

Microsurgical and laser ablation analysis of interactions between the zones and layers of the tomato shoot apical meristem

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SUMMARY

Plants exhibit life-long organogenic and histogenic activity in a specialised organ, the shoot apical meristem. Leaves and flowers are formed within the ring-shaped peripheral zone, which surrounds the central zone, the site of the stem cells. We have undertaken a series of high-precision laser ablation and microsurgical tissue removal experiments to test the functions of different parts of the tomato meristem, and to reveal their interactions. Ablation of the central zone led to ectopic expression of the *WUSCHEL* gene at the periphery, followed by the establishment of a new meristem centre. After the ablation of the central zone, organ formation continued without a lag. Thus, the central zone does not participate in organogenesis, except as the ultimate source of founder cells. Microsurgical removal of the external L₁ layer induced periclinal cell divisions and

terminal differentiation in the subtending layers. In addition, no organs were initiated in areas devoid of L₁, demonstrating an important role of the L₁ in organogenesis. L₁ ablation had only local effects, an observation that is difficult to reconcile with phyllotaxis theories that invoke physical tension operating within the meristem as a whole. Finally, regeneration of L₁ cells was never observed after ablation. This shows that while the zones of the meristem show a remarkable capacity to regenerate after interference, elimination of the L₁ layer is irreparable and causes terminal differentiation.

Key words: Laser ablation, Meristem, *WUSCHEL*, Central zone, Peripheral zone, Meristem layer, L₁ layer, *Lycopersicon esculentum*

INTRODUCTION

The shoot apical meristem (SAM) serves as a source of cells for organ formation during postembryonic growth. In order to fulfil this function over years (or centuries in trees), the meristem has to maintain a separate population of cells that serve for self maintenance. The two functions of the meristem, organ formation and self maintenance, are associated with different areas of the meristem (Steeves and Sussex, 1989; Lyndon, 1998). Stem cells are located in the central zone (CZ). Owing to their cell division activity, daughter cells are continuously displaced from the centre towards the peripheral zone (PZ) at the flank where they become competent to form organs. Considering the dynamic properties of the meristem, the maintenance of meristem integrity requires precise coordination of cell division, cell expansion and differentiation (for reviews, see Fletcher, 2002; Weigel and Jürgens, 2002; Gross-Hardt and Laux, 2003).

Genetic analysis in *Arabidopsis* and *Petunia* has identified the *WUSCHEL* (*WUS*) and *CLAVATA* (*CLV*) genes as key players in the specification and maintenance of stem cells (Clark et al., 1997; Mayer et al., 1998; Fletcher et al., 1999; Brand et al., 2000; Stuurman et al., 2002). *WUS* is expressed in a cell cluster in the CZ, several cell layers below the summit. *WUS* function induces stem cell identity in the overlying cells

of the CZ. Because of this inductive role, the *WUS*-expressing cell cluster is referred to as the organising centre (OC) of the meristem (Mayer et al., 1998). A negative feedback loop limits *WUS* expression, thereby preventing accumulation of excess stem cells (for reviews, see Simon, 2001; Fletcher, 2002; Gross-Hardt and Laux, 2003). This negative regulation requires the function of the *CLAVATA* (*CLV*) signalling pathway. *CLV3* peptide ligand produced by the stem cells, is perceived by the *CLV1* receptor kinase which is expressed in the cells below the stem cells.

Superimposed on the functional subdivision in CZ and PZ, the meristem is organised in layers (Steeves and Sussex, 1989; Lyndon, 1998). The external L₁ layer covers the subepidermal L₂ layer and the remaining internal tissues, referred to as L₃. The layered organisation of the meristem is maintained by stereotyped cell division patterns in L₁ and L₂. This leads to separated cell lineages that can be maintained for years (Tilney-Basset, 1986). Although the layered organisation of the meristem is found in virtually all angiosperms, its functional relevance is still unclear. Since the three meristem layers cooperate in organ formation, some sort of communication is required to coordinate their development (Szymkowiak and Sussex, 1996). Most information on layer interactions comes from analysis of periclinal chimeras in which one or two of the meristem layers are mutant, and the remaining layer(s) wild

type. Such studies have revealed extensive communication between the layers. For example, in tomato flowers the number and the size of organs is largely determined by the interior layers (Szymkowiak and Sussex, 1992; Szymkowiak and Sussex, 1993). Conversely, in flowers of an *Antirrhinum* periclinal chimera, the wild-type L₁ layer could restore the mutant phenotype in the interior layers of *floricaula* mutants (Hantke et al., 1995). In this case, the L₁ layer induced the complete developmental program in L₂ and L₃ to give rise to fertile flowers. Such inductive interactions between meristem layers are reminiscent of induction between the three germ layers in animal embryogenesis (Gilbert, 2000).

Mutants have provided a wealth of information on intercellular interactions in the meristem (Simon, 2001; Fletcher, 2002; Gross-Hardt and Laux, 2003). However, in many cases, the phenotypes of meristem mutants are pleiotropic, and in some mutants such as *wuschel* or *shoot meristemless*, a normal meristem is never established (Long et al., 1996; Mayer et al., 1998). This limits the use of such mutants for studies on dynamic intercellular interactions. In such cases, it is helpful to induce controlled lesions that are limited temporally and spatially. Physical ablation of cells has successfully been employed to reveal cell-to-cell communication in the root meristem (van den Berg et al., 1995; van den Berg et al., 1997). The advantage of such experiments is that they start from a normal meristem which can, after experimental interference, reorganise itself according to the natural regulatory mechanisms.

Classical microsurgical experiments have shown that needle pricking of the CZ did not lead to meristem arrest (Pilkington, 1929; Loiseau, 1959; Sussex, 1964). In all these studies, some kind of regeneration was reported; however, the results and their interpretation differed considerably. Pilkington mentions briefly that after pricking of the centre 'regeneration followed in nearly every case' (Pilkington, 1929). Loiseau gives a detailed description of the peripheral expansion, the regeneration of several new meristem centres and of fasciations after destruction of the CZ (Loiseau, 1959). He takes these results as evidence for the importance of the PZ, and for the dispensability of the CZ ('La destruction des cellules apicales n'interrompt pas le fonctionnement de l'apex; ces cellules ne sont donc pas indispensables'). Finally, Sussex reports that after puncturing of the apex 'axial growth ceased and one of the apical flanks grew out as the new meristem' (Sussex, 1964).

Despite the initial differences in interpretation, these classical studies have led to the widely accepted notion that destruction of the meristem centre leads to the establishment of one or more new growth centres at the periphery (Steeves and Sussex, 1989). It is of considerable interest to interpret the surgical experiments in the framework of the recent molecular models, and vice versa. With this in mind, we revisited the classical surgical experiments. We used technical innovations from the last half-century, such as tissue culture, high-resolution stereo light and scanning electron microscopy, and laser-based ablation techniques to increase the temporal and spatial resolution of the experiments. In addition, a number of control experiments support the notion that the effects induced by the ablations are not a general stress response but specifically shed light on endogenous developmental processes. Moreover, we monitored the expression of key

developmental genes after the microsurgical manipulations and thereby enable a link to be made between the two types of experimental approaches.

MATERIALS AND METHODS

Plant growth, in vitro culture and treatment of apices

Tomato plants (*Lycopersicon esculentum* cv Moneymaker) were grown as described previously (Reinhardt et al., 1998). Shoot apices were dissected and cultured as described previously (Fleming et al., 1997) on Murashige and Skoog (MS) medium containing 0.01 µM gibberellic acid A₃ (Fluka, Switzerland) and 0.01 µM kinetin (Sigma). Salicylic acid (Sigma), jasmonic acid (Sigma), hydrogen peroxide (Fluka) and Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichlorid; Fluka) were diluted in DMSO to give 100× stock solutions. These were diluted directly in prewarmed (60°C) lanolin containing 3% w/w paraffin (Merck), and applied manually with yellow plastic pipette tips. After chemical treatments and laser ablation, apices were further cultured on synthetic medium.

Laser ablations

Ablation of the meristem was conducted with a Q-switched Er:YAG laser that emits infrared radiation at a wavelength of 2.94 µm. Er:YAG laser radiation shows a high ablation efficiency and precision, which, by virtue of the high absorption coefficient in water (absorption coefficient is about 10,000 cm⁻¹, corresponding to an optical penetration depth of approximately 1 µm), leads to thermally damaged zones adjacent to the ablation side that are restricted to a few micrometers (Frenz et al., 1996). Q-switching was performed by a FTIR-modulator as described previously (Könz et al., 1993). The pulse duration was 60 nseconds. The laser was operated at a repetition rate of 2 Hz. The radiation was guided from the laser to the operating microscope through an optical sapphire fibre with a core diameter of 125 µm and focused with a lens system to a spot of approximately 40 µm in diameter on the surface of the meristem. The pulse energy used was 0.3 mJ for ablation of the L₁ layer (one pulse applied), and 1.5 mJ for deeper ablations (1 pulse for the ablation of the stem cells, and 10 consecutive pulses for the ablation of the entire CZ).

Cloning of *LeWUS*

A cDNA library was constructed using mRNA isolated from tomato meristems and the SMART library kit (Clontech). A 350 bp fragment of the *LeWUS* gene was obtained by polymerase chain reaction (PCR) on DNA from the meristem library using Triplex F primer (Clontech) and the WUS4R primer (5'-GCCTTCAATCTTTCCGTACTGTCT-3'), which matches the conserved region of the *Petunia PhWUS* gene (Stuurman et al., 2002) (GenBank accession number AF481951). Based on sequence information from the 350 bp fragment, we designed the WUS10F primer (5'-CAACACAACATAGAAGATG-GTGG-3'). A 1200 bp fragment amplified with the primers WUS10F and Triplex R (Clontech) was cloned into pBluescript and used for generating ³⁵S-labelled riboprobes. The sequence of *LeWUS* was deposited in GenBank (accession number AJ538329).

In situ hybridisation and microscopy

In situ hybridisations were carried out either with ³⁵S-labelled riboprobes as described by Reinhardt et al. (Reinhardt et al., 1998), or with dig-labelled riboprobes according to the method of Vernoux et al. (Vernoux et al., 2000). Silver grain signal was visualised on a Zeiss LSM310 confocal microscope as described previously (Reinhardt et al., 1998), and appears as yellow grains on a blue background. For scanning electron microscopic analysis, apices were viewed with an S-3500N variable pressure scanning electron microscope from Hitachi (Tokyo, Japan), equipped with a cool stage. In digital images lanolin paste was pseudocoloured for clarity. For live

imaging, developing tomato apices were cultured on plates and repeatedly photographed with a Sony DKC-5000 digital camera mounted on a Nikon SMZ-U stereoscope. Plastic sections were prepared as described previously (Loreto et al., 2001) with one modification: OsO₄ was omitted. Semithin sections (5 µm) were viewed on a Zeiss Axioskop2 equipped with an Axiocam camera.

RESULTS

Laser ablation at the meristem

The shoot apical meristem is organised in a central zone (CZ; Fig. 1A,B, light blue) that harbours the stem cells (dark blue), and in a peripheral zone (PZ, yellow) in which organ formation takes place. Superimposed on this functional subdivision into CZ and PZ, the meristem is organised in three cell layers (L₁, L₂ and L₃) that are clonally separated because of their stereotyped cell division patterns (Fig. 1B). In L₁ and L₂, the cells divide anticlinally, i.e. both daughter cells remain in the original layer, whereas in L₃, cell divisions occur in all planes. The three layers cooperate in organ formation (Fig. 1B, arrowhead). In the CZ, a population of cells that expresses the *WUSCHEL* gene (red) induces stem cell identity in the above cells (Mayer et al., 1998), and therefore, is referred to as the organising centre (OC). Whereas the CZ includes all undifferentiated cells distal to the region of primordium formation, the stem cells constitute only the small cell population at the summit. Each meristem layer has a few stem cells [1-3 stem cells per layer as estimated by Stewart and Dermen (Stewart and Dermen, 1970)], consequently, the meristem contains a total set of up to 10 stem cells.

Previous experiments indicated that after ablation of single cells in the L₁ layer, the dead cells were gradually displaced from the meristem without further consequences for meristem development (M. Muster and C.K., unpublished). Therefore, we wanted to ablate larger cell populations such as the entire CZ (Fig. 1C), the distal part of the CZ (Fig. 1D) or only the L₁ layer (Fig. 1E,F). To achieve this, we connected an infrared laser to an optic fibre and a lens that focused the beam to a spot of approximately 40 µm in diameter. This set-up allowed us to ablate groups of cells simultaneously and to vary the energy in a wide range. Repeated high energy pulses at the same spot ablated the entire CZ (Fig. 1C), whereas single high energy pulses ablated only the 4-5 most superficial cell sheets in the CZ (Fig. 1D; see Materials and Methods). To generate superficial lesions of only L₁ cells (Fig. 1E), single ablations at low energy were performed. For the ablation of the entire L₁ layer (Fig. 1F), we developed a microsurgical technique (see below).

Ablation of the central zone does not affect organ formation but leads to the establishment of a new meristem centre

To reveal the function of the CZ in organ formation and meristem maintenance, we generated lesions in the meristem centre that were approximately 40 µm wide and 100 µm deep (compare to a meristem diameter of approximately 150 µm). (Fig. 2A,C,D). These lesions eliminated the CZ including the *LeWUS*-expressing cells in the L₃ layer, which is located approximately 50 µm below the summit of the meristem (Fig. 2B, compare with Fig. 3A,B). After such ablations, leaf

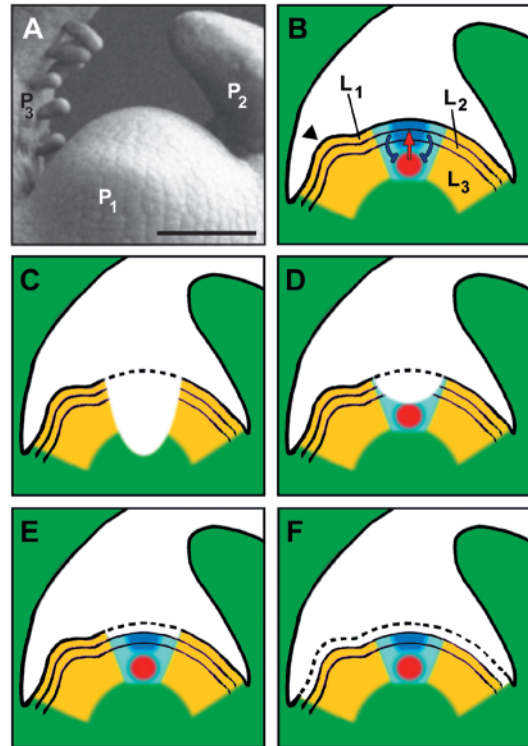
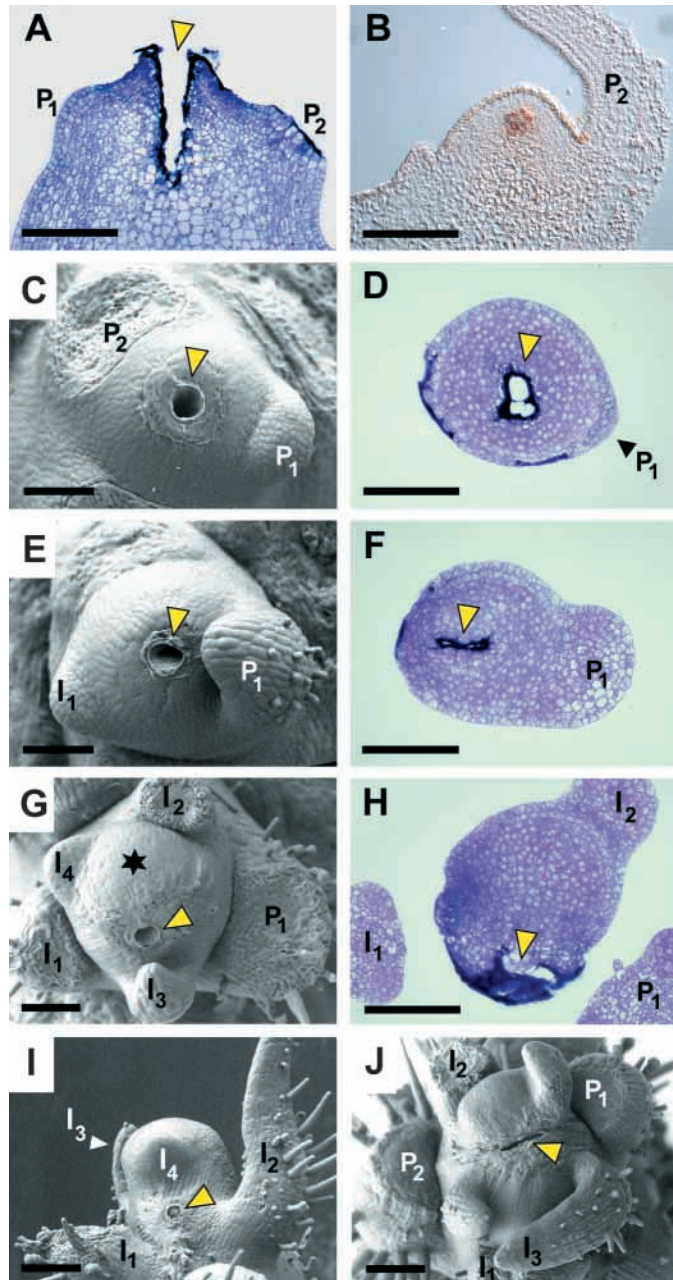


Fig. 1. Organisation of the shoot apical meristem, and schematic representation of the ablations performed in this study. (A) SEM micrograph of a vegetative shoot apical meristem of tomato. P₃, P₂ and P₁ indicate young primordia. P₁ is just being initiated at the flank. (B-F) Schematic representation of a meristem as in A. (B) The meristem consists of the central zone (CZ, light blue) that harbours the stem cells (dark blue) and the peripheral zone (PZ, yellow) in which organs are formed. An organising centre that expresses the marker gene *LeWUS* (red) induces stem cell identity in the above cells. Superimposed on the zonation (CZ and PZ), the meristem is organised in layers, namely the external L₁ layer, the subepidermal L₂ layer and the remaining cells, called L₃. All three layers cooperate in organ formation (arrowhead). (C) Ablation of the entire CZ. (D) Ablation of the stem cells. (E) Ablation of the L₁ layer in the CZ. (F) Ablation of the entire L₁ layer. Scale bar: 100 µm.

formation continued without delay (Fig. 2E,G,I). Primordium initiation rate may even have been slightly higher than in control apices, i.e. after 3 days, apices with lesions had formed 1.95 ± 0.4 (s.d.) new primordia ($n=15$) compared to 1.54 ± 0.29 (s.d.) new primordia in controls ($n=7$). Also, the position of new primordia was normal, i.e. new primordia diverged from the next older primordia by approximately 137°. In general, the hole closed within 2 days (Fig. 2F). Later, the lesions were, in most cases, gradually displaced from the meristem centre (16 out of 22; Fig. 2G,H,I). We presume that this displacement was caused by the activation of a new meristem centre at the flank. In 3 out of 22 cases, two new centres were initiated concomitantly at opposite sides of the lesion, resulting in the split of the meristem (Fig. 2J). In such cases, leaf position sometimes becomes irregular (Fig. 2J; Fig. 3G). In the remaining cases (3 out of 22), the lesion remained on the meristem. These results show that after elimination of the entire CZ, including the *LeWUS*-expressing cells, organ formation continued without an obvious lag.



Establishment of a new meristem centre is associated with ectopic induction of the *WUSCHEL* gene

The cells in the CZ and in the PZ differ in cell size, subcellular organisation, cell division rate, gene expression, and the ability to form organs (Steeves and Sussex, 1989; Lyndon, 1998; Fletcher, 2002; Reinhardt and Kuhlemeier, 2002). Therefore, if cells at the periphery are to reorganise a new meristem centre, they need to be reprogrammed. The *WUSCHEL* gene in *Arabidopsis* confers stem cell identity to the overlying cell layers (Mayer et al., 1998). Therefore, *WUSCHEL* is a good candidate for a role in reinitiation of a new centre, and a good marker for CZ identity. *WUSCHEL* expression and loss-of-function phenotypes are conserved between *Arabidopsis* and *Petunia hybrida*, a close relative of tomato (Stuurman et al.,

Fig. 2. Organ formation and meristem maintenance after ablations of the CZ. Yellow arrowheads indicate the position of the lesion. (A) Longitudinal section through a meristem immediately after ablation. The ablation eliminated the entire CZ and projected more than 100 μm into the meristem. (B) Longitudinal section through a control meristem hybridised with a dig-labelled antisense probe against *LeWUS*. The signal in the meristem centre appears brown. (D,F,H) Transverse sections; (C,E,G,I,J) scanning electron micrographs. (C) Scanning electron micrograph of a meristem with ablated CZ, in top view. (D) Transverse section of a meristem after ablation of the CZ. (E–J) Development of the meristem after ablation of the CZ. After 2 days, a new primordium was formed at the expected site (E), and the hole closed (F). After 4 days, four new primordia had been formed (G), the lesion was displaced from the centre towards the flank, and a new meristem centre (star) was established (G,H). After 6 days, the lesion was removed from the meristem and displaced onto the stem (I), or two new meristem centres had been established on either side of the lesion (J). P₃, P₂, and P₁ indicate leaf primordia that were present at the beginning of the experiment; I₁, I₂, I₃, and I₄ indicate primordia formed after the ablation. In some cases, primordia were removed to allow manipulation or visualisation. Scale bars: 100 μm .

2002). We monitored the expression of a tomato *WUS* homologue, *LeWUS*, after ablation of the CZ. Ablation of the CZ eliminated the *LeWUS*-expressing cells completely (Fig. 3A,B). However, after 1 day, *LeWUS* was ectopically induced in a ring-shaped region surrounding the lesion (Fig. 3C, arrows). Two days after the ablation, *LeWUS* expression became stronger and confined to one side of the lesion (Fig. 3D). After 4 days, a new *LeWUS*-expressing cell cluster was established, comparable in size with the *LeWUS* expression domain in control meristems (Fig. 3E, compare with 3A). After 6 days, a functional meristem was evident that grew out to the side, away from the lesion (Fig. 3F). Sometimes, two new *LeWUS*-expressing centres (*WUS* centres) were established on opposite sides of the lesion (Fig. 3G). Such meristems are likely to represent early stages of apices that would have split at later stages (compare with Fig. 2J).

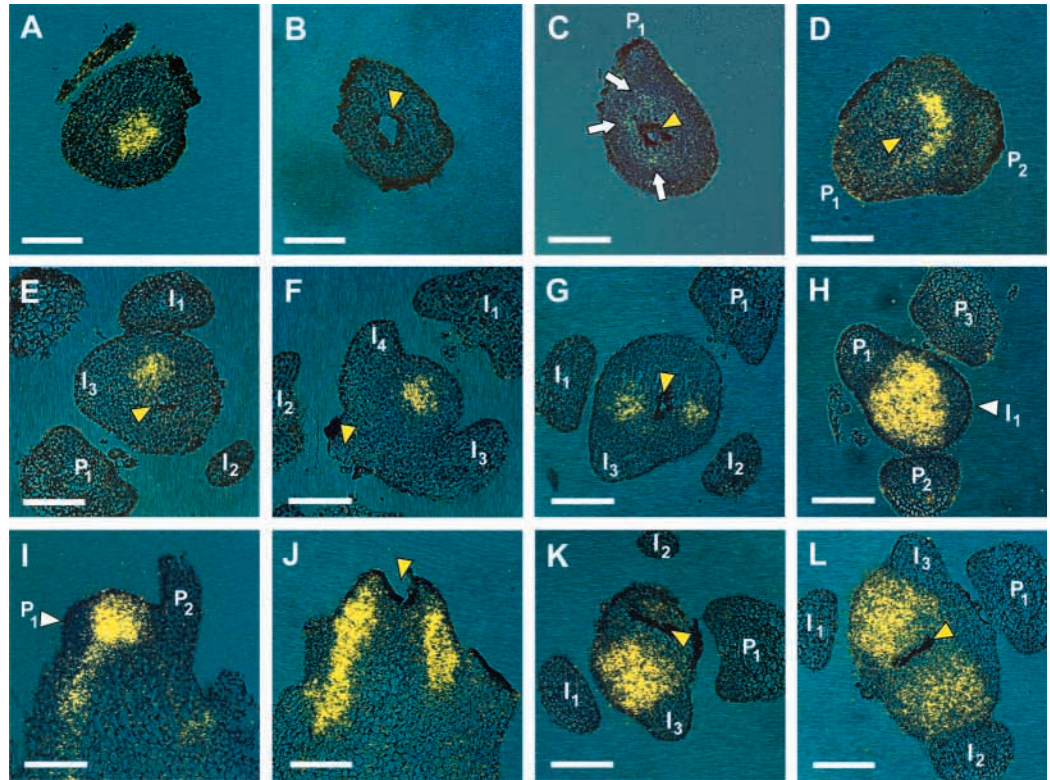
Organ formation continued after ablations of the CZ, indicating that basic meristem functions in the PZ were not affected. In order to confirm the maintenance of meristem identity in the PZ, we analysed the expression of the *LeT6* homeobox gene (also referred to as *TKn2*), a marker for meristem identity (Chen et al., 1997; Parnis et al., 1997). In untreated controls, *LeT6* was consistently expressed in the CZ and the PZ but down-regulated in the leaf primordia and the site of incipient leaf formation (I₁) (Fig. 3H,I), in a manner similar to the homologous genes *KNOTTED1* in maize (Jackson et al., 1994) and *SHOOT MERISTEMLESS* in *Arabidopsis* (Long and Barton, 2000). This is in contrast to previous reports that found *LeT6* to be expressed across the meristem, with only moderate reduction in leaf primordia (Chen et al., 1997; Parnis et al., 1997). After ablation of the CZ, *LeT6* continued to be expressed in the remaining cells at levels comparable to those in the controls (Fig. 3J). In parallel with the re-establishment of one or two new meristem centres, *LeT6*-expressing cells increased either on one (Fig. 3K), or on two opposite sides of the lesion (Fig. 3L).

Taken together, we have shown that after ablation of the CZ, *LeWUS* is induced in cells at the flank within 1 day. This increase in *LeWUS* expression is unlikely to be caused by

Fig. 3. Expression of the meristem marker genes *LeWUS* and *LeT6* in the meristem after ablation of the CZ. Gene expression was visualised by in situ hybridisation with ^{35}S -labelled riboprobes. Signal appears as yellow grains. All images are transverse sections, except for I and J which are longitudinal sections. Position of the lesion is indicated by yellow arrowheads.

(A–G) Expression of *LeWUS*. (H–L) Expression of *LeT6*. (A) Expression of *LeWUS* in a control meristem.

(B) Expression of *LeWUS* immediately after ablation of the CZ (yellow arrowhead). No *LeWUS* expression can be detected. (C) Expression of *LeWUS* 1 day after ablation of the CZ. A ring-shaped area around the lesion expresses *LeWUS* at low levels (arrows). (D) Expression of *LeWUS* 2 days after ablation of the CZ. The lesion (arrowhead) is closed, and the *LeWUS* signal



becomes confined to one side of the lesion. (E) Expression of *LeWUS* 4 days after ablation of the CZ. The *LeWUS*-expressing zone at the flank has resolved into a new WUS centre with normal dimensions (compare with A). (F) Expression of *LeWUS* 6 days after ablation of the CZ. A new functional meristem centre with a normal WUS centre has been re-established. (G) Expression of *LeWUS* 4 days after ablation of the CZ. Two new WUS centres are evident at opposite sides of the lesion. (H) Expression of *LeT6* in a control meristem. Note down-regulation of *LeT6* in the youngest primordium (P₁) and at the site of incipient leaf formation (I₁). (I) Expression of *LeT6* in a control meristem. Note down-regulation of *LeT6* in the youngest (P₁) and second youngest primordium (P₂). (J) Expression of *LeT6* 6 hours after ablation of the CZ. *LeT6* remains active at the periphery of the meristem and is only decreased in the vicinity of the lesion. (K) Expression of *LeT6* 5 days after ablation of the CZ. The lesion is displaced, and *LeT6* is expressed in the new meristem centre and excluded from leaf primordia. (L) Expression of *LeT6* 4 days after ablation of the CZ. Two new *LeT6*-expressing meristems are induced on opposite sides of the lesion. P₃, P₂, and P₁ indicate leaf primordia that were present at the beginning of the experiment; I₁, I₂, I₃, and I₄ indicate primordia formed after the ablation. Scale bars: 100 μm .

proliferation of a few *LeWUS*-expressing cells that might have escaped destruction. Also, the new *LeWUS* signal always occurred at some distance from the lesion. Therefore, the *LeWUS* mRNA at the flank appears to result from ectopic transcriptional induction of *LeWUS* in cells that did not express *WUS* before the ablation. *LeWUS* induction preceded the establishment of a new meristem centre by approximately 2 days. In parallel with ongoing organogenesis, the meristem marker gene *LeT6* continued to be expressed at the periphery, even after ablation of all *LeWUS*-expressing cells.

Ablation in the PZ and stress treatments do not affect the function of the CZ

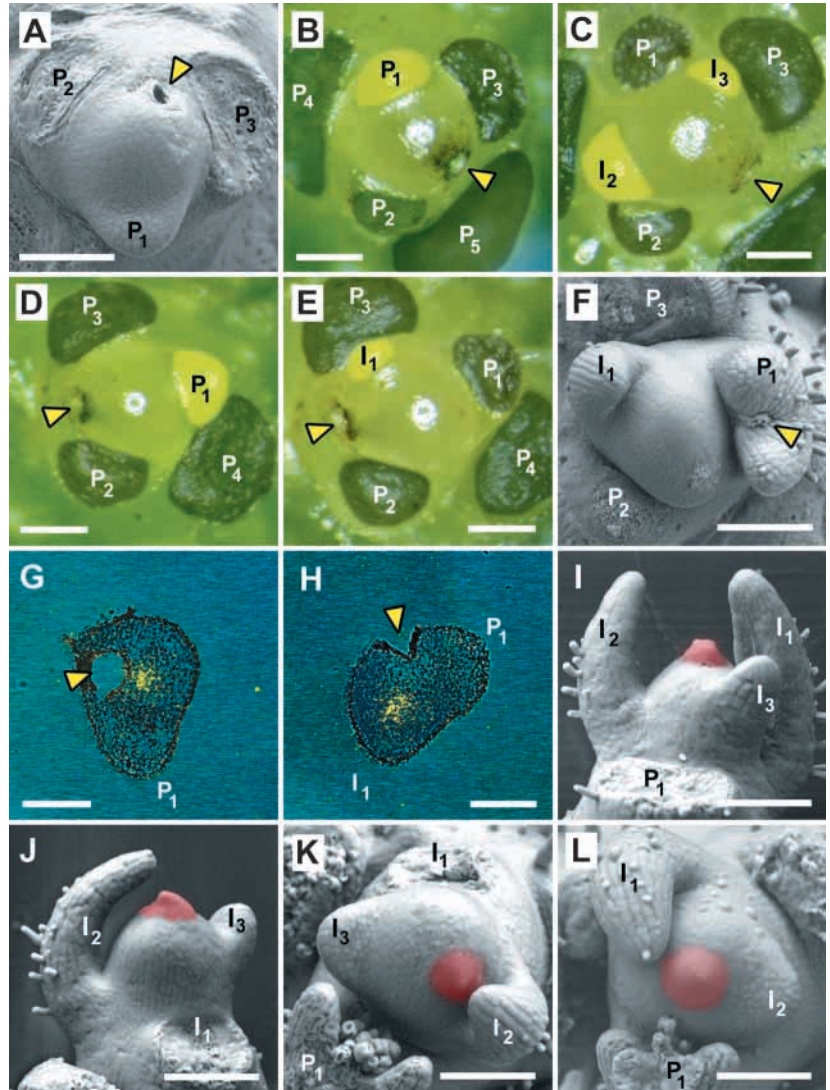
A major concern with all surgical ablations is that they may cause wound or stress responses that complicate or even invalidate the conclusions from the experiments. For example, it could be argued that ectopic induction of *WUS*, or establishment of a new growth centre, may be influenced not only by the loss of the CZ cells, but also by wound effects from the lesions, or by secondary stress signals. In order to exclude such effects, we performed a number of control experiments.

First, we performed laser ablations of similar magnitude not

in the CZ but in the PZ at the site of incipient leaf formation (Fig. 4A). Such ablations affected the positioning of new leaves in two ways. Either the site of the ablation was skipped and the next primordium was initiated at the next expected position (Fig. 4B,C), or the primordia were displaced to either side of the lesion (Fig. 4D,E), resulting in a smaller or larger divergence angle than expected. If ablations were performed in the centre of the youngest primordium (P₁), it became split (Fig. 4F). However, we never observed a reorientation of the growth axis after ablations at the PZ, indicating that despite the strong local effects on organ formation, the ablations did not affect the neighbouring CZ or lead to establishment of a new meristem centre. To confirm this, we analysed *LeWUS* expression after ablations at the PZ (Fig. 4G). *LeWUS* continued to be expressed in an area similar to that in control meristems although the expression level tended to decrease after ablation (Fig. 4H). The fact that ectopic induction of *LeWUS* was not found after peripheral ablations shows that wounding per se is not sufficient to induce ectopic *LeWUS* expression.

In order to test for effects of secondary stress signals that might be generated by ablation, we treated the centre of

Fig. 4. Control treatments do not influence the central zone. (A-H) Laser ablations (yellow arrowheads) as in Fig. 2C, but at the periphery instead of the centre. (A) Laser ablation at the periphery approximately at the site of incipient leaf formation. (B,C) Consecutive video images of a single meristem with an ablation as in (A). Cut primordia are dark green, and the youngest primordia are highlighted in yellow for clarity. (B) Immediately after ablation (t_0); (C) 2 days after ablation. Leaf formation at the site of the ablation is suppressed (arrowheads), while two new primordia (I_1 and I_2) were induced at the next two expected positions. (D,E) Consecutive video images of a single meristem with an ablation as in (A). (D) t_0 ; (E) 2 days after ablation. I_1 is initiated closer to P_1 than normal, resulting in a divergence angle of approximately 90° instead of 137° . Arrowheads point to the lesion. (F) Ablation on P_1 . 2 days after the ablation (arrowhead), the primordium has recovered and split in two halves. (G) In situ hybridisation with a *LeWUS* probe 6 hours after an ablation at the periphery. The *LeWUS* expression domain remained normal. (H) 2 days after ablation, the lesion was displaced (arrowhead), and *LeWUS* continued to be expressed in the normal area. (I-L) Effects 4 days after treatments of the CZ with stress metabolites and oxidants. (I) Control. (J) Treatment with 1 mM salicylic acid. (K) Treatment with 1 mM hydrogen peroxide. (L) Treatment with 0.1 mM Paraquat. Treatments did not affect the formation rate or the positioning of leaf primordia. Lanolin paste (red) remained in the centre, indicating that the growth centre persisted. P_5 , P_4 , P_3 , P_2 , and P_1 indicate the bases of pre-existing leaf primordia that were removed at the beginning of the experiment; I_1 , I_2 and I_3 indicate primordia formed after the ablation. Scale bars: 100 μm .



meristems with salicylic acid (Fig. 4J), H_2O_2 (Fig. 4K) Paraquat (Fig. 4L) or jasmonic acid (not shown). After all these treatments, leaves were formed at normal rates and at the normal position. In all cases, the lanolin paste was not displaced at all (Fig. 4J,L) or only slightly (Fig. 4K), indicating that the centre of the meristem was still functional and had not moved compared to controls (Fig. 4I).

From these control treatments, it can be concluded that ectopic induction of *LeWUS* and initiation of a new meristem centre after ablations of the CZ is a specific response to the removal of the CZ, and not a general stress response to wounding or secondary stress signals. It follows that under normal conditions, *LeWUS* expression at the flank is repressed by cells in the CZ, and that this block is released by the ablations.

Ablation of the distal part of the CZ does not lead to rapid ectopic induction of *LeWUS*

The ablations discussed in the previous sections removed the entire CZ including the *LeWUS*-expressing cells (Figs 2, 3). To test the effect of removal of only the distal portion of the CZ, we performed ablations at the meristem centre that consisted

of about 8 cells in diameter and reached approximately 4-5 cell layers deep. Such ablations removed most of the cells distal to the *LeWUS*-expressing cells, presumably including all stem cells, while leaving the *LeWUS*-expressing cells intact (Fig. 5A,B, compare with Fig. 2B). We assume that the rapid induction of *LeWUS* after elimination of the entire CZ is due to the release of inhibition from cells in the centre. If this inhibition originates from the distal cells of the CZ, the result of deep and partial ablations would be expected to be similar, because in both cases, the distal cells are removed. If however, cells in deeper layers were responsible for *LeWUS* limitation, then the outcome of the complete and partial CZ ablations would be expected to be different.

After ablation of the distal cells, we followed *LeWUS* expression in the meristem for a week, with emphasis on the first 3 days. It was of special interest to see whether the *LeWUS*-expressing area in the meristem expands laterally, as would be the case if peripheral cells were released from suppression after the ablation. However, the *LeWUS*-expressing domain in the CZ remained approximately the same size after 1 and 3 days (Fig. 5C,D) and after 2, 4 and 6 days (data not shown). Nevertheless, ablated meristems were able to

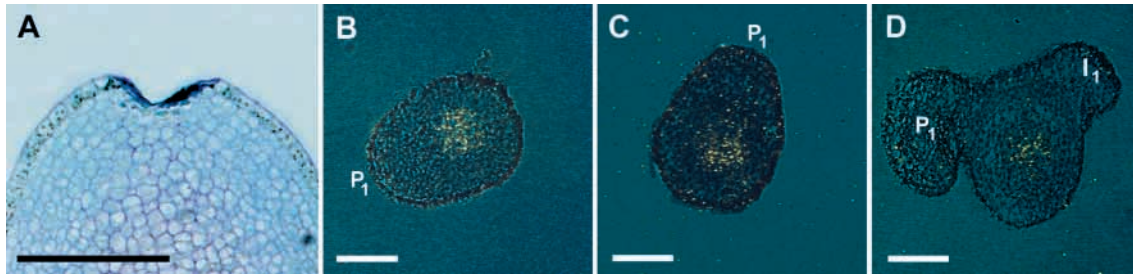


Fig. 5. Expression of *LeWUS* after ablation of the stem cells. (A) Longitudinal section through a meristem after ablation of the upper four to five cell layers. (B-D) Transverse sections showing *LeWUS* expression at various times after ablation as in A. (B) Immediately after ablation. (C) One day after ablation. (D) Three days after ablation. P₁ indicates the leaf primordium that was present at the beginning of the experiment; I₁ denotes a primordium that was formed after the ablation. Scale bars: 100 µm.

recover, and to establish a new functional growth centre (data not shown). It is possible that after partial ablations, a gradual shift of the *LeWUS*-expressing domain occurred, rather than an overall expansion. Such a shift, which would escape detection by in situ hybridisation analysis, could allow for a new growth centre to be induced beside the lesion.

The role of the L₁ layer in meristem function

The previous experiments, which involved complete and partial ablations of zones of the meristem (CZ and PZ), showed the high efficiency with which the remaining tissues compensated for the loss by adjusting their fates. In another series of experiments, we asked what the role of L₁ is in meristem development, and whether the layers of the meristem are equally flexible as the zones. By single low energy pulses of

the laser directed at the summit of the meristem, small patches of cells in the centre of the L₁ layer were ablated (Fig. 6A,B). Similar to ablations of the entire CZ (Fig. 2), such lesions did not perturb organ formation at the periphery (Fig. 6C-E). After 5 days, the meristems had formed 3.28 ± 0.93 (s.d.) new leaf primordia ($n=73$), compared to controls that had formed 2.86 ± 0.66 (s.d.) leaf primordia ($n=14$), and the primordia were initiated at the normal positions. However, the development of the cells just below the lesion was altered (Fig. 6F). Instead of dividing anticlinally to propagate the continuous layers of the meristem, they started to divide predominantly periclinally, leading to the formation of cell stacks that grew out perpendicularly to the surface (Fig. 6F). This indicates that the L₁ layer normally exerts a restriction to such cell divisions. Later, superficial lesions were displaced from the meristem, indicating that the growth centre of the meristem was displaced to the flank (data not shown). However, this shift of the growth centre appeared to be delayed compared to the establishment of a new growth centre after ablations of the entire CZ. After 6 days, only 51 out of 73 superficial lesions were displaced from the meristem (70%), whereas in the case of ablations of the entire CZ after 4 days, i.e. 2 days earlier, already 19 out of 22 lesions were displaced from the meristem (86%).

Since the L₁ layer appears to influence the development of subtending meristem cells, we were interested to see whether leaf formation required the L₁ layer. Owing to the curved surface of the meristem, the laser beam could not be focused evenly onto larger regions of the L₁ layer. Therefore, we developed a microsurgical method to ablate larger portions of the L₁ layer. Incisions were made on the abaxial (outer) side of young primordia at the base. The incisions were made as deep as possible without severing the epidermis of the adaxial

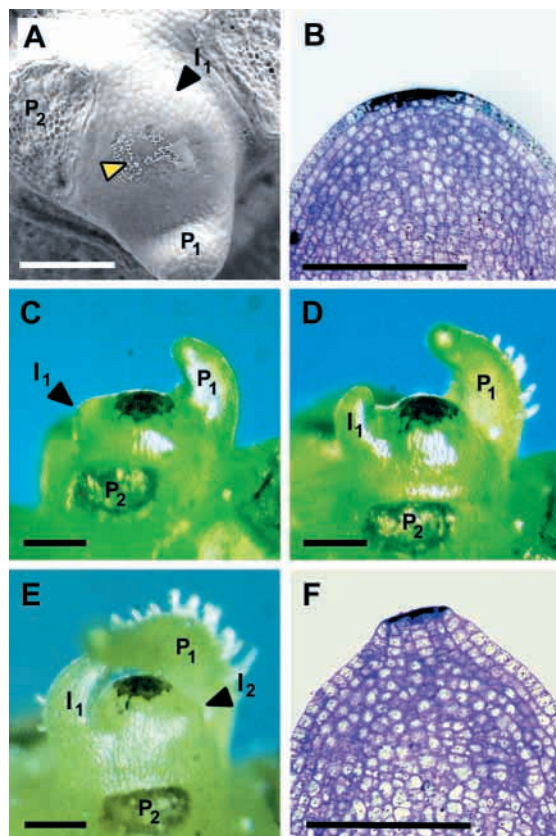
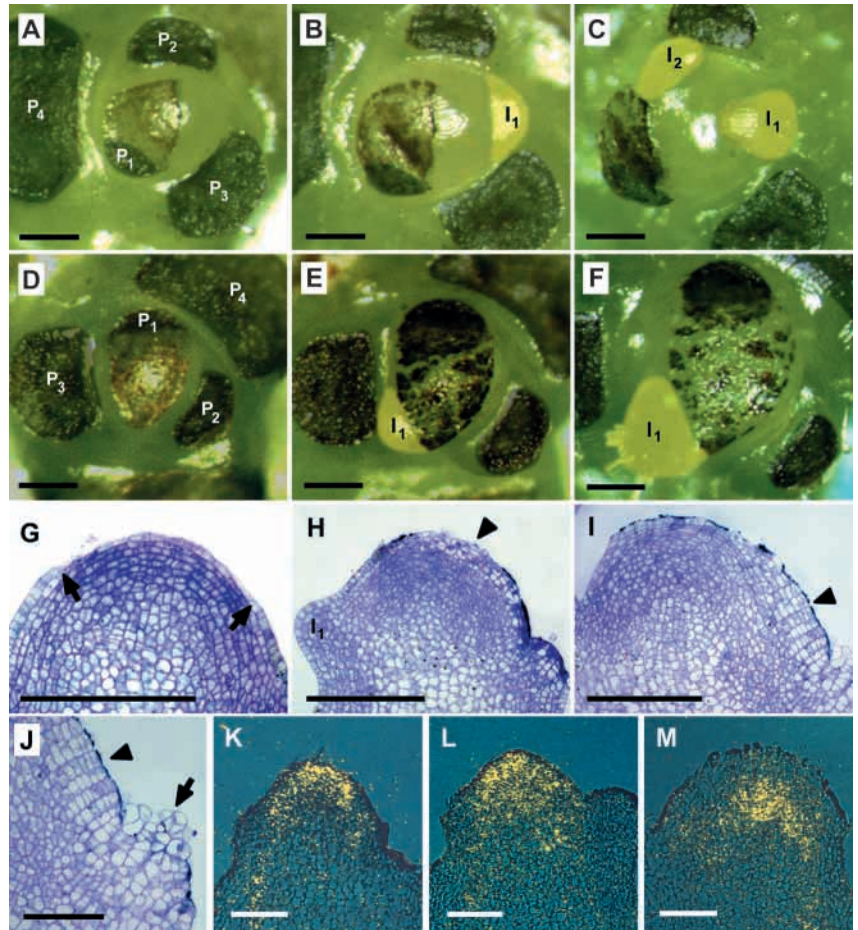


Fig. 6. Laser ablations of the L₁ layer in the centre have local effects on cell division orientation, but not on meristem maintenance and phyllotaxis. (A) Scanning electron micrograph of a meristem immediately after superficial ablation in the centre (yellow arrowhead). (B) Longitudinal section of a meristem immediately after treatment as in A. (C-E) Consecutive video images of a single meristem after ablation as in A: (C) 1 day, (D) 2 days and (E) 3 days after ablation. Note normal phyllotaxis. (F) Longitudinal section through a meristem 5 days after ablation as in A. Note periclinal divisions in cells just below the ablation. P₂ and P₁ indicate leaf primordia that were present at the beginning of the experiment; I₁ and I₂ indicate primordia formed after the ablation. Scale bars: 100 µm.

Fig. 7. Surgical ablation of the L₁ layer leads to aberrant cell division and differentiation in L₂ and L₃. (A-C) Consecutive video images of a single meristem from which the left half of the L₁ layer was removed. Cut primordia are coloured in dark green, and the youngest primordia are highlighted in yellow: (A) t₀, (B) 2 days and (C) 4 days after ablation. Organ formation continues from the unperturbed half, and the meristem centre is shifted to the right. (D-F) Consecutive video images of a meristem from which most of the L₁ layer has been removed: (D) t₀, (E) 2 days, (F) 4 days. Meristem activity ceases. Note that a final primordium is formed at the normal position (I₁). (G-I) Longitudinal sections through meristems after a surgical ablation as in D. (G) Note the continuity of the L₂ layer at the site of the ablation (between arrows). (H) Two days after ablation as in (D). A last primordium was formed (I₁), while the cells at the ablated site (arrowhead) became vacuolated and started to divide periclinally. (I) Five days after ablation as in (D). Note stacks of cells resulting from repeated periclinal division, and increasing vacuolisation (arrowhead). (J) Close up of (I). Note different cell division patterns and cell shape in the area where a primordium had been removed at the beginning of the experiment (arrow), compared to the site at which the L₁ was ablated (arrowhead). (K-M) In situ hybridisations with a ³⁵S-labelled antisense probe against *LeT6*. Tomato meristems were treated as in (D), and fixed for analysis either immediately (K), or after 2 days (L), and 5 days (M). *LeT6* signal can be observed at the ablated site until 2 days after the ablation (L). After 5 days, the vacuolated cells exhibited low levels of *LeT6* mRNA, whereas high levels of *LeT6* remained in the lower L₃ cells that exhibit less vacuolisation. P₄, P₃, P₂ and P₁ indicate the bases of pre-existing leaf primordia that were removed at the beginning of the experiment; I₁ and I₂ indicate primordia formed after the ablation. Scale bars: 100 µm (A-I, K-M); 50 µm (J).



side (towards the meristem). Then the primordia were pulled over the meristem, drawing with them a sector of the L₁ layer. This technique led to surprisingly clean removal of L₁ while leaving the L₂ layer intact (Fig. 7A,D,G).

When approximately half of the L₁ was removed (Fig. 7A-C), leaf formation continued, but only from the intact half of the meristem. During the following days, the lesions were displaced from the meristem, indicating that the growth centre of the meristem had been shifted towards the intact flank (Fig. 7C). When most of the L₁ was removed (Fig. 7D), leaf formation was abolished, either immediately, or after the formation of one last leaf (that was initiated only when a small marginal strip of L₁ was retained; Fig. 7E). After 4 days, these meristems became dramatically expanded (Fig. 7F). Histological sections revealed that the enlargement of the meristem was due both, to cell expansion and cell division at the peeled site (Fig. 7G-J). Two days after L₁ ablations, enlarged cells and periclinal divisions were evident (Fig. 7H), and after 5 days, stacks of vacuolated cells had been formed at the peeled site (Fig. 7I,J). This change in cell behaviour is similar to, although more accentuated than, that following local L₁ ablations (compare with Fig. 6F). The ordered cell stacks formed at the site of the L₁ ablations contrasted with the irregular callus formed at the cut surfaces of primordia bases (Fig. 7J).

The fact that organ formation was never observed at an area from which the L₁ layer was ablated points to a special role of this layer in organ formation. However, since the loss of L₁ also led to the loss of meristem characteristics in subtending cells, the defect in organogenesis could also be indirectly caused by the loss of meristem identity. To test this possibility, we followed the expression of the meristem marker *LeT6*. This gene continued to be expressed for several days after L₁ ablation (Fig. 7K-M), but concomitantly with vacuolization and periclinal divisions, *LeT6* expression faded away in the upper layers (Fig. 7M). Thus the loss of meristem identity developed more slowly than the immediate block of organ formation at the ablated site.

DISCUSSION

Combining classical ablation approaches with modern technology

In recent years, our understanding of the functioning of the shoot apical meristem has dramatically expanded, mostly as a result of sophisticated genetic analyses in *Arabidopsis thaliana*. The current genetics-based models do, however, incorporate conclusions based on classical tissue ablation

experiments. The latter fascinating and highly informative experiments were performed at a time when many of the tools that we now take for granted were not yet available. Our present study revisits these classical experiments. Thanks to modern tools, such as high power binocular microscopes, scanning electron microscopy, sterile meristem culture and laser-directed tissue ablation, we were able to remove smaller and better-defined pieces of tissue, and to follow the effect of the manipulations from early time points. In addition, we performed important control experiments to confirm that the responses to ablations are not due to general wound effects, an issue that today is probably considered more critical than it was half a century ago.

Another important modern tool is molecular markers, which allowed us to establish tissue identities. We note, however, that the number of markers available in tomato is rather limited. Similarly, it would have been very useful to perform these manipulations in mutant backgrounds. It is unfortunate that its small and inaccessible meristem makes *Arabidopsis* completely unsuitable for this type of experiments. Micromanipulation experiments, such as the ones presented here, can only provide indications of novel dynamic interactions in the meristem. They can direct the further genetic and biochemical experiments that are required to build definitive molecular models.

Ablation of the CZ leads to the establishment of a new meristem centre from the PZ but has no direct effect on organogenesis

Clonal analysis has demonstrated that the postembryonic leaves originate from a few stem cells in the CZ (Stewart and Dermen, 1970), emphasising the pivotal function of the CZ. The importance of the CZ is also supported by a wealth of genetic data. For instance, expression of the *WUS* gene in the CZ is necessary and sufficient for stem cell induction and maintenance (Mayer et al., 1998; Schoof et al., 2000). Confirming and extending classical ablation experiments, we show here that after removal of the CZ, including the *LeWUS*-expressing cells by infrared laser ablation, a new functional meristem centre is rapidly and efficiently established from cells in the PZ. This indicates that ablated stem cells can be replaced by cells at the periphery.

After ablation of the CZ, leaves continued to be initiated without the slightest lag. Notably, several new leaves were formed before the new growth centre became evident, indicating that despite the lack of stem cells, the pool of meristematic cells was large enough to sustain organogenesis for several plastochrons. This temporal sequence of events clearly indicates that the CZ has no direct role in organ formation and patterning of the apex, except as the ultimate source of cells (Steeves and Sussex, 1989). Mutants with perturbed CZ frequently exhibit defects in organogenesis, however, this effect is indirect. For instance, the cessation of leaf formation in the *wus* mutant is an indirect effect of stem cell depletion (Mayer et al., 1998). Similarly, the irregular phyllotaxis in the *clavata* mutants is likely to be an indirect effect of irregular enlargement of the apex (Clark et al., 1993; Clark et al., 1995).

***LeWUS* induction in the PZ precedes initiation of a new meristem centre**

Differences in the cells of the CZ and PZ have been identified

by several different means, e.g. cytological markers, gene expression profiles, cell division activity, organogenic capacity, etc. Consequently, the establishment of a new meristem centre from the periphery involves the reprogramming of cells. After ablation of the CZ, the tomato *WUS* homologue *LeWUS* was rapidly induced in the PZ, before a new meristem centre became apparent (compare Fig. 2E and Fig. 3D). We propose that *LeWUS* expression in the PZ induced the overlying cells to regenerate a new growth centre.

In *Arabidopsis*, limitation of the *WUS*-expressing OC is mediated by the CLV3 signal from the overlying stem cells (Simon, 2001; Fletcher, 2002; Gross-Hardt and Laux, 2003). The observation of ectopic *WUS* induction after ablations of the CZ is compatible with an inhibitory signal coming from the centre and acting on the periphery. However, on the basis of this experiment, it cannot be decided which cells in the CZ are responsible for *WUS* suppression. To address this question we ablated only the distal portion of the CZ, approximately eight cells wide and approximately four to five cell diameters deep (Fig. 5). This treatment left the *LeWUS* domain intact (compare Fig. 2B and Fig. 5A; 5B), but is likely to have destroyed most if not all of the overlying stem cells. Three days after ablation of the distal cells, the *LeWUS* zone had remained approximately the same size (Fig. 5), hence, ectopic induction of *LeWUS* was not observed. It is conceivable that the partial ablations led to a slower and more moderate induction of *LeWUS* or a gradual shift of the OC, which escaped detection by in situ analysis. Alternatively, considering the large difference between the responses to superficial and deep ablations, the cells in deeper layers may play a special role in preventing ectopic *LeWUS* induction. For instance, the *LeWUS*-expressing cells could inhibit *LeWUS* expression in neighbouring cells by a mechanism analogous to lateral inhibition. It is also conceivable that, as yet, unknown signals are involved. This type of micromanipulation experiments can provide useful indications of dynamic interactions that may remain hidden in genetic approaches. However, we emphasise again that with the paucity of mutants and molecular markers in tomato it is hard to arrive at conclusive molecular models.

The L₁ layer controls cell division orientation and meristem maintenance

Ablations of the L₁ layer led to local changes in cell division patterns from anticlinal to periclinal in the subtending cell layers (Figs 6, 7). This was the case irrespective of whether the ablations affected only a limited area or the entire meristem surface. This regular cell division pattern was clearly different from irregular callus-like proliferation at the base of cut primordia (Fig. 7J), indicating that it is a characteristic feature of meristem cells. Since similar aberrations in cell division patterns were not found in any other ablation, we propose that this response is not a general wound response, but is specifically due to the loss of L₁. Genetic perturbation of the embryo protodermal layer (corresponding to the L₁ layer) by L₁-specific expression of a cytotoxic gene led to defects in subtending cell layers of *Arabidopsis* root (Baroux et al., 2001). In particular, the cell division pattern was affected, resulting in supernumerary cell tiers in the embryonic root tip, similar to the development after our L₁ ablations. Thus, the L₁ layer controls cell division patterns in subtending cell layers, and prevents periclinal cell divisions.

Secondly, we observed a gradual loss of meristem identity, as judged by increasing cell expansion and decreasing *LeT6* expression. This occurred only when most of the L₁ layer was ablated. In combination with periclinal cell divisions (see above), this resulted in stacks of vacuolated cells that resembled differentiating stem cortex tissue. Therefore, L₁ not only controls cell division, but also prevents cell differentiation in lower cell layers.

Role of the L₁ layer in organ formation

It has been proposed that biophysical forces in the L₁ layer regulate organ formation and meristem patterning, with no necessity for specific chemical signals in the meristem (Green, 1996). Biophysical regulation is thought to be based on tensile and compressive forces within the meristem. According to these models, such forces result from the geometry and the growth of the apex and operate on the meristem (including the L₁) as a whole. Although computational modelling can recreate natural phyllotactic patterns (Green, 1992; Green, 1996), experimental evidence for the involvement of biophysics has proved difficult to gain. In our experiments, ablations had only local effects on organ formation and positioning, and none of the ablations had 'systemic' effects on organ formation in unperturbed parts of the meristem. Therefore, our results do not support a role for biophysical mechanisms in meristem patterning. However, we emphasise that once the site of organ formation is determined, the execution of the organogenic programme is likely to involve biophysics, particularly the modulation of cell wall properties (Fleming et al., 1997; Reinhardt et al., 1998; Pien et al., 2001).

An immediate effect of L₁ ablations was the complete block of organ formation at the ablated site, although the remaining L₂ and L₃ cells were still able to divide and to expand (see above). Could the lack of organ formation be due to the loss of meristem identity in subtending layers? We do not think so, since the loss of meristem identity developed only gradually, and after 5 days meristematic cells were still evident in L₃. In contrast, the block in organ formation was immediate and complete, since an organ was never formed at a site devoid of L₁. Therefore, the block in organ formation is unlikely to be an indirect consequence of meristem degeneration, but appears to be a direct consequence of the loss of L₁. The similarity of the defects caused by genetic L₁ ablations with the phenotypes of *bodenlos* and *monopteros* mutants may indicate a role of the L₁ layer in auxin-related patterning of the embryonic root (Baroux et al., 2001). Since leaf formation at the shoot meristem is controlled by auxin (Reinhardt et al., 2000; Kuhlemeier and Reinhardt, 2001; Reinhardt and Kuhlemeier, 2002; Stieger et al., 2002), the fact that leaf formation was blocked at sites bare of L₁, could point also to an auxin-related role of L₁ in this process. Since CZ ablations did not affect leaf formation, it is likely that the auxin-based mechanism operates in the PZ, but not in the CZ.

While leaf formation in the vegetative meristem requires the L₁ layer (see above), there is an influence of the lower layers in flower development. *lateral suppressor (ls)* mutants of tomato lack petals. However, a periclinal chimera with an *ls* mutant L₁ layer and wild type L₂ and L₃ layers has normal flowers (Szymkowiak and Sussex, 1993), indicating that in this case, the L₁ responded to organogenic signals from lower layers. It is conceivable that factors from the L₁ layers, as well

as factors from lower layers, are required to allow organ formation.

Regenerative capacities of plant cells

An important feature of L₁ ablations was the lack of regeneration. In contrast to the rapid regeneration of a new growth centre after ablation of the CZ, the L₁ could never be regenerated, even after ablations of limited extent. Although single cells that are displaced to the L₁ from the L₂ layer can adopt L₁ identity (Tilney-Basset, 1986), this depends on the presence of L₁ neighbours. Without information from neighbouring L₁ cells, L₁ identity cannot be expressed in L₂ cells, therefore, de novo formation of L₁ (or the epidermis) is not possible in plants (Bruck and Walker, 1985).

The re-establishment of a new CZ after ablation implies rapid and efficient regeneration of functional stem cells from organogenic cells at the periphery. This reveals a remarkable flexibility of plant cell fate compared to animals. It has long been assumed that adult animal stem cells cannot be replaced, once they are lost. However, evidence is now accumulating that under certain experimental conditions, stem cells can be (re)generated by dedifferentiation or transdifferentiation from cells with other (more differentiated) identities (Blau et al., 2001). However, this occurs at low frequency and may, in some cases, be due to activation of hidden pluripotent stem cells rather than to plasticity of differentiated cells (Weissman, 2000). It is therefore a matter of debate, to what extent regeneration of stem cells is relevant for animal development (Holden and Vogel, 2002). In contrast, establishment of new stem cells in plants is clearly part of normal development. Reinitiation of stem cells in axillary meristems is the basis for the branched architecture of plants, and routinely allows breeders to clonally propagate plants much more easily than animals.

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