Development 129, 3195-3206 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV0437

The *WUSCHEL* and *SHOOTMERISTEMLESS* genes fulfil complementary roles in *Arabidopsis* shoot meristem regulation

Michael Lenhard[1], Gerd Jürgens[2] and Thomas Laux[1,*]

[1]Institut für Biologie III, Universität Freiburg, Schänzlestrasse 1, D-79104 Freiburg, Germany
[2]Universität Tübingen, ZMBP – Entwicklungsgenetik, Auf der Morgenstelle 1, D-72076 Tübingen, Germany
*Author for correspondence (e-mail: laux@biologie.uni-freiburg.de)

Accepted 9 April 2002

SUMMARY

Continuous organ formation from the shoot apical meristem requires the integration of two functions: a set of undifferentiated, pluripotent stem cells is maintained at the very tip of the meristem, while their daughter cells in the periphery initiate organ primordia. The homeobox genes *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) encode two major regulators of meristem formation and maintenance in *Arabidopsis*, yet their interaction in meristem regulation is presently unclear. Here, we have addressed this question using loss- and gain-of-function approaches. We show that stem cell specification by *WUS* does not require *STM* activity. Conversely, *STM* suppresses differentiation independently of *WUS* and is required and sufficient to promote cell division. Consistent with their independent and distinct phenotypic effects, ectopic *WUS* and *STM* activities induce the expression of different downstream target genes. Finally, the pathways regulated by *WUS* and *STM* appear to converge in the suppression of differentiation, since coexpression of both genes produced a synergistic effect, and increased *WUS* activity could partly compensate for loss of *STM* function. These results suggest that *WUS* and *STM* share labour in the shoot apical meristem: *WUS* specifies a subset of cells in the centre as stem cells, while *STM* is required to suppress differentiation throughout the meristem dome, thus allowing stem cell daughters to be amplified before they are incorporated into organs.

Key words: *Arabidopsis*, *SHOOTMERISTEMLESS*, *WUSCHEL*, stem cells, shoot meristem

INTRODUCTION

Postembryonic development of higher plants is characterized by the continuous formation of organs from the shoot apical meristem (SAM) (Steeves and Sussex, 1989). The SAM serves two main functions: in the central zone, a population of undifferentiated, pluripotent stem cells is maintained, and in the peripheral zone, lateral organ primordia are initiated. While all cells of the meristem dome remain undifferentiated until they are incorporated into organ primordia, only a specialized subset functions as long-term stem cells from which all cells of the shoot and its lateral organs are ultimately derived (Satina et al., 1940; Stewart and Dermen, 1970). These stem cells are located in three cell tiers at the very apex and coincide with the domain where the *CLAVATA3* (*CLV3*) gene is expressed (Fletcher et al., 1999).

Genetic analysis in *Arabidopsis* has identified two major regulators of SAM formation and maintenance, the homeobox genes *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*). In *wus* mutants the apical stem cells are unable to self-maintain (Laux et al., 1996; Mayer et al., 1998), whereas ectopic *WUS* expression can abolish organ formation at the SAM and induce expression of the putative stem cell marker *CLV3* (Schoof et al., 2000). During embryogenesis, *WUS* mRNA can first be detected in the four inner apical cells of the 16-cell stage embryo and later becomes restricted to a small central cell group underneath the presumed stem cells in the outermost three cell layers. Thus, *WUS* expression appears to define an organizing centre whose activity establishes an apical group of long-term stem cells.

WUS expression is under negative control by the *CLAVATA* genes (*CLV1*, *CLV2* and *CLV3*), which encode components of a presumed receptor-kinase signal transduction pathway (Clark et al., 1997; Jeong et al., 1999; Fletcher et al., 1999). In *clv* mutants, the SAM enlarges progressively by the accumulation of stem cells (Clark et al., 1993; Clark et al., 1995; Fletcher et al., 1999), and this enlargement appears to be a consequence of ectopic *WUS* expression in more apical and lateral cells in *clv* mutant SAMs (Schoof et al., 2000). This has led to a model in which stem cell maintenance is regulated by a negative feedback loop mediated by the *WUS* and *CLV3* genes, with the organizing centre signalling to the apical neighbours to specify them as stem cells, which in turn signal back to restrict the size of the organizing centre (Brand et al., 2000; Schoof et al., 2000).

Loss-of-function mutations in the *SHOOTMERISTEMLESS* (*STM*) gene, which encodes a homeodomain protein of the KNOTTED class (Long et al., 1996) also result in a lack of a self-maintaining meristem. Instead of forming a SAM, the cells in the apex of *stm* mutant embryos appear to differentiate

(Barton and Poethig, 1993; Endrizzi et al., 1996). In addition, *stm* mutant seedlings exhibit fusion of the cotyledon petioles, suggesting that *STM* fulfils two functions: it inhibits differentiation of the cells in the embryo apex and prevents outgrowth of the cells separating the cotyledon primordia in the periphery. Repression of differentiation by *STM* in the SAM primordium appears to occur mainly via repression of the MYB-related gene *ASYMMETRIC LEAVES1* (*AS1*), since loss of *AS1* function in an *stm* mutant background rescues SAM formation (Byrne et al., 2000). *STM* mRNA is expressed in the shoot meristem primordium from the globular embryo stage on, and postembryonically expression is found throughout the SAM, but is excluded from incipient organ primordia (Long et al., 1996).

Whether and how the regulatory pathways defined by *WUS* and *STM* interact in SAM formation and maintenance is presently unclear. However, several lines of evidence have been taken to suggest that *WUS* is a downstream target of *STM* in functional SAMs: *wus* mutations exacerbate the phenotype of weak *stm* loss-of-function alleles, while strong *stm* mutations are epistatic to *wus* (Endrizzi et al., 1996); *STM* exhibits dosage-sensitive interactions with the *CLV* genes (Clark et al., 1996), suggesting that *STM* and *CLV* may act antagonistically on common downstream targets, one of which could be *WUS*; although *WUS* expression is initiated correctly in *stm* mutants, it is not maintained in later embryo stages (Mayer et al., 1998). However, *WUS* expression is initiated earlier in embryogenesis than *STM* expression (Mayer et al., 1998; Long and Barton, 1998), arguing that at least in embryonic SAM formation there is no linear pathway with *WUS* downstream of *STM*.

To understand how the functions of *WUS* and *STM* are integrated in SAM regulation, we have analyzed their interactions, using a combination of loss- and gain-of-function approaches.

MATERIALS AND METHODS

Mutant lines, growth conditions and dexamethasone induction

The wild type used in all experiments was the Landsberg *erecta* (L*er*) ecotype. The *wus-1* mutant has been described previously (Laux et al., 1996; Mayer et al., 1998), as well as the *stm5* mutant (Endrizzi et al., 1996). *stm-5* carries a G to A transition of the first nucleotide of the third intron, which changes the conserved GA dinucleotide of the exon-intron boundary to AA and is predicted to prevent the intron from being spliced out. This would result in a translational stop after the addition of ten unrelated amino acids, causing a loss of the C-terminal half of the homeodomain (A. Haecker and T. L., unpublished). Plant growth conditions were as described previously (Laux et al., 1996). For dexamethasone induction, plants were sprayed with a solution of 5 μM dexamethasone (Sigma Aldrich; St. Louis, USA)/0.015% Silwet L-77 (OSi Specialties; Meyrin, CH) in tap water. For the mock treatment, 0.025% ethanol/0.015% Silwet L-77 in tap water was used, since the dexamethasone stock solution was 20 mM in 100% ethanol. Seedlings were harvested 2 days after induction.

Histology, scanning electron microscopy and GUS staining

Preparation of histological sections from LR-White embedded material, DAPI staining of seedlings and scanning electron microscopy were done as described previously (Laux et al., 1996; Schoof et al., 2000). GUS staining was performed as described previously (Schoof et al., 2000). In all cases, samples to be compared where stained for the same duration.

PCR-based genotyping

Plants were genotyped for the *wus-1* allele by dCAPS (Neff et al., 1998) as described by Groß-Hardt et al. (Groß-Hardt et al., 2002).

Construction of transgenes and plant transformation

For all misexpression experiments we used the *pOpL* two-component system, where a promoter of interest controls the expression of a synthetic transcription factor, LhG4 (Moore et al., 1998). The gene to be expressed is controlled by a synthetic promoter, *pOp*, which is specifically activated by LhG4. For the sake of simplicity, we will refer to plants, for example, of the genotype *ANT::LhG4*; *pOp::STM* as *ANT::STM*.

Generation of the *pOp::WUS-pOp::GUS* (MT72) transgenic line, as well as of *ANT::LhG4* and *CLV1::LhG4* lines was described before (Schoof et al., 2000).

For the *pOp::STM* construct, the *STM* coding region was isolated from pCGN1547:*35S::STM* (kindly provided by R. Williams) by digestion with *Bam*HI and subcloned into pU-BOP (kindly provided by I. Moore) which had been digested with *Bam*HI. The resulting *pOp::STM* fragment was excised from pU-BOP:*STM* by partial digestion with *Sac*I and *Hin*dIII and subcloned into pBarM, a derivative of pGPTV-BAR (Becker et al., 1992), linearized with *Sac*I and *Hin*dIII to yield plasmid MT153. For the *pOp::STM-pOp::GUS* construct, a *pOp::GUS* fragment was isolated from plasmid MT162 by digestion with *Eco*RI and inserted into plasmid MT153 to yield MT168.

For the *35S::WUS-GR* construct, the *WUS* open reading frame was amplified using primers WUS5BAM (5′-AGT CGG GAT CCA CAC ACA TGG-3′) and WUS3BAM+2 (5′-GAG CGG ATC CAG ACG TAG CTC AAG AG-3′), digested with *Bam*HI and subcloned into the *Bam*HI site of pRS020 (kindly provided by R. Sablowski) which contains the coding sequence of the C terminus of the rat glucocorticoid receptor (GR), producing an N-terminal fusion of WUS to GR (MT141). The *WUS* fragment was sequenced to exclude amplification errors. The resulting *WUS-GR* fusion gene was inserted as an *Xba*I/*Sma*I-fragment into pBar35S (kindly provided by G. Cardon) to yield MT142.

Generation of the *WUS::NLSGUS* and *CLV3::NLSGUS* constructs have been described previously (Groß-Hardt et al., 2002).

All constructs were introduced into *Agrobacterium* strain GV3101 (pMP90) (Koncz and Schell, 1986) by electroporation. *Arabidopsis* wild-type plants were transformed by floral-dip (Clough and Bent, 1998).

KNAT1::GUS transgenic plants were kindly provided by S. Hake; the *KNAT2::GUS* line was obtained from J. Dockx and J. Traas, and the *CycB1;1::CDBGUS* line was a gift from J. Celenza. In this construct, the cyclin-destruction-box (CDB) of *CycB1;1* is fused in frame to GUS, causing the protein to be degraded at the end of mitosis, allowing visualization of cell-cycle progression by staining for GUS activity.

In situ hybridization

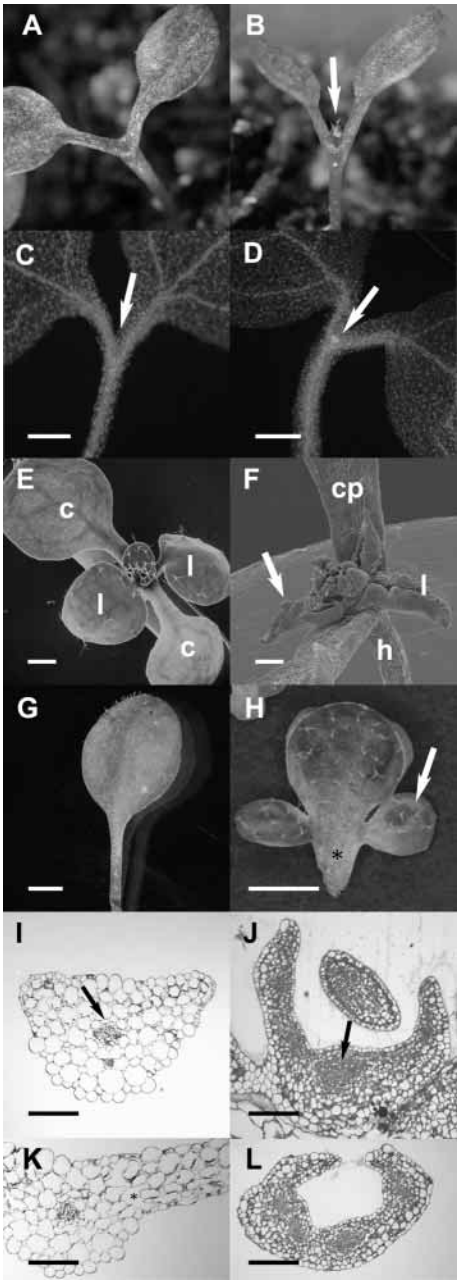
In situ hybridization for *WUS* and *CLV3* was performed as described by Mayer et al. (Mayer et al., 1998) and Schoof et al. (Schoof et al., 2000), respectively.

For the *KNAT1* riboprobe, the *KNAT1* cDNA was amplified from reverse transcribed poly(A)$^+$ RNA of Landsberg *erecta* seedlings using primers KNAT1-FOR (5′-TCT CTC GAG TCT TTA CTC ATC TGG G-3′) and KNAT1-REV (5′-AAA GGA TCC GTT GTA ACA AGA AAG C-3′). After digestion with *Xho*I and *Bam*HI, the cDNA was inserted into pBluescript II KS$^-$. The C-terminal part, containing the homeobox, was removed by digestion with *Xba*I and religation to yield ML343. For the antisense probe, ML343 was linearized with *Xho*I and transcribed with T7 RNA polymerase (Promega; Madison,

USA) using a digoxigenin-labelling kit (Roche Diagnostics; Mannheim, Germany); for the sense probe, ML343 was linearized with *Xba*I and transcribed with T3 RNA polymerase (Promega; Madison, USA).

For the *KNAT2* antisense riboprobe, plasmid pCKI-30 (kindly provided by J. Traas) which contains the full-length *KNAT2* cDNA was linearized with *Xho*I and transcribed with T7 RNA polymerase; for the sense probe, pCKI-30 was linearized with *Hin*dIII and transcribed using SP6 RNA polymerase (Promega; Madison, USA).

For all comparisons of wild-type and mutant or transgenic seedlings, sections from plants of the two genotypes under study were hybridized on the same slides, and only those slides were included in the analysis that showed clear expression in the wild-type samples. Where expression is reported, this was observed in several serial sections. The numbers given for *CLV1::WUS*-expressing *stm5* mutants refer only to those seedlings that contained an adventitious meristem.



We showed that no significant cross hybridization could occur between the *KNAT2* antisense riboprobe and *KNAT1* mRNA by a filter hybridization experiment that mimicked the conditions of in situ hybridization (data not shown).

RESULTS

Ectopic expression of *STM* in leaf primordia suppresses cell differentiation

Based on its expression pattern and loss-of-function phenotype, *STM* appears to maintain cells in an undifferentiated state, before they are incorporated into leaf primordia. To test whether *STM* was sufficient to suppress differentiation, we expressed *STM* ectopically in leaf primordia, using the *pOpL* two-component system (Moore et al., 1998; see Materials and Methods). The functionality of the *STM* transgene was confirmed by complementation of the meristem defect in *stm5* homozygous mutants (Fig. 1A-D).

We expressed *STM* under the control of the *AINTEGUMENTA* (*ANT*) promoter, which shows a complementary expression pattern to that of *STM*, i.e. it is active in primordia of cotyledons and leaves (Elliott et al.,

Fig. 1. Ectopic *STM* expression suppresses cell differentiation. (A) Light micrograph of a non-transgenic *stm5* mutant seedling 8 days after germination. Cotyledon petioles are fused and no leaves have been formed. (B) Light micrograph of an *stm5* mutant seedling expressing *CLV1::STM* 8 days after germination. The first pair of leaves formed by the SAM is visible (arrow). The bases of the cotyledon petioles are fused as in the seedling shown in A. We used the *CLV1* promoter, which is active in the centre of the embryonic shoot meristem primordium from heart-stage onward, and whose initial activation does not require *STM* function (Long and Barton, 1998), since no *STM* promoter has been described that mimics the endogenous mRNA expression pattern. (C,D) Micrographs of DAPI-stained seedlings. (C) *stm5* mutant seedling 5 days after germination. No meristematic cells are visible inside the fused cotyledon petioles (arrow). (D) *CLV1::STM*-expressing *stm5* mutant seedling 5 days after germination. A meristematic region is evident from the bright signal from cytoplasmically dense cells inside the fused petioles (arrow). (E,F) Scanning electron micrographs. (E) Wild-type seedling 10 days after germination. c, cotyledon; l, leaf. (F) *ANT::STM*-expressing seedling with a strong phenotype 21 days after germination. The petioles of the cotyledons (cp) are broader than in wild type (compare with E). Leaves (l, arrow) are not expanded and are rolled up at their margins. h, hypocotyl. (G) Light micrograph of a mature second rosette leaf of a wild-type plant. (H) Light micrograph of a mature second rosette leaf of an *ANT::STM*-expressing plant with a weak phenotype. The petiole (asterisk) is broader than wild type and lateral outgrowths have developed into leaf-like structures (arrow). (I-L) Cross-sections of plastic-embedded leaf material from seedling 12 days after germination, stained with Toluidine Blue. (I) Petiole of the first rosette leaf of a wild-type plant. A vascular bundle (arrow) with differentiated cells lacking cytoplasm is surrounded by large, vacuolated cells. (J) Basal part of the first rosette leaf of an *ANT::STM*-expressing seedling. The cells in place of the vascular strand (arrow) are cytoplasmically dense and the cells throughout the petiole are less expanded than in G. (K) The lamina of the first rosette leaf of a wild-type plant. Note the high degree of vacuolation and the large intercellular spaces (asterisk). (L) The lamina of the first rosette leaf of an *ANT::STM*-expressing seedling. Cells throughout the leaf are smaller than in I and contain more cytoplasm, indicating that differentiation is suppressed. Scale bars are 500 μm in C-H, 100 μm in I-L.

1996; Klucher et al., 1996). Staining for the activity of a linked *ANT::GUS* reporter gene confirmed expression of the transgenes in cotyledons and leaf primordia (Fig. 2D). *ANT::STM*-expressing plants showed cotyledon and leaf phenotypes of varying severity, depending on the individual *STM* target line used. The petioles of the cotyledons and of leaves were up to approximately threefold wider than in non-transgenic plants (Fig. 1E-H). Leaves were smaller than in wild type, and in the most extreme cases, were reduced to small finger-like structures (Fig. 1F, arrow). Their dorsoventrality was maintained, however, as judged from the development of trichomes only on the adaxial side of early vegetative leaves and their anisotropic growth, causing the leaves to bend over the SAM as they do in wild type. Furthermore, leaves of the transgenic plants developed lateral outgrowths from the leaf blade or petiole which was never observed in wild type (Fig. 1G,H).

Histological analysis showed that differentiation of leaf cells was suppressed in *ANT::STM*-expressing leaves compared to wild type. In the most severe cases we did not observe a vascular bundle in the finger-like structures at a time when wild-type petioles contained a well differentiated vascular strand (Fig. 1I,J). In addition, the cells throughout the leaf were small and cytoplasmically dense, resembling meristematic cells in contrast to the large, vacuolated differentiated cells of wild-type leaves (Fig. 1K,L).

Thus, *STM* is able to suppress cell differentiation in developing leaves and instead maintains the potential to form additional lateral outgrowths. These results support the reported phenotype of *35S::STM*-expressing plants which have a stunted appearance with a disorganized shoot and leaf-like bulges that do not develop into mature leaves (Williams, 1998). However, the effects of ectopic *STM* expression in leaf primordia are relatively subtle compared to those of *ANT::WUS* expression, which entirely abolishes organ formation (Schoof et al., 2000).

STM induces the expression of *KNAT* genes and *CycB1;1*, but not stem cell identity

In order to molecularly characterize the effects of ectopic *STM* activity, we analyzed the expression of several candidate downstream genes in *ANT::STM*-expressing plants.

The formation of lateral outgrowths by *ANT::STM*-expressing leaves suggested that *STM* was able to promote cell proliferation when expressed in leaves. To test this, we examined the expression of the mitotic cyclin *CycB1;1* using a promoter-GUS fusion. *CycB1;1* is expressed shortly before and during mitosis and overexpression analysis suggests it may be a limiting factor for cell division, making it a suitable marker for mitosis and cell proliferation (Doerner et al., 1996; Mironov et al., 1999).

In 10-day old wild-type plants carrying a *CycB1;1::CDBGUS* reporter gene, GUS staining was restricted to the shoot meristem and young leaf primordia, but was absent from the expanding first pair of leaves (Fig. 2A). In *ANT::STM*; *CycB1;1::CDBGUS* seedlings the first pair of leaves became visible at the same time as in wild type, yet still showed GUS staining at 10 days after germination, in addition to staining in the shoot meristem with younger leaf primordia (Fig. 2B). In older *ANT::STM*-expressing leaves, ectopic GUS staining was most pronounced in the lateral outgrowths (Fig. 2C), consistent with our observation that these arose after the main leaf had already reached a certain size (data not shown). This result suggests that ectopic *STM* expression in cells of leaf primordia promotes their proliferation.

Since the leaf phenotype of *ANT::STM*-expressing plants was similar to the effects observed when either *KNAT1* or *KNAT2*, two homeobox genes with potential regulatory functions in the shoot meristem, were overexpressed (Lincoln et al., 1994; Dockx et al., 1995; Chuck et al., 1996; Pautot et al., 2001), we addressed whether *KNAT1* or *KNAT2* was acting in one regulatory pathway with *STM*. Staining for a *KNAT1::GUS* reporter revealed ectopic expression in the vasculature of the cotyledons and in strongly affected leaves of *ANT::STM*-expressing seedlings (Fig. 2E,F), suggesting that ectopic *KNAT1* expression can be activated by ectopic *STM* activity. Similarly, the *KNAT2::GUS* reporter showed ectopic staining in the vasculature of the cotyledons and in leaves of *ANT::STM*-expressing seedlings (Fig. 2G,H).

In contrast to *KNAT1* and *KNAT2*, the stem cell marker *CLV3* was not expressed ectopically in *ANT::STM*-expressing seedlings: using in situ hybridization *CLV3* RNA was only detected in the apical stem cells of the shoot meristem, which was indistinguishable from wild type (Fig. 2I,J).

Thus, ectopic expression of *STM* in leaf primordia induces expression of two meristem genes and promotes cell proliferation, yet *STM* is not able to induce ectopic stem cell identity, based on expression of the presumed stem cell marker *CLV3*.

WUS induces ectopic stem cell identity, but not the expression of *KNAT* genes

To molecularly delimit the functions of *STM* and *WUS*, we aimed to test whether expression of the above marker genes could be induced by ectopic *WUS* activity in leaves, complementary to the analysis for *STM*. Since constitutive *ANT::WUS* expression completely suppresses leaf formation (Schoof et al., 2000), we used an inducible construct to produce leaves with ectopic *WUS* activity: we expressed a posttranslationally inducible form of WUS fused to the C terminus of the rat glucocorticoid receptor (GR) (see Sablowski and Meyerowitz, 1998) from the constitutive *Cauliflower Mosaic Virus 35S* promoter. Nuclear translocation of this fusion protein, and thus its potential to activate transcription, can be induced by addition of a GR-ligand such as dexamethasone. When germinated on dexamethasone-containing medium, *35S::WUS-GR* seedlings are indistinguishable from *35S::WUS* seedlings with suppressed differentiation, whereas in the absence of dexamethasone the transgene has no effect on plant development as has dexamethasone treatment of *35S::GR*-expressing seedlings, indicating that the fusion protein behaves as predicted and that the effects observed are due to ectopic *WUS* activity (Fig. 3A; data not shown). We introduced GUS reporter genes for *CLV3*, *KNAT1*, *KNAT2* and *CycB1;1* into *35S::WUS-GR* seedlings and analyzed GUS activity in 14-day old F_1 seedlings that had been treated for 2 days with dexamethasone or with a control solution.

Dexamethasone induction of *35S::WUS-GR* seedlings resulted in strong ectopic activation of the *CLV3::NLSGUS* reporter gene in cotyledons, leaves and hypocotyl, mainly associated with the vasculature (Fig. 3D), whereas uninduced siblings showed GUS staining exclusively in the apical stem

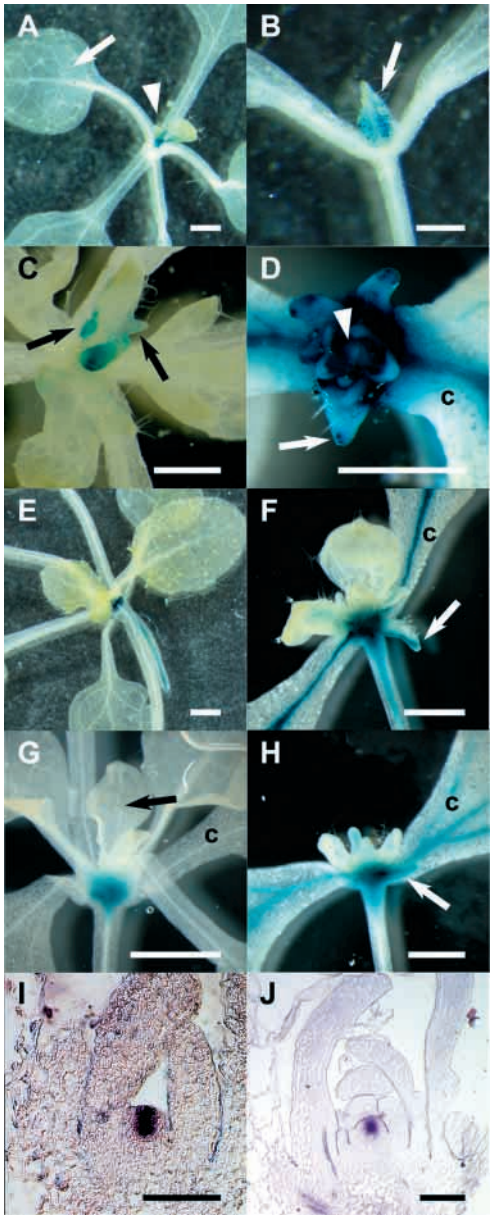


Fig. 2. Marker gene expression in *ANT::STM* plants. (A-H) Light micrographs of GUS-stained, cleared seedlings. (A) *CycB1;1::CDBGUS* expression in wild type. Staining is restricted to the SAM region and young leaves (arrowhead), but is absent from the expanded first pair of rosette leaves (arrow). (B) *CycB1;1::CDBGUS*; *ANT::STM*-expressing seedling of the same age as the one in A. Staining is seen throughout the first pair of rosette leaves (arrow). (C) *CycB1;1::CDBGUS*; *ANT::STM* seedling with intermediate phenotype. Ectopic GUS staining is observed in the lateral outgrowths of the leaves (arrows). (D) *ANT::STM*; *ANT::GUS*-expressing seedling. The transgenes are strongly expressed in the vasculature of the cotyledons (c), leaf primordia (arrowhead) and in older leaves with stronger staining at the tips (arrow), as well as in their lateral outgrowths (not visible). (E) *KNAT1::GUS* expression in wild type. Staining is restricted to the SAM region and hypocotyl, yet is absent from leaves. (F) *KNAT1::GUS*; *ANT::STM*-expressing seedling. Ectopic GUS staining is seen in the vasculature of the cotyledons (c) and in strongly affected leaves (arrow). (G) *KNAT2::GUS* expression in wild type. Staining is restricted to the SAM region and is absent from cotyledons (c) and leaves (arrow). (H) *KNAT2::GUS*; *ANT::STM*-expressing seedling. Ectopic GUS staining is observed in the vasculature of the cotyledons (c) and in leaves (arrow). (I,J) In situ hybridization with a *CLV3* antisense riboprobe. In both wild-type (I) and *ANT::STM*-expressing (J) seedlings, *CLV3* mRNA is exclusively detected in the stem cells in the three outermost layers of the SAM. Scale bars are 1 mm in A-H, 100 μm in I,J.

cells of the SAM (Fig. 3B,C). Thus, WUS appears to be sufficient to induce aspects of stem cell identity de novo in differentiated tissue. Preferential induction close to the vasculature could either be due to predominant expression of the *35S* promoter there (e.g. Chuck et al., 1996) or to a higher sensitivity of cells near the vasculature to *WUS* activity.

By contrast, expression of neither the *KNAT1::GUS* nor the *KNAT2::GUS* reporter genes could be induced ectopically by *35S::WUS-GR* (Fig. 3E-H), indicating that WUS-GR is not able to activate expression from the *KNAT1* and *KNAT2* promoters.

In dexamethasone-induced *35S::WUS-GR* seedlings carrying the *CycB1;1::CDBGUS* reporter, we occasionally detected ectopic staining in the first pair of leaves (5 out of 15 seedlings analyzed) which was never detected in uninduced seedlings of the same genotype (Fig. 3I; n=15). The ectopically stained cells were always associated with the vasculature.

In summary, *WUS* is sufficient to induce ectopic stem cell identity – as judged by *CLV3* expression – and occasional ectopic cell divisions, but is not able to ectopically activate expression of *KNAT1* or *KNAT2*. Taken together, these results suggest that ectopic expression of *STM* or *WUS* in leaf primordia activates distinct sets of downstream target genes.

Ectopic *STM* and *WUS* functions act independently of each other

To study how the activities of *WUS* and *STM* are interconnected, we analyzed whether the activity of one gene is required for the effects of ectopic expression of the other gene in leaf primordia.

To analyze whether *STM* might be a downstream target of *WUS*, we tested whether ectopic *WUS* expression could still repress organ formation in an *stm5* mutant background. While *ANT::WUS* expression in a wild-type background produced an enlarged SAM in place of leaves immediately after germination, no effect of the transgene was observed in *stm5* mutant seedlings up to 7 days after germination. However, thereafter *ANT::WUS*-expressing *stm5* mutant seedlings formed a mass of small meristematic cells inside the fused cotyledon petioles that was indistinguishable from that observed in *ANT::WUS*-expressing wild-type seedlings (Fig. 4A,B,D,E). The relatively late effect in *stm5* mutants compared to wild type appears to be due to the fact that the transgene is not expressed in *stm5* mutants up to 7 days after germination, as judged from staining for the activity of a linked *ANT::GUS* reporter gene (data not shown), and expression only becomes detectable thereafter (Fig. 4C). By contrast, non-transgenic *stm5* seedlings never produced a similar enlarged SAM, but formed adventitious leaves between the fused cotyledon petioles (Fig. 4F) (Endrizzi et al., 1996).

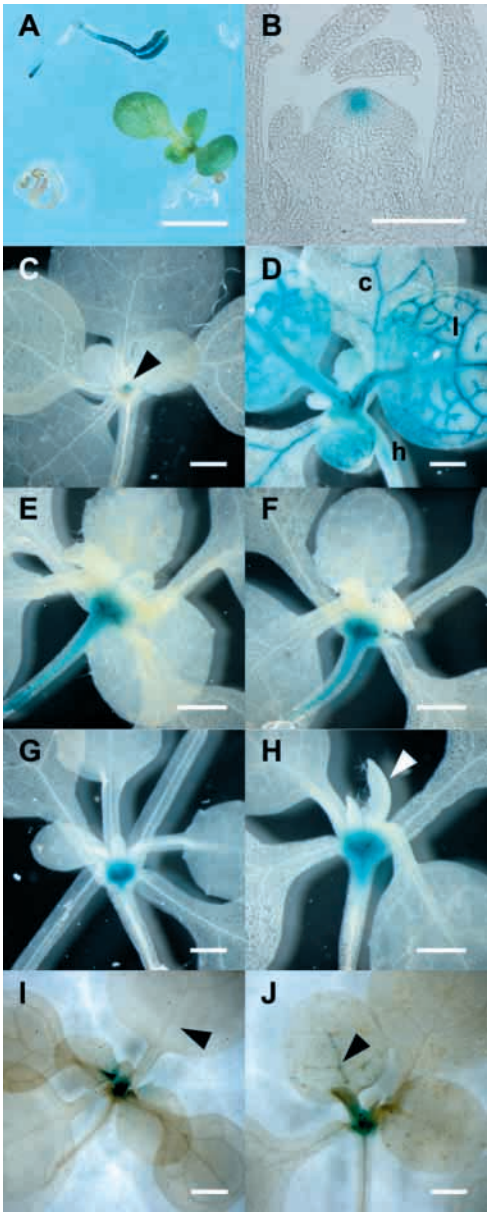


Fig. 3. Marker gene expression in *35S::WUS-GR*-expressing plants. (A) *35S::WUS-GR*-expressing seedlings (lower left) show the same phenotype with inhibition of cotyledon expansion, root growth and greening as *35S::WUS; 35S::GUS*-expressing seedlings (upper left) when germinated on dexamethasone containing medium, but not on control medium (lower right). (B) Longitudinal section through a GUS-stained *CLV3::NLSGUS*-expressing plant. Staining is restricted to the stem cells of the SAM, mirroring the *CLV3* mRNA expression pattern (compare with Fig. 2I). (C-J) Light micrographs of GUS-stained and cleared seedlings. Seedlings in C,E,G,I were treated with mock solution for 2 days, while seedlings in D,F,H,J were induced with 5 μM dexamethasone for the same time. (C,D) After dexamethasone treatment of *35S::WUS-GR; CLV3::NLSGUS* seedlings (D), strong ectopic GUS expression is observed in cotyledons (c), leaves (l) and hypocotyl (h), mainly associated with vascular strands, while expression is restricted to the stem cells of the SAM in uninduced seedlings (arrowhead, C). (E,F) No difference in the GUS staining pattern is observed between dexamethasone induced (F) and uninduced (E) *35S::WUS-GR; KNAT1::GUS*-expressing seedlings. (G,H) No difference in the GUS staining pattern is observed between dexamethasone induced (H) and uninduced (G) *35S::WUS-GR; KNAT2::GUS*-expressing seedlings, even though the first morphological effects of ectopic *WUS* activity on young leaves – reduced expansion of the lamina and upright position – are already visible (arrowhead). (I,J) Occasional ectopically staining cells are visible along the vasculature of the first pair of rosette leaves in dexamethasone-treated *35S::WUS-GR; CycB1;1::CDBGUS*-expressing seedlings (arrowhead in J), which were never observed in mock-treated seedlings of the same genotype (arrowhead in I). Scale bars are 5 mm in A, 100 μm in B and 500 μm in C-J.

These observations indicate that suppression of leaf formation by ectopic *WUS* activity does not require *STM* and suggest that *STM* is not an essential downstream target of *WUS*.

In the converse experiment, we tested whether *WUS* might be a downstream target of *STM*. To do so, we analyzed whether *WUS* is required for the effects of ectopic *STM* activity by expressing *ANT::STM* in *wus1* mutants. *ANT::STM*-expressing *wus1* mutant plants exhibited a leaf phenotype that was indistinguishable from the effect of *ANT::STM* expression in a wild-type background (Fig. 4G-J), suggesting that *WUS* is not an essential downstream target of ectopically expressed *STM*. This finding was confirmed by analyzing the expression of a *WUS::NLSGUS* reporter gene in plants with ectopic *STM* activity. *ANT::STM*; *WUS::NLSGUS* plants showed GUS staining in a small central cell group in the shoot meristem, in a pattern that was indistinguishable from that in wild type (Fig. 4K-M), but they did not show ectopic GUS staining in the cells that expressed *ANT::STM* (compare with Fig. 2D). Thus, ectopic *STM* activity does not appear to induce expression from the *WUS* promoter.

Taken together these results indicate that ectopic *WUS* and *STM* activities function independently of each other.

Coexpression of *WUS* and *STM* produces synergistic effects

Their loss-of-function phenotypes indicate that both *WUS* and *STM* activities are essential for SAM function (Barton and Poethig, 1993; Endrizzi et al., 1996; Laux et al., 1996), yet our above experiments demonstrate that their functions are genetically independent. One interpretation of these findings is that the developmental pathways regulated by them ultimately converge on some downstream process. We thus asked whether ectopic *WUS* and *STM* functions act synergistically on some shared process and coexpressed both in developing cotyledons and leaf primordia. Except for a widening of the cotyledon petioles in *ANT::STM*-expressing plants, ectopic expression of either gene alone under the control of the *ANT* promoter leaves the cotyledons largely unaffected, although staining for the activity of a linked *ANT::GUS* reporter gene showed the transgenes to be expressed throughout embryonic cotyledon primordia (data not shown). By contrast, *ANT::STM*; *ANT::WUS* coexpressing seedlings, in which the presence of both transgenes was confirmed by PCR (data not shown), showed a novel phenotype which was clearly distinct from the effects of ectopic expression of either gene alone (Fig. 5A-D): they completely lacked cotyledon petioles and had fields of small cells extending from the apex into the lamina of the cotyledons. These cells strongly resembled the dense meristematic cells in the apex of *ANT::WUS* plants as judged

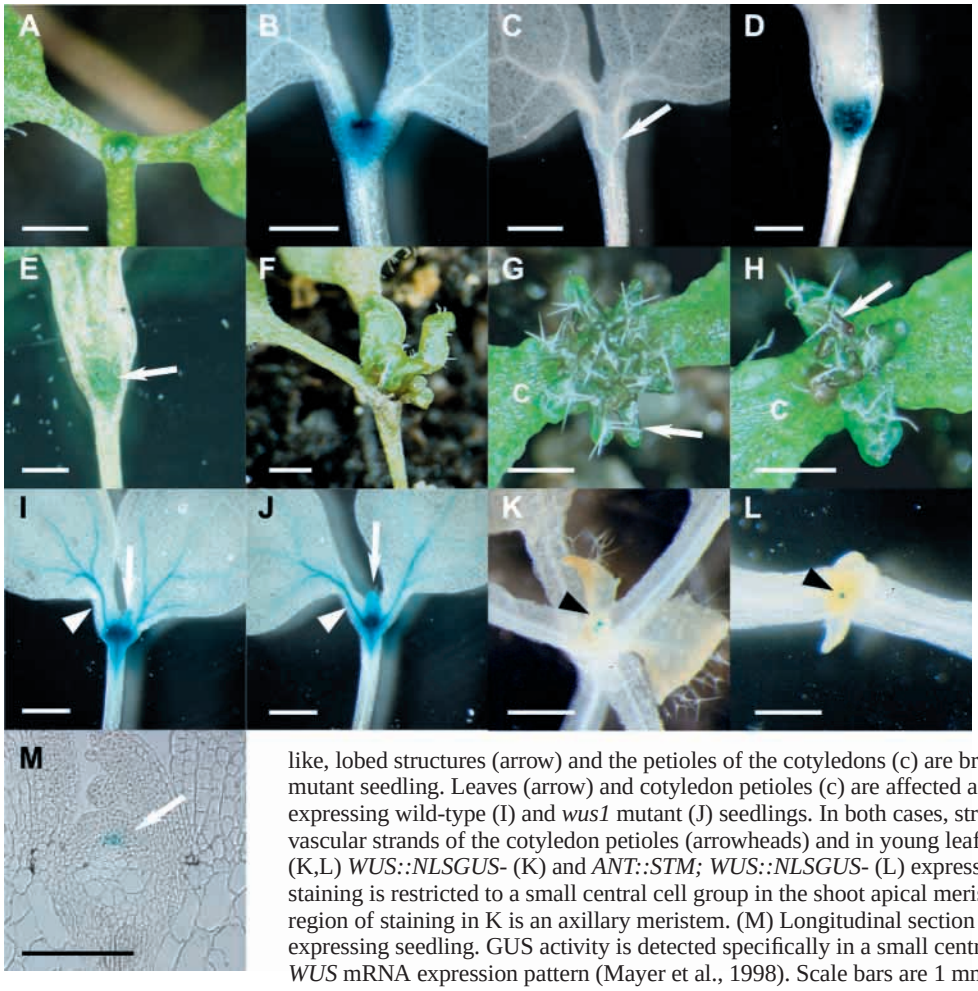


Fig. 4. Independent functions of *WUS* and *STM*. Light micrographs of live seedlings (A,E-H) and GUS-stained, cleared seedlings (B-D,I-L). (A,B) *ANT::WUS; ANT::GUS*-expressing wild-type seedlings 12 days (A) and 10 days (B) after germination. An enlarged SAM has developed in place of leaves (A) which strongly expresses the transgenes (B). (C-E) *ANT::WUS; ANT::GUS*-expressing *stm5* mutant seedlings 10 days (C) and 18 days (D,E) after germination. Transgene expression has only been initiated in a few cells (arrow) inside the fused cotyledon petioles in the seedling in C from which a mass of small meristematic cells develops subsequently (D,E arrow). In E, the fused cotyledon petioles have been cut open for clarity. (F) Non-transgenic *stm5* mutant seedling 18 days after germination. Several leaves have been formed and have ruptured the fused wall of the cotyledon petioles. (G) *ANT::STM*-expressing wild-type seedling. Leaves are reduced to finger-like, lobed structures (arrow) and the petioles of the cotyledons (c) are broadened. (H) *ANT::STM*-expressing *wus1* mutant seedling. Leaves (arrow) and cotyledon petioles (c) are affected as in G. (I,J) *ANT::STM; ANT::GUS*-expressing wild-type (I) and *wus1* mutant (J) seedlings. In both cases, strong GUS staining is visible in the vascular strands of the cotyledon petioles (arrowheads) and in young leaf primordia (arrows) at the shoot meristem. (K,L) *WUS::NLSGUS*- (K) and *ANT::STM; WUS::NLSGUS*- (L) expressing seedlings. In both cases, GUS staining is restricted to a small central cell group in the shoot apical meristem (arrowheads). The additional smaller region of staining in K is an axillary meristem. (M) Longitudinal section through a GUS-stained *WUS::NLSGUS*-expressing seedling. GUS activity is detected specifically in a small central cell group of the SAM, reflecting the *WUS* mRNA expression pattern (Mayer et al., 1998). Scale bars are 1 mm in A-L, 100 μm in M.

from their appearance under the scanning electron microscope and in histological sections (Fig. 5E,F) and showed ectopic *CLV3* expression (Fig. 5G,H).

Thus, simultaneous ectopic expression of *WUS* and *STM* produced a non-additive phenotype in that meristematic cells were induced in cotyledons which was not the case in plants expressing either gene alone. This suggests that in differentiated tissue both genes synergistically confer meristem cell identity.

Increased WUS activity can induce self-maintaining meristems in *stm* mutants, but not vice versa

We next asked whether similar to the results of the above ectopic coexpression experiment, the pathways activated by *WUS* and *STM* also converge in the regulation of SAM function. We therefore tested whether an increase of one gene's activity in the SAM could compensate for the effects of a mutation in the other gene. For this purpose we expressed *WUS* or *STM* under the control of the *CLV1* promoter in the respective other mutant.

First, we expressed *CLV1::STM* in *wus1* mutants. Since the expression patterns of transgenic and endogenous *STM* roughly overlap, this would be expected to increase the *STM* expression level throughout the apex. Expression of the *CLV1* activator line in *wus1* mutant embryos was evident from its ability to rescue the mutant phenotype when combined with a *WUS* target line (Groß-Hardt et al., 2002) and was confirmed by staining for the activity of a linked *CLV1::GUS* reporter (Fig. 6A,B). The phenotype of *CLV1::STM*; *wus1* plants was indistinguishable from that of non-transgenic *wus1* mutants: shoot development in seedlings of both genotypes arrested after the formation of two to three leaves (Fig. 6E,F). 10 days after germination, we observed strong transgene expression in what are most likely adventitious meristems (Fig. 6D; see Laux et al., 1996). Despite this, no self-maintaining meristems could be formed in a *wus1* mutant background, and *CLV1::STM*-expressing *wus1* mutant plants showed the same 'stop and go' mode of development as non-transgenic *wus1* mutants (Laux et al., 1996; data not shown). The leaves, however, showed the same wrinkled phenotype that was also observed in *CLV1::STM*-expressing wild-type plants and which appears to be due to weak expression of the transgene in leaves as judged by prolonged staining for the activity of the linked *CLV1::GUS* reporter gene (data not shown), confirming that in principle *STM* was active in *wus* mutants.

Thus, increasing *STM* expression in the shoot apex is not able to compensate for the shoot meristem defects of *wus* mutants.

Secondly, in the converse experiment, we analyzed the effects of *CLV1::WUS* expression in *stm5* mutants.

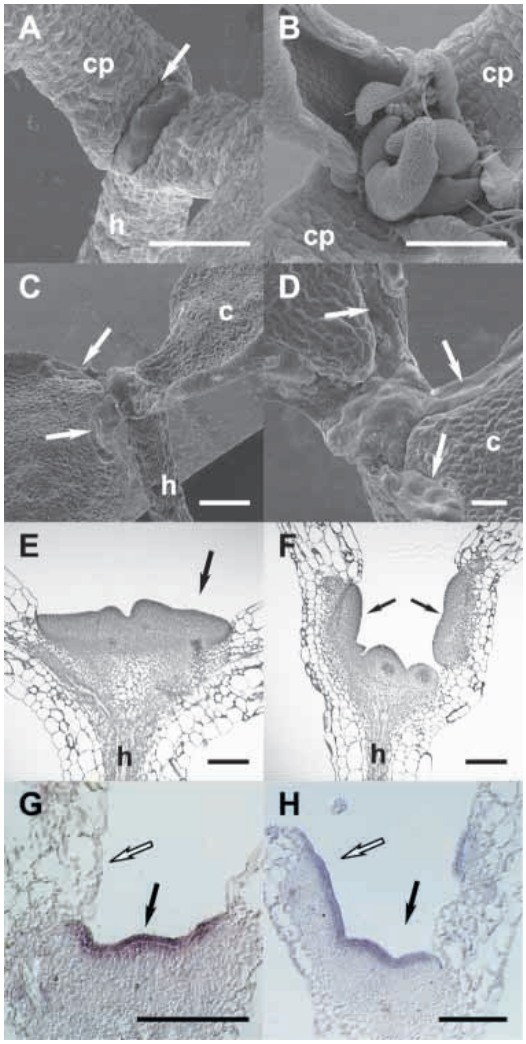


Fig. 5. Synergistic effects of coexpression of *WUS* and *STM*. (A-D) Scanning electron micrographs of seedlings 14 days after germination. (A) *ANT::WUS*-expressing seedling. An enlarged SAM has formed in place of leaves. The cotyledon petioles (cp) are unaffected and separated from the meristematic cells by a sharp boundary (arrow). h, hypocotyl. (B) *ANT::STM*-expressing seedling. Cotyledon petioles (cp) are broadened, but do not show meristem-like cells. (C,D) *ANT::WUS*; *ANT::STM* coexpressing seedlings. No cotyledon petioles have been formed and fields of small, meristematic cells (arrows) extend into the lamina of cotyledons (c). (E,F) Histological sections of plastic embedded material stained with Toluidine Blue. (E) Longitudinal section through the apex of an *ANT::WUS*-expressing seedling 8 days after germination. Note the massively overproliferated shoot meristem with small, cytoplasmically dense cells (arrow). (F) Longitudinal section through the apex of an *ANT::WUS; ANT::STM*-expressing seedling 8 days after germination. The regions of small meristematic cells are expanded into the cotyledons (arrows). The spots of darker stained cells are an artefact of processing. (G,H) In situ hybridization using a *CLV3* antisense riboprobe. (G) In *ANT::WUS*-expressing seedlings, *CLV3* mRNA is detected in the outermost cell layers of the enlarged shoot meristem (black arrow), but not in cells of the cotyledon petioles (white arrow). (H) By contrast, *ANT::WUS; ANT::STM* coexpressing seedlings show *CLV3* expression both in the enlarged shoot meristem (black arrow) and in the meristematic regions on the cotyledons (white arrow). Scale bars are 500 μm in A-C, 200 μm in D and 100 μm in E-H.

CLV1::WUS-expressing wild-type seedlings produce an enlarged meristem immediately after germination due to the enlarged *WUS* expression domain throughout the SAM (Fig. 6G-J) (Schoof et al., 2000). By contrast, 7 days after germination *stm5* mutant seedlings carrying the *CLV1::WUS* transgene lacked a recognizable shoot meristem and were indistinguishable from non-transgenic *stm5* mutant seedlings. That the *CLV1* activator was expressed in *stm5* mutants was demonstrated by its ability to rescue the mutant defect when combined with an *STM* target line (see above, Fig. 1A-D); however, even in combination with our strongest *WUS* target line, the resulting embryonic expression was only very weak as judged from staining for the activity of a linked *CLV1::GUS* reporter gene (data not shown). While such weak expression appears to be sufficient to rescue the *wus1* mutant defect (Groß-Hardt et al., 2002), it is apparently unable to overcome the lack of *STM* activity during embryogenesis. After day 7, *CLV1::WUS; CLV1::GUS*-expressing *stm5* mutant seedlings showed small clusters of GUS staining cells inside the fused cotyledon petioles and by day 12 after germination, 26 out of 40 seedlings had developed a conspicuous adventitious structure resembling a meristem surrounded by small leaf primordia (Fig. 6J,K,M,N). No similar structures were observed in any of 25 non-transgenic *stm5* mutant seedlings 12 days after germination (Fig. 6L).

To analyze whether the induced structures were meristems, we examined them for expression of the meristem marker genes *CLV3*, *KNAT1* and *KNAT2* using in situ hybridization (see above). Both *CLV1::WUS*-expressing wild-type and *stm5* mutant seedlings 10 or 14 days after germination showed strong *CLV3* expression in the outermost cell layers across their enlarged meristems and the induced structures, respectively (Fig. 7A,B). By contrast, we could not detect *CLV3* expression in any of 25 non-transgenic *stm5* mutant seedlings 10 days after germination (data not shown). While we could not detect *KNAT1* expression in the induced structures of 10-day old *CLV1::WUS*-expressing *stm5* mutant seedlings (Fig. 7E,F; n=6; see Materials and Methods), by 14 days after germination the induced structures in *CLV1::WUS*-expressing *stm5* mutant seedlings showed clear *KNAT1* expression in small patches on the flanks and at their base close to the vasculature (Fig. 7G), similar to the pattern observed in meristems of *CLV1::WUS*-expressing and non-transgenic wild-type seedlings (Fig. 7C,D) (Chuck et al., 1996). Hybridization with a *KNAT2* antisense riboprobe produced a similar result: While no *KNAT2* expression could be detected in the induced structures of 10-day old *CLV1::WUS*-expressing *stm5* mutant seedlings (Fig. 7I; n=11; see Materials and Methods), consistent weak staining was found at the flanks and base of the induced structures by 14 days after germination (Fig. 7J). *CLV1::WUS*-expressing wild-type seedlings showed virtually the same expression pattern for *KNAT2* as found for *KNAT1*, i.e. at the periphery of the enlarged SAM and at the base of young leaf primordia (Fig. 7H).

Thus, the structures induced by *CLV1::WUS* expression in *stm5* mutant seedlings showed expression of the three marker genes tested, suggesting that they represent meristems. However, these meristems never reached a size comparable to those formed by *CLV1::WUS*-expressing wild-type plants, as judged from staining for the activity of the linked *CLV1::GUS* reporter gene (Fig. 6O,P). Since the size of the cells in

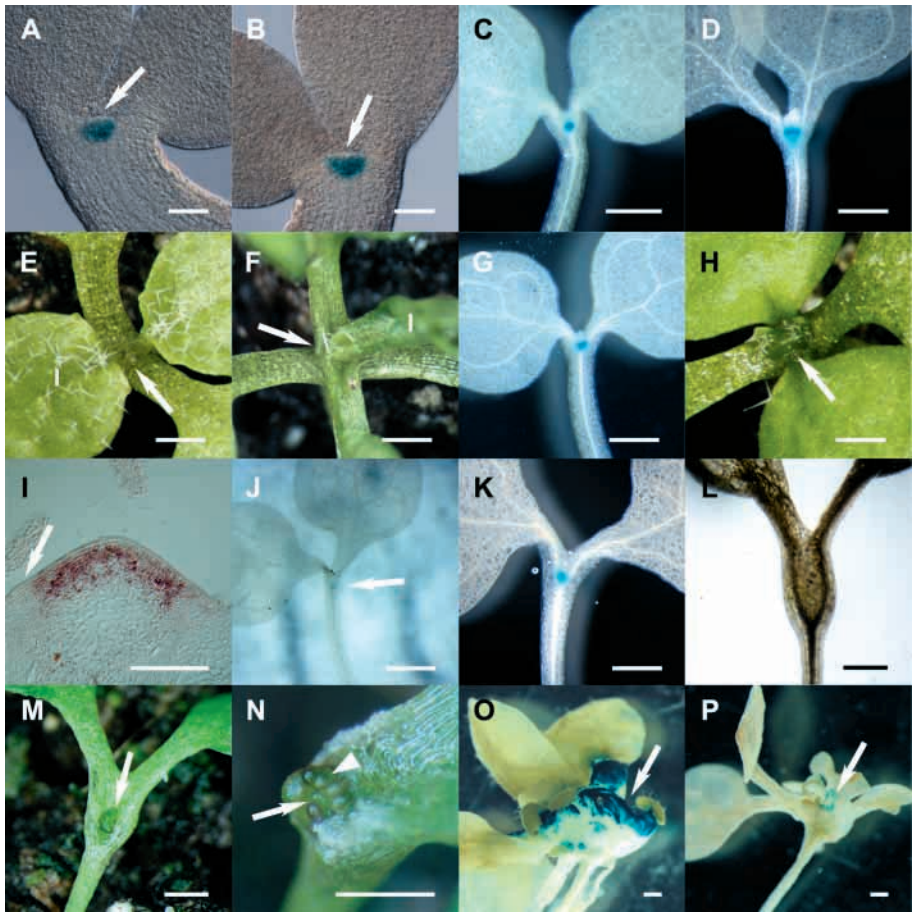


Fig. 6. The loss-of-function phenotypes of *wus* and *stm* mutants cannot be rescued by transgenic expression of the respective other gene. Light micrographs of GUS stained cleared embryos or seedlings (A-D,G,J-L,O,P) and of live seedlings (E,F,H,M,N). (A,B) The *CLV1::STM* transgene is strongly expressed in the SAM primordia (arrows) of wild-type (A) and *wus1* mutant (B) embryos as indicated by staining for the activity of a linked *CLV1::GUS* reporter. Note the flat apex of the *wus1* mutant embryo compared to the convex meristem in the wild type, suggesting that the former has terminated. (C) *CLV1::STM*; *CLV1::GUS* expression is detected in the SAM of 7-day old wild-type seedlings by GUS staining. (D) *CLV1::STM*; *CLV1::GUS*-expressing *wus1* mutant seedlings 10 days after germination show strong GUS staining at the shoot apex. (E,F) The meristems in *CLV1::STM*; *CLV1::GUS*-expressing *wus1* mutant seedlings (F) terminate indistinguishably from the meristems in non-transgenic *wus1* mutants (E) (arrows). (G,H) In *CLV1::WUS; CLV1::GUS*-expressing wild-type seedlings 7 days after germination strong GUS staining is detected at the apex (G) which causes the development of an enlarged meristem (H, arrow). (I) In situ hybridization using a *WUS* antisense riboprobe on *CLV1::WUS*-expressing seedlings confirms transgene expression specifically in the centre of the enlarged shoot meristem, yet not on the flanks (arrow) where organs are initiated. (J,K) In *CLV1::WUS; CLV1::GUS*-expressing *stm5* mutant seedlings the first GUS-staining cells are detected 7 days after germination inside the fused cotyledon petioles (arrow in J) which give rise to adventitious meristems (K, compare with M,N). (L-N) While non-transgenic *stm5* mutants 12 days after germination show no sign of a SAM inside the fused cotyledon petioles (L), *CLV1::WUS*-expressing *stm5* mutant seedlings (M,N) of the same age contain a conspicuous meristematic structure (arrows) that is surrounded by small leaf primordia (arrowhead in N). (O) In *CLV1::WUS; CLV1::GUS*-expressing wild-type plants 25 days after germination, the meristem is massively enlarged (arrow). (P) *CLV1::WUS; CLV1::GUS*-expressing *stm5* mutant plants of the same age show only small meristematic regions that express the GUS reporter gene (arrow). In addition, leaves are small and sometimes fused as in non-transgenic *stm5* mutant plants. Scale bars are 50 μm in A,B, 1 mm in C-H,J-P, and 100 μm in I.

CLV1::WUS-expressing wild-type and *stm5* mutant meristems appeared to be roughly equal (compare Fig. 7A and 7B), the reduced growth of the meristem in *stm5* seedlings likely results from fewer cell divisions, rather than from reduced cell expansion. This suggests a critical requirement for *STM* in allowing amplification of meristem cells which cannot be compensated for by increased *WUS* activity.

In summary, *CLV1::STM* expression in *wus* mutants cannot compensate for the loss of *WUS* function. However, conversely expressing *CLV1::WUS* in *stm* mutants induces the formation of adventitious shoot meristems at a high frequency, although it cannot fully rescue the *stm* mutant defect. Thus, it appears that increasing *WUS* activity can at least partly compensate for the loss of *STM* function, suggesting a convergence of the pathways activated by *WUS* and *STM* in SAM regulation.

DISCUSSION

The *WUS* and *STM* homeobox genes are both essential for the same processes, formation and maintenance of a functional shoot meristem (Barton and Poethig, 1993; Endrizzi et al., 1996; Laux et al., 1996), yet it is unknown whether and how their functions are integrated in SAM regulation. To address this issue, we have analyzed their genetic interactions using a combination of gain- and loss-of-function experiments.

STM and *WUS* function in different pathways in shoot meristem regulation

Our results suggest that *WUS* and *STM* fulfil independent, yet complementary functions in SAM regulation, for the following reasons.

(1) When expressed ectopically in leaf primordia, the effects of *WUS* and *STM* are clearly distinct. *WUS* is sufficient to completely abolish organ formation, but has little, if any, stimulating effect on cell division, as evidenced both by its inability to efficiently induce expression of the mitotic marker gene *CyclinB1;1* and by the low proportion of cells in S-phase in the enlarged central zone of *CLV1::WUS*-expressing meristems (M. L. and T. L., unpublished). By contrast, ectopic

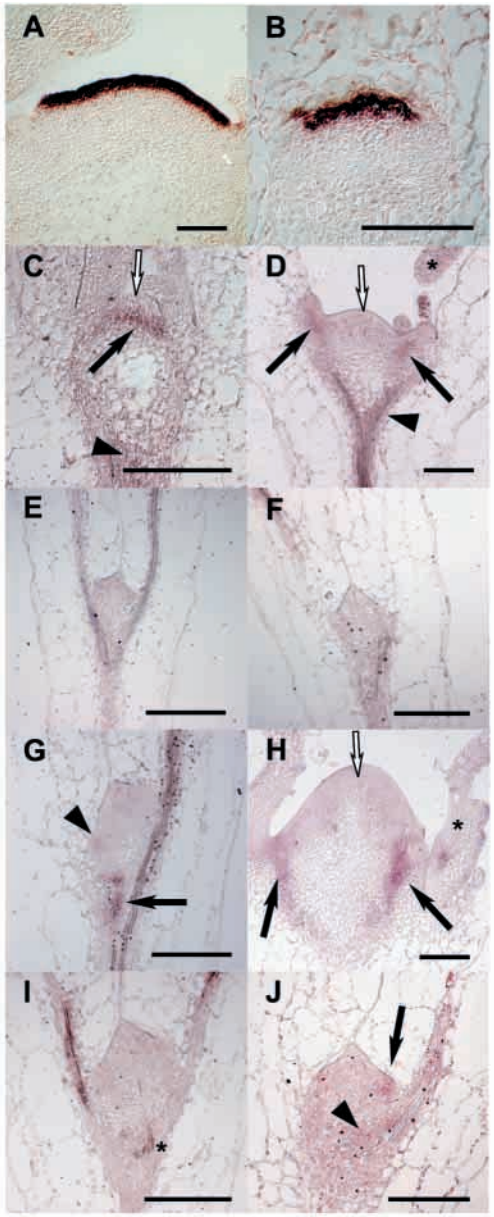


Fig. 7. Marker gene expression in *CLV1::WUS*-expressing wild-type and *stm5* mutant plants. Longitudinal sections hybridized in situ with *CLV3* (A,B), *KNAT1* (C,D,F,G), *KNAT2* (H-J) antisense and *KNAT1* sense (E) riboprobes. *CLV3* and *KNAT2* sense riboprobes did not produce any staining (not shown). (A) In *CLV1::WUS*-expressing wild-type plants 14 days after germination, cells in the three outermost layers of the meristem show strong *CLV3* expression. (B) *CLV1::WUS*-expressing *stm5* mutant plants 14 days after germination exhibit *CLV3* expression in a band at the top of the induced structure inside the fused cotyledon petioles. The same result was obtained when analyzing 10 day old seedlings (not shown). (C) In non-transgenic wild-type seedlings, *KNAT1* expression is detected at the base and periphery of the SAM and close to the base of young leaf primordia (black arrow), but is absent from the central zone of the SAM (white arrow). In addition, expression is detected in cells close to the vasculature (arrowhead). (D) In *CLV1::WUS*-expressing wild-type plants 10 days after germination, *KNAT1* expression is detected at the periphery of the enlarged meristem (black arrows) and adjacent to the vasculature (arrowhead). Although weak, this staining was consistent throughout serial sections. The central region of the meristem (white arrow) shows only weak background staining that is also found in leaves (asterisk) and in sections hybridized with a *KNAT1* sense probe (compare with E). (E) Hybridization with a *KNAT1* sense riboprobe produces only weak non-specific staining. (F,G) While no *KNAT1* mRNA can be detected in the induced structures of 10 day old *CLV1::WUS*-expressing *stm5* mutant seedlings (F), plants of the same genotype at 14 days after germination (G) exhibit clear *KNAT1* expression at the base (arrow) and in patches on the flanks of the induced structures (arrowhead). However, no expression is seen close to the vasculature in either seedling. (H) *KNAT2* mRNA can be detected in the periphery of the enlarged meristem of *CLV1::WUS*-expressing wild-type plants 14 days after germination (black arrows), while only weak and even staining is visible in the centre of the meristem (white arrow) and in leaves (asterisk) which most likely represents non-specific background staining. (I,J) In 10-day old *CLV1::WUS*-expressing *stm5* mutant seedlings (I), no *KNAT2* expression can be detected, which is however seen in seedlings of the same genotype 14 days after germination (J) on the flanks (arrow) and at the base (arrowhead) of the induced structure. The asterisk in I indicates a fragment of the vasculature which appears darker because of its secondary cell wall. Scale bars in A-J are 100 μm.

STM activity still allows organs to develop, but cell differentiation is suppressed and the cells continue to proliferate. This effect is strikingly similar to the phenotype of dominant mutations in *knotted1*, the maize ortholog of *STM*, whose misexpression in leaves leads to local overproliferation (Smith et al., 1992). At least on the basis of expression levels of the linked GUS reporter genes (Fig. 4B,I), these distinct effects do not appear to be due to strongly differing levels of transgene expression, suggesting that they reflect intrinsic functional differences between the two transcription factors.

(2) Ectopic expression of *WUS* and *STM* in leaf primordia induces the expression of distinct downstream target genes. *WUS* is able to induce expression of the presumed stem cell marker *CLV3* even in differentiated organs, but does not activate *KNAT1* or *KNAT2* expression. By contrast, expression of the latter genes can be induced by ectopic *STM* activity, which has, however, no effect on *CLV3* expression. The conclusion that, unlike *WUS*, *STM* thus does not appear to be directly involved in stem cell specification is further supported by our preliminary result that *CLV3* expression is initiated in the apex of *stm5* mutant embryos, and is lost only in late stages of embryogenesis when the apex differentiates (M. L. and T. L., unpublished).

(3) The gain-of-function phenotypes of ectopic *WUS* and *STM* expression in leaf primordia do not require the activity of the respective other gene, indicating that they function in independent genetic pathways.

(4) The shoot meristem defects of both *WUS* and *STM* loss-of-function mutants cannot be rescued by transgenic expression of the other gene: transgenic expression of *STM* in the apex is not able to compensate for the lack of self-maintaining stem cells in *wus* mutants. Conversely, even though *WUS* expression can induce the formation of meristems in *stm* mutants, these appear to grow significantly slower than the corresponding meristems in a wild-type background, suggesting that loss of *STM* function results in reduced proliferation of meristem cells and/or their premature

differentiation. Thus, *WUS* and *STM* appear to fulfil distinct functions in shoot meristem regulation.

(5) Based on the synergistic effect of ectopically coexpressing both genes in leaf primordia and on the ability of *WUS* to partly compensate for loss of *STM* activity in the apex, the developmental pathways regulated by *WUS* and *STM* appear to converge, in that both genes suppress cell differentiation.

Integration of *WUS* and *STM* in shoot meristem maintenance

Our data suggest the following model for how the independent pathways regulated by *WUS* and *STM* are integrated to produce a self-maintaining meristem. In the central region of the meristem *WUS*-dependent signalling from the organizing centre specifies an apical stem cell niche whose residents act as long-term stem cells. *STM* is not directly involved in stem cell specification, but is required throughout the meristem dome to antagonize cell differentiation and allow meristem cells to proliferate. Thus, peripheral stem cell daughters are prevented from being prematurely incorporated into organ anlagen and can amplify cell numbers. *STM* appears to act by repressing *AS1* expression and thus allowing expression of the homeobox genes *KNAT1* and *KNAT2* (Byrne et al., 2000). Local downregulation of *STM* expression in the periphery finally allows lateral organs to be formed.

The observations described here and in previous studies (Mayer et al., 1998; Fletcher et al., 1999) suggest a refinement of the classical histological zonation concept of the SAM (Steeves and Sussex, 1989). The centre of the shoot meristem, roughly equivalent to the central zone, is composed of an apical stem cell niche, whose residents express the *CLV3* gene, and the underlying *WUS*-expressing organizing centre. The peripheral zone comprises a transition zone, where differentiation is repressed by *STM*, allowing the cells to amplify, and regions where *STM* expression is discontinued and organ primordia are initiated.

Similar to other stem cell systems (Potten and Loeffler, 1990), the amplification of cell numbers by the peripheral stem cell daughters may allow the long-term stem cells to divide only relatively rarely – for example only once per 14 initiated leaves in privet (Stewart and Dermen, 1970), while still ensuring a continuous supply of sufficient cells for organ initiation. This division of labour could in turn minimize the danger for stem cells of incurring mutations associated with DNA replication and chromosome segregation. As a large portion of the plant body is ultimately derived from a single stem cell (Stewart and Dermen, 1970), mutations in them would likely be more deleterious than mutations in their daughter cells which only give rise to a more limited part of the plant.

A critical number of cells and cellular competence appear to be required for shoot meristem initiation

Our results imply two important requirements for meristem formation. First, we found that a *CLV1::WUS* transgene can induce adventitious meristems at a high frequency in *stm* mutant seedlings, which is observed to a similar extent in *stm clv* double mutants (Clark et al., 1996). In both cases, the effect is likely due to *WUS* being expressed in an enlarged domain (Schoof et al., 2000). How might this lead to more frequent meristem initiation? One conceivable interpretation is that meristems can be formed as long as there are enough undifferentiated cells, no matter whether these are produced by increasing the size of the *WUS* expression domain – as in *CLV1::WUS*-expressing plants or in *clv* loss-of-function mutants – or by a small *WUS*-expressing region in combination with *STM* activity in a larger zone as in the wild-type apex. In contrast to *WUS*, *STM* on its own does not appear to be able to induce self-maintaining meristems in the absence of *WUS* function. This could either be due to a reduced potency of *STM* in suppressing differentiation compared to *WUS* or to its inability to induce stem cells, which are lacking in *wus* mutants, or to a combination of both. Differences between the two genes in their potency to suppress cell differentiation are suggested by the different severity of the effects caused by ectopic expression of *WUS* or *STM* in leaf primordia.

Evidence supporting the above hypothesis that formation of a stable SAM requires a critical number of undifferentiated cells has also been obtained by studying the *STM* ortholog *KNOTTED1* in maize (Vollbrecht et al., 2000). The penetrance of the meristem defect in *knotted1* mutant embryos is inversely correlated with the size of the meristem primordium in wild-type embryos of the respective genetic background, such that *knotted1* mutants form meristems much more frequently in inbred lines with a large meristem primordium than in ones with a small meristem primordium.

Secondly, meristem initiation appears to depend on a competence of cells to switch to meristem identity, which they appear to gradually lose as they differentiate. While relatively undifferentiated cells in leaf anlagen can easily be respecified towards stem cell identity by *WUS* alone, the differentiated cells in cotyledons are no longer responsive to *WUS* alone. However, this block to switch to meristem identity can be overcome by the combined effects of *WUS* and *STM*, suggesting that a strongly reduced cellular competence can be compensated for by increased meristem promoting activity. This synergistic effect of coexpressing *WUS* and *STM* could have important biotechnological implications for adventitious meristem formation from differentiated cells, which could possibly be strongly enhanced by coexpression of *WUS* and *STM* orthologues.

In summary, the results presented here indicate that *WUS* and *STM* serve distinct functions in the SAM, regulation of stem cell identity and protection of meristem cells from premature differentiation, respectively, and support a division of labour between a slowly dividing set of long-term stem cells and a more rapidly proliferating population of stem cell daughters that only transiently function as initials, both of which are required for continuous organ formation from a self-maintaining meristem.

We would like to thank R. Williams for providing the pCGN1547:*35S::STM* construct, S. Hake, J. Traas and J. Celenza for providing *KNAT1::GUS*, *KNAT2::GUS* and *CycB1;1::CDBGUS* lines, respectively, V. Pautot and J. Traas for the *KNAT2* plasmid and I. Moore for the components of the *pOpL* expression system. We are grateful to Andrea Bohnert for technical assistance and to Arp Schnittger and members of the Laux laboratory for helpful suggestions on the manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft to T. L. and a PhD fellowship of the Boehringer Ingelheim Fonds to M. L.

REFERENCES

Barton, M. K. and Poethig, R. S. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the *shoot meristemless* mutant. *Development* **119**, 823-831.

Becker, D., Kemper, E., Schell, J. and Masterson, R. (1992). New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* **20**, 1195-1197.

Brand, U., Fletcher, J. C., Hobe, M., Meyerowitz, E. M. and Simon, R. (2000). Dependence of stem cell fate in *Arabidopsis* on a feedback loop regulated by *CLV3* activity. *Science* **285**, 585-587.

Byrne, M. E., Barley, R., Curtis, M., Arroyo, J. M., Dunham, M., Hudson, A. and Martienssen, R. A. (2000). *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* **408**, 967-971.

Chuck, G., Lincoln, C. and Hake, S. (1996). *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell* **8**, 1277-1289.

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1993). *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* **119**, 397-418.

Clark, S. E., Running, M. P. and Meyerowitz, E. M. (1995). *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* **121**, 2057-2067.

Clark, S. E., Jacobsen, S. E., Levin, J. Z. and Meyerowitz, E. M. (1996). The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*. *Development* **122**, 1565-1575.

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The *CLAVATA1* gene encodes a putative receptor-kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* **89**, 575-585.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735-743.

Dockx, J., Quaedvlieg, N., Keultjes, G., Kock, P., Weisbeek, P. and Smeekens, S. (1995). The homeobox gene *ATK1* of *Arabidopsis thaliana* is expressed in the shoot apex of the seedling and in flowers and inflorescence stems of mature plants. *Plant Mol. Biol.* **28**, 723-737.

Doerner, P., Jorgensen, J. E., You, R., Steppuhn, J. and Lamb, C. (1996). Control of root growth and development by cyclin expression. *Nature* **380**, 481-482.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J. and Laux, T. (1996). The *SHOOTMERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE*. *Plant J.* **10**, 967-979.

Elliott, R. C., Betzner, A. S., Huttner, E., Oakes, M. P., Tucker, W. Q., Gerentes, D., Perez, P. and Smyth, D. R. (1996). *AINTEGUMENTA*, an *APETALA2*-like gene of *Arabidopsis* with pleiotropic roles in ovule development and floral organ growth. *Plant Cell* **8**, 155-168.

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. *Science* **283**, 1911-1914.

Groß-Hardt, R., Lenhard, M. and Laux, T. (2002). *WUSCHEL* signalling functions in interregional communication during *Arabidopsis* ovule development. *Genes Dev.* (in press).

Jeong, S., Trotochaud, A. E. and Clark, S. E. (1999). The *Arabidopsis CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* **11**, 1925-1934.

Klucher, K. M., Chow, H., Reiser, L. and Fischer, R. L. (1996). The *AINTEGUMENTA* gene of *Arabidopsis* required for ovule and female gametophyte development is related to the floral homeotic gene *APETALA2*. *Plant Cell* **8**, 137-158.

Koncz, C. and Schell, J. (1986). The promoter of the T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 383-396.

Laux, T., Mayer, K. F. X., Berger, J. and Jürgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* **122**, 87-96.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K. and Hake, S. (1994). A *knotted1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* **6**, 1859-1876.

Long, J. A., Moan, E. I., Medford, J. I. and Barton, M. K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. *Nature* **379**, 66-69.

Long, J. A. and Barton, M. K. (1998). The development of apical embryonic pattern in *Arabidopsis*. *Development* **125**, 3027-3035.

Mayer, K. F. X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G. and Laux, T. (1998). Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell* **95**, 805-815.

Mironov, V., de Veylder, L., van Montagu, M. and Inzé, D. (1999). Cyclin-dependent kinases and cell division in plants – the nexus. *Plant Cell* **11**, 509-522.

Moore, I., Gälweiler, L., Grosskopf, D., Schell, J. and Palme, K. (1998). A transcription activation system for regulated gene expression in transgenic plants. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 376-381

Neff, M. M., Neff, J. D., Chory, J. and Pepper, A. E. (1998). dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J.* **14**, 387-392.

Pautot, V., Dockx, J., Hamant, O., Kronenberger, J., Grandjean, O., Jublot, D. and Traas, J. (2001). *KNAT2*: Evidence for a link between Knotted-like genes and carpel development. *Plant Cell* **13**, 1719-1734.

Potten, C. S. and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110**, 1001-1020.

Sablowski, R. W. and Meyerowitz, E. M. (1998). A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. *Cell* **92**, 93-103.

Satina, S., Blakeslee, A. F. and Avery, A. (1940). Demonstration of three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. *Am. J. Bot.* **27**, 895-905.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F. X., Jürgens, G. and Laux, T. (2000). The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the *CLAVATA* and *WUSCHEL* genes. *Cell* **100**, 635-644.

Smith, L. G., Greene, B., Veit, B. and Hake, S. (1992). A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic expression in leaf cells with altered fates. *Development* **116**, 21-30.

Steeves, T. A. and Sussex, I. M. (1989). *Patterns in Plant Development*. Cambridge, UK: Cambridge University Press.

Stewart, R. N. and Dermen, H. (1970). Determination of number and mitotic activity of shoot apical initial cells by analysis of mericlinal chimeras. *Am. J. Bot.* **57**, 816-826.

Vollbrecht, E., Reiser, L. and Hake, S. (2000). Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, *knotted1*. *Development* **127**, 3161-3172.

Williams, R. W. (1998). Plant homeobox genes: many functions stem from a common motif. *BioEssays* **20**, 280-282.