PETAL LOSS, a trihelix transcription factor gene, regulates perianth architecture in the *Arabidopsis* flower

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Summary

Perianth development is specifically disrupted in mutants of the *PETAL LOSS* (*PTL*) gene, particularly petal initiation and orientation. We have cloned *PTL* and show that it encodes a plant-specific trihelix transcription factor, one of a family previously known only as regulators of light-controlled genes. *PTL* transcripts were detected in the early-developing flower, in four zones between the initiating sepals and in their developing margins. Strong misexpression of *PTL* in a range of tissues universally results in inhibition of growth, indicating that its normal role is to suppress growth between initiating sepals, ensuring that they remain separate. Consistent with this, sepals are sometimes fused in *ptl* single mutants, but much more frequently in double mutants with either of the organ boundary genes *cup-shaped cotyledon1* or *2*. Expression of *PTL* within the newly arising sepals is apparently prevented by the *PINOID* auxin-response gene. Surprisingly, *PTL* expression could not be detected in petals during the early stages of their development, so petal defects associated with *PTL* loss of function may be indirect, perhaps involving disruption to signalling processes caused by overgrowth in the region. *PTL*-driven reporter gene expression was also detected at later stages in the margins of expanding sepals, petals and stamens, and in the leaf margins; thus, *PTL* may redundantly dampen lateral outgrowth of these organs, helping define their final shape.

Key words: *PETAL LOSS* (*PTL*), *Arabidopsis*, Trihelix, GT-factor, Flower development, Perianth

Introduction

Flowers share a common underlying architecture. In general, reproductive organs occur centrally, with the female carpels internal to the male stamens. Surrounding these are the perianth organs, which in many species are differentiated into inner, showy petals and outer, protective sepals. The number of floral organs in a flower, their spatial relationships and their degree of fusion are relatively conserved properties, presumably under genetic control.

A range of genes that regulate floral architecture has been identified in *Arabidopsis thaliana*. The *PERIANTHIA* (*PAN*) bZIP transcription factor gene ensures that flowers arise with the appropriate numbers of organs in the outer three whorls (four sepals, four petals and six stamens) (Chuang et al., 1999). In *pan* mutants there are usually five in each case. By contrast, the architecture of the second and third whorls is supported by the *UNUSUAL FLORAL ORGANS* (*UFO*) F-box gene (Durfee et al., 2003; Laufs et al., 2003). Another gene, *PRESSED FLOWER* (*PRS*), encoding a homeodomain protein, has roles in defining the lateral regions of the flower primordium rather than the radial regions (Matsumoto and Okada, 2001). In *prs* mutants, lateral sepals are frequently absent.

Other genes function to define boundaries. For example, the *SUPERMAN* (*SUP*) zinc finger gene acts to control cell proliferation in the boundary between the stamens and carpels (Sakai et al., 1995). In addition, boundaries between individual organs are controlled by CUP-SHAPED COTYLEDON (CUC) genes encoding NAC transcription factors. These act to keep the primordia of adjacent floral organs, especially the sepals, separate (Aida et al., 1997).

Genes required for the development of specific organ types have also been described. For example, another function of *UFO* is to promote petal outgrowth, perhaps by targeting an inhibitor of this process for degradation (Durfee et al., 2003; Laufs et al., 2003). Another gene that specifically promotes petal growth, *RABBIT EARS* (*RBE*), encodes a zinc finger protein (Takeda et al., 2004). In null *rbe* mutant plants, petals are mostly filamentous or absent.

The *PETAL LOSS* (*PTL*) gene of *Arabidopsis* plays a unique role in controlling perianth development (Griffith et al., 1999). In mutant plants, sepals are mis-shapen and sometimes fused with an adjacent sepal. Petals are often absent, and their mean number per flower falls progressively so that later-formed flowers usually have none. Those petals that do arise are often smaller than normal and are sometimes trumpet-shaped. Petal primordia occupy the same regions of the mutant flower primordium as in the wild type (internal to each of the inter-sepal zones), although the four regions are somewhat enlarged.

Also, their initiation may be delayed. The number of petals per flower is influenced by the presence of a dominant allele of the *PETAL LOSS MODIFIER* (*PMD*) gene, with more petals arising per flower in *pmd-1d* background.

Griffith et al. proposed that PTL normally functions to support the action of a petal initiation signal (Griffith et al., 1999). This was proposed to act in four regions of the developing flower, internal to the inter-sepal zones. These regions might be enlarged in *ptl* mutants such that response to the signal is weakened and the threshold is only occasionally reached. It was also proposed that sensitivity to the signal is boosted in the *pmd-1d* background.

The orientation of petals within the flower is also disrupted in *ptl* mutants. Some face sideways, and others are reversed. Griffith et al. suggested that the response to another signal, acting with defined polarity within the flower primordium, was being disrupted in *ptl* mutants, such that petal primordia sometimes adopted an inappropriate, or even default, orientation (Griffith et al., 1999).

In this study, we report the identification of *PTL* as a transcription factor gene of the plant-specific trihelix family. *PTL* is the first member of this family known to control morphogenesis – others known to date are associated with the regulation of light-responsive genes. *PTL* is expressed in four zones between newly arising sepals, where it may help to maintain their separation. Consistent with this idea, ectopic expression of *PTL* results in growth inhibition. In addition, some fusion of adjacent sepals occurs in *ptl* mutant plants. Surprisingly, *PTL* is apparently not expressed in developing petal primordia. This suggests that the disruptions to petal initiation and orientation in *ptl* mutants are caused indirectly, perhaps as a consequence of overgrowth in the nearby inter-sepal zones.

Materials and methods

Origin of strains and growth conditions

ptl-1 (in Columbia) was induced by ethyl methane sulphonate (EMS), and *ptl-2* (in C24) arose following transformation (Griffith et al., 1999). Three new mutants were provided by Stuart Baum (*ptl*-3) and Yuval Eshed (*ptl-4* and *ptl-5*), both from the University of California Davis, following EMS mutagenesis of Landsberg *erecta* (L*er*) plants (carrying the dominant modifier allele *pmd-1d*), and were shown not to complement *ptl-1*. The intermediate *pinoid-2* mutant allele in L*er* was from laboratory stocks (Bennett et al., 1995), and *cuc1-1* and *cuc2-1* mutants (in L*er*) were provided by Masao Tasaka (Aida et al., 1997).

Plants were grown at 20-25°C in natural daylight supplemented with continuous Cool White fluorescent light. Stages of flower development follow Smyth et al. (Smyth et al., 1990).

Cloning of *PETAL LOSS* and isolation of cDNA

Clones of Yeast Artificial Chromosomes (YACs) and TAMU Bacterial Artificial Chromosomes (BACs) for generating contigs in the region overlapping *PTL* were obtained from The Arabidopsis Biological Resource Center. *PTL* was localized to a 10.7 kb *Xba*I genomic fragment present in the right end of BAC T24M12 (Columbia ecotype). This was cloned into pBluescript SK(+) (Stratagene) for sequencing, or into the binary vector pBIN19 for complementation tests. In the latter, the construct (D289) was transferred to *Agrobacterium tumefaciens* strain AGL1, and then into *ptl-1* mutant plants by bacterial infiltration of flower buds.

A cDNA library, made from L*er* inflorescence tissue with buds up to stage 12 using λZAPII (Stratagene) (provided by Detlef Weigel, Salk Institute), was probed with BAC T14K22, and the positive clones probed in turn with the right end *Xba*I subclone of BAC T24M12. One positive clone (D171) was obtained (GenBank Accession number AY555728).

Nuclear localization

PTL coding sequences in cDNA clone D171 were translationally fused to the C terminus of the GFP coding sequence from pBIN mGFP5-ER (with ER deleted). This was inserted into plasmid pART7 (Gleave, 1992) downstream of the 35S CaMV promoter sequence. Transient expression of 35S::GFP-PTL in onion epidermal cells, and GFP fluorescence, followed the protocol of Weigel and Glazebrook (Weigel and Glazebrook, 2002).

Analysis of *PETAL LOSS* RNA

RT-PCR followed the OneStep procedure (Qiagen) starting with total RNA extracted using the RNeasy procedure (Qiagen). Primers for transcripts of *PTL* and the two control genes *APETALA3* (*AP3*) and *ACTIN2* (*ACT2*) were designed to flank or overlap introns, generating cDNA products of 738 bp (*PTL*), 356 bp (*AP3*), or 1,153 bp (*ACT2*). Each reaction started with approximately 0.5 μg total RNA, and the *PTL* and *AP3* reactions were identical (55°C annealing temperature with 20 seconds extension for 30 cycles) except that 'Q solution' (Qiagen) was added to the *PTL* reaction. The *ACT2* reaction conditions were 52°C, 30 seconds and 28 cycles.

In situ hybridization, using digoxigenin-labelled sense and antisense probes made using the cDNA plasmid D171, essentially followed the protocol of Heisler et al. (Heisler et al., 2001).

Generation and analysis of GUS reporter constructs

The *PTL* regulatory region was translationally fused with the *uid*A gene of *E. coli* encoding β-glucuronidase (GUS). The BAC clone T14K22 (Columbia genomic DNA) supplied the sequences used, either restriction fragments or PCR products. These were ligated into the shuttle vector pRITA (Eshed et al., 1999; Eshed et al., 2001) using an *Nco*I site present at the first methionine codon of GUS. After sequencing to confirm appropriate cloning, the pPTL::GUS casettes were cloned into the *Not*I site of the binary vector pART27 (Gleave, 1992) (conferring kanamycin resistance in plants), or a Basta resistance derivative of this, pMLBART. These were then transferred into Columbia plants as before.

Five PTL reporter constructs were made, three with the first exon and intron (p8.0i::GUS, p2.0i::GUS and p1.3i::GUS), and two without (p8.0::GUS and p2.0::GUS). For the first three constructs, the fusion involved either the first methionine (p2.0i) or the first tyrosine (p8.0i and p1.3i) of the second exon. For the last two (p8.0 and p2.0), the fusion was at the second methionine of the first exon. Where PCR had been used for cloning, full sequencing revealed one change in both p8.0 and p8.0i (one less T in a string of 15 located 4,957 bp upstream of the first methionine), and 9 base substitutions in p2.0i. The latter did not change the translated sequence, and as the expression pattern for p2.0i was closely similar to that of the error free p1.3i, it was assumed the changes had had no functional consequences.

GUS staining of transformed lines was carried out by briefly fixing material in 90% acetone, staining overnight in 2 mM X-Gluc in 50 mM phosphate buffer (pH 7.2) at 37°C, and then removing chlorophyll using ethanol. Most staining solution also included 6 mM potassium ferri- and ferro-cyanide to reduce bleeding. Tissues were examined as whole mounts in 70% ethanol, or were embedded in Paraplast Plus, sectioned at 8 μm, and viewed using dark-field optics. The precipitated product of the β-glucuronidase reaction appears blue in bright field, but pink in dark field.

Ectopic expression of *PTL* using the *lac* repressor/operator transactivation system

In this system (Moore et al., 1998), a driver line carries a promoter of

choice driving a hybrid transcription factor (the *lac* repressor translationally fused with the *GAL4* activation domain, LhG4). The target line carries the *lac* operator (pOp) in tandem copies upstream of a gene of interest. Expression of the target gene is induced in progeny of crosses between the two lines, here indicated as pDRIVER>>TARGET (Eshed et al., 1999; Eshed et al., 2001).

Two PTL driver constructs were made containing either 1.3 kb upstream of the first methionine codon, the first exon and the intron [pPTL(1.3i)], or 2.0 kb of the upstream region [pPTL(2.0)]. These *PTL* regulatory sequences were each inserted into the modified shuttle vector pBJ36-LhG4 (Eshed et al., 2001), and then into the *Not*I site of pMLBART. *ptl-1* plants were transformed, and 11 independent insertion lines carrying pPTL(1.3i), and seven of pPTL(2.0) were recovered. Their expression was assessed by intercrossing with a pOp::GUS target tester line (provided by Yuval Eshed, University of California Davis), and the patterns closely matched those of comparable pPTL::GUS translational fusion lines. Three other driver lines, carrying either 1.7 kb of the *APETALA1* upstream promoter region (pAP1) (Emery et al., 2003), or 0.42 kb of the *APETALA3* promoter region (pAP3), or the CaMV 35S control region present in 1.35 kb of pART7 (p35S) (Gleave, 1992), were also provided by Yuval Eshed.

A PTL target construct was made by inserting the *PTL* coding sequence from cDNA clone D171 downstream of pOp present in plasmid p12OpBJ36 (Eshed et al., 2001). The insert was excised with *Not*I and inserted into pART27. Four independent target insertion lines were obtained in *ptl-1* background, and 12 in Columbia background.

Scanning electron microscopy (SEM) was carried out as described previously (Griffith et al., 1999).

Results

Sepal fusion is increased in *ptl cuc* double mutants

Previously it was briefly reported that neighbouring sepals are sometimes fused basally along their lateral edge in *ptl-1* and *ptl-2* mutant plants (Griffith et al., 1999) (Fig. 1A,B). The three new *ptl* mutants were similarly affected. Intersepal fusions also occur in single mutants of either of the organ boundary genes *cuc1* and *cuc2* (Aida et al., 1997). We tested whether this fusion represented disruption of the same process as in *ptl* by scoring fusions in *ptl cuc1* and *ptl cuc2* double-mutant flowers. In each case there was a dramatic increase in the number and extent of fusions (Table 1; Fig. 1C-F). Levels now approached the near-complete fusion seen in *cuc1 cuc2* double-mutant plants (Aida et al., 1997). Thus the three genes *PTL*, *CUC1* and *CUC2* each apparently contribute to a process that results in sepal separation.

PTL encodes a trihelix transcription factor

The *PETAL LOSS* gene was cloned based on its map position [0.5 map units above the *TERMINAL FLOWER1* (*TFL1*) locus on chromosome 5 (Griffith et al., 1999)]. The *ptl-1* mutant in Columbia background was crossed with the *tfl1-2* mutant in Landsberg *erecta*. Using F2 plants, the *PTL* locus was mapped distal to *TFL1* (four recombinants out of 854 chromosomes) and proximal to the DNA marker UBQ6121 (one recombinant out of 220 chromosomes). A YAC contig was generated in the proximal direction from UBQ6121, and *PTL* was found to lie between the right ends of YACs CIC2B9 and CIC10H2. Using these ends, a BAC contig was assembled across this region, and *PTL* was localized to BAC T14K22. A *Hae*III polymorphism was located within this BAC using the right end of another

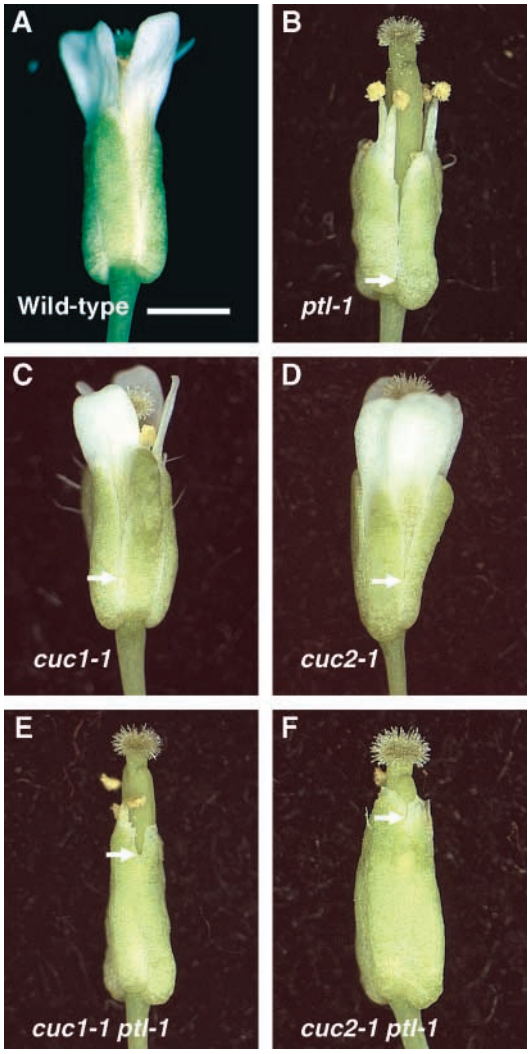

Fig. 1. Sepal fusion in mature flowers of single and double mutant combinations of *ptl-1*, *cuc1-1* and *cuc2-1*. (A) A wild-type (Columbia) flower. (B) A *ptl-1* mutant flower with some fusion (<1/4) of adjacent lateral (left) and medial (right) sepals (arrow). Note aberrant shape of sepals (deeper in profile) and the absence of petals. (C,D) Sepal fusions in *cuc1-1* (C) and *cuc2-1* (D) mutant flowers (arrows). (E,F) Near full fusion of adjacent sepals in *ptl-1 cuc1-1* (E) and *ptl-1 cuc2-1* (F) double-mutant flowers (arrows). Scale bar: 1 mm.

Table 1. Extent of lateral fusion between adjacent sepals in single- and double-mutant combinations of *ptl*, *cuc1* and *cuc2

Genotype	Number of flowers†	Proportion of total sepal length showing fusion				
		0	≤1/4	1/4-1/2	1/2-3/4	3/4-1
ptl-1	79	52.8%	25.9%	17.1%	3.8%	0.3%
cuc1-1	80	52.8%	25.3%	15.0%	6.6%	0.3%
cuc2-1	60	21.7%	29.2%	31.3%	16.3%	1.7%
ptl-1 cuc1-1	80	0.0%	0.0%	10.6%	66.6%	22.8%
ptl-1 cuc2-1	80	2.5%	8.4%	21.3%	50.3%	17.5%

*Entries show percentage of adjacent sepal positions (four per flower) with specified degree of fusion.

†The first 20 flowers formed on each of three or four plants were scored.

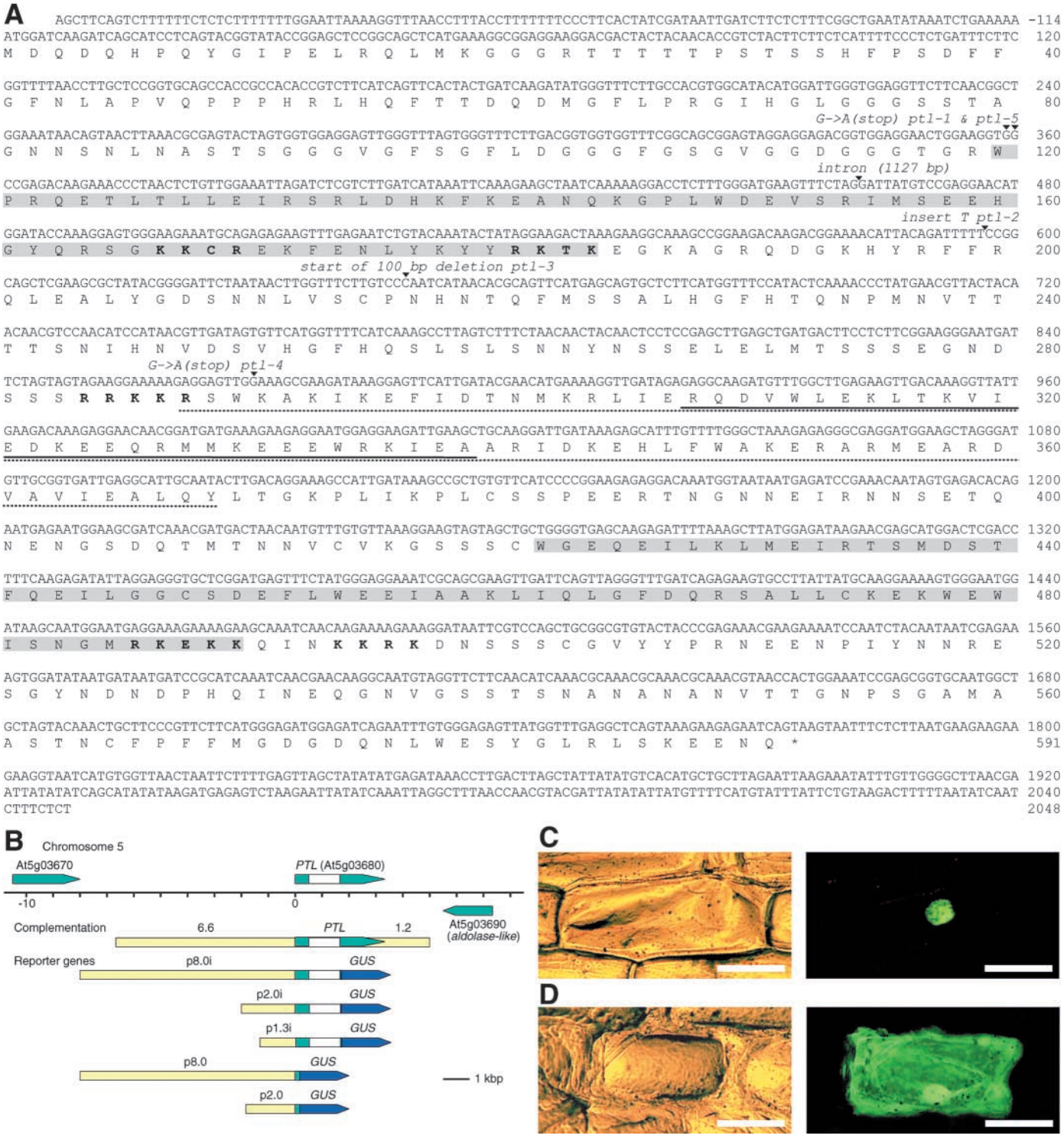

Fig. 2. cDNA sequence, genomic organization and nuclear localization of *PTL*. (A) Sequence of *PTL* cDNA D171 (Accession number AY555728), and the predicted amino acid sequence showing two trihelix DNA-binding domains (shaded), a conserved alpha-helical central domain (dotted line) that includes a predicted coiled-coil domain (solid line), and putative nuclear localization sequences (bold). The sites of the single intron, and of sequence changes in five *ptl* mutant alleles, are also shown. (The 100 bp deletion in *ptl-3* is associated with the insertion of TTTTATGT at the same site.) (B) Genomic organization surrounding *PTL*, showing putative translated regions (green) and an intron (white). The 10.7 kb clone that fully complemented the *ptl* mutant phenotype is also shown (intergenic regions – yellow). Five reporter gene constructs carry the GUS gene (blue) translationally fused downstream of *PTL* genomic regulatory sequences, in some cases including the 1,127 bp intron (white). (C,D) Onion epidermal cells showing nuclear localization of the PTL protein translationally fused downstream of GFP (C), compared with location of control GFP protein alone (D). Left, bright field; right, GFP fluorescence of same field. Scale bars in C,D: 100 μm.

BAC, T24M12, as a probe. Fortuitously, the *ptl-1* mutant was uniquely associated with loss of one of the *Hae*III sites identified by this probe.

A cDNA covering this site was isolated from an inflorescence library, and the candidate gene shown to correspond to *PTL*, in that a 10.7 kb genomic clone encompassing the gene (Fig. 2B) fully complemented the *ptl-1* mutant phenotype. Also, all five mutant alleles (of similar phenotype) were associated with sequence changes that are likely to result in loss of function of the deduced polypeptide (Fig. 2A).

The *PTL* gene (At5g03680) encodes a transcription factor of the plant-specific trihelix family. Proteins of this family were first identified through their specific binding to conserved GT boxes within the promoters of light-regulated genes (hence they are also known as GT factors) (Zhou, 1999). The trihelix region (helix-loop-helix-loop-helix), duplicated in PTL, is a conserved DNA-binding domain distantly related to that of MYB transcription factors (Nagano, 2000).

The deduced PTL protein (Fig. 2A) contains a long helical region between the two trihelices that is conserved in family members closely related to PTL (Nagano et al., 2001). The center of this is predicted to form a coiled-coil (Fig. 2A), possibly associated with multimer formation. In addition, there is a serine-threonine rich region and a glycine rich region, each commonly found in transcription factors. There are also several putative nuclear localization signals (Dehesh et al., 1995), and we have shown that the PTL protein accumulates in the nucleus (Fig. 2C,D).

PTL is expressed at relatively low levels

We were unable to detect *PTL* transcripts by northern hybridization, so we screened tissues using RT-PCR (Fig. 3). In comparison with *AP3*, a gene strongly and specifically transcribed in floral tissues (Jack et al., 1992), *PTL* is weakly expressed. The highest level was found in inflorescences (including flowers up to stage 12). Relatively high expression was also seen in 7-day-old seedlings, although not in seedlings one day younger. *AP3* transcripts also increased markedly in seedlings between 6 and 7 days, suggesting that inflorescences had commenced development. Lower levels of *PTL* transcription were detected in the other tissues examined (Fig. 3).

Detection of *PTL* transcripts by in situ hybridization was difficult. A weak *PTL* signal was observed on occasion in the flower primordium from stages 4 to 6, occurring between the developing sepal primordia and in sepal margins (Fig. 4A).

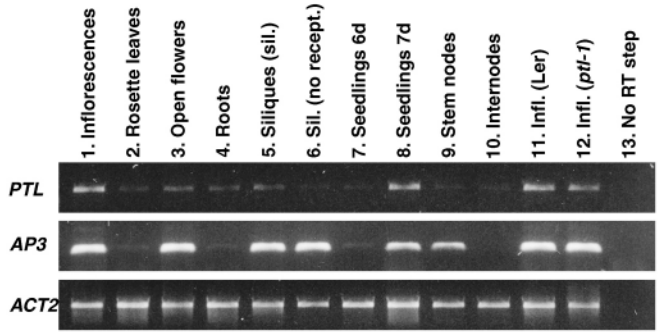

Fig. 3. Expression patterns of *PTL* assessed using RT-PCR. All tissues were from the Columbia ecotype except lane 11 (L*er*). The same total RNA samples were used for three reactions in each case, using mRNA specific primers for either *PTL* (top), flower-specific *APETALA3* amplified using essentially the same conditions (centre), or *ACTIN2* as a control (bottom). 3.5 μl of the reaction was loaded for *PTL* and *AP3* reactions, 1.5 μl for the *ACT2* control. In lane 6, the receptacle at the base of the siliques was removed before RNA extraction.

PTL is expressed in discrete zones of developing flower primordia

To assess expression patterns in detail, a reporter gene construct was made that included all of the 8 kb region upstream of the *PTL* coding sequence (up to the neighbouring gene), the first exon and the single large intron translationally fused with GUS (p8.0i::GUS) (Fig. 2B). Twelve out of 14 independent insertion lines showed a consistent pattern of GUS staining (Table 2). Closely similar patterns were seen in transformants of two similar constructs including less of the 5′ upstream region (p2.0i::GUS and p1.3i::GUS) (Fig. 2B); the results will be considered together (Table 2).

Expression occurred in a range of tissues, but the strongest signal was seen in developing flower primordia (Fig. 4B,D-H). Discrete expression zones were present on the lateral flanks of each flower primordium from the time they arose (stage 1) (Fig. 4D-H). Expression became localized to the lateral indentations where flower primordia separate from the inflorescence meristem at stage 2. As this expression faded, two dumbbell-shaped expression zones arose alongside and internal to the lateral sepals that initiated at stage 3 (Fig. 4D-H). This expression then resolved into four discrete, cone-shaped zones lying between each of the developing sepal primordia at stage

Table 2. Expression patterns of *PTL* deduced using GUS reporter lines*

		Flower primordia		Floral organs			Vegetative tissues			
Construct	Number of lines stained/total	Lateral flower primordia stage 1-2†	Inter-sepal zones stage 3-7†	Sepal margins stage 5-9†	Petal claw margins stage 8-13†	Stamen margins stage 7-8†	Leaf margins, stipules	Cauline axils, pedicel axils	Basal node, receptacle, root vasculature	Trichomes
p8.0i	12/14	++	+++	++	++	+	++	++	+	–
p2.0i	15/43	++	+++	++	++	+	++	+	(+)	+
p1.3i	26/37	++	+++	++	++	+	++	+	(+)	+
p8.0	25/32	–	–	++	–	+	++	++	++	–
p2.0	7/42	–	–	++	–	–	++	++	++	+

*Relative strength of signal is indicated by +++ (strongest) to – (no signal).
†Stages of flower development according to Smyth et al. (Smyth et al., 1990).

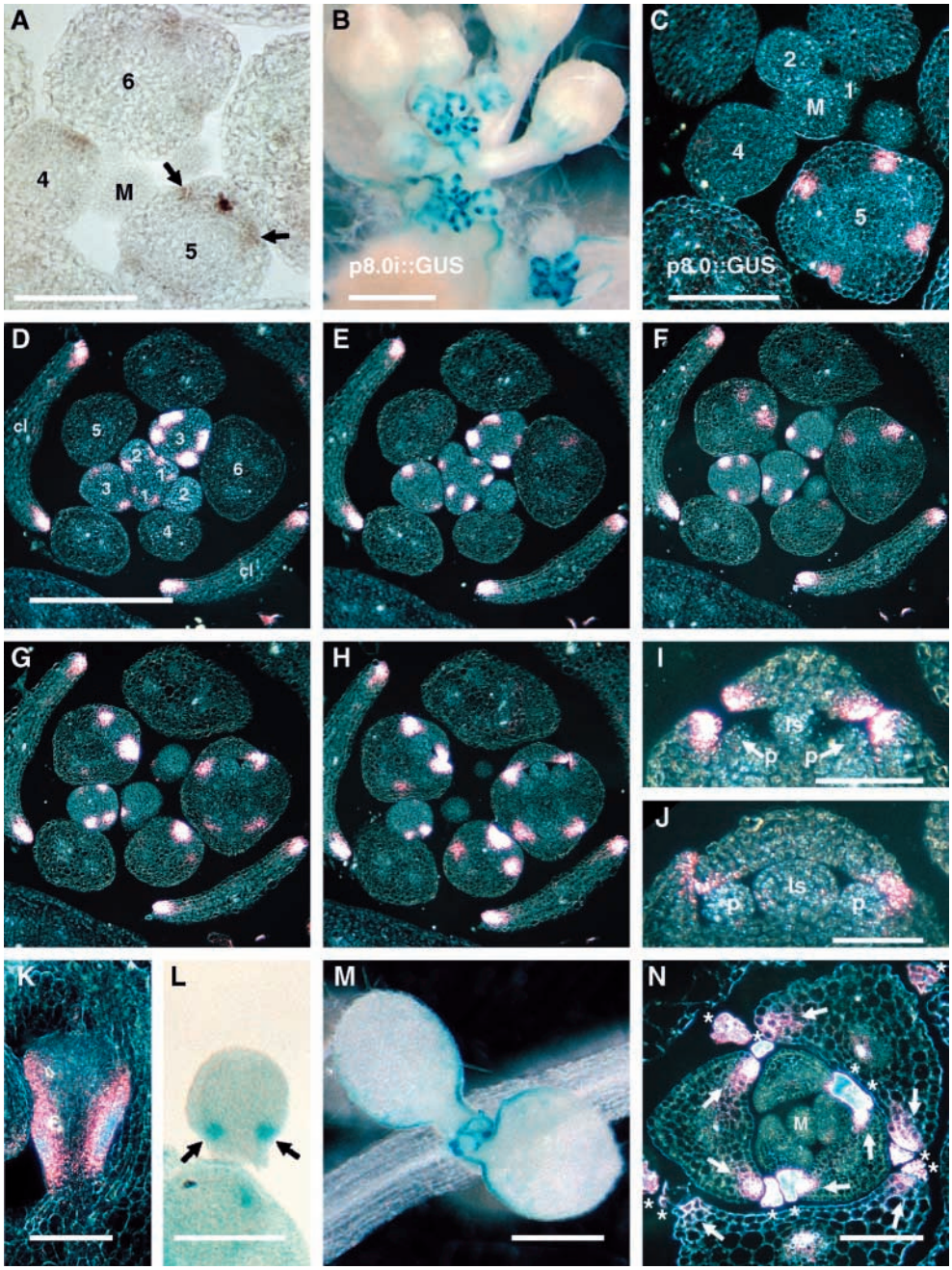

Fig. 4. Expression patterns of *PTL* using in situ hybridization and GUS reporter genes. (A) Location of DIG-labelled antisense *PTL* RNA (brown) hybridized in situ with *PTL* mRNA in transverse section of an inflorescence meristem. Label is concentrated in inter-sepal zones (arrows) and sepal margins. (The black deposit centred between the two arrows is a staining artefact.) M, shoot apical meristem; 4, 5 and 6, bud stages.
(B-N) Location of GUS reporter gene product in wholemounts (blue) or in sections (pink, dark field). (B) Side view of young inflorescences showing four spots of staining in young flower primordia (p8.0i::GUS). (C) Transverse section of inflorescence showing absence of early staining in the flower primordium without the intron, although sepal margin expression still occurs (p8.0::GUS). (D-H). Five serial transverse sections of an inflorescence showing staining patterns in buds from stage 1 to 6 (indicated in D). Staining is also present in the edges of cauline leaves (cl) (p2.0i::GUS). (I,J). Petal primordia (p) are not stained at stage 6 (I) or 7 (J). ls, lateral stamen (p2.0i::GUS). (K) Longitudinal section of stage 9 bud showing staining in the basal margins of a developing petal (p2.0i::GUS). (L) Wholemount of a stamen dissected from a stage 8 flower, showing lateral staining where developing anther and filament adjoin (arrows) (p2.0i::GUS). (M) Young seedling viewed from above showing staining in edges of developing leaves, initially all round, but later limited to basal regions (p2.0i::GUS). (N) Transverse section of shoot apical meristem of young seedling showing expression in leaf margins (arrows) and stipules (asterisks), but none in the shoot apical meristem (M) (p2.0i::GUS). Scale bars: A,C,L,N, 100 μm; B,M, 500 μm; in D, 500 μm for D-H; I-K, 50 μm.

4. This expression was relatively strong at first and matched the in situ hybridization pattern observed during stages 4-6 (Fig. 4A), but the level fell progressively until it was undetectable by the end of stage 7.

PTL is also expressed in lateral regions of developing floral organs

Expression of *PTL* was also seen fully around the edges of developing sepals (Fig. 4A,D-H) from stage 5 when they enclose the developing flower meristem. It extended several cells inward from the epidermis, particularly in adaxial regions at the sepal base, persisting until around stage 9.

Petal primordia arise between the sepals and internal to them at stage 5. It was not possible to determine whether the few petal-initiating cells carried GUS product at this stage, but, surprisingly, it was not observed in the small developing primordia at stages 6-7 (Fig. 4I,J). GUS product was first detected at around stage 8, just before petal primordia commence rapid growth (Fig. 4K). Even then, it was present only basally in the flanks, and this faded by stage 13 when petals were fully grown.

GUS product was also present in lateral regions of stamen primordia, specifically at the top of the filament and the base of the anther, from the time they differentiate from each other at stage 7 (Fig. 4L) until stage 9.

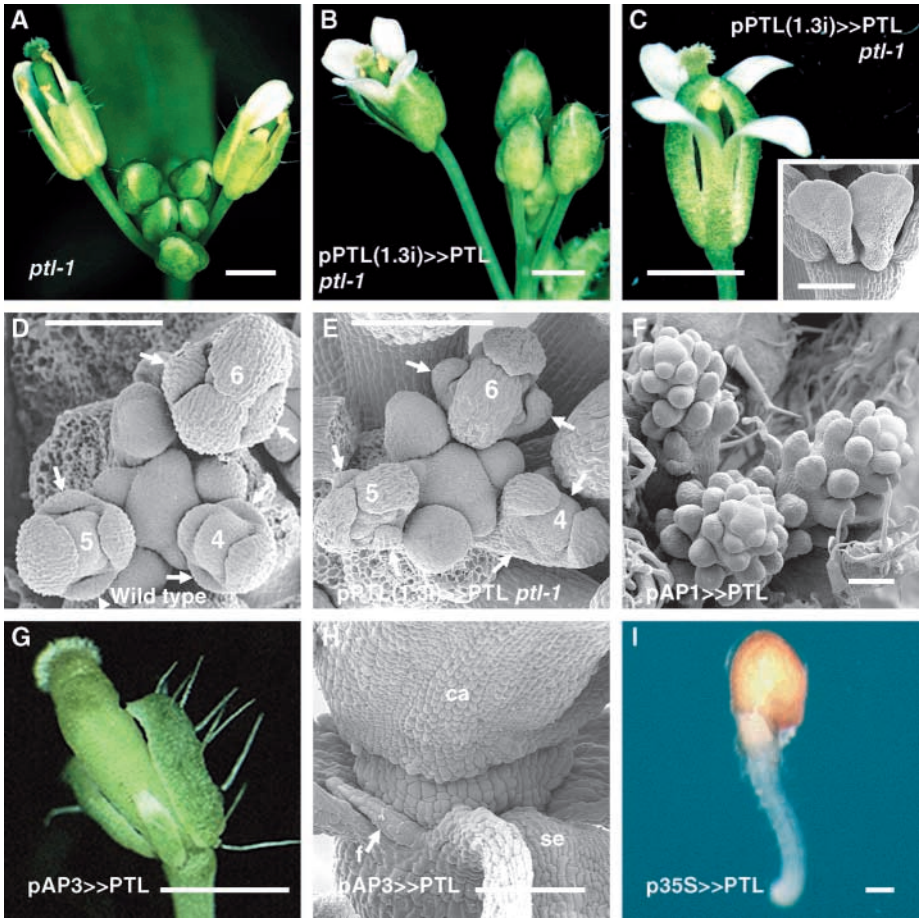

Fig. 5. Consequences of overexpression and ectopic expression of *PTL*. (A) *ptl-1* mutant inflorescence. (B) Complementation of *ptl-1* mutant phenotype in pPTL(1.3i)>>PTL plant (moderate driver line #5). (C) Gain-of-function phenotype in pPTL(1.3i)>>PTL *ptl-1* plant (strong driver line #7). Lateral sepals are absent, and four petals are restored, either separate (SEM inset), or fused along their lateral margins. (D,E) SEMs of inflorescence apex of (D) wild-type plant and (E) gain-of-function pPTL(1.3i)>>PTL *ptl-1* plant (strong driver line #1). Buds at stages 4, 5 and 6 are indicated. Lateral sepals are either absent or narrow in gain-of-function plants (E, arrows). (F) SEM of pAP1>>PTL plant with inflorescences producing flowers arrested at stage 2. (G) pAP3>>PTL flower showing absence of petals and stamens, and reduced lateral sepal. (H) SEM of pAP3>>PTL flower showing absence of petal and stamen primordia in the second and third whorls. ca, carpel; f, filamentous structure; se, sepal. (I) p35S>>PTL seedling showing final extent of growth. Strongly inducible PTL target line #10 was used for all ectopic expression plants (F-I). Scale bars: A-C,G, 1 mm; inset in C, D-F,H,I, 100 μm.

PTL is also expressed in margins of developing leaves and in some other vegetative tissues

In seedlings, *PTL* expression was first seen in developing leaves (Fig. 4M,N), commencing only after their primordia had extended over the shoot apical meristem. It was first present in a continuous band around their perimeter, extending through all three cell layers (Fig. 4N). Later, expression became limited to the basal region, including the petiole (Fig. 4M). Strong GUS staining was also seen in stipules throughout their life (Fig. 4N).

Weaker *PTL* expression was seen in the axils of cauline leaves and floral pedicels, with the intensity of GUS staining increasing as individual axils aged (Table 2). Staining intensity was somewhat lower on average in the shorter promoter lines, p2.0i::GUS and p1.3i::GUS, than in p8.0i::GUS lines. Some other vegetative regions were also stained, although relatively weakly (Table 2). These included the basal node of the rosette, the floral receptacle, and the vasculature of the root.

Overall, localization of the GUS reporter gene product is consistent with RT-PCR results (Fig. 3). Furthermore, *PTL* expression in *ptl* mutant plants, and in *pmd-1d* (L*er*) plants, was not noticeably different from the wild-type pattern in either case.

The intron is required for expression in flower primordia, but not in sepal and leaf margins

A striking difference in staining pattern was seen in reporter lines when the first exon and intron were absent (p8.0::GUS and p2.0::GUS) (Table 2). Staining in the flanks of flower primordia, and subsequently in the four inter-sepal zones, could not be detected (Fig. 4C). Staining in basal regions of developing petals was also absent. Thus, the intron is required to drive expression in these regions. (An involvement of exon 1 sequences is possible although regulatory sequences rarely occur in translated regions.) By contrast, staining around the margins of sepals and leaves was similar with or without the exon and intron (Fig. 4C; Table 2).

Expression in developing stamens was present without the intron, but only in the 8.0 kb construct (Table 2). It is apparently controlled by redundant elements present both in the intron and upstream of –2.0 kb. The intron also contains elements that dampen *PTL* expression in other vegetative regions (Table 2).

Early expression in flower primordia is required to complement the *ptl* mutant phenotype

In *ptl* mutants, visible defects were limited to sepals and petals (e.g. Fig. 5A). To test which components of the *PTL* expression pattern are associated with these defects, we carried out complementation tests using expression lines with or without the intron (i.e. with or without expression in the early flower primordium and inter-sepal zone). To do this, we used the two-component system of Moore et al. (Moore et al., 1998) (see Materials and methods). Two sets of driver lines containing

PTL regulatory sequences (one set with the intron [pPTL(1.3i)], and one without [pPTL(2.0)], were each crossed with four replicated target lines containing the *PTL* coding sequence, all in *ptl-1* mutant background. [Although the latter driver carries an additional 0.7 kb of the upstream region (–1.3 to –2.0 kb), no controlling elements were identified in this region (Table 2).]

All 11 lines containing the *PTL* promoter region and intron [pPTL(1.3i)] could fully restore the wild-type sepal and petal phenotype in combination with at least one of the target lines (Fig. 5B), whereas complementation was not seen among progeny of any of the seven driver lines that lacked the intron [pPTL(2.0); data not shown]. Thus the loss of PTL function specifically in the early flower primordium and inter-sepal zone is likely to be responsible for mutant disruptions to sepal and petal development. Sepal margin expression, conferred by both drivers, is insufficient to restore these disruptions to normal.

Interestingly, in six of the 11 combinations in which the intron was present in the driver [pPTL(1.3i)>>PTL], a new defective flower phenotype was seen (Fig. 5C). The severity of this phenotype was correlated with the strength of expression of each driver line (previously assessed by crossing them with a GUS target line). The phenotype was characterized by narrow sepals and sometimes loss of those in lateral positions (Fig. 5E, compared with 5D). The number of petals was usually restored to four, although they were often narrow and sometimes fused to each other at the base in lateral positions within the flower. Such fusion may be the consequence of petal primordia arising much closer than normal, associated with the absence of a lateral sepal (see stage 4 bud in Fig. 5E). The two lateral stamens were also often absent. This new floral phenotype was apparently controlled by regulatory sequences within the intron because similar floral defects were not seen in any of the pPTL(2.0)>>PTL combinations. One explanation is that overexpression of *PTL* in the flanks of the floral meristem and the four inter-sepal zones inhibits growth of these regions. To test this, we overexpressed *PTL* in other defined regions of the flower meristem.

Misexpression of *PTL* in other tissues results in inhibition of their growth

The *APETALA1* (*AP1*) gene is expressed predominantly in developing flower primordia from stage 1, becoming limited to sepal and petal primordia as they arise at stages 3 and 5 (Mandel et al., 1992). When an *AP1* driver line was crossed with another 12 independent PTL target lines, the pAP1>>PTL progeny revealed a spectrum of floral defects (Fig. 5F). For the most severely affected target line (#10), plants developed normally until the production of the inflorescence meristem. Flower primordia arose continuously, but their development was arrested at around stage 2 (Fig. 5F). In less severely affected target lines (e.g. #7), flower primordia were also arrested but some filamentous floral organs, mostly carpelloid, eventually arose.

A second floral gene was also tested. *AP3* drives expression specifically in the petal and stamen sectors of flower primordia from stage 3 (Jack et al., 1992). An AP3 driver line was crossed with the same 12 PTL target lines. Strikingly, in the resulting pAP3>>PTL plants, petal and stamen growth was often abolished (Fig. 5G,H), or was reduced to thin, filamentous outgrowths. In addition, the lateral sepals were often narrow and stunted (*AP3* is expressed in lateral sepal margins). The severity of the defects across the 12 target lines was strongly correlated with those seen using the AP1 driver (e.g. strongest in each case using line #10, intermediate with line #7). Interestingly, the strongest phenotype closely matched that seen when the diphtheria toxin gene *DTA* was expressed by the *AP3* promoter, ablating the tissues involved (Day et al., 1995). The weaker effect was similar to that seen in *Brassica napus* flowers when cell division was inhibited by *AP3* driven expression of *ICK1*, an inhibitor of cyclin-dependant kinase (Zhou et al., 2002).

The consequences of mis-expression of *PTL* more generally through the plant were tested using the CaMV 35S promoter. When the strongly responsive target line #10 was crossed with a 35S driver line, the p35S>>PTL progeny never developed beyond the production of a very short root (Fig. 5I). Using a less responsive target line (#7), progeny were arrested soon after the cotyledons emerged.

Thus it seems that strong expression of the *PTL* gene generally results in inhibition of growth of any tissue in which the expression occurs.

Floral expression of *PTL* is modified in mutants of the *PINOID* auxin signalling gene

Sepal development is severely disrupted in mutants of the *PINOID* (*PID*) gene (Bennett et al., 1995). This gene encodes a protein kinase associated with auxin signalling (Christensen et al., 2000). In *pid* mutant flowers, sepals can be fused laterally with each other, are irregularly spaced, and are variable in number and size (Fig. 6A). The first whorl arises initially as a ring of tissue without inter-sepal zones, and sepal primordia develop irregularly from its rim much later than normal (Bennett et al., 1995).

We were interested to see whether the apparent early loss of the inter-sepal zone in *pid* mutant flowers was associated with the loss of *PTL* expression. Surprisingly, this was not the case. Instead, ectopic *PTL* expression occurred, firstly throughout the newly arising outer ring of tissue in the flower primordia (Fig. 6B,C), and later continuously around its internal side (Fig. 6D). Thus it seems that early expression of *PTL* is negatively regulated by PINOID function. We further tested whether this ectopic *PTL* expression in *pid* mutants is associated with the delayed outgrowth of sepal primordia by looking to see if such disruptions were ameliorated in *pid-2 ptl-5* double-mutant plants. This is apparently not the case as sepal development was not significantly different between the two genotypes [mean number of sepals per flower (±s.e.m.) was 3.48±0.16 for 40 *pid-2 ptl-5* flowers compared with 3.73±0.14 for 45 *pid-2* single mutant flowers).

Discussion

PETAL LOSS represses growth in the inter-sepal zone

PTL apparently acts in the developing flower to dampen growth in four small regions lying between the sepals from the time they arise, resulting in their separate outgrowth. Evidence for this is that: (1) *PTL* expression is strongest in inter-sepal zones during early flower development (Fig. 6); (2) adjacent sepals are sometimes fused in *ptl* mutants, a likely consequence of ectopic growth occurring between them; (3) extensive sepal

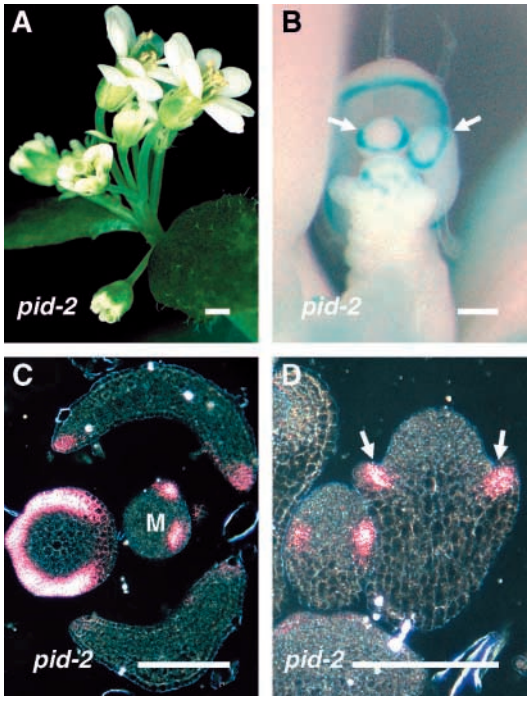

Fig. 6. Expression of *PTL* in *pinoid* mutant plants. (A) *pid-2* mutant inflorescence. (B) p2.0i::GUS expression in *pid-2* inflorescence. Rings of staining (blue) occur in the first whorl region of two buds (arrows). (C) Transverse section of a developing *pid-2* flower at the same stage as that shown in B. M, shoot apical meristem. (D) Longitudinal section of an older *pid-2* flower showing GUS staining (pink) accumulating in the inner region of the expanding first whorl (arrows). Scale bars: A, 1 mm; B-D, 100 μm.

fusion occurs in double mutants of *ptl* with either of the organ boundary genes *cuc1* or *cuc2*, as would be expected if all three genes function in keeping sepals separate; and (4) strong ectopic expression of *PTL* consistently represses growth, suggesting that growth suppression is its normal role. Concerning the latter observation, we realise that the consequences of ectopic *PTL* expression might represent a gain of function if, for example, abnormally large amounts of PTL protein sequester accessory factors required for the activity of other growth-promoting transcription factors. Alternatively, excess PTL protein might inappropriately activate growth-suppressing genes (Le Gourrierec et al., 1999). These possibilities can be tested by overexpressing *PTL* with its DNA-binding domains inactivated.

PTL does not itself define the sites of origin of the four sepals, as four still arise in appropriate positions in the absence of PTL function. Other genes are apparently involved. One of these may be *PINOID* that encodes a serine/threonine kinase implicated in auxin signalling (Christensen et al., 2000), possibly through its positive regulation of polar auxin transport (Benjamins et al., 2001). *PID* is expressed in sepal primordia as they arise and grow, but not between them (Christensen et al., 2000). Recently it has been shown that auxin is involved in defining the sites of initiation of organ primordia (Reinhardt et al., 2003). Thus, sepal initiation may be promoted by the localized concentration of auxin acting through PID. We have shown that PID excludes *PTL* expression from sepal initiation

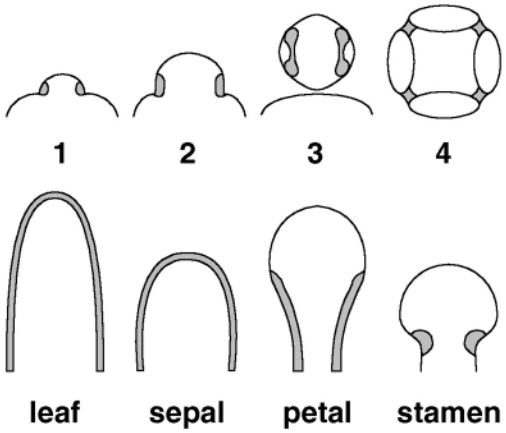

Fig. 7. Summary of expression patterns of *PTL* (shaded) in lateral regions of flower primordia (stages 1-4, vertical view), and in developing leaves and floral organs (lateral view).

regions. Furthermore, the absence of *PID* expression in inter-sepal zones would allow *PTL* to be expressed there, hence resulting in suppression of growth in these zones.

PETAL LOSS may influence petal initiation and orientation indirectly

Although loss of PTL function severely affects petal development, *PTL* is not expressed in petal primordia until they start to expand at stage 8, and even then it occurs only in their basal margins (Fig. 7). Consistent with this, when *PTL* is ectopically expressed strongly in the second floral whorl using the *AP3* promoter, petal initiation is frequently abolished, but overexpression of *PTL* controlled by its own regulatory sequences does not disrupt petal initiation. Thus it seems likely that PTL influences petal development indirectly (assuming it acts cell autonomously). It is true that *PTL* function is required for expression of the *RBE* petal development gene (Takeda et al., 2004), but this positive regulation may occur earlier (stages 3-4) in the inter-sepal zone, where their expression patterns overlap.

If PTL normally functions to inhibit growth, additional growth may be expected to occur in *ptl* mutants. Thus, the number of petals per flower might be predicted to increase if more space were available for them to arise. However, the number is observed to decrease. It may be that overgrowth in inter-sepal zones diffuses and weakens a petal initiation signal so that they arise less often, later on average, and over a wider area than usual (Griffith et al., 1999).

In *ptl* mutants, disruption of petal orientation within the flower is also seen (Griffith et al., 1999). Petal primordia normally initiate when cells in the L2 layer divide outwards (periclinally) rather than laterally (anticlinally) (Hill and Lord, 1989). Sector analysis has suggested that two adjacent cells are initially involved (Bossinger and Smyth, 1996). Presumably, these cells usually lie side by side on a circumference of the flower primordium, defining the ultimate orientation of the developing petal. In *ptl* mutants, however, petals arise in any orientation (Griffith et al., 1999). It may be that additional growth within the petal-initiating zone in *ptl* mutants has relieved the constraint that only cells lying adjacent on a circumference commence petal development.

In addition to adopting an orientation within the flower primordium, the two faces of developing petals acquire adaxial-abaxial polarity. Petal orientation, once established, may automatically define the later developing polarity, or they may be independent processes (Griffith et al., 1999). It has been proposed that polarity of lateral organs is defined by a signal emanating from the center of the shoot or flower meristem (Eshed et al., 2001; McConnell et al., 2001; Emery et al., 2003). This could ensure that adaxializing genes of the class III HD-ZIP family are expressed in adaxial regions of newly arising organ primordia. In *ptl* mutants, disruption to such a signal is also likely to be indirect because *PTL* is not expressed in petal primordia, the targets of the signal.

PETAL LOSS may regulate the marginal expansion of leaves and floral organs

Strikingly, *PTL* expression occurs in the margins of most developing lateral organs (Fig. 7). Of floral organs, only carpels lack such expression. One appealing hypothesis is that the PTL protein dampens the growth in these regions of lateral organs, helping sculpt their final shape. Thus, PTL may moderate extension around the edges of leaves and sepals, ensuring that they keep pace with expansion of more centrally located tissue. Also, constriction in the region of the petiole of leaves, the claw of petals, and the filament/anther boundary of stamens may be the consequence of growth suppression. Flower primordia, too, may be constrained from lateral expansion by early *PTL* expression in their flanks.

Even so, loss of PTL function does not seem to be associated with any obvious phenotypic consequences in these regions. Other genes with the same growth suppression function may still be active (see below). The homeodomain-encoding gene *PRESSED FLOWER* is expressed specifically in lateral regions of primordia of all of these organ types, and in newly arising flower primordia (Matsumoto and Okada, 2001). However, it apparently promotes growth in lateral regions, and it will be interesting to assess whether it competes or otherwise interacts with PTL.

PETAL LOSS is the first trihelix transcription factor known to play a role in morphogenesis

Twenty-eight trihelix genes have been identified in the *Arabidopsis* genome (Riechmann et al., 2000), but only three others have been characterized in detail to date. All were identified as encoding proteins that bind specifically to promoter elements required for light responsiveness. The GT-2 protein of rice binds to GT boxes in the promoter of the *phytochrome A* gene and is associated with its dark induction (Dehesh et al., 1990). A closely related protein, DF1 from pea, also binds to regulatory elements necessary for dark induction (Nagano et al., 2001). Contrastingly, GT-1a from tobacco binds promoter elements associated with induction by light (Gilmartin et al., 1992; Perisic and Lam, 1992).

In addition to *PTL*, six of the 28 trihelix genes in *Arabidopsis* have (or probably once had) duplicated trihelix domains. These include AtGT-2 (Kuhn et al., 1993) and AtDF1 (Nagano et al., 2001), but not AtGT-1a (Hiratsuka et al., 1994). Even so, neither AtGT-2 nor AtDF1 is closely similar to PTL (60-65% identical in amino acid sequence in the N-terminal trihelix, and 42-45% in the C-terminal trihelix). This divergence is apparently reflected by divergence in the targets of regulation, as PTL does not play any obvious role in light response.

Expression of *PTL* in early flower primordia and the inter-sepal zone is controlled intragenically, probably by sequences within the intron. Other genes are known that are regulated by intronic elements (e.g. Sieburth and Meyerowitz, 1997). Interestingly, an intron occurs at this position only in those 7 members of the trihelix family that arose following duplication of the trihelix. The closest relative of *PTL*, At3g10000 (94% identical in the N-terminal trihelix), shares some tracts of similar sequence within its intron. It will be of interest to see whether it shares functions with *PTL* in defining the inter-sepal zone. Also, it may be that this and other very close relatives of *PTL* act redundantly in lateral organ margins where loss of PTL function is apparently without effect.

In conclusion, this study has revealed that the *PETAL LOSS* gene helps to control morphogenesis of the perianth. It may repress growth between sepal primordia keeping them separate, and later allow petal developmental signals to be perceived appropriately. This represents a new developmental function for a family of transcription factors previously known only for their role in regulating light response.

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