

Pattern formation during de novo assembly of the *Arabidopsis* shoot meristem

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Most multicellular organisms have a capacity to regenerate tissue after wounding. Few, however, have the ability to regenerate an entire new body from adult tissue. Induction of new shoot meristems from cultured root explants is a widely used, but poorly understood, process in which apical plant tissues are regenerated from adult somatic tissue through the de novo formation of shoot meristems. We characterize early patterning during de novo development of the *Arabidopsis* shoot meristem using fluorescent reporters of known gene and protein activities required for shoot meristem development and maintenance. We find that a small number of progenitor cells initiate development of new shoot meristems through stereotypical stages of reporter expression and activity of *CUP-SHAPED COTYLEDON 2* (*CUC2*), *WUSCHEL* (*WUS*), *PIN-FORMED 1* (*PIN1*), *SHOOT-MERISTEMLESS* (*STM*), *FILAMENTOUS FLOWER* (*FIL*, also known as *AFO*), *REVOLUTA* (*REV*), *ARABIDOPSIS THALIANA MERISTEM L1 LAYER* (*ATML1*) and *CLAVATA 3* (*CLV3*). Furthermore, we demonstrate a functional requirement for *WUS* activity during de novo shoot meristem initiation. We propose that de novo shoot meristem induction is an easily accessible system for the study of patterning and self-organization in the well-studied model organism *Arabidopsis*.

KEY WORDS: Auxin, Callus, Cytokinin, Regeneration, Self-organization, Shoot meristem, *Arabidopsis thaliana*

INTRODUCTION

Regeneration of a patterned multicellular organism from isolated pieces of adult somatic tissues is a remarkable phenomenon that occurs both in plants and animals (Morgan, 1901). The small Cnidarian, *Hydra*, for example, can self-assemble a new correctly patterned body from re-aggregated cells derived from dissociated somatic cells of adult tissue (Gierer et al., 1972). Recently, the observation that several genes critical for proper embryonic development in higher animals are expressed during de novo *Hydra* head regeneration has led to important insights into the molecular basis of animal self-organization (Hobmayer et al., 2000). However, animal model systems for studying de novo patterning, such as *Hydra*, are not well developed for molecular analysis or genetics compared to classical model organisms with established collections of mutants, transgenic lines and protocols (Lowenheim, 2003; Wittlieb et al., 2006).

Assembly of a complete organism from fragments of adult somatic tissue is rare among animals, but many plants are capable of this type of regeneration. A half century ago Skoog and Miller demonstrated an in vitro system for regenerating flowering plants from fragments of adult somatic tissue (Skoog, 1950; Skoog and Miller, 1957). Remarkably, the identity of induced tissues in this in vitro system was shown to be driven by the ratio of two plant hormones: auxin and cytokinin. It was shown that transfer of tissue explants to medium with higher levels of auxin induced development of root regenerative tissues, whereas transfer of explants to medium with higher levels of cytokinin induced new shoot regenerative tissues, and inductive media containing both auxin and cytokinin induced a proliferation of cells termed callus.

During post-embryonic development in flowering plants such as *Arabidopsis thaliana*, all above ground organs of the plant originate from stem cells within the apical tip of the shoot meristem. The origin of the primary shoot meristem during embryogenesis can be traced back to a small group of apical precursors (West and Harada, 1993). Throughout embryogenesis the apical lineage is marked by precisely regulated expression of many genes, which are required for proper patterning of the shoot meristem (Aida et al., 1997; Barton and Poethig, 1993; Laux et al., 1996; Long et al., 1996). For example, early patterning during embryogenesis is recognizable by expression of the auxin transporter, *PIN-FORMED 1* (*PIN1*), required for the initiation and maintenance of auxin gradients within various tissues of the plant (Friml et al., 2003; Heisler et al., 2005). In the two-cell pro-embryo, *PIN1* expression coincides with an initial differential activation of auxin response in the apical cell. Expression of the homeodomain transcription factor *WUSCHEL* (*WUS*) begins in the 16-cell stage embryo in two inner apical cells and maintains a tightly restricted pattern throughout embryogenesis (Mayer et al., 1998). The dynamic expression of the redundant transcription factors *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *CUC2*) and the homeodomain transcription factor, *SHOOT MERISTEMLESS* (*STM*), marks a small number of apical cells in the mid-globular stage embryo that are required for meristem initiation (Aida et al., 1997; Aida et al., 1999; Long and Barton, 1998).

Although much is known about patterning of the shoot meristem during embryogenesis, there is little understanding of patterning that must occur during de novo induction of plant tissues in culture (Cary et al., 2002; Long and Barton, 1998). The cell proliferation observed during callus formation ensures that the ordered morphology of normal tissue is severely disrupted (Cary et al., 2002; White, 1939). Furthermore, new shoot meristems can be induced from root-derived explants, which differ in cell lineage, gene expression, and tissue structure from the shoot meristem (West and Harada, 1993), thus raising the question of how root cells react to changes in environment and initiate patterned shoot tissues.

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Live imaging of the *Arabidopsis* meristem has been recently applied to the analysis of cell lineage and cell fate during active growth of the shoot meristem, to understand genetic control of meristem size, and to cell type specification leading to flower primordium initiation and patterning (Heisler et al., 2005; Reddy et al., 2004; Reddy and Meyerowitz, 2005). In this study we use a live imaging approach to characterize stage-specific molecular patterning events during de novo organization of the shoot meristem from callus (Fig. 1).

MATERIALS AND METHODS

Plant materials

All plants used in this study were in the Landsberg *erecta* (*Ler*) ecotype except when stated otherwise. Plants and tissue cultures were grown at 22°C under continuous light. Transgenic plants were produced using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). The strong *wus-1* mutant allele and the strong *pin1-4* allele have been described previously (Bennett et al., 1995; Mayer et al., 1998).

Construction of GFP reporters

The translational protein fusion constructs including the *pPIN1::PIN1-GFP*, *pSTM::STM-VENUS*, *pREV::REV-VENUS*, and *pCUC2::CUC2-VENUS* constructs have been described previously (Heisler et al., 2005). The upstream regulatory sequence reporters including the *pDR5rev::3XVENUS-N7*, *pCUC2::3XVENUS-N7* and the *pFIL::DsRED-N7* markers were described previously (Heisler et al., 2005; Sieber et al., 2007). The transcriptional *pCLV3::GFP-ER* reporter was described previously in plants bearing a construct consisting of a 35S promoter driving 29.1 plasma membrane-localized yellow fluorescent protein (YFP) (Reddy and Meyerowitz, 2005). The *pARR5::GFP* reporter in the *Ler* ecotype has been described previously (Yanai et al., 2005) and was generously provided by Joseph Kieber (Department of Biology, University of North Carolina, Chapel Hill, USA).

The previously published *pWUS::mGFP5-ER* construct (Jonsson et al., 2005) contains 3 kb of upstream and 1.5 kb of downstream *WUS* genomic regulatory sequences separated by the *mGFP-ER* coding sequence in the T-DNA vector *pPZP222* conferring gentamycin resistance in plants (Hajdukiewicz et al., 1994). The *pWUS::DsDed-N7* construct, also in *pPZP222*, is composed of 4.4 kb of upstream and 1.5 kb of downstream *WUS* genomic regulatory sequences separated by the *DsRed* coding region fused to the N7 nuclear localization sequence. The *pWUS::DsDed-N7* construct was transformed into *Ler* harboring the *pCLV3::GFP-ER* reporter. The *pWUS::DsDed-N7* reporter line gave a pattern of expression confined to the rib zone of shoot meristems and floral meristems. A putative additive signal or strong autofluorescence was detected in the older leaves of the *pWUS::DsDed-N7* transformants, which was not found in *pWUS::mGFP5-ER* transformants. Spatial expression of the *pWUS::DsRed-N7* marker was verified by semi-quantitative RT-PCR to strictly correspond to areas of callus samples with *WUS* transcript (see Fig. S1A in the supplementary material), in contrast to random samples of callus.

The *pRIBO::2XCFP-N7* construct in the T-DNA vector *pPZP222* was composed of 2.6 kb of upstream regulatory sequence from the 60S ribosomal protein L2 gene (At2g18020) fused to two tandem copies of eCFP (Clontech) followed by the N7 nuclear localization sequence (Cutler et al., 2000).

The *pML1::GFP-ER* construct in the T-DNA vector *pPZP222* was composed of 3.4 kb of upstream regulatory sequence from the *ML1* gene containing a fragment demonstrated to drive L1-specific expression, fused to *mGFP-ER* (Sessions et al., 1999).

The *pPIN1::PIN1-CFP* construct was created by substituting the CFP coding sequence for the GFP coding sequence in the published *pPIN1::PIN1-GFP* construct. Plants bearing multiple transgenes and the mutant alleles were combined by genetic crossing.

Regeneration conditions

Root explants were harvested from 2-week-old seedlings grown in sterile culture on Murashige and Skoog basal salt mixture (MS) plates. Explants were cultured on callus-inducing medium (CIM) consisting of modified

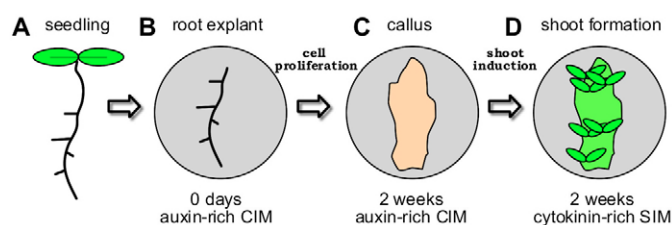


Fig. 1. Overview of the de novo shoot induction system. (A) Root explants were harvested from 2-week-old seedlings and (B) transferred to auxin-rich CIM, which induces cell proliferation, resulting in (C) callus formation. (D) Transfer of callus to cytokinin-rich SIM induces greening and induction of shoot meristems from callus often in clusters (marked by two green leaves).

Gamborg's B-5 medium (Sigma) containing 20 g/l glucose, 0.5 g/l MES (Sigma) and supplemented with 1× Gamborg's vitamin solution (Sigma), 500 µg/l of 2,4-D (Sigma) and 50 µg/l of kinetin (Sigma). Samples were incubated on CIM tissue culture plates for 2 weeks. Callus samples were cut into 2 cm length sections which were cultured on shoot-inducing medium (SIM) plates, consisting of MS medium containing 10 g/l sucrose, 0.5 g/l MES and supplemented with 1× Gamborg's vitamin solution, 2 µg/ml zeatin (BioWorld, Dublin, OH, USA), 1 µg/ml d-biotin (Sigma), and 0.4 µg/ml indole-3-butyric acid (IBA; Sigma).

For quantifying shoot meristem induction, samples were cultured in tall tissue culture plates (USA Scientific) for a further 2 weeks, at which point the number of shoots per 2 cm callus explant was recorded. Shoots were defined as described previously (Daimon et al., 2003). Each experiment contained independent wild-type controls using the same batch of medium and growth conditions.

Exogenous application of IAA

Indole-3-acetic acid (IAA) lanolin paste from Carolina Biological Supply Company at a concentration of 500 ppm labeled with 1 µg/ml of propidium iodide was applied directly to callus in the vicinity of developing shoot meristems.

Imaging conditions

Callus and regenerating shoots were imaged directly on respective media. For each marker line, at least 25 samples were imaged to confirm that observed patterns were representative of respective markers. Propidium iodide for staining root cell outlines of root tissues was applied to samples at a concentration of 10 µg/ml 10 minutes prior to imaging. The lipophilic dye FM4-64 (Molecular Probes) was used at a concentration of 10 µg/ml to demarcate cell membranes and specifically labeled regenerating shoot tissues initiating from root-derived callus.

All imaging was done using a Zeiss 510 Meta laser scanning confocal microscope with either a 10× air objective, 20× air objective, or a 40× 0.8 NA water dipping lens using the multi-tracking mode. Specific sets of filters used for the respective markers were similar to those already described (Heisler et al., 2005; Reddy and Meyerowitz, 2005). Projections of confocal data were exported using Zeiss LSM software. Alternatively, volume renderings were made using Amira (Mercury Computer Systems).

RESULTS

Auxin/cytokinin response and gene expression during callus formation

Our first goal was to determine a correlation between callus induction and distribution of auxin response during callus formation from root explants on auxin-rich CIM. Auxin response was visualized using the auxin responsive *DR5* element (Casimiro et al., 2001; Ulmasov et al., 1997) driving expression of tandem VENUS yellow fluorescent protein localized to the cell nucleus, *pDR5rev::3XVENUS-N7*. In non-induced root explants, the *DR5* reporter (green) marked root pericycle

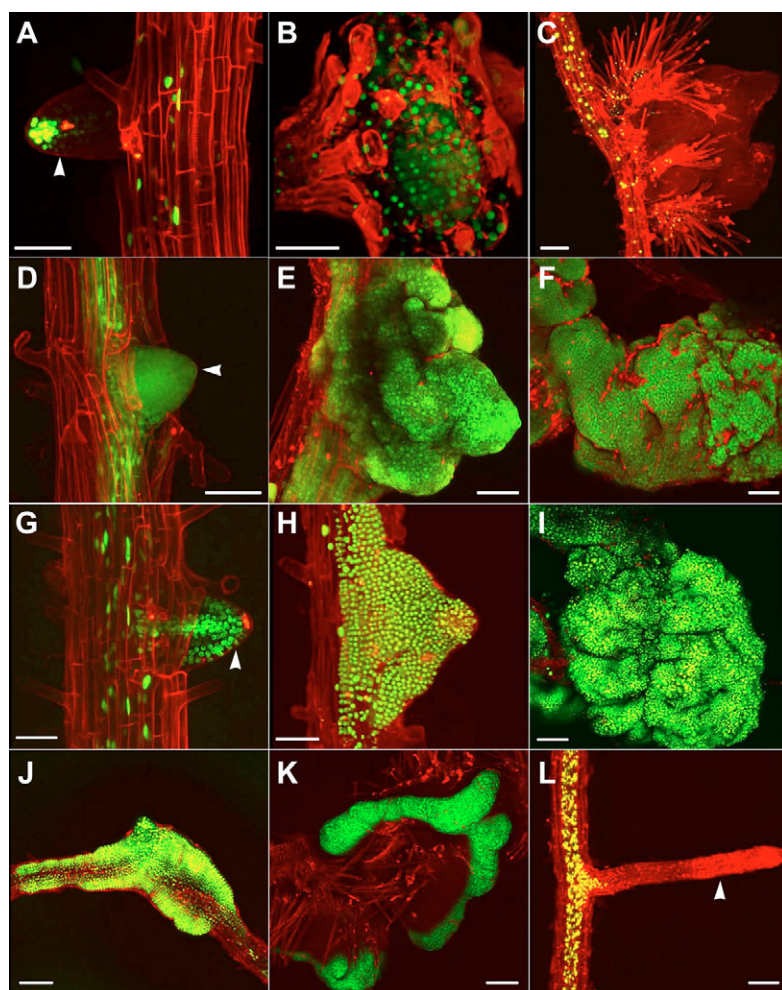


Fig. 2. Hormone response and gene expression during callus induction. All samples were stained with propidium iodide (red) to stain cell walls. (A) In wild-type roots, the auxin-responsive reporter, *pDR5rev::3XVENUS-N7* (green), was present in a subset of cells in the root vasculature, lateral root progenitors and columellar root cap cells. (B) Clusters of small cells marked by *pDR5rev::3XVENUS-N7* reporter (green) proliferate to form callus, 5 days after induction on CIM. (C) After 8 days of CIM induction, the *pDR5rev::3XVENUS-N7* reporter was weakly expressed in callus. (D) Pre-CIM cytokinin-responsive *pARR5::GFP* reporter expression (green) in the root stele, and lateral root progenitors. (E) *pARR5::GFP* reporter expression, 8 days and (F) 2 weeks after CIM induction, was visible in proliferating callus cells. (G) Pre-CIM *pCUC2::3XVENUS-N7* reporter expression (green) in a subset of cells within the root stele and lateral root meristems. (H) *pCUC2::3XVENUS-N7* reporter expression 8 days and (I) 2 weeks after CIM induction marked proliferating callus cells originating from sites of lateral root formation, root meristems and pericycle. (J, K) Two weeks induction on CIM without cytokinin, resulted in cell proliferation and expression of the *pCUC2::3XVENUS-N7* (J) and *pARR5::GFP* reporters (K). (L) Two weeks after induction on CIM without 2,4-D, callus was not induced and expression of the *pCUC2::3XVENUS-N7* reporter was faint and confined to the vasculature of the primary root. Scale bars: 50 μ m (A,B,D,E,G,H); 100 μ m (C,F,I-L). Arrowheads indicate lateral roots.

cells, a subset of lateral root progenitors, and the distal tip of lateral roots including columellar root cap cells (Fig. 2A), as previously reported (Benkova et al., 2003). However, after 5 days incubation on CIM, proliferative growth was marked by the *DR5* reporter and was initiated in the vicinity of lateral roots, root meristems and to a lesser extent, the root pericycle (Fig. 2B). *DR5* response diminished over time and was not observed within large callus outgrowths after 1 week of culture (Fig. 2C). In addition, after 2-3 days induction on CIM, a reporter for the auxin efflux carrier *PIN-FORMED 1* (*PIN1*), was induced in callus outgrowths (green in Fig. S1B in the supplementary material), but was later downregulated and was not detected after 10 days of induction.

We next investigated the spatial distribution of cytokinin response within root explants on CIM. The *ARABIDOPSIS RESPONSE REGULATOR 5* gene (*ARR5*) has been shown to be transcriptionally responsive to cytokinin, and its level of expression correlates with cytokinin content in various tissues (Aloni et al., 2004). We used transgenic plants bearing *ARR5* regulatory sequences driving GFP expression, *pARR5::GFP*, to dynamically monitor cytokinin response. In the non-induced root, *pARR5::GFP* activity was observed in the root stele, root meristems and lateral root progenitor cells (Fig. 2D, green). After 8 days of induction on CIM, signal from the *ARR5* reporter was detected in the root explant vasculature and strongly marked proliferating callus cells (Fig. 2E), and after 2 weeks of induction had expanded throughout callus (Fig. 2F).

A recent study using an enhancer trap for *CUC1* demonstrated that *CUC1* upregulation is associated with callus formation on CIM (Cary et al., 2002). We determined if transcription of the partially redundant gene *CUC2* is also upregulated on CIM. Prior to induction, a reporter consisting of *CUC2* regulatory sequences driving tandem VENUS expression localized to the cell nucleus, *pCUC2::3XVENUS-N7*, was active in a subset of cells of the root vascular cylinder and lateral root primordia founder cells (Fig. 2G, green). After 8 days of induction on CIM, the *CUC2* reporter was upregulated in small proliferating callus cells (Fig. 2H) and was later observed throughout the callus (Fig. 2I). By contrast, *WUS*, *STM* and *CLV3* were not expressed in callus, consistent with previous RT-PCR data (Cary et al., 2002), and no *FILAMENTOUS FLOWER* (*FIL*) and *REVOLUTA* (*REV*) reporter activity was observed.

CIM contains tenfold higher levels of the synthetic auxin 2,4-D, than of the cytokinin, kinetin. We next investigated which of these hormones was responsible for callus induction and upregulation of the *CUC2* reporter. Modified CIM, containing 2,4-D as the sole hormone, induced callus and *CUC2* reporter expression (Fig. 2J, green). On the same medium, the cytokinin responsive *pARR5::GFP* reporter was upregulated at sites of callus formation (Fig. 2K). By contrast, culture of explants on CIM containing only kinetin did not lead to callus proliferation and *CUC2* reporter expression was faint and did not expand outside the vasculature of the primary root (Fig. 2L). Expression of the auxin-responsive *DR5* reporter also did not expand on this medium (data not shown).

Callus induction is associated with the proliferation of multipotent cell types such as cells of the root meristems, lateral root progenitors and pericycle cells. To test the hypothesis that these cells were capable of responding to respecification cues without an intermediate culture on CIM, we cultured root explants directly on cytokinin-rich shoot inducing medium (SIM). After 5 weeks, an average of 3.9 ± 0.2 shoots were induced per 2 cm root explant, compared to an average of 5.1 ± 0.3 after 2 weeks of culture on auxin-rich CIM followed by a subsequent 4-week induction on SIM. In addition, we observed that shoots arose from proliferating cells originating from lateral root meristems labeled by the *CUC2* reporter.

Partition of cell identity and hormone response within callus

The *CUC2* reporter was active throughout 2-week-old root callus explants on auxin-rich CIM. Within 24–48 hours after transfer to cytokinin-rich SIM, *CUC2* reporter expression regressed within callus. After 1 week of culture on SIM, clusters of small dividing *CUC2*-positive cells (Fig. 3A, green) were observed in some regions of callus while absent from others. These cells developed into new shoot meristems with high frequency (Fig. 3B–D, green). As the expression of *CUC2* is known to be post-transcriptionally regulated by the MIR164 family of microRNAs (Baker et al., 2005; Sieber et al., 2007), we investigated the spatial distribution of *CUC2* protein. The expression of a translational *CUC2*-VENUS fluorescent protein fusion driven by *CUC2* upstream regulatory sequences, *pCUC2::CUC2*-VENUS was detected at low levels within shoot progenitor cells in a similar expression pattern to that of the *CUC2* transcriptional reporter (red in Fig. S1C–E in the supplementary material).

Downregulation of the *CUC2* reporter from non-progenitor cells lead us to question if these cells had changed identity, marked by concomitant activation of other gene regulators. RT-PCR and oligonucleotide arrays have previously shown that *WUS* expression is upregulated in callus after 3 days induction on SIM (Cary et al., 2002). We documented the expression of a transgene containing *WUS* regulatory sequences driving GFP expression localized to the endoplasmic reticulum (ER), *pWUS::mGFP-ER*. The *WUS* reporter was upregulated after 3 days on SIM and its expression spread throughout large domains of callus by 5 days of induction (Fig. 3E, green), and declined after 10 days culture. We observed that the *WUS* reporter was initially expressed in cells peripheral to shoot meristem progenitor cells but was later upregulated within the center of the phyllotactic shoot meristems (Fig. 3F,G). We investigated the relative expression domains of *CUC2* and *WUS* activity using a *pCUC2::3XVENUS-N7*; *pWUS::DsRed-N7* marker line. These markers formed non-overlapping domains of activity within callus (Fig. 3H,I). As described above, small rapidly dividing cells labeled by the *CUC2* reporter (green) gave rise to shoot meristem progenitor cells whereas the *WUS* reporter (red) was expressed in peripheral cells that did not rapidly divide (see Fig. S1F in the supplementary material). At later stages, the *CUC2* reporter was expressed in a radial pattern and the *WUS* marker was upregulated in the future rib zone of the developing shoot promeristem (Fig. 3J).

Our results show that induction on cytokinin-rich SIM leads to partitioning of cell identity and cell behavior within callus. We next questioned if hormonal response was partitioned within the callus in similar fashion. The cytokinin responsive *ARR5* reporter was expressed in areas of shoot meristem initiation and within developing shoot meristems, but was downregulated in organ primordia (Fig. 4A, green). *ARR5* reporter expression was absent from areas of callus that initiated root tissues or that did not

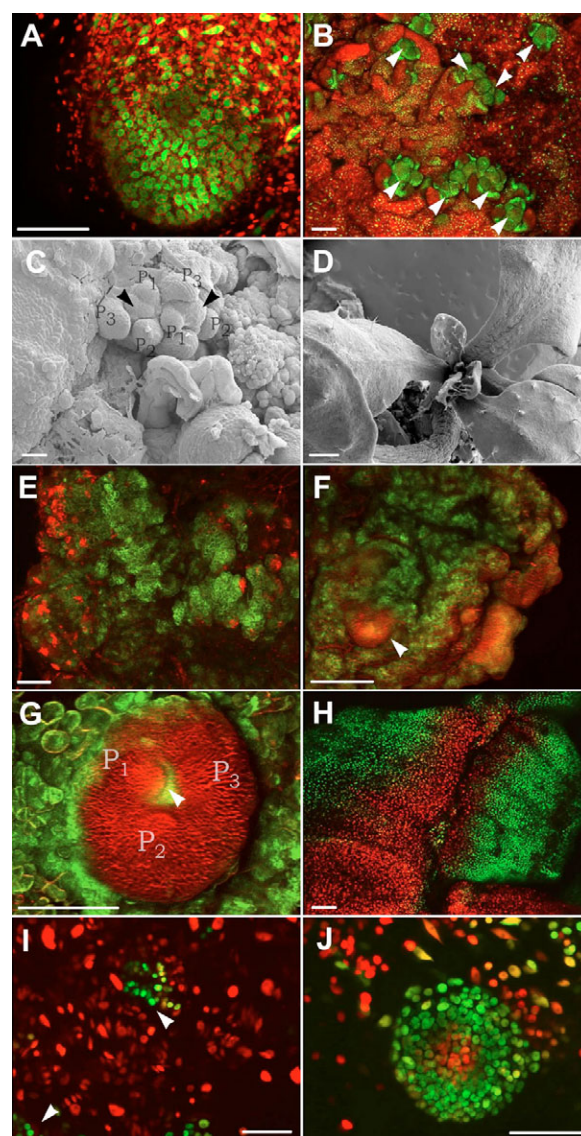


Fig. 3. Partition of gene expression and cell identity within callus. (A) Mounds of small, dividing cells marked by the *pCUC2::3XVENUS-N7* reporter (green) formed and (B) gave rise to new shoot meristems (arrowheads), often observed in clusters. Chlorophyll autofluorescence is in red. (C,D) Scanning electron micrographs of early regenerating meristems (arrowheads, C), and (D) a late stage regenerated shoot emerging from callus. (E) The *pWUS::mGFP-ER* reporter (green) was expressed in callus cells poorly stained by FM4-64 dye (red) following 5 days induction on SIM. (F,G) Shoot progenitors (F, arrowhead, 12 days on SIM) were labeled with FM4-64 dye, and emerged from regions with peripheral *pWUS::mGFP-ER* expression, and formed mature shoot meristems (G), also strongly stained by FM4-64 dye. The *pWUS::mGFP-ER* reporter was upregulated in the center of the developing meristem (arrowhead). (H) *pCUC2::3XVENUS-N7* (green) and *pWUS::DsRed-N7* (red) reporters were active in opposing domains of cells, sometimes in gradients, shown after 10 days on SIM. (I) Higher magnification after 11 days on SIM, showing clusters of cells expressing the *CUC2* reporter (arrowheads) surrounded by *pWUS::DsRed-N7* expressing cells. (J) At later stages, *WUS::DsRed-N7* expression was initiated in the center of shoot progenitors while *pCUC2::3XVENUS-N7* was restricted to the future peripheral zone, shown here after 12 days on SIM. Scale bars: 50 μ m (A,C,I,J); 100 μ m (B,E-H); 300 μ m (D). P_n, primordia.

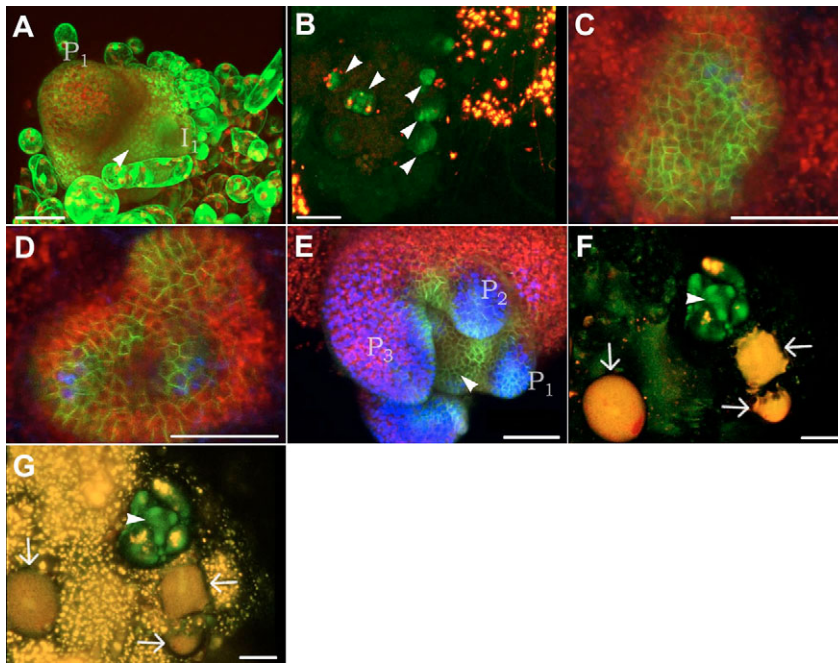


Fig. 4. Partition of hormone response within callus. (A) The *pARR5::GFP* reporter (green) was active in callus forming shoot meristems (arrowhead), but downregulated in primordia [incipient primordia (I_1) and primordia (P_1)]. Chlorophyll autofluorescence is in red. (B) Regenerating meristems (arrowheads) marked by *pPIN1::PIN1-GFP* (green) expression, emerged from callus with low *pDR5rev::3XVENUS-N7* (red) expression, while *pDR5rev::3XVENUS-N7* signal was observed in peripheral callus and within initiating primordia flanking meristems. (C) *pPIN1::PIN1-GFP* reporter expression (green) within the shoot progenitors. (D,E) Strong *pDR5rev::3XVENUS-N7* expression (blue) was later detected during the initiation (D) and outgrowth of organ primordia (P_1 - P_3 ; E). Chlorophyll autofluorescence is in red. (F) *pDR5::3XVENUS-N7* signal (red) was not observed 1 minute after application of auxin paste (arrows) to callus initiating shoot meristems (arrowhead), marked by *pPIN1::PIN1-GFP* (green). (G) Four hours after application of auxin paste (arrows) *pDR5::3XVENUS-N7* signal (red) was observed throughout the callus in contrast to the meristem itself (arrowhead), in which *pDR5::3XVENUS-N7* signal was only in developing primordia. Scale bars: 50 μ m (A,C-E); 100 μ m (B,F,G).

regenerate at all, but strongly labeled regenerating root meristems (see Fig. S1G in the supplementary material). By contrast, auxin-responsive *DR5* reporter signal (red, Fig. 4B) was low or undetectable in areas of shoot initiation, but marked surrounding regions of callus that did not initiate shoot tissues. In areas of low *DR5* reporter signal, shoot meristems and shoot promeristems were marked by expression of a *PIN1* reporter (green), consisting of *PIN1* regulatory sequences driving expression of a *PIN1-GFP* fusion protein. Higher magnification images show that *PIN1-GFP* expression (green) initiates in cells with low *DR5* activity (blue, Fig. 4C). *DR5* signal was observed within the developing shoot meristem after *PIN1* reporter upregulation at future sites of leaf primordium formation (Fig. 4D). As primordia grew outward *DR5* signal increased at the primordium tip (Fig. 4E).

Local application of auxin paste to areas of shoot meristem development induced strong *DR5* signal (red) from callus surrounding the meristems marked by the *PIN1* reporter (green, Fig. 4F,G), while no response was observed in control experiments. Only cells directly associated with the mature meristem did not show *DR5* reporter upregulation. Thus, lack of strong *DR5* signal in the vicinity of shoot meristem initiation is probably due to lower auxin concentrations in such areas and not simply due to an inability of the cells to respond to auxin.

Pattern formation within the shoot promeristem

PIN1 reporter upregulation was associated with the morphogenesis and patterning of phyllotactic shoot meristems from mounds of promeristem cells. We therefore investigated *PIN1* expression relative to expression of other developmental genes: *CUC2*, *REV*, *FIL*, *STM*, *CLAVATA 3 (CLV3)*. We documented simultaneous *PIN1* and *CUC2* reporter activity using *pPIN1::PIN1-GFP*; *pCUC2::3XVENUS-N7* transgenic plants. *PIN1* (green) was upregulated in the superficial layers of shoot promeristem labeled by the *CUC2* reporter (red, Fig. 5A). Higher levels of *PIN1-GFP* were observed at future sites of primordium initiation. As primordia initiated growth, the *CUC2* reporter was expressed in the primordial-meristem boundary (Fig. 5B). Using plants transgenic for

pPIN1::PIN1-GFP; *pWUS::DsRed-N7* reporters we observed that *PIN1-GFP* protein (green) was localized within the cell membrane directed towards the apical tip of the shoot promeristem and away from non-progenitor cells (blue, Fig. 5C).

At the periphery of the *Arabidopsis* meristem, organ primordia are specified with adaxial and/or abaxial polarity with respect to the shoot meristem, in part by the HD-ZIP gene *REV* and the YABBY transcription factor *FIL*. Early *PIN1* reporter expression (green) was closely followed by upregulation of a *pREV::REV-VENUS* reporter (red) in a subset of internal cells of the developing shoot meristem (Fig. 5D). After 24 hours, as *PIN1* reporter expression was upregulated at sites of primordium initiation, *REV* expression extended toward the adaxial side of initiating primordia (Fig. 5E) and later was observed in the adaxial side of leaf primordia (Fig. 5F). Fluorescent signal from a *FIL* reporter (red), *pFIL::DsRed-N7*, was first observed at the periphery of the early *PIN1* domain and clearly demarcated early primordia (Fig. 5G), and its expression was later maintained in the abaxial sides of primordia (Fig. 5H,I).

Upregulation of *PIN1* reporter expression within shoot promeristems was also associated with upregulation of the *STM* gene. Time-lapse imaging of transgenic plants containing reporters for *PIN1*, *STM* and *WUS* showed that the *STM* reporter (blue) is upregulated during the onset of *PIN1* reporter expression (green). *STM* was expressed in a ring of cells surrounding the shoot promeristem and a subset of cells within the promeristem as the *PIN1* reporter was upregulated in primordia initials (I_1 and I_2 ; Fig. 5J and see Fig. S1H-S in the supplementary material). After 24 hours, the *PIN1* reporter marked growing primordia (P_1 and P_2) while the *STM* reporter became upregulated through the center of the shoot promeristem (Fig. 5K) and was maintained in this domain through 48 hours of observation (Fig. 5L).

Stem cells of the shoot reside at the apical tip of the meristem, marked by expression of the *CLV3* gene (Fletcher et al., 1999; Reddy and Meyerowitz, 2005). We observed that *CLV3* expression (green), was absent from shoot progenitor cells, which were marked by the *pPIN1::PIN1-CFP* reporter (white) in plants transgenic for

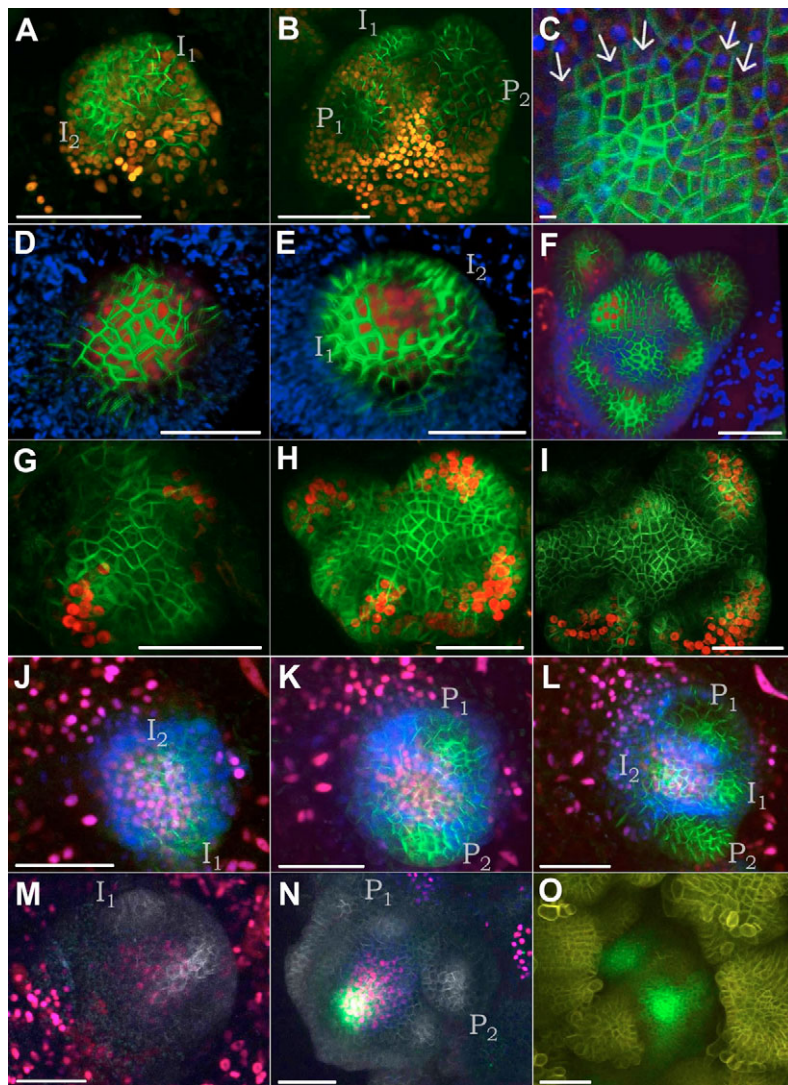


Fig. 5. Pattern formation within the shoot

promeristem. (A) *pPIN1::PIN1-GFP* expression (green) was upregulated in the superficial layer of shoot meristem progenitor cells marked by *pCUC2::3XVENUS-N7* reporter expression (red), and in labeled primordium initials (*I*₁ and *I*₂). (B) After 24 hours, primordium initials grew into primordia (*P*₁ and *P*₂) and *pCUC2::3XVENUS-N7* (red) was expressed in the meristem boundaries. (C) PIN1-GFP protein was (green) polarized towards the apex of the shoot progenitors (arrows) and away from peripheral cells marked by the *pWUS::DsRed-N7* reporter (blue). (D) Early *pREV::REV-VENUS* expression (red) was observed in the center of the progenitors underneath the *pPIN1::PIN1-GFP* (green) domain. Chlorophyll autofluorescence is in blue. (E) 24 hours later in the same developing meristem, *pREV::REV-VENUS* expression (red) was expressed in the adaxial sides of initiating primordia (*I*₁ and *I*₂), and (F) was similarly expressed in primordia within later stage shoot meristems. (G-I) *pFIL::DsRed-N7* expression (red) was upregulated in areas flanking the early *pPIN1::PIN1-GFP* (green) domain (G) and was later upregulated on the abaxial side of early primordia (H) and older primordia (I). (J) *pSTM::STM-VENUS* (blue) was expressed in a ring surrounding shoot progenitors and a subset of cells within the promeristem (11 days on SIM) while local *pPIN1::PIN1-GFP* reporter (green) upregulation marked sites of primordium initiation (*I*₁ and *I*₂). *pWUS::DsRed-N7* reporter (red) was expressed in peripheral cells and upregulated in the center of the developing meristem. (K,L) 24 hours later in the same shoot progenitors, *pSTM::STM-VENUS* (blue) was upregulated within the meristem between the developing primordia (*P*₁ and *P*₂; K) and was maintained through 48 hours of imaging during which primordia grew and two new primordia were initiated (*I*₁ and *I*₂; L). (M) *pCLV3::mGFP5-ER* expression (green) was absent from shoot progenitors marked by *pPIN1::PIN1-CFP* expression (white) and peripheral cells marked by the *pWUS::DsRed-N7* reporter (red). (N) *pCLV3::mGFP5-ER* expression (green) was detected after primordial outgrowth from the periphery of the developing meristem. (O) *pCLV3::mGFP5-ER* expression (green) was also observed in later stage shoot meristems which expressed a *p35S::YFP 29-1* transgene (yellow). Scale bars: 50 μm (A,B,D-O); 5 μm (C).

pCLV3::GFP-ER, *pPIN1::PIN1-CFP* and *pWUS::DsRed-N7* reporters (Fig. 5M). *CLV3* reporter expression appeared during upregulation of *WUS* reporter expression (red) within the center of the new meristem and the initiation of primordia (*P*₁ and *P*₂) from the meristem periphery (Fig. 5N). *CLV3* reporter activity was confirmed in plants bearing a *p35S::YFP 29-1* transgene (yellow), which express membrane-localized YFP within all cells of the mature meristem (Fig. 5O).

L1 layer specification and development of meristem structure

The homeodomain transcription factor *ARABIDOPSIS THALIANA* MERISTEM L1 LAYER (*ATML1*) is redundantly required for specification of the epidermal layer in *Arabidopsis* (Abe et al., 2003) and is restricted to the protodermal layer at the 16-cell stage onwards (Lu et al., 1996). We used a transgenic line containing *pATML1::GFP-ER* and *pCUC2::3XVENUS-N7* reporters in order to understand relative timing of L1 cell-type specification with regards to meristem organization. The *ATML1* reporter was restricted to a subset of superficial cells within the shoot

promeristem marked by the *CUC2* reporter (Fig. 6A,B). By contrast, the *ATML1* reporter was often not L1 specific when expressed in callus (Fig. 6C). Primordium initiation began after approximately 72 hours of development and was associated with homogenous expression of the *ATML1* reporter within the protoderm (Fig. 6E).

We further followed the shoot regeneration process in a *pPIN1::PIN1-GFP*; *pSTM::STM-VENUS*; *pRIBO::2XCFP-N7* marker line. The *pRIBO::2XCFP-N7* marker labeled all cells within a callus, enabling us to observe that shoot promeristems were composed of variable numbers of cells (Fig. 6G). Shoot promeristems composed of smaller numbers of cells developed into shoot meristems with fewer initial leaf primordia compared to larger promeristems (Fig. 6J).

Quantification of regeneration in *wus-1* and *pin1-4*

To determine if *WUS* and *PIN1* are necessary for efficient initiation of new shoot meristems, we quantified the number of shoots formed from 2 cm callus explants in the strong *wus-1* and *pin1-4* mutants

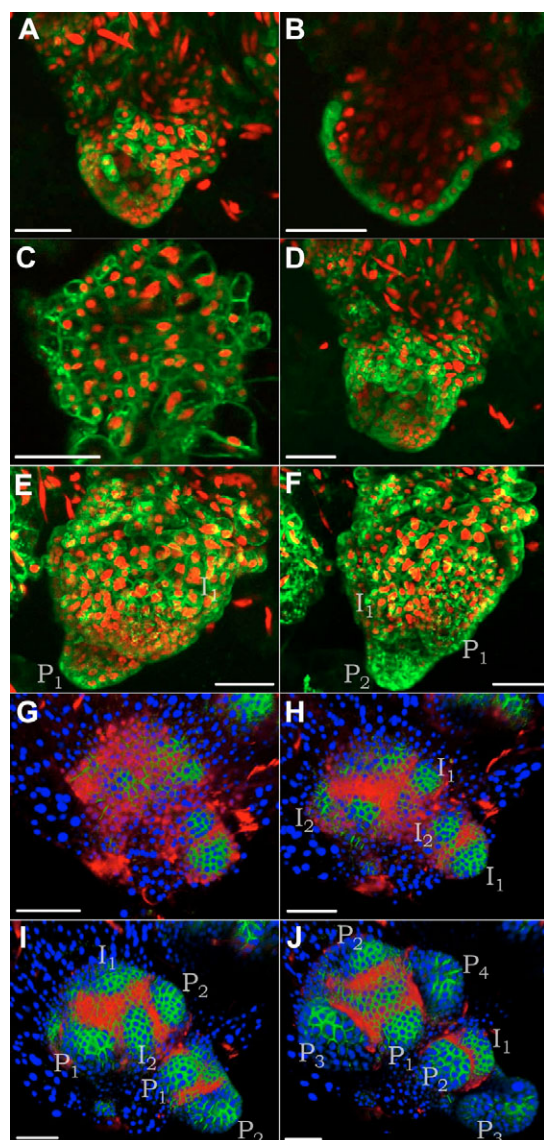


Fig. 6. L1 layer specification and development of meristem structure. (A) *pML1::GFP5-ER* reporter (green) was upregulated in a subset of superficial shoot meristem progenitors, marked by the *pCUC2::3XVENUS-N7* (red) marker. (B,C) Expression of the *pML1::GFP5-ER* reporter (green) was L1 specific within the shoot progenitors (B) but not L1 specific in cross-sections of callus (C). (D) 24 hours later, *pML1::GFP5-ER* expression was upregulated in the shoot progenitors. (E) 72 hours later, *pML1::GFP5-ER* expression was homogeneously expressed within the L1 of the meristem as primordia (I_1 and P_1) were initiated. (F) 96 hours later, two early primordia (P_1 and P_2) were evident. (G) *pPIN1::PIN1-GFP* (green) and *pSTM::STM-VENUS* (red) were upregulated with similar timing in small patches of cells marked by the ubiquitous *pRIBO::2XCFP-N7* marker (blue). (H) 24 hours later, *pPIN1::PIN1-GFP* marked initiating primordia (I_1 , I_2). (I) After 48 hours, primordia (P_1 and P_2) labeled by *pPIN1::PIN1-GFP* have grown outwards and *pSTM::STM-VENUS* was expressed in the developing meristem. (J) After 72 hours of observation, meristems derived from small numbers of initial cells gave rise to fewer primordia than meristems with more cells. Scale bars: 50 μ m (A–J).

after 4 weeks of growth on SIM. The average number of shoots formed in the *wus-1* mutant ($n=91$) decreased to 5% of wild-type number of shoots ($n=106$; 0.25 ± 0.08 versus 5.06 ± 0.04), whereas the

average number of shoots formed in the *pin1-4* mutant ($n=174$) decreased to approximately 20% of wild type numbers ($n=166$; 0.90 ± 0.07 versus 5.16 ± 0.24 ; Fig. 7A).

Quantification of an early versus late defect in *wus-1*

The decrease in the number of shoot meristems observed in the *wus-1* mutant could be due to an early defect, in which fewer shoot promeristems are initiated, or a late defect, in which shoot promeristems arrest at later stages of development prior to quantification. We differentiated between these two possibilities by examining the number of early shoot promeristems, marked by *pPIN1::PIN1-GFP* and *pSTM::STM-YFP* co-expression, formed in *wus-1*. The number of early shoot promeristems was decreased in the *wus-1* mutant ($n=45$) to only 20% of wild-type promeristems ($n=48$; 7.63 ± 0.92 versus 1.67 ± 0.43 ; Fig. 7B). However, we observed that in those shoot promeristems that do form, the *PIN1* and *STM* reporters are initially expressed in similar relative domains to those of wild-type shoot meristem assembly (Fig. 7C–E).

DISCUSSION

Characterization of hormone response and gene expression during callus induction

Prior studies have shown that good auxin efflux substrates, such as indole-3-acetic acid (IAA) or α -naphthalene acetic acid (NAA), induce lateral root growth in wild-type root explants, but callus-like proliferation in mutants for *pin* auxin efflux carriers (Benkova et al., 2003). Furthermore, a reporter for *CUC3* expression was expanded in roots simultaneously treated with IAA and the auxin transport inhibitor NPA. 2,4-D is an auxin analog that is poorly transported by the auxin efflux system (Delbarre et al., 1996). Our data shows that CIM containing 2,4-D as the sole added hormone in the growth medium is sufficient to induce callus formation, which involves proliferation of multipotent cell types including root pericycle cells, lateral root progenitors and cells of the root meristems. Combined, these findings suggest that callus induction is due to an inability of root tissue to regulate auxin distribution, leading to unrestrained proliferation of multipotent cells of the root.

Recently, it has been shown that pericycle cells uniquely continue division through the elongation and differentiation zones of the root after exit from the root meristem (Dubrovsky et al., 2000). Later, a subset of these cells gives rise to lateral root primordia. The ability of these cells to continue division may be linked with their enhanced response to environmental stimuli, such as the availability of hormones. Consistent with this model, we observe that most cells initiating and proliferating as callus are marked by expression of the auxin-responsive *DR5* and cytokinin-responsive *ARR5* reporters. The enhanced capacity to divide in response to hormone induction and the ability to give rise to multiple cell types may explain the preferential proliferation of these cells on CIM and their plasticity during induction of shoot tissues when transferred to a high cytokinin environment.

The different quantitative requirements for auxin and cytokinin in order to induce various tissues in culture is probably in part due to different endogenous concentrations of these hormones within explants (Skoog, 1950). Root meristems are sites of endogenous cytokinin production (Aloni et al., 2005; Nordstrom et al., 2004). The upregulation of the cytokinin responsive *ARR5* reporter within callus forming on CIM containing 2,4-D but no exogenous cytokinin suggests that callus induced from root meristems may endogenously produce cytokinin.

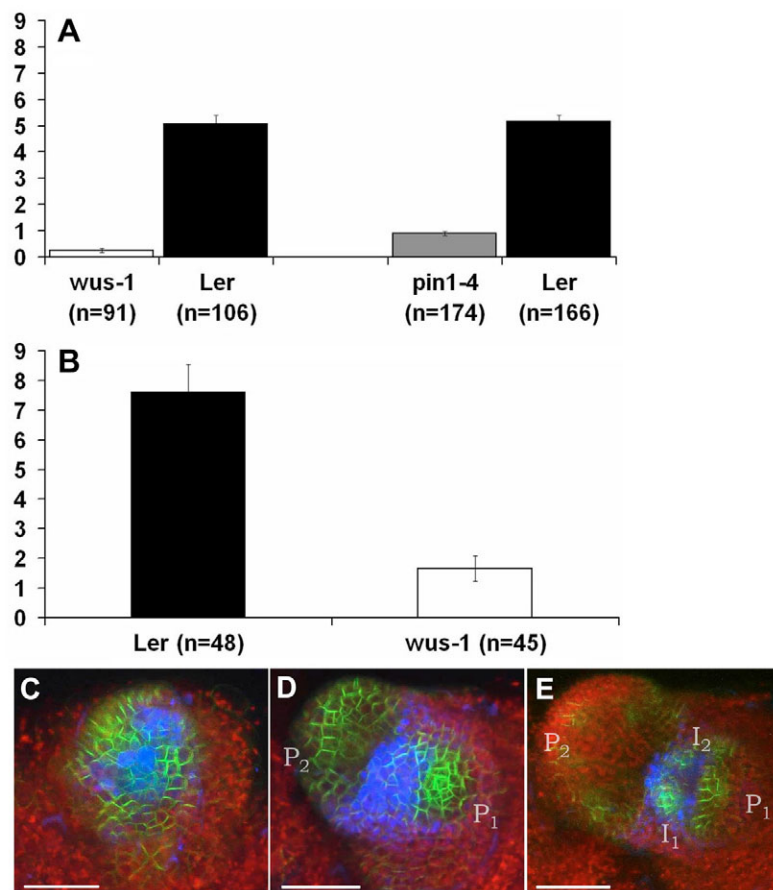


Fig. 7. *WUS* and *PIN1* are functionally required for efficient shoot meristem induction. (A) Bar graph showing average numbers of shoots formed after 4 weeks of induction on SIM from 2 cm callus explants in wild type (*Ler*) versus *wus-1*, and in a separate experiment, *Ler* versus *pin1-4*. (B) Bar graph showing number of shoot promeristems, marked by *pPIN1::PIN1-GFP* and *pSTM::STM-VENUS* coexpression, in *Ler* versus *wus-1*. (C) *pPIN1::PIN1-GFP* (green) and *pSTM::STM-VENUS* (blue) expression in a shoot promeristem initiated in the *wus-1* mutant. Chlorophyll autofluorescence is in red. (D) 24 hours later, the shoot promeristem initiated early primordia (P_1 and P_2) marked by *pPIN1::PIN1-GFP*, while *pSTM::STM-VENUS* marked the presumptive shoot meristem. (E) 48 hours later, primordia (P_1 and P_2) have further developed and two early primordia initials (I_1 and I_2) have formed near the apex of the meristem. Scale bars: 50 μ m.

Partition of cell identity and hormone response within callus during shoot meristem initiation

Previous studies have shown that mosaic overexpression of either of the redundant transcription factors *CUC1* or *CUC2* is sufficient to enhance the number of shoots initiated in culture whereas the respective mutants are deficient in this process (Daimon et al., 2003). Another recent study has shown that broad expression of a *CUC1* enhancer trap on auxin-rich CIM is progressively restricted within callus upon transfer to cytokinin-rich SIM (Cary et al., 2002). We show similar dynamics for the partially redundant gene *CUC2*. In addition, we show that *CUC2* downregulation within cells during induction on cytokinin-rich SIM is synchronized with upregulation of *WUS* expression, leading to a partition of cell identity and behavior within callus (i.e. progenitor/not progenitor). We, therefore, propose that the dynamic partitioning of *CUC2* and *WUS* expression may underlie the gradual localization and promotion of shoot meristem cell fate within callus tissue.

It was recently reported that *WUS* overexpression downregulates expression of *ARR* genes, which negatively regulate cytokinin signaling (Leibfried et al., 2005). However, *CUC2* has been shown to be downregulated in mutants defective in auxin transport (*PIN1*) and auxin-regulated gene activation (*MONOPTEROS*) (Aida et al., 2002; Leibfried et al., 2005). Indeed, we observed that a *CUC2* transcriptional reporter is upregulated on auxin-rich CIM medium and downregulated on cytokinin-rich SIM medium. Furthermore, expression of *CUC2* is maintained in shoot promeristems which express *PIN1-GFP*, polarized such that it is predicted to transport auxin into the shoot promeristem from surrounding cells. By contrast, *WUS* is induced only after culture on cytokinin-rich SIM medium and the *WUS* reporter forms gradients of expression relative

to the *CUC2* reporter in non-overlapping domains. We show that shoot meristems initiate in areas of low auxin and high cytokinin response. Our data is therefore consistent with a model in which gradients of auxin and cytokinin specify cell identities within callus through induction of gene regulators.

WUS and *WOX* genes in diverse regeneration processes

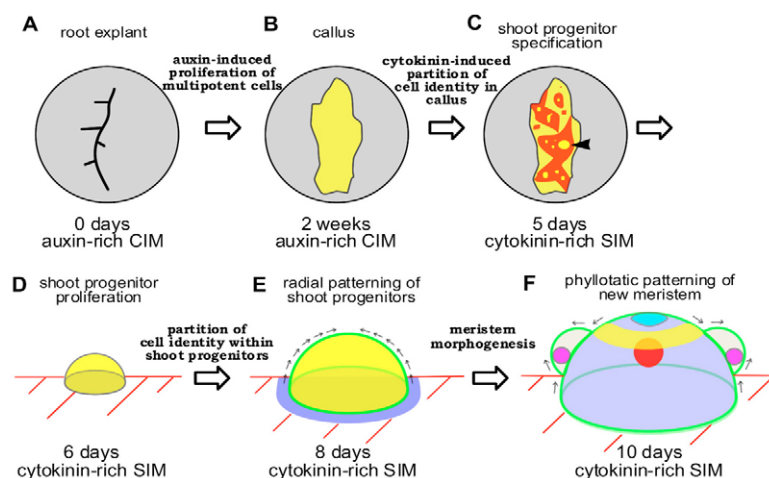
Our observations of *WUS* reporter expression in callus is consistent with previous studies which have described ectopic induction of *WUS* during cell respecification after cell ablations in the shoot meristem (Reinhardt et al., 2003). Furthermore, the *WUSCHEL related homeobox 5* gene (*WOX5*), normally active in the quiescent center (QC), is ectopically induced in surrounding cells after QC ablation in the root meristem (Haecker et al., 2004; Xu et al., 2006). In addition, mosaic over-expression of *WUS* has been shown to induce shoot tissues directly from root explants (Gallois et al., 2004). Thus it appears that broad induction of *WUS* and related *WOX* genes may be a general phenomenon associated with regeneration of specific tissues in plants.

Necessity of *WUS* and *PIN1* function for proper shoot formation

The strong *wus-1* mutant regenerated only 5% of the number of shoots observed in wild-type samples and *WUS* expression was required for initiation of wild-type numbers of shoot promeristems, marked by coexpression of the *PIN1* and *STM* markers. These data support a model in which early *WUS* expression within callus is required to promote shoot meristem progenitor cell identity, and late *WUS* expression is required for further shoot development. However,

Fig. 8. Schematic of *de novo* shoot meristem organization from callus.

Auxin-rich CIM (A) induces proliferation of multipotent cells in the root leading to callus formation (B). (C) Transfer to cytokinin-rich SIM induces partition of cell identity and behavior within callus marked by the *CUC2* (yellow) and *WUS* (red) reporters. Arrowhead indicates shoot progenitors. (D) Clusters of *CUC2*-labeled shoot progenitors proliferate among neighboring *WUS* expressing (red lines) non-progenitor cells in areas of high cytokinin and low auxin response. (E) 24–48 hours later, *PIN1* and *ML1* reporters (both green) are upregulated within the superficial layer of the shoot promeristem while *STM* (blue) is upregulated in a ring of surrounding cells and within the promeristem. Within the membrane of shoot progenitors, *PIN1* protein is directed towards the apex of the promeristem (arrows), and thus is predicted to transport auxin into the promeristem from surrounding cells. (F) 48–96 hours later, *PIN1* becomes locally upregulated within the peripheral zone and marks sites of primordial initiation. *PIN1* protein becomes locally polarized towards sites of primordia formation (arrows). *FIL* (magenta) is expressed in the abaxial sides of newly initiated primordia. *CLV3* expression (teal) is initiated within the central zone after *WUS* expression (red) initiates within the center of the meristem. *pSTM::STM-VENUS* is expressed within the meristem.



once shoot promeristems are initiated, they are largely autonomous in their development and express *PIN1* and *STM* in a pattern that is initially similar to that of wild type. Other factors may compensate for loss of *WUS* function to initiate shoot promeristem development, such as members of the *WOX* gene family or *ENHANCER OF SHOOT REGENERATION* (*ESR1*), which confers cytokinin-independent shoot regeneration (Banno et al., 2001). The *pin1-4* mutant was also deficient in shoot regeneration, though this was not as severe as in *wus-1* mutant tissue. The *pin1-4* deficiency produced a phenotype that was similar to that previously reported for *stm-1* mutant tissue (Barton and Poethig, 1993). *PIN1* activity may be more dispensable for shoot induction than *WUS*, because of its greater redundancy including other PIN proteins (Vieten et al., 2005), consistent with higher levels of NPA-blocking shoot regeneration (Christianson and Warnick, 1984; Murashige, 1965) and redundancy of *PIN* family members during embryogenesis (Friml et al., 2003).

Model for *de novo* shoot regeneration

Our observations demonstrate that cytokinin-rich SIM induces a partition of cell identity within callus marked by expression of the early developmental regulators, *CUC2* and *WUS*. *CUC2* expression marks a small number of progenitor cells that proliferate to form a relatively homogeneous cell mass, which is then later patterned into a new shoot meristem *de novo*. Patterning of the shoot promeristem involves local upregulation of genes expressed in the mature shoot meristem such as *PIN1*, *STM*, *REV*, *FIL*, *ATML1* and *CLV3* and the progressive refinement of their expression to domains found during later development (Heisler et al., 2005). We, therefore, can break the shoot organization process into distinct events: callus induction, cytokinin-induced partition of cell identity within the callus, radial patterning within shoot progenitors, and meristem morphogenesis (Fig. 8).

Classical tissue culture methods for studying developmental patterning

Over a century ago, Haberlandt noted the possible utility of tissue and cell culture for understanding development. He pointed out that cell culture was particularly well suited to determine the potential of individual cells as well as their reciprocal influences on each other

(Haberlandt, 1902). Our study represents an early step towards realizing this potential. In vitro culture experiments support the idea that cell identity in plants is largely governed by positional cues mediated by specific hormones (Steward et al., 1964). We propose a model in which partition of cell identity within a callus on SIM is mediated through non-homogeneous distributions of auxin and cytokinin, which are initially broadly distributed and therefore induce broad *CUC2* and *WUS* expression, respectively. The expression of these genes may further feed back on hormone synthesis, transport or perception, to enhance gradients of hormone signaling, which then alters *CUC2* and *WUS* expression. This feedback could lead to self-organizing patterns observed during *de novo* shoot meristem initiation. If this is the case, the primary difference between shoot meristem initiation in planta and shoot meristem induction in culture is the initial distribution of auxin and cytokinin. Auxin and cytokinin distribution is tightly controlled at all stages during development in planta, whereas this distribution must be gradually reorganized from disrupted initial conditions during shoot induction in culture. In vivo imaging of this dynamic process during gene and hormone perturbations should test the validity of this model.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/19/3539/DC1>

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