Dual role for *fimbriata* in regulating floral homeotic genes and cell division in *Antirrhinum*

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The *fimbriata* (*fim*) gene of *Antirrhinum* affects both the identity and arrangement of organs within the flower, and encodes a protein with an F-box motif. We show that FIM associates with a family of proteins, termed FAPs (FIM-associated proteins), that are closely related to human and yeast Skp1 proteins. These proteins form complexes with F-box-containing partners to promote protein degradation and cell cycle progression. The *fap* genes are expressed in inflorescence and floral meristems in a pattern that incorporates the domain of *fim* expression, supporting an *in vivo* role for a FIM–FAP complex. Analysis of a series of novel *fim* alleles shows that *fim* plays a key role in the activation of organ identity genes. In addition, *fim* acts in the regions between floral organs to specify the correct positioning and maintenance of morphological boundaries. Taking these results together, we propose that FIM–FAP complexes affect both gene expression and cell division, perhaps by promoting selective degradation of regulatory proteins. This may provide a mechanism by which morphological boundaries can be aligned with domains of gene expression during floral development.

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Introduction

Floral meristems produce four types of organs in concentric whorls in the order sepals, petals, stamens and carpels. The identity of the organs is controlled by the combinatorial action of genes which are expressed in specific regions of the floral meristem (Schwarz-Sommer *et al.*, 1990; Coen and Carpenter, 1993; Weigel and Meyerowitz, 1994). Mutations in these organ identity genes can be divided into three classes: in class *A* mutants, carpels replace sepals and stamens replace petals; in class *B* mutants, sepals replace petals and carpels replace stamens; in class *C* mutants, petals replace stamens and carpels are

replaced by reiterative growth of sepals and petals. Based on these mutants, three genetic functions, *a*, *b* and *c*, have been proposed to specify organ identity in the combination *a*, *ab*, *bc* and *c*, in whorls 1–4 respectively (Coen and Meyerowitz, 1991). In most cases, the genes required for these functions are expressed in domains that are precisely aligned with the morphological boundaries between whorls of organs, ensuring discrete changes in organ type from whorl to whorl (Jack *et al.*, 1992; Schwarz-Sommer *et al.*, 1992; Bradley *et al.*, 1993). However, the mechanisms responsible for this alignment between morphology and gene expression boundaries are unclear. A candidate gene involved in this process is the *fimbriata* (*fim*) gene of *Antirrhinum*, and its *Arabidopsis* orthologue, *UFO*, which affect both the identity and arrangement of organs within the flower (Simon *et al.*, 1994; Ingram *et al.*, 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995).

The FIM protein contains a novel structural motif, the F-box, which is present in a range of proteins, including cyclin A, cyclin F and *CDC4*, that are involved in cell cycle control in yeast (Yochem and Byers, 1987; Zhang *et al.*, 1993, 1995; Bai *et al.*, 1994, 1996). Some F-box proteins, including Met30p and *GRR1*, act as transcriptional inhibitors and may also play a role in cell cycle control (Flick and Johnston, 1991; Thomas *et al.*, 1995). The precise role of *fim* in flower development is, however, unclear. We have used a combination of molecular and genetic approaches to address this problem in *Antirrhinum*.

Flowers of *fim* mutants described so far have organs consisting of petal/sepal tissue in the second whorl and petal/sepal/carpel tissue in the third whorl. In addition, mutant flowers sometimes fail to produce carpels and instead produce large numbers of mosaic organs (apical indeterminacy). These phenotypic effects can be accounted for by reduced expression of the *b* function gene *deficiens* (*def*) and *c* function gene *plena* (*ple*), suggesting that one role of *fim* is to promote transcription of organ identity genes (Simon *et al.*, 1994). Consistent with this, expression of *fim* is detected in meristems before any organ primordia are visible, shortly before the onset of *b* and *c* organ identity gene expression. Early *fim* expression encompasses the presumptive *b* and *c* domains: it is first seen in a ventral (abaxial) region of the floral meristem and then in the central region, after which it resolves into a broad ring internal to the sepal primordia, consistent with its role in regulating organ identity. At later stages, however, *fim* expression becomes localized to the boundary regions around the base of developing petal primordia and does not overlap with most of the domain of *b* or *c* activity. This raises the question of how *fim* interacts with the organ identity genes at different stages of development. In addition to affecting organ identity, *fim* mutants also show altered organ arrangement, united growth of organs and the occasional production of meristems in the axils

of floral organs (lateral indeterminacy). Thus, *fin* also seems to play a role in establishing organ and whorl boundaries during floral development.

Here we describe two complementary approaches that have been used to study the action of *fin*. One involves isolating proteins that interact with FIM during flower development using the yeast two-hybrid system. The second approach involves generating a series of alleles with altered levels of *fin* activity. This is particularly important because previously analysed *fin* alleles are not nulls, obscuring the extent to which *fin* is required for the proper arrangement and identity of organs (Simon *et al.*, 1994). To isolate a range of novel mutations at the *fin* locus, including null alleles, we exploited a weak *fin* allele (*fin*-619), which carries an insertion of the Tam3 transposon in the *fin* promoter. Tam3 is known to generate deletions and rearrangements in genes and can therefore be used as a localized mutagen (Martin *et al.*, 1988; Almeida *et al.*, 1989; Coen *et al.*, 1989; Martin and Lister 1989; Hudson *et al.*, 1990). Such transposon-induced derivatives can be screened for by PCR, as described in *Caenorhabditis* and maize (Rushforth *et al.*, 1993; Das and Martienssen, 1995).

We show that FIM associates with a family of proteins, termed FAPs (FIM-associated proteins), closely related to Skp1 proteins. In yeast and humans, Skp1 proteins interact with F-box-containing proteins, directly or indirectly, to form a complex needed for protein degradation and cell cycle progression (Zhang *et al.*, 1995; Bai *et al.*, 1996; Connelly and Hieter, 1996). Thus, the similarity between FIM and F-box proteins is further substantiated by their ability to interact with similar partners. The *fap* genes are expressed in inflorescence and floral meristems in a pattern that incorporates the domain of *fin* expression, supporting the conclusion that FIM forms a complex with one or more FAPs *in vivo*. Analysis of a series of novel *fin* alleles shows that *fin* plays a key role in the activation of the *b* genes *def* and *globosa* (*glo*), and is needed to activate expression of the *c* gene *ple* in the centre of the floral meristem at the correct time during flower development. In addition, *fin* acts in the regions between floral organs to specify the correct positioning and maintenance of morphological boundaries. Taking these results together, we propose that a FIM–FAP complex affects both gene expression and cell division, perhaps by promoting selective degradation of regulatory proteins. This may provide a mechanism by which morphological and gene expression boundaries can be aligned with each other during floral development.

Results

Molecular characterization of novel *fin* alleles

The role of *fin* in the control of flower development was investigated by site-selected mutagenesis, using *fin*-619 as the starting material. The *fin*-619 allele carries a Tam3 insertion 976 bp upstream of the start of the longest *fin* cDNA (start of the cDNA is denoted position 0). Ten events were detected from a PCR-based screen of 4020 progeny of *fin*-619 (M_1 generation) and the corresponding seed was sown to give 10 M_2 families. Screening the plants in these families by PCR, showed that seven of the 10 events were transmitted to the M_2 . The three

untransmitted events could either have been somatic, or present in M_1 plants which did not set seed. The seven transmitted alleles, named *fin*-676 to *fin*-682, were further characterized both by PCR amplification and by cloning and sequencing the PCR products. M_2 plants homozygous for the new alleles were then analysed by DNA blot hybridization.

The seven alleles carried deletions extending to the right of the Tam3 by various amounts. Two of the alleles had retained the original Tam3 insertion, whereas the five other alleles had lost Tam3 (Figure 1A). These types of deletion have previously been observed as the result of plant transposon activity and have been attributed to aberrant transposition events (McClintock, 1953, 1954; Martin *et al.*, 1988; Coen *et al.*, 1989; Federoff, 1989; Martin and Lister 1989; Hudson *et al.*, 1990; Robbins *et al.*, 1990). There were no striking similarities between the sequences around the right breakpoints of the seven deletions, although the breakpoints found in the *fin*-676 and *fin*-678 alleles were separated by only 2 bp. Four of the five deletion events in which Tam3 was lost had the same left breakpoint, 11 bp away from the transposon (bases –976 to –987, Figure 1B), indicating that they may have arisen from the same type of event.

Deletion of the *fin* open reading frame gives rise to a novel phenotype

Plants homozygous for the deletions in *fin*-677, *fin*-679, *fin*-681 or *fin*-682 all gave a similar phenotype (Figure 2D). This was more extreme than had previously been observed for any *fin* allele, confirming that previously described alleles were not null. At least half of the *fin* open reading frame (ORF) had been deleted in these new alleles, and DNA blots confirmed that no duplications of *fin* were present in the genome of these plants. Thus, the phenotype shown in these plants represented the null mutant phenotype of *fin*. Detailed analysis of the phenotype revealed the extent to which *fin* was involved in: (i) organ identity; (ii) whorl integrity; and (iii) determinacy.

Changes in floral organ identity. All null *fin* mutants produced flowers that showed changes in organ identity that were consistent with reduced *b* and *c* activity (Figure 2D and H). Flowers usually had a first whorl of sepals, and most organs internal to these were also sepal-like. Petal tissue was rare and was only present in small localized patches on the edges of internal sepals; stamoid tissue was never observed, even under glasshouse conditions where the phenotype was less extreme than in plants grown in the field. Carpel tissue and ovules were produced towards the centre of many flowers, especially flowers produced higher on the inflorescence spike (see later). The carpels were commonly united with adjacent organs and split or distorted.

Disruption of whorl integrity. Dissection of mature flowers from *fin* nulls suggested that organs internal to the first whorl were not arranged in regular whorls. To determine the developmental origin of this change in phyllotaxy, early floral meristems were examined by scanning electron microscopy. Flower meristems arose in the axils of bract primordia on the periphery of the inflorescence apex. The meristems were numbered sequentially, starting with the

Fig. 2. Phenotypes conferred by deletions at the *fim* locus. Flowering spikes from wild-type (A), *fim-619* (B), *fim-676* (C) and the *fim-677* null (D). A typical flower is shown below each spike (E, F, G and H). Each spike shows a region of abortion (arrow), probably caused by environmental fluctuation. Decreasing amounts of petal and stamen tissue with increasing allele severity are apparent from left to right, as are increases in sepal tissue production. A high degree of apical and lateral indeterminacy is visible in the basal region of the null *fim* spike (D, indeterminate flower indicated by an arrowhead).

were observed in *fim* nulls. Wild-type flowers are apically determinate, producing only four whorls of organs, and laterally determinate because no meristems arise in the axils of floral organs. In *fim* nulls, a gradient in the degree of indeterminacy was apparent along the inflorescence spike from lower to upper regions (Figure 4). Flowers produced low down on the inflorescence spikes (flower 0–10), commonly showed both lateral and apical indeterminacy (Figure 4). The internodes of these flowers became elongated after the production of 10–24 sepals, and there was a concurrent switch to spiral phyllotaxy. Such flowers produced no carpel-like structures on the main floral axis. Some of these flowers were also laterally indeterminate, commonly having 2–12 secondary flowers, each subtended by a sepal. The secondary flowers were determinate and resembled those produced higher up on the inflorescence spike. In combination, these traits gave some early flowers many of the characteristics of an inflorescence (Figure 2D, arrowhead).

The phenotype of upper flowers was more uniform than that of lower flowers (an example is shown in Figure 2H). They rarely showed lateral or apical indeterminacy and consequently contained fewer organs than lower flowers. In addition, the proportion of carpel tissue was considerably greater. Upper flowers were usually composed of a first whorl of sepals, followed by 4–10 sepals and then carpeloid organs in the centre of the flower. In many cases,

the internal organs of the flower were united with each other and, very occasionally, flowers terminated with united carpels similar to those found in the third whorl of *def* or *glo* mutants.

Phenotypes conferred by partial deletions of *fim*

Although the *fim-680* deletion extended into the ORF, its mutant phenotype was less extreme than that of the null alleles: it showed greater determinacy, more petal and carpel tissue, and less internode elongation (data not shown). This weaker phenotype might be explained by the presence of an in-frame ATG, situated 38 amino acids (aa) into the ORF; the deletion in *fim-680* extended only 108 bp (36 aa) into the ORF so the mutant could have produced a protein with 38 aa missing from the N-terminus (Figure 1B). The missing N-terminus is the region of FIM that is least conserved with its homologue from *Arabidopsis*, UFO (Ingram *et al.*, 1995). It is therefore probable that this part of the protein is not absolutely required for FIM activity.

Two deletions gave very different phenotypes but had almost identical breakpoints in the 5' promoter region of *fim*. The main difference in the structure of these alleles was whether or not they retained Tam3. In *fim-678*, which had lost Tam3 and 770 bp of promoter DNA, the deletion breakpoint was at –206, and in *fim-676*, which retained Tam3, the breakpoint was at –204. Homozygous *fim-678*

Fig. 3. The development of *fim* null mutant flowers. Floral meristems from wild-type and null *fim-681* mutant plants are shown at nodes 13, 15, 17 and 19. At node 13 (stage 4), the dorsal region of the mutant flower appears normal but an extra primordium is visible between the ventral two organs (marked with an arrow in **B**). By node 15 (early stage 5) defects in the mutant are more apparent, illustrated by the extra ventral sepal which is united with a more internal organ (arrow in **D**, the two dorsal sepals are absent). At node 17 in wild-type, the second whorl petal primordia are small and stamen primordia are clearly visible (**E**, all but the most dorsal sepal removed). By contrast, at a comparable node in the *fim* mutant, organ primordia (probably sepals) internal to the first whorl are relatively large and those further in are hardly visible (**F**, all but the most dorsal first whorl organs removed). Whorl structure has also started to deteriorate. By node 19, retarded development of internal primordia is even more noticeable in the mutant (**H**, first whorl sepals have been left in place but have been partially dissected to reveal internal primordia) as compared with wild-type (**G**, all sepal primordia removed). United ventral sepals (**H**, arrow) and a filament structure (labelled with an asterisk in **H**) are also apparent in the mutant.

plants gave a wild-type phenotype, suggesting that the 770 bp 5' region deleted in these plants was not necessary for *fim* transcription. However, plants homozygous for *fim-676*, which retained Tam3, had a mutant phenotype with sepal/petal mosaic organs in the second whorl and a third whorl comprising one to two deformed stamens together with petal/sepal/carpel mosaic organs. The pheno-

type of plants carrying *fim-676* was more extreme than that of the *fim-619* progenitor which only showed slight abnormalities in the second whorl (Figure 2). Therefore, the presence of Tam3 in the *fim* promoter, in combination with the loss of promoter sequence to the right of Tam3, caused considerable disruption of *fim* function. This effect of Tam3 could be due to transcription factors or transposase

Fig. 4. Loss of determinacy in *fim-677* flowers. Proportion of indeterminate flowers on 87 spikes from null *fim* mutants grown under field conditions. Flower number was counted from the lowest flower upwards, each bract being counted as a floral node. For each range of flower numbers, the proportion of indeterminate (left bar) and determinate (right bar) flowers was measured. The abortion rate in the first 15 nodes was considerably higher (up to 5%) than in concurrently grown wild-type plants (1–2%).

proteins binding to its ends and interfering with *fim* expression. Alternatively, the effects of Tam3 could be due to physical distancing of 5' regulatory elements from the start of *fim* (Bradley *et al.*, 1993; Chatterjee *et al.*, 1996).

Expression of the *fim* alleles

The effects of the various deletions on *fim* expression were analysed by RNA *in situ* hybridizations on inflorescence apices. In wild-type, *fim* is first expressed in the ventral region of floral meristems and then in their centre before any floral organ primordia are visible (stage 2). The expression of *fim* then appears to spread outwards and, by the florotypic stage (stage 4), becomes localized as a ring around the centre of the floral meristem, adjacent to the sepal primordia (Simon *et al.*, 1994). By stage 5, when petal primordia become visible, *fim* transcripts are present in domains around the base of each developing petal (Figure 5A). Throughout the rest of floral development *fim* remains localized at the junctions between sepals and petals, and between petals and stamens (Simon *et al.*, 1994).

The deletion mutants showed reductions in *fim* expression which correlated with the severity of their phenotypes. The *fim-678* allele, which had a 768 bp 5' deletion and a wild-type phenotype, showed wild-type *fim* expression (data not shown). In contrast, *fim-676*, which had a similar deletion but which retained Tam3 and showed a mutant phenotype, had a reduced level of transcript, although the distribution appeared to be temporally and spatially similar to wild-type (Figure 5D). Plants homozygous for *fim-680*, which gave an almost null phenotype, showed a very low level of *fim* transcript, although the spatial domain of expression seemed normal (not shown). Detection of *fim*

transcripts in *fim-680* was delayed compared with wild-type: transcripts first became visible at around node 12 compared with node 9 in wild-type. Plants homozygous for *fim-679* or *fim-681*, which showed a null phenotype, had very low levels of transcript with the same spatial and temporal distribution as in *fim-680* (not shown). The probe used for *in situ* analysis extended 3' of the deletion breakpoints in all of these alleles and could therefore detect transcripts truncated at the 5' end. No *fim* transcripts were detected in plants homozygous for the null alleles *fim-682* and *fim-677*, which had lost the entire region covered by the probe (Figure 5G).

The results indicated that even when the entire 5' region between *fim* and Tam3 was deleted, information conferring spatially specific expression of *fim* was still present. Sequences 5' of the Tam3 excision site and/or in the 3' of the gene therefore play an important role in *fim* regulation. A candidate regulatory element is a putative MADS box protein-binding site 39 bp 5' of the Tam3 insertion site, which was not lost in any of the deletions studied (Figure 1B) (Schwarz-Sommer *et al.*, 1990).

Expression of *glo* and *def* in *fim* mutants

The observed decrease in petal and stamen tissue in *fim* mutants suggested that *b* activity was reduced. In wild-type, the *b* function gene *def* is first expressed in the central region of floral meristems at node 10 just before the florotypic stage (stage 4). As second and third whorl primordia arise, *def* expression is lost from the centre of the floral meristem and becomes localized in the developing petal and stamen primordia (Schwarz-Sommer *et al.*, 1992) (Figure 5B). Previous analysis of the *fim-620* allele had shown that reduced *fim* activity led to a decrease and delay in the expression of *def* (Simon *et al.*, 1994). A further reduction in *def* expression was observed in the *fim* nulls we generated in this study. About half of all floral meristems analysed showed no detectable *def* expression and in those that did show expression, it was delayed and restricted to small patches of cells in the central regions of young floral meristems. At later stages, small patches of *def* expression were occasionally detected in organs internal to the sepal whorl but these patches were very rare (Figure 5H).

In *fim* null mutants, expression of another *b* function gene, *globosa* (*glo*), was also found to be severely diminished. The *glo* expression pattern in *fim* nulls was very similar to that of *def*: it was absent from many floral meristems, and very much reduced and delayed in meristems where it was detected (data not shown). The temporal and spatial expression pattern of *glo* is known to be closely correlated to that of *def* in wild-type plants. However, probing adjacent sections of *fim* null buds at the late florotypic stage (nodes 13–14) indicated that both *def* and *glo* expression was found in small patches of cells, but that these patches only occasionally overlapped. This indicated that these genes were being independently activated in a stochastic manner in small areas within the *b* function domain at an early stage of meristem development. In later buds, probing of adjacent sections showed that the rare patches of *glo* expression observed did correlate with patches of *def* expression. This is consistent with studies which show that it is only after stage 6 of wild-type that mutual activation by DEF/GLO

Fig. 5. Expression of *fim*, *def* and *ple* in *fim* mutants. Expression pattern of *fim*, *def* and *ple* in floral meristems at around node 16–17 of wild-type (A–C), *fim-676* (D–F) and the *fim-677* null (G–I). Expression of *fim* in *fim-676* (D) is considerably reduced compared with wild-type (A), although the expression domain remains essentially unchanged. No *fim* transcripts were detected in *fim-677* (G). The domain of *def* expression in *fim-676* (E) is similar to wild-type (B), although large areas lacking *def* expression are observed within some second and third whorl organs. Two such organs are visible in transverse section (arrowed in E), one showing *def* expression at both lateral margins and the other showing a small amount of expression at one lateral margin. A very low level of *def* expression can be seen near the centre of the *fim-677* null floral meristem (H), although many *fim* null floral buds show no *def* expression. Expression of *ple* in *fim-676* (F) was slightly delayed and restricted as compared with wild-type (C). Expression of *ple* in *fim-677* (I), when present, was delayed and restricted to a small region in the centre of the floral meristem. Less than 50% of *fim-677* flowers showed any *ple* expression at node 16. The positions of sepal (s), petal (p), stamen (st) and carpel (c) primordia in wild-type meristems are shown in (A). Scale bar is 100 μ m.

heterodimers becomes necessary to maintain expression of *def* and *glo* (Zachgo *et al.*, 1995).

In *fim-680*, which showed an almost null phenotype, the amounts of *def* and *glo* transcript detected, although still very low, were higher than in the null mutants, confirming that there was some *fim* activity in these plants (data not shown).

In *fim-676* mutants, mosaic organs composed of sepal and petal tissue were produced in the second and third whorls. Similar types of mosaic organs were also reported in the *fim-620* mutant, where it was shown that regions of sepal and petal tissue separated by lines of only one or two cells of intermediate identity (Simon *et al.*, 1994). Since both *fim-620* and *fim-676* carry transposon insertions in the *fim* promoter, it is possible that patches of *b* function expression were being caused by transposon excision during early flower development. However, the mosaic organs appeared at a reproducible frequency, independent

of temperature, whereas Tam3 excision is known to be temperature-sensitive, suggesting that transposition events are an unlikely explanation for most patches.

A more likely explanation for the mosaic organs was that they reflected an early role of *fim* played in allocating cells in the floral meristem to express *b* function genes. In weak *fim* alleles, such as *fim-676* and *fim-620*, only a subset of cells might be allocated, giving rise to discrete sectors with *b* activity. To investigate this possibility, we studied the expression of *def* and *glo* in *fim-676* plants. Expression was very variable in early floral meristems (nodes 12–14, stage 4), with some floral meristems showing almost wild-type *def* and *glo* transcript levels and others showing very reduced levels. Expression always occurred within the *b* domain and was not appreciably delayed compared with wild-type. Early patches of *glo* expression were always completely overlapped by *def* expression, although the domain of *glo* appeared to be

Fig. 6. Expression of *def* and *glo* in organs of *fim-676*. Adjacent sections of a *fim-676* floral meristem (stage 5) probed with *def* (A) and *glo* (B). Expression of these *b* function genes is variable and reduced compared with wild-type (compare with Figure 5B). The patch of *def* expression in (A) completely overlaps the smaller patch of *glo* seen in (B). An older floral meristem of *fim-676* probed with *def* is shown in (C). Mosaic organ primordia can be seen with distinct patches of cells expressing *def* adjacent to non-expressing regions (arrowhead). Note that primordia are thicker in non-expressing patches than in adjacent expressing regions, consistent with their developing with sepal, rather than petal identity (at this stage in wild-type, sepals are thicker in cross-section than petals). Expression of *glo* at this stage was found to be identical to that of *def*. Scale bar is 100 μ m.

slightly more restricted than that of *def* (Figure 6B). The coordinated expression of both genes early in development indicated that their activation was most likely not stochastic. In older buds (stages 5 and onwards), expression of the two genes was coincident and maintained a patchy pattern. Some areas of second and third whorl organs showed a wild-type level of *def* and *glo*, while adjacent areas of the same organs showed no expression. When organs were sectioned transversely, patches of *def* and *glo* expression were found to be most common on the lateral edges of organs rather than in the midrib region (Figure 5E). In wild-type meristems at later stages, developing sepals were considerably thicker than petals. Consistent with their development as mosaics, many second whorl organs of *fim-676* flowers showed variations in width within each organ: thinner areas (assumed to be developing petal tissue) showed *def* and *glo* expression whereas thicker regions (assumed to be developing sepal tissue) showed no expression.

Activation of *ple* and *flo* varies between early and late flowers of *fim* mutants

The presence of carpel tissue in flowers higher up on the inflorescence of *fim* nulls suggested that the *c* function was active in these flowers. However, flowers lower down showed considerable apical indeterminacy, similar to flowers lacking *c* function. To clarify these effects on *c* function, expression of *ple* was investigated in the *fim* mutants.

Decreased and delayed *ple* expression was observed in *fim-676*, *fim-680* and all *fim* null flowers. In wild-type flowers, expression of *ple* is first observed at late stage 3 in a central region of the floral meristem and then in the presumptive stamen and carpel primordia as they form (Figure 5C) (Bradley *et al.*, 1993). In *fim-676* plants, which have a reduced level of *fim* expression, *ple* transcripts were present at a reduced level, and the domain of *ple* expression was restricted to a slightly more central area of the meristem than normal, similar to the pattern reported in *fim-620* plants (Figure 5F) (Simon *et al.*, 1994). In *fim* null mutants, *ple* expression was never observed until primordia internal to the first whorl were clearly visible (stage 5), a delay of five nodes compared with wild-type (Figure 7A) (Bradley *et al.*, 1993). In some floral buds, *ple* expression was entirely absent, even at late stages (Figure 7B). The variable timing and levels of *ple* expression correlated with the variation in phenotype observed along the inflorescence of *fim* nulls. Sections of floral meristems lacking *ple* expression at late stages tended to show inflorescence-like characters typical of flowers low down on the inflorescence.

The inflorescence-like characteristics of some flowers on *fim* nulls was further analysed by studying expression of the meristem identity gene *floricaula* (*flo*) (Coen *et al.*, 1990). In wild-type meristems, *fim* and *flo* have a complementary expression pattern (Simon *et al.*, 1994). In *fim* nulls, the expression pattern of *flo* was indistinguishable from wild-type up to stage 5, showing that *fim* does not affect the early expression pattern of *flo*. At later stages, indeterminate flowers showed strong *flo* expression in the same domains as observed in the main inflorescence apex: *flo* was expressed in the youngest organ primordia (morphologically comparable with bracts) and in the secondary meristems, but was absent from the apical region (Figure 7C).

Identification of FIM-associated proteins (FAPs)

The analysis of the nulls indicates that *fim* plays a role both in regulating expression of organ identity genes and in the pattern of meristem growth. To gain further insights into how *fim* might affect these processes, we used the yeast two-hybrid system to identify proteins that interacted with FIM. A fusion protein, comprising FIM and the GAL4 DNA-binding domain, was made by cloning the entire FIM-coding region into the vector pGBT9, to give plasmid pBD/FIM. This plasmid did not itself activate transcription: when yeast transformants were grown on medium lacking histidine, growth was much reduced, similar to untransformed cells, showing that pBD/FIM was unable to activate transcription of the *HIS3* selectable marker.

To identify proteins that interact with FIM, the HF7c(BD/FIM) strain was transformed with an expression

Fig. 7. Expression of *ple* and *flo* in flowers of *fim* null mutants. Flowers from a *fim-677* null at around node 30–35 probed with *ple* (A and B) and with *flo* (C). The meristem in (A) shows lateral indeterminacy but also shows apical *ple* expression and would presumably have formed an apically determinate flower. In contrast, the meristem shown in (B) shows no *ple* expression and would probably have formed an indeterminate inflorescence-like structure. This is confirmed by probing the adjacent section with *flo* (C), revealing strong *flo* expression in young primordia and axillary meristems, typical of the *flo* expression pattern of wild-type inflorescence apices. Scale bar is 100 μ m.

library (Davies *et al.*, 1996) in which a plasmid vector carrying the GAL4 activation domain had been coupled to *Antirrhinum* floral cDNA. A total of 2.4×10^6 transformants were screened for enhanced growth in the absence of histidine. The 340 resulting candidates were then tested for activation of the *LacZ* gene. This gave 27 positive colonies, from which the plasmids were isolated and re-transformed into another yeast strain (SFY526) carrying pBD/FIM to confirm that they could still activate *LacZ*.

Of the resulting transformants, only 16 could activate *LacZ* conditional on pBD/FIM also being present in the yeast host. All 16 plasmids were then partially sequenced.

The predicted proteins encoded by the 16 activation domain-containing plasmids, termed Fim-associated proteins (FAPs), came from three closely related genes: *fap1* (seven plasmids), *fap2* (seven plasmids) and *fap3* (two plasmids) (Figure 8). From the sequence available, the predicted amino acid sequence of FAP1 was 84% identical to FAP2, and FAP1 and FAP2 were both 70% identical to FAP3 (Devereaux *et al.*, 1984). The fact that only proteins belonging to the same family came through the screen confirmed that they had been selected on the basis of association with FIM, rather than through any hypersensitivity in the yeast two-hybrid system. The sequences were most highly conserved at their C-terminal ends and contained a variable region in the centre. Database searching using the 'Blast' algorithm revealed that the FAP family showed strong similarity to the human p19^{Skp1} protein and SKP1 from yeast, which are required for protein degradation and cell cycle progression (Altschul *et al.*, 1990; Zhang *et al.*, 1995; Bai *et al.*, 1996; Connelly and Hieter, 1996).

Expression of FAPS in floral meristems

If the FAP–FIM interaction is of functional significance *in vivo*, there should be an overlap between the expression domains of the *fim* and the *fap* genes. To test this, *in situ* hybridizations were carried out on wild-type *Antirrhinum* inflorescences using the inserts from the 16 *fap* clones. All *fap* RNA probes gave a very similar expression pattern. High expression was seen in inflorescence and floral meristems and provascular tissue (Figure 9A and B), all areas known to be in a state of active cell division (Fobert *et al.*, 1994; V.Gaudin *et al.*, submitted). The earliest overlap between *fim* and *fap* expression in the flower occurred at about stages 2–3, before the sepals had formed and when *fim* was expressed in the central dome of the floral meristem. At stage 4, *fap* expression was observed throughout the floral meristem (Figure 9B), whereas *fim* was restricted to a ring around the periphery of the central dome. At later stages, as the floral primordia emerged, *fap* expression was mainly within primordia and to a lesser extent between primordia (Figure 9C), whereas *fim* expression was restricted to the base of petal primordia (Figure 5A). Expression of the *faps* continued to be seen in later stages of organ development, particularly in actively dividing ovule and anther tissue (Figure 9D). To summarize, the *fap* expression domain was broader than, and incorporated, that of *fim* throughout floral development.

Discussion

We have shown that FIM associates specifically with a class of proteins implicated in protein degradation during the cell cycle of yeast and humans. The FIM-associated proteins (FAPs), isolated by the yeast two-hybrid system, fall into a family comprising three related proteins, FAPs 1–3. The FAP family shows strong similarity to Skp1 proteins from yeast and human. In yeast and humans, Skp1 proteins interact with F-box-containing proteins, to form a complex needed for protein degradation and cell

FAP1	 L K T K K I	T L R S S D G E V F	E V D E S T A L L S	Q T I K H M I E . .	 D D C A D N V	T P L P N V H G K I	51
FAP2	S S S D N A S K K I	T L R S S D G E V F	E V D E A T A L L S	Q T I K H M I E . .	 D D C A D N V	T P L P N V H G K I	55
FAP3							12
Skp1 HS	 M P S I	K L Q S S D G E I F	E V D V E I A K Q S	V T I K T M I E D L	G M D D G D G D D	M P L P N V N A A I	54
Skp1 SC	 M V T S N V	V L V S G E G E R F	T V D K K I A E R S	L L L K N Y L N D M	D D D D D D D D I V	M P L P N V R S S	56
FAP1	L S K V I E Y C K R	H V D A A A A K A D	D K L A S T G T S D	D E L K A F D A E F	V K V D Q A T L F D	L I L A A N Y L N I	111
FAP2	L S K V I E Y C K R	H V D A D A A K S D	D K V A A A A A G D	D D L K A F D A E F	V K V D Q A T L F D	L I L A A N Y L N I	115
FAP3	L A K V I E Y C K R	H V D A A A A K T D A	D D . . . G Q G D R	D A L K V E D A E F	V K V D Q G T L F D	L I L A A N Y L N I	69
Skp1 HS	L K K V I Q C T H	H K D D P P P E D	D E . . . N K E K R T	D D L P V D D Q E E	V K V D Q G T L F D	L I L A A N Y L N I	112
Skp1 SC	L Q K V I E A E H	H R D S N F P D E D	D D D S R K S A P V	D S . . . D R E E	V K V D Q E M L F D	L I L A A N Y L N I	113
FAP1	K T L L D L T C Q T	V A D M I K G K T P	E E I R K T F N I K	N D F T F E E E E E	V R R E N Q W A F E	*	.161
FAP2	K T L L D L T C Q T	V A D M I K G K T P	E E I R K T F N I K	N D F T F E E E E E	V R R E N O W A F E	*	.165
FAP3	K S L L D L T C Q T	V A D M I K G K T P	E E I R K T F N I K	N D F T F E E E E E	V R R E N A W A F E	*	.119
Skp1 HS	K G L L D V T C K T	V A N M I K G K T P	E E I R K T F N I K	N D F T F E E E A Q	V R K E N O W C E E	K	.163
Skp1 SC	K P L L D A G C K V	V A E M I R G R S P	E E I R R T F N I V	N D F T F E E E A A	V R R E N E W A E D	R	*.164

Fig. 8. Sequence of FAPs. Predicted amino acid sequences for FAP1, FAP2 and FAP3 compared with the human (HS) and yeast (SC) Skp1 proteins. Amino acid identities are boxed in black and conservative amino acid changes in grey. The black triangle represents an additional 30 amino acids present in the yeast protein. The genes are more highly conserved at their 3' ends and are more variable in the central region. The *fap* clones sequenced are not complete at their 5'-ends.

Fig. 9. Expression of *faps* in wild-type inflorescences. Expression pattern of *faps* in the inflorescence apex (A); stage 4 floral meristem (B); stage 6 floral meristem (C) and later floral bud (D). All *faps* give a similar expression pattern; those shown are *fap1* (A–C) and *fap2* (D). Expression correlates with regions where cells are actively dividing: the inflorescence meristem (I), bracts (Br), early floral meristems (F), sepals (S), petals (P), stamens (St), carpels (Ca), ovary (O) and anthers (An). Some expression is also seen in provascular tissue. Scale bar is 100 μm.

cycle progression (Zhang *et al.*, 1995; Bai *et al.*, 1996; Connelly and Hieter, 1996). Thus, the similarity between FIM and F-box proteins is further substantiated by their ability to interact with similar partners. A role for the FIM–FAP complex in flower development is supported by the RNA expression pattern of the *fap* genes. These genes are expressed in inflorescence and floral meristems in a pattern that incorporates the domain of *fin* expression. Based on the phenotypic and expression analysis of the various *fin* alleles generated by site-selected mutagenesis, the FIM–FAP complexes might play two roles in flower

development: (i) regulation of organ identity gene expression; and (ii) establishing morphological boundaries.

Regulation of organ identity gene expression

The *b* function is largely absent from the flowers of *fin* null plants: they contain almost no petal or stamen tissue and the *b* function genes, *def* and *glo*, are both expressed at greatly reduced levels compared with wild-type. In those cases where *def* and *glo* transcripts are detected, they first appear as small patches within the *b* function domain (stage 4). There is little overlap between early

patches of *def* and *glo* expression, indicating that both genes are activated in a stochastic manner in *fim* nulls. An early role of *fim* may therefore be to coordinate activation of *def* and *glo* expression, although the detectable level of *b* activity in the nulls indicates that *fim* is not the only means for promoting *def* and *glo* expression. At later stages, interactions between *def* and *glo* appear to be involved in maintaining their expression so that by about stage 6, the rare patches of *def* and *glo* expression in *fim* null mutant flowers are found to coincide. This is consistent with previous results which show that auto-regulatory control of *def* and *glo* expression does not become important until around stage 6 (Tröbner *et al.*, 1992; Zachgo *et al.*, 1995).

The homology between FIM–FAP and proteins from other systems suggests a possible mechanism by which *fim* might influence *b* activity. F-box proteins and their partners in other systems have been shown to affect the activity of cell cycle proteins and transcription factors, most probably by promoting protein degradation (Flick and Johnston, 1991; Barral *et al.*, 1995; Kumar and Paietta, 1995; Thomas *et al.*, 1995; Bai *et al.*, 1996). By analogy, the FIM–FAP complex might act by promoting degradation of a transcriptional inhibitor of *def* and *glo*. As expression of *fim* moves out from the centre of the meristem, the inhibitor could be removed in its wake, allowing the *b* genes to be activated. This is consistent with the pattern and timing of gene expression in the meristem: *fim* transcripts are first detected in the central dome, one to two nodes before expression of *def* and *glo*; expression of *fim* then spreads out to the periphery of the dome, with *def* and *glo* expression following behind.

A key requirement of any model for FIM function is to explain how the effects of *fim* on *b* activity persist until late stages of development, even though *fim* is only transiently expressed in the central dome of wild-type floral meristems. The persistent effects of *fim* might have two explanations: (i) early transient expression of *fim* might allocate cells to express *b* genes and this pattern is then maintained during later stages independently of *fim*; or (ii) late expression of *fim* at the boundaries of primordia might act non-autonomously to promote *b* expression throughout the primordia.

Analysis of the development of mosaic organs in *fim-676* mutants supports a transient role for *fim* in activating *b*. Expression of *def* and *glo* initiates in distinct patches in second and third whorl organ primordia of *fim-676* flowers. These patches may represent subsets of primordium cells that have been allocated to express *b* function genes early on. At later stages, expression of *b* function genes is still observed in discrete patches with sharp boundaries, correlating with areas of petal tissue observed in the mature flowers of *fim-676* plants. These discrete regions of *b* activity presumably include the clonal descendants of patches seen earlier on in development. The pattern of *b* activity in *fim-676* flowers can therefore be explained by allocation of cells early on in development which then persists through cell division as the flowers develop. According to this view, *fim* would act transiently, ensuring that cells within the *b* domain of wild-type meristems are allocated to express both *def* and *glo* at early stages.

Allocation might be explained if the FIM–FAP complex

promotes degradation of a transcriptional inhibitor of *b* genes early on, such that an active transcriptional state is then maintained through subsequent development. The existence of this sort of de-repression mechanism is supported by the analysis of *CURLY LEAF* in *Arabidopsis*, a gene which inhibits organ identity gene expression and encodes a product with homology to the Polycomb group of proteins, thought to be involved in cell allocation during *Drosophila* development (Goodrich *et al.*, 1997). However, *CURLY LEAF* mainly affects *c* activity in floral meristems at later stages of development, so other genes would presumably have to play a comparable role in repressing *b* function early on. Early allocation is also compatible with studies of cell lineages in *Antirrhinum* petal primordia which have shown that lineage restrictions occur between petals and adjacent organs at about the time that *fim* expression starts to diminish in organ primordia (Vincent *et al.*, 1995).

In addition to affecting *b*, the *fim* nulls also show reduced and delayed *c* activity. There is a decrease in reproductive tissue and an increase in organ number, correlating with delayed expression of the *c* function gene *ple*. In the absence of *fim*, there appears to be a gradient of *ple* activation along the inflorescence, with upper flowers showing *ple* expression earlier in their development than lower flowers. This is similar to the graded increase in *c* function activity observed towards the top of *Arabidopsis* inflorescences carrying mutations in meristem identity genes (Irish and Sussex, 1990; Huala and Sussex, 1992; Weigel *et al.*, 1992; Schultz and Haughn, 1993; Wilkinson and Haughn, 1995). It is unlikely that *fim* directly maintains *ple* expression since, by the time that *ple* expression is clearly detected, *fim* shows a complementary expression pattern. A more likely explanation is that early transient expression of *fim* in the central dome of the floral meristem is needed to activate *ple* at the right time, perhaps by a similar mechanism to that proposed for regulation of *b* genes.

The availability of null alleles also allowed the interaction of *fim* with the meristem identity gene *flo* to be investigated. These two genes show complementary expression patterns: expression of *fim* initiates in the central dome of the floral meristem at about the same time that *flo* declines in this region (stages 2–3), and at later stages *flo* is expressed within petal primordia whereas *fim* is expressed at their boundaries. This raises the possibility that *fim* and *flo* might inhibit each other's expression. In *fim* nulls, however, early *flo* expression is not significantly altered, indicating that *fim* is not necessary for restricting *flo*. Nevertheless, it is still possible that *flo* inhibits *fim* expression: the restriction of *fim* to the central dome at stage 3 could, for example, reflect the decline of *flo* expression specifically in this region. This is not easy to test in *Antirrhinum* because *flo* is also needed earlier on to activate *fim* in the central dome of floral meristems. In *Arabidopsis*, where *LFY* (the *flo* orthologue) is not needed to activate *UFO* (the *fim* orthologue), expression of *UFO* has been shown to become more extensive in *lfy* mutants (Lee *et al.*, 1997), consistent with *LFY* playing a role in restricting the domain of *UFO*.

Growth at morphological boundaries within the flower

Two aspects of the null phenotype indicate that *fim* plays a role at morphological boundaries in the flower. First,

meristems are often produced at the boundaries between floral organs in null flowers, giving rise to axillary shoots (lateral indeterminacy). Secondly, the boundaries between organs are often displaced, resulting in united organs and a loss of whorled phyllotaxy. The expression of *fim* in wild-type correlates with these effects. For instance, an extra ventral organ is frequently observed in the first whorl of *fim* null mutants, correlating with early expression of *fim* in this region of wild-type. Thus, localized *fim* expression in early meristems may repress growth and/or primordium initiation. Later expression of *fim* at the boundaries between primordia might also repress growth, ensuring lateral determinacy and whorled phyllotaxy. In affecting boundaries between primordia, *fim* shows similarities to the *no apical meristem (nam)* gene of petunia, which is also expressed between floral organs where it is thought to reduce growth (Souer *et al.*, 1996).

The similarities between *fim* and F-box genes from yeast suggest that *fim* may influence growth by modulating the cell cycle. This is also consistent with down-regulation of the *cyclin D3b* gene in boundary regions expressing *fim* (V.Gaudin *et al.*, submitted). One possibility is that the FIM-FAP complex is involved in degrading a regulatory protein needed for cell cycle progression. Another possibility is that FIM competes for a FAP needed for cell cycle progression.

Although *fim* regulates boundary growth, it is not absolutely required for the formation of morphological boundaries because they still form in null mutants even though they are in abnormal positions. Furthermore, over-expression of the *fim* orthologue, *UFO*, in *Arabidopsis* does not result in any obvious reduction in growth, indicating that growth effects of *fim* may depend on other spatially restricted factors (Lee *et al.*, 1997).

The significance of *fim* affecting both growth at boundary regions and organ identity gene expression may be that it provides a means of coupling the patterns of meristem growth to the regulation of gene activity. For example, the outer boundary of *b* gene expression appears to be centred within a domain of *fim* expression by stage 5. If the effects of *fim* on growth also ensure that morphological boundaries are centred within the *fim* domain, the anatomical and expression boundaries would be automatically aligned. This dual aspect of *fim* may be comparable with the role of *GRR1* in yeast, which encodes an F-box protein thought to coordinate gene expression with cell growth and division (Barral *et al.*, 1995). The *superman* gene of *Arabidopsis* also appears to have a dual role in *b* gene expression and cell proliferation (Schultz *et al.*, 1991; Bowman *et al.*, 1992; Sakai *et al.*, 1995). It is therefore possible that coupling between transcriptional regulation and growth, as exemplified by the FIM-FAP complex, is a general mechanism for aligning expression domains in meristems.

Materials and methods

Strategy for site-selected mutagenesis of the *fim* gene

To generate deletions and rearrangements caused by Tam3, several plants homozygous for *fim-619* (the M_0 generation) were placed at 15°C, a temperature that favours Tam3 activity (Carpenter *et al.*, 1987; Carpenter and Coen 1990). The *fim-619* allele carries a Tam3 insertion 976 bp upstream of the longest *fim* cDNA (start of the cDNA is denoted position 0). This allele is unstable due to the excision of Tam3,

Fig. 10. The *fim* locus. The position covered by the *fim* cDNA is shown as a box, with the solid region indicating translated sequence and the direction of transcription shown by a large arrow. The Tam3 insertion in *fim-619* is indicated as a black triangle. Positions of oligos A–K (used in PCR screening) are indicated by small arrows and probes X and Y are shown hatched.

giving germinal revertants at a rate of ~1–2% under field conditions (Simon *et al.*, 1994). The M_0 plants were self-pollinated and 268 seed capsules individually harvested. Each capsule was sown to give a family of 15 M_1 plants (total of 4020 plants). The M_1 plants were also self-pollinated and seed pooled within each family.

Plants in the M_1 generation were expected to be heterozygous for any germinal events which had occurred in the M_0 . To allow these events to be detected by PCR, leaves were harvested and pooled from each M_1 family of 15 plants and genomic DNA was extracted from the pools. Three sets of oligonucleotide primers (oligos) were designed: oligos A and B were complementary to each end of Tam3, C and D were based on sequences near the Tam3 insertion site, and E–K corresponded to sequences within the 6.5 kb genomic region containing *fim* and its 5' and 3' regions (Figure 10). Screening with Tam3 oligos (A and B) in combination with *fim*-specific oligos (E–K) should detect events in which Tam3 had transposed to a new site within *fim*, or deletions adjacent to Tam3. In addition, *fim*-specific oligos in combination with oligos adjacent to the Tam3 insertion site (C and D) should detect deletions in which Tam3 had excised. The sensitivity of PCR for detecting rare events was tested by making serial dilutions of genomic DNA from homozygous *fim-619* individuals in an excess of wild-type DNA. Dilutions were tested with oligos A and E or with B and F. In control reactions, both combinations gave the expected products of ~1 kb with undiluted *fim-619* DNA, and no products with wild-type DNA. Amplification of a 1:1000 dilution of *fim-619* wild-type DNA with either oligo combination gave 1 kb PCR products which were visible on an ethidium bromide-stained gel and which strongly hybridized to a *fim* probe on DNA blots (probe Y, Figure 10; data not shown). A dilution of 1:10 000 also gave bands detectable on DNA blots probed with *fim*, although they were not visible on ethidium bromide gels. Screening DNA pooled from 500 plants in one PCR reaction therefore allowed ready detection of heterozygous events by ethidium bromide gel staining.

To facilitate screening, DNA from M_1 families was further pooled in a combinatorial fashion so that each family was represented in a unique combination of two large pools. This minimized the number of reactions needed to identify the family in which an event had occurred. The large pools contained DNA from a maximum of 11 families (165 plants) so that new events were well within the limit of detection (1 in 500 plants). Only events found in two large pools were considered to be genuine.

DNA preparation and PCR screening

For DNA extraction, leaf samples were ground in liquid nitrogen and added to extraction buffer [50 mM EDTA, pH 8; 0.1 mM NaCl; 0.1 M Tris-HCl, pH 8; 1% (w/v) sodium dodecyl sulfate (SDS)]. Phenol/chloroform extractions were performed and DNA was precipitated with 10% (vol.) sodium acetate and 70% (vol.) isopropanol, washed in 70% ethanol, air-dried and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing 5 µg/ml RNase A. DNA concentrations were determined by optical density at 260 nm and gel analysis.

Oligonucleotides were synthesized and purified in a synthesizer according to the manufacturer's instructions (Pharmacia Biotech) and were as follows: (A) 5'-CAC GGC CCA ATT CAC ATC TTT A-3'; (B) 5'-CTC GGC ACG TTT CAC ATC TTT A-3'; (C) 5'-GTT TGA TGT GTA CTC CTT TTT GCC-3'; (D) 5'-CTT GTC GTC CTT CAC

TCC CC-3'; (E) 5'-TTC TTC TTT TTT CCC TCC ACT CC-3'; (F) 5'-GTT TGT TTT GGA GTG GGA GGA C-3'; (G) 5'-GTG CAG TTA CTT TGC CCA-3'; (H) 5'-CCG CTT TAT CGT ACG ATC C-3'; (I) 5'-CAA ACT ACG CAG GCT ACC-3'; (J) 5'-ATT CTG CTA ATG TTT TTG AGG C-3'; (K) 5'-TGC CAG TTT TGA GGT TTT ACC-3'.

PCR was carried out in a programmable thermal cycler (Perkin Elmer Cetus, Munich, Germany). Reactions were made in 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin; 0.2 mM each of dATP, dCTP, dTTP and dGTP; 0.002% (w/v) Tween 20, 100 ng DNA and 2 U *Taq* polymerase were added per 50 µl reaction. Samples were heated to 95°C before addition of polymerase to minimize primer dimerization. Reactions were overlaid with 20 µl of mineral oil and 40 cycles of 1 min at 94°C, 2 min at 60°C and 5 min at 72°C were performed. Samples were then heated to 72°C for 10 min before being stored at 4°C. PCR products were electrophoresed on 1.2% agarose gels and blotted onto Hybond-N+ membranes (Amersham International, UK) under alkaline conditions according to the manufacturer's instructions. Probes X and Y (Figure 10) were prepared by labelling the inserts from plasmids pJAM137 and pCTW6 (Simon *et al.*, 1994) respectively, using a random hexanucleotide labelling kit (Pharmacia Biotech). Hybridization was performed at 65°C with 2×SSC, 1% (w/v) SDS and 0.5% (w/v) dried milk powder and two 30 min washes were performed at 65°C with 0.1× SSC and 0.5% (w/v) SDS.

Molecular and phenotypic analysis of new *fim* alleles

PCR products were cloned into pGEM4z (Promega) by filling overhangs using T4 DNA polymerase and then ligating into *Sma*I-cut vector under blunt-end conditions. Plasmids were isolated using Qiagen columns (Qiagen, Hybaid Ltd, UK) and sequenced by dideoxynucleic acid methods using the Sequenase II kit according to the manufacturer's instructions (US Biochemicals). Methods for isolation of genomic DNA for gel blot analysis and preparation of DNA probes were as described previously by Bradley *et al.* (1993).

Plants for SEM analysis were grown in growth chambers at 20°C. SEMs were prepared as described in Green and Linstead (1990). The mutant *fim-619* (originally called *sep-619*) was derived from stock JI.98 (*nivea*^{recurrens}-98) during a large-scale mutagenesis programme (Carpenter and Coen, 1990; Simon *et al.*, 1994).

Yeast two-hybrid screening and further analysis

The FIM bait was generated by subcloning the full-length *fim* open reading frame into the vector pGBT9, which encodes the yeast GAL4 DNA-binding domain. PCR amplification was used to engineer *Sa*I and *Bam*HI restriction sites into the *fim* sequence, allowing an in-frame fusion of the open reading frame to the GAL4-binding domain. The resulting clone, pJAM2036, was fully sequenced and is referred to as BD/FIM.

The prey was kindly provided by Hans Sommer (Davies *et al.*, 1996) and consisted of a cDNA library from floral tissue, directionally cloned into the vector pGAD424 which carries the yeast GAL4 activation domain. 240 µg of the library was then used to transform the yeast strain HF7c carrying BD/FIM (Gietz *et al.*, 1992). Colonies that contained activation plasmids were selected by their ability to grow more quickly on plates lacking histidine. Candidates were also tested for β-galactosidase production using a filter assay (Breedon and Naysmith, 1985). A crude yeast DNA preparation from the remaining positive colonies was transformed into the *Escherichia coli* strain HB101 (Hoffman and Winston, 1987). Candidate positives were re-transformed into HF7c (Feilottter *et al.*, 1994) or SFY526 (Bartel *et al.*, 1993), with or without the BD/FIM bait and tested for β-galactosidase activity. Those activation domain plasmids that were only positive by filter assay in the presence of the BD/FIM bait were sequenced using the automated ABI PRISM™ Dye Terminator Cycle sequencing Ready Reaction Kit system (Perkin Elmer). The primers used for sequencing flanked the pGAD424 cloning sites, as described previously (Davies *et al.*, 1996).

In situ hybridization

Methods for tissue preparation, digoxigenin labelling of RNA probes and *in situ* hybridization were as described in Bradley *et al.* (1993). The probe used to detect *fim* transcript was prepared from pCTW6 as described in Simon *et al.* (1994). Probes used to detect *ple*, *def* and *flo* transcripts were prepared as described in Bradley *et al.* (1993). The probe used to detect *glo* transcript was prepared as described in Tröbner *et al.* (1992). The probes used to detect the *fap* genes were from PCR-amplified products, using primers to the pGAD424 vector, of which one primer included the T7 promoter. The purified PCR products were used as templates for T7 RNA polymerase.

Accession numbers

The sequence information presented in this paper has been assigned the following EMBL database accession numbers: *Antirrhinum majus* mRNA for fimbriata-associated protein 3 (Y14856), *Antirrhinum majus* mRNA for fimbriata-associated protein 2 (Y14857) and *Antirrhinum majus* mRNA for fimbriata-associated protein 1 (Y14858).

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