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# *PETAL LOSS* gene regulates initiation and orientation of second whorl organs in the *Arabidopsis* flower

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## SUMMARY

***PETAL LOSS* is a new class of flower development gene whose mutant phenotype is confined mostly to the second whorl. Two properties are disrupted, organ initiation and organ orientation. Initiation is frequently blocked, especially in later-formed flowers, or variably delayed. The few petals that arise occupy a wider zone of the flower primordium than normal. Also, a minority of petals are trumpet-shaped, thread-like or stamenoid. Studies of *ptl* combined with homeotic mutants have revealed that the mutant effect is specific to the second whorl, not to organs with a petal identity. We propose that the *PTL* gene normally promotes the induction of organ primordia in specific regions of the second floral whorl. In *ptl* mutants, these regions are enlarged and organ induction is variably reduced, often falling below a threshold. A dominant genetic modifier of the *ptl* mutant phenotype was found in the Landsberg *erecta* strain that significantly boosts the mean number of petals per flower, perhaps by reinforcing induction so that the threshold is now more often reached. The second major disruption in *ptl* mutants relates to the orientation adopted by second whorl organs from early in their development. In single mutants the full range of orientations is seen, but when B function (controlling organ identity) is also removed, most second whorl organs now face outwards rather than inwards. Orientation is unaffected in B function single mutants. Thus petals apparently perceive their orientation within the flower primordium by a mechanism requiring PTL function supported redundantly by that of B class genes.**

Key words: *Arabidopsis thaliana*, Flower development, Organ initiation, Orientation, Perianth, *PETAL LOSS*, *PETAL LOSS MODIFIER*

## INTRODUCTION

Floral organs usually occur in concentric whorls within a flower, with each whorl containing a defined number of organs. Typically, there are several whorls of sterile organs, sepals and petals, surrounding and protecting the reproductive organs, stamens and carpels. The arrangement of floral organs within a flower, the floral ground plan, is defined early in flower development when the exact number and placement of organs is established.

The relative constancy of the floral ground plan in Angiosperms implies that it is under strict genetic control. While much has been learned about genes that control the identity of floral organs once they have arisen, less is known about genes that control when and where floral organs arise (Irish, 1999; Meyerowitz, 1997). In *Arabidopsis thaliana*, mutants of several genes have been described that, when mutated, consistently result in changes to the numbers of organs initiated. *Arabidopsis* flowers normally contain four outer sepals surrounding four petals in positions alternate to them. Internal to these six stamens are present, two in lateral positions and four inserted medially. The two-carpelled gynoecium occupies the centre of the flower. In mutants of three *CLAVATA* (*CLV*) genes, however, there is an increase in the number of all four floral organ types, particularly the inner whorls of stamens and carpels. Two of these genes have been cloned. One (*CLV1*) encodes a receptor kinase likely to be involved in signalling within the flower meristem that keeps in check the proliferation of the undifferentiated central zone (Clark et al., 1997). Another (*CLV3*) encodes a small extracellular protein that may act as a ligand in the same process (Fletcher et al., 1999).

On the other hand, mutations of the *SHOOT MERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*) genes can generate flowers with the complementary phenotype, reduced numbers of organs especially in the inner whorls. Both *STM* and *WUS* encode homeodomain proteins that regulate the expression of other genes, and it has been proposed that they normally promote proliferation of the central zone of the flower meristem (Long et al., 1996; Mayer et al., 1998). For both

classes of gene, therefore, mutant changes in floral organ number may reflect the secondary consequence of disruption to the balance between the dampening and promotion of proliferation of the undifferentiated central meristem. Increased or reduced numbers of organs may reflect changes in the amount of material upon which the ground plan genes can act.

One *Arabidopsis* gene likely to play a more direct role in generating the floral ground plan is *PERIANTHIA* (*PAN*) (Running and Meyerowitz, 1996). In this case, mutant flowers frequently demonstrate five-fold symmetry in their outer three whorls rather than the normal bilateral symmetry seen in the wild type. Importantly, unlike the genes described above, no changes to the size of the mutant flower primordium were detected. This is consistent with a role for *PAN* in defining the bilateral ground plan that defaults to a more regular radial pattern in the absence of PAN function (Chuang et al., 1999).

In the present study, we describe a different category of floral organ mutant. Mutations of the *PETAL LOSS* (*PTL*) gene result in the loss of just one type of floral organ. Even so, *PTL* is not absolutely required for petal initiation as some petals may arise in *ptl* mutants, especially in the first-formed flowers. In addition, a dominant modifier specific to *ptl* mutants was discovered that boosts organ production in the second whorl. The *PTL* gene (and its modifier) may support the action of an inducer that is required above a threshold level for these primordia to be initiated. At the same time, genetic evidence suggests that PTL has another unique function, that of allowing petal primordia to perceive their orientation within the flower primordium. This function is redundantly supported by the products of B function organ identity genes.

## MATERIALS AND METHODS

The *ptl-1* mutant was isolated by John Alvarez (Alvarez and Griffith, 1994) in a screen of the Columbia strain mutagenised with ethyl methane sulphonate. The *ptl-2* allele arose in the C24 strain following regeneration from root tissue transformed with T-DNA, but was unlinked to kanamycin resistance encoded by the T-DNA construct. The ecotypes Columbia (Col-3), Landsberg *erecta* (L*er*-0) and Niederzenz (Nd-1) were obtained from Elliot Meyerowitz, Bensheim (Be-0) and Enkheim (En-2) from the Arabidopsis Information Service, and Kiruna (Kr-2) from Liz Dennis. The floral homeotic mutants *agamous-1* (*ag-1*), *apetala3-1* (*ap3-1*), *ap3-3* and *pistillata-1* (*pi-1*), all in L*er* background, were available from laboratory stocks (Bowman et al., 1991). Recombinant inbred lines were obtained from Caroline Dean (Lister and Dean, 1993).

Plants were grown in seed-raising mix and perlite, and sub-irrigated with nutrient solution. Most were grown in glasshouse conditions at 22-24°C under daylight combined with continuous fluorescent lighting (Cool White). Some were grown in incubators at 16°C or 29°C using the same artificial lighting conditions. The first 20 flowers on the main inflorescence stem of 4 to 20 plants were scored unless otherwise mentioned. Comparisons were based on plants raised together. Crosses were carried out in duplicate, and genotypes confirmed by progeny testing if appropriate.

For scanning electron microscopy, samples were fixed in 2% glutaraldehyde in 25 mM sodium phosphate buffer (pH 6.8), infiltrated with 0.5% osmium tetroxide, dehydrated through an ethanol series, and critical point dried using liquid carbon dioxide. After coating with gold, samples were viewed using a Hitachi s570 scanning electron microscope. For light microscope sections, material was fixed in glutaraldehyde and dehydrated as above, embedded in LR White resin, sectioned at 2 μm, and sections stained with 0.3% toluidine blue in 1% tetraborate buffer (pH 9.0).

## RESULTS

### *petal loss* mutants result in aberrant petal development

The most obvious effect of the *ptl-1* and *ptl-2* mutations is a reduction in the number of petals per flower (Fig. 1A-C). Wild-type flowers always have four petals, one between each of the sepals. The first formed flowers in *ptl-1* mutants may have two or three petals, but the mean number per flower falls progressively such that most later arising flowers have none (Fig. 2A). The regions where petals normally arise are vacant. In the second mutant allele, *ptl-2*, flowers have more petals than *ptl-1* on average initially, but the number falls to a similar baseline level (Fig. 2A). This difference between alleles may reflect the relative strength of the mutational changes, or it may be a consequence of the difference in their genetic background. The number of petals per flower is also influenced by temperature. Both *ptl-1* and *ptl-2* plants raised at 16°C have about one more petal per flower on average in early flowers compared with those raised at 22-24°C, but plants raised at 29°C have almost no petals in any flower (data not shown). As the two mutant alleles respond similarly, the initiation process they disrupt may itself be influenced by temperature.

Strikingly, the orientation of petals within the mutant flower may also be disrupted. In wild-type petals the concave adaxial surface normally faces inward (Fig. 1B), but in *ptl* mutants it may face any direction (e.g. Fig. 1C). Control experiments show that the direction of lateral curvature of the petal blade accurately reflects the petal's orientation. In the wild type (Fig. 1D), epidermal cells of the concave (adaxial) surface are consistently more dome-shaped, with radial ridges that are more evenly spaced (Fig. 1F) than those of the convex (abaxial) outer surface (Fig. 1E). When abnormally oriented *ptl* petals are examined (Fig. 1G), the absolute association between curvature and surface structure remains. If petals now face outwards, the outer concave surface typically shows ridged conical-shaped cells (Fig. 1H) while cells of the inner convex surface are block-shaped and irregularly sculptured (Fig. 1I). Importantly, close examination of cell files in the claw of mature mutant petals reveals that they do not twist significantly.

In *ptl* mutant flowers, the majority of mutant petals are still normally oriented, but about 15-20% are reversed, and around 20-25% face sideways, mostly medially (see Table 2). The patterns of disorientation are similar in earlier- and later-formed flowers (data not shown).

The form of petals that arise in *ptl* mutant flowers is also frequently abnormal (Table 1). About one quarter are trumpet-shaped or tubular (Fig. 1J). In these, the outer surface has cells characteristic of the normally abaxial surface, and the inner surface cells are of the adaxial type. Another frequent abnormality is the occurrence of thread-like organs in petal positions. These do not have petalloid surface cells, and they are thinner than the adjacent stamen filaments (Fig. 1K). Less frequently (<10%), petals arise that contain mosaic regions of stamenoid tissue, or occasionally a petal position may be occupied by a stamen. Despite these abnormalities, more than one third of second whorl organs are petals with the normal

**Table 1. Percentage of organ types among second whorl organs of *petal loss* mutant flowers**

| Type of organ | *ptl-1* (Col) | *ptl-2* (C24) | *ptl-1 PMD** | *ptl-1 pmd-1d** |
|---|---|---|---|---|
| Petals | 35% | 44% | 66% | 79% |
| Tubular petals | 25% | 23% | 14% | 12% |
| Petal/stamen mosaics, stamens | 8% | 2% | 7% | 5% |
| Threads/filamentous organs | 32% | 31% | 13% | 4% |
| Number of organs | 174 | 203 | 231 | 926 |

*Plants segregating from a cross between Columbia and Landsberg *erecta* backgrounds; *pmd-1d* plants carry the dominant modifier, *PMD* plants are unmodified controls.

shape (Table 1), although they are often smaller, especially in later flowers. Overall the proportions of the abnormal types are similar in *ptl-1* and *ptl-2* mutants, but there are fewer stamenoid organs in the latter (Table 1).

Other organ whorls in *ptl* mutant flowers are much less affected, if at all. The first whorl sepals arise as the usual four per flower, although compared with the wild type they are more infolded, deeper in profile (Fig. 1A-C), and are sometimes fused along their edges towards the base (Fig. 1L), especially in *ptl-2* mutants. Also, in nearly one in five sepals of *ptl-1* flowers, carpelloid structures develop from their edges. These structures are mostly found in the central region, and include stigmatic papillae and ovules whose development is often arrested (Fig. 1M). In later-formed flowers, the medial sepal closest to the inflorescence stem occasionally contains patches of petal tissue along its flanks. Third whorl stamens, too, may show some abnormalities. These are manifest as an occasional additional stamen in either lateral or medial positions (seen in 9 out of 398 flowers), or a fused or bifid stamen with a single filament but with two anthers. The presence of additional stamens does not vary acropetally. The fourth whorl gynoecium is apparently normal.

Both *ptl* mutants are fully recessive, and the *PTL* locus was mapped at the top of chromosome 5, 0.5 map units above *TERMINAL FLOWER1* (4 recombinant chromosomes out of 854).

## A dominant modifier of the *ptl* mutant phenotype, *pmd-1d*, is present in Landsberg *erecta*

The *ptl-1* mutant allele was isolated in the Columbia genetic background, and *ptl-2* in C24. When either was crossed into Landsberg *erecta*, plants showing a marked increase in the number of petals per flower were observed among the $F_2$ progeny. In the first formed flowers, between five and eight petals per flower were now seen (Fig. 3A,B), although the number decreased to two or three by the twentieth flower on the inflorescence (Fig. 2B).

To deduce the genetic basis of this increase, 275 $F_2$ plants were raised, and 216 had four petals per flower and were indistinguishable from wild type, 38 had significantly more than four petals in the first flowers, while 21 had fewer than four. This is consistent with the increase being caused by an unlinked dominant modifier gene with an effect only in plants homozygous for *ptl* (the classes are not significantly different from a 12:3:1 ratio ($\chi^2_{[2]}$=4.87, 0.05<*P*<0.1)). To confirm this, 15 of the *ptl-1* $F_2$ plants with more than four petals were allowed to self-fertilise. Of these, 7 generated $F_3$ families in which all plants had more than four petals in early flowers, whereas 8 generated $F_3$ progeny one quarter of which now had significantly fewer than four. The mean numbers of petals produced by these 7 presumed $F_2$ homozygotes and 8 presumed heterozygotes had already been recorded for each of the first 20 flower positions, and the distributions were now compared and found not to differ significantly (data not shown). Thus the action of the modifier allele is fully dominant.

The gene was called *PETAL LOSS MODIFIER* (*PMD*), and the dominant *pmd-1d* allele was discovered in the Landsberg *erecta* strain. The Columbia and C24 strains are homozygous for the recessive *PMD* allele. To determine the modifier genotype of other strains, *ptl-1* (in Columbia) was crossed with the Bensheim, Enkheim, Kiruna and Niederzenz strains. In each case, no modified plants were seen among $F_2$ progeny, indicating that the modifying *pmd-1d* allele is restricted to Landsberg *erecta*. This strain also carries the recessive *erecta* (*er*) mutation, reducing plant height and pedicel elongation, but *PMD* is inherited independently of *er*, and the *er* mutation had no significant effect on the numbers of petals produced by unmodified *ptl-1* flowers. In modified flowers, however, the average number of petals was slightly higher initially, and the rate of loss somewhat faster in *er* mutant plants compared with *ER* controls (data not shown).

The *PMD* locus was mapped utilising recombinant inbred lines derived from a cross between Lansdsberg *erecta* (*pmd-1d*) and Columbia (*PMD*) (Lister and Dean, 1993). The original *ptl-1 PMD* mutant line was crossed with a selection of 32 highly recombinant lines, and $F_2$ progeny scored in each case to determine if the recombinant inbred line carried the *PMD* or *pmd-1d* allele. The results were then matched against the distribution of known molecular markers in the lines. In this way, the *PMD* locus was assigned to the middle of chromosome 1 between markers m235 and GAPB, and closer to the former.

## The modifier boosts the number of petals, and affects their form but not their orientation

Counts of the first twenty flowers in plants from an $F_2$ family segregating for both *ptl-1* and *pmd-1d* revealed that while the mean number of petals is increased in modified *ptl pmd* flowers, the number falls away acropetally in a similar pattern to that seen in unmodified flowers (Fig. 2B). [Note that the number of petals in unmodifed mutant flowers in this segregating family is higher than reported earlier for the original unmodified mutant (Fig. 2A). This is likely due to the hybrid genetic background (Columbia and Landsberg *erecta*) of the present plants.] When there are more than four petals in a *ptl-1 pmd-1d* flower, the additional petals also arise from the alterni-sepalous regions such that some now contain two petals. These arise as inner and an outer petal (Fig. 1G), with the former almost always larger than the latter. Overall, the phenotypes at each of the four petal positions within a flower are independent. For example, when there are four petals in *ptl pmd* flowers, one position may have two petals whereas another may be vacant.

The modifier also influences the proportions of the various forms of mutant petals. Relatively few thread-like organs are

present, and there is a consequent increase in the number of normal-shaped petals (Table 1).

The effect of the modifier is also strongly temperature dependent. The higher the temperature, the fewer petals seen for all flower positions within the inflorescence. For example, the first formed flower of modified mutant plants raised at 16°C had, on average, 7.3±0.3 petals, but 6.6±0.3 when raised at 22-24°C, and 4.8±0.5 when grown at 29°C. By the twentieth flower, the mean number of petals per flower was still 4.9±0.4 for the 16°C plants, whereas at 22-24°C it had fallen to 2.8±0.4 (Fig. 2B), and at 29°C there were only 0.2±0.2 petals per flower. Thus the rate of acropetal reduction is faster at higher temperatures. This is consistent with the process that is influenced by the modifier also being influenced by temperature.

Even though the modifier boosts the number of petals, it does not further disrupt their orientation (Table 2). 60% of petals are still normally oriented, and the other three orientations are represented in the same relative proportions. Because the number of petals that arise in *ptl* mutants is sensitive to *pmd* and to temperature, yet their orientation is relatively insensitive to *pmd*, we tested whether orientation is also temperature independent. This proved to be the case, as the spectrum of orientations in *ptl pmd* plants raised at 16°C was similar to that seen in plants raised at 22-24°C (Table 2).

Finally, the effects of *pmd* are apparently specific to the second whorl. Sepals and stamens of modified *ptl* mutants are similar in number and phenotype to those of unmodified plants, and the carpels remain normal.

**Table 2. Percentage of second whorl organs in each orientation in *petal loss* mutant plants**

| | 24°C | | | 16°C |
|---|---|---|---|---|
| Orientation | *ptl-1* | *ptl-2* | *ptl-1 pmd-1d** | *ptl-1 pmd-1d** |
| Normal | 60% | 56% | 65% | 68% |
| Medial-facing | 19% | 15% | 15% | 13% |
| Reversed | 17% | 19% | 17% | 14% |
| Lateral-facing | 4% | 9% | 4% | 6% |
| Number of organs | 250 | 155 | 495 | 247 |

*Modified plants segregating from a cross between Columbia and Landsberg *erecta* backgrounds.

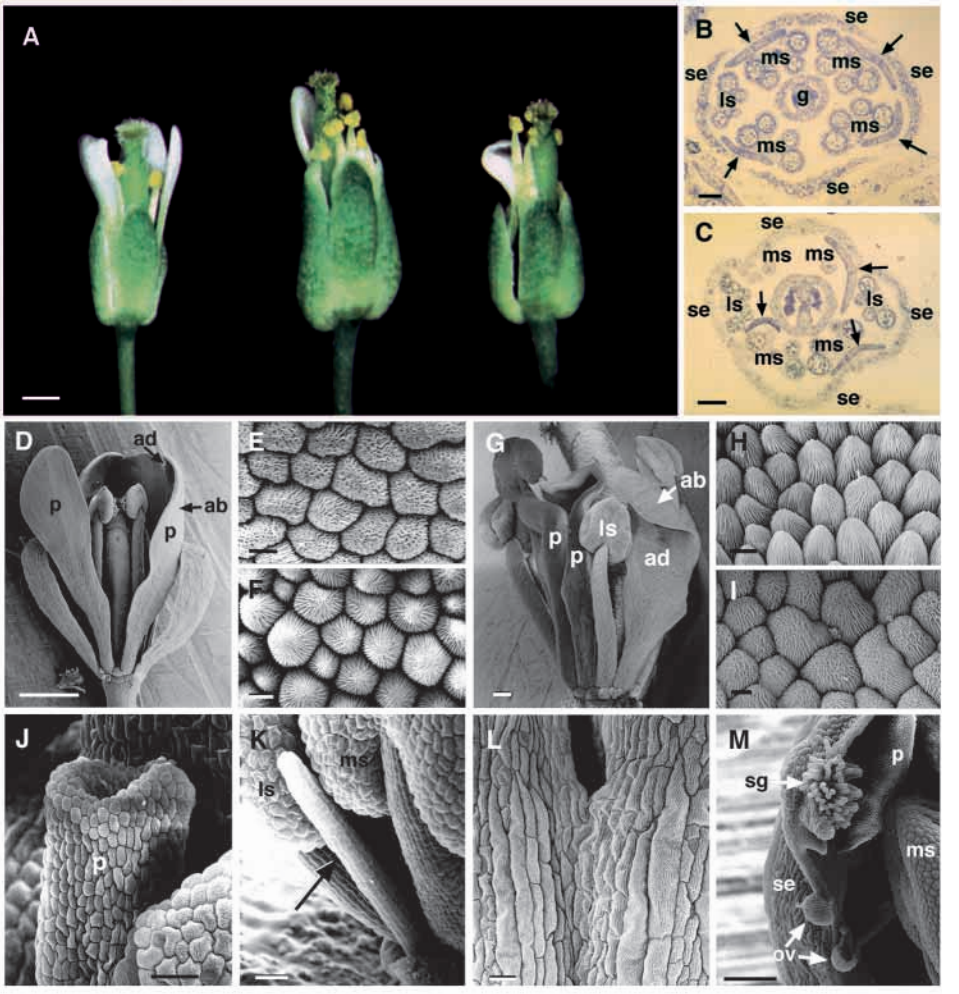


**Fig. 1.** Structure of wild-type and *petal loss* mutant flowers. (A) Mature flowers of wild type (ecotype Columbia) (left), *ptl-1* (centre) and *ptl-2* (right). (B) Transverse section of stage 12 wild-type flower (Landsberg *erecta*), showing the positions and curvature of the four petals (arrows indicate the convex, abaxial surface). (C) Transverse section of late stage 12 *ptl-1* mutant flower. The three petals show disruptions to their orientation (arrows show convex surface), with two now facing the medial stamens and one reversed. Also, the sepals are deeper in profile than wild type. (D) SEM of wild-type stage 15 flower in which petals (p) are at maximum expansion. The lateral sepal has been removed and a lateral stamen is absent. Ad, adaxial surface; ab, abaxial surface. (E,F) SEMs of petal epidermal cells from a wild-type stage 15 flower. Cells on the abaxial (outer convex) surface (E) are cobble-stone shaped with irregular epicuticular ridges, while cells from the adaxial (inner concave) surface (F) are conical and display more regular radial ridges. (G) SEM of modified (*pmd-1d*, see below) *ptl-1 er* flower, showing two petals (p) originating from the same location (left), and a petal in reversed orientation (right, see base of organ). Ab, surface resembling wild-type abaxial epidermis; ad, adaxial-like epidermis. (H,I) SEMs of petal surfaces from the reversed petal in G. The epidermis resembling the wild-type adaxial surface (H) now occupies the outer surface, whereas the surface facing inwards now resembles the wild-type abaxial epidermis (I). (J) SEM of a tubular *ptl-1* petal. The outer surface is typical of abaxial cells of wild-type petals (E). (K) SEM of a typical filamentous organ (arrow) of a *ptl-1* flower. Ls, lateral stamen; ms, medial stamen. (L) SEM of fused medial and lateral sepals of a *ptl-2* mutant flower. (M) SEM of carpelloid outgrowths from the margin of a *ptl-1* mutant sepal (se), including vestigial ovules (ov) and stigmatic papillae (sg). p, petal; ms, medial stamen. Bar: A, D 500 μm; B, C, G, M 100 μm; E, F, H-L 25 μm.

### Developmental defects in organ initiation and orientation

Next, the development of *ptl-1* mutant flowers was examined to determine where and when petals arise, and to identify any changes in their pattern of growth compared with wild type (Hill and Lord, 1989; Smyth et al., 1990). No overt differences were detected up to developmental stage 4 (Fig. 4A,B). At stage 5 in the wild type, four petal primordia appear simultaneously between the sepals. These continue to grow slowly as small mounds through stages 6 (Fig. 4C), 7 and 8 (Fig. 4E,G), before rapid growth as tongue-shaped organs commences in stage 9 (Fig. 4I) and continues through stage 11 (Fig. 4K) to maturity (Fig. 1D).

In *ptl-1* mutants, on the other hand, petal primordia are present sporadically, and they are more variable in size throughout their development (e.g. stages 6 (Fig. 4D) and 8 (Fig. 4F)). Counts of the number of primordia per petal position show a progressive increase throughout flower development in samples of both unmodified (*ptl-1 PMD*) and modified (*ptl-1 pmd-1d*) mutant flowers (Fig. 5), unlike the wild type where every position is occupied from stage 5. Thus it seems there is a staggered delay in the initiation of petal primordia, a conclusion supported from sections of individual flowers (e. g. Fig. 4M,N). It seems likely that the smaller petals seen in mature flowers arise later than the larger petals, and that their growth and maturation are correspondingly delayed, although they eventually reach the same end point of differentiation. The site of initiation of petal primordia is also somewhat disrupted in *ptl* mutants, especially in modified plants. For example, they may arise from the base of a sepal rather than between them (Fig. 4F,N). This is especially true of the outer petal in cases where two share the one position.

The abnormal orientation of *ptl* mutant petals can be seen to result from their abnormal insertion on the floral receptacle (Fig. 4H,J,L), and this can be traced back to at least stage 8 (Fig. 4H). In the wild type, petal primordia are flattened radially in relation to the flower primordium from the time they arise (Fig. 4G), whereas in *ptl* mutants flattened primordia can be seen in any one of a full range of other orientations from early in their development. Significantly, by careful examination of cell file patterns at the base of petals at all stages of their growth, it can be seen that these abnormal orientations are not the result of twisting or rotation (e.g. Fig. 4H,J,L).

Finally, trumpet-shaped petals apparently arise and grow as radially symmetric domes rather than from flattened primordia. These are apparent from stages 6-8, and from stage 9 some are seen to have begun tubular growth from their apical rim (Fig. 4L). Threads develop in a similar way except they are smaller, maintain solid, cylindrical growth, and do not differentiate petal-like cells.

### Loss of organs is position dependent, not identity dependent

To test if disruption to PTL function is specific for petals, or localised to the second whorl where they normally occur, homeotic mutants that change organ identity were used. Firstly, third whorl petals were produced in place of stamens using the *agamous-1* (*ag-1*) mutant (Bowman et al., 1991). Double mutants of *ptl-2* with *ag-1* (in *pmd-1d* background) were carefully observed, and among 119 flowers, 111 (93%) had six

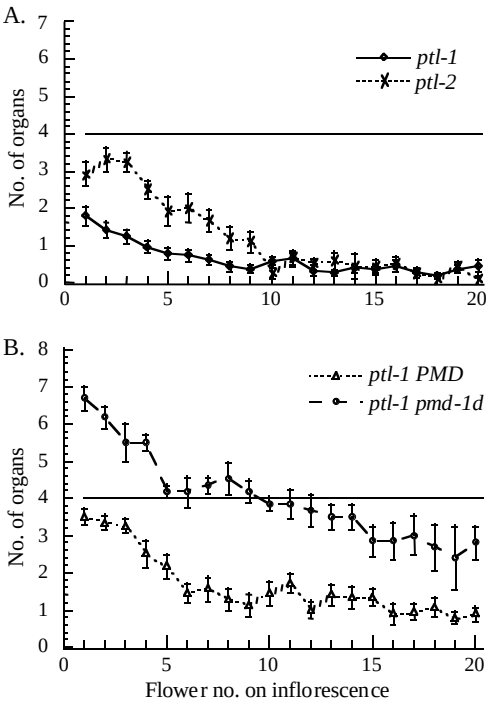


**Fig. 2.** Mean number of second whorl organs per flower for the first 20 flowers of *ptl* mutants. (A) *ptl-1* and *ptl-2* mutants (in Columbia and C24 background, respectively). (B) Unmodified (*PMD*) and modified (*pmd-1d*) *ptl-1* mutants from a segregating $F_2$ family.

third whorl petals, closely similar to the number seen in 52 *ag-1* single mutant controls (46, or 88%). (The other flowers had five or seven third whorl organs.) On the other hand, second whorl organs still displayed disruptions to their number and shape similar to those seen in single mutants. Thus organs with a petal identity are insensitive to the effects of *ptl* mutation if they arise outside the second whorl.

The second test involved changing the identity of second

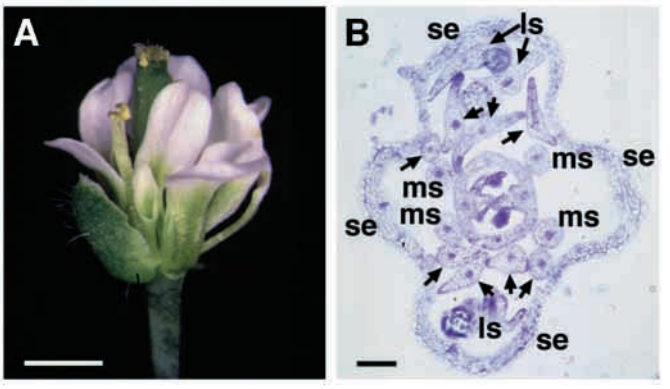


**Fig. 3.** Phenotype of the modified *ptl* mutant flower (*ptl-1 pmd-1d*). (A) Early *ptl-1 pmd-1d er* flower showing numerous extra petals. The medial and right lateral sepals have been removed. (B) Transverse section of stage 12 *ptl-1 pmd-1d er* mutant flower showing eight petals (arrows show convex abaxial surface) lying at various orientations. The two lateral stamens (ls) each contain mosaic patches of petal tissue. se, sepal; ms, medial stamen. Bar: A, 500 μm; B, 100 μm.

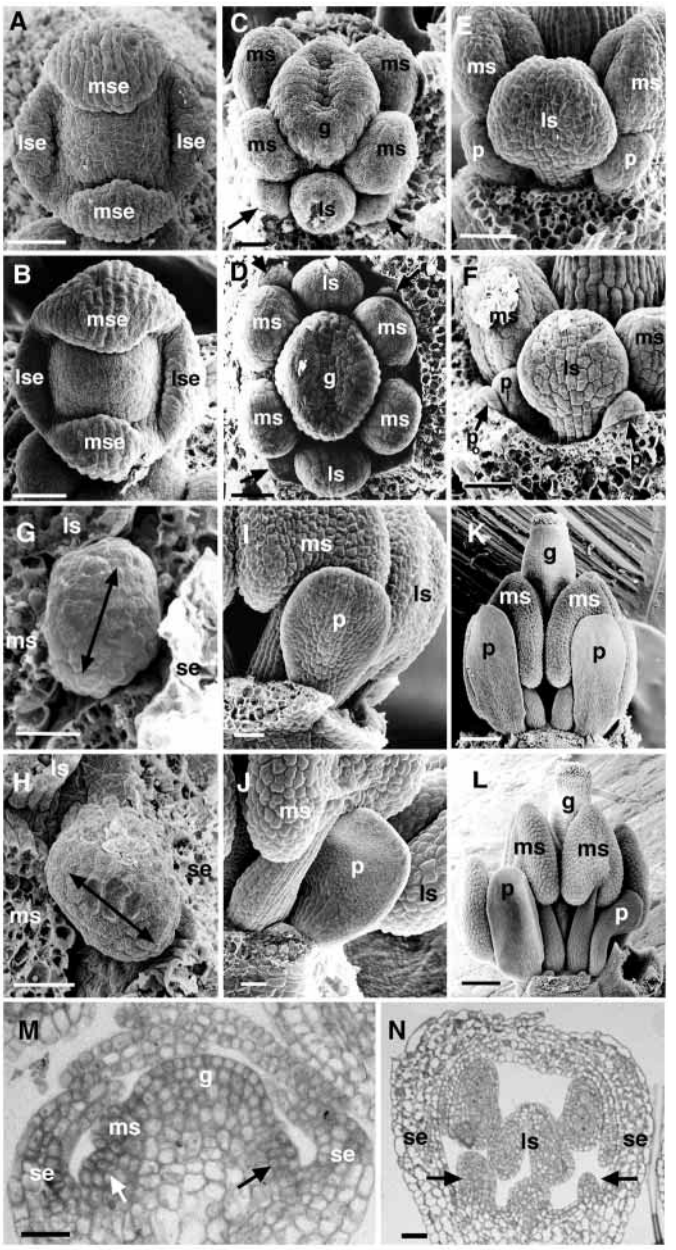


**Fig. 4.** Comparison of petal development in wild-type (Columbia) and *ptl-1* mutant flowers. A-L are SEMs; M and N are longitudinal sections. (A,B) Stage 4 flowers of wild-type (A) and the *ptl-1* mutant (B) just prior to the initiation of petals and stamens. No major differences are apparent. Mse, medial sepal; lse, lateral sepal. (C,D) Stage 6 flowers of wild-type (C) and the *ptl-1* mutant (D) in lateral view with the sepals removed to expose primordia of the petals (arrows), medial (ms) and lateral (ls) stamens, and the gynoecium (g). In the *ptl-1* mutant (D), petal primordia are variable in size or absent (bottom right). (E,F) Wild-type (E) and *ptl-1 pmd-1d* modified mutant (F) flowers at early stage 9 and late stage 8, respectively, in lateral view. In the mutant, a well developed inner petal ($p_i$) and a much less advanced outer petal ($p_o$) is visible in the left position. The right hand petal primordium (p) is developing in an abnormal location, on the base of the sepal and closer to the lateral stamen (ls) than the medial stamen (ms). (G,H) Orientation of petal primordia at stage 8 in dissected buds of wild type (G) and *ptl-1* (H) displayed in equivalent orientations of the flower. In the wild type (G), the elongated petal primordium (arrows) lies flat against the medial (ms) and lateral (ls) stamen primordia, whereas in *ptl-1* (H) it lies nearly at right angles to the stamens. se, medial sepal (removed). (I,J) Stage 9 wild-type (I) and *ptl-1* mutant (J) flowers in medial view, showing the abnormal orientation of the mutant petal (p) that is inserted between the lateral (ls) and medial (ms) stamens at 90° compared with the wild type. (K,L) Stage 11 flowers of wild type (K) and the *ptl-1 pmd-1d* mutant (L) in medial view, the latter showing one petal in reversed orientation (left, concave surface now facing outward) and a tubular petal (right). p, petal; ms,– medial stamen; g, gynoecium. (M) A *ptl-1 pmd-1d* flower at stage 5 showing staggered initiation of petals. The left petal primordium has already formed (arrow on left) while on the right a periclinal cell division in the sub-epidermal layer (arrow) signifies the onset of petal initiation (Hill and Lord, 1989). se, sepal; ms, medial stamen; g, gynoecium. (N) A *ptl-1* mutant flower at early stage 9, showing that the left petal (left arrow) is at a more advanced stage of development than the right petal (right arrow) (confirmed in adjacent sections), and that the right petal has arisen abnormally from the base of the sepal (se). ls, lateral stamen. Bar: A-F, I, J, M, N 25 μm; G, H 10 μm; K, L 100 μm.

whorl organs to sepal-like organs using the *apetala3-3* (*ap3-3*) mutant (Bowman et al., 1991; Fig. 6A). Observations of the first 20 flowers of *ptl-1 ap3-3* double mutants (in *pmd-1d* background) revealed the same acropetal reduction in the number of second whorl organs as seen in matched *ptl-1* single mutant flowers. The mean number fell from 7.4±0.2 in the first formed flowers to 2.5±0.4 by flower 20 in *ptl-1 ap3-3* double mutants, compared with a reduction from 6.7±0.3 to 3.0±0.5 for equivalent flowers in *ptl-1* single mutant plants (*ap3-3* mutant flowers always have four second whorl organs). Thus the effects of *ptl* mutations are specific to organs that arise in second whorl positions, not to petals *per se*.

### Orientation is further disrupted when *ptl* is combined with B function mutants

While observing double mutants of *ptl-1* with the B function mutant *ap3-3*, a striking observation was made. Sepalloid second whorl organs are now almost always fully reversed (Fig. 6D-H; Table 3). In mature flowers, both the shape of the organs and their epidermal identities were consistently reversed (Fig. 6D-F compared with Fig. 6A-C). Again no evidence of twisting of the organs was seen, an observation supported by the pattern of expression of the normally abaxially expressed gene *FILAMENTOUS FLOWER* (*FIL*). In *ptl-1 ap3-3* double mutant flowers, *FIL* is already expressed adaxially in the young second whorl organs by at least stage 8 (Siegfried et al., 1999).

The reversal in second whorl organ orientation seen in *ptl-1 ap3-3* mutants is apparently the consequence of removing B function, as a similar change was also seen when *ptl-1* mutants were combined with the null B function mutant *pistillata-1* (*pi-1*) (Bowman et al., 1991; Hill and Lord, 1989; Table 3). Both *ap3-3* and *pi-1* mutants result in aggregation of third and fourth whorl organs into a central gynoecium. To test if this modification in floral architecture is responsible for the further effect on orientation, *ptl-1* was combined with the weaker *ap3* allele, *ap3-1*, in which third whorl organs are now mostly separate from those in the fourth whorl (Bowman et al., 1991). Clearly the re-establishment of third whorl organs does not fully ameliorate the B function disruption (Table 3). As a final test of the influence of inner whorls on orientation, they were converted from carpels to sepal-like organs by incorporating

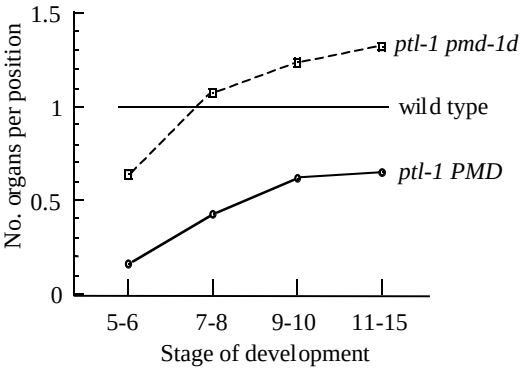


**Fig. 5.** Increase in the mean number of petal primordia occupying each available position over developmental time in unmodified (*PMD*) and modified (*pmd-1d*) *ptl-1* mutant flowers. At least 8 buds were scored for each genotype at each stage.

the *ag-1* mutant into the *ptl-1* B function double mutants. In these triply mutant plants, the second whorl organs were still almost always reversed (data not shown) so disruption does not depend upon the internal organs having a carpelloid identity. The same pattern of reversal was seen in internal flowers.

Finally, pairs of second whorl organs that occupy the one position may adopt the same orientation (e.g. Fig. 6H), but they do not necessarily do so. In fact, in *ptl-1 pmd-1d* flowers they adopted different orientations in 103 cases among 209 pairs observed (54%). The orientation of the smaller, outer organ is less often disrupted, especially in *ptl* B function double mutants. In 77 doubly occupied positions in *ptl-1 pmd-1d ap3-3* flowers, for example, nearly all (98%) of the inner organs but only 57% of the outer organs showed the reversed orientation.

In conclusion, 30% to 40% of second whorl organs apparently arise in a sideways or reversed orientation in *ptl* mutants, but they are almost all fully reversed when B function is also removed.

## DISCUSSION

Two different disruptions of second whorl organ development are seen in *petal loss* mutants, reduced number and variable orientation. That these have a different mechanistic basis is highlighted by their differential sensitivity to modifying influences. Whether or not an organ arises is dependent upon where the flower arises within the inflorescence (i.e. there is an acropetal effect), the presence of a dominant modifier gene

**Table 3. Percentage of second whorl organs in each orientation in *ptl-1* mutant flowers in combination with B function mutants**

| Orientation | *ptl-1* | *ptl-1 ap3-3* | *ptl-1 pi-1* | *ptl-1 ap3-1* |
|---|---|---|---|---|
| Normal | 69% | 6% | 3% | 23% |
| Medial-facing | 12% | 4% | 4% | 21% |
| Reversed | 15% | 87% | 92% | 53% |
| Lateral-facing | 4% | 3% | 2% | 3% |
| Number of organs | 272 | 284 | 258 | 295 |

Results are from the first 10 flowers of 6 plants in each case. All plants were *pmd-1d*.

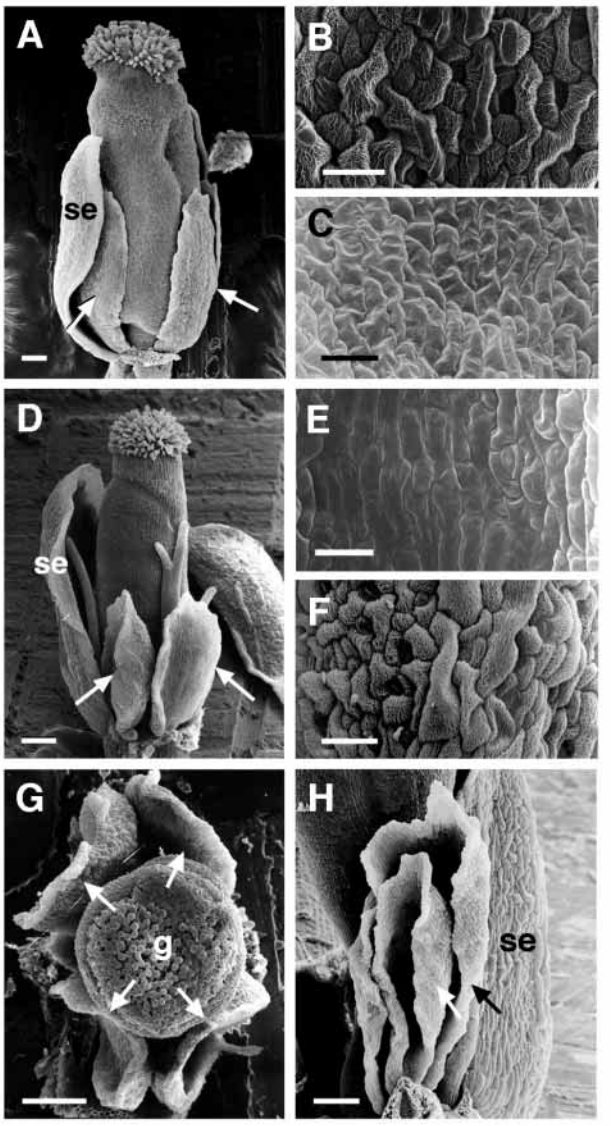


**Fig. 6.** SEMs of flowers of the homeotic B function mutant *ap3-3*, alone and in combination with *ptl-1*. (A) Mature *ap3-3* flower with two first whorl organs removed. The second whorl organs have been transformed into sepals (arrows) and the centre of the flower is typically occupied by a large multicarpellate gynoecium. (B,C) Cell surfaces of second whorl sepals of an *ap3-3* mutant flower. They have adopted the normal orientation; the outer surface (B) and inner surface (C) resemble the abaxial and adaxial surfaces of normal first whorl sepals. (D) Mature *ptl-1 pmd-1d ap3-3* flower with two of the outer sepals removed. The two second-whorl sepals show a sideways (left) and reversed (right) orientation as judged from their curvature (arrows indicate concave surface). (E,F) Cell surfaces of the reversed second whorl sepal of a *ptl-1 ap3-3* mutant flower (D). The outward facing surface (E) closely resembles the normally inward facing adaxial surface of *ap3-3* single mutants (C), whereas the inward facing surface (F) is now similar to the normal outer (abaxial) surface (B). (G) Vertical view of a mature *ptl-1 pmd-1d ap3-3* mutant flower with the first whorl sepals removed. The reversed curvature of all four second-whorl sepalloid organs is indicated (arrows). G, gynoecium. (H) Two second whorl sepalloid organs (arrows) arising from the one position in a *ptl-1 pmd-1d ap3-3* mutant flower, each showing a reversed orientation. Se, first whorl sepal. Bar: A, D, G, H, 100 μm; B, C, E, F, 25 μm.

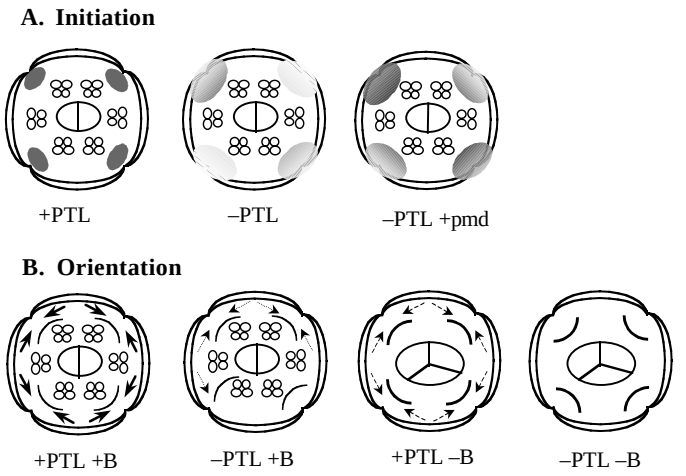


**Fig. 7.** Proposed roles of the *PETAL LOSS* gene in controlling initiation (A) and orientation (B) of second whorl organs. (A) In the wild type (left), an inducer promotes the initiation of petals in defined zones between the sepals (ovals). In *ptl* mutants (centre), the zone is enlarged and there is a variable weakening of the response to the inducer (indicated by lighter shading). At some sites, the response threshold is not met and petals are absent. In modified *ptl* mutants (right), the action of the inducer is uniformly boosted (darker shading), although its expanded zone of influence, and its variability, remain. (B) In the wild type (left), petals perceive their orientation as they arise (arrows). In *ptl* mutants (centre left), this perception is apparently variably reduced (dotted arrows) or absent such that petals sometimes arise sideways (lower left petal now faces medial stamens), or reversed (lower right petal). In B function mutants (centre right), the perception may be uniformly reduced (dashed arrows) but it remains adequate to maintain the normal orientation. In *ptl* B function double mutants (right), however, the perception is no longer sufficient, and a default, reversed orientation is adopted uniformly. (For simplicity, one organ is shown at each second whorl position.)

*pmd-1d*, and the growth temperature. In contrast, organ orientation does not change acropetally, it is not influenced by the modifier, and it is not temperature dependant. However, orientation but not organ number is further disrupted when B function is removed. Thus initiation and orientation are separable phenomena, and they will be discussed separately.

## Initiation

### *PTL* regulates induction of second whorl organs

To account for our data, we propose that an inducer of petal initiation, supported by PTL function, is normally active within the floral primordium. A discrete number of cells respond to a threshold level of signal by initiating organs in four zones within the flower (Fig. 7A, left). In unmodified *ptl* mutants (Fig. 7A, centre), induction is disrupted in two ways. Firstly, cells are unable to respond as effectively to the inducer so that an active threshold is achieved only infrequently, and hence fewer petals are initiated. Secondly, the inducer's sphere of action is somewhat enlarged, accounting for the observed disturbance to the sites of petal initiation. In modified *ptl* mutants (Fig. 7A, right), the only change proposed is a uniform boost to the level of induction without any changes to its variability or site of action. Thus the threshold is more often reached, and, owing to the expanded zone, two primordia may sometimes arise within the one zone. Weaker induction at the periphery of the flower would account for the apparently reduced number and later initiation of the smaller outer primordia in *ptl pmd* plants. Finally, the level of induction is markedly influenced by growth conditions, in that it falls progressively in later-arising flower primordia, and it is also reduced in flowers developing at higher temperatures.

### Influence of differential growth within the floral meristem

Changes to the size and shape of the floral primordium in various mutants may result in changes to the number of floral organs that arise (see Introduction). In *ptl* mutants, however, if there are any changes to flower meristem growth, they are relatively subtle as preliminary size measurements revealed no major differences. Even so, the occasional congenital fusion of adjacent sepals in *ptl* mutant flowers may be the consequence of a slightly smaller floral meristem. The development of aberrant structures, particularly threads, in the second whorl of *ptl* mutant flowers, too, might be the result of them arising from a constrained input of cells. On the other hand, fusion of stamens primarily occurred when there were seven stamens in the flower, and this part of the flower meristem may be slightly larger.

One way *ptl* mutants may reduce second whorl initiation is by allowing ectopic AG function in this region. AG normally inhibits growth in the centre of the floral meristem. In *apetala2* (*ap2*) mutants, however, it is ectopically expressed in the outer whorls (Drews et al., 1991), resulting in carpelloidy of the first whorl and the loss of almost all second whorl organs. That the latter is the result of AG function is inferred from organ restoration when *AG* is also inactive in *ap2 ag* double mutant plants (Bowman et al., 1991). However, this is unlikely to be the primary cause of petal loss in *ptl* mutants. When the null mutant *ag-1* was bred into *ptl-1* or *ptl-2* mutant plants, the mean number of second whorl organs per flower increased somewhat, but it was not restored to the normal four per flower, the number of organs decreased acropetally, and small petals and tubular petals continued to arise (results not shown). In addition, we could detect no differences in the pattern of *AG* expression in young *ptl* mutant flowers compared with the wild type (M. E. G., unpublished).

### Role of the *PETAL LOSS MODIFIER* gene

Two properties of *pmd-1d* requiring explanation are its dominance and the lack of detectable effects in wild-type plants. The *pmd-1d* allele is restricted to the Landsberg *erecta* background, being absent in the other six ecotypes tested, suggesting that the dominant form is a mutant or variant allele. If so, it may result from a gain of function (although we cannot exclude the possibility that its function has been lost in all strains except Landsberg). In the context of our model (Fig. 7), *PMD* may be a component of the induction process, with the frequency of the outcome (petal initiation) being amplified by the presence of one or more *pmd-1d* alleles. The dominance of pmd could result from its now constitutive activation of a signalling pathway, for example. Amplification might also occur in wild-type *PTL* plants, but the threshold of petal initiation would already have been met, and the site of initiation restricted such that only one organ can arise at each location. It is also possible that once a petal primordium has begun to

form, PTL function may directly prevent the triggering of further primordia.

### Role of boundaries within the floral meristem

Studies of cell lineages have shown that boundaries between whorls are established relatively early in the development of *Antirrhinum* flowers (Vincent et al., 1995). One gene that may play a specific role in setting up petal whorl boundaries is *FIMBRIATA* (*FIM*) of *Antirrhinum* and its *Arabidopsis* ortholog *UNUSUAL FLORAL ORGANS* (*UFO*; Ingram et al., 1995). After activating B function organ identity genes, *UFO* expression is subsequently limited to a small region immediately between the sepals at the base of each developing petal. The number of petals in *ufo* mutant flowers is reduced somewhat, petal/sepal and petal/stamen mosaics are frequent, and filamentous organs are common in petal positions. Thus there are some parallels between *ufo* and *ptl* mutant phenotypes, although the orientation of second whorl organs is apparently not disrupted in *ufo* flowers.

One gene apparently involved in setting boundaries between adjacent organs within a whorl is the *NO APICAL MERISTEM* (*NAM*) gene of *Petunia* (Souer et al., 1996). In developing flowers, *NAM* expression encircles the stamen primordia, and in *nam* mutants additional petals arise adjacent to each stamen primordium as well as between them. The setting of boundaries between organs may also be the function of two *CUP-SHAPED COTYLEDON* (*CUC*) genes in *Arabidopsis*. In *cuc1 cuc2* double mutants, fusion of adjacent sepals and adjacent stamens is frequent (Aida et al., 1997), and, interestingly, *CUC2* also encodes a protein related to NAM. It will be important to observe the phenotype of mutants that combine *ptl* with *ufo* or *cuc* to test if *PTL* shares organ boundary functions with these genes.

## Orientation

### PTL regulates organ orientation

In the present study, we distinguish two aspects of organ development – polarity and orientation. We follow the conventional definition of polarity as the abaxial-adaxial differentiation that occurs within flattened organs. However, we define organ orientation as the angle adopted by flattened, polarised organs in relation to the centre of the floral meristem. While a range of genes has been identified that regulate organ polarity (see below), to our knowledge *PTL* is the first to be described that influences organ orientation.

In *ptl* B function double mutants, second whorl organs are usually reversed, and it could be argued from this that PTL simply controls polarity. That is, such organs may arise in the normal orientation but their abaxial and adaxial surfaces are interchanged. However, the presence of organs showing any one of a continuous 360° range of abnormal orientations, as is seen in *ptl* single mutants and in *ptl ap3-1* double mutants, strongly suggests that orientation rather than polarity is disrupted.

Among genes known to regulate polarity, the *PHANTASTICA* (*PHAN*) gene of *Antirrhinum* is apparently involved in establishing adaxial identity (Waites and Hudson, 1995). In *phan* mutants, leaves and petals are filamentous, and the surface cells are usually only of the abaxial type. The *PINHEAD*/*ZWILLE* and *ARGONAUTE1* genes of *Arabidopsis*, too, may share functions in controlling adaxial tissue fate, at least within petals (Lynn et al., 1999). On the other hand, in the dominant *phabulosa* (*phb*) mutant of *Arabidopsis*, the leaves and petals are also radialised but are now covered with adaxial cells suggesting that abaxiality has been affected (McConnell and Barton, 1998). Recently, a group of new abaxial identity genes has been described in *Arabidopsis* (Siegfried et al., 1999). Several *YABBY* family genes, including *FIL*, are preferentially expressed in abaxial regions of all lateral organ primordia from an early age, and the effects of loss or gain of their function are consistent with an abaxial identity role.

Even though orientation and polarity can be distinguished, they could arise from a common mechanism. For example, Waites and Hudson (1995) propose that the lateral outgrowth of an organ primordium is the consequence of the sensing of an already-established adaxial-abaxial boundary within it. This accounts for the radial growth of organs whose adaxial (or abaxial) identity has been disrupted. Under this scenario, PTL would control organ orientation by defining the position of the abaxial-adaxial boundary in the petal anlagen before flattened growth begins. Furthermore, the occasional production of tubular petals in *ptl* mutants might reflect displacement of such a boundary.

### Signalling of petal orientation within the flower meristem

In general, to account for our observations we propose that the orientation of petal primordia is somehow signalled to precursor cells (Fig. 7B, left), and that the *PTL* gene product plays a role in the perception of, or response to, this signal. In *ptl* mutants, the signal is perceived to be generally weaker and variable, so that the orientation of some petals is distorted (Fig. 7B, centre left), perhaps partly as a consequence of expansion in the zone of cells within which primordia may arise.

In B function single mutants, where sepals arise in place of petals, signalling may be reduced but it remains effective (Fig. 7B, centre right). In *ptl* B function double mutants, however, the signal is usually ineffective, and most second whorl organs are now reversed (Fig. 7B, right). Thus the role of B function in signalling is fully covered by that of PTL function, although not vice versa. This reversal in organ orientation may reflect the influence of another signal, or it may be the default orientation adopted in the absence of signal. Such a default orientation has also been proposed to account for the reversed orientation of bristles in *Drosophila* mutants that lack Frizzled activity (Shulman et al., 1998).

The existence of signals that control leaf orientation has already been deduced from surgical experiments on the shoot apical meristem (e.g. Sussex, 1955). A range of different cuts disturbs the orientation of newly arising leaves in a pattern consistent with a controlling signal emanating from the meristem apex. It is possible that an equivalent signal controls the orientation of second whorl organs and originates from the centre of the flower meristem. If so, we have shown that it is unaffected if the third and fourth whorl organs are fused together, or if the central organs are no longer carpelloid.

In conclusion, discovery of the *PTL* gene has provided us with an entry point into gaining a fuller understanding of two fundamental processes in floral architecture, initiation of individual organs within a floral whorl, and specification of their orientation within the flower. The roles of a proposed

inducer of petal initiation, and of possible signals that determine their orientation, will be important aspects of future work.

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