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Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in *Arabidopsis*

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SUMMARY

Almost all aerial parts of plants are continuously generated at the shoot apical meristem (SAM). To maintain a steady pool of undifferentiated cells in the SAM while continuously generating new organs, it is necessary to balance the rate of cell division with the rate of entrance into differentiation pathways. In the *Arabidopsis* meristem, *SHOOT MERISTEMLESS (STM)* and *WUSCHEL (WUS)* are necessary to keep cells undifferentiated and dividing. Here, we tested whether ectopic *STM* and *WUS* functions are sufficient to revert differentiation and activate cell division in differentiating tissues. Ectopic *STM* and *WUS* functions interacted non-additively and activated a subset of meristem functions, including cell division, *CLAVATA1* expression and organogenesis, but not correct phyllotaxy or meristem self-maintenance. Our results suggest that *WUS* produces a non-cell autonomous signal that activates cell division in combination with STM and that combined *WUS/STM* functions can initiate the progression from stem cells to organ initiation.

Key words: *Arabidopsis thaliana*, Meristem establishment, *WUS*, *STM*

INTRODUCTION

The aerial part of plants has a modular structure. The basic module, called a phytomer (Gray, 1885; Weatherwax, 1923), consists of a region of stem, a leaf, and at the base of the leaf, an axillary meristem (a small group of undifferentiated cells that can initiate new branches or flowers). These modules originate periodically at the growing tip, from a group of undifferentiated and actively dividing cells called the shoot apical meristem (SAM).

To generate new phytomers continuously, two main functions have to be balanced in the SAM (Brand et al., 2001; Clark, 2001; Fletcher and Meyerowitz, 2000; Lenhard and Laux, 1999). One is the initiation of organ primordia on the flanks of the meristem, with predictable size, position and timing (thus determining the arrangement of leaves around the stem, or phyllotaxy). The other is to maintain the pool of undifferentiated cells, with new cell divisions in the meristem replacing the cells allocated to organ primordia. These two functions roughly correspond to histologically distinguishable regions of the meristem. New organs are initiated in the peripheral zone (PZ). The cell population in the PZ is maintained by continuous recruitment of stem cells from the central zone (CZ). The shift of cells from the CZ to the PZ and the transition from the PZ to organ primordia are genetically separable. Mutations in *CLAVATA* (*CLV*) genes increase the size of the CZ and have been proposed to delay the transition from CZ to PZ (Clark et al., 1993; Clark et al., 1995; Laufs et al., 1998b). Mutations in *MGOUN* genes delay the transition to organogenesis and increase the size of the PZ (Laufs et al., 1998a).

The balance between the rate of cell division and differentiation is crucial for the ordered transition of cells between meristem zones. In *Arabidopsis*, two genes have been implicated in the maintenance of undifferentiated cells in the meristem: *SHOOT MERISTEMLESS (STM)* and *WUSCHEL (WUS)*, both of which encode homeodomain proteins (Long et al., 1996; Mayer et al., 1998). *STM* is thought to prevent premature recruitment of cells into differentiation pathways. In strong *stm* mutants, the meristem is absent at the end of embryogenesis (Barton and Poethig, 1993); weak *stm* mutants fail to maintain the meristem after germination (Clark et al., 1996; Endrizzi et al., 1996). The *STM* mRNA accumulates in both the CZ and PZ of the meristem but is repressed in organ primordia, in accordance with a role in maintaining cells in an undifferentiated state (Long et al., 1996). *WUS* is required to keep the pool of stem cells in the CZ of the meristem (Laux et al., 1996; Mayer et al., 1998). In *wus* mutants, the meristem is not established during embryogenesis; after germination, axillary meristems are initiated and aborted repeatedly. This repeated termination of the meristem has been attributed to a failure to specify the central stem cells that are required to re-populate the peripheral meristem. *WUS* is expressed in a small group of cells below the CZ, indicating that *WUS* must signal across cell layers to specify the stem cells.

WUS and *STM* are activated independently during

embryogenesis (Mayer et al., 1998) and although both genes are ultimately required to prevent differentiation and maintain cell division, it is unclear how their functions interact. It is also unknown whether ectopic expression of WUS or STM can revert cell differentiation and re-initiate cell division. We investigated these questions by activating *STM* and *WUS* functions in differentiating tissues, after embryogenesis. The combined effect of ectopic *WUS* and *STM* functions was more than additive: a subset of meristem functions were activated, including cell division, expression of a CZ marker and initiation of organ primordia, but not meristem self-maintenance and correct phyllotaxy. Our results suggest that *WUS* signals across cells to enable STM-expressing cells to initiate cell division and meristem activity.

MATERIALS AND METHODS

Arabidopsis lines

Immature flowers were emasculated and manually cross-pollinated to combine transgenes and mutations. *wus-1* (Laux et al., 1996), *stm-1* (Barton and Poethig, 1993) and *stm-11* (Long and Barton, 1998) were all in Landsberg *erecta* background and were maintained as heterozygotes, identified by PCR-primer-introduced restriction analysis (Jacobson and Moscovits, 1991) using leaf or root pieces (Klimyuk et al., 1993). Amplification was in all cases initiated by adding *Taq* polymerase at 94°C, followed by 35 cycles of 94°C for 30 seconds, 54°C for 60 seconds and 94°C for 90 seconds. For detection of *stm-1*, the primer sequences were 5′GTCGATATG-AACAATGAATTTGTAGATGCA3′ and 5′GTATAAGGGAAGAGA-GTTACCGAAG3′; the 221 bp product was cut to 193 bp with *Nsi*I if amplified from *stm-1*, but not from the wild type. For *stm-11*, the primers were 5′AGCTTACTGTGAAATGCTCGTTAAGTACTAG3′ and 5′TTCCTCATCTTACGTTTAATTTGACGCCAT3′; the 235 bp product was cut to 204 bp with *Spe*I only if amplified from *stm-11*. Both *stm* PCR products spanned an intron-exon boundary to prevent interference by the wild-type *STM* cDNA contained in *35S::STM-GR*.

The *cycB1::uidA* (line FA4C), *KNAT2::GUS*, *LOB* and *hsp18.2::Cre* lines have been described previously (Colón-Carmona et al., 1999; Laufs et al., 1998a; Pautot et al., 2001; Shuai et al., 2002). The J2341 gene trap line was generated by Jim Haseloff (http://www.plantsci.cam.ac.uk/Haseloff/Home.html) and was obtained from the NASC Arabidopsis Stock Centre (http://nasc.nott.ac.uk/). The *cycD3::uidA* reporter was derived from the cyclin D3b gene from *Antirrhinum majus* (Gaudin et al., 2000) and transformed into *Arabidopsis thaliana* Landsberg *erecta* (L*er*). The *cycD3::uidA* reporter mimicked the expression pattern of *Arabidopsis* cyclin D3:2 (Swaminathan et al., 2000) shown by in situ hybridisation (C. W., R. S. and J. D., unpublished). *35S::STM-GR*, *35S::lox-uidA-NOS-lox-GFP and 35S::lox-uidA-NOS-lox-WUS* lines were generated as described below.

Plasmid construction

Standard molecular biology techniques were used (Sambrook et al., 1989). For cDNA synthesis, RNA was extracted from inflorescences of *Arabidopsis* L*er*, using TRIZOL (Sigma), following the manufacturer's instructions. DNase I-treated total RNA was reverse-transcribed using MMLV reverse transcriptase (Stratagene). The *STM* and *WUS* coding sequences were amplified from inflorescence cDNA using *Pwo* polymerase (Stratagene). The primers for *STM* were 5′ATCTGGATCCATGGAGAGTGGTTCCAACAGC3′ and 5′AAAGTCTAGATCAAAGCATGGTGGAGGAGATG3′; *WUS* primers were 5′AGTCGGATCCAACACACATGGAGCCGCCAC3′ and 5′GCGACACTAGTGTAAGAGCTAGTTCAGACG3′. Both PCR products were cloned in pBluescript KS(–) (Stratagene), as a *Bam*HI-*Xba*I insert (*STM*) or *Bam*HI-*Spe*I (*WUS*), and their sequences were confirmed using the ABI Big Dye kit and an ABI 3700 sequencer.

To create the STM-GR fusion, the *STM* cDNA was re-amplified from the cloned product with primers 5′AAGGTCTAGATCTAG-CATGGTGGAGGAGATGTGAT3′ and 5′ATCTGGATCCATGG-AGAGTGGTTCCAACAGC3′. This replaced the *STM* stop codon with *Bgl*II site, which was ligated to the *Bam*HI site at the start of the sequence encoding the steroid-binding domain of the rat glucocorticoid receptor from pBI-ΔGR (Lloyd et al., 1994). The STM-GR fusion was re-sequenced and inserted as a *Bam*HI-*Xba*I fragment into pCGN18 (Krizek and Meyerowitz, 1996), to create p35S::STM-GR.

To create the mosaic expression vector, the *uid*A coding sequence of pRAJ275 (Jefferson, 1988) and the NOS terminator (NOS) from pCGN18 were cloned in pBluescript KS(–) as *Eco*RI-*uidA*-*Sal*I-NOS-*Pst*I. Oligonucleotides containing *lox*P sequences (Hoess et al., 1982) were cloned on both ends of *uid*A-NOS, with a *Bgl*II site added upstream of *lox*-*uid*A-NOS-*lox*. Relative to the *uid*A coding sequence, both *lox*P sequences were in the orientation 5′GACCTAAT-AACTTCGTATAGCATACATTATACGAAGTTATATTAAGGGTTG3′. After sequences were confirmed, the *lox*-*uid*A-NOS-*lox* cassette was cloned between *Bam*HI and *Xba*I sites in pCGN18 to generate pGUSMOS (in the orientation 35S promoter::*lox*-*uid*A-NOS-*lox*). pGUSMOS was cut with *Bam*HI and *XbaI* to receive mGFP5-ER (Haseloff, 1999) as a *Bam*HI-*Xba*I fragment, or the *WUS* cDNA as *Bam*HI-*Spe*I. To allow transformation into kanamycin-resistant plants, the 35S::*lox*-*uidA*-NOS-*lox*-WUS-NOS cassette was removed from the pCGN18 derivative as an *Asp*718-*Hind*III fragment and cloned into pPZP222, which confers gentamycin resistance to plants (Hajdukiewicz et al., 1994).

The *CLV1::GFP* construct was derived from pKR126 (containing the *CLV1* gene in pCGN1547). The mGFP5-ER from pBINmGFP5-ER (http://www.plantsci.cam.ac.uk/ Haseloff/Home.html) was used as a *Bam*HI fragment to replace the *CLV1* coding sequence in pKR126, retaining 5642 bp upstream and 729 bp downstream of the *CLV1* ORF.

Plant transformation and growth

Arabidopsis L*er* was transformed by the floral dip method (Clough and Bent, 1998). *Agrobacterium* strains were ASE for pCGN derivatives and GV3101 for pPZP222. Transformants were selected on GM medium (Valvekens et al., 1988) with 50 μg/ml kanamycin (for pCGN derivatives) or 100 μg/ml gentamycin (for pPZP200 derivatives). Transformants were moved to soil after 1-2 weeks. *35S::STM-GR* lines were selected that segregated kanamycin resistance as a single locus. *35S::lox-uidA-loxGFP* and *35S::lox-uidA-loxWUS* lines were selected with strong and widespread GUS activity and single T-DNA insertions detected by Southern blotting.

For growth on plates, seeds were surface-sterilised for 5 minutes in 50% v/v commercial bleach with 0.1% Tween 20, and plated on GM medium. Both for plate and soil growth, seeds were stratified at 4°C for 4 days and grown at 18-20°C, with 16 hours light (fluorescent lights at approximately 100 μmol photons m^{-2} s^{-1}) and 8 hours dark cycles.

Activation of STM-GR and Cre recombinase

For activation of STM-GR, seeds were plated on GM medium supplemented with 50 μg/ml kanamycin and 1 μM dexamethasone (Sigma). For activation of *hsp18.2::Cre*, surface-sterilised, stratified wet seeds were spread on the inside of Eppendorf tubes and germinated for 2 days at 20°C under continuous light (the tubes were left open inside a sealed Petri dish lined with wet filter paper). The closed tubes were incubated in a water bath at 38°C for 5 minutes. The heat shocked, germinating seeds were suspended in water and spread on GM medium (with 1 μM dexamethasone if the treatment also involved STM-GR activation).

Microscopy

For conventional scanning electron microscopy (SEM), seedlings were vacuum-infiltrated and left overnight at 4°C in a solution of 2.5% glutaraldehyde/PBS. The tissue was then rinsed 2× at 15 minute intervals with PBS followed by a further two washes with water. All rinses were at 4°C. The tissues were dehydrated through an ethanol series – 30%, 50%, 70%, 90%, 100%, 100% for 30 minutes at each stage. The samples were critical point-dried in liquid carbon dioxide and were sputter coated with gold. The specimens were mounted on aluminium specimen stubs using carbon tabs (Agar Scientific) and viewed on a Philips XL30 FEG scanning electron microscope.

For cryo-scanning electron microscopy, seedlings were frozen in nitrogen slush at –190°C. Ice was sublimated at –90°C, and the specimens were sputter coated and examined on a Philips XL 30 FEG scanning elelctron microscope fitted with a cold stage.

For confocal imaging, a Leica TCS SP microscope was used, with excitation by an argon laser (488 nm) and emission filters set at 500-550 nm (for GFP) and 600-660 nm (for chlorophyll fluorescence). The image settings gave no signal in the GFP channel for GFP-negative controls. The images shown are projections of up to twelve 8 μm optical sections, combining the GFP and chlorophyll channels.

Histological techniques

GUS staining was as described previously (Sieburth and Meyerowitz, 1997).

In situ hybridisation was carried out as described by Long et al. (Long et al., 1996) (detailed on http://www.wisc.edu/genetics/CATG/barton/protocols.html).

The template used to transcribe the WUS probe was derived from the WUS cDNA cloned in pBluescript KS(–) (described above), by deletion of a *Hin*dII fragment, leaving nucleotides 545-1004 in the EMBL entry AJ012310.

RESULTS

Steroid-dependent STM-GR rescued meristem development in *stm* mutants

To test the effect of STM activation after embryogenesis and in differentiated tissues, we generated plants with inducible STM function, expressing a fusion between STM and the steroid-binding domain of the rat glucocorticoid receptor (GR), as done previously for other plant transcription factors (Lloyd et al., 1994; Sablowski and Meyerowitz, 1998; Simon et al., 1996; Wagner et al., 1999). The STM-GR fusion protein was expected to remain in the cytoplasm until application of steroid (dexamethasone) triggered transport to the nucleus, allowing access to genes controlled by *STM*.

In the absence of dexamethasone, STM-GR under the widely expressed *35S* promoter had no effect (Fig. 1A). When *35S::STM-GR* seeds were germinated on medium containing dexamethasone, leaf development and growth of the cotyledons and hypocotyl were inhibited; these effects are described in more detail below. In spite of the disruption of leaf development, leaf primordia emerged from the meristem with the normal spiral phyllotaxy (Fig. 1C). This phenotype was seen in five independent *35S::STM-GR* lines; 15 other lines showed similar but weaker phenotypes that could be reproduced by treatment of the strong lines with low concentrations of dexamethasone (not shown). The phenotype seen in the strong lines was also observed in seedlings with STM expressed under control of the 35S promoter, although severe *35S::STM* lines could not be maintained because the plants could not set seed (not shown). For further analysis, we used two strong *35S::STM-GR* lines.

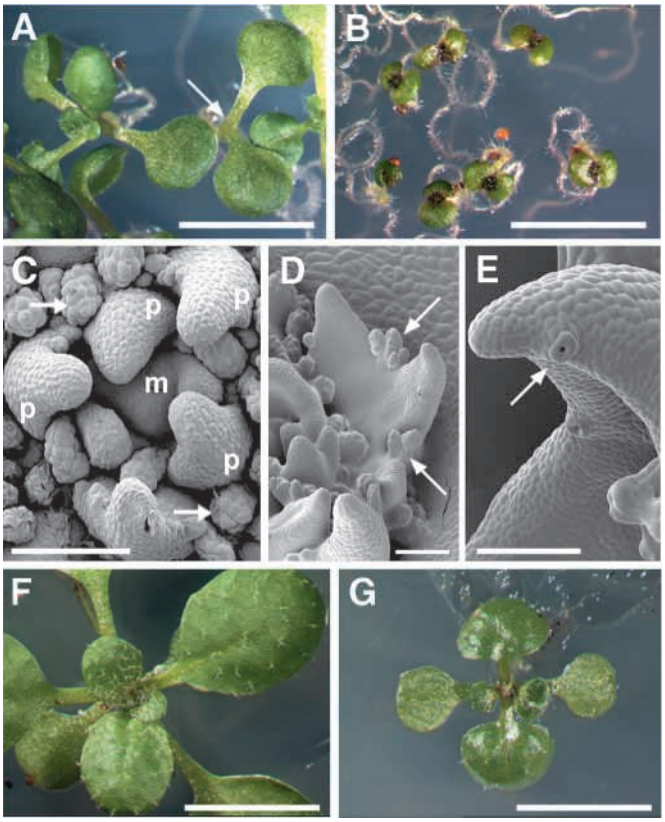

Fig. 1. Activated STM-GR rescued the meristem in *stm1* mutants and inhibited leaf development. (A) *35S::STM-GR* seedlings grown on medium without dexamethasone. One of the seedlings is homozygous for *stm1* (arrow). Bar: 5 mm. (B) *35S::STM-GR* seedlings (including one-quarter of *stm1* homozygotes), grown on medium with 1 μM dexamethasone. Bar: 5 mm. (C) Scanning electron micrograph of the apex of a *35S::STM-GR* seedling two weeks after germination on medium with 1 μM dexamethasone. The meristem (m) is surrounded by leaf primordia (p) in the normal spiral phyllotaxis. The arrows indicate stipules, whose identity was confirmed using a stipule-specific reporter gene (not shown). Bar: 100 μm. (D) Cryo-scanning electron micrograph of a leaf from a 2-weeks old *35S::STM-GR* seedling grown on medium with 1 μM dexamethasone. Arrows indicate ectopic stipules between leaf lobes. Bar: 100 μm. (E) Tip of a leaf from a *35S::STM-GR* seedling grown for 2 weeks on medium with 1 μM dexamethasone. The arrow indicates a pair of guard cells. Bar: 100 μm. (F,G) *35S::STM-GR* plants grown for 2 weeks on soil, sprayed once a day for 4 days with 0.015% v/v Silwet L-77 (F) or 0.015% v/v Silwet L-77 with dexamethasone 0.1 μM (G) and photographed 20 days after germination (moved to agar medium only for photography). The area around in the centre or the rosette in G is contains arrested leaf primordia similar to those seen in B, and early floral buds. Seedlings similar to the one shown in G were seen after spraying with 0.1-10 μM dexamethasone, although the degree of growth arrest was more severe at higher steroid concentrations. Bar: 5 mm.

To test whether STM-GR could replace *STM* function, *35S::STM-GR* was crossed to plants heterozygous for the strong *stm* mutants, *stm-1* (Barton and Poethig, 1993) and *stm-11* (Long and Barton, 1998). In the absence of dexamethasone, the kanamycin-resistant progeny of *35S::STM-GR*; *stm*/+

plants segregated one-quarter of *stm* mutants, showing that the *35S::STM-GR* and *STM* loci were not linked. When germinated in the presence of dexamethasone, however, all kanamycin-resistant seedlings formed a meristem and leaf primordia like those formed after activation of STM-GR in a wild-type background (Fig. 1A-C). PCR genotyping confirmed that some of these seedlings were indeed homozygous *stm* mutants (not shown). Thus *35S::STM-GR* provided dexamethasone-dependent *STM* function that was sufficient to restore meristem development in strong *stm* mutants. In spite of the widespread expression directed by the 35S promoter, meristems only formed in the area where a meristem normally forms. This suggested that additional functions that are independent of *STM* provided the cells in the presumptive meristem area with competence to fully respond to STM. This is consistent with the observation that some meristem genes are still weakly expressed in the presumptive meristem area of strong *stm* mutants (Long and Barton, 1998).

Additional evidence that STM-GR provided *STM* function came from ectopic activation of genes that are normally expressed in the meristem. One of these (Laufs et al., 1998a) was the *KNAT2* promoter fused to the *uidA* reporter, which encodes β-glucuronidase (GUS). *KNAT2* encodes a homeodomain protein that functions downstream of *STM* and is normally expressed in the periphery of the meristem but absent or much reduced in organ primordia (Byrne et al., 2000; Laufs et al., 1998a; Lincoln et al., 1994; Pautot et al., 2001). When treated with dexamethasone, *35S::STM-GR* plants expressed *KNAT2::uidA* in cotyledons, leaf primordia and roots (Fig. 2A,B). In cotyledons, expression was not uniform, but concentrated on spots that did not seem to correlate with a particular tissue or cell type. The same pattern (not shown) was obtained with a reporter gene directed by the promoter of *KNAT1*, which is related to *KNAT2* (Lincoln et al., 1994; Ori et al., 2000). The meaning of this expression pattern is not clear; it could reflect a positive feedback loop initiated in random cells that reach a threshold level of *STM* activity. Other meristem markers, however, showed uniform ectopic activation by STM-GR. These included *LOB* (*LATERAL ORGAN BOUNDARIES*), which is normally expressed in the boundaries around organ primordia (Shuai et al., 2002) and the gene trap line J2341, which is expressed around primordia in the shoot meristem (not shown).

Ectopic STM activity inhibited differentiation but did not activate cell division

We next examined the effects of ectopic STM activity on cell division and differentiation. In *35S::STM-GR* seedlings treated with dexamethasone, leaves were small, deeply lobed and frequently had ectopic stipules (Fig. 1D). The epidermal cells of these leaves were nearly the same size as meristematic cells and lacked any signs of specialisation. Occasionally, isolated guard cells or trichomes developed and reached normal size while still surrounded by small, undifferentiated cells, suggesting that reduced cell size was associated with a failure to differentiate and not simply caused by a general inhibition of cell expansion (Fig. 1E). The fact that the isolated trichomes and guard cells reached normal size also suggests that the final size of these cell types is set cell-autonomously. In cotyledons and hypocotyls, in which cells were at least partially differentiated at the time of STM-GR activation, growth was also restricted, owing to inhibition of cell expansion (not shown). When the plants were allowed to produce several leaves before STM-GR was activated, further leaf growth was arrested or deformed. In contrast with previous examples of ectopic *knox* gene expression (Sinha et al., 1993; Chuck et al., 1996), ectopic meristems were not seen on the leaves (Fig. 1F,G).

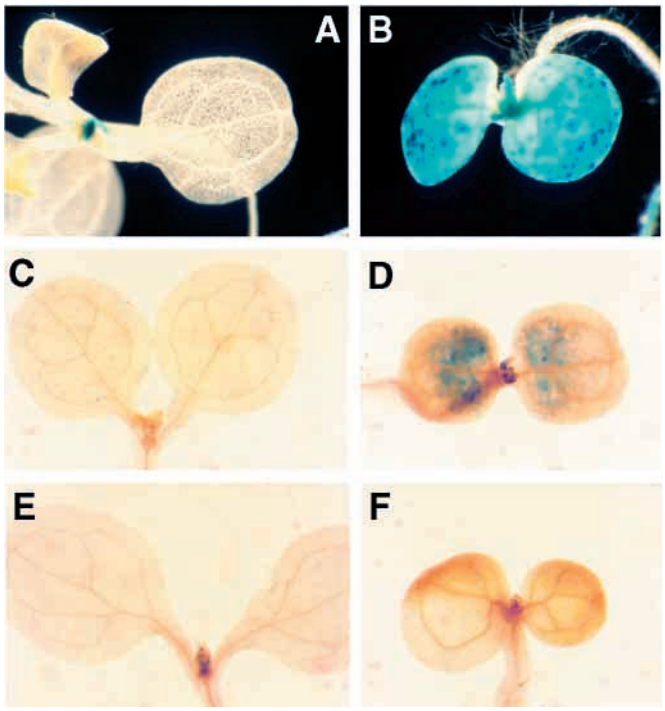

Fig. 2. STM-GR activated ectopic expression of *KNAT2::uidA* and *cycD3::uidA*, but not *cycB::uidA*. *35S::STM-GR* seedlings were grown for 9 days on medium without dexamethasone (A,C,E) or with 1 μM dexamethasone (B,D,F). The seedlings also contained the reporters *KNAT2::uidA* (A,B), *cycD3::uidA* (C,D) or *cycB::uidA* (E,F).

To monitor the effects of ectopic STM-GR on cell division, we used cyclin D and cyclin B reporter genes. D-cyclins are thought to control primarily the G_1-S transition; B-cyclins control the G_2-M transition and were used as markers for mitotic activity (Meijer and Murray, 2001). In wild-type background, both *CycD3::uidA* and *CycB1::uidA* were expressed in scattered cells within the meristem and young leaves (Fig. 2C,E). In seedlings with activated STM-GR, *CycB1::uidA* expression was comparable to wild-type (Fig. 2F). *CycD3::uidA* expression was also similar to wild type in meristem and organ primordia, but in addition, it was activated in cotyledons (Fig. 2D). The ectopic expression of the *CycD3::uidA*, but not of *CycB1::uidA*, showed that activation of STM-GR in differentiated tissues partially activated the cell division machinery, but was not sufficient to trigger mitosis.

Combined STM-GR and WUSCHEL induced ectopic organogenesis

The *KNAT1::uidA*, *KNAT2::uidA* and *LOB* marker genes that were ectopically activated by STM-GR are expressed mainly in the periphery of the wild-type meristem (Laufs et al., 1998a; Ori et al., 2000; Pautot et al., 2001; Shuai et al., 2002). A

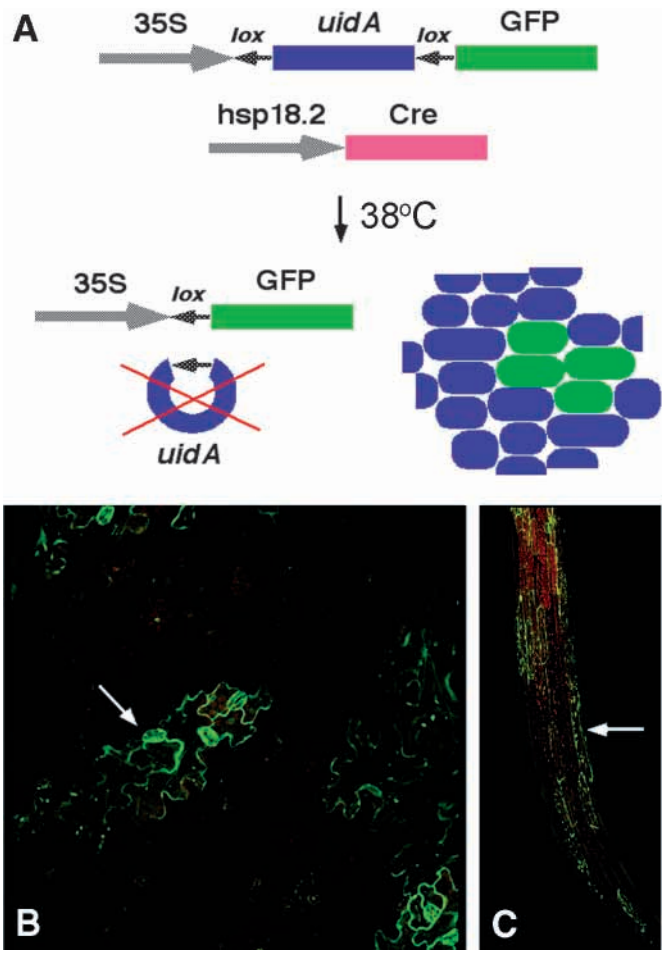

Fig. 3. Cre-*loxP*-based system for mosaic expression. (A) The system was tested in plants containing heat-shock-inducible Cre recombinase (*hsp18.2::Cre*) and a reporter construct consisting of *lox*P-flanked *uidA* inserted between the 35S promoter and GFP (*35S::lox-uidA-lox-GFP*). After a brief heat shock, transient induction of Cre caused excision of *uidA* and GFP activation in random cells and their descendants. (B,C) Optical sections of GFP-expressing sectors (arrows) on the cotyledon epidermis (B) and in hypocotyls (C) were recorded 7 days after heat shock.

reporter gene that is expressed in the central zone of the meristem (*CLV1::GFP*; see below) was not activated by STM-GR outside the shoot apex (not shown). It has been proposed that *STM* has distinct roles in the central and peripheral regions of the meristem (Long and Barton, 1998). Our results raised the possibility that the partial meristem identity induced by STM-GR on cotyledons and hypocotyls corresponded to the meristem peripheral zone, and that full meristem activity could be induced if central zone functions were added. Since *WUS* controls central zone identity (Mayer et al., 1998), we generated plants in which WUS could be expressed in differentiated tissues. In wild-type development, *WUS* is expressed in a small group of cells in the SAM and has a short range, non cell-autonomous effect (Mayer et al., 1998). To mimic this situation, we used the *Cre-loxP* recombination system (Sternberg and Hamilton, 1981) to generate random WUS-expressing sectors outside the meristem.

The vector used for mosaic expression (Fig. 3A) had the *uidA* reporter flanked by *lox*P sequences, inserted between the 35S promoter and the coding sequence to be expressed in random sectors. Plants with this construct were crossed to a line containing the Cre recombinase directed by a heat shock-inducible promoter (Sieburth et al., 1998). Heat-shock-induced Cre catalysed recombination between the *loxP* sequences, removing the *uidA* reporter and activating expression of the downstream coding sequence. The system was tested initially with the GFP reporter. In *35S::lox-uidA-lox-GFP* seedlings that were not heat shocked, GUS was widely expressed and no GFP was detected; after heat shock, sectors developed that expressed GFP but not GUS (not shown). The *35S::lox-uidA-lox-GFP* line was used to optimise the conditions for induction of mosaic expression in seedlings (Fig. 3B,C) and as a negative control in subsequent experiments with WUS expression.

Plants were then generated in which WUS replaced GFP in the mosaic expression construct (*35S::lox-uidA-lox-WUS*). The results shown here are based on two representative lines with strong, widespread GUS activity. *35S::lox-uidA-lox-WUS* was crossed into the *hsp18.2::Cre* line. Without heat shock, *hsp18.2::Cre; 35S::lox-uidA-lox-WUS* seedlings were indistinguishable from the wild type (not shown). Heat shock had no morphological effect on wild type or *hsp18.2::Cre*; *35S::lox-uidA-lox-GFP* controls (Fig. 4A,E). When heat shocked during germination, however, *hsp18.2::Cre; 35S::lox-uidA-lox-WUS* seedlings had misshapen cotyledons and hypocotyls, with aberrant cell expansion and a limited amount of cell division (Fig. 4B,F). On approximately 3% of seedlings (1/42, 4/101, 2/83 in three experiments), outgrowths formed on the surface of hypocotyls (Fig. 4G). In contrast to subsequent experiments in which ectopic WUS and STM were combined (below), these outgrowths did not behave as organ primordia.

To test the effect of combined STM and WUS functions, lines homozygous for *35S::lox-uidA-lox-WUS* and *hsp18.2::Cre* were crossed to *35S::STM-GR* plants. Progeny seedlings were heat shocked to induce WUS sectors and subsequently plated on medium with dexamethasone. Controls that were not heat shocked, or that were heat shocked but lacked *35S::lox-uidA-lox-WUS*, showed the same phenotypes described above for *35S::STM-GR* seedlings when plated on dexamethasone-containing medium. Controls that were heat-shocked and plated without dexamethasone showed the effects described above after mosaic activation of WUS.

After heat shock and dexamethasone treatment, *35S::STM-GR; 35S::lox-uidA-lox-WUS*; *hsp18.2::Cre* seedlings had a novel phenotype: in approximately 35% of seedlings (15/45, 45/125, 16/29 in three experiments), outgrowths emerged on the surface of cotyledons and hypocotyls (Fig. 4C,D). These outgrowths appeared both on the abaxial and adaxial sides of cotyledons, and at any position on hypocotyls. Scanning electron microscopy (Fig. 4K), confocal microscopy (Fig. 6D) and conventional sections of hypocotyls (not shown) suggested that each outgrowth originated from a single epidermal cell. Although ectopic cell divisions were also seen in the cortex and endodermis, outgrowths never seemed to originate from these tissues. The outgrowths usually developed into organs that resembled the abnormal leaf primordia seen at the apex of seedlings after STM-GR activation, with leaf-like features such as dorsoventral assymetry and trichomes (Fig. 4C,D,J).

The ectopic organs induced when STM-GR was activated in heat-shocked *35S::lox-uidA-lox-WUS* had GUS activity,

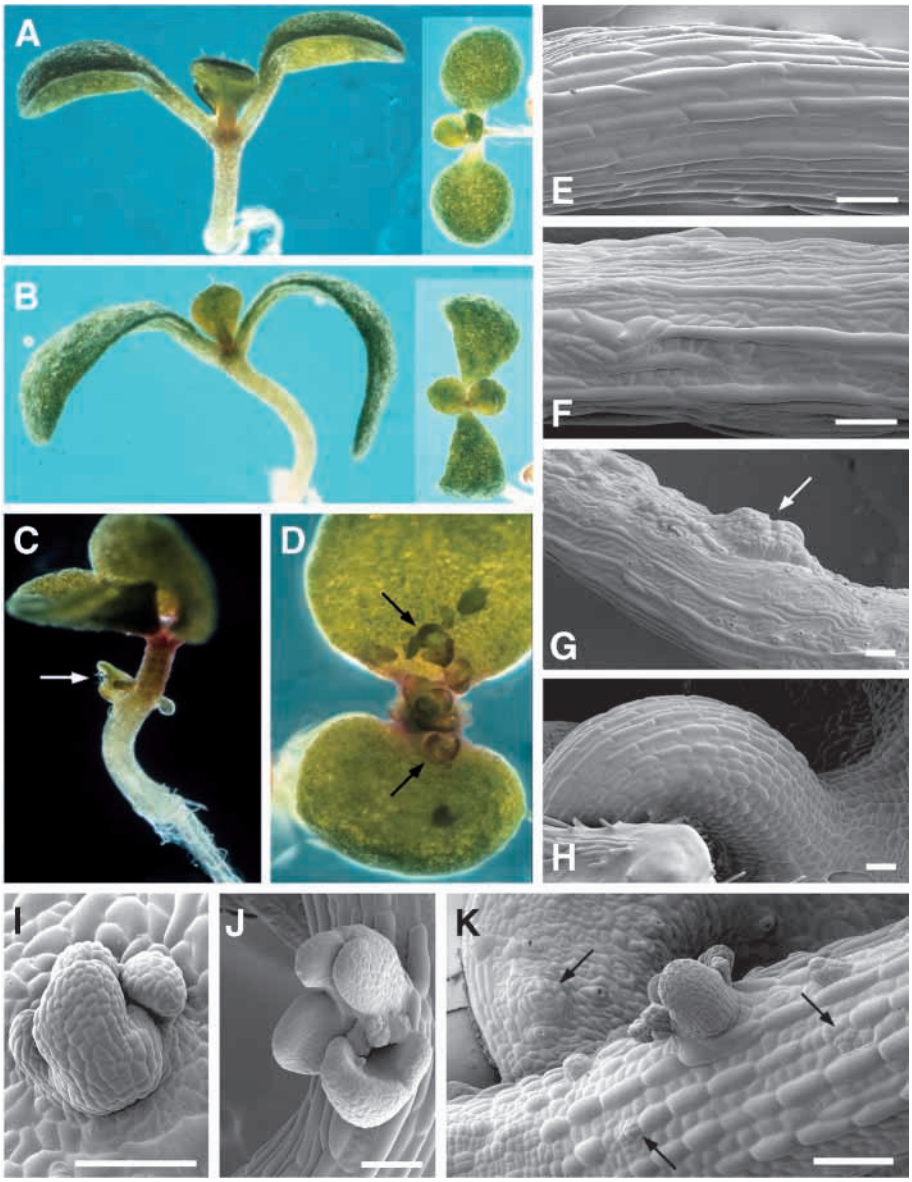

Fig. 4. Mosaic WUS expression combined with STM-GR activation caused ectopic organogenesis. (A) Control seedling (*35S::lox-uidA-lox-GFP; hsp18.2::Cre*) with no morphological effects 7 days after heat shock induction of GFP sectors (see Fig. 3). Inset: top view of comparable seedling. (B) Phenotype caused by mosaic WUS expression in *35S::lox-uidA-lox-WUS; hsp18.2::Cre* seedlings, 7 days after heat shock. Inset: top view of similar seedling. (C,D) Phenotype after induction of mosaic WUS expression and STM-GR activation, in *35S::lox-uidA-lox-WUS; hsp18.2::Cre; 35S::STM-GR* seedlings grown on medium with 1 μM dexamethasone 10 days after heat shock. The arrows indicate ectopic organs on the hypocotyl (C) and cotyledons (D). (E-H) Cryo-scanning electron micrographs of hypocotyls. (E) Hypocotyl from a control seedling as in A; (F) hypocotyl after WUS induction as in B; (G) example of outgrowth (arrow) observed on the hypocotyl at low frequency 2 weeks after WUS induction as in B; (H) hypocotyl after activation of STM-GR but not WUS (*35S::STM-GR* seedling grown for 10 days on medium with 1 μM dexamethasone). Bar: 100 μm. (I-K) Cryo-scanning electron micrographs of ectopic organs induced by combined WUS and STM-GR activation (as in C,D). (I) Outgrowth on cotyledon (as in D); (J,K) outgrowths on hypocotyls (as in C). Arrows in K indicate outgrowths at early stages. Bar: 100 μm.

indicating that they developed from cells that still contained the *uidA* reporter and therefore should not express WUS (Fig. 5A-C). This indicated that the outgrowths developed from cells that received a signal from WUS-expressing cells, consistent with the non cell-autonomous activity of *WUS* in the meristem. In situ hybridisation was used to verify that outgrowths did not express *WUS* but appeared in the vicinity of *WUS*-expressing cells (Fig. 5E-G). Not every patch of *WUS*-expressing cells in cotyledons and hypocotyls, however, was associated with an outgrowth. As mentioned above, outgrowths seemed to originate from epidermal cells, suggesting that different tissues and cell types responded differently to the WUS signal. It cannot be excluded, however, that production of the WUS signal could also be limited to specific tissues or cell types.

Ectopic organs induced by WUS and STM originated from transient meristem activity

The appearance of organ primordia on cotyledons and hypocotyls suggested that combined *WUS* and *STM* activity initiated ectopic meristem activity, but self-perpetuating meristems were not established. To verify whether the ectopic organs originated from cells that expressed a meristem marker gene, we monitored expression of *CLV1::GFP*. *CLV1* encodes a receptor kinase and functions in a pathway that controls the size of the stem cell population in the centre of the meristem. Accordingly, *CLV1* is expressed in the meristem central zone (Clark et al., 1997). Confocal microscopy of the apex of germinating seedlings showed that *CLV1::GFP* was expressed in the meristem CZ (Fig. 6A,B), in a few expanded cells below the meristem and in the developing vasculature in the vicinity of the meristem (not shown).

Plants homozygous for *35S::lox-uidA-lox-WUS* and *hsp18.2::Cre* were crossed to *35S::STMGR; CLV1::GFP* plants. The progeny was heat shocked 2 days after germination and plated on dexamethasone-containing medium. Two days after heat shock, ectopic cell divisions were detected on the hypocotyl epidermis (not seen in

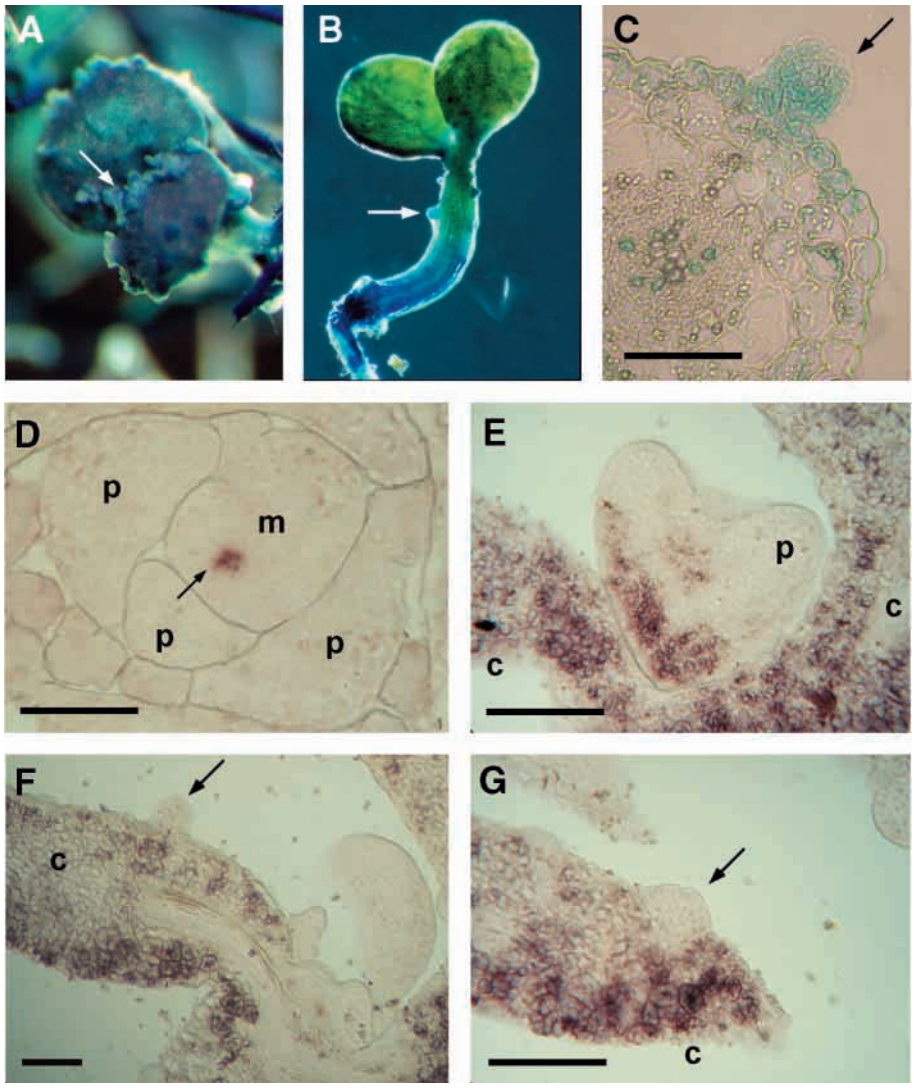

Fig. 5. Ectopic organogenesis was a non cell-autonomous response to WUS. (A,B) Whole seedlings stained for GUS activity after induction of ectopic organogenesis. *35S::lox-uidA-lox-WUS; hsp18.2::Cre; 35S::STM-GR* seedlings were grown on medium with 1 μM dexamethasone for 10 days (A) or 7 days (B) after heat shock. Arrows indicate GUS-expressing outgrowths. (C) Transverse section through the hypocotyl of a seedling treated as described in B, showing an outgrowth with GUS-expressing cells. Bar: 100 μm. **(**D-G) Detection of WUS mRNA by in situ hybridisation. (D) Control showing endogenous *WUS* expression (arrow) below the centre of the meristem, in a transverse section through the apical meristem (m) and leaf primordia (p) of a *35S::STM-GR* seedling (equivalent to the seedlings in Figs 1B and 2B). (E-G) Patches of ectopic WUS mRNA in sections through a leaf primordium (p) and cotyledons (c) in seedlings with mosaic WUS expression combined with STM-GR activation (equivalent to the seedlings shown in Fig. 4C,D). The arrow indicates outgrowths on cotyledons, consisting of cells that did not express WUS. Bar: 100 μm.

controls that were not heat shocked). These newly dividing cells expressed *CLV1::GFP* (Fig. 6C). Seven days after heat shock, ectopic outgrowths were visible. Like meristems, these outgrowths contained small, isodiametric, *CLV1::GFP*-expressing cells (Fig. 6D). In the areas where the outgrowths were bulging out of the hypocotyl surface, the cells were still small and seemed to be actively dividing, but no longer expressed *CLV1::GFP*. In outgrowths with leaf-like features (10 days after heat shock), expression of *CLV1::GFP* was detected mostly at the base and occasionally in a fraction of the cells within the ectopic organs (Fig. 6E). These GFP-expressing cells were fewer and larger than in earlier stages, suggesting that they were no longer actively dividing. Controls with induction of STMGR only or WUS only did not activate *CLV1::GFP* in cotyledons (not shown) or in the hypocotyl (Fig. 6G,H), except in the outgrowths seen at a low frequency on the hypocotyl after activation of WUS alone (not shown).

These results suggested that combined WUS and STM-GR initiated meristem activity (based on activated cell division and *CLV1::GFP* expression), but the meristematic cells were consumed in the development of ectopic organs.

DISCUSSION

The continuous and reiterative nature of plant development requires the maintenance of undifferentiated cells at the growing tips throughout the life of the plant. Genetic and cell ablation experiments suggested that meristem cells differentiate by default unless they receive specific signals that prevent differentiation (Mayer et al., 1998; van den Berg et al., 1997). In the shoot meristem, *STM* and *WUS* antagonise differentiation. An important open question, however, is whether activation of these genes in differentiating tissues can revert differentiation and restore competence to initiate organs. Previous studies have shown that ectopic expression of meristem regulators such as KNAT1 and WUS cause ectopic meristem activity (Chuck et al., 1996; Schoof et al., 2000). In these studies, however, meristem regulators were expressed from early stages in meristem-derived organs, and therefore reversion to meristematic identity could not be distinguished from maintenance of meristem functions in developing organs. In contrast, most of our experiments focussed on the effect of activating WUS and STM in cotyledons and hypocotyls, which are the only shoot organs believed not to derive from the shoot

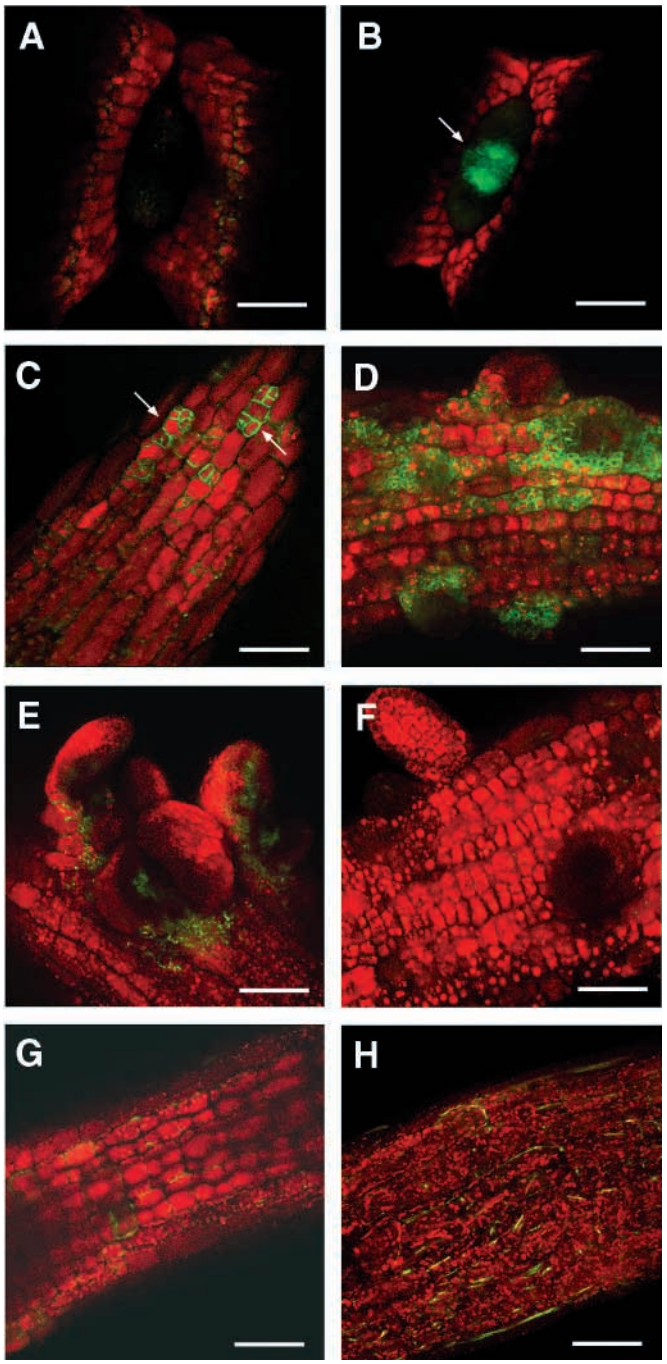

Fig. 6. The *CLV1* promoter was transiently activated during ectopic organogenesis. (A,B) Optical sections through the apex of 3-day old control seedlings. (A) Seedling lacking *CLV1::GFP*. The red chlorophyll fluorescence marks the base of the cotyledon petioles; the space in the centre is occupied by the meristem and the first two leaf primordia. (B) *CLV1::GFP* seedling. GFP signal is visible in the meristem (arrow); the dark areas adjacent to the meristem is occupied by leaf primordia. (C-F) Longitudinal optical sections through the hypocotyls of seedlings with ectopic organs (*35S::lox-uidA-lox-WUS; hsp18.2::Cre; 35S::STM-GR*, heat shocked and plated on medium with 1 μM dexamethasone; equivalent to the seedlings shown in Figs 4C,D and 5B). (C-E) *CLV1::GFP* seedlings, (C) 2 days, (D) 7 days and (E) 10 days after heat shock. The arrows in C indicate the division of epidermal cells seen in early stages of outgrowth formation. (F) Control seedling lacking *CLV1::GFP*, 10 days after heat shock. (G,H) Longitudinal optical sections through the hypocotyls of control seedlings (*35S::lox-uidA-lox-WUS; hsp18.2::Cre; 35S::STM-GR*, *CLV1::GFP*) with activation of STM-GR or WUS alone. (G) seedling that was not heat shocked, after growth for 7 days in medium with 1 μM dexamethasone (STM-GR activation alone); (H) seedling that was heat shocked and grown for 7 days on medium without dexamethasone (WUS activation alone). The green/yellow signal seen along part of the walls of the large cells in H is an artefact of light refraction, also seen in seedlings that lacked *CLV1::GFP*. Bar: 100 μm.

apical meristem (Jürgens, 2001; Long and Barton, 1998). This way, we could more easily study the ectopic establishment of meristem functions.

Ectopic activation of STM-GR inhibited cell expansion and the development of specialised cell types, but did not activate cell division. The previously reported effects of ectopic expression of *STM* homologues included delayed differentiation, increased subdivision of the leaf lamina, ectopic meristems on the adaxial surface of leaves, or a combination of these phenotypes (Reiser et al., 2000). Ectopic meristems were seen on the leaves of plants overexpressing KNAT1 in *Arabidopsis* and in tobacco plants with high levels of expression of the maize *STM* homologue, *KNOTTED1* (Sinha et al., 1993). In contrast, ectopic activation of *KNAT1* and *KNAT2* by STM-GR (our results) or in *asymmetric leaves1-1* (*as1-1*) and *as2-2* mutants (Ori et al., 2000) did not cause ectopic meristems [although occasional ectopic meristems were reported in *as1-1* by Byrne et al. (Byrne et al., 2000)]. One possible explanation for this discrepancy is that higher levels of STM (and consequently KNAT1 and KNAT2) would be required to initiate meristems. The level of STM-GR expression in our experiments, however, appeared to be physiologically relevant, because it was sufficient to rescue the meristem in *stm* mutants and to initiate ectopic meristem activity in combination with WUS. In addition, a more severe *35S::STM* phenotype has been reported, with inhibition of growth and leaf development, disruption of the SAM, but no ectopic meristems (Williams, 1998). The less severe effect in our experiments could be due to the fact that STM-GR was activated only after embryogenesis. Our most severe *35S::STM* lines, however, were similar to dexamethasone-treated *35S::STM-GR* lines (not shown); seedlings with more severe inhibition of growth may have been missed during the selection of kanamycin-resistant transformants.

In spite of the failure to induce ectopic meristems or mitosis, activation of the cyclin D3 promoter suggested a link between *STM* and the cell division machinery. This link, however, may be indirect. Expression of cyclin D3 is activated by cytokinin (Riou-Khamlichi et al., 1999), and *STM* may increase cytokinin levels, as seen after ectopic expression of KNOTTED1 in tobacco leaves (Ori et al., 1999). D-cyclins have been proposed to function at a point when differentiation decisions are made (Meijer and Murray, 2000) so activation of cyclin D3 may be related to the delay in differentiation caused by ectopic STM activity.

Our data support the previous suggestion that *STM* has separable PZ and CZ functions. These different functions have been proposed on the basis of the mutant phenotypes and on the expression pattern of *STM* during embryogenesis. The

partial fusion of cotyledons seen in *stm* mutants suggested that *STM* inhibits growth in the areas between emerging organs, while in the centre of the meristem, *STM* is required to maintain cell proliferation (Long and Barton, 1998). In our experiments, activation of genes that are expressed in the periphery of the meristem, but not of the central zone marker *CLV1::GFP*, suggested that STM outside the shoot apex activated peripheral zone functions. The prediction that the missing central zone functions could be provided by WUS was confirmed here: combined WUS and STM-GR triggered meristem activity, expression of a CZ marker (*CLV1::GFP*) and organogenesis.

An interesting feature of the results of combined STM and WUS activity was that ectopic organs were induced on both the adaxial and abaxial sides of cotyledons. This contrasts with previous reports where misexpression of genes caused ectopic organs specifically on the adaxial surface of leaves (Chuck et al., 1996; McConnell and Barton, 1998; Sinha et al., 1993). These previous results indicated that the adaxial side has special competence for meristematic activity, consistent with the fact that axillary meristems normally form on the adaxial side of the leaf (McConnell and Barton, 1998). Our work suggest that combined WUS and STM function can bypass the factor(s) that confer special meristematic competence to the adaxial side of lateral organs (assuming that these factors function similarly in leaves and cotyledons).

Our results are consistent with the suggestion that *WUS* is required to produce a signal that specifies stem cells across cell layers (Mayer et al., 1998). When *STM* and *WUS* functions were combined, cell division was activated in cells that did not express WUS. This observation was supported by experiments in which no ectopic organs were initiated after STM-GR activation and ubiquitous WUS expression caused by high levels of Cre induction (not shown). In seedlings with mosaic WUS expression, however, not every WUS-expressing sector was associated with ectopic organs. The ability to initiate organs seemed limited to epidermal cells that did not express WUS, but we cannot exclude the possibility that the ability to produce the WUS signal may also be limited to specific cell types or tissues.

With the caveat that additional factors may affect the ability to produce or respond to the *WUS* signal, our results suggest that this signal triggers division in STM-expressing cells. This is consistent with the observation that in late *wus-1* embryos, the cells in the area normally occupied by the meristem still express *STM* but fail to divide (Laux et al., 1996; Mayer et al., 1998). Instead, these cells enlarge, show signs of differentiation and, after germination, no longer express *STM*. The fact that *wus-1* mutants re-initiate organogenesis days after germination, however, shows that cell division can occur at the shoot apex independently of *WUS* (Laux et al., 1996). This organ re-initiation may rely on the mechanism that establishes axillary meristems at the base of leaves. The tomato *lateral suppressor* mutant showed that establishment of the apical and axillary meristems are genetically separable (Schumacher et al., 1999). We propose that during axillary meristem development, other genes can transiently replace the *WUS* signal to trigger cell division in response to *STM*.

The ectopic organs in seedlings with combined WUS and STM activity initiated with localised activation of cell division and *CLV1::GFP* expression, indicating that the outgrowths initially had characteristics of the CZ of the meristem. Subsequently, *CLV1::GFP* was down-regulated and leaf-like organs developed. This suggested that the outgrowths progressed through the states normally assumed by cells in different meristem areas: central zone (marked by *CLV1* expression), peripheral zone (active cell division but no *CLV* expression, and ability to initiate organs), and finally organ primordia. We propose two hypotheses to explain this result. The response to the *WUS* signal could be distance-dependent: in the vicinity of WUS-expressing cells, STM-expressing cells respond to a strong signal and acquire central zone identity. As cell division increases the number of cell layers from the WUS source, STM-expressing cells continue dividing but shift to peripheral zone functions and acquire the ability to initiate organs. Alternatively, combined WUS and STM could trigger a cascade of signals, starting with activation of central zone identity by a WUS-dependent signal. Central zone functions would include the mechanism to trigger the transition to peripheral zone identity, possibly stimulated by the *CLV* pathway (Clark et al., 1997); peripheral zone identity would trigger the transition to organogenesis.

If combined ectopic STM and WUS provided both central zone and peripheral zone functions, why were self-maintaining meristems not established? One possibility is that additional genes need to be activated or repressed independently of *STM* and *WUS* for full meristem activity. Another possibility may be that in the ectopic induction experiments, WUS expression was insensitive to the delicate regulatory mechanisms that maintain the meristem, in particular the feedback loop between *WUS* and *CLV* genes (Schoof et al., 2000). An early effect of ectopic WUS was activation of the *CLV1* promoter. Since *WUS* was also reported to activate *CLV3* (Schoof et al., 2000), it is reasonable to expect that the whole *CLV* pathway was activated. The *CLV* pathway has been proposed to have two functions, which are not mutually exclusive: to inhibit cell proliferation in the central zone of the meristem, and to promote the transition of cells from the central to the peripheral zone (Clark et al., 1996; Clark et al., 1997). In addition, *CLV* activity negatively regulates *WUS* at the transcript level; this negative feedback loop prevents excessive growth of the central zone in response to *WUS* (Brand et al., 2000; Schoof et al., 2000). In our experiments, *CLV1* may not be able to moderate its own expression by repressing its activator, WUS, because the latter is controlled by a heterologous promoter. The build-up of high *CLV* activity may eventually inhibit cell proliferation and promote the transition to peripheral zone function and organogenesis. Both effects would explain why expression of *CLV1::GFP* and organogenesis were transient in our experiments.

In conclusion, we have shown that ectopic *STM* and *WUS* functions interact non-additively to activate a subset of meristem functions, including cell division, *CLV1* expression and organ initiation. Our results also suggest that *WUS* and *STM* not only maintain meristem cells undifferentiated, but can also revert cells to a CZ-like, undifferentiated and actively dividing state, from which they can follow an alternative differentiation pathway (from hypocotyl to leaf-like identity). The ectopic induction of meristem CZ identity was followed by organogenesis, suggesting that the mechanisms that establish the CZ and organogenesis are coupled.

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