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 lead 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 (Frimerl 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 wass-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, pPREV::REV-VENUS, and pCU2C::CU2-VENUS constructs have been described previously (Heisler et al., 2005). The upstream regulatory sequence reporters including the pSTM::STM-VENUS reporter was described previously (Yanai et al., 2005) and was generously provided by Meyerowitz, 2005). The pSTM::STM-VENUS reporter was described previously (Bennett et al., 1995; Mayer et al., 1998).

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 mGFP5-ER coding sequence in the T-DNA vector pZPP222 conferring gentamycin resistance in plants (Hajdukiewicz et al., 1994). The pWUS::DsRed-N7 construct, also in pZPP222, 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 pZPP222 was composed of 2.6 kb of upstream regulatory sequence from the 60S ribosomal protein L2 gene (At2g18020) fused to two tandem copies of cCFP (Clontech) followed by the N7 nuclear localization sequence (Cutler et al., 2000).

The pML1::GFP-ER construct in the T-DNA vector pZPP222 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 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-butryic 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...
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
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 progenitors 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 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 meristems 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 (I1 and I2; Fig. 5J and see Fig. S1H-S in the supplementary material). After 24 hours, the PIN1 reporter marked growing primordia (P1 and P2) 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
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 (P1 and P2) 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 meristem 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; pSTL::STM-VENUS; pRIBO::2XCFP-N7 marker line. The pRIBO::2XCFP-N7 marker labeled all cells within a callus, enabling us to observe that shoot meristem primordia were composed of variable numbers of cells (Fig. 6G). Shoot meristem primordia composed of smaller numbers of cells developed into shoot meristems with fewer initial leaf primordia compared to larger meristems (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.

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**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 (I1 and I2). (B) After 24 hours, primordium initials grew into primordia (P1 and P2) 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 (I1 and I2), and (F) was similarly expressed in primordia within later stage shoot meristems. (G-J) 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 (I1 and I2). 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 (P1 and P2) and was maintained through 48 hours of imaging during which primordia grew and two new primordia were initiated (I1 and I2). (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 peripheral 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).
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 5.06±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 meristems are initiated, or a late defect, in which shoot meristems arrest at later stages of development prior to quantification. We differentiated between these two possibilities by examining the number of early shoot meristems, marked by pPIN1::PIN1-GFP and pSTM::STM-YFP co-expression, formed in wus-1. The number of early shoot meristems was decreased in the wus-1 mutant (n=45) to only 20% of wild-type meristems (n=48; 7.63±0.92 versus 1.67±0.43; Fig. 7B). However, we observed that in those shoot meristems 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 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. 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 (I1 and P1) were initiated. (F) 96 hours later, two early primordia (P1 and P2) 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 p Rubio::2XCFP-N7 marker (blue). (H) 24 hours later, pPIN1::PIN1-GFP marked initiating primordia (I1, I2). (I) After 48 hours, primordia (P1 and P2) 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 μm (A-I).
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 meristems which express pPIN1-GFP, polarized such that it is predicted to transport auxin into the shoot meristem 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 (Gallosi 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,
De novo shoot meristem formation

once shoot meristems 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 meristem 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 expressed by cytokinin accumulation in 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 meristem 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

References


