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Short communication

Dynamic expression reveals a two-step patterning of *WUS* and *CLV3* during axillary shoot meristem formation in *Arabidopsis*

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ABSTRACT

Seed plants have a remarkable capability to produce axillary meristems (AM) in the leaf axils, however, the dynamic establishment of a stem cell niche in AM is largely uncharacterized. We comprehensively examined the dynamic patterning of *WUSCHEL* (*WUS*) and *CLAVATA3* (*CLV3*), the two key marker genes defining the shoot stem cell niches, during AM formation in *Arabidopsis*, and we found that a two-step patterning of *WUS* and *CLV3* occurred during AM stem cell niche establishment. Our further work on the *wus* and *clv3* mutants implicates that such two-step patterning is likely critical for the maintenance of AM progenitor cells and the specification of AM stem cell niche. These data provide a cytological frame for how a stem cell niche is established during AM formation.

1. Introduction

Seed plants initially exhibit a single growth axis by forming a primary shoot apical meristem (SAM) during embryogenesis. The diversity of plant architectures is largely dependent on the postembryonic formation of the new shoot apical meristems, named axillary shoot meristems (AMs), which finally develop into branches. Both SAM and the developed AM have a functional stem cell niche organized by the central zone (CZ), which resides at the summit of the meristem and harbors the stem cells, and the organizing center (OC) underneath the CZ that is required to maintain the stem cells and determine their fates. The stem cells in the CZ continuously divide and provide initials for the peripheral zone (PZ), which generates lateral organs at the flanks of the meristem. Extensive studies on *Arabidopsis* have revealed that the homeobox gene *WUSCHEL* (*WUS*) expressed in OC and the peptide gene *CLAVATA3* (*CLV3*) expressed in CZ form a negative feedback loop to dynamically maintain the stem cell niche within SAM (Gaillochet et al., 2015). Therefore, the dynamic and spatial patterning formations of *WUS* and *CLV3* in embryonic SAM and postembryonic AM represent the central events for establishment of the shoot stem cell niche in plants.

During embryogenesis, the expression of *WUS* is initiated in the four inner cells of 16-cell embryos, which finally give rise to the OC by a series of asymmetric divisions (Mayer et al., 1998). Interestingly, the *CLV3* mRNA is detectable as early as heart-stage embryos and ultimately restricted to the CZ (Fletcher et al., 1999; Tucker et al., 2008). Consistent with this, the earliest abnormal structure of SAM primordium is observed in the heart-stage embryos of the *wus* mutants (Boscá et al., 2011). It appears that *WUS* and *CLV3* expression exhibit few overlaps but are restricted to a few cells that are subsequently defined into OC and CZ at later embryogenic stages, respectively (Boscá et al., 2011), suggesting that spatial patterning of *WUS* and *CLV3* during embryogenesis might be critical to specify the OC and CZ. Moreover, the expanded domain of *WUS* expression is found in the *clv1*, *clv2*, and *clv3* mutant embryos from heart stage (Schoof et al., 2000), and *CLV3* expression is absent in the matured *wus-1* embryos (Brand et al., 2002), implying that WUS is required for *CLV3* expression and their feedback loop is critical for appropriate SAM stem cell niche formation during embryogenesis.

Unlike the SAM that forms in embryogenesis, the AM initiates from the adaxial side of leaf axils by forming a bump and then develops into axillary buds. Genetic studies on *Arabidopsis* and rice have identified a large number of transcription factors that function in AM initiation, including *SHOOT MERISTEMLESS* (*STM*), *REVOLUTA* (*REV*), *REGULATOR OF AXILLARY MERISTEM FORMATION* (*ROX*), *LATERAL SUPPRESSOR* (*LAS*), *RAX1*, *TILLERS ABSENT1 (TAB1*, also known as

Abbreviations: SAM, shoot apical meristem; AM, axillary shoot meristems; CZ, central zone; OC, organizing center; WUS, WUSCHEL; CLV3, CLAVATA3; STM, SHOOT MERISTEMLESS

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OsWUS), and *MONOCULM1* (*MOC1*) (Greb et al., 2003; Li et al., 2003; Müller et al., 2006; Otsuga et al., 2001; Tanaka et al., 2015; Yang et al., 2012; Yang and Jiao, 2016). Moreover, recent studies on *Arabidopsis* also demonstrate that cytokinin signaling and auxin depletion in the leaf axil are required for AM initiation (Wang et al., 2014a,b), demonstrating that microenvironmental phytohormone signaling is also critical for AM formation.

Despite the fact that the regulatory network, including phytohormone signals and multiple transcription factors, has been shown to regulate the initiation of AM (Yang and Jiao, 2016), the cytological feature of dynamic patterning of *WUS* and *CLV3* during AM formation is not fully characterized, and to what extent this early AM patterning resembles that of embryonic SAM remains to be explored. In this study, we describe the dynamic characteristics of *WUS* and *CLV3* expression during AM formation in *Arabidopsis*. Our results reveal a two-step patterning of *WUS* and *CLV3* during AM formation. Further characterization of the *wus* and *clv3* mutants suggests that such patterning is essential for establishing AM stem cell niches. These data provide a cytological basis for dissecting the molecular regulation of AM formation.

2. Material and methods

2.1. Plant materials and growth condition

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used in this study, except in the experiments performed with the *clv3-2*, in which Landsberg (*Ler*) background was used (Clark et al., 1995). The *wus* T-DNA insertion mutant (CS349353) was obtained from Arabidopsis Biological Resource Center as described previously (Daum et al., 2014). The plants were grown under short-day conditions (8 h light/16 h dark at 22 °C) for 28 d and then shifted to long-day conditions (16 h light/8 h dark at 22 °C) for 2–6 d for histological analyses of AM development and three weeks for determination of the formed axillary buds, respectively.

2.2. RNA in situ hybridization

Plant samples were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) plus 0.01% Triton X-100 overnight at 4 °C and then washed twice in cold PBS buffer, dehydrated in a graded ethanol series, and embedded in Paraplast Plus (Sigma-Aldrich) after replacement with xylene. RNA *in situ* hybridization was performed with 8-μm sections of samples as described previously (Mayer et al., 1998). For RNA probes, a 974-bp *WUS* cDNA was amplified with the primers 5 'GAATCAAACACACATGG-AGCC 3′ and 5′ CGAAGCATAGTTGTGAACATACG 3′; a full-length *CLV3* cDNA was amplified with the primers 5′ TCACTCAGTCACTTTCTCTCTAAAA 3′ and 5′ GAAAATCATGAGATATAATAGTGCG 3′, and a 318-bp *STM* fragment was amplified with the primers 5′ GTTGCTTCTTCTTCTTCTCCTTC 3′ and 5′ AGCTT-CCATGAATTGATCAAGCCC 3′. The resulting cDNAs were cloned into the pGEM-T easy vector. The PCR products containing a T7 or SP6 promoter sequence amplified from recombinant plasmids were used as templates for probe synthesis. None of the probes were hydrolyzed.

2.3. Confocal microscopy

For confocal microscopy, aerial parts of a plant without leaf blades were collected and prepared according to the method described previously (Wang et al., 2014b), with the slight modification of adding 0.01% Triton X-100 to the fixative solution. Sections of 30–50 μm were made using a Leica VT1200S vibratome. For high-resolution images, the sections were stained with 20 μg/mL propidium iodide (Sigma-Aldrich) and observed under an Olympus FV1000MPE confocal microscope. The GFP signal was detected with a 488-nm laser excitation line, and emission was determined at 505–525 nm. For detecting the propidium iodide staining, a 543-nm laser line was used for excitation, and emission was determined at 560–660 nm.

2.4. Histological analysis

Plant tissues were fixed in FAA (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) overnight at 4 °C, dehydrated in a graded ethanol series, and embedded in Technovit 7100 resin. 3-μm-thick sections were made with a Leica RM2255 microtome. Sections were stained with 0.5% toluidine blue (Sigma-Aldrich) and photographed under an Olympus BX51 microscope with bright-field illumination.

3. Results and discussion

3.1. Developmental stages and cytological characteristics of AM formation

The initiation of *Arabidopsis* AM has been divided into the four developmental stages, stage 1 to stage 4 (S1–S4), by the spatially expressed *SHOOT MERISTEMLESS* (*STM*) gene and the AM morphological changes (Long and Barton, 2000). To define the dynamic expression of *WUS* and *CLV3* during AM formation, we expanded the AM developmental stages from stage 0 (S0), for the leaf axils showing no sign of AM initiation, to stage 5 (S5), for a well-developed AM with initiated leaf primordia. We first compared the *STM* expression by RNA *in situ* hybridization and the cytological characteristics at these AM developmental stages under our growth conditions (Fig. 1). Briefly, at S0, the *STM* expression was restricted to the boundary between SAM and leaf axils showing no sign of cell division (Fig. 1). At S1, *STM* expression was enriched in the medial area of leaf axils where cell division was occurring, and, in the S2 axils, the expression region of *STM* extended to the adaxial base above the insertion point of the leaf where a mass of dividing cells was visible (Fig. 1) (Long and Barton, 2000). In S3 axils, a distinguishable bump was formed on the adaxial base of leaf axils with a uniform expression of *STM*, while the S4 AMs displayed a domed morphology with *STM*-negative regions, an indicator of leaf primordium formation (Fig. 1) (Long and Barton, 2000). Finally, the S5 AM exhibited the newly initiated leaf primordia with a typical *STM* expression pattern, as did the primary SAM (Fig. 1). Furthermore, the developmental stages of rosette leaf primordia corresponding to S0–S5 were P_{1-6}, P_{7-8}, P_{9-10}, P_{10-12}, P_{12-13}, and $P_{\geq 14}$, respectively (Long and Barton, 2000) (Fig. 1A).

3.2. Dynamic expression patterning of WUS and CLV3 in AM formation

We next carefully investigated the spatiotemporal expression of *WUS* and *CLV3* in the leaf bases during AM formation by *in situ* RNA hybridization. As expected, neither *WUS* nor *CLV3* transcription was detected in the S0 leaf axils (Fig. 2A,B). The *WUS* mRNA was initially detectable at S1 in the inner junction site of the leaf and SAM, and, at S2, the *WUS* expression expanded largely to the adaxial base of leaf axils, which obviously overlapped with the cell division region (Fig. 2A), implying that the onset of *WUS* expression might be associated with the dividing meristematic cells whose fates were determined to form AM. Subsequently, the *WUS* expression was continuously observed in a broader region beneath L1 and L2 within the developing bump throughout S3 and S4, but finally restricted to the OC in a well-developed AM at S5 (Fig. 2A). However, the *CLV3* mRNA was first detectable at S2, and, surprisingly, the onset of *CLV3* expression was also observed in the central region of the dividing cells by being spatially activated within the *WUS*-expressed domains (Fig. 2B). This central expression pattern was even more obvious at early S3 in the bump where the *CLV3* mRNA was still absent in the layer 1 and layer 2 cells and overlapped with *WUS* (Fig. 2A). Subsequently, the expression of *CLV3* underwent a dynamic shift upward to L2 and L1 from late S3 and finally localized at the CZ at S5 (Fig. 2B). These observations demonstrate that *WUS* and *CLV3* are initially activated in

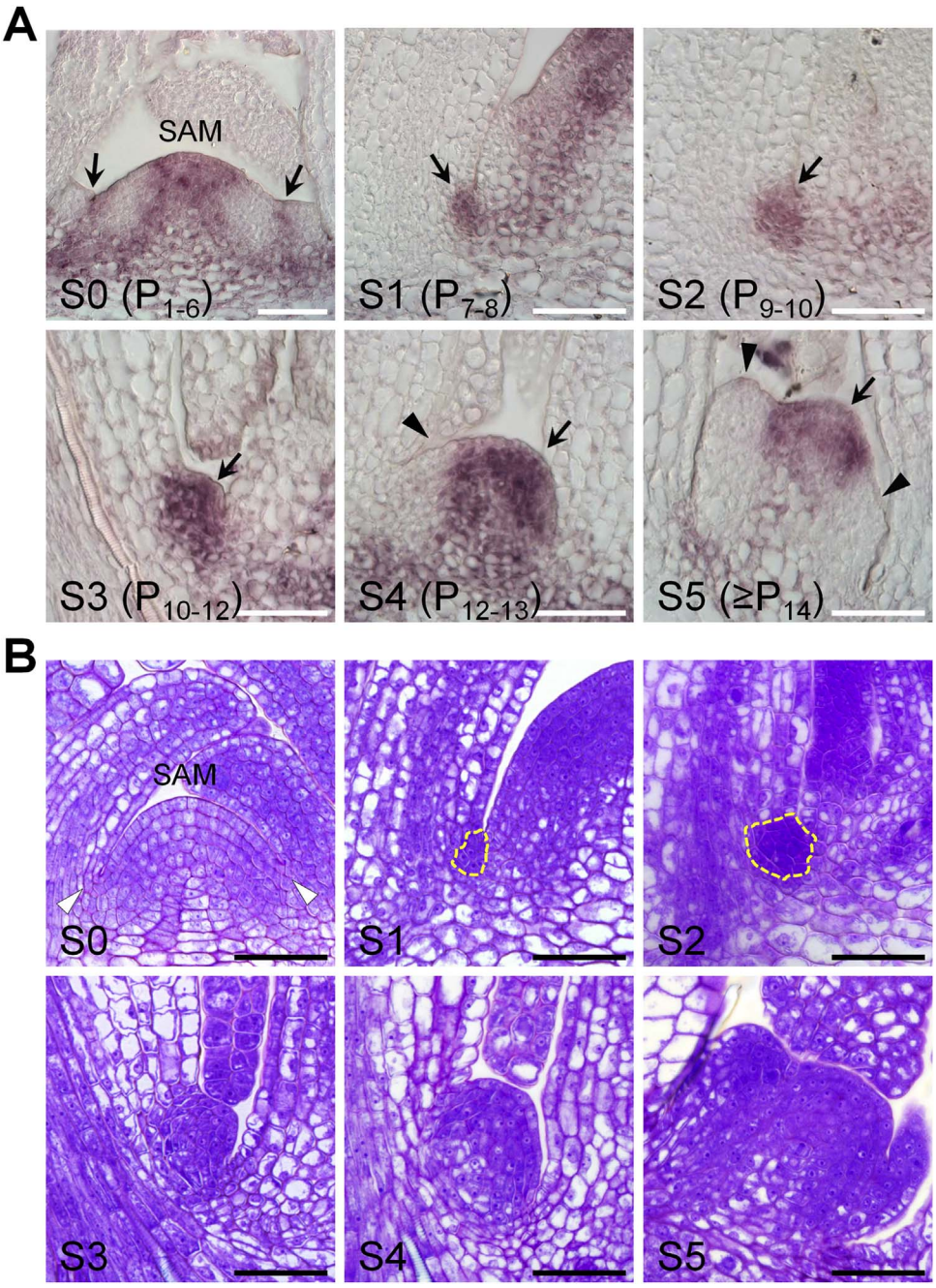

Fig. 1. Developmental stages of AM formation.(A) *STM* expression pattern during axillary meristem development from stage 0 to stage 5 (S0-S5). Arrows note the *STM* expression region, and the arrow heads show the negative *STM* expression regions. The developmental stages of rosette leaf primordia corresponding to S0-S5 are shown in the brackets. (B) Cytological characterization of AM formation. Note that the cell division is activated at S1 and S2 leaf axils where AM bumps are not morphologically detectable. The white arrow heads indicate the S0 leaf axils. Bars = 40 μm.

the AM progenitor cells and a dynamic patterning shift occurs during the AM stem cell niche formation.

To verify this, we further visualized the WUS protein accumulation and *CLV3* expression in the transgenic plants respectively harboring a *pWUS:WUS-GFP* construct in *wus* background and *pCLV3:GFP* construct in Col-0 background. WUS has been shown to act as a cell-layer moving protein to mediate stem cell homeostasis in the *Arabidopsis* SAM (Daum et al., 2014; Yadav et al., 2011). As expected, the WUS-GFP protein was visualized in almost all the dividing cells from S1 to early S3, by a broader region than did its transcripts (Fig. 2C). As the AM bump developed, the WUS-GFP protein gradually concentrated in CZ and OC from late S3–S5. In contrast, the GFP signals in the transgenic *pCLV3:GFP* plants clearly showed that the *CLV3* expression was initially activated in the central region of the developing bump but then dynamically shifted into the CZ when the AM stem cell niche formed (Fig. 2D), confirming the two-step patterning of *WUS* and *CLV3* during AM formation.

3.3. *WUS* is required for maintenance of AM progenitor cells and activation of *CLV3* expression

To further investigate the possible function of *WUS* and *CLV3* and their interaction during AM stem cell niche formation, we obtained a T-DNA insertion mutant of *WUS* and the *clv3-2* mutant (Clark et al., 1995; Daum et al., 2014) and examined their AM formation respectively. When compared to that in WT, the AM initiation was largely disrupted

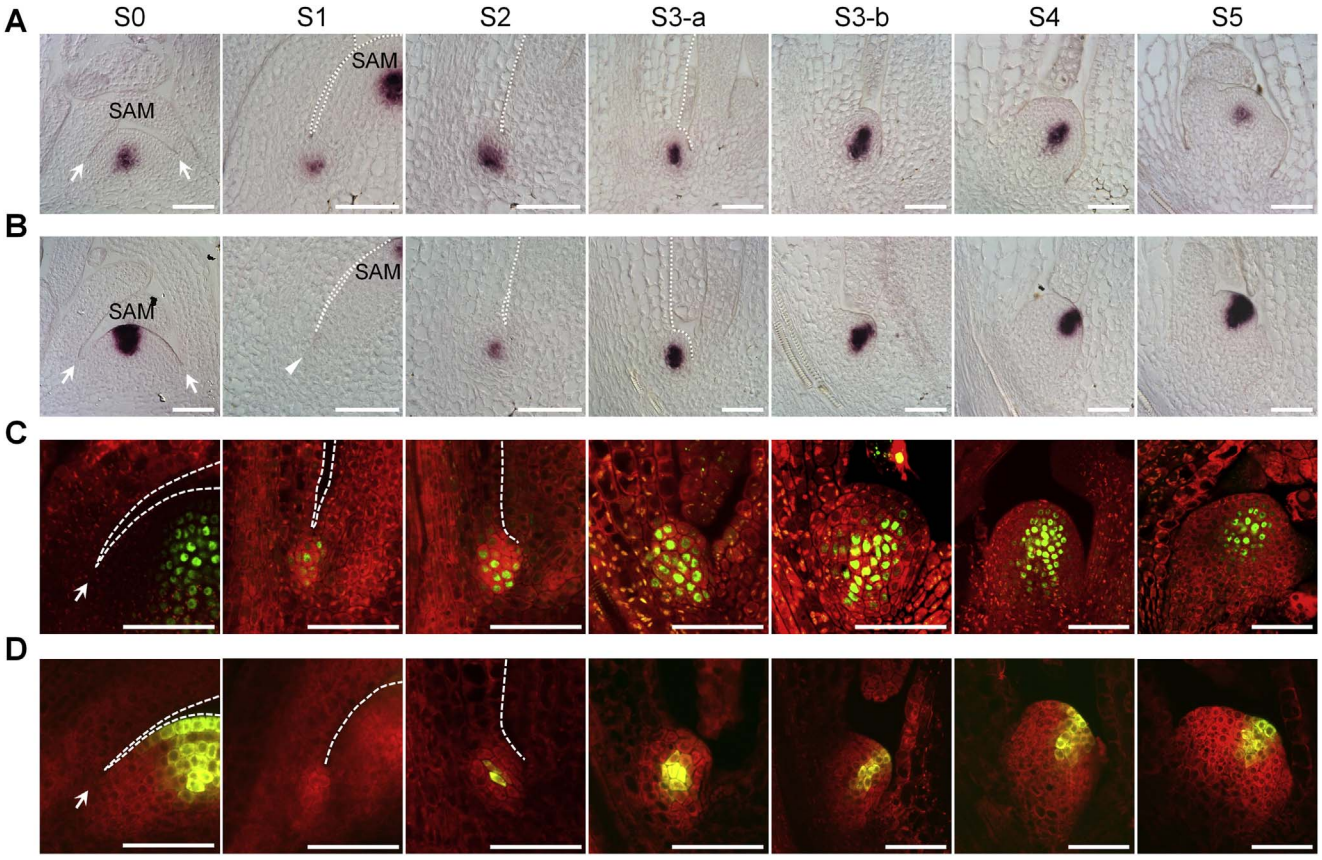

Fig. 2. Dynamic patterning of *WUS* and *CLV3* in AM stem cell niche formation.(A, B) The spatiotemporal expression of *WUS* (A) and *CLV3* (B) by RNA *in situ* hybridization during AM formation. S3-a and S3-b indicate the early and late stage 3, respectively. Arrows note S0 axils, and arrow heads indicate S1 axils without *CLV3* expression. (C) WUS-GFP protein distribution in AM formation. Longitudinal sections across leaf axils of the *pWUS:WUS-GFP*;*wus* plants were visualized under a confocal microscope. (D) Dynamic patterning of *CLV3* in AM formation assayed with the *pCLV3:GFP* plants. Bars = 40 μm.

in the leaf axils of the *wus* mutant (Fig. 3A,B). By contrast, the AM formation in the *clv3-2* leaf axils was slightly dampened when compared to that in WT (Fig. 3C,D). Interestingly, cytological analysis revealed that a mass of differentiated cells were still observed in some of the *wus* leaf axils where AMs were expected to be initiated (Fig. 3E), suggesting that *WUS* may not be required for activation of cell division but is likely to be involved in the maintenance of the fate or identity of the AM progenitor cells. Furthermore, RNA *in situ* hybridization revealed that *STM* was still weakly expressed at S0 but undetectable thereafter, and that *CLV3* mRNA was not detectable in the *wus* leaf axils (Fig. 3F,G), indicating that *WUS* is required for activation of *CLV3* expression during AM initiation. By contrast, the *WUS* expression was highly elevated and expanded as early as S2 in the *clv3-2* leaf axils, in which AM primordium was larger than that in wide-type (Fig. 3H), implicating that CLV signaling already plays a role in restricting or balancing the *WUS* expression for proper AM size determination at early developmental stages.

In summary, our data reveal a two-step patterning of *WUS* and *CLV3* in AM formation (Fig. 4), which provides further cytological insights into how the AM stem cell niche is established. Firstly, the *STM* is expressed in the meristematic cells of leaf axils that define to initiate the AM, and its continuous expression is likely required for both AM initiation and stem cell niche formation (Shi et al., 2016). Secondly, the onset of WUS, and subsequent *CLV3*, implicates that the identity of AM stem cells are defined in this process. Because the *CLV3* is the direct target of WUS (Yadav et al., 2011), the fact that *CLV3* is expressed after *WUS* and that their expression overlaps at early stages suggests that *CLV3* transcription may be directly activated by WUS, which differs from the delayed activation of *CLV3* expression during embryogenesis (Boscá et al., 2011; Fletcher et al., 1999; Tucker et al., 2008), although WUS seems to be required for the activation of *CLV3* in both cases. Thirdly, the overlapped expression of these two genes and the differentiated cells observed in the *wus* leaf axils imply that the interaction between WUS and CLV signaling is likely required for maintenance of AM progenitor cell identity and population. Therefore, it appears that the *WUS* and *CLV3* patterning and their roles during AM formation may not fully recapitulate those in embryonic SAM formation. In contrast, during *in vitro* shoot regeneration, *WUS* expression throughout a large domain within the callus at early stage is required for promoting shoot progenitor cell identity, and the subsequent WUS activity is required for *de novo* assembly of shoot meristem (Gordon et al., 2007). Recent studies also suggest that the high cytokinin but low auxin gradient within the leaf axil is necessary for the initiation of AM (Wang et al., 2014a; Wang et al., 2014b). Thus, it is also an open question whether the dynamic interaction of WUS and CLV3 during AM formation shares some features with *de novo* shoot regeneration. Moreover, disruption of *WUS* neither completely prohibits the onset of cell division in AM initiation nor fully blocks AM formation, implying that other factors also function critically in AM formation (Brand et al., 2002). Indeed, *STM* has been shown to be essential for AM formation (Shi et al., 2016), and it could activate the expression of *CycB1;1* (Lenhard et al., 2002), which might also explain why a mass of differentiated cells is still observed at some of the leaf axils in the *wus* mutant. Therefore, it is of more interest to link other regulatory networks, including hormonal signaling, into this patterning to determine the onset of *WUS* and the strict regulation of WUS/CLV3 during AM formation.

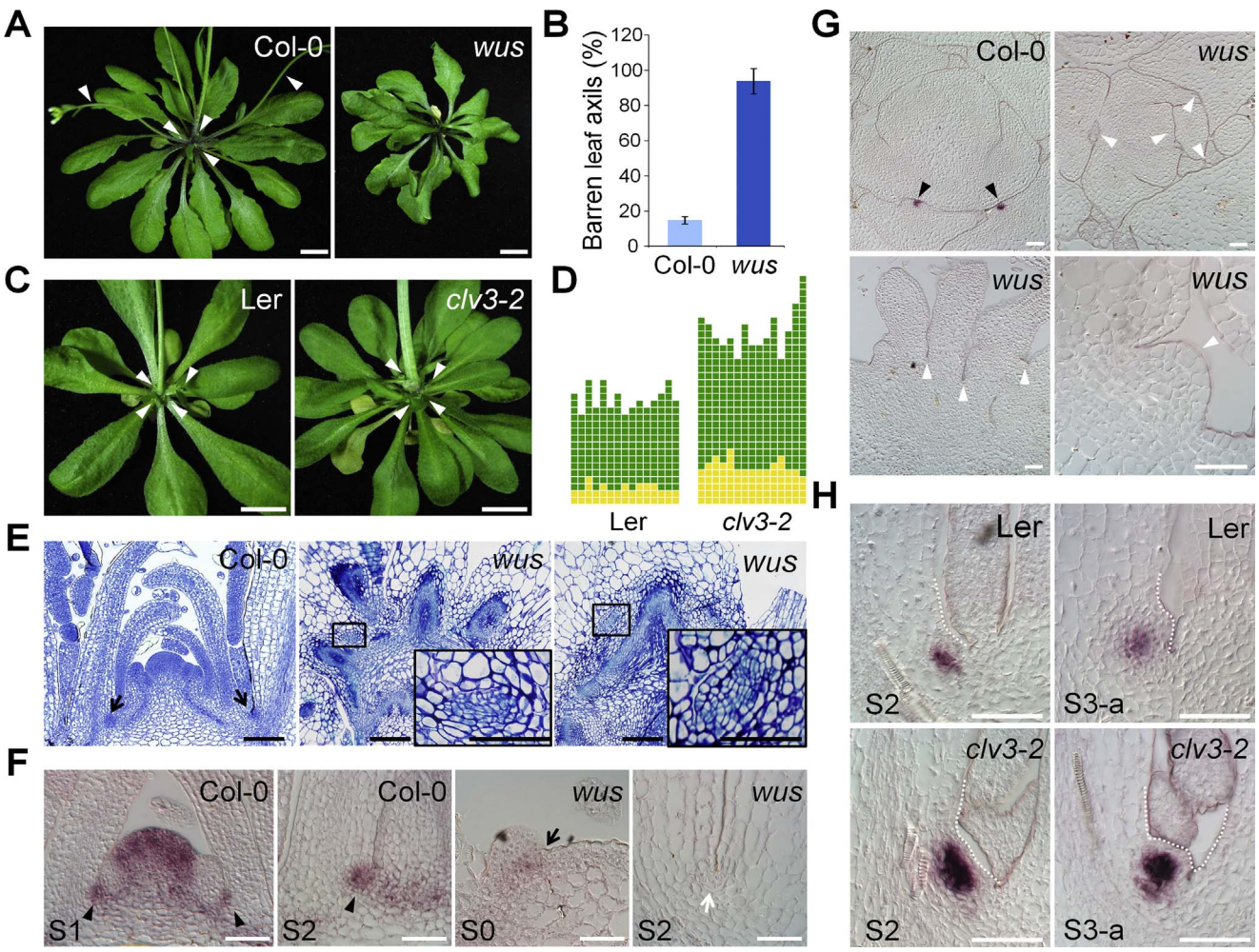

Fig. 3. AM formation and interaction of *WUS* and *CLV3* in the *wus* and *clv3* mutants.(A, B) AM formation (A) and proportion of barren rosette leaf axils (B) in wild-type (Col-0, n = 16) and *wus* (n = 26) plants. Arrow heads note the axillary buds formed in Col-0. The error bars show the SD. (C, D) Phenotypes (C) and schematic illustration (D) of axillary buds formation in the rosette leaf axils of wild-type (*Ler*) and *clv3-2*. Arrow heads note axillary buds. Each column represents a single plant, and each square within a column stands for an individual leaf axil. Green denotes the presence of an axillary bud and yellow the absence of an axillary bud in any particular leaf axil. (E) Cytological characterization of Col-0 and *wus* leaf axils. Arrows indicate that the dividing cells for AM initiation were stained densely in Col-0. Black boxes show the leaf axils with a mass of differentiated cells in leaf axils of the *wus* mutant. Bars = 100 μm. (F) *STM* expression in the *wus* mutant. Note that *STM* mRNA was still detectable at S0 leaf axils and undetectable thereafter. Bars, 40 μm. (G) Undetectable *CLV3* expression in leaf axils of the *wus* mutant. The black arrow heads indicate the *CLV3* expression regions in Col-0, and the white arrow heads indicate no *CLV3* mRNA was detected in the *wus* mutant by transverse and longitudinal sections. (G) Expanded *WUS* expression in the *clv3-2* leaf axils at S2 and early S3. Bars in (A) and (C), 1 cm, and in (F) and (G), 40 μm.

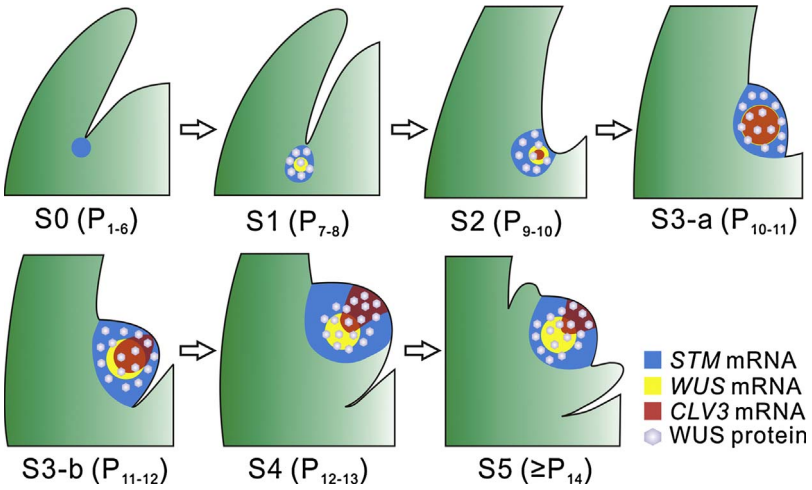

Fig. 4. Schematic illustration of the dynamic patterning of *STM*, *WUS*, *CLV3* mRNA, and WUS protein during AM stem cell niche formation.The developmental stages of rosette leaf primordia corresponding to AM S0-S5 are shown in the brackets.

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