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Development 124, 000-000 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV0127



# The induction and maintenance of flowering in *Impatiens*

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## SUMMARY

**The mechanisms that establish the floral meristem are now becoming clearer, but the way in which flowering is maintained is less well understood. *Impatiens balsamina* provides a unique opportunity to address this question because reversion to vegetative growth can be obtained in a predictable way by transferring plants from inductive to non-inductive conditions. Following increasing amounts of induction, reversion takes place at progressively later stages of flower development. Partial flower induction and defoliation experiments show that a floral signal is produced in the cotyledon in response to inductive conditions and that this signal progressively diminishes after transfer to non-inductive conditions, during reversion. Therefore reversion in *Impatiens* is most likely due to the failure of leaves to become permanent sources of inductive signal in addition to the lack of meristem commitment to flowering. Analysis of the expression of the *Impatiens* homologues of the meristem identity genes *floricaula* and *squamosa* indicates that a change in *floricaula* transcription is not associated with the establishment or maintenance of the floral meristem in this species. *Squamosa* transcription is associated with floral development and petal initiation, and is maintained in existing petal or petaloid primordia even after the meristem has reverted. However, it is not expressed in the reverted meristem, in which leaves are initiated in whorled phyllotaxis and without axillary meristems, both characteristics usually associated with the floral meristem. These observations show that *squamosa* expression is not needed for the maintenance of these floral characters. The requirement for the production of the floral signal in the leaf during the process of flower development may reflect an additional function separate to that of *squamosa* activation; alternatively the signal may be required to ensure continued transcriptional activation in the meristem.**

Key words: flower reversion, flower induction, flower commitment, meristem identity, *floricaula*, *squamosa*, *Impatiens*

## INTRODUCTION

Flowering is controlled by an interaction between the leaf, where a floral signal is generated in response to inductive conditions, and the meristem, where this signal evokes flower development (Vince-Prue, 1975; Bernier et al., 1981). In *Antirrhinum majus* (Scrophulariaceae) and *Arabidopsis thaliana* (Brassicaceae), the events in the meristem involve activation of the *floricaula* (*flo*)/*leafy* (*lfy*) and *squamosa* (*squa*)/*apetala1* (*ap1*) genes (Coen et al., 1990; Irish and Sussex, 1990; Schultz and Haughn, 1991; Huala and Sussex, 1992; Huijser et al., 1992; Mandel et al., 1992; Weigel et al., 1992). However, the events in the leaf and the role of the floral signal in flower development are less clear. Circumstantial evidence suggests that in many plants the signal acts as a switch, causing a stable change in the meristem (flower commitment) so that a constant supply of signal from the leaf is not required to maintain flowering once it has been initiated (Bernier et al., 1981; McDaniel, 1996). However, in some plants flower commitment does not occur: removal of plants from inductive conditions causes reversion to vegetative growth (Battey and Lyndon, 1990). A simple hypothesis to account for this is that in these plants floral signal needs to reach the meristem continuously during flower development. This type of action of the floral signal we paraphrase here as 'maintenance' activity.

*Impatiens balsamina* (Balsaminaceae) is a species that does not become committed to flower and is able to revert to leaf initiation (Krishnamoorthy and Nanda, 1968; Debraux and Simon, 1969). In the cv Dwarf Bush Flowered, three states of the terminal meristem, *vegetative*, *flowering* and *reverted*, can be obtained in a predictable way by manipulating the photoperiod in which plants are grown (Battey and Lyndon, 1984, 1990; Pouteau et al., 1995). Flowering in *Impatiens* is induced in short days (SD) and plants remain vegetative in long days (LD). The vegetative meristem is characterised by indeterminate growth and the production of leaves arranged in spirals with axillary meristems. The flowering meristem is determinate forming several whorls of floral organs lacking axillary meristems before it stops growth. Interruptions of the SD induction by transfer into LD result in flower reversion. The reverted meristem is characterised by the production of whorls of leaves lacking axillary meristems and separated by long internodes. It behaves differently from a vegetative meristem because it immediately resumes flower development without a

lag period when transferred back into inductive conditions (Battey and Lyndon, 1986, 1988).

We wanted to know whether the molecular control of flower development involved similar mechanisms in *Impatiens*, where the floral signal apparently exercises 'maintenance' control; and in *Antirrhinum*, where daylength transfer experiments suggest a 'switch' control, expression of *flo* and *squa* correlates with commitment to flower development, and flower reversion is not observed (Bradley et al., 1996b). Here we present evidence that strongly supports the idea that failure to maintain flowering in *Impatiens* (reversion) occurs because floral signal ceases to reach the meristem. We find that expression of the *Impatiens flo* homologue (*Imp-flo*) does not change during flowering or reversion, and that expression of the *squa* homologue (*Imp-squa*) is correlated with the initiation and development of petals.

## MATERIALS AND METHODS

### Plant material

A uniform, determinate (producing a terminal flower), red-flowered line isolated from the original mixed seeds of *I. balsamina* cv Dwarf Bush Flowered (Battey and Lyndon, 1984) was used. Seed imbibition, sowing, and plant growth were as previously described. Plant growth after sowing was in LD of 24 hours under a total photon flux density, as measured at the top of the plants on Day 0, of 260-280 μmol/$m^2$/second during the day (8 hours) and 3-5 μmol/$m^2$/second during the night (16 hours), and a temperature of 21±1°C.

### Photoperiodic treatments

The relationship previously found between the size of the first true leaf and the number of primordia initiated by the shoot apical meristem during early stages of development in the LD growth conditions (Pouteau et al., unpublished) was used to select the young seedlings with 9 primordia on average on Day 0 (7 to 8 days after sowing). Vegetative growth in LD, flowering in SD (referred to here as continuous SD), flower reversion in LD after 4 SD to 18 SD, and re-flowering in SD after 5 SD + 5 LD were performed as previously described (Battey and Lyndon, 1984, 1986, 1988). SD conditions consisted of an 8 hour period of illumination identical to that applied in LD but complete darkness was maintained during the 16-hour long night. No plant grown in constant LD developed any floral features over 3 months at least.

Plants under different photoperiodic treatments were randomly sampled at different times for the preparation of material for wax embedding and 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.

Leaf removals during reversion were performed after 5 SD and 8 SD. Some of the plants had their SD leaves removed on the day of transfer to LD (5 SD-SLR and 8 SD-SLR). Other plants had their young unfolded LD leaves removed for 15 days after transfer to LD (5 SD-LLR and 8 SD-LLR). Control plants had no leaves removed after transfer to LD (5 SD-C and 8 SD-C). All plants in this experiment were submitted to 14 hours 30 minutes complete darkness during the night between Day 17 and Day 18 due to a power failure. Vegetative controls grown under continuous LD did not show any flowering features in response to this long night and the 5 SD + LD and 8 SD + LD reversion controls were as in previous experiments. It was thus assumed that the long night did not interfere with the leaf removal treatments.

### Partial flowering experiments

Plants were grown under continuous LD and had one cotyledon covered with a light-proof bag during the 16-hour night, so that this cotyledon was maintained under SD for 40 days after Day 0 (Cov treatment). Some of these plants had the other cotyledon removed on Day 0 (Cov-CR treatment). Others had one cotyledon removed on Day 0 and young unfolded leaves were removed for 18 days after Day 0 (Cov-LR treatment). In Experiment 1, Cov-CR and Cov-LR plants were subjected to 9 hours 25 minutes complete darkness during the night between Day 29 and Day 30 due to a power failure of the growth cabinet. To confirm the validity of this experiment, a second experiment was run in which there was no power failure. Comparisons of the results obtained in both experiments showed that floral conversion in the Cov-CR and Cov-LR treatments was in fact more pronounced in Experiment in 2 than in Experiment 1. This suggests that the power failure of the growth cabinet in Experiment 1 did not have much influence on partial flowering. In both experiments, control plants submitted to the same treatments as in the Cov, Cov-CR, and the Cov-LR treatments (except that they did not have one cotyledon maintained under SD) remained vegetative.

### Gene cloning

Fragments homologous to *flo/lfy* and *squa/ap1* were amplified by RT-PCR using RNAs extracted from *Impatiens* apices on Day 12, i.e. during the phase of petal initiation in continuous SD. Four degenerate oligonucleotides (5′-CGGAATTCATGC/AGICAT/CTAT/CGTICATT/CGT/CTAT/CGC-3′, 5′-CCGAATTCACIAAC/TCAA/GGTITTC/TA/CGITA-3′, 5′-CGGGATCCGGT/CTTA/GTAA/GCAIGCT/CTGICT/GCCA-3′, and 5′-TTGGATCCIT/CT/GIGTIGGIACA/GTACCAA/TAT-3′) corresponding to conserved domains in the coding sequence of *flo* in *Antirrhinum* (position 901-1177; Coen et al., 1990) and *lfy* in *Arabidopsis* (position 938-1214; Weigel et al., 1992) were used to amplify homologous sequences from *Impatiens*. Two overlapping 169 and 142 bp long fragments were amplified and cut with *Eco*RI and *Bam*HI for ligation into KS+ and SK+ Bluescript vectors cut with the same enzymes. The sequences of one clone of each fragment containing an insert in KS+ pBluescript, pflo1 and pflo19, and an insert in SK+ pBluescript, pflo7 and pflo13, were analysed by dideoxy methods using a Sequenase II kit according to the manufacturer's instructions (US Biochemicals) and universal primers in the Bluescript vector.

One *Antirrhinum squa*-specific oligonucleotide (5′-ACTGATTCTTGCATGGACAGGA-3′, position 339; Huijser et al., 1992) and one degenerate oligonucleotide (5′-GCGAATTCT/CTTIAGIGCIGTA/GTCIAGT/CTGITGT/CTC-3′) corresponding to conserved domains in the coding sequence of *squa* in *Antirrhinum* (position 574; Huijser et al., 1992) and *ap1* in *Arabidopsis* (position 411; Mandel et al., 1992) were used to amplify an homologous sequence from *Impatiens* cDNA by RT-PCR after amplification with a tailed oligo-dT (5′-CGGATATCGAATTCTCGAGAAGCTT-$(T)_{16}$-3′) and a degenerate MADS-box oligonucleotide (5′-AATIG/CICAA/GGTIA/CITT/AT/CIG/CIAAA/GC/AG-3′). A 186 bp long fragment was amplified and its extremities were filled with T4 DNA polymerase for ligation into KS+ and SK+ Bluescript vectors cut with *Eco*RV. The sequences of two clones containing an insert in KS+ pBluescript, psquaK2 and psquaK3, were analysed as indicated above. The sequence obtained was used to design an *Impatiens*-specific oligonucleotide (5′-GGTCTAGAGCAGAGAAACAAGTCAATGC-3′) corresponding to the region downstream to the MADS-box (positions 389 and 226 in *Antirrhinum* and *Arabidopsis* cDNA sequences, respectively; Huijser et al., 1992; Mandel et al., 1992). This specific oligonucleotide and the tailed-oligo-dT were used to amplify a 687 bp long fragment by RT-PCR. This fragment was cut with *Xho*I and *Xba*I for ligation into SK+ pBluescript vector cut with the same enzymes and the sequence of one clone, psquaS1, was analysed.

### In situ hybridisation

The methods for digoxigenin labelling of RNA probes, tissue preparation, and in situ hybridisation were as described by Bradley et al. (1993). pflo1 cut with *Eco*RI and psquaS1 cut with *Xba*I were used as templates for T7 RNA polymerase to generate antisense *Imp-flo* and *Imp-squa* RNA probes, respectively. Sense *Imp-flo* and *Imp-squa* RNA probes were synthesised from pflo7 cut with *Bam*HI and psquaS1 cut with *Xho*I by using T7 and T3 RNA polymerases, respectively.

## RESULTS

### Analysis of reversion at different times during flower development

Photoperiodic control of the vegetative, flowering, and reverted states of the terminal meristem of *Impatiens* has been previously described (Battey and Lyndon, 1984; Pouteau et al., 1995). Plants remain vegetative in continuous LD and are induced to flower by transfer to SD (on Day 0, when 9 leaves and primordia on average have been initiated). The terminal flower that develops in continuous SD (from Day 0) is composed of bracts (sepaloid organs), petals with some leaf features but more than 50% anthocyanin pigmentation, true petals (petals with 100% petal shape and pigmentation), staminate petals, stamens, and carpels. Reversion of the terminal flower is obtained by transferring plants induced for a period of time in SD back to LD. To determine the progress of flowering in the terminal meristem, batches of plants induced for different periods of time from 4 SD to 18 SD were transferred back to LD. Reversion responses were recorded using a reversion scale from R0 (most extreme reversion) to R8 (least reversion), based on the extent of floral development occurring before return to leaf initiation (Battey and Lyndon, 1984; this work). The results obtained show that reversion occurs at progressively later stages following increased periods of SD induction (Table 1; Fig. 1).

### Analysis of the role of leaf signals during flowering and reversion

Flower reversion may occur when the level of floral signal is insufficient for the completion of flower development and the suppression of indeterminacy (Hempel, 1996). To test this possibility, partial flower induction experiments based on spatial restriction of the inductive treatment were compared to the reversion experiment (which involved temporal restriction of the inductive treatment) described above. Plants were maintained in non-inductive LD, but one cotyledon on each was given SD by being covered for 16h every day (**Cov**ered treatment). Some of the plants also had the second cotyledon removed (**Cov-C**otyledon **R**emoved treatment). In a third treatment, the second cotyledon was removed and young leaves starting to unfold were progressively removed for 18 days (**Cov-L**eaf **R**emoval treatment). Control plants maintained in LD and subjected to the same treatments but without giving the cotyledon SD did not show any sign of flowering and remained vegetative (Fig. 1F). The Cov, Cov-CR, and Cov-LR treatments resulted in a variety of partial flowering phenotypes which were very similar to reversion phenotypes, showing that a flower-promoting signal is formed in the cotyledon in SD. The partial flowering response was

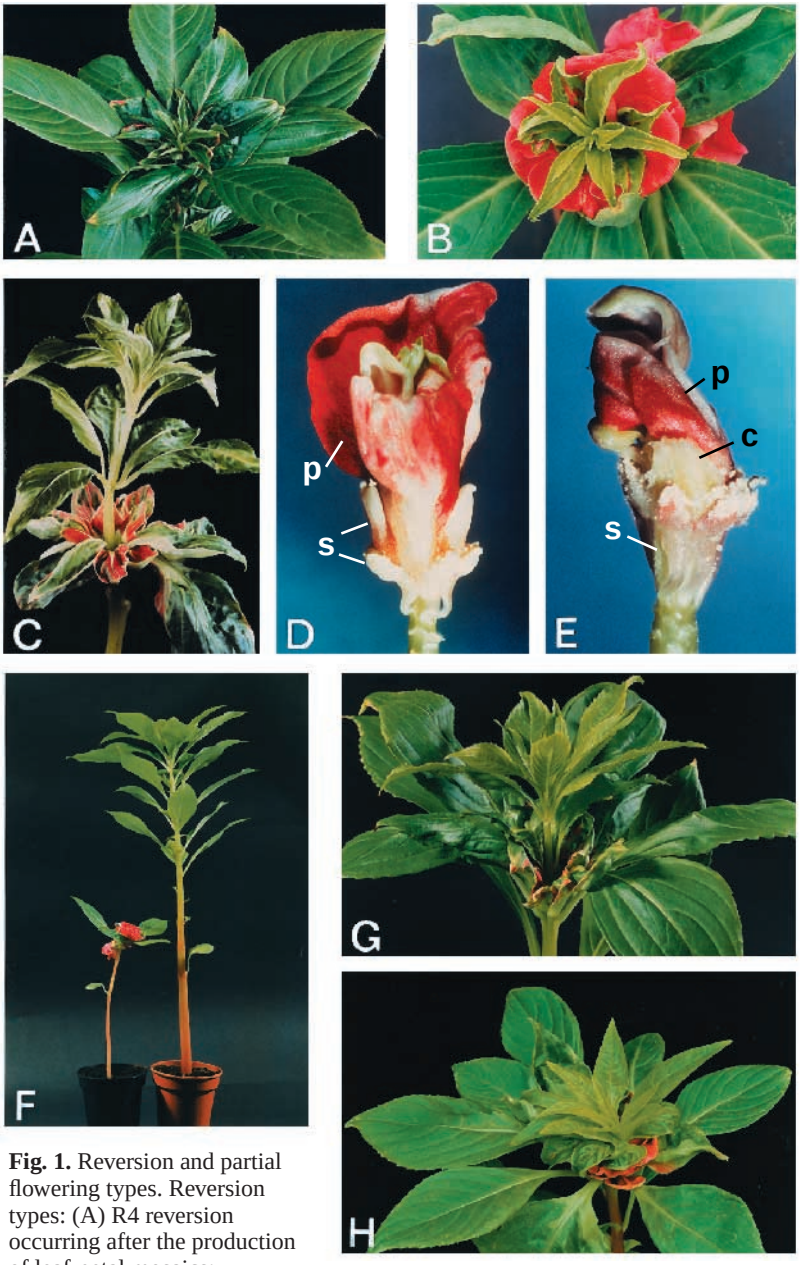


**Fig. 1.** Reversion and partial flowering types. Reversion types: (A) R4 reversion occurring after the production of leaf-petal mosaics; (B) Strong R5 reversion occurring after the production of true petals; (C) Weak R5 reversion occurring after the production of petals (but no true petals); (D) R7 reversion occurring after the production of stamens; (E) R8 reversion occurring after the production of carpels. Partial flowering types: (F) PF7 partial flowering obtained in the Cov-LR treatment (left) and vegetative plant obtained in the control treatment with no cotyledon covering during the night (right); (G) PF4 partial flowering obtained in the Cov-CR treatment; (H) PF7 partial flowering obtained in the Cov-LR treatment. p, petal; s, stamen; c, carpel.

**Table 1. Reversion at different times during flower development**

| Reversion type | | Number of inductive SD before transfer to LD | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 4 | 5 | 6 | 8 | 9 | 12 | 13 | 15 | 18 |
| R0 | Vegetative | 1 | - | - | - | - | - | - | - | - |
| R1 | Virescent axillary structures | 2 | - | - | - | - | - | - | - | - |
| R2 | No axillary structures | 2 | - | - | - | - | - | - | - | - |
| R3 | No internode | 15 | 5 | - | - | - | - | - | - | - |
| R4 | Modified venation, petal pigment | - | 13 | 7 | - | - | - | - | - | - |
| R5 | Petals | - | 2 | 13 | 10 | 9 | 2 | - | - | - |
| R6/R7 | Stamens | - | - | - | - | 1 | 10 | 10 | 1 | - |
| R8 | Carpels | - | - | - | - | - | 3 | 3 | 1 | - |
| F | Flowering after partial reversion | - | - | - | - | - | 2 | 1 | - | - |
| FLOWERING | | - | - | - | - | - | 3 | 5 | 18 | 18 |

For each treatment, data from 18-20 plants (10 plants in 8 SD and 9 SD treatments) from two independent experiments were recorded. Reversion types were classified on a scale from R0 to R8 according to the degree of flower development observed before return to leaf initiation. R3 reversion: plants reverted after the production of a cluster of leaves, some having modified shape and venation, with no axillary structures and not separated by internodes. R4 reversion: plants reverted after the production of petal-pigmented organs (leaf-petal mosaics) and no 'bracts' were produced (Fig. 1A). R5 reversion: plants reverted after the production of petals; in the 6 SD + LD treated plants, only 4 petals on average were produced and no 'true' petals were observed, i.e. all petals exhibited some degree of leaf features (weak R5 reversion; Fig. 1C); in the 8 SD + LD and 9 SD + LD treated plants a large number of petals (22.5 and 18.0 on average, respectively) including 'true' petals were produced (strong R5 reversion; Fig. 1B). R6 and R7 reversion: plants reverted after stamen production (Fig. 1D); during the transition from stamen to leaf identity only petal mosaics were produced in R6 reversion whilst petals in addition to petal mosaics developed in R7 reversion. R8 reversion: plants reverted after the formation of 1 or 2 carpels (Fig. 1E). Plants showing an F phenotype produced several petals after stamens but failed to return to leaf initation and, instead, resumed stamen and carpel initiation.

**Table 2. Partial flowering in LD by maintaining one single cotyledon in SD**

| Partial flower type | | Experiment 1 | | | Experiment 2 | | |
|---|---|---|---|---|---|---|---|
| | | Cov | Cov-CR | Cov-LR | Cov | Cov-CR | Cov-LR |
| PF0 | Vegetative | 10 | - | - | 1 | - | - |
| PF1 | Virescent axillary structures | 2 | 1 | - | 1 | - | - |
| PF3 | No internode | 1 | 3 | - | 3 | 3 | - |
| PF4 | Modified venation, petal pigment | - | 4 | - | 5 | 5 | - |
| PF5 | Petals | - | 5 | 1 | - | 2 | - |
| PF6/7 | Stamens | - | - | 6 | - | - | 2 |
| PF8 | Carpels | - | - | 2 | - | - | 1 |
| FLOWERING | | - | - | - | - | - | 5 |

Plants were grown in continuous LD and had one single cotyledon covered in a light-proof bag during a 16-hour night period for 40 days. They were divided into three groups: (1) plants that had no leaf removed (Cov treatment), (2) plants that had the second cotyledon removed (Cov-CR treatment), and (3) plants that had the second cotyledon removed and, for 18 days, also had their leaves removed when these started to unfold (Cov-LR treatment). For each treatment, control plants that had no cotyledon covered with a bag showed no sign of flowering and remained vegetative. Two independent experiments are presented (see Materials and Methods).

weakest in the Cov treatment, and strongest in the Cov-LR treatment where plants showed PF6/7 partial flowering (Fig. 1F, H) or full flowering (Table 2). The Cov-CR response was intermediate between the Cov-LR and the Cov responses (PF4/PF5 partial flowering; Fig. 1G). These data show that the second cotyledon as well as leaves developing in LD opposed the flower promoting effect of the covered cotyledon.

To establish the basis for this inhibition, leaf removal experiments were carried out on plants transferred to LD after 5 SD or 8 SD. In some of these plants, one cotyledon and the leaves that had expanded in SD were removed (**S**hort Day **L**eaf **R**emoval treatment). In others, the leaves unfolding in LD were progressively removed during a period of 15 days (**L**ong Day **L**eaf **R**emoval treatment). The overall reversion response was as described in the previous experiment (Table 1); results were similar in the SLR, LLR and control treatments (Table 3). However, in the SLR treatment the reversion began at a slightly lower node (Table 3), suggesting that normally SD leaves and cotyledons continue to supply the terminal meristem with the floral signal for a short time after transfer of plants to LD, so delaying the process of reversion. LD leaves caused very limited or no detectable inhibition, indicating that their negative effect in the cotyledon-covering experiment was most likely due to their dilution of a limited dose of floral signal from the induced cotyledon.

## PCR cloning of *flo* and *squa* homologue fragments in *Impatiens*

The results described above show that there is a leaf-based mechanism that underlies reversion events occurring in the meristem. It is important to understand how this affects the expression of genes that control flower development. *Flo/lfy* and *squa/ap1* are required for floral conversion in *Antirrhinum* and *Arabidopsis*, and to analyse the regulation of these two genes during reversion of the terminal meristem of *Impatiens*, PCR fragments were amplified from this species by RT-PCR using *flo/lfy*- and *squa/ap1*-specific oligonucleotides. The homologies with the corresponding *Antirrhinum* and *Arabidopsis* sequences (Figs 2 and 3) suggest that the fragments are derived from the *flo* and *squa* homologues in *Impatiens.* They were therefore called *Imp-flo* and *Imp-squa*. The larger fragments were used to analyse *Imp-flo* and *Imp-squa* tran-

**Table 3. Influence of leaf removal on reversion**

| | First node with | | | | |
|---|---|---|---|---|---|
| | no internode | a pigmented organ | a petal | an internode | a normal leaf |
| 5 SD - C | 12.1 | 18.4 | - | 27.0±3.0 | 28.7±3.0 |
| 5 SD - SLR | 11.9 | 16.5 | - | 21.7±2.7 | 25.4±4.8 |
| 5 SD - LLR | n.d. | 21.8 | - | 28.3±3.9 | 28.5±2.9 |
| 8 SD - C | 11.6 | 15.4 | 18.6 | 40.0±3.3 | 45.9±4.8 |
| 8 SD - SLR | 12.7 | 14.6 | 19.8 | 36.3±2.5 | 39.0±3.0 |
| 8 SD - LLR | n.d. | n.d. | 21.0 | 42.0±5.2 | 46.3±8.0 |

Plants were induced for 5 SD or 8 SD after Day 0 and transferred to LD. Young leaves that unfolded in SD (SD leaves) and one cotyledon were removed from one batch of plants (SLR treatment) after transfer. In total 3.8±0.4 and 5.0±0.0 SD leaves were removed in the 5 SD - SLR and the 8 SD - SLR treatments, respectively. Young leaves unfolding after transfer to LD (LD leaves) were removed for 15 days from another batch of plants (LLR treatment). In total 12.7±1.6 and 12.1±2.4 LD leaves were removed in the 5 SD - LLR and the 8 SD - LLR treatments, respectively. A control batch had no leaves removed (C treatment). Mean values ± standard deviations are given where appropriate.

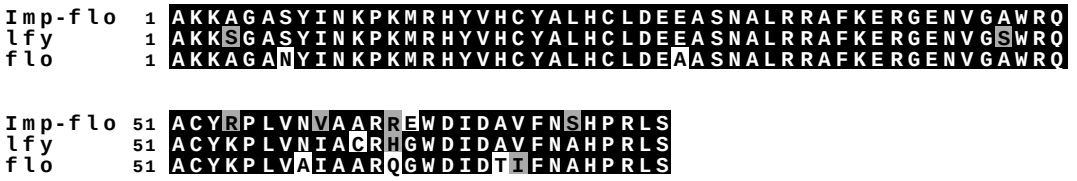


**Fig. 2.** Comparison of the *Impatiens flo* sequence with its homologues in *Antirrhinum* and *Arabidopsis*. Alignment of the partial *Impatiens flo* (*Imp-flo*) protein sequence with the corresponding *Antirrhinum flo* (*flo*) and *Arabidopsis lfy* protein sequences. Amino acids at positions of identity and similarity between two or three of these proteins are blocked in black and grey, respectively. The total *Imp-flo* sequence obtained corresponded to 235 bp of the *flo/lfy* coding sequence. The identity at the nucleotide level was 71% with *flo* and 72% with *lfy*. At the amino acid level, the identity was 87% and 90% (similarity 92% and 95%) with *flo* and *lfy* respectively. These homologies are similar to the level of homology between *flo* and *lfy* in the regions covered by the clones (Coen et al., 1990; Weigel et al., 1992).

scription 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-flo* and *Imp-squa* transcription under different photoperiodic treatments

#### *Imp-flo* transcription

*Imp-flo* transcription in *Impatiens* apices is shown in Fig. 4. *Imp-flo* was transcribed in the vegetative terminal meristem in LD (Fig. 4A). Its transcripts accumulated at the base of leaf primordia as well as within primordia but not in the central zone of the meristem. The signal was often stronger in the epidermal (L1) layer, and was also present in axillary meristems (Fig. 4A). After transfer to continuous SD, no change in the distribution pattern of *Imp-flo* transcripts was detected (Fig. 4B-D). *Imp-flo* was transcribed in all floral organs (bracts, petals, stamens, and carpels). However, during petal initiation (from 8 SD to 14 SD; Fig. 4B,C), the intensity of the signal was apparently higher than in the vegetative

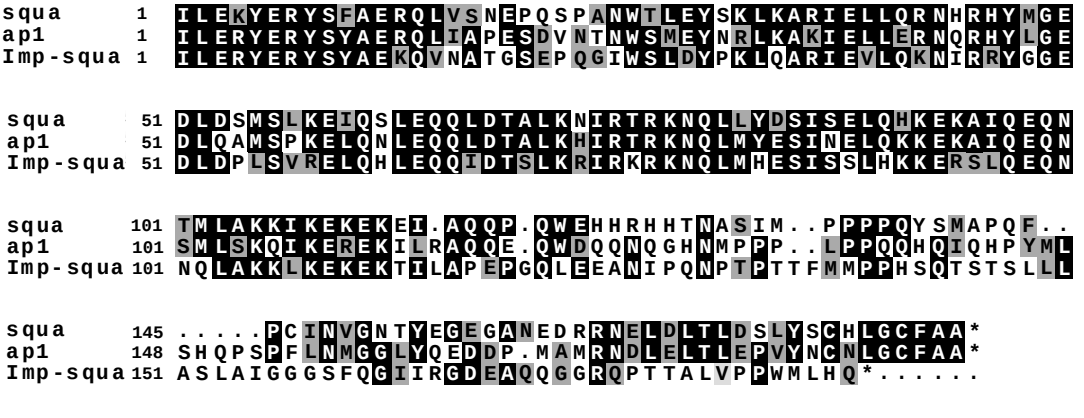


**Fig. 3.** Comparison of the *Impatiens squa* sequence with its homologues in *Antirrhinum* and *Arabidopsis*. Alignment of the partial *Impatiens squa* (*Imp-squa*) protein sequence with the corresponding *Antirrhinum squa* (*squa*) and *Arabidopsis ap1* protein sequences. Amino acids at positions of identity and similarity between two or three of these proteins are blocked in black and grey, respectively. The total *Imp-squa* sequence obtained (787 bp) corresponded to the entire coding sequence downstream of the *squa/ap1* MADS box. The identity at the nucleotide level was 58% with *squa* and with *ap1*. At the amino acid level, the identity was 45% and 43% (similarity 67% and 66%) with *squa* and *ap1* respectively. These homologies are a little less than the level of homology between *squa* and *ap1* in the regions covered by the clones (59% identity and 76% similarity at the amino acid level; Huijser et al., 1992; Mandel et al., 1992), but similar levels of homology are found between *squa/ap1* homologues from tomato and white campion and those from *Antirrhinum* and *Arabidopsis* (Pnueli et al., 1991; Hardenack et al., 1994).

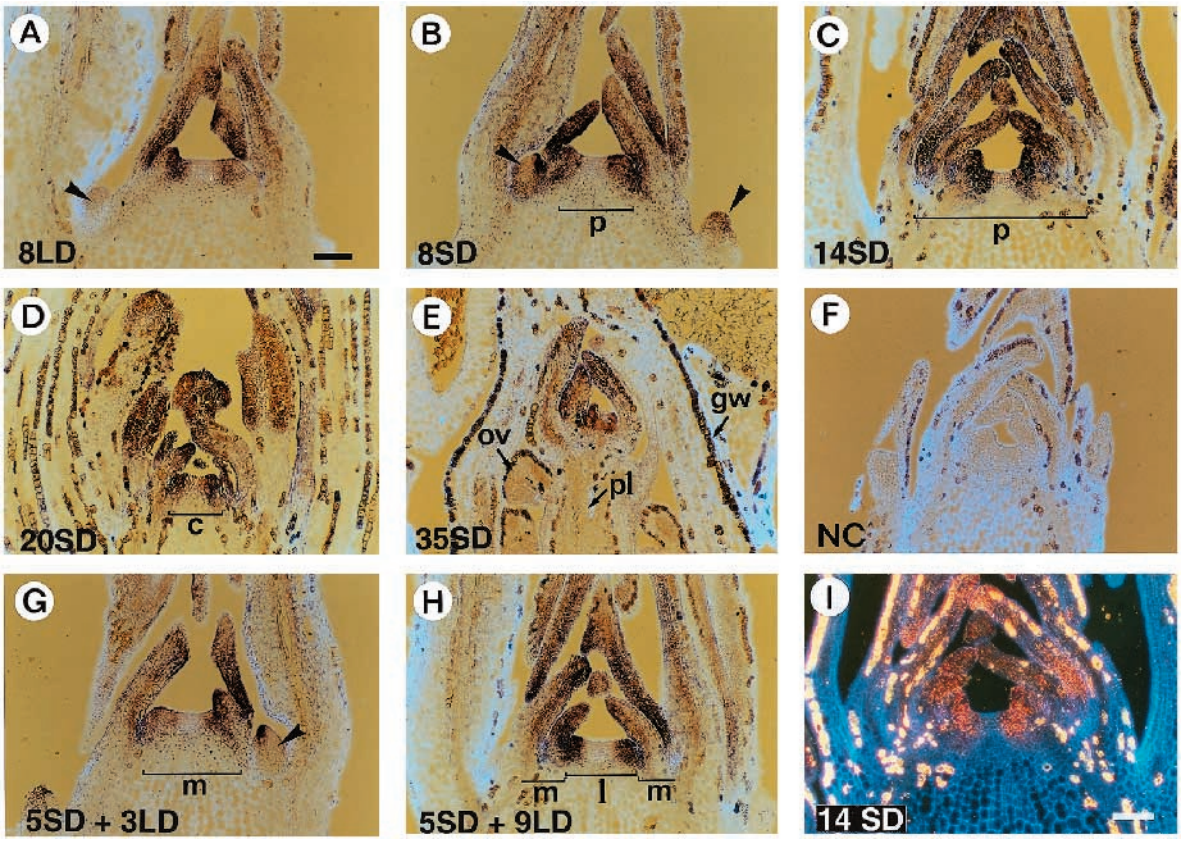


**Fig. 4.** In situ hybridisation analysis of *flo* transcription during flower development and reversion. (A) Vegetative (8 LD); (B- E,I) flowering (8 SD to 35 SD); (G-H) reverted (5 SD + 3 LD and 5 SD + 9 LD) apices are shown. Longitudinal sections were probed with digoxigenin-labelled *Imp-flo* antisense RNA and viewed under light-field optics (RNA signal is purple on a white tissue background). Dark-field control (I) emphasises the contrast between stained granules in the meristem (in white) and the *Imp-flo* signal (in orange). (F) The negative control (NC) was hybridised with a digoxigenin-labeled *Imp-flo* sense RNA. All photos under light-field optics were taken at the same magnification. Scale bars, 100 μm. The identity of primordia is indicated as follows: p, petals; c, carpels; l, leaves; m, leaf-petal mosaics; ov, ovule; pl, placental column; gw, gynoecium wall. Axillary meristems are arrowed.

meristem. This may reflect the decreased plastochron (0.33 day/primordium) observed during this phase compared to the vegetative plastochron (1.08 day/primordium): *Imp-flo* transcripts were more apparent because of the greater number of primordia visible in sections of meristems initiating petals than in vegetative meristems. During gynoecium development, meristematic activity from the top of the placental column (Pouteau et al., 1995) resulted in a reiteration of the flowering programme and the initiation of floral organ primordia in which *Imp-flo* was also found to be transcribed (Fig. 4E). During reversion after 5 SD, no difference in the pattern of transcription could be observed in plants transferred to LD (Fig. 4G,H).

### *Imp-squa* transcription

*Imp-squa* transcription in *Impatiens* apices is shown in Fig. 5. No *Imp-squa* transcripts were detected in the vegetative apex in continuous LD (Fig. 5A). *Imp-squa* transcripts remained essentially undetectable for the first days after transfer to inductive SD. After 8 SD, corresponding to the initiation of the fourth petal primordium on average, *Imp-squa* was strongly expressed in the youngest petal primordia; expression can also be seen in the uppermost lateral flower primordia (Fig. 5B). Transcripts accumulated in the abaxial halves of the petal primordia and the signal also extended to the bases of the primordia. Vascular bundles connecting the newly formed primordia and lateral flowers also showed low levels of *Imp-squa* transcription. *Imp-squa* expression remained at a high level with the same pattern during the petal initiation phase. After 14 SD, *Imp-squa* RNA was mostly distributed in the abaxial halves of developing petals, but no transcription was detected in primordia of staminate petals and stamen primordia in the youngest whorl (Fig. 5C). After 20 SD, when carpel primordia were initiated, no transcription could be detected in the meristem or young primordia (not shown). Therefore, *Imp-squa* transcription was associated with the initiation of petal primordia but not the initiation of reproductive organ primordia.

Three days after transfer to LD (5 SD + 3 LD) only faint *Imp-squa* transcription could be detected in primordia that would later develop as leaf-petal mosaics (Fig. 5D). After 5 SD + 15 LD, *Imp-squa* transcript was absent from the reverted meristem and leaf primordia initiated in whorls without axillary meristems (Fig. 5E). In re-flowering experiments, plants were given a second inductive treatment in SD after 5LD. After 5 SD + 5 LD + 4 SD, the same pattern of

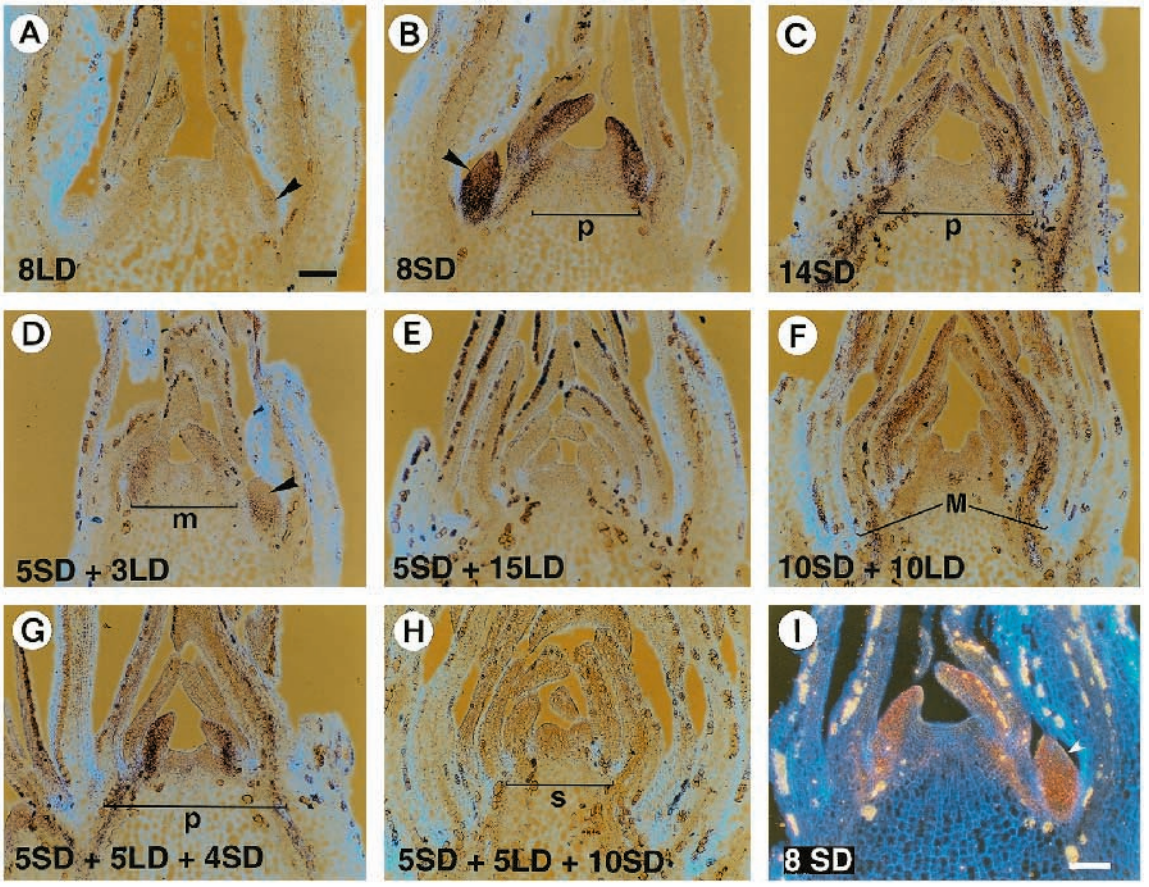


**Fig. 5.** In situ hybridisation analysis of *squa* transcription during flower development and reversion. (A) Vegetative (8 LD); (B-C,I) flowering (8 SD to 14 SD); (D-F) reverted (5 SD + 3 LD, 5 SD + 15 LD, and 10 SD + 10 LD); (G-H) re-flowering (5 SD + 5 LD + 4 SD and 5 SD + 5 LD + 10 SD) apices are shown. Longitudinal sections were probed with digoxigenin-labelled *Imp-squa* antisense RNA and viewed as in Fig. 4. Scale bars, 100 μm. The identity of primordia is indicated as follows: p, petals; s, stamens; m, leaf-petal mosaics; M, petal-leaf mosaics. Leaf-petal mosaics were formed during R4 reversion (no true petals produced). Petal-leaf mosaics were formed after petals during R5 reversion. Axillary meristems are arrowed.

*Imp-squa* transcription as after 8 SD was observed, with pronounced expression in the abaxial halves of petal primordia (Fig. 5G). After 5 SD + 5 LD + 10 SD, by which time stamen initiation had begun, *Imp-squa* transcripts were not detected in primordia of staminate petals or in stamen primordia (Fig. 5H).

The reduced *Imp-squa* transcription after 5 SD + 3 LD could be due to the removal of the floral signal in LD. It could also reflect the absence of normal petals in R4 plants. To distinguish between these possibilities, plants induced by 10 SD and initiating petals were transferred to LD. This resulted in the production of many petals followed by a range of petal-leaf mosaics before leaf initiation resumed (R5 reversion). On the day of transfer to LD, *Imp-squa* was transcribed at a high level in petal primordia. Ten days after transfer to LD, the plants had nearly returned to leaf initiation and in primordia of petal-leaf mosaics in the two or three youngest whorls, *Imp-squa* transcription was faint or undetectable (Fig. 5F); however, developing petal primordia in the three older whorls showed the same pattern of *Imp-squa* transcription as petal primordia developing in continuous SD. Therefore, accumulation of *Imp-squa* transcripts was not prevented in primordia initiated after transfer to LD where it was strongly associated with petal identity.

## DISCUSSION

*Imp-flo* is constitutively transcribed in the vegetative, flowering, and reverted states of the terminal meristem of *Impatiens*. This is very similar to expression of the *flo* homologue reported in tobacco (Kelly et al., 1995) but contrasts with the situation in *Antirrhinum*, *Arabidopsis*, and cauliflower, where the *flo* homologues are not expressed in vegetative, nor, in the case of cauliflower, in reverted meristems (Coen et al., 1990; Weigel et al., 1992; Anthony et al., 1996). The *Imp-flo* transcript is excluded from the central zone of the meristem but accumulates at the base of young primordia in a region encircling the central zone that might correspond to the ring of transcription observed in transverse sections of tobacco vegetative meristems. This continuous pattern of transcription associated with primordium initiation suggests that *Imp-flo* may have a function in partitioning primordia from the peripheral zone (anneau initial) of the meristem in *Impatiens*.

The constitutive expression of *Imp-flo* may be because, like the *centroradialis/terminal flower* (*cen/tfl*) mutants in *Antirrhinum* and *Arabidopsis* (Alvarez et al., 1992; Bradley et al., 1996a, 1997), *I. balsamina* cv. Dwarf Bush Flowered and tobacco are characterised by the formation of a terminal flower.

It is thus possible that the *cen/tfl* function is altered in *Impatiens* and tobacco. Analysis of *Imp-flo* expression in indeterminate lines of *Impatiens* similar to the 'Rose' cultivar used in initial reversion experiments (Krishnamoorthy and Nanda, 1968) should indicate if one function of *cen/tfl* is to exert a negative regulation on *Imp-flo* expression in the apex.

As observed in tobacco, transcription of *Imp-flo* in the meristem is not sufficient for its conversion into a floral meristem since plants grown under continuous LD remain vegetative. This contrasts with the finding that constitutive *lfy* expression is sufficient to accelerate floral initiation in transgenic *Arabidopsis* and aspen (Weigel and Nilsson, 1995). A possible explanation for this discrepancy is that in the latter cases *lfy* was overexpressed in the whole plant. It may also be that the lack of increase in *Imp-flo* expression on flowering leads to a failure of meristem commitment in *Impatiens*: recent evidence suggests that threshold levels of *flo* are associated with flower commitment in *Antirrhinum* (Bradley et al., 1996b). Finally, it is possible that the transcription pattern of *Imp-flo* does not reflect its protein activity because important post-transcriptional regulations may be involved.

In contrast to *Imp-flo*, *Imp-squa* is not transcribed in the apical meristem of *Impatiens* during vegetative growth and is activated after about 1 week of flower induction. This is similar to *squa* and *ap1* expression in *Antirrhinum* and *Arabidopsis* (Huijser et al., 1992; Mandel et al., 1992). *Imp-squa* transcript accumulates in petal primordia initiated in the terminal flower during flower development, reversion, and re-flowering. This suggests that *Imp-squa* is involved in the specification of petal identity in *Impatiens*, as are *squa/ap1* in *Antirrhinum* and *Arabidopsis* (Huijser et al., 1992; Mandel et al., 1992). Petal identity was also found to correlate with transcription of the *fimbriata (Imp-fim)* gene within primordia during flower development and reversion (Pouteau et al., unpublished). It is thus likely that *Imp-fim* acts together with *Imp-squa* to affect petal identity.

A possible explanation for the differences in expression pattern of *Imp-flo* and *Imp-squa* compared to their homologues in other species, is that other copies of these genes exist in *Impatiens*. However, only one copy of *flo* per haploid genome, or per homeologous genome in tobacco, has been reported (Coen et al., 1990; Weigel et al., 1992; Kelly et al., 1995). In white campion two homologues of *squa* have been found (Hardenack et al., 1994), but because their transcription seems to be essentially the same, and in all other species only one copy per haploid genome has been reported (Pneuli et al., 1991; Huijser et al., 1992; Mandel et al., 1992), the results for *Imp-squa* are likely to represent expression of the *squamosa* homologue in *Impatiens*.

By maintaining one single cotyledon in SD, partial flowers that were phenotypically very similar to pseudo-flowers formed during reversion were produced. This suggests that, as in other species tested so far (Evans, 1969; Zeevaart, 1976; O'Neill, 1992), a floral promoting signal is produced in cotyledons of *Impatiens* in response to inductive photoperiods. Persistence of the induced state in leaves that have been exposed to inductive photoperiod has been clearly documented in *Perilla*, and in *Xanthium* indirect induction has the effect of keeping the plant induced even in non-inductive conditions (Vince-Prue, 1975; Zeevart, 1976; Bernier et al., 1981). To determine if the induced state is lost in *Impatiens* leaves during reversion, SD induced leaves were removed at the time of transfer of the plants to LD. SD leaf removal resulted in a slightly earlier return to leaf production, compared to controls, indicating that a floral signal from leaves can continue to stimulate flowering at the terminal meristem for a short time after transfer to LD and that it then rapidly reduces (or the meristem becomes insensitive to it). This suggests that reversion results from insufficient floral signal and thus is virtually identical to the partial flowering that arises when a single cotyledon is given inductive SD. The possibility of inhibitory mechanisms acting via non-induced LD leaves was tested by removing LD leaves in these experiments, and in reversion experiments. Partial flowering is strongly enhanced by removing LD cotyledons and leaves, but LD leaf removal has no detectable effect on reversion responses. This suggests that the inhibitory effect of LD leaves during partial flowering is most likely to be caused by dilution of the floral signal. During reversion, a dilution effect would be expected to have little influence because in this situation the floral signal declines rapidly.

The expression pattern of *Imp-flo* suggests that a change in its transcription is not associated with the establishment of floral identity, or with reversion, in the meristem of *Impatiens*. *Imp-squa* seems to play a role in petal identity, but its transcription is not needed for the persistence of other floral characteristics (whorled phyllotaxis, absence of axillary meristems) that are uncoupled from floral organ identity during reversion. The striking absence of expression of both *Imp-flo* and *Imp-squa* in the central zone of the meristem may be associated with the lack of commitment to flower, or it may be a feature of the terminal flower in *Impatiens*. The expression of *Imp-flo* and *Imp-squa* that does occur is apparently not sufficient to confer floral identity in the absence of the continued supply of floral signal by the leaf.

We are grateful to people in E. S. C. and N. H. B.'s labs for their support and encouragement. Particular thanks to Coral Vincent for the dark-field photographs in Figs 4 and 5. Thank you to Liz Wheeler (Zoology, Reading) for her help with microtome sectioning. We are grateful to BBSRC Stem Cell Molecular Biology Initiative for funding this work (grant number AT45/559), D. N. and F. T.; and to INRA, Versailles for supporting S. P.

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(Accepted 25 June 1997)