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.

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<sup>tsM</sup>) (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<sup>tsM</sup>. 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<sup>tsM</sup> 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<sup>tsM</sup> allele is considerably less active than the wild-type DT-A allele, and that DT-A<sup>tsM</sup> 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

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Long Days</th>
<th></th>
<th>Short Days</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% (n)</td>
<td>RL (range)</td>
<td>CL (range)</td>
<td>% (n)</td>
</tr>
<tr>
<td>Wild type</td>
<td>100 (32)</td>
<td>11.5 ± 1.3</td>
<td>2.9 ± 0.6</td>
<td>100 (32)</td>
</tr>
<tr>
<td>LFY::DT-A</td>
<td>1.2 (1)</td>
<td>n/a</td>
<td>n/a</td>
<td>1.6 (1)</td>
</tr>
<tr>
<td>Class I</td>
<td>66.7 (54)</td>
<td>12.0 ± 1.6</td>
<td>3.9 ± 0.6</td>
<td>49.3 (33)</td>
</tr>
<tr>
<td>Class II</td>
<td>32.1 (26)</td>
<td>n/a</td>
<td>n/a</td>
<td>37.3 (25)</td>
</tr>
</tbody>
</table>

RL, rosette leaves; CL, cauline leaves; n/a, not applicable. Measurements are the average ± standard deviation. (n) indicates number of plants.

*Class III transformants did not show internode elongation and therefore did not produce cauline leaves.

*Since the number of arrested leaves in the most severely affected plants could not always be accurately determined, an average was not calculated.

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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 transformants during the vegetative phase. The flower-bearing part of the inflorescence of the LFY::DT-A transformant has been replaced by a pin-formed structure (arrowhead).

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 struc-
tures 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 inflores-
cences of strong Ify mutants (Huala and Sussex, 1992;
Schultz and Haughn, 1991; Weigel et al., 1992), the scales
of LFY::DT-A transforms initiated as triangular primordia
(Figure 3e). Obvious scars indicating the presence of
ablated cells were not observed, in agreement with previ-
ous 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 Ify mutants,
in which LFY activity is very much reduced, flowers are
often subtended by reduced bracts which arise concomit-
antly 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 pro-
moter activity are killed in LFY::DT-A plants, and the adja-
cent 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
depended 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 inhibit-
ing 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 485–2755 of GenBank accession number M91208) and *LFY* 3′ sequences (nucleotides 5348–7503). The final fusion product was generated in the plant transformation vector pCGN19547 (McBride and Summerfelt, 1990). Transformation vectors pEW3 (LFY::DT-A) and pEW4 (LFY::DT-A<sup>th</sup>) 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 µM 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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**References**


