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Non-reversion of *Impatiens* in the absence of meristem commitment

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Abstract

Purple-flowered plants of *Impatiens balsamina* maintained floral development on transfer from inductive short days (SD) to long days (LD), a treatment in which red-flowered plants of *Impatiens* are known to revert to leaf production. An investigation into the non-reverting nature of purple-flowered plants was carried out to establish whether these plants achieved meristem commitment or whether their non-reverting state was controlled by the leaves. When the leaves that had unfolded during the inductive SD treatment were removed at the time of transfer to LD, the purple-flowered plants did revert. This result suggests that, as in red-flowered *Impatiens*, meristem commitment is absent, but that purple-flowered plants maintain flowering in LD conditions because of a more permanent supply of signal from their leaves than occurs in red-flowered plants. A working hypothesis is proposed to explain how a signal from the leaves can retain a controlling role during flower development.

Key words: Floral commitment, *Impatiens*, floral reversion, *floricaula*.

Introduction

Commitment (or determination) of the meristem to a floral state appears, in many plants, to be a prerequisite for floral development. At this point the meristem becomes autonomous for flowering and the signals produced by the leaves are no longer significant to development (McDaniel, 1992). In red-flowered plants of *Impatiens balsamina*, a point of commitment does not exist and development of the terminal flower proceeds as dictated by the inductive status of the leaves, which is controlled by their external environment (Battey and Lyndon, 1986, 1990; Pouteau *et al*., 1995, 1997). Thus, plants transferred from inductive short days (SD) to non-inductive long days (LD) revert to leaf production (Krishnamoorthy and Nanda, 1968; Battey and Lyndon, 1984). Such an ability, alongside the potential which exists for normal flowering when the plant is maintained in continuous SD, implies that meristem commitment is not required for flowering (Battey and Lyndon, 1990; Lyndon, 1990), but that a continuous supply of floral stimulus is sufficient to activate the succession of floral development steps. Recent experiments demonstrate that a supply of a leaf-derived signal is needed to maintain flowering in the *Impatiens* meristem (Pouteau *et al*., 1997).

Meristem identity and organ identity genes are two sets of genes involved in the flowering process (Ma, 1994). Members of the former group function in the transition from vegetative to reproductive growth whilst organ identity genes are thought to control flower development in the manner outlined in the ABC model (Coen and Meyerowitz, 1991). The transcription of the floral meristem identity gene, *floricaula* (*flo*) of *Antirrhinum majus* has been linked with the point of commitment to flower (Bradley *et al*., 1996) and overexpression of its homologue, *leafy*, in *Arabidopsis thaliana* has been shown to be sufficient to initiate flowering (Weigel and Nilsson, 1995). In *Impatiens*, however, the transcription of the *Impatiens flo* homologue, *Imp-flo* was strikingly different, with the *flo* transcript found in the vegetative as well as the floral and reverting meristems (Pouteau *et al*., 1997). This pattern of vegetative transcription has also been observed in *Nicotiana* and pea (Kelly *et al*., 1995; Hofer *et al*., 1997) and suggests that transcription of *flo* is

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insufficient to cause flowering or act as a marker for commitment in these species.

In this paper, the floral development of a purple-flowered line of *Impatiens* is described. In common with red-flowered plants, purple-flowered plants were found to lack meristem commitment but, in contrast to red-flowered plants, were able to maintain their floral state on transfer to LD. Plants were thus non-reverting but uncommitted, with non-reversion being reliant on the presence of leaves that remain induced on transfer of plants to LD. The results for purple-flowered *Impatiens* support the idea that a leaf-derived signal can directly control the developmental phases of the floral meristem, possibly through direct interaction with organ identity genes.

Materials and methods

Plant growth

Seed was collected from purple-flowered plants originally derived from the same batch of mixed flower colour seeds of *Impatiens balsamina* cv. Dwarf Bush Flowered as the red-flowered plants used in previous experiments (obtained from William K McNair, Portobello, Edinburgh, UK). After a period of at least 4 weeks cold storage, seeds were imbibed on moist filter paper in Petri dishes for 72 h in 16 h photoperiods at 23 °C. Seeds with a radicle length of 3–4 mm after this time were selected and sown at a depth of 1 cm in F1 compost (Levington) with 35 seeds per tray. After sowing, plants were placed in growth cabinets under controlled light regimes. The temperature was maintained at 21 °C. Each tray received 200 cm^3 of tap water and was rotated (both the tray itself and around the cabinet) daily.

Plants were initially grown in LD, given by 8 h of light provided by tungsten bulbs and fluorescent tubes at 263–280 $\mu mol\ m^{-2}\ s^{-1}$ followed by 16 h of light from tungsten bulbs only, at 3–4 $\mu mol\ m^{-2}\ s^{-1}$ measured at the top of the plants on day 0. After around 8–10 LD, when a sufficient number of plants had a first true leaf between 7 and 11 mm in length, as uniform a population as possible was selected and used for experimentation. The plants had initiated 8.9 ± 0.79 and 9.8 ± 0.98 leaves and primordia for red and purple-flowered plants, respectively. This day was designated day 0. SD treatments began on this day; 8 h of tungsten and fluorescent light as above followed by 16 h of darkness.

On day 0, and at all transfer and fixation times, at least five plants were selected at random and dissected to determine the number of leaves and primordia present. On day 14 and day 35, plants were potted into 9 cm and 12.5 cm pots, respectively, using M2 compost (Levington). In one purple-flowered experiment of those used to calculate the combined data presented in Table 2, plants experienced darkness during the day on day 17 for less than 3.5 h, due to a power failure.

Leaf removal

Leaf removal experiments were carried out to establish the basis for non-reversion of purple-flowered *Impatiens* (see Results for details). Treatments involved in this experiment were:

(i) 5 SD + LD: no leaf removal.

(ii) 5 SD-SLR (SD Leaves Removed): After 5 SD, one cotyledon and any leaves which had unfolded during the 5 SD treatment were removed. The plants were then transferred to LD and no more leaves were removed.

(iii) 5 SD-LLR (LD Leaves Removed): Leaves unfolded during 5 SD were left on the plants. Plants were transferred to LD and any leaves unfolding during the LD component of the treatment were removed on a daily basis for 15 d.

(iv) 5 SD + SD-LR (5SD followed by more SD and Leaf Removal): Leaves unfolded during the first 5 SD were left on the plant. Plants were kept in SD and any leaves unfolding after the first 5 SD were removed on a daily basis for 15 d.

These treatments are summarized in Fig. 1. The number of leaves removed is given in Table 1. 5 SD + LD, 5 SD-LLR and 5 SD-SLR mature plants were dissected on days 55–56. SD and 5 SD + SD-LR plants were dissected when the terminal flower was fully opened (from day 40 onwards).

Results

Flowering in red-flowered and purple-flowered plants

In our original work on *Impatiens balsamina*, the process of flowering and reversion was described in a population of plants from mixed seed that included a range of flower colours (Battey and Lyndon, 1984). Red-flowered plants showed the most uniform reversion whilst purple-flowered plants did not revert on transfer to LD (Battey, 1985; Battey and Lyndon, 1986). Subsequent work has focused on reversion in the red-flowered genotype (Battey and Lyndon, 1986, 1988; Pouteau *et al.*, 1995, 1997). The aim of this work was to study non-reversion in purple-flowered plants in order to illuminate further the physiological basis for reversion in *Impatiens*.

The rate of primordium initiation was slightly faster in purple-flowered than in red-flowered plants. The number of leaves and primordia present on days 0, 5, and 8 in purple-flowered plants corresponded approximately to the number present on days 2, 7 and 10 in red-flowered plants. The process of flowering at the terminal meristem of plants of the purple-flowered line proceeded along closely similar lines to that in red-flowered plants (Table 2). Purple-flowered plants exposed to continuous SD from day 0 produced a fully-opened terminal flower after approximately 42 d (Fig. 2C), whilst red flowers opened after around 50 d. The terminal flower consisted of bracts (defined as <5 cm long with modified venation, loss of leaf serrations, $<50\%$ petal pigmentation and no petiole—see Battey and Lyndon, 1984), petals, petals with some staminate characteristics, stamens and, in most cases, carpels. The number of each organ type was variable. However, purple-flowered plants produced approximately twice as many stamens as red-flowered plants. In some plants, petal and stamen development was reiterated and stamen initiation was sometimes still in progress at the time of dissection (Fig. 3A). In contrast to red-flowered plants which remain completely vegetative

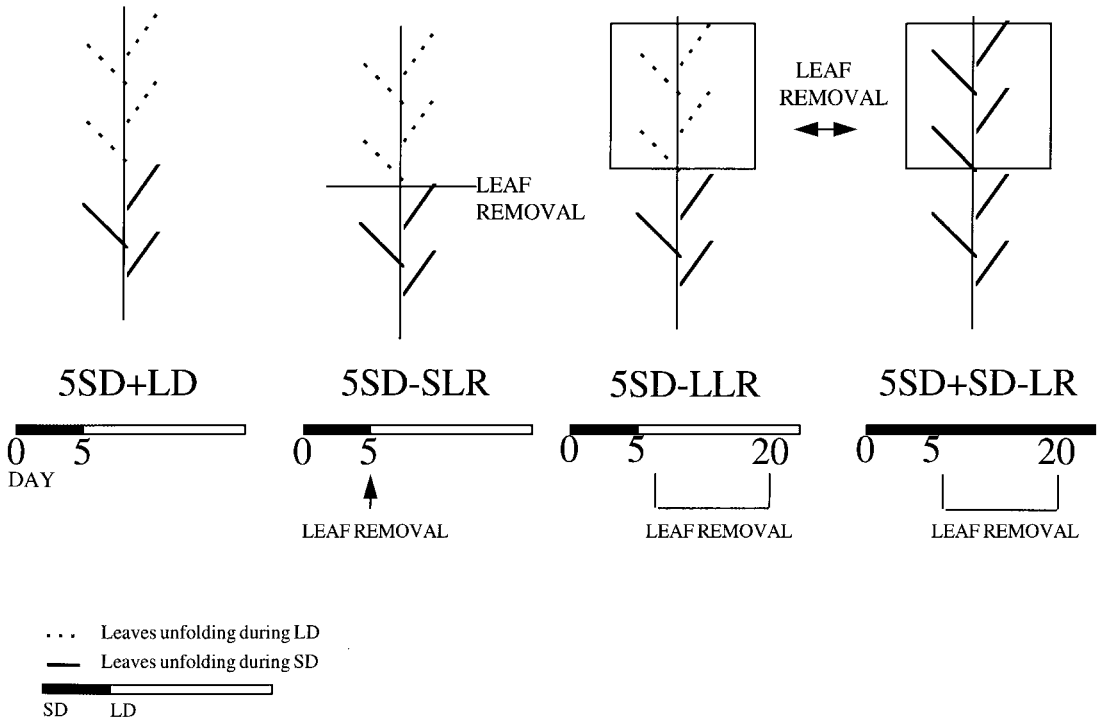

Fig. 1. Diagram of experimental treatments carried out in the leaf removal experiment. Treatments are described in detail in the Materials and methods and the Results sections of the text. The diagrams indicate which leaves were removed; for the numbers of leaves removed refer to Table 1.

Table 1. *Number and type of leaves removed during leaf removal treatments*

Treatment	Cotyledon	Unfolded in 5 SD	Unfolded after 5 SD	*n*
5 SD-LLR	–	–	14.7±2.2[a] (LD)	10
5 SD-SLR	1	4.0±0.0	–	9
5 SD+SD-LR	–	–	11.5±0.5[a] (SD)	6

[a]Total removed in these treatments may have included some of the outer bracts.

Table 2. *Development of red and purple-flowered plants grown under continuous SD*

The starting node of various phases of floral development at the terminal meristem is indicated. On commencing flowering the meristem stops initiating axillary structures and internodes are no longer elongated. Combined data from four experiments. $n=31$ and $n=24$ for purple and red-flowered plants, respectively, unless otherwise stated. n=Number of plants.

	Node of first occurrence of		Bract	>50% Petal pigment	Stamen	Carpel
	No axillary structure	Internode loss				
Purple	11.6±1.5	10.2±1.3 $n=30$	13.5±1.5	16.6±1.7	40.5±7.7 $n=30$	75.3±16.4 $n=14$[a]
Red	10.4±1.1	9.3±1.2	12.9±1.0	15.4±1.2	35.4±4.2	49.3±4.3

[a]Carpels were not always visible at time of dissection. Approximately 25% purple plants showed some petal-stamen reiteration.

in LD, purple-flowered plants exposed to continuous LD flowered, though much later than SD plants. The terminal flower of these plants opened around day 70 and the first bract was initiated between nodes 30–40 (as opposed to nodes 11–15 in SD). Floral development was characterized by a prolonged phase of initiation of leaves with modified venation and/or shape prior to the first bract and development of fully pigmented petals was accomplished over a greater number of nodes than in SD plants. Stamens and carpels were not always visible at the time of dissection on days 69–70.

Non-reversion of purple-flowered plants

Purple-flowered plants were given a treatment of 5 SD, followed by LD until dissection. In red-flowered plants

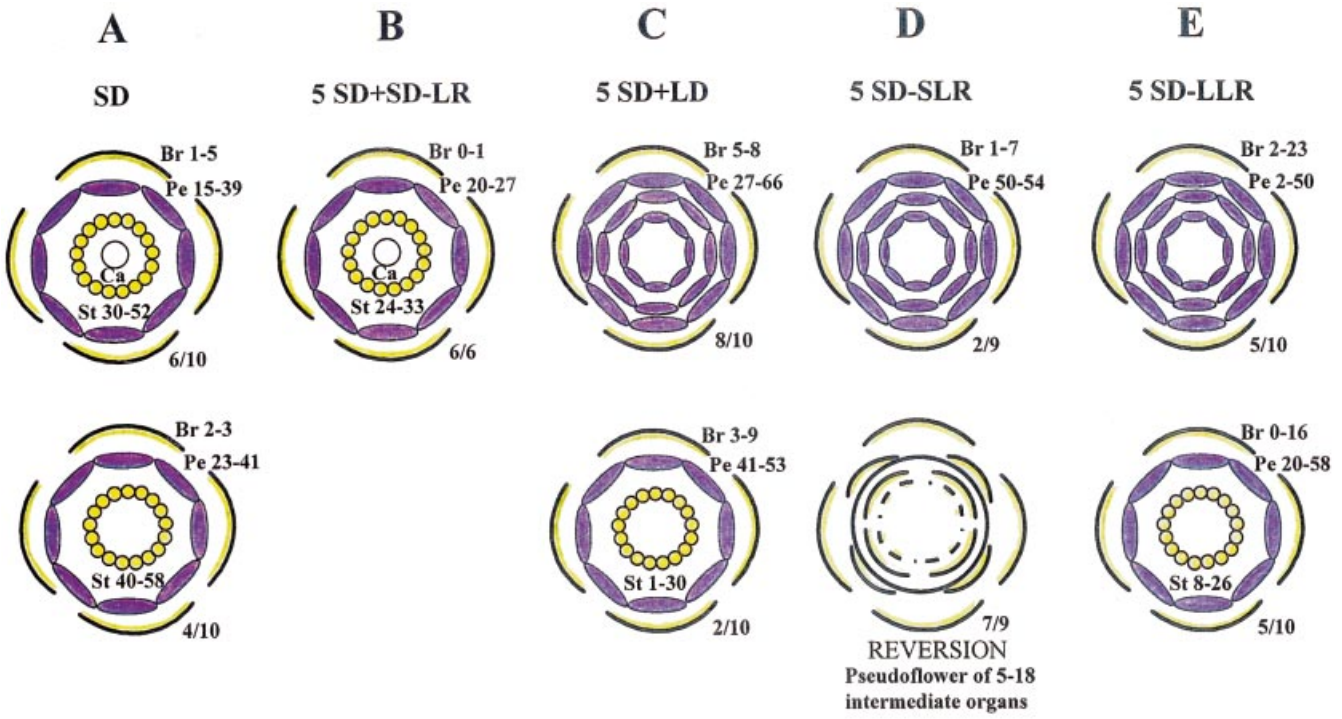

Fig. 3. Floral diagrams depicting development at the terminal meristem of purple-flowered *Impatiens* in SD and 5 SD treatments. Diagrams are representations constructed using data collected from the dissection of mature plants. For clarity one whorl of each class of organ identity is shown except in C, D and E where continued petal initiation and indeterminacy is illustrated by many whorls. (Normally there are 5–6 organs per whorl.) The number at the bottom right of each floral diagram indicates the proportion of plants in the treatment which developed in the manner indicated by the diagram. Br, bract; Pe, petal; St, stamen; Ca, carpel. Numbers given after these initials correspond to the range of organ number of this type found in the plants developing in this manner. Intermediate organs are leaves with some petal features. Bract number in 5 SD-LLR and 5 SD+SD-LR plants may be an underestimate as a result of removal of some bracts during leaf removal in these treatments. Where petal initiation was still in progress at the time of dissection, the total given is to the last pigmented petal (young petals at the centre were often green and unexpanded at the time of dissection). Where no carpels are shown, petal or stamen or a repeating pattern of petal-stamen-petal was continuing at the time of dissection.

this treatment results in reversion of the terminal meristem to leaf production after floral development has begun; flowering is halted and leaf production resumed after the production of a pseudoflower of organs containing some areas of petal pigment (Battey and Lyndon, 1984; Pouteau *et al*., 1997) (Fig. 2B; compare with Fig. 2A). In contrast to red-flowered plants, purple-flowered plants did not revert on transfer to LD, but instead continued flower development (Fig. 2D; compare with Fig. 2C). Similar results were obtained in a 2 SD+LD and 8 SD+LD treatment (data not shown), both known reversion treatments in red-flowered plants (Pouteau *et al*., 1997, and unpublished data). In 5 SD+LD purple-flowered plants internodes were lost and flowers had, at first sight, a similar phenotype to SD plants. On closer inspection, the effect of the LD component of the treatment was evident through the lack of visible stamens at the centre of most plants (Fig. 2D). The number of floral organs in this treatment was greater than in SD flowering plants (mean petal number in SD plants was 30.3 ± 7.9 and in 5 SD +LD plants, in which petal initiation was often continuing at the time of dissection, 46.6 ± 11.0).

Mechanism of floral maintenance of purple-flowered plants

In red-flowered and purple-flowered plants, a floral stimulus is produced in the leaf in SD (Pouteau *et al*., 1997, unpublished results). SD leaves of red-flowered plants lose their induced state rapidly on transfer to LD and this, because of the absence of meristem commitment, causes reversion (Pouteau *et al*., 1997). The non-reversion of purple-flowered plants suggested that either meristem commitment occurred in these plants or the induced state of the leaves could persist in LD. In order to determine the likely maintenance mechanism, a leaf removal experiment was carried out. If leaves remained in control of

Fig. 2. Development of the terminal meristem of *Impatiens*. Red-flowered plants: (A) terminal flower of SD plant; (B) reversion to leaf production occurring after 5 SD+LD. Purple-flowered plants: (C) terminal flower of SD plant and (D) 5 SD+LD plant. (E) Reversion of the terminal meristem in 5 SD-SLR treatment. (F) Terminal flower of 5 SD-LLR plant and (G) 5 SD+SD-LR plant. (H) Petal-leaf and petal-bract organs as observed in a number of plants from 5 SD+LD, 5SD-LLR treatments.

flowering, either by persistence of the inductive ability of SD leaves or as a result of a quantitative build-up of stimulus in LD leaves, their removal would be expected to affect flowering. In contrast, flowering would be unaffected by leaf removal if the meristem was committed. All plants were given 5 SD and transferred to LD as above. At the time of transfer, in some of the plants one cotyledon and the leaves that had unfolded during the 5 SD period (SD leaves) were removed (5 SD-SLR). In other plants, SD leaves were left intact on the plant but those leaves unfolding in LD (LD leaves) were removed for a period of 15 d after transfer (5 SD-LLR) (plants were inspected daily and any unfolding leaves were removed). No leaves were removed from a further set of plants (5 SD + LD controls) (Fig. 1; Table 1).

The results of this experiment are summarized in Fig. 3. When SD leaves were removed (5 SD-SLR treatment), 7 out of 9 plants reverted (Fig. 3D). At the time of dissection (when SD controls had developed fully-opened terminal flowers) these 7 plants had little or no pigmentation of any organ, no petals except small, green unexpanded petals in 2 plants and in all plants internodes were lost then reinstated creating a pseudoflower of between 5 and 18 intermediate (leaves with petal features) organs (Fig. 2E). Finally, from the centre of the pseudoflower leafy organs, often with hairy abaxial surfaces and serrated edges were emerging. The other 2 plants in this treatment flowered, but produced approximately twice as many petals as SD plants and no stamens were visible at the time of dissection (Fig. 3D).

In contrast all the 5 SD-LLR plants were able to maintain floral development (Fig. 2F) and were developmentally similar to those in the 5 SD + LD treatment (described above) though only half of the 5 SD-LLR plants lacked visible stamens (Fig. 3E). Within the 5 SD-LLR and 5 SD + LD treatments, a few plants had mosaic organs consisting of a leaf-like tip with a petaloid base (Fig. 2H).

This leaf removal experiment suggested that purple-flowered plants were able to maintain flowering on transfer to LD through the retention of an induced state in the SD leaves. The LD influence did not cause reversion, but did cause flowering to deviate from that of SD plants suggesting that the leaves unfolded in SD produced a lower level of stimulus on transfer of the plants to LD. To test this, plants were grown in SD and leaves unfolding during the first 5 SD of the treatment were left intact, but those unfolding subsequently were removed on a daily basis for 15 d. Plants remained in SD for the whole of the experiment (5 SD + SD-LR, see Fig. 1 and Table 1). The results are shown in Figs 3B and 2G (compare with 5 SD-LLR, Fig. 3E). 5 SD + SD-LR plants produced smaller numbers of bracts and petals than 5 SD-LLR plants, reflecting a more rapid transition through the steps of flower development. In addition, all 5 SD + SD-LR plants had visible stamens whilst this was true for only half of the 5 SD-LLR plants at the time of dissection. These results suggest that 5 SD + SD-LR plants produced floral stimulus at a higher level than those maintained in LD (5 SD-LLR), but undergoing the same leaf removal treatments, and also suggest that the level of floral stimulus produced by leaves unfolded in SD is higher in SD than in LD.

Discussion

When leaves that had unfolded in SD were removed from purple-flowered plants on transfer into LD after 5 SD (5 SD-SLR treatment), reversion occurred at the terminal meristem. This is evidence that meristem commitment does not occur in purple-flowered plants. However, the lack of reversion in 5 SD + LD plants demonstrates that there is a commitment of the whole plant to the floral state. This result supports the idea, implied by red-flowered plants, that meristem commitment is not a prerequisite for flowering (Battey and Lyndon, 1990). Expression of the meristem identity gene *floricaula* above a threshold level correlates with meristem commitment in *Antirrhinum* (Bradley *et al*., 1996). However, in red-flowered plants of *Impatiens* the *flo* homologue is expressed in vegetative, floral and 5 SD + 3 LD meristems (Pouteau *et al*., 1997). Similarly in meristems of purple-flowered plants sampled after 8 LD, 8 SD or 5 SD + 3 LD transcription of *Imp-flo* could be seen in bands at the base of the primordia (data not shown). This expression pattern was identical to red-flowered plants. It may be that the absence of meristem commitment in both these lines of *Impatiens* is a result of a failure to achieve a threshold level of *flo*.

In the absence of meristem commitment, the leaves of *Impatiens* retain a dominant control over the developmental state of the terminal meristem and persistence of the induced state of leaves is an adequate substitute for apical autonomy in floral development. Whilst in red-flowered plants the ability of leaves to maintain an induced state in LD is absent, giving rise to reversion (Pouteau *et al*., 1997) the requirement for these leaves (SD leaves) to be intact for flowering of the purple-flowered line to occur implies that they are capable of maintaining an induced state in LD. In the work described here, an enhanced induced state was apparent in those leaves unfolding during SD. However, even in continuous LD purple-flowered plants eventually achieved an anomalous type of flowering so presumably LD leaves achieved a low level of induction. This implies that the reverting 5 SD-SLR plants would be expected eventually to flower under the control of the LD leaves. The results of this experiment also imply that the stimulus-producing ability of leaves induced in SD diminished to a lower level on transfer to LD. Whilst the retention of the induced state

of the leaves has been documented for a number of species (Bernier *et al*., 1981), the nature of determination to this state and the specific role its persistence plays in flowering is largely unknown.

In *Antirrhinum* and *Arabidopsis* leaf induction appears to lead to the expression of an irreversible floral pathway involving meristem and organ identity genes (Ma, 1994). On this basis a logical explanation for reversion might be that, in the absence of meristem commitment a signal derived from induced leaves must persist in order to allow the continued expression of this pathway. Removal of the signal would eradicate floral meristem identity with a consequent loss of organ identity gene expression. This stimulus-dependent interpretation accounts for the continued requirement for induced leaves (purple-flowered plants) or SD (red-flowered plants) for the continuation of floral morphogenesis. However, the concept that in *Impatiens* it is merely the presence or absence of floral stimulus that controls an all-or-nothing flowering response is an oversimplification. The inadequacy of this explanation is made apparent by the incomplete eradication of floral meristem features upon reversion (Battey and Lyndon, 1984) and by the diversions from the SD flowering pattern found when purple-flowered plants were given treatments involving a LD component.

These observations and the overall development of *Impatiens* during flowering and reversion suggest that the flowering process is not only stimulus-dependent, but also driven by the attainment of critical threshold levels of stimulus; levels which are necessary in order to initiate and maintain expression of specific elements of the floral process. Thus our working hypothesis is that there is a quantitative relationship between the level of floral stimulus and the progress of floral morphogenesis. Using this hypothesis, for example, the prolonged petal initiation of 5 SD + LD plants (Fig. 3C) is interpreted as an arrest of the floral process arising from insufficient stimulus for activation of stamen initiation when plants are transferred from SD to LD. The swift transition to stamen initiation in SD plants (Fig. 3A) reflects the stronger stimulus-producing ability of leaves in SD than in LD. This hypothesis also explains why the extent of flower development found in red-flowered plants increases with the number of SD (Battey and Lyndon, 1984; Pouteau *et al*., 1997). A similar situation, also implying involvement of the level of floral stimulus is the whorl-by-whorl determination of flowers of *Silene coeli-rosa in vitro* (Donnison *et al*., 1991; Donnison and Francis 1993, 1994). In *Impatiens*, further evidence that genes influencing organ determination may be affected by the level of floral stimulus is the non-appearance of stamens in most 5 SD + LD and some of the 5 SD-LLR purple-flowered plants at the time of dissection, and the 'superflowering' described by Simon (1973) which occurred when plants were exposed to many SD and high temperatures. Such developmental plasticity suggests that determination of floral fate in *Impatiens* does not conform to spatial models in which the flower is structured by the rigid confines of domains and compartments (see Bowman *et al*., 1989).

Conclusion

If this model of flowering in *Impatiens* is correct then the stable flowering normally observed in purple-flowered plants is a result of the leaves maintaining a stimulus level sufficient to impose a set of floral features on the meristem; there is no evidence that this requires prior commitment to a floral meristem identity. Therefore, the stability of flowering can be considered to be largely superficial because the fate of each successively initiated organ is dictated by the stimulus level emitted by the leaves. Thus the overall flowering state achieved by the plant (leaf and meristem) is only as stable as the leaf-induced status. The demand for the leaf-derived signal for floral maintenance in *Impatiens* raises the possibility that this signal has a direct involvement in the expression of the organ identity genes as well as developmental processes that precede this. The detailed mechanism by which the leaf determines organ fate is currently being studied in red-flowered plants of *Impatiens*.

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