of a de novo organogenesis stage. This capacity is probably attributable to the common principle shared by the shoot and root meristems for maintaining meristem organization (21, 133).

6.1.1. Signal perception to initiate shoot regeneration. Shoot regeneration from excised roots is directed by exogenous phytohormones and wounding stimuli. Auxins and CKs act together to promote cell division by activating or repressing core cell cycle regulators (58). Auxins, via AUXIN RESPONSE FACTOR (ARF) TFs and the downstream LATERAL ORGAN BOUNDARIES DOMAIN (LBD) TFs, induce expression of the E2F TFs required for entry to the S phase of the cell cycle. In addition, auxins suppress the *KIP-RELATED PROTEIN* (*KRP*)

genes encoding cell cycle inhibitors (95). CKs, via type-B ARRs, induce expression of *CYCD* genes encoding the D-type cyclins required for progression into S phase (55). Nevertheless, it was recently reported that type-B ARR1 inhibits callus formation by counteracting the positive effect of type-B ARR12 (80), indicating that not all genes downstream from CKs promote cell proliferation.

Numerous reports describe loss- and gain-of-function mutations in genes involved in the auxin- or CK-signaling pathways, resulting in impaired or enhanced callus formation and shoot regeneration. For example, the *arr1 arr10 arr12* triple mutant exhibits a dramatic reduction in callus formation on CIM (83), whereas proliferation and shoot regeneration are enhanced in the quintuple mutant of *KRP* genes (27).

Wounding by itself can reactivate cell proliferation to form callus and/or to regenerate. However, there is no consensus on the necessity of wounding as a signal for regeneration. Using the two culturing steps approach to induce shoot regeneration from roots of intact seedlings results in callus formation but—most intriguingly—with hardly any shoot regeneration (11, 60). This raises the question of whether wounding is needed to initiate wounding signal cascades or whether it is required for disruption of a plausible long-distance signal from the shoot, allowing autonomous hormone regulation. On the one hand, application of an auxin transport inhibitor to the junction between roots and shoots of intact seedlings resulted in shoot regeneration, suggesting that shoot-derived auxins inhibit the process (11). On the other hand, the WOUND-INDUCED DEDIFFERENTIATION1 (WIND1) TF is rapidly upregulated upon wounding and activates CK biosynthesis. WIND1 was shown to play a pivotal role in callus formation and regeneration (60). Plants expressing the dominant-negative form of WIND1 do not regenerate shoots, and constitutive expression of *WIND1* enhances shoot regeneration without wounding (55, 57, 60).

Further studies are needed to clarify the role of wounding in shoot regeneration and to discriminate between the dual effect of wounding, namely, the activation of the CK pathway and detachment of the explant, keeping it away from shoot-derived signals to allow its autonomy.

6.1.2. Reentering the cell cycle or reactivating cell division. Cells that retain the capacity to divide can serve as a source for the proliferating cells that form the callus. They can derive from preexisting proliferating tissues such as meristems, from reactivating cell division in quiescent cells or SCs, or from differentiated cells that reenter the cell cycle.

In *Arabidopsis* excised roots, the xylem pole pericycle (XPP) cells that are paused in G2 phase of the cell cycle are the only cells that respond to CIM (7, 9). Consistently, ablation of XPP cells completely abolishes callus formation (25). The dividing XPP cells do not revert to a less differentiated state within their own lineage and do not regain the gene expression profile of their progenitor cells. As they divide and proliferate, they rather acquire callus cell identity of increased developmental potency. Therefore, no consensus has yet been reached on the use of the term dedifferentiation to describe this process (for a recent review of the term's relevance to plant cells, readers are referred to Reference 41).

6.1.3. Reprogramming to acquire callus cell identity and regenerative competence. Culturing of excised roots on SIM is not sufficient to induce shoot regeneration. The goal of the preincubation step on the CIM is to acquire SAM organogenesis competence. When XPP cells start to divide on CIM, they acquire a new cell identity, which is achieved by reprogramming, i.e., turning hundreds of genes on or off, resulting in a massive change in gene expression.

Acquisition of competence for SAM organogenesis requires the activation of genes affecting auxin and CK biosynthesis, transport, and signaling, as well as of genes encoding for the TFs essential for meristem organization and function (111). It might also require the setting of a permissive chromatin state at the loci of essential genes to facilitate their later activation on SIM (59).

For example, CIM activates the expression of the *PLETHORA* (*PLT*) gene family of TFs. PLTs are required for future shoot regeneration, as shown by the *plt3 plt5 plt7* triple mutant that is capable of forming callus but fails to regenerate shoots (61). Upon SIM culturing, PLT TFs induce the expression of *PIN-FORMED 1* (*PIN1*), which encodes for a polar auxin efflux carrier (15) that initiates and maintains auxin gradients. The failure of *plt3 plt5 plt7* roots to establish proper *PIN1* expression on SIM, and to generate auxin response domains (61), might explain the incapability to regenerate shoot. This is further supported by the severe reduction in shoot formation in a *pin1* mutant (47).

The expression of multiple TFs and setting up the machinery for the future partitioning of auxins and CKs on SIM are therefore prerequisites for regeneration. Yet it should be borne in mind that no SAM will be established in the absence of WUS. The WUS TF is required for de novo SAM formation, as the *wus* mutant fails to regenerate shoots in any tissue culture system (23, 145). Moreover, ectopic expression of *WUS* is sufficient to induce shoot regeneration from roots or calli in the absence of other stimuli (45, 91, 150) (**Figure 3*d***). Thus, to prevent ectopic expression and SAM formation, *WUS* must be kept silenced outside of its true domains.

Accordingly, it is reasonable to argue that the competence to regenerate shoots starts with providing the right conditions for *WUS* expression and action. These include the expression of genes that generate hormone gradients and CK-dominated domains, chromatin remodelers to further remove any epigenetic barriers from the *WUS* locus, genes required for *WUS* expression, and genes encoding for WUS interactors.

6.1.4. Patterning and acquiring competence to express WUS. Among the most intriguing questions in the field of regeneration are, How does patterning occur during regeneration, and what are the instructional cues? In the excised root system, some positional cues might derive from the roots. But in the case of an established callus that has no traces of the initial organ (**Figure 3*f***), and patterning occurs in what seems to be an unorganized mass of proliferating cells, the questions become even more puzzling. What initiates *WUS* expression, and how are the sites of its expression and the further positioning of the SC domains determined? Inducing shoot regeneration from excised roots or from fully developed callus results in multiple shoot formations (**Figure 3*a*,*f***). What mechanism secures sufficient spacing between *WUS* expression foci to ensure the formation of discrete and functional SAMs?

Induced by SIM, the auxin-CK partition provides the first cues to initiate the patterning required for de novo organogenesis. SIM enhances the expression of multiple CK-regulated genes (24), including the *PIN* auxin efflux carrier genes (47, 112), thereby modulating the direction and distribution of auxin flow. Extensive auxin-CK crosstalk (for a review, see 22) leads to the spatial partitioning of auxin and CK responses, thus enabling key regulatory genes to be expressed in nonoverlapping domains (47).

Outside the meristem, the *WUS* locus is silenced by DNA methylation and repressive histone modifications (64, 78, 109, 145). Therefore, CKs do not induce *WUS* expression prior to removal of epigenetic barriers, even when the required TFs are present. Epigenetic marks can be removed, either actively by the action of designated enzymes or passively via reduction in the expression of maintenance enzymes through replication-dependent dilution.

Callus derived from a mutant of *METHYLTRANSFERASE1* (*MET1*), which encodes for enzymes maintaining DNA methylation at CG sites, exhibits earlier expression of *WUS* and accelerated formation of shoots when cultured on SIM (78). Similarly, the *chromomethylase 3* (*cmt3*) mutant, which exhibits significant reduction in CHG methylation, readily regenerates shoots directly on SIM via direct organogenesis (109) (**Figure 3*c***).

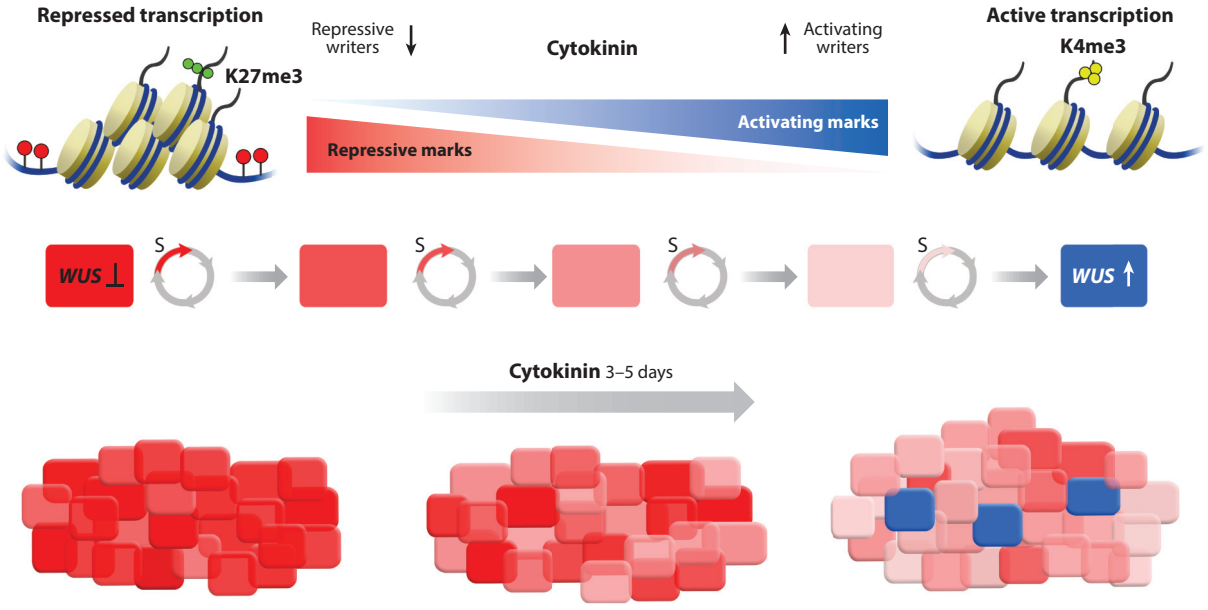

Figure 4

Schematic representation of the proposed model of stochastic cell division events leading to random epigenetic switches activating *WUSCHEL* (*WUS*) in a few foci in the callus. On callus-inducing media (*left*), *WUS* is silenced by repressive epigenetic marks (*red*). Upon transferring to cytokinin-rich shoot-inducing media, the expression of maintenance enzymes is reduced, leading to a progressive dilution in the repressive marks in each cell division (*red* represents the repressive chromatin state; *gradual lightening* represents the dilution of the marks). Random cell division events generate a cell population that has differential chromatin states on the *WUS* locus and plausibly on other genes as well. Cells within the callus that underwent the minimal number of rounds required for sufficient dilution of the repressive marks and are found within the cytokinin field activate *WUS* expression (*blue*). Once *WUS* is expressed, it moves to adjacent cells to suppress cell division, leading to the development of only a few foci of *WUS* within the callus.

In a study conducted on shoot regeneration from hypocotyls, CK was shown to promote the division-dependent removal of the H3K27me3 repressive mark at the *WUS* locus, to allow its activation by type-B ARR TFs (145). On SIM, the CK field, as detected by the signal of the fluorescent cytokine synthetic reporter TWO-COMPONENT OUTPUT SENSOR (TCS), and the downstream type-B ARRs domain, detected by in situ hybridization, were both much wider than the foci of *WUS* expression. Therefore, the authors sought to find the factor that confines *WUS* (145). Possible candidates are the *class III HD-ZIP* genes that were found by in situ hybridization to be enriched in small patches of cells, and their proteins were shown to interact physically with type-B ARRs. These findings led to the proposal that class III HD-ZIP and type-B ARR TFs interact to activate *WUS* expression and that their colocalized spots determine the sites of *WUS* expression (145). Yet, there are many more and much wider expression domains of the *class III HD-ZIP* than those of *WUS*. Therefore, in the following section, I propose a different model to explain how the sites of *WUS* expression are determined to generate only a few foci within a callus (**Figure 4**).

6.2. Stochastic Cell Division Events Leading to Random Epigenetic Switches Activating *WUS*

When root-derived callus is transferred to SIM, the H3K27me3 at the *WUS* locus is gradually reduced in a cell division–dependent manner (78, 145). Shemer et al. (109) and Zhang et al. (145), studying the effect of DNA methylation and H3K27me3 marks, respectively, on the capacity to

regenerate shoots, proposed that a few rounds of cell division in the absence of maintenance enzymes are required to switch the *WUS* locus from a repressive to a permissive state. In addition, they showed that the expression of several epigenetic maintenance enzymes are suppressed in a CK-rich environment (109, 145).

WUS::GUS and in situ hybridization analyses demonstrate that very few foci of *WUS* are developed on the callus cultured on SIM. I suggest that within the callus cell population there is considerable variation, thus far uncharacterized, in cell division rate. In that scenario, stochastic cell division events on SIM lead to an epigenetic switch in the *WUS* locus from repressive to permissive, only in those few cells that underwent the minimal number of rounds required for sufficient dilution of the repressive marks (**Figure 4**). Cells that are embedded within the CK field, and that express all the factors required for *WUS* expression, are now ready to activate *WUS*. More evidence is required to support this model, for example, by following the epigenetic state of the *WUS* locus and the *WUS* expression in tissue (excised roots or callus) exhibiting synchronized cell division. This can be achieved by alternating between arresting and releasing cell divisions.

The initial patterning steps of setting the spatial partition of auxin and CK responses set the CK fields that activate *WUS* expression. This is followed by WUS function that activates and suppresses numerous genes (17) to specify stem cell fate, setting up the CLV3 feedback signal and promoting a regulatory cascade that refines the SAM fate.

7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The past few years have seen significant advances in our understanding of the gene regulatory network that controls the establishment and maintenance of the SAM. Innovative imaging technologies and next-generation sequencing technologies have changed the kinds of questions we can address, exposing principles and mechanisms of patterning and reprogramming that act both at the SAM and during shoot regeneration. Nevertheless, many questions remain. Advances in single-cell approaches now enable us to perform multiomics analyses at single-cell resolution, dissect cell behaviors in distinct domains, and gain insights into genetic interactions controlling the initiation and maintenance of the SAM. Adopting the transgenic multicolor approach (26) combined with single-cell omics might have the potential to fill the gaps in our knowledge with regard to the role, the extent, and the nature of molecular trafficking at the meristem and in the callus during shoot regeneration.

DISCLOSURE STATEMENT

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