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SHORT COMMUNICATION

Genetic ablation of flowers in transgenic *Arabidopsis*

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Summary

We have created transgenic *Arabidopsis* plants in which a gene encoding the cell-autonomous diphtheria toxin A chain (DT-A) was expressed under the control of the *LEAFY (LFY)* promoter. This promoter is active both in emerging leaf primordia and young flowers, with the highest activity in flowers. The majority of *LFY::DT-A* plants had normal vegetative development but lacked flowers, demonstrating that relatively widespread activity of a promoter does not exclude its possible use for ablating selected tissues, as long as differences in activity levels between different tissues are significant. We also found that flowers were replaced by empty bracts in *LFY::DT-A* plants, suggesting that flower-derived signals normally suppress bract development in *Arabidopsis*.

Introduction

Genetic studies in model species such as *Arabidopsis* have led to the identification of a number of genes that control various aspects of flower development, although to date no single gene that is absolutely required for flowering has been isolated. Among the genes controlling flower development in *Arabidopsis* are several meristem-identity genes that are expressed very early during flower development (Weigel, 1995). One of these is the *LEAFY (LFY)* gene, whose RNA is detected in floral primordia before they become morphologically distinct from the shoot apical meristem (Weigel *et al.*, 1992), suggesting that the *LFY* promoter is potentially useful for flower-ablation strategies. However, recent studies have revealed that the *LFY* promoter is also active during the vegetative phase, although its activity is substantially up-regulated upon floral induction (Blázquez *et al.*, 1997; Hempel *et al.*, 1997). Here, we show that despite its vegetative activity, the *LFY* promoter can be combined with the gene encoding the A chain of diphtheria toxin (DT-A) to generate transgenic plants that completely lack flowers, but are vegetatively normal. Thus, a threshold effect can be exploited to adopt plant promoters for specific cell ablation strategies, if differences in levels of activity between different tissues are high enough.

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Results and discussion

Generation of LFY::DT-A *transformants*

We constructed two transgenes in which DT-A coding sequences encoding either the wild-type form (DT-A) or a temperature-sensitive derivative (DT-A^{tsM}) (Bellen *et al.*, 1992) are flanked by the 2.3 kb *LFY* promoter as well as 2.2 kb of *LFY* 3′ sequences including the poly-adenylation site (Figure 1). DT-A has been shown to be active in plants (Day *et al.*, 1995; van der Geest *et al.*, 1995; Thorsness *et al.*, 1991). Transgenic *Arabidopsis* plants carrying the *LFY::DT-A* fusion genes were generated by the vacuum-infiltration method. Although the exact timepoint of transformation is unknown, seeds derived from such infiltrated plants are not chimeric, constitute independent transformation events, and are heterozygous for transgene insertions (Bechtold *et al.*, 1993).

One hundred and forty-eight primary transformants were obtained for *LFY::DT-A* and 50 for *LFY::DT-A^{tsM}*. The transformation efficiencies for both constructs (in the 1% range) were similar to those that we routinely obtain with various innocuous reporter-gene constructs that do not affect plant morphology or development, indicating that the *DT-A* constructs did not confer any significantly deleterious phenotype up to the stage of seed germination. None of the *LFY::DT-A^{tsM}* transformants showed any abnormal morphology, either at normal (22–23°C) or reduced growth temperatures (16–18°C). This observation indicates that even at the restrictive temperature, the *DT-A^{tsM}* allele is considerably less active than the wild-type *DT-A* allele, and that *DT-A^{tsM}* is of limited usefulness in *Arabidopsis*, and possibly other plants as well.

Phenotypic classes of LFY::DT-A *transformants*

Phenotypic analyses were carried out with primary *LFY::DT-A* transformants, as the majority were sterile and did not produce any progeny. Since photoperiod modulates the time to flowering in *Arabidopsis*, *LFY::DT-A* transformants were selected both in long days, in which wild-type plants

produced about 14 leaves before the first flower was formed on the main shoot, and in short days, in which wild-type plants produced about 47 leaves. Under both photoperiods, a similar range of phenotypes was observed (Table 1).

According to the severity of the phenotype, three classes of transformants could be defined. The most infrequent group was class I, which was phenotypically normal and had normal seed set. Class I accounted for 1% of transformants in long days and 13% in short days.

The majority of transformants (67% in long days and 49% in short days) fell into class II. These plants showed severe floral defects, although they had largely normal vegetative development, including a near-normal number of leaves (Figure 2a–c; Table 1). In long days, class II transformants lacked flowers completely. As in wild-type plants, internodes between the last leaves elongated, producing what resembled the basal part of a normal inflorescence spike. However, the normally flower-bearing apical part of the inflorescence was replaced by a scale-bearing pin-formed structure (Figure 2a,b). Only the first one to three internodes on each pin-formed structure elongated significantly, such that the final size of the pin-formed structures was a few millimetres, instead of the normal 10–40 centimetres. Secondary and higher-order shoots repeated the pattern of the main shoot, which was especially striking in short days (Figure 2c). In contrast to long days, in which none of the class II transformants showed any macroscopic signs of flower development, a minority of class II plants (15%) sporadically produced a small number of reduced and abnormal flowers in short days.

Class III transformants were most severely affected, and were arrested during vegetative development. The main shoots of class III plants, which accounted for 32% of transformants in long days and 37% in short days, never showed any signs of internode elongation (Figure 2d). In long days, the main shoots produced 0–14 leaves before growth ceased, while in short days 0–40 leaves were produced. Lateral shoots were never observed.

The *LFY* promoter is active in both leaf and flower primordia (Blázquez *et al.*, 1997) and even in the embryo (I. Lee and D. Weigel, unpublished observations). The levels of *LFY::GUS* activity, as well as the steady-state levels of endogenous *LFY* RNA in newly emerging leaf primordia, increase continuously with the age of the plant (Blázquez *et al.*, 1997). Thus, growth arrest of class III *LFY::DT-A*

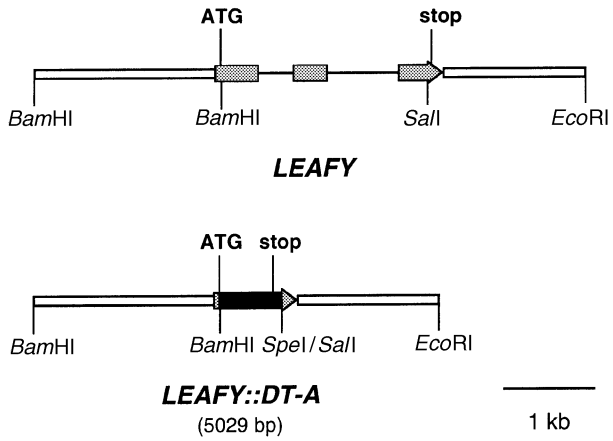

Figure 1. Diagram of *LFY::DT-A* transgenes.
The top section shows the structure of the genomic *LFY* fragment that rescues the *lfy* mutant phenotype in transgenic plants (Blázquez *et al.*, 1997). 5′ and 3′ untranscribed regions are indicated by thin open bars, exons are indicated by a thick shaded bar, and introns by a thin line.
The bottom section shows the *LFY::DT-A* chimeric construct. A thick black bar indicates *DT-A* sequences.

Table 1. Class frequency and leaf numbers of *LFY::DT-A* transformants

Genotype	Long Days			Short Days		
	% (*n*)	RL (range)	CL (range)	% (*n*)	RL (range)	CL (range)
Wild type	100 (32)	11.5 ± 1.3 (10–14)	2.9 ± 0.6 (2–4)	100 (32)	39.8 ± 3.6 (31–44)	7.3 ± 3.6 (4–9)
LFY::DT-A Class I	1.2 (1)	12 (n/a)	4 (n/a)	13.4 (9)	43.2 ± 21.5 (16–75)	7.7 ± 1.5 (4–9)
LFY::DT-A Class II	66.7 (54)	12.0 ± 1.6 (11–13)	3.9 ± 0.6 (3–5)	49.3 (33)	48.6 ± 9.9 (27–75)	13.0 ± 3.2 (6–18)
LFY::DT-A Class III	32.1 (26)	n/a[b] (0–14)	– [a]	37.3 (25)	n/a[b] (0–40)	– [a]

RL, rosette leaves; CL, cauline leaves; n/a, not applicable. Measurements are the average ± standard deviation. (*n* indicates number of plants).
[a]Class III transformants did not show internode elongation and therefore did not produce cauline leaves.
[b]Since the number of arrested leaves in the most severely affected plants could not always be accurately determined, an average was not calculated.

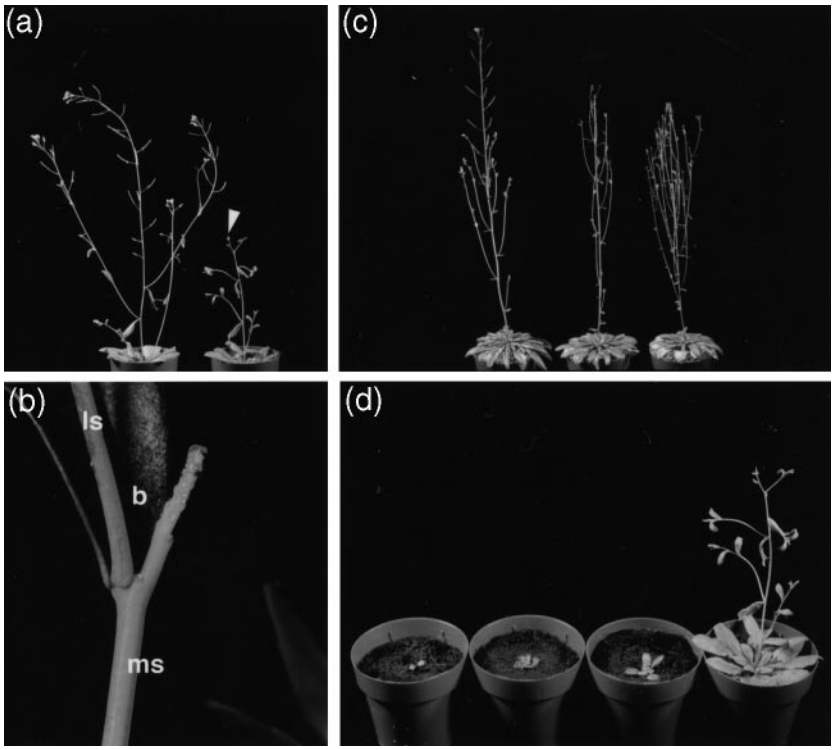

Figure 2. Macroscopic appearance of *LFY::DT-A* plants.
(a) 35-day-old wild-type plant (left) and *LFY::DT-A* class II transformant grown in long days. The flower-bearing part of the inflorescence of the *LFY::DT-A* transformant has been replaced by a pin-formed structure (arrowhead).
(b) Close-up of pin-formed structure on a different 35-day-old *LFY::DT-A* transformant grown in long days. A lateral shoot (ls) subtended by a bract (b) has grown out and overtaken the main shoot (ms).
(c) 92-day-old wild-type plant (left) and *LFY::DT-A* class II transformants grown in short days. Note normal vegetative growth as well as prolific growth of secondary and higher-order shoots in *LFY::DT-A* transformants.
(d) Three 35-day-old *LFY::DT-A* class III transformants grown in long days, with a *LFY::DT-A* class II transformant shown on the right for comparison. Vegetative growth has been arrested at different stages in the class III transformants.

transformants during the vegetative phase is consistent with the known activity of the *LFY* promoter, and is likely caused by significant expression of DT-A toxin in emerging leaves of class III transformants.

Expression of *LFY* is up-regulated upon the transition to flowering, and the steady-state levels of *LFY* RNA in emerging flowers appears to be significantly higher than in previously formed leaves (Blázquez *et al.*, 1997). The majority of *LFY::DT-A* transformants (class II) was normal during the vegetative phase but lacked flowers, suggesting a strong threshold effect of DT-A in *Arabidopsis*, assuming that the *LFY::DT-A* transgene was similarly expressed as a *LFY::GUS* transgene (Blázquez *et al.*, 1997).

While the vegetative phase of *LFY::DT-A* transformants was principally normal, the rosette leaf number of both class I and class II transformants in short days was much more variable than in wild-type plants. Several transformants produced a very high number of leaves (more than 70), while others produced fewer leaves than wild-type plants (Table 1). It is unlikely that this variability was caused by selection of transgenic plants on kanamycin-containing medium and subsequent transplantation to soil, as the controls were kanamycin-resistant plants that carried an innocuous reporter gene fusion (DW228; *UFO::GUS*) and were treated in the same way as the *LFY::DT-A* transformants. It is possible that stress caused by residual *Agrobacterium* load carried over from the vacuum-infiltration procedure led to a decrease in leaf number compared to wild type. An increase in leaf number, on the other hand, is more difficult to explain, as it seems to imply that weak *LFY::DT-A* expression can delay the transition to flowering. One possibility is that the toxin slows primordium formation, and that primordia only grow out once the activity of *LFY* and other meristem-identity genes has declined within these primordia to levels that are too low to over-ride the default leaf fate.

Replacement of flowers with empty bracts in LFY::DT-A *transformants*

The flower-bearing part of the inflorescence was replaced by scale-bearing, pin-formed structures in class II trans-

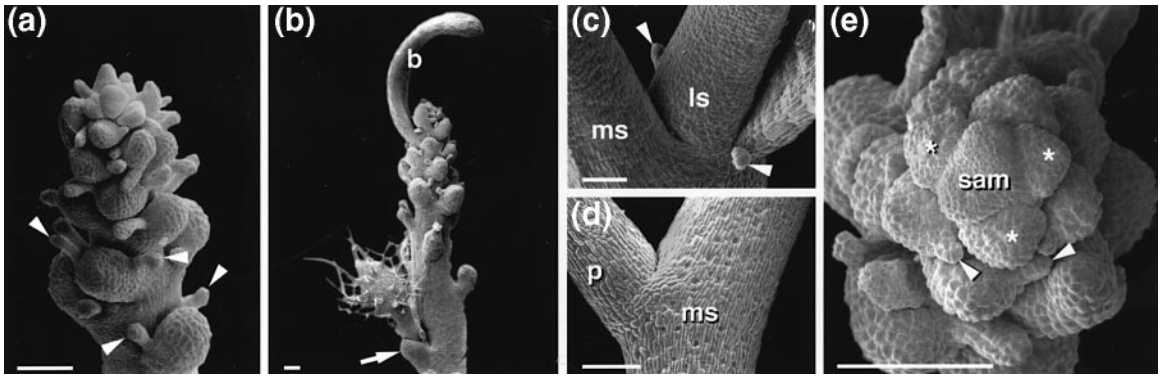

Figure 3. Morphology of *LFY::DT-A* plants as seen in the scanning electron microscope.
(a,b) Scale-bearing pin-formed structures from *LFY::DT-A* class II transformants. Arrowheads indicate stipules. In part (b), a bract leaf (b) has grown out. Note a scale (arrow) subtending the last lateral shoot produced.
(c) Base of a wild-type inflorescence bract leaf, which is flanked by stipules (arrowheads) and in whose axil a lateral shoot (ls) has grown out. The main shoot (ms) is to the left.
(d) Base of the pedicel (p) of a wild-type flower. Note the absence of stipules. The main shoot is to the right.
(e) Close-up view of the shoot apex of a *LFY::DT-A* class II transformant. Arrowheads indicate developing stipules. Note the triangular shape of the primordia (asterisks) forming at the flanks of the shoot apical meristem (sam). Bars equal 100 μm.

formants, which were examined in more detail under the scanning electron microscope. We found that the scales were flanked by two filamentous organs (Figure 3a,b) resembling wild-type organs called stipules, which are associated with inflorescence bracts (cauline leaves), but not flowers, of *Arabidopsis* (Figure 3c,d). The apparent presence of stipules differentiates the pin-formed structures replacing the inflorescence in *LFY::DT-A* plants from the pin-formed structures in which the main shoots of *pin-formed* or *pinoid* mutants terminate (Bennett *et al.*, 1995).

The stipules suggested that the scales in *LFY::DT-A* plants were reduced bracts, which was further supported by the observation that in rare cases the scales grew out to form macroscopic bract leaves, and that occasionally the last lateral shoot to be produced on the main shoot was subtended by a scale instead of a normal cauline leaf (Figure 3b). Similar to bract primordia seen on the inflorescences of strong *lfy* mutants (Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel *et al.*, 1992), the scales of *LFY::DT-A* transformants initiated as triangular primordia (Figure 3e). Obvious scars indicating the presence of ablated cells were not observed, in agreement with previous studies in which DT-A was expressed from the petal- and stamen-specific *APETALA3* promoter (Day *et al.*, 1995).

As a member of the Brassicaceae, *Arabidopsis* differs from most other dicots in that macroscopic leaves subtend only secondary inflorescence shoots (paraclades), but not flowers. We have previously argued that the primordia of leaf/paraclades and of flowers are equivalent, and that threshold levels of *LFY* activity transform what would otherwise become a leaf/paraclade into a bract-less flower (Blázquez *et al.*, 1997). One way to explain the formation of bracts in *LFY::DT-A* plants would be if *LFY* expression in newly emerging primordia was not entirely uniform. In this scenario, cells with the lowest level of *LFY* expression would have the potential to give rise to bracts, but either their proliferation is normally prevented by a signal from the adjacent flower, or they become incorporated into the flower. Although obvious differences in *LFY* RNA or protein expression have not been reported (Levin and Meyerowitz, 1995; Weigel *et al.*, 1992), support for such a scenario comes from the observation that the first flower of wild-type plants is occasionally subtended by a reduced bract (Hempel and Feldman, 1995), and that in weak *lfy* mutants, in which *LFY* activity is very much reduced, flowers are often subtended by reduced bracts which arise concomitantly with the flower (Weigel *et al.*, 1992). While the bract primordium would be suppressed in wild type by the adjacent flower, the cells with highest levels of *LFY* promoter activity are killed in *LFY::DT-A* plants, and the adjacent bract primordium can grow out. An alternative scenario is that the toxicity of DT-A slows the development of individual primordia, such that these only grow out once the activity of *LFY* and other meristem-identity genes has declined within each primordium to levels that are not any longer sufficient to impart floral fate (see above).

Conclusions

Despite extensive genetic analysis of floral induction in *Arabidopsis*, single-gene mutations that prevent flower formation completely have not been found. Thus, complete suppression of flower formation by inactivating or inhibiting individual genes is not possible at present. We have explored an alternative strategy and found that a *LFY::DT-A* transgene can be used to eliminate flowers completely, while allowing apparently normal vegetative development. Our results also indicate that gradual differences in activity

levels of a promoter can be exploited to achieve cell ablation that is restricted to the tissues with the highest levels of promoter activity. In addition, the unexpected replacement of flowers with empty bracts suggests that flowers normally inhibit the formation of bracts in *Arabidopsis*.

There is considerable applied interest in producing plants that never form floral structures while retaining normal vegetative development. A major public concern regarding the cultivation of transgenic plants is the risk of transgene spread to wild relatives. A recent study has documented that a transgene conferring herbicide resistance can spread from oilseed rape, *Brassica napus*, to its weedy relative *B. campestris*, confirming that the risk of transgene spread is not merely hypothetical (Mikkelsen *et al.*, 1996). One way to address such concerns is through engineering genetic sterility that is caused by a complete ablation of floral structures. In species where fruits or seeds are not the reason for cultivation, sterility would not be a disadvantage. On the contrary, complete ablation of flowers might actually stimulate growth in plants whose vegetative growth is terminated by flowering, or in species such as forest trees, for which it is widely believed that the production of reproductive structures and seeds occurs at the expense of vegetative structures (Strauss *et al.*, 1995).

Experimental procedures

Transgene construction and plant material

The DT-A open reading frames along with 3′ untranslated sequences were isolated from plasmids pSK-DT-A or pSK-DT-M (Bellen *et al.*, 1992), and inserted between the *LFY* promoter (nucleotides 465–2755 of GenBank accession number M91208 (Weigel *et al.*, 1992), and *LFY* 3′ sequences (nucleotides 5348–7503). The final fusion product was generated in the plant transformation vector pCGN1547 (McBride and Summerfelt, 1990). Transformation vectors pEW3 (LFY::DT-A) and pEW4 (LFY::DT-A^{tsM}) were transformed into *Agrobacterium tumefaciens* strain ASE (Fraley *et al.*, 1985), and introduced into *Arabidopsis* ecotype Columbia by vacuum infiltration (Bechtold *et al.*, 1993). Transgenic seedlings were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 50 μg ml^{-1} kanamycin. Antibiotic-resistant seedlings were transferred to soil after 1 week. Plants were grown at 23°C in long-day (16 h light/8 h dark) or short-day cycles (9 h light/15 h dark), under a mixture of Cool White and Gro-Lux fluorescent lights (Osram, Sylvania).

Scanning electron microscopy

Plant material was dissected and fixed in 4% glutaraldehyde overnight at 4°C, followed by several days of postfixation in 1% osmium tetroxide. Specimens were critical-point dried, coated with gold/palladium on a Technics Hummer 1 sputter coater, and viewed in a Cambridge 360 scanning electron microscope, at accelerating voltages of 10–20 kV.

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