Microsurgical and laser ablation analysis of leaf positioning and dorsoventral patterning in tomato

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Summary

Leaves are arranged according to regular patterns, a phenomenon referred to as phyllotaxis. Important determinants of phyllotaxis are the divergence angle between successive leaves, and the size of the leaves relative to the shoot axis. Young leaf primordia are thought to provide positional information to the meristem, thereby influencing the positioning of new primordia and hence the divergence angle. On the contrary, the meristem signals to the primordia to establish their dorsoventral polarity, which is a prerequisite for the formation of a leaf blade. These concepts originate from classical microsurgical studies carried out between the 1920s and the 1970s. Even though these techniques have been abandoned in favor of genetic analysis, the resulting insights remain a cornerstone of plant developmental biology.

Here, we employ new microsurgical techniques to reassess and extend the classical studies on phyllotaxis and leaf polarity. Previous experiments have indicated that the isolation of an incipient primordium by a tangential incision caused a change of divergence angle between the two subsequent primordia, indicating that pre-existing primordia influence further phyllotaxis. Here, we repeat these experiments and compare them with the results of laser ablation of incipient primordia. Furthermore, we explore to what extent the different pre-existing primordia influence the size and position of new organs, and hence phyllotaxis. We propose that the two youngest primordia (P_1 and P_2) are sufficient for the approximate positioning of the incipient primordium (I_1), and therefore for the perpetuation of the generative spiral, whereas the direct contact neighbours of I_1 (P_2 and P_3) control its delimitation and hence its exact size and position. Finally, we report L_1-specific cell ablation experiments suggesting that the meristem L_1 layer is essential for the dorsoventral patterning of leaf primordia.

Key words: Tomato, Meristem, Phyllotaxis, Laser ablation, Dorsoventral, Patterning, Meristem layer, L_1 layer

Introduction

The shoot apical meristem of plants is the ultimate source of all aerial parts that arise after germination (Steeves and Sussex, 1989; Weigel and Jürgens, 2002). The meristem is a specialized tissue, which continuously produces lateral organs such as leaves and flowers. These are arranged in a specific pattern, called phyllotaxis (Steeves and Sussex, 1989; Reinhardt and Kuhlemeier, 2002). It is generally assumed that pre-existing leaf primordia influence the site of future organ formation, thus resulting in a reiterative propagation of phyllotactic patterns (reviewed by Reinhardt and Kuhlemeier, 2002).

It has been proposed that the primordia are the source of a diffusible inhibitor of organ formation (Schoute, 1913) (reviewed by Steeves and Sussex, 1989). According to this idea, inhibitory fields emanate from the primordia, thus allowing new primordia to be formed only at certain minimal distances from pre-existing ones. However, recent evidence has identified an inverse mechanism in which the primordia act as sinks for the organ inducer auxin rather than as sources of an inhibitor (Reinhardt et al., 2003a). The result of this scenario is similar: new organs can only be formed at certain minimal distances from pre-existing ones, thus leading to regular arrangement of leaves.

Given the proposed role of primordia in phyllotaxis, isolating a young primordium from the meristem is expected to change the position of subsequent primordia, allowing them to arise closer to the operated site. It has been attempted to experimentally interfere with leaf positioning by separating incipient primordia from the remainder of the meristem through tangential incisions (Snow and Snow, 1931). When P_1 was isolated from the meristem by a tangential incision, I_1 arose at its normal position relative to P_1, but the angle between I_1 and I_2 increased. This may indicate that the position of I_1 was developmentally fixed at the time of the operation, whereas the position of I_2 could still change once the influence from P_1 was eliminated. I_1 has been well characterized by its distinct pattern of gene expression. It differs from the surrounding cells of the peripheral zone in that it expresses organ marker genes, such as *PINFORMED1* (Vernoux et al., 2000; Reinhardt et al., 2003a), *REVOLUTA* (Otsuga et al., 2001), *LEAFY* (Weigel et

al., 1992), and *ZWILLE/PINHEAD* (Moussian et al., 1998; Lynn et al., 1999), whereas KNOTTED1-type transcription factors, markers for meristem identity, are repressed (Jackson et al., 1994; Long and Barton, 2000). This indicates that the I_1 cells are committed to organogenesis. Gene expression analysis suggests that I_2, and perhaps even incipient primordia as early as I_3 or I_4, is distinct from the surrounding cells by the expression of organ marker genes (Otsuga et al., 2001). However, the Snow experiments have shown that the position of I_2 can be changed by the isolation of P_1, and therefore these cells are not determined (even though initial steps of commitment may have been taken). Surprisingly, when I_1 was isolated by similar tangential incisions, the position of I_2 was not affected, while I_3 was displaced. So, if I_2 is not determined (concluded from P_1 isolation), why did it not change its position after I_1 isolation?

Taken together, the experiments of the Snows supported a negative influence of pre-existing primordia on I_1, but they did not evaluate the relative influence of the different pre-existing primordia on I_1, and they opened the question of whether P_1 affects the positioning of I_1 at all. To address these issues, we decided to reassess the experiments of the Snows using the tomato in vitro meristem culture system (Reinhardt et al., 2003b). In a first set of experiments we repeated the original experiments. Our results are in line with the old data, but they also uncover an effect on elongation growth that may require a more cautious interpretation. Therefore, we used infrared laser technology to precisely ablate incipient primordia and to reduce the experimental interference to a minimum. Finally, we isolated meristems from the influence of all primordia but P_1, in order to assess the influence of older primordia on phyllotaxis.

While the young primordia influence organ positioning in the meristem, the meristem in its turn influences the development of organ primordia after their initiation. For example, the dorsoventral patterning of the leaves, that is the formation of different upper and lower leaf tissues, depends on the activity of the meristem. This was first demonstrated by the finding that the young or incipient leaf primordia of potato developed as radially symmetric finger-like structures when they were surgically separated from the meristem (Sussex, 1951; Sussex, 1955). However, these experiments were controversial at the time (Snow and Snow, 1954a; Snow and Snow, 1954b; Sussex, 1954), and have not been repeated since, neither in potato nor in other species.

Genetic analysis has identified several putative transcription factors that are required for the specification and development of the upper (adaxial) and lower (abaxial) leaf surface (Bowman et al., 2002). Recent evidence indicates that microRNAs (miRNAs) act in the abaxial domain of the leaf primordia by silencing adaxializing transcription factors (Emery et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004). By contrast, the proposed meristem-borne signal that instructs adaxial cells to adopt their correct identity remains elusive. Here, we report on new evidence supporting a function of the meristem in the specification of the adaxial leaf domain, and we explore the role of the L_1 layer in this process.

Materials and methods

Plant growth and in vitro culture

Tomato plants (*Lycopersicon esculentum* cv Moneymaker) were grown as described previously (Reinhardt et al., 1998). Shoot apices were dissected and cultured according to Fleming et al. (Fleming et al., 1997) on MS medium containing 0.01 μM giberellic acid A_3 (Fluka) and 0.01 μM kinetin (Sigma).

Microsurgery and laser ablations

Separation of the site of incipient primordium formation from the remainder of the meristem was carried out with small pointed scalpel blades (Bard-Parker® #11, Becton Dickinson, New Jersey, USA). Removal of the L_1 layer was carried out as described (Reinhardt et al., 2003b). Superficial ablation of the L_1 layer between the site of primodium formation and the meristem was carried out with drawn glass needles. The ultimate tip of the needle was removed, and the sharp edge of the remaining tip was used to superficially scratch the L_1 layer. Laser ablation of the site of incipient leaf formation was performed essentially as described (Reinhardt et al., 2003b). A Q-switched Er:YAG laser emitting infrared radiation at a wavelength of 2.94 μm was used to direct 10 consecutive pulses (2 Hz) of 1.5 mJ per pulse at a circular area of approximately 40 μm in diameter on the surface of the meristem.

Microscopy

Scanning electron microscopic analysis and time-lapse photographic analysis of living tomato apices was carried out as described (Reinhardt et al., 2003b).

Results

Effects on phyllotaxis of the isolation or laser ablation of the incipient leaf primordium

Classical microsurgical studies on *Lupinus albus* have indicated that the isolation of young or incipient leaf primordia from the meristem leads to an altered phyllotaxis in a way that is compatible with an inhibitory influence originating from primordia (Snow and Snow, 1931). However, the Snows assessed only the final outcome of the experiment 3-5 weeks after the operation, and emphasized the circumferential aspect of phyllotaxis, while the vertical growth of the apex received little attention. In addition, the question remained open as to what extent the described effects of the operations on phyllotaxis could have been influenced by wound-related effects. We therefore decided to reassess the Snows' conclusions by repeating their experiments in tomato, and by comparing them with other surgical and laser ablation techniques.

Before we describe the experiments, it is useful to define the nomenclature used in this study. We designate the youngest primordium as P_1, and the older primordia as P_2-P_n, according to increasing age. Correspondingly, I_1 designates the first incipient primordium, and I_2-I_n designates the following primordia in the succession of their appearance. In order to avoid confusion, this nomenclature was applied to the initial situation at the beginning of the experiment (t_0) and remained unchanged during the course of the experiment.

First, we repeated the experiment of the Snows. We dissected tomato apices and separated the region of presumptive leaf formation (I_1) from the remainder of the meristem shortly before primordium emergence (indicated by the fact that P_1 was well developed; Fig. 1A,B). Despite this interference, 89% of the isolated initials grew out at the expected position (16 out of 18) (Fig. 1B-D). We then determined the successive divergence angles between I_1 and subsequent primordia (I_2, I_3, etc.), each at the time of

primordium emergence. This immediate determination avoids errors due to later distortions caused by the interaction with neighbouring leaves, or errors due to wound responses (e.g. callus growth).

Like I_1, the next primordium after the operation, I_2, was initiated at the expected position (Fig. 1D, Fig. 2). However, the second next primordium, I_3, was consistently formed at an increased divergence angle relative to I_2 (Fig. 1E, Fig. 2). Furthermore, I_3 grew to be larger than normal in 17% of the cases (3 out of 18; Fig. 1G). Notably, when I_3 was formed, the apex had grown considerably in the vertical direction, thus leading to an increased vertical distance between I_1 and I_3 (Fig. 1H, compare with Fig. 1I). Thus, isolating I_1 from the meristem has two effects: an increased divergence angle between I_2 and I_3, and an increase in vertical growth of the apex. In addition, the rearrangement of the apex resulted in post-meristematic changes of divergence angles (compare Fig. 1B with 1D)

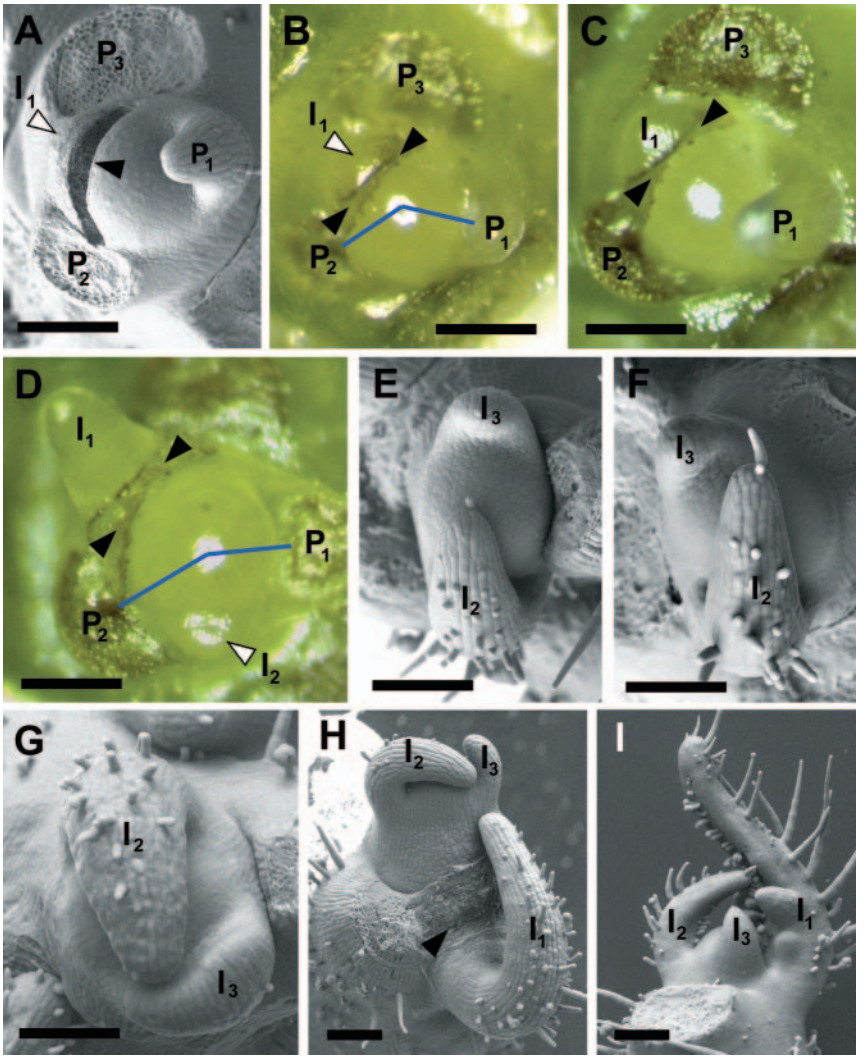

Fig. 1. Isolation of the site of incipient leaf formation (I_1) from the meristem affects phyllotaxis. (A,E-I) Scanning electron micrographs (SEM); (B-D) light stereomicrographs. (A) Tomato meristem in top view. The site of incipient leaf formation (I_1), which can be predicted to be on the upper left part of the meristem (white arrowhead), was separated from the remainder of the meristem by an incision (black arrowhead) just before imaging. (B) Tomato apex just after operation as in A. (C) The same apex as is shown in B 1 day after operation. (D) The same apex as is shown in B, 3 days after operation. P_1 was removed to expose the meristem. I_2 has initiated at the expected position. Note an apparent post-meristematic increase in the divergence angle between P_1 and P_2 (blue lines), compare with (B). (E) The same apex as is shown in B, 6 days after operation. Note that I_2 (bottom) and I_3 (top) diverge by approximately 180°. (F) Control apex with normal divergence angle between I_2 and I_3. (G) Tomato apex 6 days after operation as in A. Note the extended width of the I_3 primordium. (H) Apex 6 days after operation as in A. The vertical distance between I_1 and I_2, is strongly increased compared with a control (I). P_3 and P_2 indicate the bases of pre-existing leaf primordia that were removed at the beginning of the experiment, and P_1 represents the youngest primordium; I_1, I_2 and I_3 indicate primordia formed after the operation. Black arrowheads indicate the incisions. Blue lines in B and D represent the divergence angle between P_1 and P_2. Scale bar: 100 µm.

Incisions such as the ones performed by Snow and Snow, and those in the present study (Fig. 1), do not only interrupt signalling between primordia and the meristem, but inevitably cause major tissue damage of the meristem. It therefore seems plausible that the effects of incisions could have been influenced by wound responses. Laser ablation has successfully been employed to study interactions between cells and tissues in meristems of the root and the shoot (Van den Berg, 1995; Van den Berg, 1997; Reinhardt et al., 2003b). The precision of the laser allows ablations to be performed with a minimum of tissue destruction. To test the effect of primordium elimination, we performed laser ablations at the meristem periphery at the position of incipient leaf primordium formation (I_1). The development of two representative examples is documented in Fig. 3. In 50% of the cases (n=26), the new primordium (I_1*) was initiated in the vicinity of the lesion, resulting in an increased divergence angle (Fig. 3A-D), or in a decreased divergence angle (data not shown) between P_1 and I_1*. However, this deviation was transient, and the meristem rapidly re-established normal spiral phyllotaxis (Fig. 3D). In 12% of cases, I_1* formed at an ectopic position between the ablated I_1 position and I_2 (Fig. 3F). This led to the reversal of the phyllotactic spiral (Fig. 3G,H). In the remaining cases (38%), the primordium was initiated below the lesion, with no significant deviation in divergence angle, and with no recognizable consequences for further phyllotaxis. Notably, vertical elongation, as seen after incision into the meristem (Fig. 1), was not observed after laser ablation.

In conclusion, after laser ablation of the I_1 position, the next primordium was formed either in close vicinity of the lesion, or at an ectopic position (but not at I_2). Therefore, precise removal of incipient primordia by laser ablation yielded results that were different from those obtained by tangential incisions (Fig. 1) (Snow and Snow, 1931). Conceivably, laser ablation revealed a flexibility of the meristem that was unnoticed after surgical incisions,

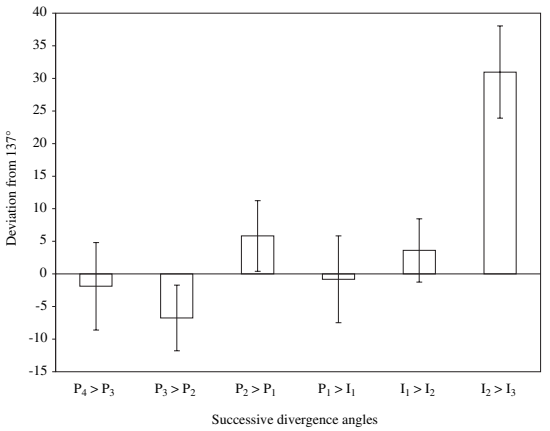

Fig. 2. Effect of I_1 isolation from the meristem on the divergence angles. Each angle was determined at the actual time point of primordium initiation, not at the end of the experiment. The angle between I_2 and I_3 deviates by approximately 30° from the mean phyllotactic divergence angle (137°).

because the fragmentation of the peripheral zone (PZ), and the resulting indirect growth response, influenced the effects on phyllotaxis in specific ways, and restricted the potential of the meristem to respond with ectopic organogenesis. The tendency of I_1* to be formed in proximity to the original I_1 position indicates that the mechanism of leaf positioning remained active after laser ablation at I_1.

The effect on phyllotaxis of removing all primordia but P_1

An important observation after isolation of I_1, both in this study as well as in the study of the Snows, was the fact that I_2 did not respond to the isolation of I_1. Could the position of I_2 have already been fixed? The fact that I_2 was displaced when P_1 was isolated (Snow and Snow, 1931) does not support this possibility. If the isolation of I_1 has no effect on the position of I_2 (although the latter is not fixed), does this mean that under natural conditions, primordia are not influenced by their next older predecessor? If this were the case, which would be the primordia that determine leaf position?

Two principal scenarios could be envisaged: A new primordium (I_1) could be influenced by the two previous primordia (P_1 and P_2), with P_1 having the stronger effect so that I_1 comes to lay closer to P_2. Alternatively, the position could be determined by the two immediate neighbours, which would be P_2 and P_3 in the case of tomato. In very large meristems, as in the case of the sunflower capitulum, it is likely that the latter mechanisms applies, as the distance between P_1 and I_1 is very large compared with the distance of P_1 to its immediate contact neighbours. By contrast, in small meristems, such as in tomato and most other plants, the meristem appears small enough to permit an influence of P_1 on I_1.

To distinguish between these two possibilities, we isolated tomato meristems from the apex in a way that left only one primordium (P_1) attached to them. Although this operation inevitably creates a large wound, it differs in two important ways from the tangential incisions carried out by the Snows and in this study. First, the meristem as such is not damaged, as the cut was made just below the peripheral zone, and secondly, any wound effect would act equally on the entire circumference of the meristem. Hence, effects like a shift of the growth axis or distortion of divergence angles are not expected to occur. Isolated meristems were cultured on MS medium and further organogenesis was observed. Eighteen of 64 isolated meristems (28%) developed in culture and continued to form leaf primordia. The first new primordium (I_1) was formed at the expected position (Fig. 4B, compare with 4A). However, in many cases the primordia were oversized (n=10; Fig. 4B,C). This was true particularly for P_1, which in five cases grew approximately to double width with two tips (Fig. 4D). Such wide and fused primordia resemble the primordia induced by the ectopic application of IAA to tomato meristems (Reinhardt et al., 2000). The next

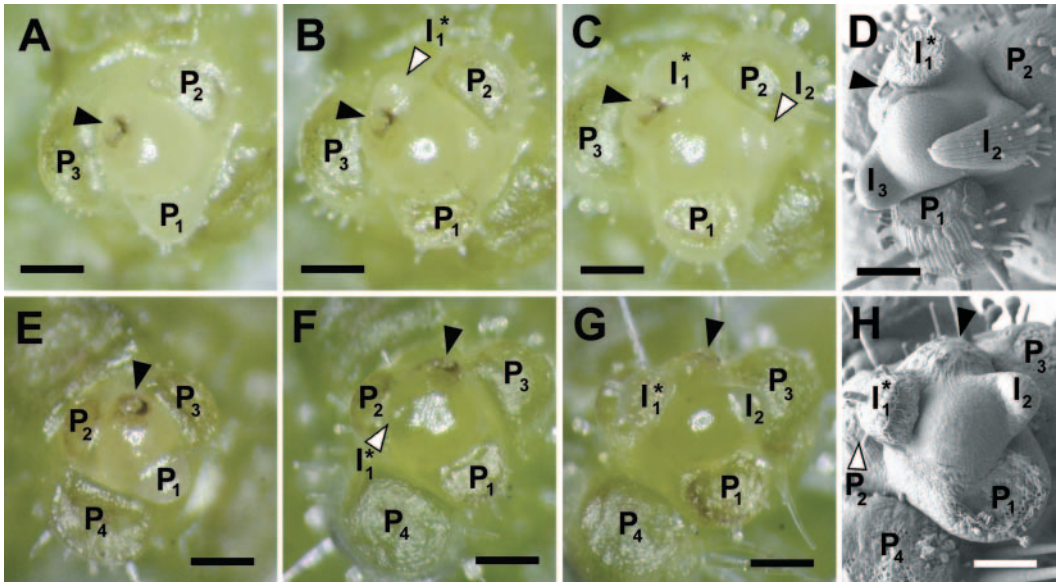

Fig. 3. Laser ablation of the site of incipient leaf formation (I_1) affects phyllotaxis. (A-H) Two individual tomato apices imaged from the top, 1 day (A,E), 2 days (B,F), 3 days (C,G) and 4 days (D,H) after laser ablation of I_1. (A-C,E-G) light stereomicrographs; (D,H) SEM images. (A) The site of incipient leaf formation (black arrowhead) was ablated with 10 successive laser pulses as described previously (Reinhardt et al., 2003b). (B) I_1* initiates adjacent to the ablation. The divergence angle between P_1 and I_1* is increased to approximately 175°, whereas the angle between I_1* and I_2 (C) is smaller than normal (107°). The following angle between I_2 and I_3 (D) is normal again (138°). (E) Ablation as in A. I_1* was formed just above P_2 (F), resulting in the reversal of the phyllotactic spiral from an anti-clockwise to a clockwise direction (G,H). P_4, P_3 and P_2, indicate the bases of pre-existing leaf primordia that were removed at the beginning of the experiment, and P_1 represents the youngest primordium; I_1, I_2 and I_3 indicate primordia formed after the ablation. Scale bar: 100 μm.

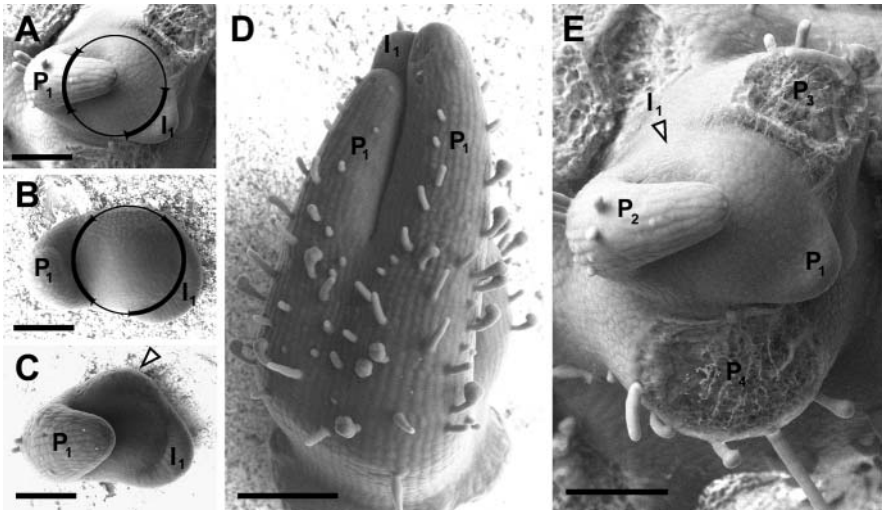

Fig. 4. Isolation of the meristem from all primordia but P_1 leads to wider leaves. (A) Control tomato apex in top view. The approximate delimitation of the meristem is represented by a circle. The arc encompassed by the primordia is represented by thickened portions of the circles. (B) Top view of a meristem 2 days after its isolation. Note the increased lateral width of P_1 and I_1 (compare with A). (C) Meristem as shown in B, with I_2 becoming visible as a small bulge (white arrowhead). (D) An oversized P_1 primordium with two tips, one week after isolation of the meristem. (E) Control apex shown in A showing the direct contact neighbours of P_1 (P_3 and P_4) and of I_1 (P_2 and P_3). P_4, P_3 and P_2, indicate the bases of pre-existing leaf primordia that were removed at the beginning of the experiment, and P_1 represents the youngest primordium; I_1, I_2 and I_3 indicate primordia formed after the ablation. Scale bar: 100 μm.

primordium (I_2) was formed approximately at the expected position, thus the direction of phyllotaxis was not reversed (Fig. 4C). However, its divergence angle was more variable, conceivably as a consequence of the changes in size of P_1 and I_1. Taken together, these results indicate that P_1 and I_1 are sufficient to determine the approximate region of I_2 (and therefore to propagate the generative spiral), whereas the older primordia (P_2-P_4), which are the direct neighbours of P_1 and I_1 (Fig. 4E), are necessary to determine the boundaries of P_1 and I_1, and thereby their exact width and position. The large increase in the width of P_1 demonstrates that it was able to recruit excess cells, even after the onset of outgrowth, if its contact neighbours P_3 and P_4 were absent.

Regulation of dorsoventral leaf patterning and lateral leaflet formation by the meristem

After its initiation, a leaf is subject to dorsoventral patterning, which leads to the formation of a specialized upper (adaxial) and lower (abaxial) leaf surface. In potato, surgical isolation of young or incipient primordia from the meristem leads to the formation of radialized primordia that lack a leaf blade (Sussex, 1955). This indicates that a signal from the meristem is required for dorsoventral patterning of the primordia, and for the formation of the leaf lamina. Although the initial observations were at first controversial (Snow and Snow, 1954a; Snow and Snow, 1954b; Sussex, 1954), the concept of a meristem-borne signal that confers adaxial identity to the portion of the primordium that is adjacent to the meristem became widely accepted (Steeves and Sussex, 1989; Bowman et al., 2002). Tomato has compound leaves, i.e. the leaves consist of a central rachis with a terminal leaf blade, and two rows of lateral leaflets (Sinha, 1999). It was therefore of interest to see whether compound leaf architecture also depends on signals from the meristem. Overexpression of the meristem gene Tkn1 in leaves leads to supercompound leaf architecture (Hareven et al., 1996); however, this is probably due less to the direct regulation of leaflet initiation than to the spatial and temporal extension of a state of meristematic competence that allows repetitive leaflet formation.

We surgically separated, by tangential incisions, young leaf primordia (or the site of incipient leaf formation) from the meristem (Fig. 1A), and followed their development. However, before reporting on the effect of this operation, it is useful to give a detailed description of normal leaf development in tomato (Fig. 5A). The first obvious sign of dorsoventral patterning of tomato leaf primordia is their curvature towards the centre of the meristem (e.g. P_1 in Fig. 1A). Later, the primordia form characteristic trichomes on the adaxial (towards the stem, upper side of the mature leaf) and abaxial (away from the stem, lower side of the mature leaf) surface of the primordium. The abaxial side exhibits three types of trichomes: long linear, short linear, and few globular trichomes, which are distributed relatively sparsely (Fig. 1I, Fig. 5A). By contrast, the adaxial side has only globular trichomes, which are concentrated in a dense stripe along the central axis of the primordium (Fig. 5A). Thus, adaxial and abaxial identity can clearly be distinguished by their trichomes. After the onset of trichome formation, the leaf primordia initiate lateral leaflets in a basipetal fashion, i.e. new pairs of leaflets are successively formed at the base of the primordium (Fig. 1I, Fig. 5A) (Sinha, 1999). The leaflets grow towards the adaxial side of the primordium, i.e. they point to the meristem (Fig. 1I, Fig. 5A).

When incipient tomato leaf primordia were separated from the meristem, 15 out of 23 (65%) exhibited a partial or complete loss of lateral leaflets (Fig. 5C-F, compare with 5A,B). Sometimes only one central fused leaflet was formed on the adaxial side of the primordium (Fig. 5C), while the distal portion of the leaf developed normally. In 7 out of 23 cases (30%), the primordium developed apparently normal, and one apex died (4%) (data not shown). These results are in line with earlier reports that a signal from the meristem is required for normal dorsoventral patterning (Sussex, 1951; Sussex, 1955). Cases in which the loss of dorsoventral patterning was incomplete, i.e. a small dorsoventral portion of the primordium was retained at the distal end, suggest that the adaxial domain was lost (Fig. 5D). This notion was confirmed by the morphology and distribution of the trichomes on radialized primordia. They were primarily long and linear, with fewer interspersed short linear and globular trichomes, like on the abaxial side of normal primordia (compare Fig. 5F with 5A). Thus, separation from the meristem has the same consequence as do loss-of-function mutations in the *Antirrhinum* MYB transcription factor PHANTASTICA (Waites and Hudson, 1995; Waites et al., 1998), and as does the overexpression of genes of the *YABBY* and *KANADI* families in

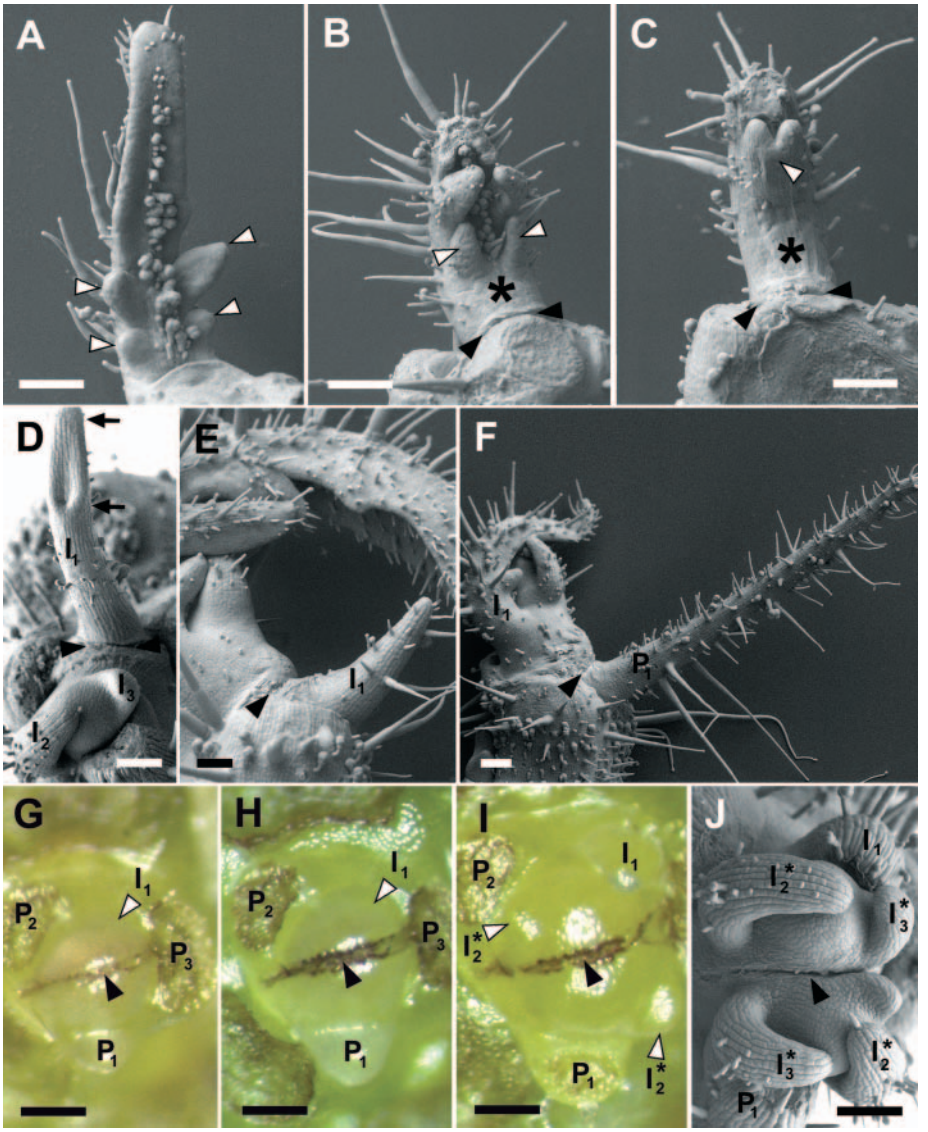

Fig. 5. Isolation of the site of incipient leaf formation (I_1) from the meristem leads to defects in dorsoventral patterning of the isolated primordium. (A) Leaf primordium of an untreated control apex. Note the difference in trichome morphology on the adaxial side compared with on the abaxial side. Abaxial trichomes are mostly long and linear, with interspersed short linear and globular trichomes. Adaxial trichomes are exclusively globular, and are arranged in a row along the central axis of the primordium. The lateral leaflets (white arrowheads) emerge from the edge between the adaxial and abaxial domain, and point to the meristem (removed for better visibility). (B) Leaf primordium with weak dorsoventral defects. The upper half of the primordium was removed for better visual access. Note the more central position of the second leaflet pair (white arrowheads), and the absence of globular trichomes on the basal portion of the adaxial side (asterisk). (C) Leaf primordium with intermediate dorsoventral defects. The upper half of the primordium was removed for clarity. Note the fused single leaflet in the centre (white arrowhead), and the absence of globular trichomes below the leaflet (asterisk). (D) Leaf primordium with strong dorsoventral defects from the same apex as is shown in Fig. 1B-E. I_1 is completely radialized, except for a small distal portion (between arrows). (E,F) Leaf primordia that lack any sign of dorsoventral patterning. The primordium in E is retarded, whereas the primordium in F grew out to a normal length. Note that the trichomes around the entire circumference of the primordia correspond to abaxial trichomes (compare with A and B). (G-J) Development of an apex after incision through the meristem centre. The meristem continues to grow and to form leaf primordia (H, 1 day; I, 3 days), and finally splits (J). I_1 and the following primordia exhibit normal dorsoventral curvature. 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 operation. Black arrowheads indicate the incision. Scale bar: 100 μm.

Arabidopsis (Bowman et al., 2002). In all of these cases, adaxial tissues were converted into abaxial tissues.

The defects in dorsoventral patterning could potentially be caused by the wound itself, rather than by the isolation from a specific patterning signal. To control for wound effects, we performed incisions of a similar extent through the centre of the meristem (Fig. 5G; n=12). Such meristems continued to develop and to form leaf primordia (Fig. 5H-J). The primordia exhibited normal dorsoventral patterns, even if they were initiated next to the lesion (Fig. 5J). This was evident by their characteristic curvature towards the meristem (Fig. 5J), and later by the formation of lateral leaflets and normal trichomes (data not shown). As a result of the incision, the two halves reorganized into two new meristems. The result of this control experiment shows that wounding is not sufficient to induce radialized primordia, indicating that the loss of dorsoventral pattern in surgically isolated primordia is a consequence of the lack of contact with the meristem, and is not due to wounding. Thus, our results are in agreement with those of Sussex (Sussex, 1951; Sussex, 1955), and similarly, we conclude that dorsoventral patterning is dependent on a signal from the meristem. Taken together, separation of primordia from the meristem caused two defects: the loss of lateral leaflets and the loss of dorsoventral patterning.

A role for the meristem L_1 layer in dorsoventral patterning of the leaf primordia

The meristem consists of different subdomains, the central zone (CZ) and the peripheral zone (PZ), and it can be subdivided in three clonally isolated cell layers, L_1, L_2 and L_3 (Steeves and Sussex, 1989). Therefore, a certain region of the meristem could potentially play a principal role in the production or transmission of the adaxializing signal. Alternatively, the meristem as a whole could produce and release the signal. Laser ablations of the central zone (CZ) did not affect dorsoventral patterning in a measurable way (Reinhardt et al., 2003b), therefore, the CZ cannot be the exclusive source of the adaxializing signal.

Table 1. Formation of radial symmetric leaf primordia after ablations of the L_1 layer

Treatment	Number of apices	Radially symmetric P_1	Radially symmetric I_1	Meristem termination
Controls	10	–	–	–
0-25% ablated	15	–	1	–
25-50% ablated	10	–	–	–
50-75% ablated	22	–	4	8
75-100% ablated	27	3	5	26

The L_1 layer, which is clonally separated from the subtending cell layers by its stereotypical, anticlinal cell division pattern, plays important roles in meristem function and organ development (Baroux, 2001; Abe et al., 2003; Reinhardt, 2003a; Reinhardt, 2003b). The cells that give rise to the meristem L_1 layer (and the entire epidermis) are set aside early during embryogenesis at the dermatogen stage (Jürgens, 2003). These cells attain a specific identity that is characterized by the expression of L_1-specific genes, such as AtMERISTEM LAYER1 (AtML1), FIDDLEHEAD (FDH), PROTODERMAL FACTOR1 (PDF1) and PDF2, (Lu et al., 1996; Yephremov et al., 1999; Abe et al., 2001; Abe et al., 2003). We wanted to assess the role of the L_1 layer in dorsoventral patterning of primordia.

The L_1 layer can be surgically removed from the meristem (Reinhardt et al., 2003b). Removal of the entire L_1 layer results in a gradual degeneration of basic meristem functions, and in the inhibition of organ formation (Reinhardt et al., 2003b). In order to assess the role of the meristem L_1 layer in dorsoventral patterning of adjacent leaf primordia, we removed the L_1 layer to various extents, while leaving the youngest primordium (P_1) intact. We then followed in detail the consequences for the development of P_1, and for further primordium formation (Table 1; Fig. 6). Removal of up to 50% of the L_1 layer had only local effects. P_1 developed normally and leaf formation continued, but only from the area with an intact L_1 layer. The meristem rapidly shifted its centre away from the wound, and continued to form normal leaf primordia at a normal rate (Reinhardt et al., 2003b). Owing to the ablation and to the shift of the growth centre, the phyllotactic angles were sometimes irregular (data not shown). In only one case did a primordium exhibit a dorsoventral patterning defect after removal of less than 25% of the L_1 surface (Table 1). When 50-75% of the L_1 layer was removed, 36% of the apices (8 out of 22) terminated after the formation of one new primordium (I_1). In half of these cases (4 out of 8), this primordium was radially symmetric (Fig. 6B; Table 1), while P_1 developed normally. Removal of 75-100% of the L_1 layer resulted in termination of almost all meristems (26 out of 27; Table 1). However, 38% of these apices (10 out of 26) formed a last primordium (I_1) before termination, which was, in 50% of the cases, radially symmetric (5 out of 10). Of the P_1 primordia, only 11% (3 out of 27) developed as partially or entirely radialized primordia (Fig. 6C-E; Table 1), whereas the others developed normally (Fig. 6F).

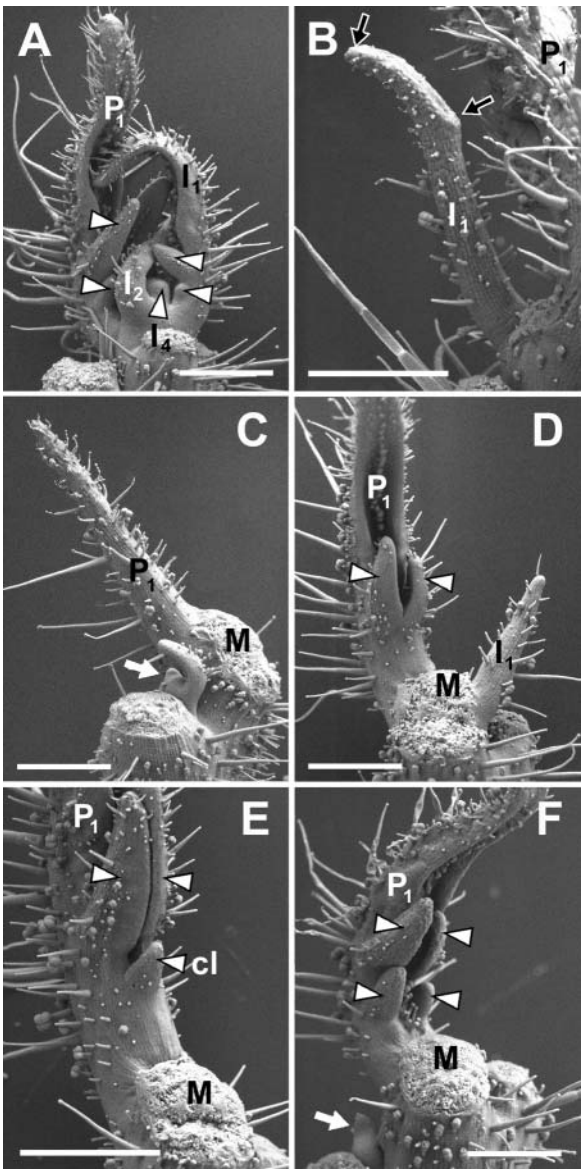

Fig. 6. Formation of radially symmetric primordia after ablations of the L_1 layer. (A) Control tomato apex after 7 days in tissue culture. Besides P_1, which was the youngest primordium at the beginning of the experiment, four new primordia were formed in clockwise phyllotaxis. The youngest (I_4) is just becoming evident at the flank of the meristem. (B-F) Tomato apices 7 days after the removal of 75-100% of the L_1 layer. The apices had one preformed primordium (P_1) at the time of the operation. (B) I_1 has developed into a radially symmetric organ with a small adaxial domain at the distal end (between arrows). (C) P_1 has developed into a radially symmetric primordium. The trichomes on its surface are typical for the abaxial side of normal leaf primordia, indicating that the radially symmetric primordium has only abaxial identity. The white arrow indicates an axillary meristem growing from the leaf base of P_3. (D) P_1 developed almost normally in the distal part; however, at the base, the adaxial domain is lost after the formation of one pair of leaflets (white arrowheads; compare with P_1 in Fig. 5A). One additional primordium was initiated (I_1), which developed to be completely radially symmetric with only abaxial identity, based on the morphology and the distribution of the trichomes. (E) P_1 has developed similarly to the primordium shown in D. After the formation of one pair of leaflets (arrowheads), the adaxial domain terminates with a single central leaflet (cl). (F) P_1 has developed normally (compare with P_1 in A), and exhibits two pairs of leaflets (white arrowheads). Axilllary meristems of older primordia are induced to grow out (arrow). M, meristem; P_1, youngest preformed primordium; I_1, I_2, I_3 and I_4, first, second, third and fourth primordium, respectively, formed after the ablation. Scale bar: 200 μm.

These results show that even preformed leaf primordia can loose their initial dorsoventral pattern once the L_1 layer is lost. However, even small portions of the L_1 layer appear to be sufficient to promote dorsoventral patterning, even in situations where the meristem aborts (Fig. 6F). As in the case of surgical separation from the meristem (Fig. 5C,D), primordia of apices with L_1 ablations were frequently radialized only in the proximal parts (including the lack of leaflets), whereas the distal portion exhibited a normal dorsoventral pattern with the initiation of the leaf blade (Fig. 6B,D,E). Primordia that were radialized exhibited abaxial identity, as determined by the morphology and distribution of their trichomes (Fig. 6C,D compare with Fig. 5A).

It could be argued that the loss of dorsoventrality after L_1 ablations was simply due to the degeneration of the meristem, and that it does not suggest a specific role of the L_1 layer. However, the similar range of patterning defects (compare Figs 5, 6), as well as the comparable frequences of dorsoventral defects between incised and L_1-ablated meristems (Table 1), indicates that the L_1 ablations had an effect equivalent to the surgical separation, which is likely to be effective immediately. By contrast, the degeneration of the meristem after L_1 ablations proceeded slowly over several days (Reinhardt et al., 2003b). These results are compatible with a direct role of the L_1 layer in the establishment and maintenance of the dorsoventral pattern in leaves. For example, a signal for dorsoventral patterning could be produced and transported to the developing primordia through the L_1 layer.

To test this possibility more directly, we performed narrow, superficial ablations of the L_1 layer that left the majority of the L_1 layer intact, but separated the L_1 layer of I_1 or P_1 from the L_1 layer of the remainder of the meristem by a small corridor (further referred to as corridor ablations; Fig. 7A,B). This operation was expected to have no consequences for meristem function and maintenance, but it would interrupt the adaxializing signal, if it were transported through the L_1 layer. We operated 30 apices shortly before (I_1), and 10 shortly after, primordium initiation (P_1). Four apices operated at P_1 (40%) showed varying degrees of dorsoventral patterning defects (Fig. 7C-F). Of the apices operated at I_1, 13% showed partial defects in dorsoventral pattern (similar to Fig. 6D,E), and one was completely radialized (Fig. 7G). Because, unexpectedly, the percentage of effects was lower when the corridor ablation was performed at the I_1 stage than at the P_1 stage, we repeated the experiment with 40 apices from which 20 were operated at the I_1 stage and 20 at the P_1 stage. To assess the extent of the defects more precisely, we distinguished between partially radialized primordia, which were dorsoventral in their distal portion and radialized in their proximal portion, and fully radialized primordia, which failed to exhibit any sign of dorsoventral patterning and had only abaxial trichomes (Table

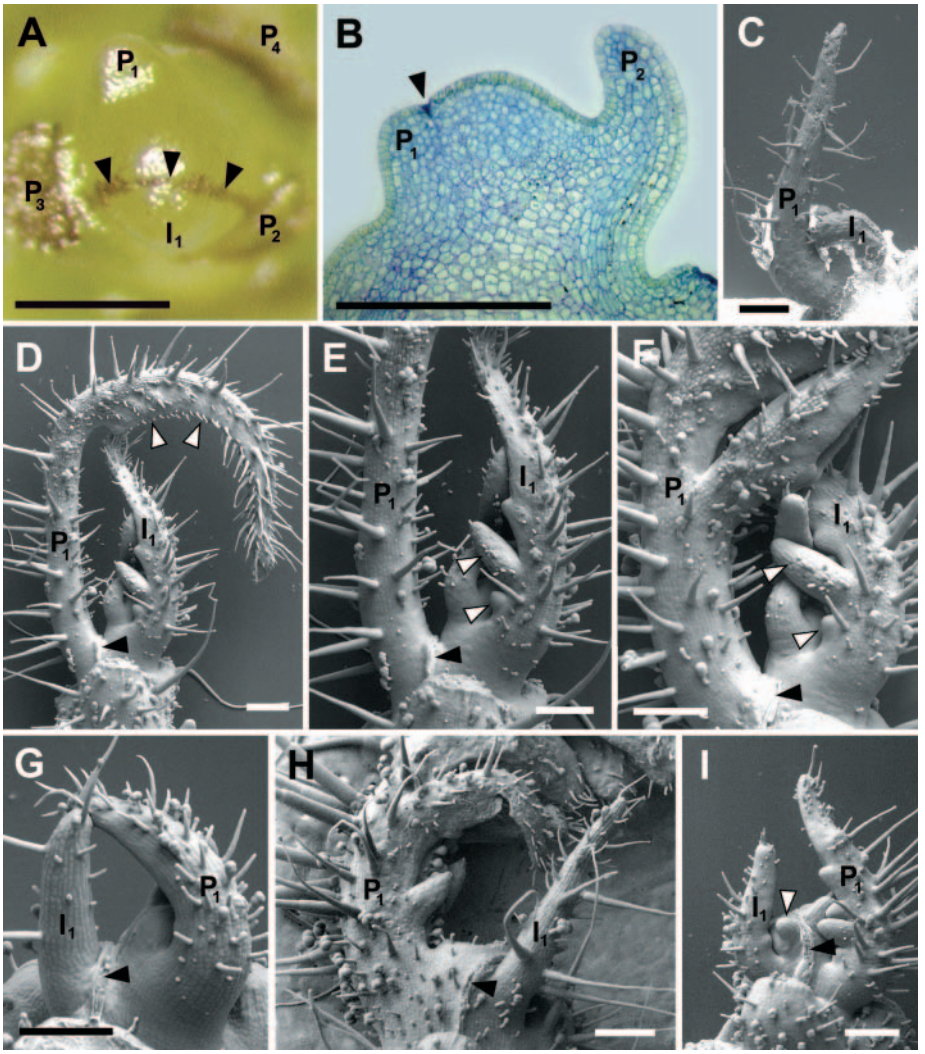

Fig. 7. Partial isolation of the site of incipient leaf formation (I_1), or of the youngest primordium (P_1), from the meristem by a superficial incision leads to defects in dorsoventral patterning of the isolated primordium. (A) Tomato apex immediately after isolation of I_1 by an ablation of the superficial L_1 layer (black arrowheads). (B) Semi-thin section of a tomato apex immediately after isolation of P_1 by an ablation as in A (black arrowhead). (C) Tomato apex 8 days after isolation of P_1 as described in A. P_1 lacks any sign of leaflets or of a developing leaf blade. The trichomes exhibit only abaxial features (compare with Fig. 5A). (D-I) Tomato apices 8 days after operation as in A. (D) P_1 lacks leaflets but has developed a leaf blade (white arrowheads). (E) Close up view of D. Note the lack of leaflets in P_1, compared with I_1 (white arrowheads). (F) P_1 exhibits only one pair of leaflets, compared with I_1, which has already formed two pairs of leaflets (white arrowheads). (G) I_1 is retarded and lacks any sign of dorsoventral patterning. (H) Completely radialized I_1 of approximately normal size. (I) Initiation of an accessory meristem (white arrowhead) above the operated I_1 position. P_4, P_3 and P_2 indicate the bases of pre-existing leaf primordia that were removed at the beginning of the experiment, and P_1 represents the youngest primordium; I_1 indicates the first primordium formed after the ablation. Scale bar: 200 μm.

Table 2. Formation of radial symmetric leaf primordia after corridor ablations of the L_1 layer

Treatment	Number of apices	Normal	Partially radialized	Completely radialized
I_1	20	12 (60%) 9 split	7 (35%) 2 split	1 (5%)
P_1	20	9 (45%) 1 split	3 (15%)	8 (40%)

2; Fig. 7). Again, primordia operated at the I_1 stage showed defects at a lower rate and of weaker severity (35% partially, 5% completely radialized; Fig. 7H), compared with primordia operated at the P_1 stage, where 40% were fully, and a further 15% partially, radialized (Table 2; Fig. 7). Interestingly, more than half of the apices operated at I_1 initiated an accessory meristem above the primordium (11 of 20; Fig. 7I). In such cases, the primordium was frequently normal (9 cases), and in only two cases was it partially radialized. Such an accessory meristem was only formed once in apices operated at the P_1 stage. It thus appears likely that the accessory meristems formed after operations at I_1 provided the dorsoventral signal, thereby lowering the frequency of radialized primordia. In conclusion, the fact that corridor ablations provoked similar defects at comparable frequencies to deep tangential incisions or complete L_1 ablations demonstrates that the continuity of the L_1 layer is essential for the establishment of the adaxial domain of leaf primordia.

Discussion

The positioning of new primordia is influenced by the pre-existing primordia

Early studies on *Lupinus albus* showed that microsurgical separation of incipient leaf primordia (I_1) from the meristem influenced the positioning of new primordia (Snow and Snow, 1931). Whereas the next primordium (I_2) was formed at the expected position with a divergence angle of approximately 137°, the following primordium (I_3) was 'displaced' in the circumferential dimension towards the isolated I_1, resulting in an increased divergence angle between I_2 and I_3. This led to the interpretation that the isolation of I_1 created more space in its vicinity, which could then be occupied by the adjacent I_3 primordium.

One general limitation of the Snows' study was that they analyzed only the final outcome of the experiments in transverse sections of the shoot prepared 3-5 weeks after the operation. Hence, the divergence angles were measured several plastochrons after the initiation of the respective primordia. Therefore, some of the reported effects on phyllotaxis may have been influenced by wound reactions. We have detected, within 4 days after the operation, shifts of the meristem centre away from the wound, and this response clearly affected apparent divergence angles post-meristematically (Fig. 1B,D). To record the changes in divergence angles with confidence, we followed in our experiments the divergence angles starting immediately after the operation, thus, secondary distortions of the divergence angles can be ruled out.

Concerning the effects on divergence angles, we obtained results that are in agreement with the findings of the Snows. However, in addition to changes in divergence angles, we found major changes in the vertical growth (Fig. 1 and Fig. 2). The Snows analyzed the result of incisions at a time at which even I_5 and I_6 had developed into leaves with a well-developed leaf blade (Snow and Snow, 1931). Hence, initial changes in vertical growth, such as the ones reported here, may well have occurred in *Lupinus albus* as well, but could have been obscured at the time of analysis.

Upon isolation of I_1, both in the experiments of the Snows and in ours, the first angle was normal and only the second primordium was displaced. One possible explanation is that the position of I_2 is already fixed. This, however, is ruled out by the P_1 isolations, which clearly demonstrate that I_2 is not fixed, because it was displaced in this case (Snow and Snow, 1931). Another possibility is that the inflexibility of I_2 after I_1 isolation is related to wound effects that influence the response of the meristem. The incision causes major damage to the peripheral zone and thus restricts the space available for a new primordium. In contrast to the tangential incisions, the laser ablations deleted I_1 with a minimum of tissue damage to the meristem. Importantly, increased vertical growth was not observed. Thus, laser ablation did not affect the geometry and organisation of the meristem, and it allowed us to assess, more directly, the question of how spiral phyllotaxis responds to the specific elimination of a primordium.

After laser ablation of I_1 the next primordium (I_1*) was formed ectopically, either in direct proximity of the lesion (50%) or at a new position between the lesion and I_2 (Fig. 3). Hence, under these conditions, the position of the first primordium forming after the lesion was not fixed but displayed remarkable flexibility. Such changes in organ position are in line with theoretical predictions that elimination of a primordium should affect further organ formation immediately after ablation. Furthermore, the tendency of I_1* to be formed at a position close to the original I_1 position indicates that I_1 normally suppresses organogenesis in its vicinity, similar to P_1 (Reinhardt et al., 2000). After initial deviations caused by I_1 ablations, phyllotactic patterning rapidly returned to normal spiral phyllotaxis, either in the original, or in the reverse direction. This demonstrates the strong tendency to revert to spiral phyllotaxis in the case of disturbance, and underscores the stability of the spiral pattern, once the disturbance is overcome.

The contribution of the different primordia to organ positioning

Theoretical consideration, as well as experimental evidence, suggests that the positioning of new primordia is influenced by pre-existing primordia (see above). However, it is not known to what extent the different primordia contribute to this mechanism. It seems likely that the influence of a primordium decreases with its distance to the site of incipient organ formation (I_1). In a large meristem, such as the capitulum of the sunflower, where organ formation proceeds from the edge towards the centre, the closest neighbours of a new primordium are not the predecessors in the generative spiral (P_1 and P_2), but the direct contact neighbours. If the capitulum exhibits a 34:55 phyllotactic system, the direct neighbours are P_{34} and P_{55}, which are 34 and 55 plastochrons older than I_1, respectively. By comparison, P_1 and P_2 of such a system are positioned much more remotely. Therefore, it is likely that I_1 is positioned according to P_{34} and P_{55}, rather than according to

P_1 and P_2. However, in a phyllotactic system, as in tomato and many other plants, the meristem is small enough that not only the direct neighbours (in tomato which exhibits a 2:3 phyllotaxis, this is P_2 and P_3), but also P_1 could influence the positioning of a new organ.

In the experiments of the Snows, the first primordium to arise after operations was not affected. Does this mean that the youngest primordium has no effect on the positioning of the following one? We have previously proposed that the two youngest primordia influence the positioning of the following primordium, with P_1 having the stronger effect, so that I_1 comes to lay closer to P_2 than P_1, thus resulting in the characteristic divergence angle of 137° (Reinhardt and Kuhlemeier, 2002). In agreement with this idea, the youngest primordia express auxin transport proteins in a way that suggests a strong sink function for auxin (Reinhardt et al., 2003a). To distinguish between the influence of the direct predecessors and that of the direct contact neighbours on organ positioning, we isolated meristems from all influence but that of P_1. The assumption was that I_1, whose position is likely to be determined, would be formed at its normal position. However, I_2 could potentially respond to the experimental isolation with a shift. If it was positioned ectopically, this would indicate that the direct neighbours (P_1 and P_2) are important for organ positioning. If however the positioning of I_2 were not affected, then the interpretation would be that the youngest primordia (I_1 and P_1) are sufficient for leaf positioning. Taken together, our results suggest a compromise of the two possibilities. The generative spiral did not reverse in isolated meristems, indicating that I_2 was always formed in the correct direction. This finding is in accordance with surgical experiments in which isolated meristems of *Primula* formed leaves in continuity with the original phyllotactic pattern (Wardlaw, 1950), whereas similar isolations in *Lupinus* interrupted the original spiral, and led to the establishment of a new spiral system (Ball, 1952).

After isolation of the meristem, P_1 and I_1 grew considerably wider than normal (Fig. 4), indicating that in the absence of older primordia, the primordia could recruit more cells than normal. Hence, the following picture emerges for phyllotaxis under natural conditions. Because I_1 appears to be fixed, it is at the I_2 stage that organ position becomes determined. We propose that I_1 and P_1 determine the approximate location of I_2, thereby dictating the direction of the generative spiral. After the approximate positioning in the meristem, the direct neighbours (P_1 and P_2) delimit its exact boundaries, hence determining its final size and its precise radial position.

The fact that P_1 grew wider in the absence of its contact neighbours indicates that lateral restriction (and fine positioning) is a prolonged process that continues after the initiation of a primordium. Although it may seem counterintuitive at first, the conclusion therefore is that organ positioning and organ outgrowth occur concomitantly, and not sequentially. Such a mechanism would allow for the feedback mechanism that is predicted to operate in phyllotaxis and other models of pattern generation in living organisms (Meinhardt, 1994).

Control of leaflet formation and dorsoventral patterning in tomato leaves

The surgical separation of tomato leaf primordia from the meristem caused two defects. The loss of lateral leaflets and the loss of dorsoventral patterning. This raises the question whether the two processes are linked. For example, it is conceivable that leaflets, like the leaf lamina, can be formed only where the adaxial (dorsal) and the abaxial (ventral) domain of the primordium are juxtaposed. This view is compatible with the occurrence of single leaflets in a central position on the adaxial side of the rachis, instead of in a lateral position (Fig. 5C, Fig. 6E). A similar case is represented by the variably sized distal patches of adaxial identity found on primordia of *phantastica* mutants in *Antirrhinum*. In this case, the lamina of the distal leaf portion is joined on the adaxial side of the primordium, presumably marking the course of the adaxial/abaxial boundary (Waites and Hudson, 1995). This position corresponds to the position of the single central leaflets (Fig. 5C, Fig. 6E). It is therefore likely that in such cases, the central leaflet marks the boundary between the extended abaxial domain, and the reduced adaxial domain in the distal portion of the primordium. Taken together, these data indicate that lateral leaflet formation, like lamina outgrowth, occurs only at the boundary between adaxial and abaxial domains.

Dorsoventral patterning of leaves is thought to be influenced by the meristem (Bowman et al., 2002). Evidence for this notion came from early microsurgical analysis in potato (Sussex, 1951; Sussex, 1955). These experiments were challenged in the following years, and had not been repeated in other plant species since. We confirm in the present study, using three different microsurgical techniques (vertical incision, complete L_1 ablation, corridor ablation), that the meristem provides information that is required for the dorsoventral patterning of the primordia (Figs 5-7). It has been proposed that a factor from the meristem induces adaxial identity in the upper part of leaf primordia, while the lower part adopts abaxial fate by default (Bowman et al., 2002). However, despite the identification of a number of putative transcription factors, which are required for the establishment of adaxial and abaxial identities (Bowman et al., 2002), the nature of the adaxializing signal remains elusive. Recently, miRNAs have been implicated in the control of abaxial identity. These are expressed just below incipient primordia (i.e. on their abaxial side), and in the abaxial side of the leaves. There, they determine abaxial cell fate by downregulating the levels of adaxializing proteins, such as PHABULOSA and ROLLED LEAF1 on the abaxial side of the primordia (Kidner and Martienssen, 2004; Juarez et al., 2004). Thus, miRNA may represent an abaxializing signal that, in concert with the adaxializing signal from the meristem, establishes dorsoventral polarity.

We have previously shown that surgical removal of the L_1 layer from the meristem leads to a progressive degeneration of the meristem (Reinhardt et al., 2003b). However, removal of L_1 also abolished dorsoventral polarity of the last one or two primordia before the meristem arrested (Fig. 6). This radialization was observed at a similar extent and frequency to in the case of surgical separation from the meristem (50% versus 65%; compare with Fig. 5), indicating that removal of L_1 is equivalent to an immediate interruption of the adaxializing signal. This is in contrast to meristem degeneration, which proceeded slowly over several days (Reinhardt et al., 2003b). Hence, the loss of dorsoventrality after L_1 ablation preceded the loss of meristem identity in the

remaining tissue, rendering it improbable that the loss of dorsoventrality is due, indirectly, to the degeneration of the meristem. This evidence suggests a special role for the L_1 layer in the determination of dorsoventral polarity. For example, the adaxializing signal could be transported through the L_1 layer to the young primordia to induce adaxial fate in the adjacent portion of the primordium. Indeed, our data show that the continuity of the L_1 layer between the meristem and the site of primordium formation is relevant for dorsoventral patterning, as corridor ablation caused defects of a similar extent and at similar rates to the vertical incisions. Nevertheless, it remains to be clarified whether the L_2 and L_3 layer also contribute, directly or indirectly, to dorsoventral patterning.

It is noteworthy that in all experiments only a minority of the primordia were completely radialized. In most cases, the young primordia had a dorsoventral distal portion, and a proximal radialized portion of different extent. Often, this partial loss of dorsoventral pattern was acccompanied by a partial or complete loss of lateral leaflets. In tomato, the leaf primordia develop in a basipetal fashion, i.e. new leaflets are successively formed at the leaf base (Sinha, 1999). Therefore, the partially radialized primordia consist of an older distal portion with normal dorsoventrality, and a younger proximal portion that has lost its dorsoventral pattern during development. The range of dorsoventral defects observed included all possible intermediates between completely normal and completely radialized. This indicates that the adaxializing signal needs to be present during an extended period of leaf development, and that a loss of the signal during this process can abolish the dorsoventral pattern in the proximal parts at various stages of development. Thus, the establishment of dorsoventral polarity appears to be a continuous process. Such a scenario is compatible with the genetic models for dorsoventral polarization, which envisage a self-reinforcing mechanism based on the mutual inhibition of adaxial and abaxial determinants that leads to the gradual separation of the domains with adaxial and abaxial identity (Bowman et al., 2002). Furthermore, the frequent occurrence of partially radialized primordia with a normal distal portion shows that radializations are not due to the destruction of the (predetermined) adaxial domain of the I_1 position. Partially radialized primordia always exhibit a normal distal portion. As this is the part of the leaf that is formed first, the primordia must have started with an intact adaxial domain, which later lost its adaxial identity during the course of leaf development.

This work and our previous study (Reinhardt et al., 2003b) demonstrate that microsurgical techniques continue to be useful tools for studying plant development. They complement genetic analyses, and they are particularly useful in cases where it is desirable to restrict functional interference tightly in space and time. Now the challenge will be to develop new genetically based tools to elucidate the mechanisms underlying phyllotaxis and dorsoventral patterning in more detail. Such tools will be developed when we know more about the nature of the components in the signal chains. For example, once we know the nature of the adaxializing signal, its production, transport or destruction could be influenced in a tissue-specific manner. Similarly, the tissue-specific expression in subdomains of the meristem of genes involved in auxin biosynthesis, metabolism, transport and perception will allow us to rigorously test models of phyllotaxis.

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