**The Stem Cell Niche in Leaf Axils Is Established by Auxin and Cytokinin in Arabidopsis**

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Plants differ from most animals in their ability to initiate new cycles of growth and development, which relies on the establishment and activity of branch meristems harboring new stem cell niches. In seed plants, this is achieved by axillary meristems, which are established in the axil of each leaf base and develop into lateral branches. Here, we describe the initial processes of *Arabidopsis thaliana* axillary meristem initiation. Using reporter gene expression analysis, we find that axillary meristems initiate from leaf axil cells with low auxin through stereotypical stages. Consistent with this, ectopic overproduction of auxin in the leaf axil efficiently inhibits axillary meristem initiation. Furthermore, our results demonstrate that auxin efflux is required for the leaf axil auxin minimum and axillary meristem initiation. After lowering of auxin levels, a subsequent cytokinin signaling pulse is observed prior to axillary meristem initiation. Genetic analysis suggests that cytokinin perception and signaling are both required for axillary meristem initiation. Finally, we show that cytokinin overproduction in the leaf axil partially rescue axillary meristem initiation-deficient mutants. These results define a mechanistic framework for understanding axillary meristem initiation.

**INTRODUCTION**

As sessile organisms, plants have evolved remarkable developmental plasticity to adapt to the environment. Distinct from the majority of animals, plants have the potential to initiate new organs during postembryonic development. The shoots of most vascular plants can give rise to a succession of branches postembryonically, making the whole shoot a ramifying system. Along with the main shoot, each shoot branch generates new leaves from its apex (Domagalska and Leyser, 2011). In the seed plants, branching is typically lateral (Hagemann, 1990); that is, secondary growth axes arise from buds situated in or near the axils of leaves. Bud formation requires the establishment of axillary meristems (AMs), which are leaf axil stem cell niches. The developmental flexibility of shoot systems is largely determined by AM specification and activation. AM activity has long been a target of breeding scientists to increase crop yield by influencing both tiller and panicle/ear number and panicle/ear branching complexity.

AM specification occurs during modular plant growth into phytomers, which consist of a leaf, an AM on the adaxial side of the leaf base (i.e., the side of the leaf toward the shoot), and a stem segment. AMs in seed plants typically arise in close physical association with the base of the subtending leaf (Snow and Snow, 1942; Grbić and Bleecker, 2000; Long and Barton, 2000). Clonal analysis indicated that the AM and the subtending leaf share a common pool of ancestral cells (Furner and Pumfrey, 1992; Schnittger et al., 1996). AM initiation is closely associated with adaxial leaf fate. When adaxial sides of leaves are abaxialized, either in mutants or by microsurgical incisions between leaf primordia and meristems, AM initiation is compromised (Snow and Snow, 1942; Lynn et al., 1999; Eshed et al., 2001). Conversely, ectopic AMs arise on the underside of fully adaxialized leaves, such as in the *phabulosa-1D* mutant (McConnell and Barton, 1998). During AM initiation, axillary cells first divide, following which the dividing cells produce a raised meristem. After this, the meristem forms its first axillary leaf primordia (Supplemental Figure 1). After initiation, axillary buds may either continue to develop into branches (i.e., undergo outgrowth) or may remain dormant for different periods of time (Stimberg et al., 2002). Expression of the shoot meristem marker **SHOOT MERISTEMLESS (STM)** persists in the boundary region at the leaf axils, initially at low levels and then at increased levels during AM initiation (Grbić and Bleecker, 2000; Long and Barton, 2000). The STM expression pattern can be explained by both the “detached meristem” model, in which a small number of stem cells detach from the shoot apical meristem (SAM) and associate with the leaf axil as the leaf differentiates from the SAM, and the “de novo induction” model, in which an AM initiates from leaf cells that have lost stem cell identity and differentiated into leaf cells (Long and Barton, 2000).

Phytohormones play central roles during lateral organ formation. Leaf and floral organogenesis at the SAM is triggered by the accumulation of auxin (Reinhardt et al., 2000), which depends largely on the auxin efflux transporter PIN1 for its distribution.
(Reinhartd et al., 2003; Heisler et al., 2005). Lateral root initiation depends on the interplay of two phytohormones: auxin and cytokinin. Auxin promotes early events during lateral root initiation (Benková et al., 2003; De Smet et al., 2007; Dubrovsky et al., 2008; Swarup et al., 2008), with both polar auxin transport and signaling involved. On the other hand, cytokinins constrain lateral root development (Werner et al., 2003; Li et al., 2006). More recently, it was reported that cytokinins inhibit lateral root initiation (Laplaze et al., 2007; Ruzicka et al., 2009; Bielach et al., 2012).

The effect of phytohormones on AM outgrowth, during which a bud grows out to produce a branch, has been extensively studied (reviewed in Domagalska and Leyser, 2011). Auxin and cytokinin have antagonistic roles in the regulation of AM outgrowth. Auxin inhibits AM outgrowth, whereas cytokinins antagonize auxin in promoting bud activation. Strigolactones, a group of newly identified phytohormones, act together with auxin to inhibit AM outgrowth.

It is currently unclear whether phytohormones regulate AM initiation. In this study, we report that AM initiation requires a domain of low auxin, although an established AM produces auxin. We also show that AM initiation is associated with a cytokinin signaling pulse and that perturbation of cytokinin perception or signaling affects AM initiation. In addition to identifying a large number of genes affecting AM initiation, we propose a mechanistic framework for AM initiation.

RESULTS

Low Auxin in the Leaf Axil during Leaf Formation

AMs are initiated at the axil of subtending leaves in Arabidopsis thaliana during early leaf development. The expression of meristem marker STM is elevated in the center of the leaf axil around stage P9, which designates the eighth youngest primordium, and AMs are morphologically distinguishable by P11 (Long and Barton, 2000). To monitor auxin concentration in early leaf axes, we visualized the activity of the auxin concentration sensor DII-Venus (Vernoux et al., 2011). We found that DII-Venus had a strong signal, indicating low auxin, in the boundary region of emerging leaf primordia, starting from P7 (Figures 1A and 1B). This signal spreads into the adaxial domain of the leaf primordium as well. As a negative control, mDII-Venus, which harbors a mutation and does not activate auxin in promoting bud activation. Strigolactones, a group of newly identified phytohormones, act together with auxin to inhibit AM outgrowth.

To dynamically monitor the AM initiation process, we performed live imaging of DII-Venus expression dynamics during AM initiation. Using an auxin signaling reporter DR5:GFP-ER (Benková et al., 2003), we found an absence of signals in the leaf axil, although strong signal was associated with leaf primordium formation and in the SAM, as well as in stipules (Figure 1C).

Low Auxin in the Leaf Axil before AM Initiation

To dynamically monitor the AM initiation process, we performed live imaging of axes of leaf primordia that were isolated from the shoot apex. Consistent with a previous report (de G Alvarez et al., 2006), we found that AMs were efficiently initiated in cultured leaf primordia from P9 to P11 in the absence of any exogenous phytohormone, in Columbia-0 (Col-0) and Landsberg erecta (Ler) ecotypes (Supplemental Table 1). In fact, AM initiation was enhanced in isolated leaves because multiple AMs Occasionally initiated at the axil of one single leaf. To determine if these meristems were AMs or adventitious meristems, we first used scanning electron microscopy to examine the origin of these meristems. AMs are initiated in close association with the leaf axil epidermis (Grbić and Bleecker, 2000; Long and Barton, 2000; Greb et al., 2003), whereas adventitious meristems derived from callus originate from pericycle (Atta et al., 2009; Sugimoto et al., 2010). The meristems at the adaxial base of isolated leaf primordia were initiated from cells above the vasculature (Supplemental Figure 1), suggesting that they have similar origins to AMs. In addition, the formation of meristems in isolated leaf primordia was compromised in AM initiation mutants but not in the aberrant lateral root formation4-1 mutant defective in callus formation (Sugimoto et al., 2010; Supplemental Table 1), implying that this process is mediated by pathways that regulate AM initiation, rather than callus and adventitious shoot formation. Finally, we found that only the region within 50 μm of the leaf insertion site had the potential for AM initiation. Taken together, these data indicate that isolated leaf primordium culture provides a good system in which to live-image AM initiation.

Using live-imaging of DII-Venus expression dynamics during AM initiation, we found that cells with low auxin concentration are AM progenitors. At the point a leaf primordium was isolated, DII-Venus signal was observed only in cells close to the incision line (a P3 stage primordium is shown in Figure 1D). Although DII-Venus expression expanded into additional adjacent cells within a few hours (Figures 1E and 1F), only some of the cells with the original DII-Venus signals divided after 72 h in culture (Figure 1G). These dividing cells soon organized into a meristem within 96 h in culture (Figure 1H), and new leaf primordium were formed within 120 h in culture (Figure 1I).

Elevated Auxin Concentration in the Leaf Axil Perturbs AM Initiation

To determine whether low auxin is necessary for AM initiation, we ectopically expressed the Agrobacterium tumefaciens auxin indole-3-acetic acid (IAA) biosynthesis enzyme iaaM in the leaf axil region. This was achieved by targeting the expression of a pOp: iaaM transgene using the leaf axil region-specific pLAS:LhG4 or pCUC2:LhG4 drivers (Goldshmidt et al., 2008). When homozygous pLAS:LhG4 or pCUC2:LhG4 drivers transformed with a pOp: iaaM transgene were grown for 4 weeks under short photoperiods and then shifted to long days to induce flowering, a significantly higher proportion of the first six leaf axils could not produce AMs, in contrast to wild-type-like plants harboring the pLAS:LhG4 or pCUC2:LhG4 drivers alone (Figure 2A). These transgenic plants exhibited no defects in AM formation in cauline leaves. In wild-type-like pLAS:LhG4 or pCUC2: LhG4 driver plants, a cluster of small and proliferating cells at the adaxial leaf base was the first visible evidence of AM formation when analyzed by scanning electron microscopy (Figure 2B). The small clusters of proliferating cells characteristic of the early stages of AM formation were completely absent in most early rosette leaf axils in pCUC2>iaaM (i.e., pCUC2:LhG4 pOp:iaaM) or pLAS->iaaM plants (Figure 2C; Supplemental Figure 3A). As expected, we observed activated auxin signaling in pCUC2->iaaM and
pLAS>>iaaM plants, as indicated by the DR5:Venus-N7 reporter (Supplemental Figure 4). Although the LAS promoter has highly boundary-specific expression activity, whereas the CUC2 promoter has slightly broader expression extending into leaf primordia, we observed identical AM defective phenotypes in pLAS>>iaaM and pCUC2>>iaaM. We reason that this observation was likely a consequence of polar auxin transport. Even though auxin biosynthesis by iaaM is restricted to the leaf axil in pLAS>>iaaM, PIN protein-mediated auxin transport would lead to a broader auxin distribution as in pCUC2>>iaaM plants (Figures 2D and 2E). Ectopic production of IAA in the boundary region also caused abnormal boundary enlargement and more active auxin efflux mediated by PIN1 from the boundary region (Figures 2D and 2E). No AM initiation or boundary formation defects were observed in plants with pOP:iaaM alone or when LhG4 drivers were crossed out. This suggested that low auxin in the leaf axil is necessary for full levels of AM initiation from early rosette leaves.

The need for an auxin minimum for complete AM initiation was further supported by microapplication experiments in tomato (Solanum lycopersicum). AMs are morphologically visible in tomato leaf axils by stage P₆. We ectopically applied lanolin paste containing IAA to the axil of tomato leaves at P₄ through P₇ stages. After 8 days, we found the treated leaf axils remained barren for all P₄-5 leaves and ~70% of P₆-7 leaves (Figure 2G), while AMs were visible in all leaves mock-treated with lanolin alone (Figure 2F). In addition, the IAA-treated leaf axils were wider than those given mock treatment.

**Low Auxin Concentration Is Required for STM Expression but Not LAS Expression at Leaf Axils**

Expression of the shoot meristem marker STM is activated during AM initiation (Grbić and Bleecker, 2000; Long and Barton, 2000; Greb et al., 2003). We followed the spatio-temporal expression pattern of STM using a pSTM:GUS (β-glucuronidase) reporter. In wild-type plants, STM is expressed in the boundary zone between the SAM and leaf primordia. During AM initiation, STM transcripts accumulate in a small group of cells at the center of the leaf axil.
In pCUC2>>iaaM and pLAS>>iaaM plants, STM expression remained visible until P7 but diminished in later stages (Figure 2I). In addition, the STM signal in the SAM was comparable to or slightly weaker than in wild-type plants, consistent with a SAM size reduction in pCUC2>>iaaM and pLAS>>iaaM plants. Reduced STM expression in pCUC2>>iaaM plants was further confirmed by RT-PCR analysis (Supplemental Figure 5).

AM initiation depends on the specification of leaf boundary identity (Schmitz and Theres, 2005), and expression of LAS at the boundary zone between the SAM and leaf primordia is required for AM formation (Greb et al., 2003). We therefore examined LAS expression in pCUC2>>iaaM and pLAS>>iaaM plants and compared it with the wild type. Similar LAS expression was observed as a continuous band separating early leaf primordia from the SAM with or without ectopic iaaM expression in the leaf axil (Figures 2J and 2K; Supplemental Figure 3B), suggesting that LAS expression is independent of the leaf axil auxin minimum.

The Leaf Axil Auxin Minimum Requires Polar Auxin Transport

The direction of auxin transport depends largely on the polar subcellular localization of PINFORMED (PIN) auxin efflux transporters in the plasma membrane (Petrásek and Friml, 2009). At the tissue level, coordinated polarization of PIN proteins was found critical for auxin distribution, which directs organogenesis and pattern formation.

To test whether PIN-mediated auxin transport was involved in the formation of the leaf axil auxin minimum, we first visualized the expression and localization of PIN1 in the leaf axil prior to AM initiation. In the vegetative meristem, PIN1 is localized in the L1

\( (n > 20) \) that formed an axillary bud in a specific position along the shoot axis.

(B) and (C) Scanning electron micrograph of a rosette leaf axil in Ler wild type with a developing AM (arrowhead) (B) and in pCUC2>>iaaM with a bare axil (arrowhead) (C).

(D) and (E) Longitudinal section of the boundary region of pCUC2:LhG4 (D) and pCUC2>>iaaM (E) with immunolocalization of PIN1 (green) in longitudinal sections of vegetative shoot apex stained with propidium iodide (red). Arrowheads indicate leaf axils.

(F) and (G) Close-up of mock-treated (F) and 100 mM IAA-treated (G) tomato leaf axils showing presence (F, arrowhead) and absence (G, arrowhead) of an axillary bud, respectively.

(H) and (I) Patterns of STM-promoter driven GUS expression in longitudinal sections through vegetative shoot apex of 25-d-old wild-type-like pOp:iaaM (H) and pCUC2>>iaaM (I) plants. GUS signal is reduced in leaf axils of pCUC2>>iaaM (I) plants. For the purpose of comparing signals, these plants were stained in parallel, and sections were placed on the same slides for detection.

(J) and (K) Patterns of LAS transcript accumulation (arrows) in transverse sections through vegetative shoot apex of 28-d-old pOp:iaaM (J) and pCUC2>>iaaM (K) plants. LAS accumulation in pCUC2>>iaaM (K) is similar to pOp:iaaM (J). For the purpose of comparing signals, sections of these plants were placed on the same slides for hybridization and detection. Bars = 200 μm in (B) and (C), 50 μm in (D), (E), and (H) to (K), and 1 mm in (F) and (G).
surface layer. Whereas stereotypical PIN1 polarity changes lead to cycles of auxin buildup, which accompanies leaf primordium initiation (Reinhardt et al., 2000, 2003; Heisler et al., 2005), we and others have observed that epidermal PIN1 adaxial to the primordium reversed polarity from being directed toward the primordium to being directed back toward the meristem center after the I1 stage, which designates the oldest incipient primordium (Figure 3A; Supplemental Figure 6) (Heisler et al., 2005; Bayer et al., 2009). On the other side of the boundary region, PIN1 also mediates auxin flow in the adaxial domain away from the leaf axil toward the tip of the primordium. Therefore, PIN1-mediated auxin flow would predict an auxin minimum at the leaf axil.

To provide direct evidence for the dependence of auxin minima on auxin efflux, we visualized auxin reporter DII-Venus signals in the strong pin1-1 mutant. In contrast to the clear DII-Venus signals observed in the leaf axil region early during leaf formation in wild-type plants (Figures 1A, 1B, and 3B), we found the DII-Venus signal was remarkably reduced in pin1-1 plants without clear leaf axil enrichment (Figure 3C), demonstrating that the leaf axil low auxin niche depends on PIN1 activity.

To further understand whether auxin efflux is critical for AM initiation, we next examined AM initiation in auxin efflux mutants. In mutants of pin1 and pinoid (pid), a regulator of PIN protein activity, AM initiation was often compromised (Figures 3D to 3G). These data indicated that PIN1-dependent auxin efflux plays a role in the formation of the auxin minimum at the leaf axil, which subsequently affects AM initiation.

**Cytokinin Pulse during AM Initiation**

Cytokinins are crucial signaling molecules regulating meristem activity and are involved in the control of gene expression in the SAM (Jasinski et al., 2005; Yanai et al., 2005; Kurakawa et al., 2007; Gordon et al., 2009). To investigate whether cytokinin is also involved in AM initiation, we visualized cytokinin signaling at high resolution using a synthetic reporter, pTCS::GFP-ER (Müller and Sheen, 2008), which reports downstream activity of the cytokinin signaling pathway. In the Col-0 ecotype, although no enhanced GFP signals were found in the axil for leaves earlier than P4, we found strong induction of GFP signals at the axil of leaves from P6 to P9 (Figures 4A and 4B). The GFP-expressing cells at the leaf axil extended above the insertion point of the leaf, showing a similar pattern to the STM-positive cells at this stage (Long and Barton, 2000; Greb et al., 2003). Whereas pTCS::GFP-ER expression was also observed in the SAM (Gordon et al., 2009; Chickarmane et al., 2012), the GFP maxima at leaf axils were substantially stronger than the SAM signals. The strength of this localized cytokinin signaling maximum in the leaf axil after P11 was reduced to levels comparable to that in the SAM. After that, the AM formed a morphologically detectable bump around P21 (Greb et al., 2003).

![Figure 3. Leaf Axil Auxin Minimum Requires Polar Auxin Transport.](image)
Because the leaf axil cytokinin pulse appeared later than the leaf axil auxin minimum, we tested whether the cytokinin response depends on the earlier auxin minimum. To this end, we crossed the pTCS:GFP-ER reporter into pCUC2>>iaaM and pLAS>>iaaM lines overproducing auxin in the leaf axil region. We were not able to find the leaf axil TCS signal foci in any leaves examined (Figures 4C and 4D), although TCS signal in the SAM and leaf vasculature remained detectable. Sibling plants harboring only LhG4 or pOp:iaaM transgene showed normal TCS signal. These results demonstrate the dependence of the leaf axil cytokinin pulse on the auxin minimum.

**Leaf Axil-Enriched Cytokinin Signaling**

In the current model, cytokinin signal is perceived by three ARABIDOPSIS HISTIDINE KINASEs (AHK2, AHK3, and AHK4/CRE1/WOL) that act as receptors and is transferred through a two-component signaling pathway via phosphorelay to activate TCS signal. These results demonstrate the dependence of the leaf axil cytokinin pulse on the auxin minimum.

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**Figure 4.** Enhanced Cytokinin Biosynthesis, Perception, and Signaling during AM Initiation.

- **(A)** and **(B)** Expression of pTCS:GFP-ER (green) in longitudinal sections of vegetative shoot apex showing TCS signals (arrows) initially detected in P_6 leaf axil (A) and later enhanced in P_9 leaf axil (B). TCS signals in SAM cells are much weaker than in AM progenitor cells.
- **(C)** and **(D)** Expression of pTCS:GFP-ER (green) in longitudinal sections of vegetative shoot apex in pLAS>>iaaM (C) and pCUC2>>iaaM (D) plants showing absence of leaf axil TCS signals (arrows).
- **(E)** Patterns of AHK4 promoter–driven GUS expression in transverse sections through the vegetative shoot apex of 28-d-old Col-0 wild-type plants. Arrowheads indicate GUS signals in leaf axils.
- **(F)** Immunolocalization of PIN1 (green) showed polarization away from a leaf axil toward the tip of the primordium as well as the center of the meristem in a longitudinal section of a ar1-4 plant stained with propidium iodide (PI) (red), similarly to wild-type plants (Figure 3A). The region within the yellow dotted line is enlarged to highlight polar localization of PIN1 in each cell.

Bars = 50 μm in **(A)** to **(E).**
the type-B primary ARABIDOPSIS RESPONSE REGULATOR (ARR-B) transcription factors (Hwang et al., 2012).

We found cytokinin receptors also had strong expression prior to and during AM initiation. We analyzed the expression of genes encoding cytokinin receptors (Higuchi et al., 2004; Nishimura et al., 2004). The expression of GUS under the control of the AHK4/CRE1 promoter was detected at high levels in the leaf axil region of leaf primordia at similar stages as pTCS:GFP-ER signal was observed (Figure 4E). Thus, cytokinin perception and signaling were both activated in leaf axils prior to the appearance of axillary buds.

**Cytokinin Perception and Signaling Promote AM Initiation**

Analysis of AM initiation in *ahk* receptor mutants showed that this developmental process was substantially compromised (Figure 5A). In the *ahk2-5 ahk3-7* and *ahk2-5 ahk3-7 cre1-2* mutants, AM initiation was affected in early rosette leaves (Figure 5A). AM initiation was reduced in each single *ahk* mutant, suggesting their overlapping roles in controlling AM initiation.

In addition to cytokinin receptors, B-type ARR transcription factor mutants also showed AM initiation defects (Figures 5B to 5D). Two different *arr1* mutant alleles showed AM initiation defects (Figure 5B). Around one-third of the leaf axils were barren in homozygotes for the *arr1-4* allele. The AM initiation defect was enhanced by mutations in additional B-type *ARR* genes. More barren leaf axils were observed when *arr1-3* was combined with *arr10-5*, *arr11-2*, or *arr12-1* in double and triple mutants (Figure 5B). In addition, lateral shoots from cauline leaf axils, which are separated from the primary axis in the wild type, were sometimes attached to the primary axis in several single, double, or triple *arr* mutants. A similar lateral shoot fusion phenotype was also occasionally observed in the *las* and *cuc3* mutants with AM initiation defects (Greb et al., 2003; Hibara et al., 2006).

Expression of the shoot meristem marker *STM* is reduced in leaf axils in *arr1-4* plants. In *arr1-4* plants, *STM* expression remains visible until ~P10, but gradually diminishes in later stages, although the *STM* signal in the SAM is comparable to that in wild-type plants (Figures 5E and 5F). On the other hand, PIN1-mediated auxin transport away from the leaf axil is not affected in *arr1-4* plants (Figure 4F; individual leaf axil. The bottom row represents the oldest rosette leaf axil, with progressively younger leaves above. Green indicates the presence of an axillary bud, and yellow indicates the absence of an axillary bud in any particular leaf axil.

(C) and (D) Scanning electron micrograph of a rosette leaf axil in Col-0 wild-type with a developing AM (arrow) (C) and in the *arr1-4* mutant with a bare axil (arrow) (D).

(E) and (F) Patterns of *STM* transcript accumulation in transverse sections through the vegetative shoot apex of 30-d-old Col-0 (E) and *arr1-4* (F) plants. *STM* accumulation is reduced in leaf axils of *arr1-4* (F) plants. Arrows indicate leaf axils. For the purpose of comparing signals, these plants were stained in parallel, and sections were placed on the same slides for detection.

(G) Schematic representation of axillary bud formation in leaf axils of *rax1-3*, *pLAS: IPT8 rax1-3*, *rax1-3 rax2-1 rax3-1*, and *pLAS: IPT8 rax1-3 rax2-1 rax3-1* plants. The same color code is used as in (A). Bars = 200 μm in (C) and (D) and 50 μm in (E) and (F).
Supplemental Figure 6), supporting the observation that leaf axil cytokinin pulse appeared later than the leaf axil auxin minimum.

**Overproduction of Cytokinin in Leaf Axils Rescues AM Initiation Deficiency in rax mutants**

A few genes have been identified as regulators of AM initiation, among them are the Arabidopsis REGULATOR OF AXILLARY MERMISTEMS genes (Keller et al., 2006; Müller et al., 2006). The rax1-3 mutant has a reduced number of AMs due to initiation defects, which is further enhanced in the rax1-3 rax2-1 rax3-1 triple mutant. To test whether cytokinin could rescue the AM initiation defects in rax mutants, we exogenously treated the shoot apex region of rax1-3 and rax1-3 rax2-1 rax3-1 mutants and found restoration of AM initiation. Further confirmation was provided by expressing a cytokinin synthase gene IPT8 in the leaf axil region. We found the AM initiation defect was largely rescued in rax1-3 plants with a pLAS::IPT8 transgene in different transgenic lines (Figure 5G). A pLAS::IPT8 transgene could also rescue the more severe rax1-3 rax2-1 rax3-1 triple mutant (Figure 5G). These results imply that RAX transcription factors influence AM initiation through promoting cytokinin biosynthesis or/and signaling.

**DISCUSSION**

**Plasticity of Shoot Development Depends on AM Initiation**

Different from most animals, plants form ramifying systems with continuous production of lateral organs, such as leaves, and branch meristems, such as AMs. This key characteristic of plant development leads to a major distinction from animal development and is a central mechanism that allows plants to adapt to their changing local environment. Each branch meristem gives rise to a whole new cycle of growth and development, responsive to the environment that exists at the time of growth. Lateral branching, which relies on the activities of the AM, is typical for seed plants, whereas other branching mechanisms are adopted by other lineages of land plants (Harrison et al., 2007; Tomescu, 2009). The study of how plant branch meristems initiate remains rudimentary. Forward and reverse genetic studies have identified several transcription factors affecting AM initiation (Talbert et al., 1995; Schumacher et al., 1999; Chuck et al., 2002; Schmitz et al., 2002; Komatsu et al., 2003; Hibara et al., 2006; Lee et al., 2009), implying that complex regulatory networks underlie AM initiation. In this study, we provide a mechanistic framework to explain how plant hormones regulate AM initiation (Figure 6). Our hormonal action-based framework not only leads to the identification of a number of AM initiation-defective mutants, it also integrates previously reported genes in the AM initiation pathway, such as LAS, RAX, and STM.

**Sequential Auxin and Cytokinin Activities at the Leaf Axil Regulate AM Initiation**

Roles for auxin and cytokinin in lateral root and leaf initiation have started to be uncovered in recent years. In lateral root initiation, the distribution of auxin in protoxylem cells spatially produces recurrent local auxin maxima, which define and promote lateral root priming, founder cell specification, initiation, and patterning (Benková et al., 2003; De Smet et al., 2007; Dubrovsky et al., 2008; Swarup et al., 2008; Ivanchenko et al., 2010). Cytokinin activities also influence lateral root development, mostly antagonistically to auxin activities. It has been reported that repressed cytokinin response is necessary for lateral root initiation and patterning (Werner et al., 2003; Li et al., 2006; Laplaze et al., 2007; Bielach et al., 2012). Similarly to lateral root organogenesis, auxin maxima generated by dynamic auxin transport in the epidermis of the SAM activate new leaf primordia (Reinhardt et al., 2000, 2003; Heisler et al., 2005), whereas cytokinin activity is suppressed during leaf maturation (Efroni et al., 2013).

A large body of physiological, genetic, and molecular cell biological evidence supports the idea that auxin and cytokinin are both major regulators of axillary bud activation (reviewed in Domagalska and Leyser, 2011). Here, we demonstrated that auxin and cytokinin also influence AM initiation. We found that an auxin minimum is established at each leaf axil, where an AM will later initiate, and that cells within an auxin minimum have increased potential to form an AM (Figure 1). Auxin efflux carrier PIN1 localization suggests that this auxin minimum depends on auxin transport (Figure 3). Our data further showed that disruption of the auxin minimum, either by ectopic expression of an auxin biosynthetic gene or by local auxin application, compromised AM initiation (Figure 2). Consistent with the idea that the auxin minimum depends on PIN-mediated auxin transport, we similarly observed AM initiation defects in pin1 and pid mutants (Figure 3).

AMs develop in close association with adaxial leaf fate and are found only on the adaxial side of leaf axils (Snow and Snow, 1942; McConnell and Barton, 1998). The finding that AM initiation depends on the establishment of a low auxin region at the leaf axil provides an explanation for the association between

**Figure 6. Conceptual Summary of Hormonal Regulation of AM Initiation.**

(A) During leaf development, an auxin minimum (gray) is first observed at the leaf axil due to PIN1-mediated auxin flow, which is followed by a transient cytokinin signal (green) pulse at the same location between P$_6$ and P$_7$. Both hormonal niches are required for proper AM initiation during vegetative development.

(B) During reproductive development, auxin (red) promotes FM development through LFY (Li et al., 2013; Yamaguchi et al., 2013).
AMs and leaf adaxial fate. Before leaf initiation, PIN1-mediated auxin flow is directed toward an incipient primordium from the surrounding area (Heisler et al., 2005). Epidermal PIN1 proteins adaxial to, but not abaxial to, the primordium reverse polarity from being directed toward the primordium to being directed back toward the SAM center right before the leaf primordium forms its initial bulge (Heisler et al., 2005). Thus, the auxin minimum forms only on the adaxial leaf side. It remains to be tested if mutants with fully adaxialized leaves form auxin minima also in the abaxial side, so as to induce abaxial AM initiation.

In addition to auxin, we found a clear pulse of cytokinin signaling prior to AM initiation, which is developmentally after, and depends on, the earlier auxin minimum (Figure 4). Leaf axil expression of cytokinin receptor genes implied that the cytokinin signaling pulse came from the activation of cytokinin signal transduction in the leaf axil (Figure 4). We further demonstrated that AM initiation was affected in cytokinin perception and signaling mutants (Figure 5). Because STM directly activates cytokinin biosynthesis (Jasinski et al., 2005; Yanai et al., 2005), and the leaf axil cytokinin pulse accompanies enhanced leaf axil STM expression during leaf maturation (Long and Barton, 2000; Greb et al., 2003), it is plausible that STM activates cytokinin biosynthesis to promote leaf axil cytokinin signaling. This enhanced leaf axil cytokinin pulse depends on the leaf axil auxin minimum established earlier because activated auxin inhibits STM expression (Figure 2). Cytokinins can subsequently promote functional shoot meristem establishment by activating expression of the stem cell marker WUS (Gordon et al., 2009). On the other hand, as cytokinins can activate expression of STM (Rupp et al., 1999), it is possible that a positive feedback loop amplifies STM expression and cytokinin signaling during AM initiation. Our finding that ectopic cytokinin biosynthesis in the leaf axil can rescue AM initiation-deficient rax mutants indicates a possibility that RAX may be upstream of cytokinin signaling to promote the expression of the shoot meristem marker gene STM (Müller et al., 2006).

Conservation and Divergence of Hormonal Regulation of Shoot Stem Cell Niches

Production of most plant organs is dependent on the activity of meristems, which maintain stem cells. The embryonic shoot meristem is produced only once during embryogenesis, whereas AMs, as postembryonic meristems, repetitively arise during modular plant growth. In addition, adventitious shoot meristems may form from differentiated organs or calli (Gordon et al., 2007; Sugimoto et al., 2010). In this study, we identified sequential phytohormone actions on the initiation of AMs, the branching meristem of seed plants. The actions of auxin and cytokinins are likely conserved in the embryonic and adventitious shoot meristems regenerated in vitro. A low auxin window has been observed during embryogenesis between the 32-cell stage and the triangular stage (Friml et al., 2003), as well as during adventitious shoot meristem formation (Cheng et al., 2013). A transient TCS signal in the shoot meristem at the heart stage has been reported (Müller and Sheen, 2008; Züchter et al., 2013), and transient enhancement of TCS signaling is also reported during adventitious meristem formation (Cheng et al., 2013), consistent with cytokinin induction of shoot meristem formation in callus tissue (Skoog and Miller, 1957). Further study on how auxin minima and cytokinin pulses affect embryonic shoot meristem formation and adventitious meristem formation would provide more comprehensive comparison with their regulation of axillary meristem formation.

It should be noted that a different hormonal regulation mechanism is likely employed after the floral transition (Figure 6). The finding that auxin biosynthesis and signaling mutants are defective in inflorescence development in maize (Zea mays) and Arabidopsis led to the proposal that an auxin maximum is required for branching during inflorescence development (Przemeck et al., 1996; Cheng et al., 2007; Barazesh and McSteen, 2008; Gallavotti et al., 2008). It has also been noted that AM initiation during the vegetative stage and the reproductive stage requires different sets of genes (Schmitz and Theres, 2005). This discrepancy has recently been reconciled by the finding that auxin promotes the expression of LEAFY (FLY), a key regulator of the floral transition, and that FLY feeds back to the auxin pathway (Li et al., 2013; Yamaguchi et al., 2013). FLY further activates the expression of RAX1 to promote inflorescence branching (Chahtane et al., 2013).

As a key factor for plant architecture, the AM has profound influence on crop yield (Wang and Li, 2008; Thompson and Hake, 2009). The mechanistic framework underlying AM initiation presented in this study is expected to be conserved across flowering plants and may be useful as a conceptual framework to assist breeding practices.

METHODS

Plant Strains, Growth Conditions, and IAA Treatment Conditions

Arabidopsis thaliana ecotypes Col-0 and Ler were used as wild-type controls. Arabidopsis plants were grown in short-day conditions (8 h light at 22°C and 16 h dark at 18°C) for 28 or 30 d and induced to flower under long-day conditions (16 h light at 22°C and 8 h dark at 18°C). The transgenic lines pDR5rev:GFP-ER, p35S:DiI-Venus, pTCS:GFP, pSTM:GUS, and pAHK4:GUS plants are in the Col-0 background (Hay et al., 2002; Benková et al., 2003; Higuchi et al., 2004; Miyawaki et al., 2004; Müller and Sheen, 2008; Verroux et al., 2011), and pCUC2:LhG4 and pLAS:LhG4 are in the Ler background (Goldshmidt et al., 2008). The athk2-5, athk3-7, cre1-2, arr1-3, arr1-4, arr1-5, arr1-12, arr1-1, rax1-3, and rax1-1 mutants are in the Col-0 background (Müller et al., 2006; Riefler et al., 2006; Argyros et al., 2008), the rax1-1 mutant is in the Wassilewskija background, and the pin1-1 mutant is in the Enkheim background (Blilou et al., 2005).

Tomato (Solanum lycopersicum) cultivar Castlemart was used and grown in Murashige and Skoog (MS) medium under controlled conditions (25°C at 60% humidity, with 16 h light and 8 h dark) until the fifth to seventh plastochron stage was reached. For IAA treatment, 1 M stock solutions of IAA (Sigma-Aldrich) in DMSO were dissolved in lanolin (Sigma-Aldrich) prewarmed at 50°C to a final concentration of 100 mM. IAA paste, or lanolin paste with DMSO only as control, was manually administered to leaf axils.

For leaf culture, seedlings were grown in MS medium in short-day conditions (8 h light at 22°C and 16 h dark at 18°C) for 15 d after seed stratification. Leaves between P5 and P11 were then detached from seedlings, laid flat on MS medium supplemented with 0.1 mg/L folic acid and 1 mL/L Linsol (Steeves et al., 1957), and grown for a period of up to 30 d under the same conditions.

Construction of Transgenic Plants

The pOP:iaaM construct was made by inserting the Agrobacterium tumefaciens iaaM gene (Romano et al., 1995) between the pPst and Acc65 sites of the construct BJ36-pOP6 (Jiao and Meyerowitz, 2010). The
construct was then transformed into pCUC2::LH4 and pLAS::LH4 plants. Multiple transgenic lines (>20 for each activator) were obtained, and lines with representative phenotypes were used in the analysis. For constructing pLAS::IPT8, a 3.9-kb fragment upstream of the LAS coding region (Goldshmidt et al., 2008) was amplified and inserted into BJ36. The Arabidopsis IPT8 gene cdNA was cloned downstream of the LAS promoter. The construct was then transformed into ras1-3 and ras1-3 ras2-1 ras3-1 plants. Primers used to make constructs are listed in Supplemental Table 2.

Immunolocalization and in Situ Hybridizations

For immunolocalization, shoot apices were fixed in fresh FAA solution (3.7% formaldehyde, 50% ethanol, and 5% acetic acid) under vacuum and embedded in Steedman’s wax composed of polyethylene glycol 400 diesterate and 1-hexadecanol (Sigma-Aldrich). After rehydration, 6-µm sections were pretreated 1 h with 2% BSA in PBS and incubated overnight with the anti-PIN1 (aP-20) antiserum (Santa Cruz Biotechnology) or the anti-ARR1 antiserum (Roche) diluted 1:500 and 1:750, respectively, in PBS containing 0.1% BSA. After three washes in PBS with 0.1% (v/v) Tween 20, sections were incubated for 1 h with the secondary antibodies Alexa Fluor 488 donkey anti-goat IgG or Alexa Fluor 555 donkey anti-mouse IgG (Life technologies) diluted 1:1000 in PBS supplemented with 0.1% (w/v) BSA. After additional rinses in PBS plus 0.1% Tween 20, sections were mounted in ProLong Antifade (Life Technologies) under cover slips and examined using a confocal laser scanning microscope.

Nonradioactive in situ hybridization was performed as previously described (Jiao and Meyerowitz, 2010). Details of methods used for fixation of plants, embedding in paraffin, and in situ hybridization can be found at http://www.its.caltech.edu/~plantlab/protocols/insitu.html. Sections (8 µm thick) were cut with a Leica RM2255 rotary microtome. LAS probe contained nucleotides 2 to 1348 of LAS cdNA amplified by PCR with primer 1 (5'-TGCTTACTCTTCTTAAATCCTCTAGCTC-3') and primer 2 (5'-CTTCTCTTGATGATCCATTCCAGGCG-3') and cloned into the pEASY-Blunt vector (TransGen Biotechnology). The STM probe included nucleotides 436 to 1143 of the STM open reading frame and has been described before (Long et al., 2002).

Tissue Preparation for Confocal Analysis

Shoot apices with leaves removed were collected and immediately placed in 2.5% paraformaldehyde (PFA; Sigma-Aldrich) at pH 7.0 at 4°C, vacuum infiltrated for 30 min, and stored overnight at 4°C. Fixed tissue samples were washed with 10% sucrose and 1% PFA at pH 7.0 for 20 min, with 20% sucrose and 1% PFA at pH 7.0 for 20 min, 30% sucrose and 1% PFA at pH 7.0 for 30 min. Samples were then embedded in 5 to 7% LM agarose (Promega) liquid at 30°C and placed at 4°C for 15 min to solidify. Sections of 40 to 70 µm were made using a Leica VT1000S vibratome. For high-resolution images, samples were stained with 50 µg/mL propidium iodide (Sigma-Aldrich).

Confocal Microscopy, Optical Microscopy, and Scanning Electron Microscopy

Images were taken with a Zeiss LSM 510 Meta or a Nikon C2 Si confocal microscope. Excitation and detection windows setups for GFP, Venus, Alexa Fluor 488, and Alexa Fluor 555 were as previously described (Heisler et al., 2005). To detect the signal of propidium iodide staining, a 488-nm laser line was used for excitation and a 585- to 615-nm band-pass filter was used for detection. Auto-fluorescence was excited at 488 nm and detected in the 680- to 700-nm range. Optical photographs were taken with a Nikon SMZ1000 stereoscopic microscope or an Olympus BX60 microscope equipped with a Nikon DS-Ri1 camera head.

Scanning electron microscopy was performed using a Hitachi S-3000N variable pressure scanning electron microscope after standard tissue preparation (Jin et al., 2011).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome initiative or GenBank/EMBL databases under the following accession numbers: At1g35750 (AHK2), At1g27320 (AHK3), At2g01830 (AHK4), At3g16857 (ARR1), At4g31920 (ARR10), At1g67710 (ARR11), At1g25180 (ARR12), At5g3950 (CUC2), At3g19160 (IFT8), At1g55580 (LAS), At2g34650 (PID), At1g73590 (PIN1), At5g23000 (RAX1), At2g36890 (RAX2), At3g49690 (RAX3), At1g62360 (STM), and AB025110 (iaaM).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. AM Initiation in Cultured Leaf Bases.

Supplemental Figure 2. Expression of mDII-Venus in the Vegetative Shoot Apex and Leaf Primordia.

Supplemental Figure 3. Phenytoin Characterization and LAS Expression in pLAS::iaaM Plants.

Supplemental Figure 4. Distribution of Auxin Response in the Leaf Axil.

Supplemental Figure 5. Expression of STM in pCUC2::iaaM Plants.

Supplemental Figure 6. Leaf Axil PIN1 Localization.

Supplemental Table 1. Number of AMs Produced in Leaf Culture Using AM- and Lateral Root-Defective Mutants.

Supplemental Table 2. Primers Used to Make Constructs and for RT-PCR.

ACKNOWLEDGMENTS

We thank M. Aida, J.L. Celenza, Y. Eshed, T. Kakimoto, M. Tatsaka, K. Theres, M. Tsiantis, C. Ueguchi, J. Zuo, and the ABRC for seeds and plasmids and C. Li for allowing us to use his tomato growth facility. We also thank J. Zuo and two anonymous reviewers for their constructive comments. This work was supported by National Natural Science Foundation of China Grant 31222033, National Basic Research Program of China (973 Program) Grant 2014CB943500, Strategic Priority Research Program of CAS XDA020105, the Hundred Talents Program of CAS to Y.J., and U.S. National Science Foundation 2010 Project Grant MCB-0929349 to E.M.M. and Y.J. The Meyerowitz Laboratory is also supported by funds from the Howard Hughes Medical Institute and the Gordon and Betty Moore Foundation (through Grant GBMF3406). Y.W. received a fellowship from the China Postdoctoral Science Foundation, and J.W. was a recipient of the Syngenta Friendly Laboratories Scholarship.

AUTHOR CONTRIBUTIONS


Received January 16, 2014; revised April 18, 2014; accepted April 29, 2014; published May 21, 2014.
REFERENCES


The Stem Cell Niche in Leaf Axils Is Established by Auxin and Cytokinin in *Arabidopsis*
Ying Wang, Jin Wang, Bihai Shi, Ting Yu, Jiyan Qi, Elliot M. Meyerowitz and Yuling Jiao
*Plant Cell* 2014;26;2055-2067; originally published online May 21, 2014;
DOI 10.1105/tpc.114.123083

This information is current as of June 29, 2017

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