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Sylvie Pouteau, D. Nicholls, F. Tooke, E. Coen, N. Battey, . University of Reading

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Transcription pattern of a *FIM* homologue in *Impatiens* during floral development and reversion

Sylvie Pouteau^{1,*†}, David Nicholls¹, Fiona Tooke¹, Enrico Coen² and Nicholas Battey¹

¹Plant Science Laboratories, University of Reading, Whiteknights, Reading RG6 6AS, UK, and

²John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Summary

Flowering and reversion in *Impatiens* are characterised by gradual transitions of organ identity and constitute a unique system for the molecular and physiological study of floral organogenesis. The authors have isolated an *Impatiens* homologue of the *FIM* gene of *Antirrhinum* (*UFO* in *Arabidopsis*), *Imp-FIM*, and analysed its expression in three states of the terminal meristem: vegetative, floral, and reverted. In floral meristems, *Imp-FIM* transcription is associated with petal identity, as in *Antirrhinum* and *Arabidopsis*, but this is achieved through a novel transcription pattern, characterised by a high level of transcript within petal primordia. This novel transcription pattern could contribute to the more diffuse boundaries between organ types in *Impatiens*. In vegetative meristems, *Imp-FIM* is expressed in the axils of leaf primordia which are arranged in a spiral. A similar pattern is observed in reverted meristems in which leaf primordia are initiated in a whorled arrangement. This result indicates that the maintenance of floral phyllotaxis is not associated with a specific pattern of *Imp-FIM* transcription. Transcription of *Imp-FIM* in a non-reverting line is no different from that in the reverting line. Therefore, the lack of floral commitment in the reverting line does not seem to be responsible for *Imp-FIM* transcription within petals. The novel transcription pattern in petals, together with features of *Impatiens* that are reminiscent of *fim* and *ufo* mutant phenotypes suggest an evolutionary divergence for *Imp-FIM* regulation in this species.

Introduction

In a large number of plants, flowers are characterised by discrete whorls of organs arranged from outside to inside in the sequence: sepals, petals, stamens and carpels. The genetic control of floral organogenesis in two such plants,

Antirrhinum (*Scrophulariaceae*) and *Arabidopsis* (*Brassicaceae*), has attracted much attention based on the existence of a range of floral homeotic mutants. The molecular genetic characterisation of these mutants has allowed the identification of two main types of genes. The first type is represented by early acting genes involved in the control of meristem identity, such as *floricaula* (*FLO*) in *Antirrhinum*, and *leafy* (*LFY*) in *Arabidopsis* (Coen *et al.*, 1990; Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel *et al.*, 1992). The second type are later acting genes involved in the control of organ identity. Organ identity mutants are characterised by the partial or total replacement of one class of floral organ by another class. Their analysis led to the ABC model (reviewed in Coen and Meyerowitz, 1991; Ma, 1994; Weigel and Meyerowitz, 1994) which proposes that three classes of genes corresponding to three functions, called A, B and C, act together in overlapping domains. Four combinations resulting from their interactions, A, AB, BC, and C, specify sepals, petals, stamens, and carpels, respectively. However, it is still unclear how discrete boundaries are established between organs and what mechanisms prevent organ identities from overlapping and mixing to give mosaic organs (Meyerowitz, 1996).

The function of organ identity genes depends on the activity of meristem identity genes. Recently, a mediator between meristem and organ identity genes, called *fimbriata* (*FIM*), has been identified in *Antirrhinum* and its homologue in *Arabidopsis*, called *unusual floral organs* (*UFO*), has also been characterised (Ingram *et al.*, 1995, 1997; Levin and Meyerowitz, 1995; Simon *et al.*, 1994; Wilkinson and Haughn, 1995). The *fim* and *ufo* mutants are affected in both meristem and organ identity. Their flowers often have increased inflorescence characteristics and reduced B and C functions. *FIM* and *UFO* genes show extensive homology and their expression patterns are very similar (Ingram *et al.*, 1995; Simon *et al.*, 1994). However, some functional differences between the two genes suggest that *FIM* and *UFO* probably mediate between meristem identity genes and organ identity genes in slightly different ways. More recently, Lee *et al.* (1997) suggested that *UFO* is not a simple mediator but a partially dispensable co-regulator of *LFY* in *Arabidopsis*. In addition to this mediator or co-regulator function, the *fim* and *ufo* mutant phenotypes suggest that *FIM* and *UFO* may also have roles in establishing a whorled phyllotaxis and defining boundaries for the domains of function of organ identity genes (Levin and Meyerowitz, 1995; Simon *et al.*, 1994).

The terminal flower of the Dwarf Bush Flowered cultivar

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*For correspondence (fax +33 13083 3099;

e-mail pouteau@versailles.inra.fr).

†Permanent address: Laboratoire de Biologie Cellulaire, INRA, Route de St Cyr, F78026 Versailles cedex, France.

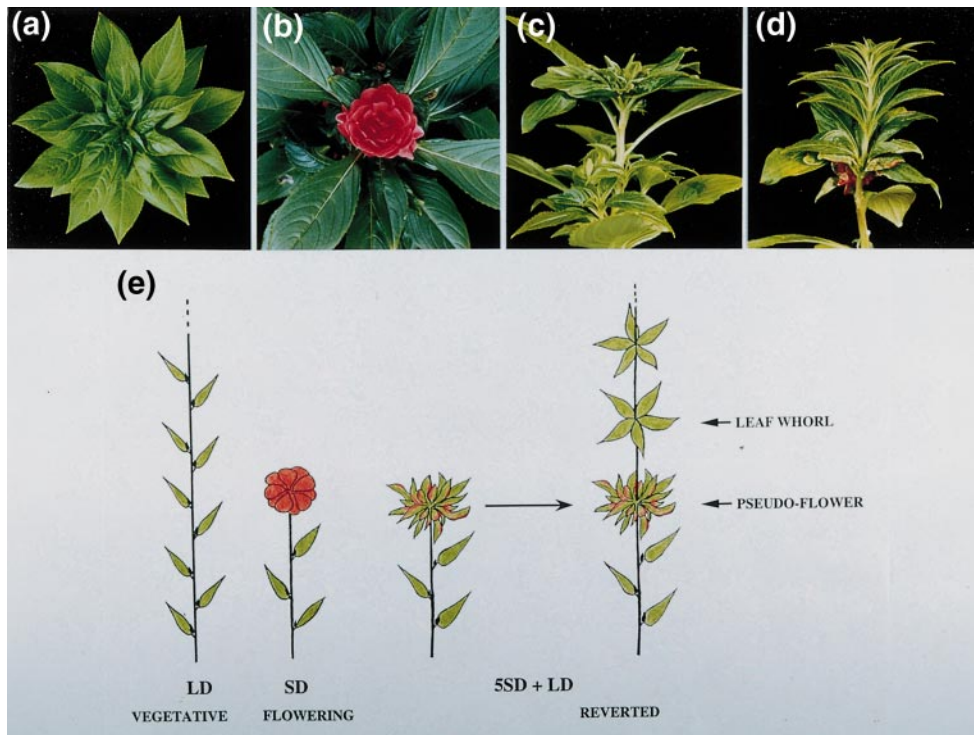


Figure 1.

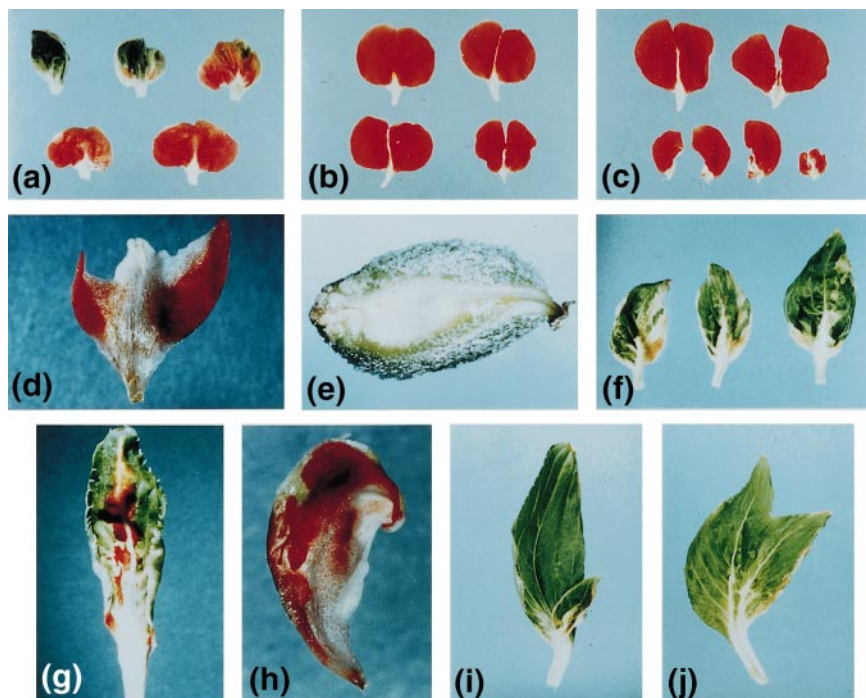


Figure 2.

of *Impatiens balsamina* (*Balsaminaceae*) does not have discrete whorls of floral organs and the total number of organs is not fixed. In addition, there is a progressive transformation of one class of floral organ into the next class through a continuum of floral organ identity (Battey and Lyndon, 1984; Pouteau *et al.*, 1995; this work). The absence of clear boundaries between the different organ identity domains results in the production of a large number of mosaic organs, especially petal mosaics. This phenotype suggests that there is an alteration in the definition of organ identity domains in *Impatiens*.

Impatiens has attracted much interest because three states of the shoot apical meristem, vegetative, flowering, and reverted (Figure 1), can be obtained in a predictable way by manipulating the photoperiod in which plants are grown (Battey and Lyndon, 1984, 1990; Debraux and Simon, 1969; Krishnamoorthy and Nanda, 1968; Pouteau *et al.*, 1995, 1997). Flowering in *Impatiens* is induced in short days (SD) and plants remain vegetative in long days (LD). Interruption of the SD induction by transfer into LD results in flower reversion and return to leaf production. The reverted meristem behaves differently from a vegetative meristem because it immediately resumes flower development without a lag period when transferred back into inductive photoperiod (Battey and Lyndon, 1986, 1988), and thus constitutes a new developmental state which has not been observed in floral homeotic mutants isolated thus far.

The relationship between organ arrangement, determinacy and organ identity can be studied through reversion because reverted meristems have a combination of floral (whorled arrangement, absence of axillary meristems) and vegetative (indeterminate number of leaves, long internodes) features. A combination of floral and vegetative features is also observed in *fim* and *ufo* mutants, so to study the molecular basis of reversion in *Impatiens* we set out to isolate the *FIM* homologue in this species (*Imp-FIM*) and analyse its transcription in the different states of the meristem. *Imp-FIM* transcripts were detected in the vegetative, flowering, and reverted states of the meristem but not during reproductive organ initiation.

As in *Antirrhinum* and *Arabidopsis*, *Imp-FIM* transcription was associated with petal identity but this was achieved through a novel transcription pattern, characterised by a high level of transcript within petal primordia. A similar pattern of *Imp-FIM* transcription was observed in a non-reverting line, suggesting that a lack of commitment to flowering may not be responsible for this new pattern of transcription. Similarities of the *Impatiens* phenotype to *Antirrhinum fim* and *Arabidopsis ufo* mutants suggest that this pattern could result from an evolutionary divergence in *Imp-FIM* regulation. This could account for the pronounced developmental plasticity of *Impatiens* and the absence of discrete boundaries between organ identity domains.

Results

Morphological description

The vegetative meristem. Flowering was prevented by the continuous application of low light intensity throughout the night period (Battey and Lyndon, 1984; Pouteau *et al.*, 1995, 1997). Under these conditions, referred to as LD, plants remained vegetative (Figure 1). The vegetative meristem was characterised by the initiation of leaves with axillary meristems and spiral phyllotaxis (Battey and Lyndon, 1984; Pouteau *et al.*, 1995, 1997).

The flowering meristem. Flowering was induced by transfer from LD to SD after an average of nine leaves had been initiated (day 0). Continuous SD were required after day 0 to allow the formation of a complete flower (Figure 1). The transition from spiral phyllotaxis to a whorled arrangement first became obvious after ≈ 8 SD. The whorled pattern was gradually established and was usually imperfect; the whorls were not discrete and there was sometimes a small degree of internode elongation between adjacent organs.

Organ identity in the terminal flower gradually changed with successive organs from leaf to sepal, petal, stamen, and carpel identities. No true sepals were formed but

Figure 1. The three states of the meristem: vegetative, flowering, and reverted.

(a) Top view of a vegetative plant grown in continuous LD; (b) top view of the terminal flower formed in continuous SD; (c) R4 reverting plant induced for 5 SD and then transferred to LD, showing a pseudo-flower with leaf-petal mosaic organs; (d) R4 plant at a later stage in LD, showing whorls of leaves lacking axillary meristems and separated by long internodes above the pseudo-flower; (e) diagram summarising the main features of the plants illustrated above.

Figure 2. Morphology of mosaic floral organs.

Mosaics produced during flower development in continuous SD: (a) leaf-petal mosaics formed during the transition from bracts to petals with chlorophyll-rich sectors; (b) and (c) transition from true petals to staminate petals; (d) stamen exhibiting vestigial petal lobes; (e) staminate gynoecium.

Mosaics produced during flower reversion: (f) R4 reversion: leaf-petal mosaics formed in the pseudo-flower; (g) R5 reversion: petal-leaf mosaic formed during the transition from petal identity to leaf identity; (h) stamen-petal-leaf mosaic formed during the transition from stamen identity to leaf identity in a plant showing R6/R7 reversion. (i) Organ fusion between two leaf-petal mosaics from different whorls formed in the pseudo-flower during R4 reversion. (j) Organ fusion between two modified leaves from the same whorl formed in the pseudo-flower during R4 reversion.

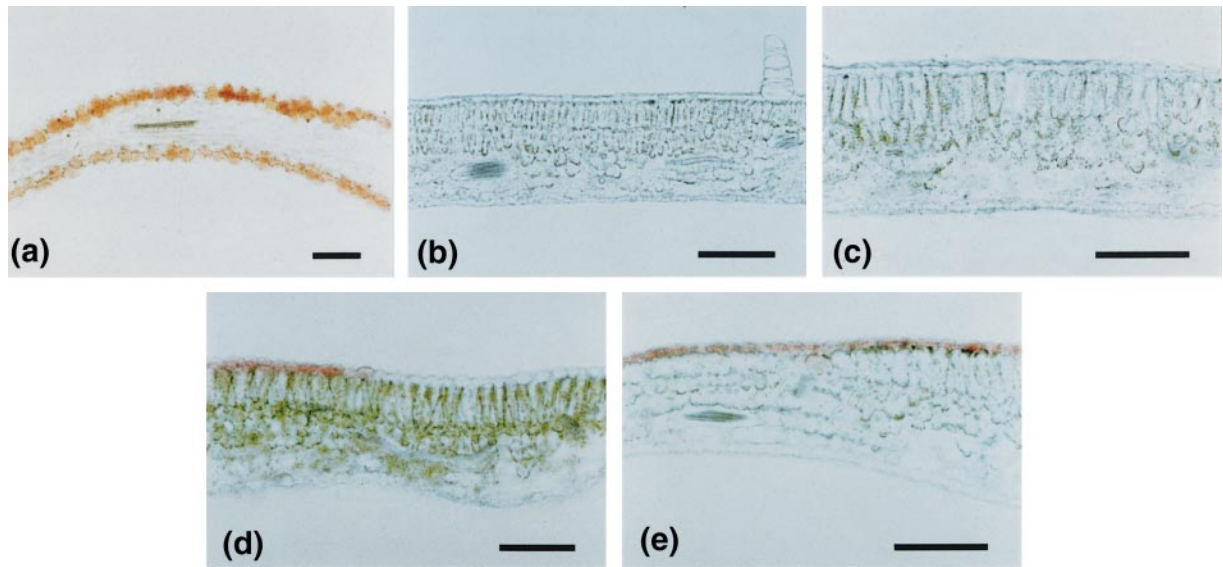


Figure 3.

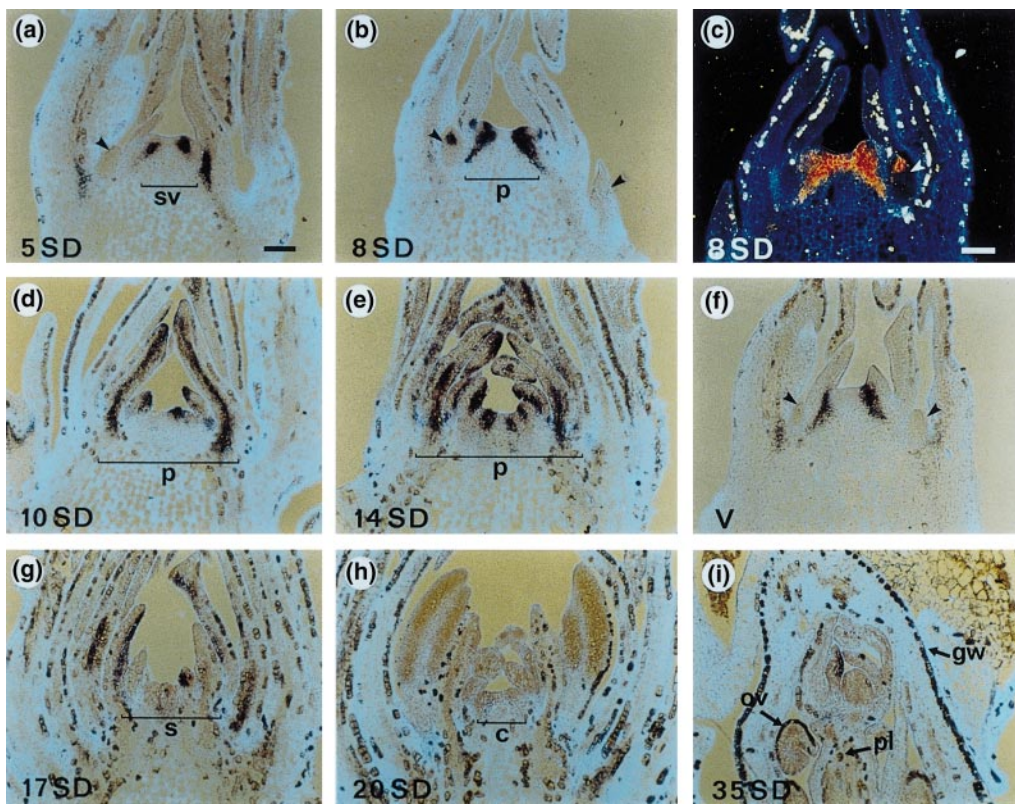


Figure 5.

organs with sepaloid features, called bracts (Battey and Lyndon, 1984), were produced between leaves and petals. The gradual change of organ identity resulted in the occurrence of bract-petal mosaics, petal-stamen mosaics and stamen-carpel mosaics. Anthocyanin pigmentation appeared in organs that had been initiated after ≈ 5 SD (Figure 2a). An average of 21 petal primordia were initiated between 7 SD and 14 SD. Only about seven of the resulting petals were classified as true petals, i.e. exhibited 100% petal tissue, whilst the remaining petals were mosaic for bract or stamen identity (Figure 2a–c). The first five petals exhibited varying levels of bract features with mosaics of chlorophyll and anthocyanin pigmentation in the mesophyll and the epidermis, respectively. The last nine petals had progressively more stamen tissue; at the same time, the petal lobes became distorted and decreased in size. An average of 13–14 stamen primordia were initiated between 14 SD and 20 SD. Some of the first stamens still had petal features such as vestigial petal lobes and anthocyanin-pigmented filaments and anthers (Figure 2d). Mosaics between stamens and carpels were also observed and the carpels that fused to form the wall of the gynoecium often included one or more stamens (Figure 2e).

The reverted meristem. Interruption of the SD induction by transfer to LD resulted in reversion. Different reversion phenotypes were obtained by increasing the inductive period in SD and were classified on a scale from R0 to R8, based on the increasing extent of flower development taking place before a return to leaf initiation (Battey and Lyndon, 1984; Pouteau *et al.*, 1997). A typical reversion treatment used in the experiments to be described below consisted of a five SD period of induction followed by transfer to continuous LD (Figure 1). The 5 SD + LD treatment was chosen because it caused the most uniform reversion response and resulted in most of the plants having an R4 reversion phenotype (Battey and Lyndon, 1984; Pouteau *et al.*, 1997). R4 reversion occurred in two stages: (i) the pseudo-flower stage, and (ii) the leaf-whorl stage.

The pseudo-flower stage of R4 reversion was characterised by the production of leaf-petal mosaic organs arranged in whorls. Six days after transfer to non-inductive LD, most of the pseudo-flower had been formed (on average 12.5 primordia in total) with an average 4.6 primordia developing as leaf-petal mosaics at maturity. The extent of anthocyanin pigmentation in these mosaics varied from one plant to another but organs with less than 50% anthocyanin pigmentation were typical (R4 reversion type, Figure 2f). Petal sectors in these mosaics showed different combinations of leaf and petal histological structures: some sectors had spongy and palisade leaf mesophylls underlying a petal epidermis, and other sectors had only spongy leaf mesophyll underlying a petal epidermis but no palisade mesophyll (Figure 3d and e).

The leaf-whorl stage of R4 reversion started 7 days after transfer to non-inductive LD, and was characterised by the resumption of internode elongation and the production of whorls of leaves, each comprising five or six leaves lacking axillary meristems. Hand sectioning of fresh tissue and SEM revealed no difference between the histological structures (Figure 3b and c) and epidermal surfaces (not shown) of the reverted leaves and the vegetative leaves. This suggests that both types of leaves were essentially identical. The terminal meristem continued to initiate whorls of leaves lacking axillary meristems for at least 30 LD after transfer from SD. After several weeks, the phyllotaxis progressively returned to the spiral arrangement characteristic of the vegetative plant and axillary meristem initiation resumed.

Expression of petal identity in the terminal meristem could be prolonged by inducing reversion during petal initiation, such as after 10 SD. The 10 SD + LD reversion phenotype showed a proliferation of petals before a return to leaf initiation through a series of mosaics (R5 reversion type). These mosaics, called petal-leaf mosaics, differed consistently from the mosaics produced during flowering and R4 reversion as they retained petal characteristics along the mid-vein whilst the edges adopted leaf identity (Figure 2g). In some cases a few

Figure 3. Hand sections of organs produced during reversion.

(a) True petal; (b) leaf produced during vegetative growth; (c) leaf produced during the leaf-whorl stage of R4 reversion; (d) leaf-petal mosaic produced during R4 reversion, showing a petal sector with an upper petal epidermis above a normal leaf mesophyll; (e) same as (d), showing the transition of a normal leaf mesophyll (right) to a petal spongy mesophyll accumulating chlorophyll through the gradual loss of the palisade mesophyll (left). Scale bars = 500 μ m.

Figure 5. *In situ* hybridisation analysis of *Imp-FIM* transcription during flower development.

(a)–(e) and (g)–(i) Sections of flowering apices fixed at different times (5 SD to 35 SD); (f) vegetative apex (V, fixed after 14 LD). Apical sections were probed with digoxigenin-labeled *Imp-FIM* antisense RNA and viewed under light field (RNA signal is purple on a blue tissue background). Leaf tissue and, in a more pronounced way, floral tissues remained strongly pigmented after fixation and embedding due to the accumulation of brown-stained granules. The dark field image (c) emphasises the contrast between the stained granules in the meristem and young primordia (white) and the *Imp-FIM* signal (orange). All photos under light field were taken with the same magnification factor. Scale bars = 100 μ m.

The identity of primordia is indicated as follows: sv = leaves with a modified shape and/or venation; p = petals; s = stamens; c = carpels; ov = ovule; pl = placental column; gw = gynoecium wall. Axillary meristems are indicated with arrowheads.

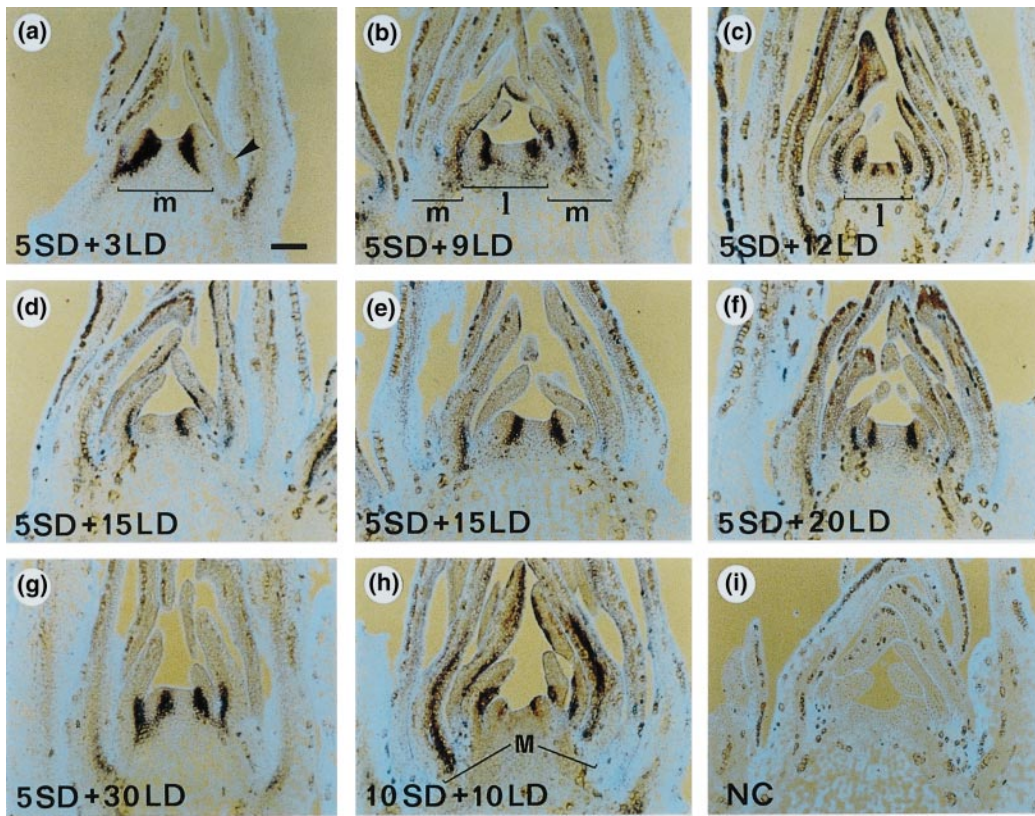


Figure 6.

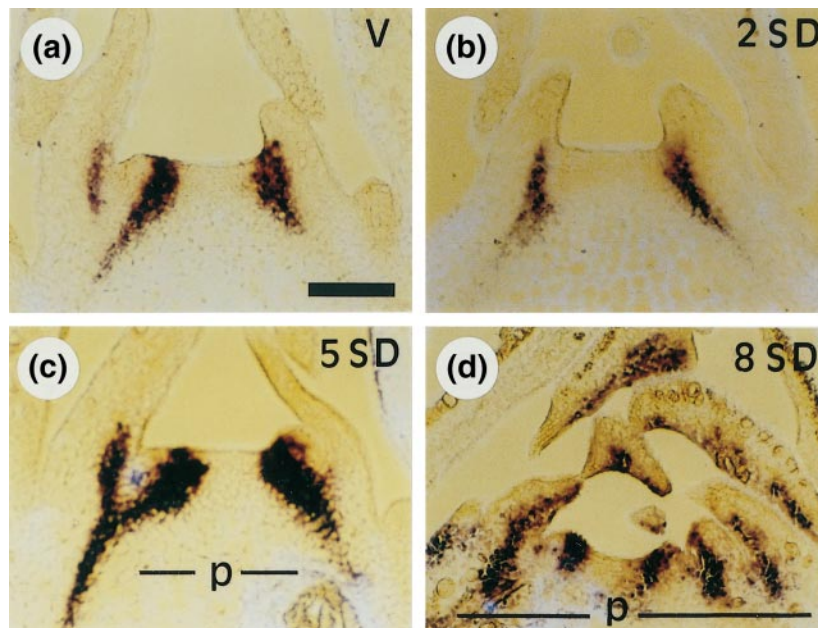


Figure 7.

stamens were initiated (R6/R7 reversion type) before a return to leaf initiation. This caused the production of a new type of mosaic which had mixed stamen, petal, and leaf tissues (Figure 2h).

Impatiens phenotypic features related to *fim* and *ufo* mutations

The terminal flower of *Impatiens* exhibited several similarities to *fim* and *ufo* mutant phenotypes in *Antirrhinum* and *Arabidopsis* (Ingram *et al.*, 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). One characteristic of *ufo* mutants is that their flowers contain mosaic organs and fused organs. In *Impatiens*, most of the organs that were formed in the terminal flower under SD or in the pseudo-flower stage of R4 reversion were mosaic, as described above (Figure 2a–j). Fused organs were also commonly produced in *Impatiens* during flowering and reversion (Figure 2i–j). Fusion was often observed between adjacent organs from the same whorl, whether they had the same identity or not, but it occurred less frequently between organs in different whorls.

The pseudo-flower stage of R4 reversion was very reminiscent of the phenotype of some of the *fim* mutants in *Antirrhinum* which display a proliferation of sepals in the

centre of the flower (Simon *et al.*, 1994). Furthermore, even under continuous SD, *Impatiens* terminal flowers exhibited reduced determinacy. This was illustrated by the variability in numbers of each organ type in different flowers: the number of petals varied from 12 to 34 (on average 20.8), and of stamens from 5 to 23 (on average 13.5). In addition, resumption of meristematic activity occurred very late in development at the top of the placental column and resulted in the production of a new gynoecium or more often of a new flower (Pouteau *et al.*, 1995; unpublished data).

PCR cloning of *Imp-FIM*, a FIM homologue fragment in *Impatiens*

The phenotypic analysis of flowering and reversion in *Impatiens* suggested that *FIM* function may be altered in this species. For molecular analysis, a 538 bp fragment was amplified from genomic DNA of *Impatiens* using *FIM*- and *UFO*-specific oligonucleotides, and sequenced. The fragment corresponded to approximately 40% of the *FIM* open-reading frame and was in the middle of the coding sequence. The identity at the nucleotide level was 58% with *FIM* and 51% with *UFO*. At the amino acid level, the identity was 56% and 48% (similarity was 72% and 69%)

| | | |
|----------------|-----|---|
| Imp-FIM | 1 | LYLQV PNK LHTW F IF FL MON .VRAASMTSD GIN SR RY K PTE E AY LL NPD EP |
| FIM | 1 | LHLQA SP IRHW .F MF FK Q SIKH HI YNN STN AR P . TNY E GY L FDP Q TL |
| UFO | 1 | TYLQL LP LR H NC F LF FK H KT L KS YI Y KR G TN DD DS NA K . EG FL FDP NE I |
| Imp-FIM | 50 | SWHRI H FPM I PP GY SPA AS SG GLIC WVS NE PG SK S LL LS N PI I GS I S PL P |
| FIM | 48 | KWYRI S FPL I PP GF SPA SS SG GLIC WV SE DS GP KNI LL SN PL INT AI QLP |
| UFO | 50 | RWYRL S FAY I PS GF YPS SS SG GL V SW VSE E AG L KT IR KAL AL ILV A TDSR |
| Imp-FIM | 100 | PTLV P R L F P SV GL TVT NA SV D VT V AG DD MV SP YAV K NLS SE SF H L D NG GF |
| FIM | 98 | STLE P R L C P T I GL T IT NS SI D LS F AG DD LIS P YAV K NLS SE SF H ID V GG F |
| UFO | 100 | IRQ E GY T R P S I GL S V T P T SI D VT V AG DD LIS P YAV K NLS SE SF H V D AG GF |
| Imp-FIM | 150 | YSV W G T T S C L P R L C S L ES G Q M VH A R G K F Y C |
| FIM | 148 | YSI W N T T S S L P R L C S L ES G R M VH V O G R F Y C |
| UFO | 150 | FSL W A M T S S L P R L C S L ES G K M VY V O G K F Y C |

Figure 4. Comparison of the *Impatiens* FIM sequence with its homologues in *Antirrhinum* and *Arabidopsis*.

Alignment of the partial *Impatiens* FIM protein sequence with the corresponding *Antirrhinum* FIM (amino acid 86–262; Simon *et al.*, 1994) and *Arabidopsis* UFO (amino acid 89–267; Ingram *et al.*, 1995) proteins. Amino acids at positions of identity and similarity between these proteins are blocked in black and grey, respectively.

Figure 6. *In situ* hybridisation analysis of *Imp-FIM* transcription during flower reversion.

(a–h) Sections of reverted apices fixed at different times (5 SD + 3 LD to 30 LD; and 10 SD + 10 LD) were hybridised and viewed as in Figure 5. (i) The negative control (NC) was hybridised with a digoxigenin-labeled *Imp-FIM* sense RNA. Scale bar = 100 μ m.

The identity of primordia is indicated as follows: m = leaf-petal mosaics; l = leaves; M = petal-leaf mosaics. Leaf-petal mosaics were produced during R4 reversion (no petals produced). Petal-leaf mosaics were formed after petals during R5 reversion. Young primordia in (d–g) are all leaves. Axillary meristems are indicated with arrowheads.

Figure 7. *In situ* hybridisation analysis of *Imp-FIM* transcription in a non-reverting line.

(a) Vegetative apex (V, fixed after 8 LD); (b–d) Sections of flowering apices fixed at different times (2 SD to 8 SD) were hybridised and viewed as in Figure 5. Scale bars = 100 μ m. Petal identity (p) of primordia is indicated.

with FIM and UFO, respectively (Figure 4). This is similar to the level of homology between FIM and UFO in the region covered by the clone (Ingram *et al.*, 1995). Therefore, the fragment isolated was considered likely to be part of the FIM homologue in *Impatiens* and called *Imp-FIM* (for *Impatiens FIM*). High stringency genomic Southern blotting showed one single *Imp-FIM* sequence in the genome of *Impatiens*. The *Imp-FIM* fragment was used to analyse FIM transcription in the apex of *Impatiens* at different stages during flower development and reversion by generating antisense digoxigenin-labelled RNA probes and performing *in situ* hybridisation on longitudinal sections of apices.

Imp-FIM transcription during vegetative growth in continuous LD

In situ hybridisation on the vegetative apex at different stages of vegetative growth showed that *Imp-FIM* was transcribed in the apical meristem (Figure 5f). The distribution of the *Imp-FIM* transcript was investigated by the analysis of serial sections and by whole mount *in situ* hybridisation (not shown). The signal formed a crescent shape in the axils of the young primordia. It extended several cell layers deep into the meristem, giving a stripe of expression on longitudinal sections.

Imp-FIM transcription during the transition to flowering in continuous SD

During the first week after transfer from LD to SD, *Imp-FIM* transcription in the apical meristem was very similar to that in the vegetative meristem (Figure 5a). It started to change after about 8 SD when most of the petals, which would at maturity contain some chlorophyll, had been initiated (Figure 5b and c). The intensity of the signal increased slightly in the apical meristem at this stage and there were fusions between the extremities of the stripes of signal in the two youngest whorls. The pattern of *Imp-FIM* transcription started to change drastically after 10 SD, when approximately four true petal primordia had been initiated. The new pattern was maintained during the period of initiation of staminate petals (exhibiting a white region), between 11 SD and 14 SD (Figure 5d and e). During this period, *Imp-FIM* transcription was located mostly within petal primordia and a little further down in the axils. *Imp-FIM* RNA accumulated in the adaxial halves of the young petal primordia. For the oldest petal primordia, the distribution of the signal was more complex, as can be seen on occasional transverse sections of primordia. *Imp-FIM* RNA still accumulated in the adaxial halves of the petal primordia but not in the median region, resulting in two stripes of signal within the primordia, in addition, *Imp-FIM* was transcribed in the midvein (Figure 5e).

Imp-FIM transcription during reproductive organ initiation

During stamen initiation, *Imp-FIM* signal progressively disappeared in the primordia and the meristem. After 17 SD, the time of initiation of the 11th stamen primordium on average, the terminal meristem still displayed patches of *Imp-FIM* signal within growing petal primordia but stamen primordia in the two youngest whorls had very little signal (Figure 5g). After 20 SD, the time of carpel initiation, no *Imp-FIM* signal could be detected in mature stamens or in carpel primordia (Figure 5h).

After 35 SD, the gynoecium had formed by united growth of the carpel primordia, and differentiation of the placental column and ovules was visible (Pouteau *et al.*, unpublished observations). At this stage several primordia had already been initiated from the top of the placental column. *Imp-FIM* was transcribed in some of the primordia that would later develop as petals or petaloid organs (Figure 5i).

Imp-FIM transcription during the pseudo-flower stage of R4 reversion

After 5 SD + 3 LD, when a whorl of about seven modified leaf and leaf-petal mosaic primordia had been formed in the pseudo-flower, *Imp-FIM* transcription was similar to that in the flowering meristem at the same stage (i.e. after 8 SD, Figure 6a). After 5 SD + 9 LD, when all the pseudo-flower had been formed and a first whorl of six or seven leaf primordia had been initiated above it, the pattern of *Imp-FIM* transcription was intermediate between the flowering and the vegetative patterns (Figure 6b). The *Imp-FIM* signal was found in the axils of primordia in the four youngest whorls and was also detected within primordia that would later develop as leaf-petal mosaics. *Imp-FIM* transcript was located along the adaxial side of these primordia but, in contrast to *Imp-FIM* transcription in petal primordia, the signal was limited to only a few cell layers or sometimes only to the epidermis and was occasionally restricted to patches at the adaxial side of the primordia. At a later stage of development of the pseudo-flower, after 5 SD + 12 LD, *Imp-FIM* transcription could still be observed in growing primordia of leaf-petal mosaics (Figure 6c).

Imp-FIM transcription during the leaf-whorl stage of R4 reversion

After 5 SD + 15 LD, when approximately two whorls of normal leaf primordia had been initiated, the expression of *Imp-FIM* had returned mostly to its vegetative pattern (Figure 6d and e). *Imp-FIM* transcript was mainly located in the axils of primordia, the signal extending deeper inside the meristem than in the flowering meristem (Figure 6e).

However, one apex still had a few patches of *Imp-FIM* transcription along the adaxial side of some of the primordia (Figure 6d). After an additional period of 5–15 LD (i.e. 5 SD + 20 LD to 30 LD), *Imp-FIM* transcript was no longer in the primordia but was restricted to their axils in all cases (Figure 6f and g).

Imp-FIM transcription during R6/R7 reversion after petal initiation

After 10 SD + 10 LD, during the initiation of petal-leaf mosaic primordia, the *Imp-FIM* transcription pattern was essentially the same as that during the petal initiation stage of normal flower development, except that expression in the primordia of the two youngest whorls was more restricted (Figure 6h). Dissection of mature plants showed that a few plants had already resumed leaf initiation by this stage and that, on average, the first true leaf primordium was initiated soon after, suggesting that the reduced *Imp-FIM* transcription in these primordia correlated with a reduction of their petal features and an increase in leaf characteristics.

Imp-FIM transcription in a non-reverting line

The data described above show that a specific *Imp-FIM* pattern of transcription, distinct from the one observed in *Antirrhinum* and *Arabidopsis* (i.e. at the junction between primordia) (Ingram *et al.*, 1995; Simon *et al.*, 1994), is established during petal production in *Impatiens*. To test the possibility that this transcription pattern could be associated with the lack of floral commitment, *Imp-FIM* expression was analysed in the terminal flower of a non-reverting line of *Impatiens*.

The non-reverting line used did not revert, even after inductive treatments as short as 2 SD (Tooke *et al.*, submitted for publication). Its vegetative growth and flower development were more rapid than in the reverting line: floral stages after 2 SD, 5 SD, and 8 SD in the non-reverting line corresponded approximately to floral stages after 5 SD, 8 SD, and 10 SD in the reverting line. However, the terminal flower was essentially similar to that of the reverting line, with diffuse boundaries between organ types.

In situ hybridisation on vegetative and flowering apices of the non-reverting line at different stages showed that *Imp-FIM* was transcribed in the same way as in the reverting line (Figure 7). *Imp-FIM* was transcribed in the axils of leaves during vegetative growth and shortly after the beginning of the inductive period (Figure 7a and b). The intensity of the signal increased at the beginning of the petal production period (after 5 SD; Figure 7c) and there were fusions between signals in the two youngest whorls. Finally, during the petal production period *Imp-FIM* transcript accumulated a little further down in the axils and

was mostly located in the adaxial halves of the young petal primordia (after 8 SD; Figure 7d).

Discussion

Analysis of the domains of floral organ identity in relation to *Imp-FIM* transcription shows that petal identity correlates with a shift in the distribution of the *Imp-FIM* transcript in the terminal meristem. In the vegetative terminal meristem, *Imp-FIM* transcripts accumulate in the axils of the leaf primordia whilst during flowering they accumulate mostly within the primordia that would give rise to petaloid organs or petals.

Transcription of *Imp-FIM* in *Impatiens* shows some similarities and differences compared to *FIM* and *UFO* transcription in *Antirrhinum* and *Arabidopsis*. Like *Imp-FIM*, *UFO* is transcribed in the shoot apical meristem during vegetative growth (Lee *et al.*, 1997). The *Imp-FIM* transcription pattern in the shoot apical meristem of *Impatiens* is very similar to the *FIM* and *UFO* patterns observed during flower development in *Antirrhinum* and *Arabidopsis* (i.e. at the junctions between primordia) (Ingram *et al.*, 1995; Simon *et al.*, 1994). However, the pattern of *Imp-FIM* transcription that correlates with the production of petals in *Impatiens* is not observed in *Antirrhinum* and *Arabidopsis*. This suggests that *FIM* functions may be achieved through different means in the different species.

One function of *FIM* and *UFO* is the regulation of petal development (Ingram *et al.*, 1995; Lee *et al.*, 1997; Levin and Meyerowitz, 1995; Simon *et al.*, 1994). Another function of *FIM* and *UFO* in the establishment of a whorled phyllotaxis has been raised based on the observation of a disrupted whorled organisation or spiral phyllotaxis in *fim* and *ufo* mutant flowers (Ingram *et al.*, 1995; Levin and Meyerowitz, 1995). These two possible functions of *Imp-FIM* were tested in *Impatiens* by transferring plants induced in SD to non-inductive LD.

During the pseudo-flower stage of R4 reversion, the *Imp-FIM* pattern of transcription is intermediate between flowering and vegetative patterns. *Imp-FIM* RNA accumulates in the axils of primordia but is also found within sectors of primordia that will later develop as leaf-petal mosaics. During the leaf-whorl stage of R4 reversion, *Imp-FIM* transcription returns to the vegetative pattern. Absence of *Imp-FIM* transcript in the reverted leaf primordia shows that *Imp-FIM* transcription within primordia is strictly correlated with the specification of petal identity rather than primordium position. This result suggests that *Imp-FIM* is needed for the regulation of petal development. In contrast, the return to a vegetative pattern of transcription several weeks before the leaf arrangement returns to spiral indicates that the floral *Imp-FIM* transcription pattern is not specifically associated with whorled phyllotaxis and the suppression of axillary meristems.

Another suggested function of *FIM* and *UFO* is the setting of boundaries for the growth of cells and establishment or maintenance of boundaries between domains of organ identity (Levin and Meyerowitz, 1995; Simon *et al.*, 1994; Vincent *et al.*, 1995). *FIM* and *UFO* expression at the junctions between primordia could help to define boundary domains and to delimit expression of downstream organ identity genes. In *Impatiens*, floral organogenesis under continuous inductive SD is characterised by graded transitions in organ identity. The domains of organ identity lack sharply defined boundaries, and a variety of mosaic organs with mixed identities are produced. In particular, the domain of petal identity is enlarged and spans 3–5 whorls (about 20 organs in a total of 40 floral organs). It overlaps with bract and stamen identity domains and only about seven of the organs in this domain exhibit 100% petal tissue. The change in *Imp-FIM* transcription could contribute to the more diffuse boundaries between organ identity domains and between organs.

There are several possibilities that could explain the novel transcription pattern of *Imp-FIM* in the terminal flower of *Impatiens*. One is that it is associated with the lack of floral commitment in the reverting line. However, a similar pattern is observed in plants of a non-reverting line (Tooke *et al.*, submitted for publication; this work). This suggests that the state of floral commitment in *Impatiens* does not influence *Imp-FIM* transcription.

Another possibility is that this may reveal more ancestral conditions that are also typical of members of the *Magnoliaceae*, *Ranunculaceae* and *Nymphaeae* families. These families produce large flowers with numerous organs that are typically arranged in spirals, although perianth organs are often in whorls, and show some similarities with the terminal flower of *Impatiens*. Evolutionary divergence for *Imp-FIM* regulation can also be invoked to explain similarities in *Impatiens* to *fim* and *ufo* mutants of *Antirrhinum* and *Arabidopsis*. These similarities involve mosaic organs and fusions between organs in the same or different whorls, as well as evidence for altered B and C functions. Altered B function is suggested by a reduction of sepal features and an enlarged petal identity domain. Altered C function is shown by petal identity consistently overlapping the stamen identity domain and by the weak imposition of determinacy in meristems. This is further demonstrated by the variable number of petals and stamens in different flowers and resumption of meristematic activity from the top of the placental column (Pouteau *et al.*, 1995; unpublished data). It would be interesting to determine if the expression of B and C genes is altered in relation to the different *Imp-FIM* pattern of transcription in *Impatiens*. The evidence suggests that *Imp-FIM* is, most likely, the *FIM* and *UFO* orthologue. However, evolutionary divergence of *FIM* in *Impatiens* could have occurred

through gene duplication, and it would also be interesting to determine if other *FIM* relatives exist in the genome of *Impatiens*.

Finally, the differences in *Imp-FIM* transcription may be caused by alterations in some of its regulators. Evidence that altered expression of *LFY* can affect the spatial pattern of transcription of *UFO* exists in *Arabidopsis* (Lee *et al.*, 1997). In *Impatiens*, the homologue of *FLO*, *Imp-FLO* is constitutively transcribed in the different states of the meristem (Pouteau *et al.*, 1995, 1997). The absence of regulation of *Imp-FLO* transcription, in contrast to the transcription of its homologues in *Antirrhinum* and *Arabidopsis*, could be responsible for the modified regulation of *Imp-FIM* transcription. Another possibility is that the terminal flower phenotype of the Dwarf Bush Flowered cultivar of *Impatiens* is caused by a defect in the *centroradialis* (*CEN*)/terminal flower (*TFL*) function (Alvarez *et al.*, 1992; Bradley *et al.*, 1996). Terminal flowers of *cen* and *tfl* mutants of *Antirrhinum* and *Arabidopsis* exhibit variable organ numbers and arrangement, and frequently have mosaic organs as in *Impatiens* (Alvarez *et al.*, 1992; Bradley *et al.*, 1996). The *CEN/TFL* function might be necessary for the control of *Imp-FIM* and the formation of regular flowers with discrete whorls. One way to test this possibility is to analyse *Imp-FIM* expression in an indeterminate line of *Impatiens*.

Experimental procedures

Plant material

In previous work (Battey and Lyndon, 1984) mixed seeds of *I. balsamina* cv Dwarf Bush Flowered were used. Because the red-flowered plants gave the most uniform reversion response, in this work we used a uniform, determinate (producing a terminal flower), red-flowered line isolated from the original mixed seeds. This line was isolated from mixed seed obtained from W.K. McNair, Edinburgh, UK: plants showing an R4 reversion phenotype were induced to re-flower and seeds from determinate, red plants were collected. Second and third generations of seeds were obtained in the same way to provide the material used in this work. The flowering and reversion responses were analysed in the red-flowered line and compared to previous descriptions obtained with mixed seeds (Battey and Lyndon, 1984). Petal, stamen, and carpel initiation were slightly delayed by about 1 or 2 days. The transition from leaf identity to petal identity via bracts was essentially as described previously, except that anthocyanin pigmentation appeared slightly later, on the fifth or sixth primordium, which was initiated after approximately 5 SD. Reversion after 5 SD was as described with red-flowered plants grown from mixed seeds.

Batches of seeds were imbibed on moist filter paper at 23°C in LD (16 h illumination period) for 72 h. For each experiment, about 280 seeds with emerged radicles (approximately 4 mm long) were sown at a depth of 1 cm in moist F1 compost (Levington, UK) in individual pots (4 × 4 × 4.8 cm) placed in trays (36 × 23.5 × 6 cm). Plant growth after sowing was in LD of 24 h as described in Battey and Lyndon (1984), except that the total photon flux density during

the day (8 h) and night (16 h) was slightly less (260–280 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ and 5 $\mu\text{mol m}^{-2} \text{sec}^{-1}$, respectively, at the top of the plants on day 0, see below), warm white rather than white tubes were used as the fluorescent light source, and the temperature was also slightly lower (21 + 1°C). The compost was kept moist by application of 200 ml of tap water per tray every day.

Photoperiodic treatments

During the early stages of development in the LD growth conditions described above, there was a reliable relationship between the size of the first true leaf and the number of primordia initiated by the shoot apical meristem. This relationship was used to select the young seedlings for developmental uniformity on day 0 (7–8 days after sowing). Plants with nine primordia on average were selected, by discarding all plants in which the first true leaf was not 7–11 mm. Vegetative growth in LD, flowering in SD, and flower reversion in LD after 5 SD or 10 SD were performed as described previously (Battey and Lyndon, 1984). SD conditions consisted of an 8 h period of illumination identical to that applied in LD, but complete darkness was maintained during the 16 h long night. This experimental procedure allowed the application of a similar overall quantity of light in both non-inductive LD and inductive SD, so that there would be little modification of photosynthesis due to photoperiodic changes. The plants were potted on into fresh M2 compost (Levington, UK) in larger pots on days 14 or 15 and days 35 or 36. No plant grown in constant LD developed any floral features over at least 3 months.

Plants under different photoperiodic treatments were randomly sampled at different times for the preparation of hand sections of fresh tissues and wax embedding for *in situ* hybridisation assays. The number of nodes and primordia initiated by the shoot apical meristem was determined in 10 plants at each sampling time. An average of 10 plants were grown until maturity to record the characteristics of organ identity, axillary shoot identity, phyllotaxis and internode elongation at each node.

Hand sections of fresh tissue

Fresh organ tissue was prepared using polystyrene blocks as a support for sectioning with a Reichert sliding microtome. The sections were collected in distilled water, de-aerated under vacuum for approximately 30 min, and mounted in water on microscope slides.

Gene cloning

Two degenerate oligonucleotides (5'-TTCTCCA(A/C)CAC(A/C)TT-CCTCGA-3' and 5'-ACGCTAAA(A/C)GGGCT(A/G)TAGTTCAT-3') corresponding to two conserved domains in the coding sequence of *FIM* in *Antirrhinum* (position 331–350 and 883–905, respectively; Simon *et al.*, 1994) and *UFO* in *Arabidopsis* (Ingram *et al.*, 1995) were used to amplify homologous sequences (*Imp-FIM*) from *Impatiens* genomic DNA by PCR. An approximately 550 bp long fragment was amplified and its extremities were filled with T4 DNA polymerase for ligation into KS+ and SK+ Bluescript vectors cut with *EcoRV*. The sequences of two clones containing an insert in KS+ pBluescript, psep1–9 and psep2–4, and one clone containing an insert in SK+ pBluescript, psep3–1, were analysed by dideoxy methods using a Sequenase II kit according to the manufacturer's instructions (US Biochemicals). Universal primers in the Bluescript vector as well as internal specific oligonucleotides were used. The three sequences were identical.

In situ hybridisation

The methods for digoxigenin labelling of RNA probes, tissue preparation, and *in situ* hybridisation were as described in Bradley *et al.* (1993). psep1–9 cut with *HindIII* and psep3–1 cut with *EcoRI* were used as templates for T7 RNA polymerase to generate antisense and sense RNA probes, respectively.

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