# Stem cells within the shoot apical meristem: Identity, arrangement and communication.

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**Running title:** Stem cells in the SAM

**Abstract**

Stem cells are specific cells that renew themselves and also provide daughter cells for organ formation. In plants, primary stem cell populations are nurtured within shoot and root apical meristems (SAM and RAM) for the production of aerial and underground parts, respectively. This review article summarizes recent progress on control of stem cells in the SAM from studies of the model plant *Arabidopsis thaliana*. To that end, a brief overview of the RAM is provided first to emphasize similarities and differences between the two apical meristems, which would helps better understanding of stem cells in the SAM. Subsequently, we will discuss in depth how stem cells are arranged in an organized manner in the SAM, how dynamically the stem cell identity is regulated, what factors participate in stem cell control, and how intercellular communication by mobile signals modulates stem cell behaviors within the SAM. Remaining questions and perspectives are also presented for future studies.

**Key words:** Central Zone; Organizing center; Peripheral zone; Shoot apical meristem; Stem cell: Tissue layer

## Introduction: stem cells within apical meristems of plants

Stem cells are broadly defined by two abilities; to renew themselves and to provide daughter cells that differentiate into other cell types [1]. In plants, two apical tissues, shoot and root apical meristems (SAM and RAM, respectively), contain primary stem cell populations that continuously produce cells for organ formation and growth throughout the life [2, 3]. The main purpose of this review article is to summarize recent progress on control of stem cell identity, spatial organization of the stem cell population and intercellular communication for stem cell regulation in the SAM, from studies of the model plant *Arabidopsis thaliana*. To that end, it is worth beginning with a brief overview of the RAM for better understanding of a general concept of plant stem cells because, compared with the SAM, the spatial arrangement of stem cells and their daughter cells has been clearly understood in the *Arabidopsis* RAM where different cell types can be easily and visually distinguished due to its well-organized tissue structure (Fig. 1) [4, 5]. Overlooking similarities and differences between the two apical meristems would deepen the discussion about stem cells in the SAM.

## Stem cell niche in the RAM

Figure 1 shows the stereotypical arrangement of cells at the *Arabidopsis* root tip. Stem cells (also called 'initial' cells) are located around the quiescent center (QC) and directly contact the QC. These stem cells are mitotically less active and divide infrequently [6]. The QC is considered as a signaling center or an organizer of the RAM, sending non-cell-autonomous signals toward the surrounding stem cells to regulate their maintenance and asymmetric cell division [7]. The stem cells give rise to all root tissues by dividing asymmetrically to renew themselves and produce

daughter cells that undergo several times of amplifying cell divisions before final differentiation into specialized cells. Importantly, under normal conditions, differentiation potency of stem cells in the RAM is strictly limited based on their position. Each stem cell contributes to the production of only one or two specific cell types, resulting in the formation of an array of specialized cell files originated from respective initial cells (Fig. 1). Thus stem cells in the RAM act as 'lineage-specific' stem cells. So far no universal genes specifically marking all stem cells around the QC have been established.

A part of the QC function requires *WUSCHEL RELATED HOMEOBOX 5* (*WOX5*), a transcription factor gene specifically expressed in the QC [8]. The loss of function of *WOX5* causes a defect in the maintenance of columela stem cells (CSCs) beneath the QC. Interestingly, although *WOX5* is specifically transcribed in the QC, the WOX5 protein moves from the QC into the CSCs and represses chromatin-mediated differentiation programs in the CSCs [9]. It is noteworthy that, while other stem cells around the QC except for CSCs do not show obvious phenotypes in the *wox5* mutant, the WOX5 protein appears to move not only to CSCs but also to other stem cells adjacent to the QC as shown by the *WOX5* genomic construct fused with yellow fluorescent protein (*gWOX5-YFP*) [9]. Therefore *gWOX5-YFP* may be able to serve as a universal reporter for all stem cells around the QC, though it shows fluorescence also in the QC cells. Roles of the WOX5 protein in non-CSC stem cells are unknown.

It has been proposed that another role of the QC is to serve as a starting point or an end point of concentration gradients of several factors [4]. In addition to the phytohormone auxin [10] and peptide hormones such as CLAVATA3/EMBRYO-SURROUNDING REGION 40 (CLE40) [11] and ROOT

MERISTEM GROWTH FACTORs (RGFs) [12], some transcription factors including PLETHORA (PLT) family proteins are also distributed in a concentration gradient according to the distance from the QC [13-15]. Some of such gradients affect one other to form complex regulatory networks [12-14, 16, 17], which output feedback signals to control the QC activity and stem cell behaviors [11, 18].

**Stem cell identity in the SAM**

As described above, there exist several types of specialized stem cells with different differentiation potencies at the root tip, and each stem cell produces only one or two specific cell files as a 'lineage-specific' stem cell. In contrast, the SAM maintains a group of pluripotent stem cells that give rise to all aerial tissues of the body [19]. These stem cells are located in the central zone (CZ) of the SAM structure (Fig. 2) [20] and divide slowly, providing daughter cells outward [21]. The daughter cells in the peripheral zone (PZ) surrounding the CZ undergo several round of cell divisions as 'transit amplifying cells' before they completely lose the indeterminate state and are eventually incorporated into lateral organ primordia [21, 22].

Maintenance of stem cell population and specification of stem cell identity in the SAM largely depend on the homeodomain transcription factor, WUSCHEL (WUS) [23, 24]. The expression domain of the *WUS* gene is restricted to a group of cells underneath the CZ (Fig. 2) [24]. Because *WUS* non-cell-autonomously promotes the proliferation of stem cells and specifies the stem cell identity in the CZ, the *WUS*-expressing cells are defined as the organizing center (OC) of the SAM. The molecular nature of the non-cell-autonomous effects derived from the *WUS* gene will be discussed in the later section. The stem cells express *CLAVATA3* (*CLV3*) [25, 26], which encodes a secreted peptide [20, 27, 28] acting to suppress the *WUS* expression

via several classes of CLV3 receptor proteins such as CLAVATA1 (CLV1) [29-38]. This *WUS*-*CLV3* negative feedback circuit plays a crucial role to maintain stem cell homeostasis. The *CLV3* expression serves as a reliable marker for stem cells within the SAM due to its specific expression in the CZ. However, it should be noted that, as *clv3* mutants retain functional stem cells, *CLV3* itself is not essential for the specification of stem cell identity [20, 39].

*WUS* not only regulates stem cells in the CZ but also affects cell behaviors in the PZ. It has been established that local accumulation of auxin within the PZ and the activation of auxin signling at the accumulation site specify lateral organ founder cells [40-42]. Intriguingly, transient down-regulation of *WUS* leads to enlarged organ primordia with an increase in the number of cells responding to auxin within the PZ, suggesting that *WUS* non-cell-autonomously controls the allocation of PZ cells to a differentiation pathway by decreasing either local auxin accumulation or auxin signaling [43]. Recently, HECATE (HEC) family transcription factors were reported to negatively modulate auxin signaling in the PZ by physically associating with MONOPTEROS (MP), a key transcription factor of auxin signaling in the SAM [44, 45]. It is worthwhile to examine whether the *WUS* down-regulation in the OC affects the *HEC* function in the PZ.

**Dynamic re-specification of stem cell identity in the SAM**

It was shown that expression of endogenous *CLV3* or *CLV3*-promoter-based reporters expands into the PZ in mutants of *CLAVATA* signaling pathway such as *clv1* and *clv3* [20, 43]. This observation raises two possibilities. One is the expansion of stem cell population caused by enhanced cell division of stem cell itself. The other is the re-specification of cells in the PZ back to stem cell identity. Live imaging

following induced down-regulation of *CLV3* or induced enhancement of *WUS* activity revealed that the latter is the case [43, 46]. Upon the induction, re-specification of stem cell identity gradually and radially expands from the original CZ toward the adjacent PZ cells, indicating that the re-specification likely requires unknown factors that exist in the CZ or unidentified short-range signals derived from the CZ.

Although the *CLV3* expression covers almost the entire SAM in *clv* mutants, the expression is excluded from the outer narrow domain of the PZ (Fig. 2) [20, 43], indicating that the stem cell identity cannot be re-specified in the outer PZ even in *clv* mutants, and that cellular situations are not uniform throughout the entire PZ. Mechanisms that determine the border between the inner and outer PZ in terms of the capability of re-specification of stem cell identity remain to be revealed. However, ectopic overexpression of *WUS* is able to completely convert the entire PZ into stem cell identity including the outer PZ [43], suggesting that, under normal conditions, the range of unknown non-cell-autonomous signals derived from the original *WUS* expression domain may define the border between the inner and outer PZ. Single cell transcriptome analysis of the SAM may reveal stepwise or gradual changes in cell state from the inner PZ toward the outer PZ. A recent study suggests a gradual transition from stem cell to differentiation state in the RAM. Specifically, transcriptome analysis of root cell subpopulations separated according to their distance from the QC demonstrated that, with increasing distance from the QC, stem cell-enriched transcripts are gradually decreased and differentiation-associated transcripts are inversely increased [15]. A similar gradual progression may occur from the center of the SAM toward the edge of the PZ. In this scenario, the distance from the OC may control the progression via non-cell-autonomous effects originated from the *WUS* function in the OC.

Re-specification of cells in the PZ back to stem cell identify was also proposed by classical microsurgical ablation experiments using tomato shoot apex [47]. After ablation of the entire CZ and OC, the expression of tomato *WUS* homologue gene was rapidly induced in the PZ, and eventually the functional SAM was re-established, indicating that, most likely, stem cells can be regenerated from the PZ. Later ablation experiments using the *Arabidopsis* SAM clearly showed re-specification of cells in the PZ into stem cell identity [48]. *CLV3*-expressing cells appeared in the PZ after the ablation, following the re-establishment of *WUS* expression. Thus, cells in the PZ retain a potency to be re-specified into stem cell identity even in the wild-type situation. Ablation experiments with the *Arabidopsis* RAM were also reported [49]. The complete removal of the QC and all surrounding stem cells led to the appearance of the QC marker *WOX5* in the endodermis cell file, followed by re-construction of a functional root tip containing re-established stem cells. Therefore, the appearance of *WUS*/*WOX* family genes prior to stem cell regeneration is common between the two apical meristems and likely a prerequisite step for stem cell re-specification.

**Layered arrangement of stem cell subpopulations in the SAM**

The SAM is composed of three tissue layers: the epidermal L1, the sub-epidermal L2 and further inner L3 layers (Fig. 3A), and each tissue layer shows distinct characteristics [50]. Cells in the L1 and L2 layers divide in an anticlinal manner, producing clonal cell layers. In contrast, cells in the L3 tissue divide both anticlinally and periclinally, giving rise to internal tissues. In particular, since the L1 layer is exposed to the environment, it plays a role as a mechanical barrier with some special properties [51] such as thicker cell walls on the outer surface [52, 53]. Genes

that exhibit epidermis-specific expression have been extensively identified [54], including *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*AtML1*) that is widely used as an L1 identity marker in shoot tissues [55, 56].

Although the three tissue layers of the SAM play distinct roles during development, stem cells spread between all the layers within the CZ (Fig. 3B) [20]. It is noteworthy that the OC, which is defined by the *WUS* expression, resides within the L3 tissue [24] (Fig. 3A, B) and the lowermost layer of the stem cell population overlaps with the uppermost layer of the OC (Fig. 3B). Thus, there exist at least three distinct stem cell subpopulations within the SAM tissue layers (Fig. 3C); stem cells with the L1 identity, those with the L2 identity and those with the OC identity. This layer arrangement of stem cells within the SAM is in sharp contrast with the situation in the RAM, where only cells adjacent to the QC act as stem cells (Fig. 1). Specific marker genes for each stem cell subpopulation located in respective tissue layers of the SAM have not been established. According to the reported *Arabidopsis* transcriptome data of SAM cell subpopulations separated by FACS using several established domain-specific markers including *CLV3*, *WUS* and *AtML1* [57, 58], *At1g04880* and *At5g06270* may be candidates for specific marker genes for stem cells with the L1 identity, and *At4g17710* for those with the L2 identity. Future single cell transcriptome analysis of the CZ cells would further facilitate the identification and establishment of specific markers for each stem cell subpopulation.

**WUS distribution for the proper arrangement of stem cells in the SAM**

One of key questions raised by the layered arrangement of stem cells in the SAM is how the OC in the L3 zone specifies stem cell identity of cells in the entire CZ, especially those in the L1 and L2 layers which do not overlap with the OC. As

mentioned in the above section, *WUS* plays a central role to specify the stem cell identity of the CZ cells though it is specifically expressed in the OC [24]. Intriguingly, WUS protein moves from the OC to the entire CZ via plasmadesmata [59, 60] and activates *CLV3* expression by directly binding to the *CLV3* promoter [61]. This is similar to the case of the WOX5 movement from the QC to surrounding stem cells in the RAM [9]. However, it remained to be answered why the *CLV3* expression is only seen in its uppermost layer of the OC despite that the *WUS* is expressed in the entire OC (Fig. 3B). Very recently, it was revealed that the activity of HAIRY MERISTEM (HAM) family proteins, GRAS-domain transcription factors, prevents the *CLV3* activation in the lower OC [62]. Expression patterns of *HAM* family members and *CLV3* are nearly complementary along the apical-basal axis, and the *HAM* family is highly expressed in the lower part of the SAM [62-64]. Furthermore, HAM proteins interact with WUS [65]. Combined, these data suggest that HAM proteins inhibit the *CLV3* activation by WUS in the lower OC via forming a protein complex with WUS. Accordingly, in the absence of the *HAM* family activity, the *CLV3* expression expands into the lower part of the SAM [62, 64]. Thus, the WUS protein distribution and the inhibitory effect by HAM collectively define the *CLV3* pattern.

An important remaining question is why the WUS proteins move only acropetally, not toward all direction from the OC. HAM may also inhibit WUS movement by forming a complex with WUS. Distribution pattern of WUS proteins in mutants of *HAM* family may provide a hint to answer this question. Furthermore, interestingly, attenuation of the *HAM* family activity leads to an increase in the number of tissue layers exhibiting anticlinal cell division to several layers, or sometimes more than ten, from the original two (one L1 and one L2) [63, 64]. Since, even in the mutant SAM, expression of the L1 marker *AtML1* is detected only in the

outermost layer, the supernumerary layers are likely a consequence of either amplified L2 layers or extra anticrinally-dividing layers with the L3 identity. Live imaging following induced down-regulation of *HAM* family would reveal how supernumerary SAM layers are formed and how the stem cell identity expands into the lower region of the SAM.

Unusual expression patterns of *WUS* and *CLV3* were also seen in the double mutant of *PLT*-clade genes *AINTEGUMENTA* (*ANT*) and its closely-related gene *AINTEGUMENTA-LIKE6*/*PLT3* (*AIL6*/*PLT3*) [66]. In *ant ail6* mutants, *CLV3* expression is shifted downward, and the peak of the expression overlaps with the region where *WUS* is normally expressed. Inversely, the *WUS* expression is shifted upward and expands into the L1 and L2 layers. Thus *WUS* and *CLV3* expression domains are almost reversed in the mutant. However, considering this unusual situation, it may be worth examining whether the altered expression pattern of *CLV3* in the *ant ail6* mutant correctly reflects the position of stem cells, that is, whether the *CLV3* expression pattern correctly marks stem cells even under the *ant ail6* mutant backgrounds. Future research on the molecular mechanism as to how *ANT* and *AIL6*/*PLT3* regulate SAM function will provide mechanistic insights into this interesting phenomenon. In the RAM, RGF peptides, which are also called GOLVEN (GLV) or CLE-LIKE (CLEL) peptides [67-69], control stem cell activity by modulating stability of PLT family proteins PLT1 and PLT2 [12, 70]. Because several *RGF*/*GLV*/*CLEL* family genes are expressed in the SAM [71], they may regulate stem cell arrangement via stability control of ANT and/or AIL6/PLT3 proteins like their roles in the RAM. However, no shoot phenotype has been reported in known *RGF*/*GLV*/*CLEL* family mutants, possibly due to high redundancy among the family genes. Generation of higher order mutants in the family by CRISPR/Cas9-mediated

gene editing may reveal functions of the family in the SAM.

### ***WUS*-independent maintenance of stem cells in the SAM**

Recent studies started highlighting that stem cells in the SAM can be maintained even in the absence of *WUS* [72-74]. Loss-of-function mutations in *ERECTA* (*ER*) family receptor kinase genes, *Class III Homeodomain Leucine Zipper* (*HD-ZIPIII*) family transcription factor genes or the putative glutamate carboxypeptidase *ALTERED MERISTEM PROGRAM1* (*AMP1*) gene lead to the recovery of stem cells even in the *wus* mutant SAM, suggesting that there should exist mechanisms that maintain stem cells in a *WUS*-independent manner. Given that *ER* and *HD-ZIP III* genetically act in parallel [75, 76] and that the *HD-ZIP III* activity can be controlled downstream of *AMP1* [77], at least two pathways, *ER* family pathway and *AMP-HD-ZIPIII* pathway, impact the *WUS*-independent maintenance of stem cells. Interestingly, in addition to SAM development, the *ER* family also regulates serration formation [78] and procambial development [79-81], which involve *WUSCHEL RELATED HOMEOBOX 1* (*WOX1*) [82] and *WUSCHEL RELATED HOMEOBOX 4*/*14* (*WOX4/14*) [79, 83, 84], respectively. Although it remains unclear whether the *ER* family also participates in regulating the RAM where *WOX5* plays a crucial role, it is attractive to speculate that such co-recruitment of the *ER* family and *WUS*/*WOX* family modules in multiple developmental contexts in Arabidopsis may have been originated from their ancestral but yet-unrevealed roles in early land plant lineages.

### **Layer-specific regulation of stem cells in the SAM**

In the viewpoint of the layered structure of stem cell population in the SAM

(Fig. 3C), phenotypes seen in the complete absence of three *ER*-family genes (hereafter *er*-family mutant) should be noted. In the *er*-family mutant, the *CLV3* expression is highly up-regulated [85-87] and also expands into the epidermis of the PZ of the SAM [72]. Surprisingly, the *CLV3* expression in the L1 layer of the *er*-family mutant does not require the *WUS* activity, as observed in the multiple mutant lacking both *ER*-family and *WUS* functions. On the other hand, the *CLV3* expression in inner L2 and L3 tissues still depend on *WUS*. This observation suggests that there exists the mechanism that maintains stem cells in the L1 layer in a *WUS*-independent manner and that *ER* family suppresses the mechanism under normal conditions. In general, receptor signaling is modulated by specific ligand molecules [88]. Although a ligand for ER-family receptor proteins in stem cell control remains unclear, it is possible that *WUS*-independent stem cells are activated by reducing the level of the ER-family receptor signaling via expression changes of such an unknown ligand in response to some stimuli or under specific conditions. Since the L1-specific expression of *ER* is sufficient to rescue the dysregulation of stem cells in the mutant L1, *ER* acts cell-autonomously in this mechanism, consistent with its molecular nature as a transmembrane receptor kinase [89]. Further details of this mechanism remain to be revealed.

Interestingly, although the canonical stem cell marker *CLV3* is detected only in the epidermal L1 layer of the SAM of the multiple mutant of *er*-family and *wus*, the mutant SAM still retains well-organized sub-epidermal layer that appears to divide anticlinally like the normal L2 layer [72]. This raises an intriguing possibility that *CLV3*-negative stem cells might exist in the inner layer of the mutant SAM lacking the *ER* family activity, although no such evidence exists thus far. Development of alternate reliable stem cell markers other than *CLV3* would be required to examine

316 this possibility.

317

318 **Communication between tissue layers in the SAM**

319 Although stem cells in each tissue layer of the SAM provide cells for their
320 own layer and they do not move between layers in general (Fig. 3A) [50], whole stem
321 cells coordinately behave as one group (Fig. 3B, C) [20, 25, 26]. This coordination
322 between the SAM layers requires a variety of inter-layer communication in addition to
323 the CLV3-WUS circuit (Fig. 4). Acropetal and basipetal signals have been reported,
324 and most of these signaling pathways are interconnected to achieve well-organized
325 and flexible behaviors of stem cells [90-92]. Details about these signals are described
326 in the following sections.

327

328 **Modulation of WUS activity by inter-layrer cytokinin signaling**

329 As described, one of central mobile signals in stem cell maintenance is
330 WUS protein that moves from the OC to the CZ via plasmadesmata [59, 60] and
331 activates *CLV3* expression in the CZ [61]. WUS protein level and spatial distribution
332 pattern are controlled by protein destabilization [93], and phytohormone cytokinin
333 stabilizes the WUS protein [94]. As activation of cytokinin signaling occurs in the OC
334 but is excluded from the L1 and L2 layers [94, 95], WUS protein level decreases
335 according to the distance from the OC [94]. The degradation machinery that directly
336 degrades the WUS protein remains elusive. In addition to the WUS protein level,
337 cytokinin signaling also promotes *WUS* transcription through type-B ARABIDOPSIS
338 RESPONSE REGULATORs (ARRs) [96-99], transcription factors that directly
339 activate cytokinin-induced gene expression. Thus, cytokinin positively regulates WUS
340 at both transcription and protein levels. Interestingly, the *WUS* up-regulation is not

directly reflected in an increase in the *CLV3* expression. Although exogenous cytokinin application remarkably increases the *WUS* expression, its effect on the *CLV3* transcription is moderate in wild type conditions [100], implicating that there exist mechanisms that buffer the cytokinin effect. CLV3- and ER-family pathways appear to act in these buffering mechanisms, as the *CLV3* promoter activity drastically expands in response to cytokinin in *clv3* and *er*-family mutants [87, 100]. Further details of these mechanisms remain to be elucidated.

The production site of active cytokinin species in the SAM is presumed to be the epidermal L1 layer because the expression of *LONELY GUY 4* (*LOG4*), which encodes an enzyme catalyzing the final step of cytokinin biosynthesis, is restricted to the L1 [95, 100]. On the other hand, cytokinin receptor genes, *ARABIDOPSIS HISTIDINE KINASE 2* (*AHK2*), *AHK3* and *AHK4* are expressed in inner tissues including the OC, but excluded from the L1 and L2 layers [95, 100, 101]. Cytokinin response, as reflected by two component signaling sensor *TCSn::GFP* [102], is strongly detected in the OC and weakly beneath the OC, overlapping with the upper part of the expression region of cytokinin receptors [94, 100, 101]. Thus, the currently proposed model is that active cytokinin molecules produced in the L1 layer move basipetally and activate primary cytokinin responses in the OC, where WUS function is enhanced by the activation of cytokinin signaling.

**Modulation of cytokinin signaling by multiple inputs and feedbacks**

The level of cytokinin signaling in the OC is modulated by multiple inputs and feedbacks. WUS directly represses expression of *type-A ARR* genes, *ARR7* and *ARR15*, which are negative regulators of cytokinin signaling, resulting in enhanced cytokinin responses in the OC [103]. Auxin also reinforces cytokinin signaling in the

SAM in addition to its well-known role to promote initiation of lateral organs in the PZ. A low but certain level of auxin signaling input is detected in the CZ by the synthetic DII-VENUS reporter [104] that reflects the activation of TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX family auxin receptor proteins [105]. A key transcription factor of auxin signaling in the SAM, MP, directly represses *ARR7/15* expression in the CZ, hence strengthening cytokinin signaling [106]. Therefore, WUS and auxin both act as positive regulators of cytokinin pathway in the OC through modulation of the *ARR7/15* expression. It remains unclear whether there is a direct molecular link between the two transcription factors, WUS and MP, in the *ARR7/15* regulation. Furthermore, it is known that MP directly represses transcription of *DORNRÖSCHEN* (*DRN*), which activates *CLV3* expression [107]. Thus auxin signaling may also enhance the *WUS* expression by negatively regulating *CLV3*. Collectively, cytokinin and auxin cooperate to promote the WUS function in the SAM. It is reported that the repression of *ARR7/15* expression by auxin is released upon treatment with the auxin transport inhibitor N-1-naphthylphthalamic acid [106], suggesting that auxin transport contributes to the *ARR7/15* regulation. However, unlike the well-established mechanism of auxin accumulation in the PZ for primordium initiation, where the auxin efflux transporter PIN-FORMED1 play a key role [40-42], how auxin is delivered toward the center of the SAM remains elusive. Because some *YUCCA*-family genes, which encode flavin monooxygenases for auxin biosynthesis, are expressed in the SAM [97, 108-110], auxin synthesized in the SAM may also activate auxin signaling at the production site.

**Non-cell-autonomous secondary effects triggered by the primary cytokinin response**

391 Although the primary cytokinin response is specifically activated in the OC
392 [95, 100, 101], attenuation of the cytokinin signaling leads to an overall reduction in
393 SAM size [103, 111-114] and, accordingly, the expression pattern of the stem cell
394 marker *CLV3* in all SAM tissue layers also shrink [72]. This suggests that there must
395 exist OC-derived secondary effects that non-cell-autonomously affect cells within the
396 whole SAM in response to the change of the primary cytokinin response in the OC.
397 Since WUS function is enhanced by the cytokinin signaling [94, 96-99], WUS protein
398 movement from the OC [59] could act as such a non-cell-autonomous signal
399 downstream of the primary cytokinin response. However, given that WUS protein
400 does not move beyond the CZ [59] and that WUS affects expression of hundreds of
401 genes [115], WUS may regulate gene expression that leads to production of other
402 secondary signals that spread beyond the CZ. Whereas the molecular nature of these
403 secondary signals remains unclear, ER-family pathway may modulate the
404 responsiveness of the SAM to such signals in a tissue layer-specific manner. In the
405 absence of ER-family activity, expression of stem cell marker in the epidermal L1
406 layer turn resistant to the attenuation of cytokinin signaling, while that in the internal
407 L2/L3 tissues decreases in response to the reduced cytokinin signaling [72]. It will be
408 interesting to further characterize these cytokinin-triggered and OC-derived
409 non-cell-autonomous effects in future studies.
410
411 **Mobile small RNAs for control of stem cell activity in the SAM**
412 Small RNAs also act as mobile molecules that non-cell-autonomously
413 control gene expression of their target genes [116, 117]. Two families of microRNAs
414 are reported to affect stem cell activity in the SAM. One is miR165/166 that are
415 derived from outside of the SAM, mainly from abaxial domain of leaf primordia and

provascular tissues [118-120] and target *HD-ZIPIII* family genes [121-124]. Interestingly, SAM phenotypes vary depending on combinations of mutations in *HD-ZIPIII* family members [125, 126]. Some combinations result in the loss of the SAM, while others conversely enhance the SAM activity, suggesting not only overlapping but also antagonistic gene functions among *HD-Zip III* family members. It remains to be elucidated how *HD-ZIPIII* family genes control stem cell activity in the SAM. The other is miR394, which acts as a mobile signal between SAM tissue layers [127]. In the SAM, *miR394* is transcribed in the L1 layer and the mature miR394 molecules spread into inner L2 and L3 cells to repress its target *LEAF CURLING RESPONSIVENESS* (*LCR*). miR394-resistant *LCR* leads to premature termination of stem cell activity. *LCR* encodes an F-box-domain-containing protein. In general, F-box proteins promote degradation of their targets by recruiting them to the ubiquitin-proteasome pathway [128]. Although it is still unknown whether LCR is actually involved in protein degradation, identification of LCR-interacting proteins would facilitate the elucidation of the molecular function of LCR. Plants overexpressing miR394-resistant *LCR* resemble *hd-zipIII*-family multiple mutants [127]. Also, the phenotype of miR394-resistant *LCR* is exaggerated by mutations in the *ARGONAUTE10*/*ZWILLE* gene [127] that enhances the *HD-ZIPIII* activity by antagonizing miR165/166 [118, 122]. Thus, LCR pathway and HD-ZIPIII function may converge to maintain stem cells.

**Future perspectives**

While a number of factors and pathways that affect stem cell homeostasis in the SAM have been identified, in some cases it is still largely obscure how each input is connected to the core WUS-CLV3 circuit. The molecular nature of some putative

441 OC-derived non-cell-autonomous effects is also unknown. Furthermore, it remains
442 elusive how such multiple pathways and complex relationships between them are
443 integrated to achieve a coordinated behavior of a group of stem cells spreading
444 between three tissue layers within the SAM. It is important in future studies to address
445 these unresolved issues. It should be also noted that the conclusions drawn from many
446 past studies were based on the analyses of mutant plants. Such steady-state mutant
447 phenotypes could be an end-point consequence of a series of events initiated by the
448 mutation, which may not necessarily reflect the direct role of the gene of interest.
449 Time-course analyses that follow temporal induction or perturbation of gene/protein
450 functions will provide further insights into understanding of stem cell behavior. Single
451 cell transcriptome analysis of the SAM may also reveal the dynamic characteristics of
452 stem cells such as stepwise or gradual changes in cell state from the CZ toward the PZ,
453 or identification of stem cell subpopulations within the SAM with a higher resolution.
454 Although this review article mostly focuses on stem cell control within the
455 SAM, it will be also important to investigate relationships between environmental
456 responses and stem cell homeostasis. For example, it is not surprising that changes in
457 nutrient conditions affect stem cell activity. Recent studies have demonstrated that
458 cytokinin mediates the interplay between nitrate availability and SAM activity [129,
459 130]. Biosynthesis of precursors of trans-zeatin-type cytokinin is rapidly elevated in
460 roots in response to an increase in nitrate concentration [131]. The root-derived
461 precursors are transported to shoot tissues via xylem [132, 133] and in the SAM they
462 are converted to active cytokinins that promote stem cell activity via enhancement of
463 WUS function [129, 130]. Light conditions and sugar availability also change the
464 SAM activity by modulating cytokinin signaling and *WUS* expression [134, 135].
465 Thus recent studies have highlighted cytokinin as a mediator for regulating the SAM

activity in response to some environmental changes. However, it is still largely unknown how and whether each of a variety of environmental cues modifies stem cell behaviors.

Redox status affects growth and development both in shoots and roots [136]. Recently it was suggested that redox-related signaling molecules might link environmental cues with SAM functions [137, 138]. Enzyme genes for metabolism of reactive oxygen species (ROS) are expressed in distinct spatial domains within the SAM [57], and different forms of ROS play distinct roles in each domain of the SAM [139, 140]. The superoxide anion ($O_2^{\cdot -}$) is enriched in the CZ, while hydrogen peroxide ($H_2O_2$) is abundant in the PZ [140]. Pharmacological or genetic disruption of the $O_2^{\cdot -}$ accumulation in the CZ causes stem cell loss accompanied by a reduction in the *WUS* expression [140]. On the other hand, decreasing $H_2O_2$ level by a mutation in the *UPBEAT1* (*UPB1*) gene that represses peroxidases [141] leads to a reduction in the number of PZ cells without affecting the stem cell population, whereas conversely elevating $H_2O_2$ level by *UPB1* overexpression induces an increase in the PZ size [140]. Thus, the ROS level and/or balance affects SAM activities. Interestingly, increased ROS accumulation in the SAM by a mutation in the mitochondrial protease *FTSH4* causes loss of the SAM at elevated temperatures [142], indicating that the ROS level and/or balance is important for robustness of the SAM against environmental fluctuations. Because nitrate is known to affects the *UPB1* expression in roots [143], nitrate availability may also modulate SAM functions via the regulation of *UPB1* and the ROS status. Nitrate is a major source of another redox-related and environment-responsive signaling molecule, nitric oxide (NO) [144], and high nitrate promotes NO production [145, 146]. Although NO production is necessary for root growth and the RAM maintenance [147], it is unclear whether NO participates in stem

491 cell regulation in the SAM. Because NO accumulation enhances *WOX5* expression in
492 the RAM [147], it is attractive to hypothesize that NO also affects *WUS* expression in
493 the SAM and, furthermore, that nitrate-derived NO production may mediate the
494 interplay between nitrate availability and stem cell activity in the SAM. Further
495 investigation is required to elucidate how redox-related signaling is integrated into the
496 intercellular communication network within the SAM shown in Fig. 4.

497 Roles of mechanical cues are receiving increasing attention in diverse
498 aspects of plant growth and development. It has been proposed that the epidermis,
499 which is under tension, is one of major growth-limiting tissues [53, 148]. Local cell
500 wall loosening of the epidermis often specifies a position of cell fate change. In the
501 SAM periphery, accumulation of auxin directs lateral organ initiation accompanied by
502 cell wall loosening [149-151], and localized application of cell-wall-loosening protein
503 Expansin also induces the formation of an organ primordium at the application site
504 [152]. Inter-tissue-layer propagation of the elasticity change was observed in the
505 process of organ initiation in the PZ [150]. Difference in cell stiffness is detected
506 between the CZ and PZ. The epidermis in the tip region of the SAM is stiffer than that
507 in the SAM periphery [150, 151, 153, 154], and importantly a methodology, named
508 ‘quantitative tandem epifluorescence and nanoindentation’, clearly demonstrated that
509 the stiff region corresponds with the *CLV3*-expresing domain, that is the CZ [155],
510 suggesting that cell stiffness seems to be a characteristics of stem cells in the SAM.
511 These findings raise important or intriguing questions. When cells in the PZ are
512 re-specified into stem cell identity by modulating *WUS* function, does the change in
513 cell stiffness reversibly occur? Conversely, does artificial cell wall hardening in the
514 PZ lead to re-specification of stem cell identity? How does the change in stem cell
515 identity in the epidermal L1 layer by mechanical cues affect stem cell behaviors in

inner L2 and L3 tissues? Because cytokinin and miR394 are signaling molecules produced in the L1 layer, it will be interesting to examine whether their production is controlled by changes in stiffness of the epidermis.

As described in each section of this review article, there are a number of unanswered questions about control of stem cell identity and behavior. Although conventional forward genetic approaches will still be useful to address such questions, increasing new techniques and tools such as single cell transcriptome analysis, reverse genetic approaches using genome editing, live imaging in combination with new technologies, will not only facilitate the identification of missing pieces in known pathways and mechanisms but also provide novel viewpoints on stem cell biology in plants.

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**Figure Legends**

**Fig. 1**

Stem cell niche in the RAM. The stereotypical arrangement of cells at the *Arabidopsis* root tip is illustrated. Stem cells (also called 'initial' cells), which are shown as hatched cells, are located around the quiescent center (QC), and the stem cell region is surrounded by blue lines. Differentiation potency of each stem cell is strictly limited according to its position. Basically, each stem cell contributes to the formation of a specialized cell file shown in the same color. Exceptions are endodermis/cortex initial and epidermis/lateral root cap initial; the former gives rise to endodermis cell files and cortex cell files, and the later produces epidermis cell files and lateral root cap cells.

**Fig. 2**

Stem cell niche in the SAM. Photos are top-view images of the *Arabidopsis* SAM at the vegetative stage by electron scanning microscope. The left is the original image and the right is overlaid by the following zones with different colors. Yellow indicates the central zone (CZ) composed of stem cells expressing *CLV3*. Beige and dark beige indicate peripheral zone (PZ) composed of transit amplifying cells. The dark beige region shows the outer PZ. Magent indicates the organizing center (OC) defined by *WUS* expreson. Please see main text for further details.

**Fig. 3**

Layered arrangement of stem cell subpopulations in the SAM. **A** The SAM is composed of three tissue layers: the epidermal L1 (light blue), the sub-epidermal L2 layers (green) and the inner L3 tissue (grey). L1 and L2 cells divide anticlinally,

resulting in clonal cell layers. L3 cells divide both anticlinally and periclinally, giving rise to internal tissues. **B** Stem cells consisting the CZ (yellow) spread between all the layers. The OC (magenta), which is defined by *WUS* expression, is located within the L3 tissue. The lowermost layer of the CZ overlaps with the uppermost layer of the OC. **C** There are three stem cell subpopulations; stem cells with the L1 identity (striped pattern of yellow and light blue), those with the L2 identity (striped pattern of yellow and green) and those with the OC identity (striped pattern of yellow and magenta).

**Fig. 4**

Factors involved in intercellular communication between tissue layers in the SAM. Yellow indicates the central zone composed of stem cells expressing *CLV3*. Magenta indicates the organizing center defined by *WUS* expression. Light blue shows the epidermal L1 layer. Please see main text for further details about each factors and pathways.

Fig. 1

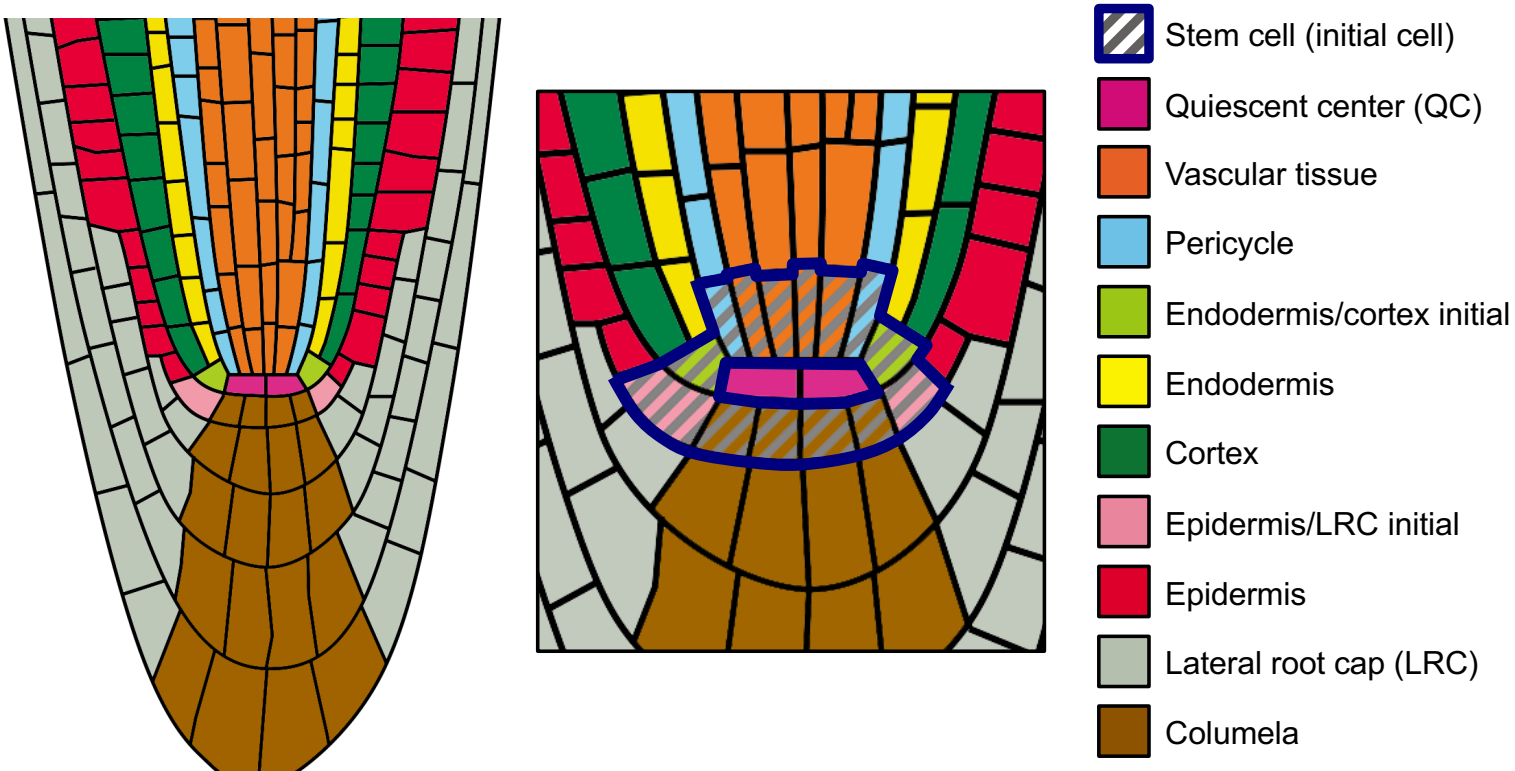
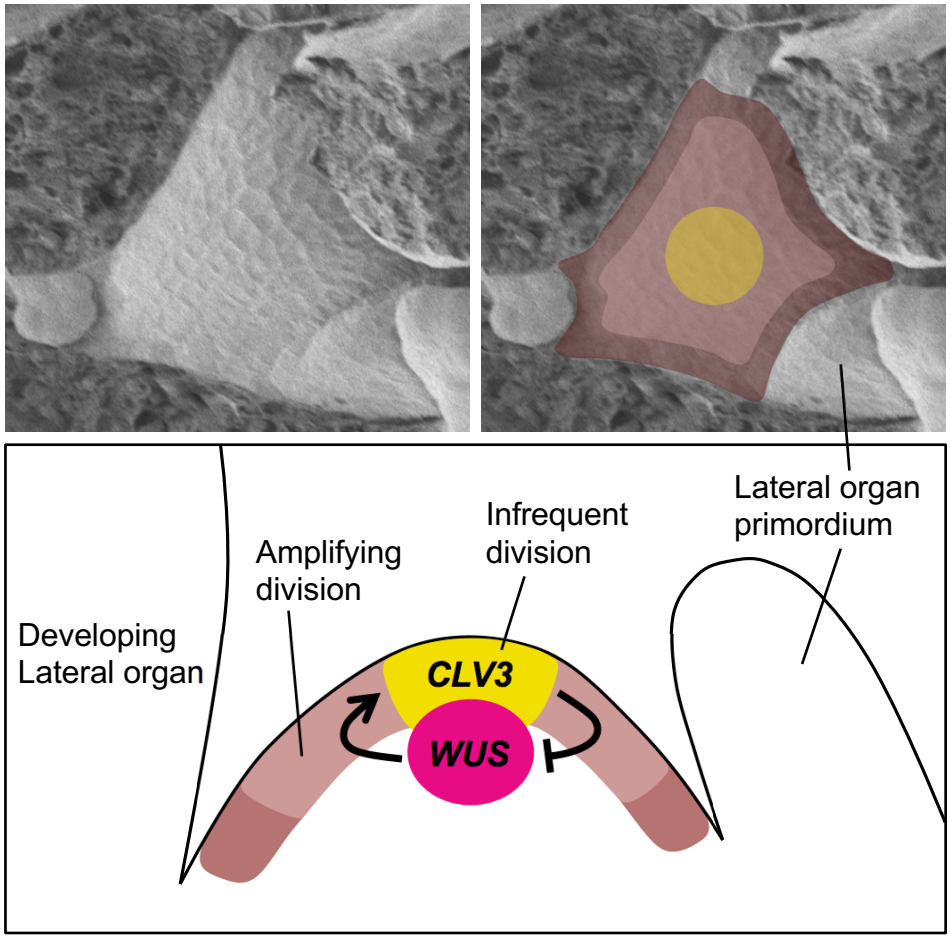

Stem cell (initial cell)
Quiescent center (QC)
Vascular tissue
Pericycle
Endodermis/cortex initial
Endodermis
Cortex
Epidermis/LRC initial
Epidermis
Lateral root cap (LRC)
Columela

Fig. 2




**Central zone (CZ)**
Slowly dividing stem cells. ***CLV3*** is expressed.

**Organizing center (OC)**
***WUS*** is expressed.

**Peripheral zone (PZ)**
'Transit amplifying cells' prior to incorporation into primordia

**Outer PZ**
Stem cell identity cannot be re-specified even in *clv* mutants.

Fig. 3

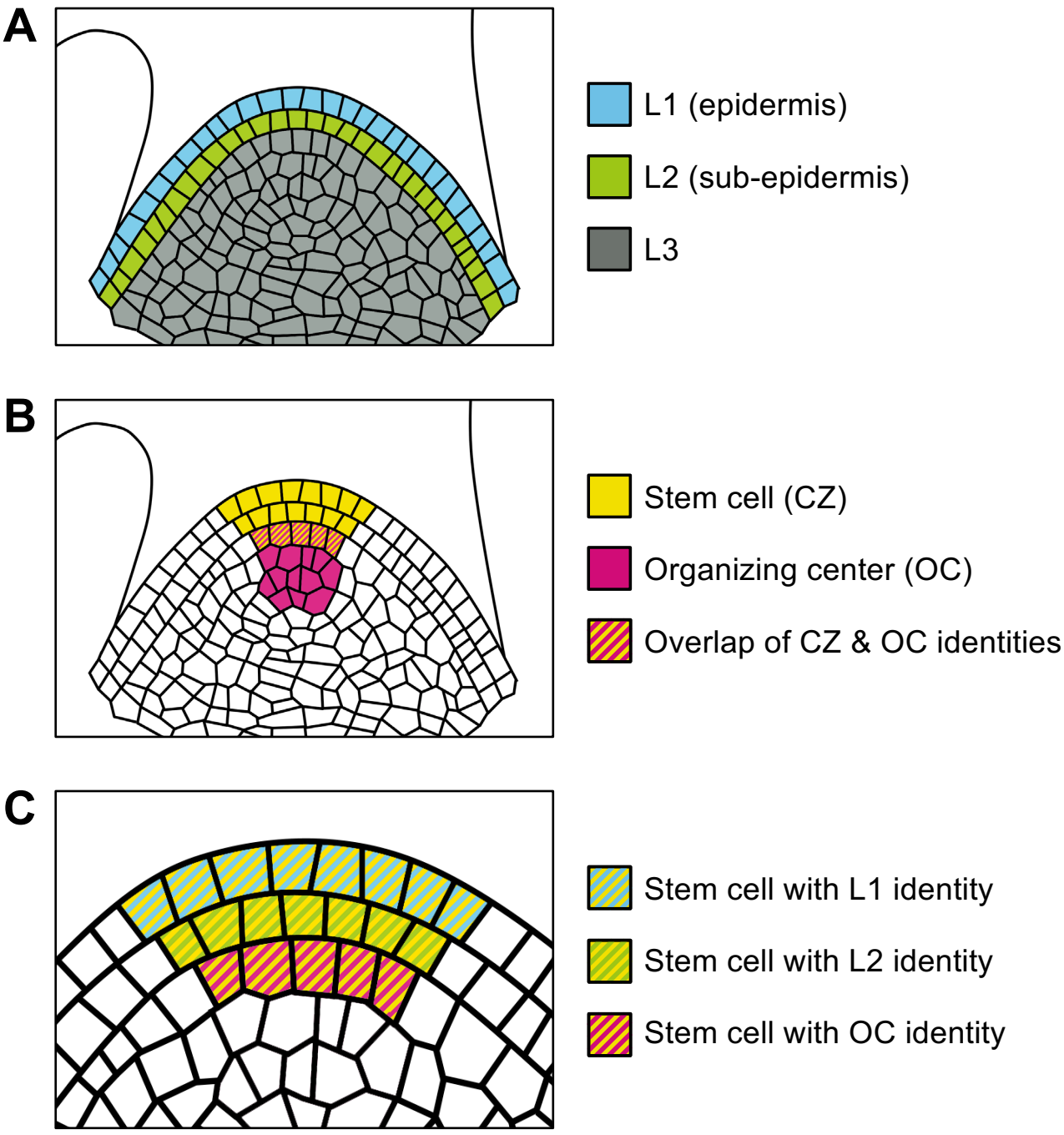
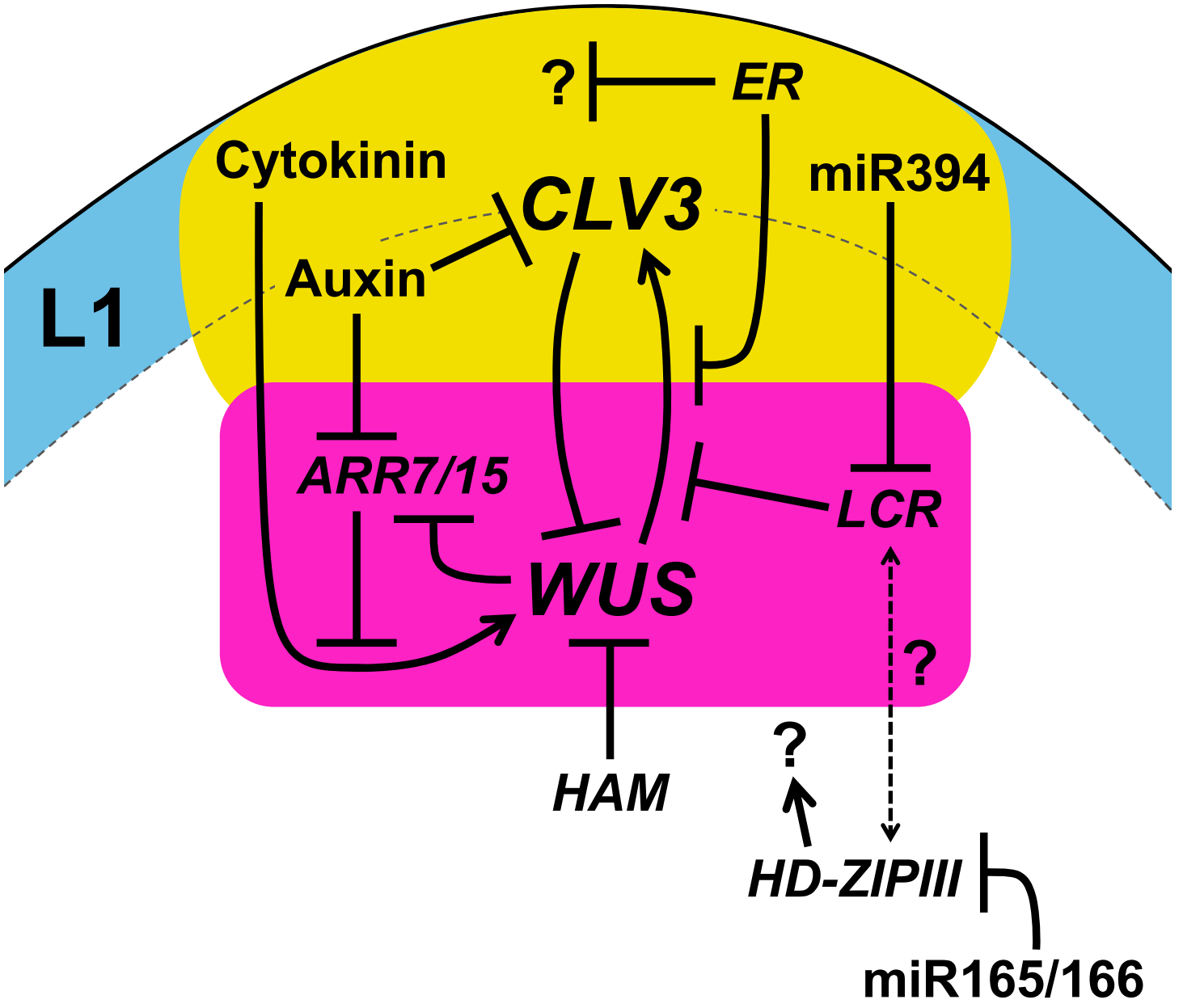

A
L1 (epidermis)
L2 (sub-epidermis)
L3
B
Stem cell (CZ)
Organizing center (OC)
Overlap of CZ & OC identities
C
Stem cell with L1 identity
Stem cell with L2 identity
Stem cell with OC identity

Fig. 4


?
ER
Cytokinin
CLV3
miR394
Auxin
L1
ARR7/15
LCR
WUS
?
HAM
?
HD-ZIPIII
miR165/166