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Mutations in the *PERIANTHIA* gene of *Arabidopsis* specifically alter floral organ number and initiation pattern

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SUMMARY

An open question in developmental biology is how groups of dividing cells can generate specific numbers of segments or organs. We describe the phenotypic effects of mutations in *PERIANTHIA*, a gene specifically required for floral organ patterning in *Arabidopsis thaliana*. Most wild-type *Arabidopsis* flowers have 4 sepals, 4 petals, 6 stamens, and 2 carpels. Flowers of *perianthia* mutant plants most commonly show a pentamerous pattern of 5 sepals, 5 petals, 5 stamens, and 2 carpels. This pattern is characteristic of flowers in a number of plant families, but not in the family Brassicaceae, which includes *Arabidopsis*. Unlike previously described mutations affecting floral organ number, *perianthia* does not appear to affect apical or floral meristem sizes, nor is any other aspect of vegetative or floral development severely affected. Floral organs in *perianthia* arise in a regular, stereotypical pattern similar to that in distantly related species with pentamerous flowers. Genetic analysis shows that *PERIANTHIA* acts downstream of the floral meristem identity genes and independently of the floral meristem size and floral organ identity genes in establishing floral organ initiation patterns. Thus *PERIANTHIA* acts in a previously unidentified process required for organ patterning in *Arabidopsis* flowers.

Key words: *Arabidopsis*, *PERIANTHIA*, flower development, pattern formation

INTRODUCTION

One general question in developmental biology is how developing tissues produce the correct number of subunits. Numbers of pattern elements are often fixed within a species and among closely related species. For instance, humans have 5 digits per appendage, and all mammals, from giraffes to mice, have the same number of cervical vertebrae. Similarly, the floral meristems of many plants initiate organ primordia in a precise number and pattern. As in most other members of the plant family Brassicaceae, *Arabidopsis* flowers initiate 4 concentric whorls, or rings, of organs, with 4 sepals present in the first (outer) whorl, then 4 petals, 6 stamens, and 2 carpels in the second, third, and fourth (central) whorls, respectively. While almost all families of flowering plants show this same order of organ types from the outside to the inside of the flower, the number of organs of each type varies widely among different families.

The mechanism by which floral organ types are specified has been the subject of study in recent years (reviewed in Weigel and Meyerowitz, 1994), but the establishment of the pattern of organ initiation in the flower has been less studied and is not understood. A number of mutations that affect floral organ number without affecting floral organ identity or floral meristem identity have been isolated. These include *clavata1*, *clavata2*, *clavata3*, *fasciata1*, *fasciata2*, *tousled*, and *revoluta*. Mutations in the *CLAVATA* (*CLV*) class genes increase organ number in all four whorls, and also lead to additional whorls (Koornneef et al., 1983; Leyser and Furner, 1992; Clark et al., 1993, 1995). The increase in organ and whorl number is correlated with an increase in floral meristem size at the time of organ initiation (Clark et al., 1993, 1995). Mutations in the *FASCIATA* (*FAS*) genes cause a lesser and more variable change in organ number in the first three whorls of the flower (Leyser and Furner, 1992). Mutations in the *TOUSLED* (*TSL*) and *REVOLUTA* (*REV*) genes lead to reduced organ numbers in all four whorls (Roe et al., 1993; Talbert et al., 1995). Each of these mutants shows phenotypes outside of the flower: the apical meristem structure is severely disrupted in *fas* and *clv* mutants, leading to fasciation and abnormal phyllotaxy, and *fas*, *tsl*, and *rev* mutants show defects in most aspects of aerial development, including leaf number, leaf and floral organ development, and flowering time. In addition, the floral phenotype is highly variable in these mutants. This suggests that the genes may play an indirect or non-specific role in determining the number and pattern of organ initiation events, such as by regulating the size or shape of meristems.

In this paper we describe the effects of mutations in *PERIANTHIA* (*PAN*), a gene specifically required for proper floral organ patterning. The typical flower in *pan* mutant plants is pentamerous, having 5 organs arranged in a stereotypical pattern in each of the first three whorls, along with 2 carpels in the fourth whorl. The mutants show no change in floral meristem identity or floral organ identity. In addition, we have

found no effect on meristem structure, nor on any other aspect of vegetative, floral, or organ development. Genetic analysis indicates that *PAN* acts in a different pathway than the *CLV* genes, and requires floral meristem identity gene activity for its function. It also acts independently of the homeotic genes in specifying floral patterning. *PAN* thus represents an example of a gene required for generating proper organ number, but not for proper organ type or meristem size.

MATERIALS AND METHODS

For all plants, seeds were sown at least 2 cm apart on a 1:1:1 mix of soil:perlite:vermiculite, imbibed at 4˚C for 4 days, then placed under 600 foot-candles of constant cool-white fluorescent light at 23˚C. Plants were fertilized at regular intervals beginning approximately 7 days after germination.

Both *perianthia-1* and *perianthia-2* were isolated in a screen of *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* seeds of ecotype Wassilewskija (Ws; Feldmann, 1992). All other mutants used in the study have been previously described, and all except *ag-2*, *clv1-4*, p35S-*AP3*, and p35S-*PI* were isolated in the Landsberg *erecta* (L*er*) ecotype. *ag-2* was isolated from Ws (Yanofsky et al., 1990). *clv1-4* was isolated in either Estland or Limburg (McKelvie, 1962), and was backcrossed to L*er* three times before use (Clark et al., 1993). p35S-*AP3* and p35S-*PI* were generated in ecotype Nossen (Jack et al., 1994; Krizek and Meyerowitz, 1996).

Double mutants were obtained by screening the progeny of F_2 *pan* plants obtained from the cross of homozygous parents, except in crosses with *ag-1* and *ag-2*, which are male and female sterile, where crosses were made using heterozygous *ag* plants. The quadruple mutant *pan-1 ag-1 ap2-2 pi-1* was constructed by using *pan-1* pollen to pollinate plants homozygous for *ap2-2* and *pi-1* and heterozygous for *ag-1*, collecting seeds from F_2 plants homozygous for *pan*, and screening F_3 plants. Control crosses were performed with wild-type Ws and all the mutants used in the study. In two cases, *ag* and *ap1*, the phenotype of the previously described mutants was affected by the Ws background; both cases have been noted in other studies. Flowers homozygous for *ag* show more pronounced internode elongation in an *ERECTA* background (Yanofsky et al., 1990), and Ws is mutant for *CAULIFLOWER*, a gene that has no phenotype on its own but mutations in which dramatically enhance the *ap1* phenotype (Bowman et al., 1993). No notable phenotypic differences due to the Ws background were seen for any of the other mutants used.

In the cross between *pan-1* and *ap1-1*, very few of the *pan-1* plants collected in the F_2 were also heterozygous for *ap1-1* (3 *pan-1/pan-1 ap1-1*/+ out of 256 plants tested), suggesting tight linkage (0.8±0.4 cm), and that *PAN* maps to chromosome 1. Linkage was also noted in crosses with *clv1-4*, *clv2-1*, and *cer6-1* (Koornneef et al., 1983).

Scanning electron microscopy was performed as described by Bowman et al. (1989). Confocal laser scanning microscopy was performed as described by Running et al. (1995). Figures were prepared digitally as described by Sieburth et al. (1995).

RESULTS

perianthia affects floral organ number

Wild-type *Arabidopsis thaliana* flowers contain 4 concentric whorls of organs, with 4 sepals present in the outer (first) whorl, then 4 petals, 6 stamens (2 short lateral and 4 long medial), and 2 carpels in the second, third, and fourth (central) whorls, respectively. Organ number varies little in wild type, except that 25% of the flowers are missing one or both lateral stamens (Smyth et al., 1990). Two non-complementing alleles of *perianthia* were identified from a T-DNA mutagenized population of *Arabidopsis* seeds based on their aberrant floral organ number (Fig. 1). Each mutation affects the number of all floral organ types (Table 1). The modal numbers of organs in *pan* flowers homozygous for either mutant allele are 5 sepals, 5 petals, 5 stamens, and 2 carpels (Fig. 1B,D). There is, however, greater variation in organ number in *pan* than in wild type. The organ number ranges from 4 to 7 in the first three whorls, and 1 to 3 in the fourth whorl. Both *pan-1* and *pan-2* have a very similar phenotype, except that *pan-2* flowers have slightly more stamens on average compared to *pan-1*. The organ number per flower does not appear to change throughout the life of the plant or on the primary versus axillary meristems: flowers on 45 day old plants on axillary meristems have a similar number of organs as the first 15 flowers on the primary meristem (Table 1). The phenotype of *pan* is sensitive to growth conditions: in adverse environments such as high temperature or crowding, the number of organs in all 4 whorls is decreased compared to those grown under optimal conditions, though fewer than 4 organs have not been observed in the first 3 whorls of *pan* mutants. Most flowers on plants heterozygous for either allele of *pan* resembled wild type, though a slight semidominance was noted in both alleles. In one study, 2 flowers out of 180 wild-type flowers had 5 sepals, but 18 out of 112 *pan-2*/+ flowers had 5 sepals. Petal, stamen and carpel number in heterozygous *pan* plants are similar to wild type.

Sepal initiation and development in *perianthia*

In mutants homozygous for either allele of *pan*, the phenotype is limited to the flower. Root structure and length are the same as in wild type, as is the number, size, and shape of rosette and cauline leaves, the time to flowering, fertility, and germination rate. The apical meristem in *pan* plants is the same size as in wild type, and flowers are produced in a normal spiral phyllotaxy in both mutants (Fig. 2A-C).

The earliest defect seen in *pan* flowers is observed at the

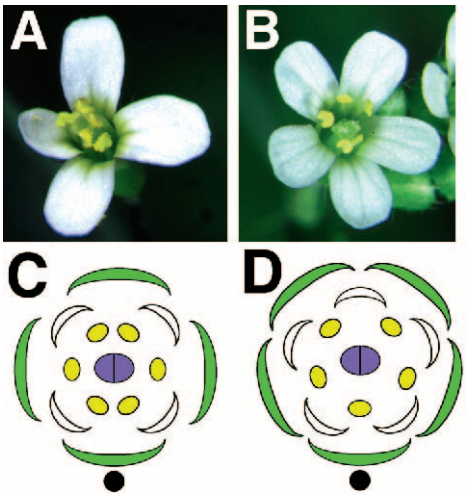

Fig. 1. (A) Wild-type flower. (B) *perianthia-1* flower. (C) Diagram of the organ positions most commonly found in wild-type *Arabidopsis* flowers. (D) Diagram of the organ positions most commonly found in *perianthia* mutants.

time of sepal initiation (stage 3; all stages according to Smyth et al., 1990). In wild-type *Arabidopsis*, the first whorl consists of four equidistant sepals, two medial and two lateral with respect to the apical meristem (Fig. 2A; Smyth et al, 1990). The sepals initially appear as a ridge surrounding the floral meristem (Fig. 2D). The abaxial medial sepal (the sepal on the opposite side of the floral primordium from the apical meristem) initiates first, with the other three sepals arising slightly later. The lateral sepals initiate lower on the floral meristem than the medial sepals. Later the abaxial sepal grows to overlie the dome-shaped floral meristem, growing to and covering the apex of the adaxial sepal (the sepal arising on the same side of the primordium as the apical meristem), with the lateral sepals meeting each other below the medial sepals (Fig. 3A; Smyth et al., 1990).

The *pan* flower usually contains 5 sepals, with 4 or 6 sepals occasionally and 7 sepals rarely seen. All sepals arise in a single whorl. In contrast to other mutations affecting organ number, such as *clv1*, *clv3*, and *tsl*, the sepals arise in a regular fashion, with the adaxial sepal always present, and the other sepals arising equidistantly around the floral meristem (Fig. 2B,C). As in wild type, sepals initiate as a ridge surrounding

Table 1A. Mean number of floral organs in wild-type (ecotype Wassilewskija), *pan-1*, and *pan-2* flowers

	First 15 flowers				Flowers from 45 day plants			
	Sepals	Petals	Stamens	Carpels	Sepals	Petals	Stamens	Carpels
Ws	4.0±0.1	4.0±0.1	5.8±0.6	2.0±0.0	4.0±0.0	4.0±0.0	5.7±0.5	2.0±0.0
pan-1	5.2±0.5	4.8±0.5	5.1±0.7	2.0±0.1	5.0±0.4	4.7±0.5	5.1±0.5	2.0±0.0
pan-2	5.3±0.6	5.0±0.6	5.4±0.8	2.0±0.1	5.1±0.4	4.8±0.5	5.3±0.7	2.0±0.0

Table 1B. Percentages of flowers with particular organ numbers

		First 15 flowers			45-day flowers		
	Number of organs	Sepals (%)	Petals (%)	Stamens (%)	Sepals (%)	Petals (%)	Stamens (%)
pan-1	4	5	23	19	10	34	12
pan-1	5	74	72	54	84	64	70
pan-1	6	21	4	23	6	2	14
pan-1	7	1	1	3	0	0	4
pan-2	4	4	19	15	6	26	10
pan-2	5	68	67	41	82	70	54
pan-2	6	24	13	36	12	4	28
pan-2	7	3	2	9	0	0	8

The first 15 flowers on the primary meristem were scored from each of 12 Ws, *pan-1*, and *pan-2* plants (total of 180 flowers for each genotype). An additional 50 flowers were scored on primary and axillary meristems from a collection of 45-day old plants. The standard error is indicated.

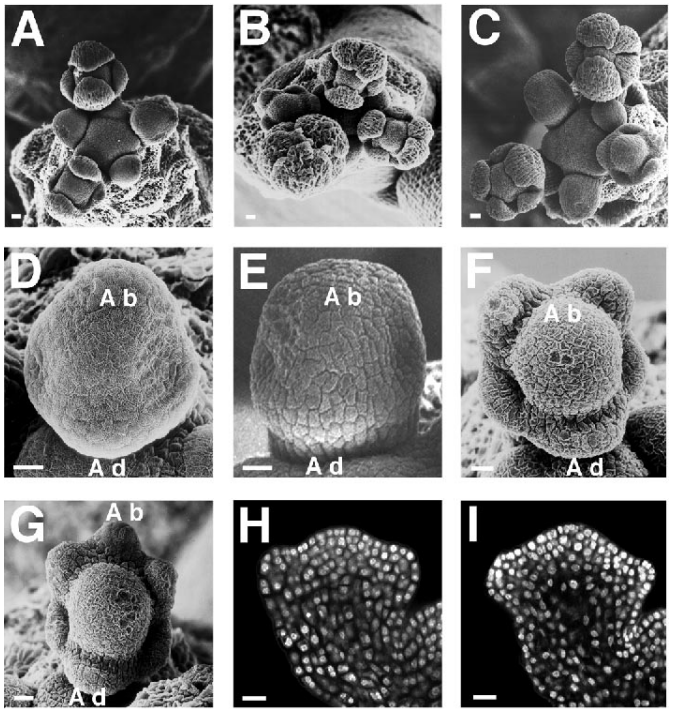

Fig. 2. Early development of *Arabidopsis* wild type and *perianthia* mutants. (A) Top view of wild-type (ecotype Wassilewskija) apex. (B) *pan-1* apex. (C) *pan-2* apex. The pattern of sepal initiation in *pan* mutants is regular, with a sepal always present in the adaxial position, and other sepals arising equidistantly around the floral meristem. Mutations in *pan* do not affect apical meristem size or phyllotaxy. (D) Wild-type early stage 3 (stages from Smyth et al., 1990), with sepals initiating as a ridge surrounding the floral meristem. (In D-G, the adaxial side of the flower is toward the bottom of the panel.) (E) *pan-2* flower at the same stage as the flower in D, also showing the ridge of cells surrounding the floral meristem predicting the site of sepal initiation. (F) *pan-1* stage 3 flower, slightly older than the flower in E. Sepals closest to the lateral positions maintain the lateral characteristics of smaller size and more basal origin. (G) *pan-1* stage 3 flower with 6 sepals. In this case both adaxial and abaxial sepals are present, and are larger and initiate higher than the 4 sepals straddling the lateral positions. (H) Confocal optical medial center section of a whole-mount early stage 3 wild-type flower (approximately the same age as the flower in D) stained with the DNA fluorescent dye propidium iodide (Clark et al., 1993; Running et al., 1995). (I) Confocal optical medial center section of a *pan-2* stage 3 flower at the same age as the flower in H. We were unable to detect changes in floral meristem size, cell number, or cell patterning in *pan* compared to wild-type flowers at the stage of sepal initiation. Ad, adaxial side of the flower. Ab, abaxial side of the flower. Bar, 10 μm.

the floral meristem before individual sepals can be identified (Fig. 2E). In flowers with 5 sepals, which represent the majority of *pan* flowers, two of the sepals arise close to the lateral positions but are offset towards the adaxial side, and two sepals arise straddling the abaxial position. In flowers with 6 sepals, both adaxial and abaxial sepals are present, with two pairs of sepals arising straddling the lateral positions (Fig. 2G). Sepals also maintain the positional identity found in wild-type flowers: those sepals initiating closer to the lateral positions arise later and lower than those sepals initiating closer to the medial positions (Fig. 2E,F,G), and the sepals growing most medially generally grow to overlie those arising more laterally (Fig. 2B). Mature *pan* sepals are indistinguishable from wild-type sepals in overall morphology and size.

Petal, stamen and carpel initiation and development in *perianthia*

In wild type, 4 petals of identical size arise at stage 5, alternate with and interior to the sepals (Fig. 3B,C,F). The petals initiate as a smaller primordium, and initially develop more slowly, than stamens (Smyth et al., 1990). In *pan*, petals also arise alternately with and interior to the sepals (Fig. 3D,E,G). In flowers in which there are fewer petals than sepals, the petals that are present still arise alternately with and interior to the sepals, with a petal failing to form in one of the predicted locations. While there is not an absolute dependence of petal number on sepal number, we have not observed any *pan* flowers with more petals than sepals, indicating that petal and sepal number are not independently determined. The size at which petals initiate and their pattern of growth are not affected by the mutation, and mature *pan* petals are indistinguishable from wild type in size and morphology.

In most wild-type flowers, 6 stamens initiate at stage 5 in a stereotypical pattern, with 4 long stamens occupying the medial positions and 2 short stamens occupying the lateral positions, though flowers missing one or both lateral stamens are also seen (Fig. 3B,C,F; Smyth et al., 1990). In contrast, stamens in *pan* flowers typically arise alternate with and interior to the petals, and as a result the stamens occupy positions aligned with the sepals. In most *pan* flowers a medial adaxial stamen is present, and the 4 remaining stamens occupy equidistant positions around the third whorl (Fig. 3D,E,G). In flowers where more stamens than sepals are seen, two stamens often co-occupy a position interior to one of the sepals, as the medial sepals do in wild-type flowers. As in the sepals, positional identity of stamens is maintained: stamens arising closer to the medial positions are taller than those arising closer to the lateral positions (Fig. 3D,E), though the difference in height among *pan* stamens is less than that seen between wild-type stamens. The number of stamens in a particular flower is independent of the number of sepals and petals. Mature stamens have wild-type morphology and are fully fertile, except that stamens lacking anthers are occasionally seen.

In wild type, two carpels initiate as a single cylinder from a central dome of cells and later fuse at their apices, creating a gynoecium with a septum oriented along the medial axis (Fig. 3F; Smyth et al., 1990). In almost all *perianthia* flowers, the carpels form in the same position as in wild type (Fig. 3D,G,H). Some aspects of gynoecium development may be disrupted, however; for instance, in some flowers the gynoecial apex remains unfused for a longer period than in wild type (Fig. 3H). Nevertheless, mature carpels show no dramatic changes in morphology and size, and are fully fertile.

Fig. 1 diagrams the organ positions most commonly found in wild-type and *pan* mutant flowers.

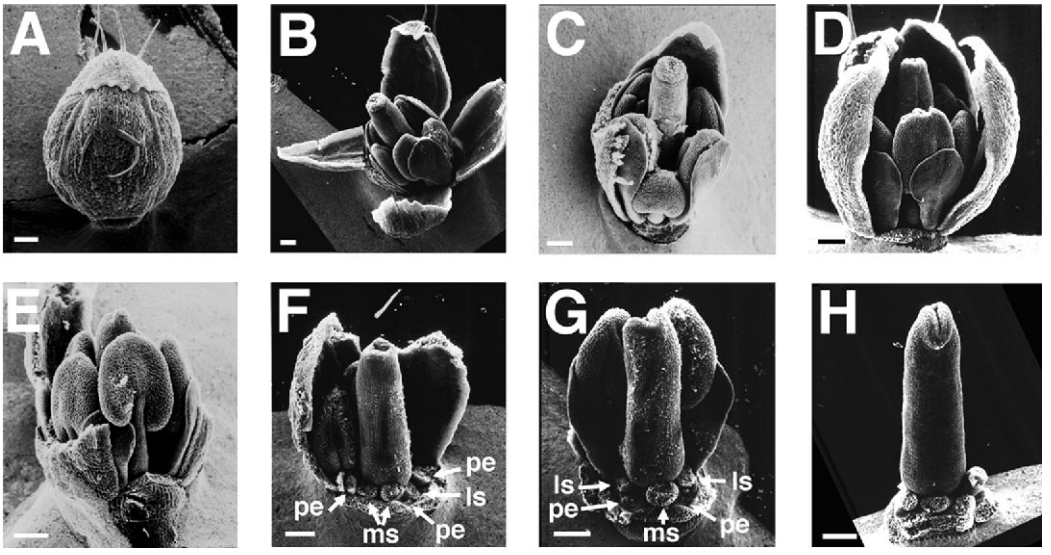

Fig. 3. Later development of wild-type and *perianthia* flowers. (A) Wild-type (ecotype Wassilewskija) flower. (In A-B and D-H, the adaxial side of the flower is toward the bottom of the panel.) The abaxial sepal grows to overlie the adaxial sepal, with the two lateral sepals meeting underneath both medial sepals. This is also true in most *pan* flowers (see e.g.Fig. 2B). (B) Wild-type flower opened to reveal the positions of interior organs. Petals arise alternate with and interior to the sepals, and 4 long medial stamens and 2 short lateral stamens arise interior to the petals. (C) Wild-type flower, with adaxial side toward the right of the panel. Here again the 4 petals and short medial stamens are visible. (D) *pan-1* flower with adaxial sepal removed. Note that two petals are visible in between the adaxial sepal and two sepals closest to the lateral positions. The adaxial stamen is also visible, and is somewhat taller than the adjacent, more lateral stamens. The margin where the carpels are fused is visible along the medial axis. (E) *pan-1* flower with the adaxial sepal and most of the two adjacent sepals removed. Here again the longer adaxial stamen is visible, with petals arising in between and interior to the sepals. (F) Wild-type flower with several internal organs removed to reveal organ arrangement. Note the margin where the carpels have fused. (G) *pan-1* flower with all the sepals, 2 petals and 3 stamens removed, showing the initiation pattern of all adaxial organs. H. *pan-1* gynoecium. In some flowers, gynoecium development is slightly abnormal, with carpels fusing later in development than in wild type. pe, petal; ms, medial stamen; ls, lateral stamen; Bar, 100 μm.

perianthia mutants are not defective in meristem structure

One potential explanation for the increased perianth organ number in *pan* is that the floral meristem size is larger at the time these organs initiate. Previous studies have shown that the increase in organ number in strong *clv1* and *clv3* flowers is correlated with a floral meristem increased in size and cell number compared to wild type at the time of sepal initiation, with more severe alleles having larger floral meristems than weaker alleles (Clark et al., 1993, 1995). Specifically, *clv* floral meristems are much taller but only slightly wider than wild type, which presumably accounts for the organ number changes seen in *clv* flowers, which have only slightly more sepals but many more carpels than wild type.

To determine if the phenotype of *pan* results from a similar mechanism, we examined the size and cell pattern of wild-type and *pan* flowers at the time of sepal initiation using confocal microscopy (Table 2, Fig. 2H,I). We chose flowers at the time the first whorl forms as a ridge, before individual sepals are distinguishable, as in flowers in Fig. 2D,E. We were unable to detect a size difference between wild-type and *pan* floral meristems in central medial sections. At the time of sepal initiation, *pan* floral meristems have a similar size, shape, and cell number per section as wild type (Table 2, Fig. 2H,I).

Table 2. Mean width and height of floral meristem, and number of cells visible in center sections of wild-type (ecotype Wassilewskija) and *pan-2* flowers at early stage 3

Genotype	Width (μm)	Height (μm)	Cell number
Ws	43±2.7	11±1.4	161±13
pan-2	42±2.8	11±1.8	165±11

15 wild-type and 13 *pan-2* flowers stained with the nuclear dye propidium iodide were analyzed by confocal laser scanning microscopy (Running et al., 1995). All measurements were based on central medial sections through the floral meristem at the stage similar to the those of the flowers in Fig. 2D,E,H, and I. Width measurements were taken between the visible ridges that will form the sepal whorl. Height measurements were taken from the point equidistant along the width to the top of the floral meristem. The total number of cells visible in the center section of the floral primordia is also presented. The standard error is indicated.

Double mutants of *perianthia* and other genes controlling flower development

perianthia-1 clavata1-4, perianthia-1 clavata3-2

The *clv* mutations lead to an increase in the number of organs in all four whorls, especially the inner whorls, presumably due to a larger floral meristem (Leyser and Furner, 1992; Clark et al., 1993, 1995). Of the *clv* mutants described in detail, *clv1-4* and *clv3-2* show the greatest departure from wild type in both organ number and meristem size (Clark et al., 1993, 1995). The *pan-1 clv1-4* and *pan-1 clv3-2* double mutants are additive with respect to organ number in the first three whorls (Table 3, Fig. 4A,B). Each double mutant has more sepals and petals and fewer stamens than either *clv* mutant alone. In addition, *pan-1 clv3-2* flowers have fewer carpels than *clv3-2* alone. Double mutants of *pan-1* in combination with *clv2-1* (Koornneef et al., 1993), as well as the intermediate alleles *clv1-1 and clv3-1*, also show additive organ number in the first three whorls.

perianthia-1 leafy-6; perianthia-1 apetala1-1

The *LEAFY* (*LFY*) and *APETALA1* (*AP1*) genes have been shown to be important in establishing floral meristem identity. Plants mutant for either gene show a partial conversion of the flower to a shoot, with a more dramatic transformation seen in the *lfy ap1* double mutant (Irish and Sussex, 1990; Schultz and Haughn, 1991, 1993; Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Shannon and Meeks-Wagner, 1993). These mutants also show a reduction of expression of flower-specific genes, as shown both by genetic and molecular methods: strong *lfy* mutations are epistatic to both *apetala3* (*ap3*) and *pistillata* (*pi*; Huala and Sussex, 1992; Weigel et al., 1992), and *AP3* and *PI* RNA transcripts are expressed at lower levels in *lfy* mutant plants (Weigel and Meyerowitz, 1993). In addition, *AP3*, *PI* and *AGAMOUS* (*AG*) mRNA expression is dramatically reduced in *lfy ap1* double mutant flowers (Weigel and Meyerowitz, 1993). Strong alleles of *lfy* and *ap1* also affect organ number and position. In *lfy-6* mutants, usually 4 but occasionally 3 or 5 organs are present in the first whorl, and a variable number of organs arise in a slightly spiral pattern in the interior (Weigel et al., 1992). *ap1-1* mutants develop 4 or fewer first whorl organs, rarely have second whorl organs, and have a slightly reduced number of third whorl organs, about 5.2 on average (Irish and Sussex, 1990; Bowman et al., 1993).

The *pan-1 lfy-6* double mutant is indistinguishable from *lfy-6* alone with respect to organ number, identity and pattern. Most significantly, we did not observe an increase over *lfy-6* in the number of first whorl organs present in the double mutant: almost all *pan-1 lfy-6* flowers had 4 first whorl organs, with 3 or 5 occasionally seen, as in *lfy-6* alone, in 63 flowers scored. In addition, the *ap1-1 pan-1* double mutant is indistinguishable from *ap1-1* alone (Fig. 4C,D). No increase compared to *ap1-1* in first and second whorl organ number and no decrease in third whorl organ number was noted in 61 *ap1-1 pan-1* flowers scored.

perianthia-1 interactions with floral organ identity mutants

APETALA2 (*AP2*) is a class A homeotic gene involved in specifying floral organ number, floral meristem identity, and floral organ identity (Bowman et al., 1989, 1991; Kunst et al., 1989). Strong alleles, such as *ap2-2*, show a severe reduction in organ number in the first three whorls, and a conversion of sepals to carpels in the first whorl. The decrease in organ number is partly attributable to the ectopic expression of *ag* in these whorls, since the *ag-1 ap2-2* double mutant shows partly restored organ number (Bowman et al., 1991), and overexpression of *ag* by a constitutive promoter causes a reduction in outer organ number (Mizukami and Ma, 1992). The *pan-1 ap2-2* double mutant was not noticeably different from *ap2-2* alone: no additional organs were formed, and no more than 4 first whorl organs were seen in any flowers.

Mutations in the class B homeotic genes *AP3* and *PI* lead to a conversion of petals to sepals, and the third whorl is either absent or converted to carpel tissue (Hill and Lord, 1989; Bowman et al., 1991, 1993). *pan-1 ap3-3* and *pan-1 pi-1* are additive, with 5 sepals usually seen in each of the first 2 whorls, and a multicarpellate gynoecium.

Mutations in the class C homeotic gene *AG* give flowers that show a conversion of stamens to petals in the third whorl, and show a repeated, indeterminate pattern of an additional whorl

of sepals and two additional whorls of petals (Bowman et al., 1989). In *ag* plants wild type for *ERECTA*, such as *ag-2*, internode elongation is seen before each sepal whorl. *pan-1 ag-1* and *pan-1 ag-2* show additive effects in the first three whorls, but have a reduction of sepal tissue in the inner whorls (Fig. 4E,F), and, in an *ERECTA* background, reduced internode elongation. Both of these traits are similar to but less dramatic than those seen in double mutants of strong *ag* and *superman* alleles (Schultz et al., 1991; Bowman et al., 1992).

Flowers of the triple mutant *ag-1 ap2-2 pi-1* produce an indeterminate number of slightly carpelloid leaves, lacking most aspects of floral organ identity, and having 4 or fewer organs in the first and subsequent whorls (Fig. 4G; Bowman et al., 1991). The *pan-1 ag-1 ap2-2 pi-1* quadruple mutants often have 5 first whorl organs instead of 4, a phenotype not seen in *ag-1 ap2-2 pi-1* (Fig. 4G,H). This suggests that *pan-1* acts independently of the homeotic genes in specifying organ number and organ initiation patterns, and that *AG* activity in outer whorls is responsible for the absense of a *pan* phenotype in *ap2-2 pan* double mutant flowers.

perianthia-1 suppresses the organ number defect in *superman-1* but not in p35S-*AP3*

Flowers of plants mutant for *superman* (*sup*, also called *flo10*) show a dramatic increase in the number of stamens and number of whorls of stamens, loss of determinacy of the floral meristem, and a decrease in carpel tissue accompanied by a loss of an organized gynoecium structure (Schultz et al., 1991; Bowman et al., 1992). The double mutant *pan-1 sup-1* is additive in the first two whorls, with 5 sepals and petals present on average, but shows a large decrease in stamen number compared to *sup* alone, and a partial restoration of carpels (Fig. 4I,J). The average number of stamens in 63 *pan-1 sup-1* flowers examined was 6.2, while *sup-1* always had at least 8 stamens and an average of 10.8 stamens in the 83 flowers examined. In addition, an increase in carpel tissue is seen in *pan-1 sup-1* compared to *sup-1* alone, though the gynoecium resembles *sup* gynoecia in that the carpels appear disorganized and are often not fully fused. *pan-1 sup-1* plants are able to produce more seed than *sup-1* plants, but are still much less fertile than wild type.

To test whether this interaction was specific to the *sup* locus, we examined the effect of *pan-1* mutants in plants that constitutively express *AP3* and both *AP3* and *PI*. Flowers from p35S-*AP3* plants resemble *sup* mutant flowers in that they have extra stamens, indeterminacy, and less carpel tissue than wild type (Jack et al., 1994). The p35S-*AP3* p35S-*PI* transgenics have an even greater increase in stamen number and reduction in carpel tissue, in addition to a conversion of sepals to petals in the first whorl (Fig. 4K, Krizek and Meyerowitz, 1996). *pan-1* p35S-*AP3* and *pan-1*

Table 3. Mean number of organs in *clv1-4*, *clv3-2*, *pan-1 clv1-4*, and *pan-1 clv3-2* flowers

	Sepals	Petals	Stamens	Carpels
clv1-4	5.0±0.7	4.5±0.6	8.1±1.0	4.6±0.9
clv3-2	4.7±0.6	4.6±0.6	8.6±1.1	5.7±0.8
pan-1 clv1-4	6.0±0.7	5.8±0.6	7.0±1.0	4.2±0.5
pan-1 clv3-2	6.0±0.6	5.4±0.7	7.4±1.0	4.2±0.4

The first 15 flowers on 6 plants of each genotype were scored (a total of 90 flowers per genotype scored). The standard error is indicated.

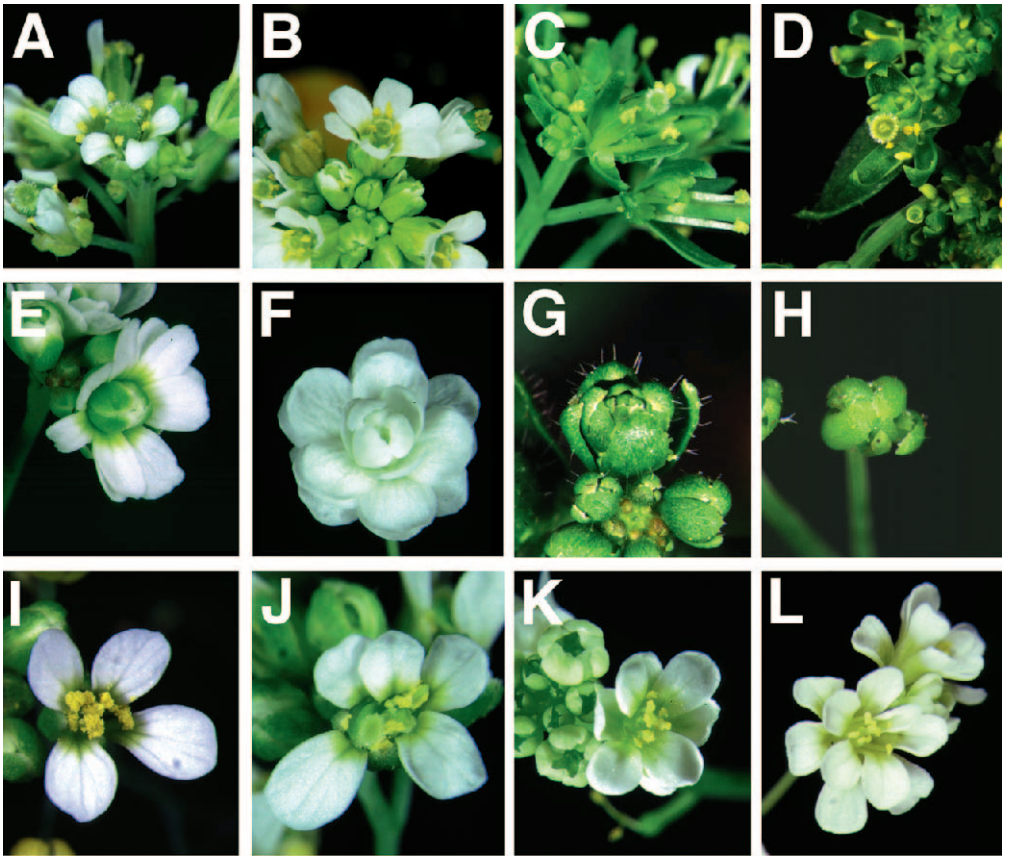

Fig. 4. Double mutants of *perianthia* with other genes affecting flower development. (A) *clv1-4* flower. (B) *clv1-4 pan-1* flower. Additive effects on organ number are seen in the double mutant flowers. (C) *ap1-1* flower. (D) *ap1-1 pan-1* flower. No differences are seen in the double mutant compared to *ap1-1* single mutants. (E) *ag-1* flower. (F) *ag-1 pan-1* flower. The inner whorl organs of *ag-1 pan-1* flowers show less sepal character compared to *ag-1*, and sometimes lack sepal tissue altogether. (G) *ag-1 ap2-2 pi-1* flower. This triple mutant has 4 or fewer carpelloid leaves in each whorl. (H) *ag-1 ap2-2 pi-1 pan-1* flower, with 5 first whorl organs. (I) *sup-1* flower. (J) *sup-1 pan-1* flower. The double mutant has dramatically reduced stamen number, increased floral meristem determinacy, and an increased amount of gynoecium tissue compared to the *sup-1* single mutant. (K) p35S-*AP3* p35S-*PI*. (L) *pan-1* p35S-*AP3* p35S-*PI*. In addition to plants mutant in *sup*, plants overexpressing *AP3* or both *AP3* and *PI* also have extra stamens and reduced carpel tissue; *pan* has no effect on this phenotype.

p35S-*AP3* p35S-*PI* flowers are strictly additive in organ number (Fig. 4L): the average stamen number in 27 p35S-*AP3* p35S-*PI* flowers is 15.3, compared to 14.4 stamens on average in 25 *pan-1* p35S-*AP3* p35S-*PI*. This suggests that the interaction seen in *pan-1 sup-1* is specific to *sup*. This supports the model that the similar phenotypes of plants mutant in *sup* and plants overexpressing B function genes are due to different mechanisms (Sakai et al., 1995).

DISCUSSION

We have investigated the effects of mutations in the *PERIANTHIA* gene on *Arabidopsis* development. The phenotype of *pan* is distinct from that of previously described mutants: *pan* does not appear to affect the growth and development of plant parts outside the flower, and floral organ identity and development are normal except for minor defects in gynoecium development. The pattern of organ initiation is constant in *pan* flowers, and indeed resembles the pattern found in a number of families with pentamerous flowers, a trait not normally found in the Brassicaceae.

PERIANTHIA is distinct from previously described genes affecting organ number

Several loci that affect floral organ number without affecting the identity of the floral meristem or floral organs have been described, including *fas1*, *fas2*, *clv1*, *clv3*, *tsl*, and *rev* (Leyser and Furner, 1992; Clark et al., 1993, 1995; Roe et al., 1993; Talbert et al., 1995). Unlike *pan*, all of these mutations have pleiotropic effects on plant development. In addition, in the mutants where organ initiation pattern was examined (*clv1*, *clv3*, and *tsl*), sepal initiation occurs without a regular pattern, and the sepals lack positional identity (Clark et al., 1993, 1995; Roe et al., 1993); in contrast the organ initiation pattern in *pan* is consistent and predictable. It is likely that the proteins encoded by these pleiotropic genes affect cell division patterns and/or apical meristem structure, which secondarily affect organ initiation, resulting in a less regular pattern of organ position.

The positional identity in the radial axis of each whorl is maintained in *pan* flowers. Sepals that initiate closer to the lateral positions arise lower and are smaller than those initiating closest to the medial positions, and grow to underlie the more medial sepals, as in wild type. Similarly, stamens that initiate on or closest to the medial axis are taller than those that arise closest to the lateral positions, though the difference in height between the stamens is not nearly as dramatic as in wild type. This suggests that the initiation of the floral organs and the radial position identity of *Arabidopsis* floral organs are genetically separable and are established by independent developmental processes. An example of a mutant that affects positional identity within a whorl is the *cycloidea* mutant of *Antirrhinum majus* (Coen and Nugent, 1994). In *cycloidea* flowers the petals and stamens lose their positional identity, resulting in a radially symmetric flower.

PERIANTHIA acts independently of genes controlling floral meristem size

Mutations in *CLV1* and *CLV3* have been shown to cause an increase in the number of organs, especially the inner whorl organs, and a corresponding increase in the number of whorls in *Arabidopsis* flowers (Leyser and Furner, 1992, Clark et al., 1993, 1995). The change in organ number is correlated with a large increase in height and a small increase in width of the floral meristem at the time of floral organ initiation. Alleles of *clv* genes that cause a larger number of organs and whorls have a more dramatic increase in floral meristem size (Clark et al., 1993, 1995). In *pan*, the change in organ number is not correlated with an increase in the size of the floral meristem, suggesting that *pan* controls floral organ number by regulating the distance (either the absolute distance or the number of cells) between initiation events, rather than increasing the total area available for initiation events. It is possible that the size change required for the change in organ number in *pan* would be too small for us to detect. We believe that this is not the case, however, since a mutation in a separate locus, *wiggum*, leads to a very similar organ number phenotype to that in *pan* (approximately one additional sepal and petal per flower), but also has an increase in floral meristem size, particularly in width, at the time of sepal initiation (M. P. R. and E. M. M., unpublished data). This indicates that a size change sufficient to cause the change in organ number seen in *pan* would be readily detectable.

PERIANTHIA acts downstream of the floral meristem identity genes

Mutations in the *LEAFY* and *APETALA1* genes lead to a partial conversion of the flower to a shoot; with a more dramatic transformation seen in the double mutant (Irish and Sussex, 1990; Schultz and Haughn, 1991, 1993; Huala and Sussex, 1992; Weigel et al., 1992; Bowman et al., 1993; Shannon and Meeks-Wagner, 1993). Also, there is a dramatic reduction in the activity of the flower-specific genes *AP3* and *PI* in *lfy* mutant plants (Huala and Sussex, 1992; Weigel et al., 1992; Weigel and Meyerowitz, 1993). Both *lfy-6* and *ap1-1* are epistatic to *pan-1*, suggesting that *PAN* is also a flower-specific gene that requires floral meristem identity for its activity, and formally acts downstream of *LFY* and *AP1*. It remains a possibility, however, that the effect on organ number caused by *pan* may be masked by the organ number defects in *lfy* and *ap1*.

PERIANTHIA acts independently of the floral homeotic genes in specifying early flower patterning

The floral homeotic genes *AP2*, *AP3*, *PI*, and *AG* all play important roles in the specification of organ identity in *Arabidopsis* flowers. In addition, the *ap2-2* mutant leads to a severe decrease in floral organ number, which has been shown genetically to be largely due to ectopic expression of *ag* throughout the flower, since in *ap2-2 ag-1* double mutants the loss of organs is partially suppressed (Bowman et al., 1991). Similarly, the *ag-1 ap2-2 pi-1* triple mutant has more organs in the first whorl than *ap2-2* mutants alone, sometimes having as many as 4 organs, but often missing lateral first whorl organs. The *pan-1 ag-1 ap2-2 pi-1* quadruple mutant also usually has reduced organ number compared to wild type, but sometimes produces flowers with 5 first whorl organs. This suggests that *pan* acts independently of the homeotic genes in specifying early floral pattern. *pan* mutations are also additive in double mutant combinations with the floral homeotic genes, except for *ap2-2*, which is not notably different from the *ap2-2 pan-1* double mutant. In this case the severe reduction in

floral organ number seen in *ap2-2*, which is in turn at least partially due to *AG* misexpression in the outer whorls, may mask the effect of *pan-1* in *ap2-2* mutants.

The role of *PERIANTHIA* in third and fouth whorl flower development

In addition to an increase in organ number in the first two whorls, *pan* leads to changes in the development of the third and fourth whorls. In *pan* single mutants, there are generally fewer stamens than in wild type, though extra stamens are sometimes seen. *pan* flowers also occasionally produce one or three carpels, and the gynoecium shows a slight deviation from wild-type development, though *pan* flowers are functionally normal and fully fertile. More dramatic effects are seen in double mutant combinations. For instance, *pan-1 clv3-2* double mutants show a small but noticeable decrease in stamen and carpel number, and *pan* almost completely suppresses the growth of additional stamens in the third whorl of *sup* mutants, coupled with an increase in gynoecium tissue. *PAN* also seems to play a role in the formation of inner *ag* flowers. The sepals in the fourth and higher order whorls of *ag* flowers are partially to fully converted into petals in *ag-1 pan-1*, and internode elongation is reduced in the double mutant compared to the single mutant alone. These results suggest that *PAN* is at least partially required for cell proliferation and organ initiation in the third and fourth whorls, especially in particular mutant backgrounds.

Evolutionary implications

One of the important distinctions between plant families is the number of the different types of organs present in the flower. Almost all members of the Brassicaceae (mustard) family have perianth organs in a tetramerous pattern, along with 6 stamens and 2 carpels (Cronquist, 1981). In *Arabidopsis* flowers mutant for *pan*, both the number of organs and the pattern by which they arise resemble those of distantly related flowering plant species. For instance, *Antirrhinum majus* (snapdragon) has pentamerous flowers arranged in the same way as the most commonly seen *pan* mutant flowers, with an adaxial sepal always present, other sepals arising equidistantly around the floral meristem, petals arising interior to and in between the sepals, and stamens arising interior to and between the petals (Coen and Meyerowitz, 1991). It is an intriguing possibility that the *PAN* gene played an important role in the evolution of the organ number and arrangement seen in mustard flowers.

Mechanism of *PERIANTHIA*

The *PAN* gene product is necessary for the establishment of tetramerous versus pentamerous flowers in *Arabidopsis*, acting at the level of altering the pattern of organ initiation. *PAN* is not required for organ initiation per se, and is not required to maintain a consistent pattern of organ initiation, since both can occur in *pan* mutants. In addition, meristem size, cell size, cell number, and cell pattern are not affected in the *pan* early floral meristem. This indicates that *PAN* acts directly in the process by which cells assess their position within the developing floral meristem, and affects the switch that commits them to enter an organ initiation program.

One potential role of *PAN* is that it may act to modify an underlying mechanism of organ initiation, affecting the signaling processes involved in organ initiation in a precise way to cause an alteration from a largely pentamerous to a largely tetramerous pattern. *PAN* may do this by acting in the abaxial side of the flower to anchor a signal required for initiation of sepals in the correct pattern. When this signal is absent, the adaxial sepal provides the only signal for organ position, and other sepals arise uniformly from this position. When the signal is present, adaxial and abaxial sepals form, providing the signal for the two remaining sepals to form in a lateral position. Signaling of the initiation of organs in the inner whorls would be secondarily affected. Molecular cloning of the gene, and studies of its expression patterns, should aid in testing this model, or in finding new models. Further study of *PAN* may also help answer one of the fundamental problems in developmental biology; that is, how cells are able to generate pattern elements with specific numbers and positions.

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