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The *Arabidopsis* floral homeotic gene *PISTILLATA* is regulated by discrete *cis*-elements responsive to induction and maintenance signals

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SUMMARY

PISTILLATA **is a B-class floral organ identity gene required for the normal development of petals and stamens in *Arabidopsis*. *PISTILLATA* expression is induced in the stage 3 flowers (early expression) and is maintained until anthesis (late expression). To explore in more detail the developmentally regulated gene expression of *PISTILLATA*, we have analyzed the *PISTILLATA* promoter using *uidA* (β-glucuronidase gene) fusion constructs (PI::GUS) in transgenic *Arabidopsis*. Promoter deletion analyses suggest that early *PISTILLATA* expression is mediated by the distal region and that late expression is mediated by the proximal region. Based on the PI::GUS expression patterns in the loss- and gain-of-function alleles of meristem or organ identity genes, we have shown that *LEAFY* and *UNUSUAL FLORAL ORGANS* induce *PISTILLATA* expression in a flower-independent manner via a distal promoter, and that *PISTILLATA* and *APETALA3* maintain *PISTILLATA* expression (autoregulation) in the later stages of flower development via a proximal promoter. In addition, we have demonstrated that de novo protein synthesis is required for the *PISTILLATA* autoregulatory circuit.**

Key words: *Arabidopsis thaliana*, *PISTILLATA*, Flower development, Homeotic gene, Transcriptional regulation

INTRODUCTION

Floral organ development in *Arabidopsis* is regulated by three classes of floral organ identity genes. Each class of organ identity genes is expressed in two adjacent regions of four concentric whorls of flower buds (the ABC model; Bowman et al., 1991; Meyerowitz et al., 1991 reviewed by Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1993). Mutations in floral organ identity genes result in homeotic changes in the flower. Thus the floral organ identity genes regulate spatial and temporal cell proliferation and cell differentiation in flower buds. Most floral organ identity genes such as *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), *PISTILLATA* (*PI*), *APETALA3* (*AP3*), and *AGAMOUS* (*AG*) encode a highly conserved DNA binding domain called the MADS domain (Goto and Meyerowitz, 1994; Jack et al., 1992; Kempin et al., 1995; Mandel et al., 1992; Schwarz-Sommer et al., 1990; Yanofsky et al., 1990) and may work as transcription factors.

The B-class organ identity genes *PI* and *AP3* are primarily expressed in the second and third whorls of the *Arabidopsis* flower and specify petal and stamen development. Mutations in either the *PI* or *AP3* gene result in similar phenotypes, with the petals being transformed to sepals and the stamens to carpels (Bowman et al., 1989; Hill and Lord, 1989; Jack et al., 1992), indicating that the activities of both the *PI* and *AP3* genes are required for B function, but that the spatial expression domains of *PI* and *AP3* are not identical. Both *AP3* and *PI* are expressed in whorls 2 and 3, but *PI* is expressed in the fourth whorl at the early stages of flower development (Goto and Meyerowitz, 1994) and *AP3* is expressed in a small number of cells in the first whorl (Jack et al., 1994; Weigel and Meyerowitz, 1993). The genomic sequences of non-coding regions of *PI* and *AP3* do not show any similarities. Based on the above, the establishment of *PI* and *AP3* transcription is thought to be regulated by different mechanisms, although the expression domain and function are similar.

Both *PI* and *AP3* are regulated in two steps; the establishment of initial expression in response to induction signals and the maintenance of their expression by their own gene products (autoregulation) (Goto and Meyerowitz, 1994; Hill et al., 1998; Jack et al., 1994; Krizek and Meyerowitz, 1996; Samach et al., 1997; Tilly et al., 1998). It has been proposed that the initial expression of *PI* and *AP3* is induced by combinations of the meristem identity genes, *AP1*, *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*), as genetic studies have shown that the expression levels of *PI* and *AP3* in the initial stages are reduced in *lfy*, *lfy*;*ap1* double, and *ufo* mutants (Lee et al., 1997; Levin and Meyerowitz, 1995; Weigel and Meyerowitz, 1993). Once *PI* and *AP3* expression are established, expression in the petals and stamens are maintained by the activities of the proteins themselves. Both *PI* and *AP3* expression are reduced when either the *PI* or *AP3*

gene is mutated (Goto and Meyerowitz, 1994; Jack et al., 1992), and constitutive expression of both *PI* and *AP3* gives rise to the ectopic expression of *PI* and *AP3* throughout the flower (Jack et al., 1994; Krizek and Meyerowitz, 1996).

The MADS proteins bind to DNA having the consensus sequence, $CC(A/T)_6GG$, referred to as the CArG box (reviewed by Riechmann and Meyerowitz, 1997). PI and AP3 form a heterodimer and bind to the CArG boxes of the *AP3* promoter in vitro (Hill et al., 1998; Tilly et al., 1998). *GLOBOSA* (*GLO*) and *DEFICIENS* (*DEF*) are orthologs of *PI* and *AP3*, respectively, in *Antirrhinum majus*, a distantly related species in which DEF and GLO form a heterodimer and bind to CArG boxes of both *GLO* and *DEF* promoters (Davies et al., 1996; Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Zachgo et al., 1995). Therefore, the direct interactions between the heterodimer of B-class gene products and the CArG boxes of their promoter are thought to be the major mechanisms by which B function is autoregulated (Samach et al., 1997). However, the genomic sequence of *PI* shows that there is no CArG-box-like sequence in the 5′ flanking region or in the introns.

In this study, we analyzed the expression patterns conferred by various fragments of *PI* promoter during flower development using fusions to the *uidA* reporter gene, which encodes β-glucuronidase (GUS); as a result, we were able to investigate the interactions between *trans*-acting factors that induce as well as maintain *PI* expression and *cis*-elements of the *PI* promoter. We have demonstrated that the *PI* promoter consists of discrete *cis*-acting elements; one in the distal region is responsive to induction signals mediated by the meristem identity genes *LFY* and *UFO*, and a second element in the proximal region is responsive to autoregulatory signals produced by the PI/AP3 complex. Furthermore, we have shown that de novo protein synthesis is required for the PI/AP3 complex to upregulate *PI* transcription via a proximal promoter. These results, together with the finding that the constitutive expression of both *PI* and *AP3* cannot give rise to the expression of *PI* in non-floral tissues, suggest that an unknown flower-specific factor is necessary to maintaining *PI* expression.

MATERIALS AND METHODS

Primer extension and S1 nuclease mapping

Poly(A)$^+$ RNA was isolated from inflorescences and 10-day-old plants (for vegetative RNA) of the Landsberg ecotype. A 5 μg sample of poly(A)$^+$ RNA was hybridized at 37°C overnight with a single-stranded *Dra*I-*Nco*I fragment of *PI* gene and was digested by S1 nuclease at 100, 200, or 500 Units/ml at 30°C for 30 minutes. For the primer extension, end-labeled oligo DNA (5′TCACCACTCTG-TTGTTTGCG3′) was annealed with 15 μg poly(A)$^+$ RNA, and complementary DNA was synthesized by reverse transcriptase. Other procedures were performed as described by Sambrook et al. (1989).

Construction of DNA

Fragments of the *PI* promoter (1G to 9G) were amplified by PCR using a *PI* genomic clone as a template with a 3′ primer containing ATG of the *PI* sequence 5′CGGGATCCCATGGTTCTCTCTCTA-TCTC3′ and a 20-mer DNA starting at each 5′ deletion point. The PCR products were subcloned into pGEM3z (Promega) and sequenced to avoid PCR error. Correct clones were ligated between the *Pst*I and *Bam*HI site of pBI221 (Clontech) so that the ATG of *PI* was in frame with the *uidA* coding sequence. To make 15G, the 940 bp *Xba*I fragment of the *PI* genomic clone was ligated to the *Xba*I site of 6G (see Fig. 2).

Transcriptional fusion constructs were also made by PCR amplification with one primer beginning just 5′ from the various transcription start sites. A *Dra*I-digested 3G promoter fragment and the PCR-amplified –300 to –201 fragment were ligated to the *Eco*RV site of the 35S promoter to make 3DmG and 32mG, respectively. These constructs start translation at ATG of the *uidA* gene.

The +1 to –600 region of the *AP3* promoter was PCR-amplified and fused translationally to the *uidA* gene by the same strategy as that used for the *PI* promoter.

All these promoter::uidA constructs were subcloned into the pCGN1547 vector (McBride and Summerfelt, 1990) for plant transformation.

Plant materials and histochemical analysis

The *Arabidopsis* Columbia ecotype was used for Agrobacterium-mediated vacuum transformation (Bechtold et al., 1993). The GUS expression pattern was surveyed using kanamycin-resistant T1 transformants, and further analyses were carried out with a homozygote carrying the transgene in a single locus.

Plant crossing was carried out by manual cross-pollination. F_1 and F_2 plants were analyzed for gain- and loss-of-function alleles, respectively. The presence of the transgenes was confirmed by PCR.

Staining for GUS activity was performed as described by Sieburth and Meyerowitz (1997). After staining, the tissue was fixed with 1.5% glutaraldehyde and 0.3% paraformaldehyde for 6 hours at 4°C and was then processed through an ethanol series. For the microscopy, whole-mount tissues were mounted in a clearing solution (72% chloral hydrate and 11% glycerol).

All photographs were taken with an HC300 digital camera (Fujifilm) connected to a Zeiss Axiophoto or a Leica MZAPO microscope and assembled using Adobe Photoshop software.

Induction of a glucocorticoid receptor and in situ hybridization

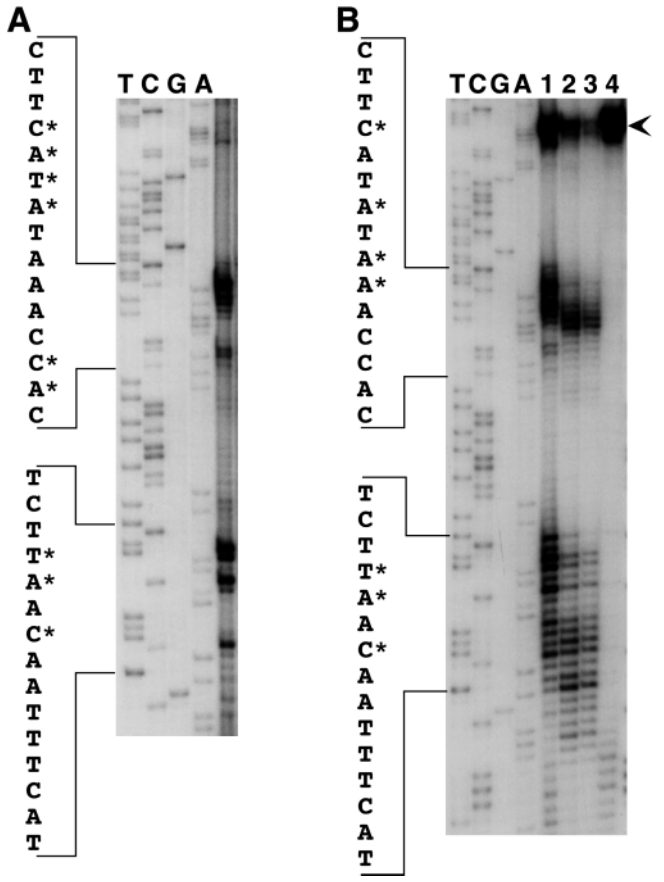
To induce the glucocorticoid receptor into its active form, plants carrying 35S::AP3-GR gene were treated with dexamethasone and cycloheximide as described by Sablowski and Meyerowitz (1998) except that the treatment duration was 24 hours.

The flowers were fixed, embedded, sectioned, hybridized, and washed as described previously (Sakai et al., 1995). The anti-*uidA* probe was made from pGUS/7z, which carries the *uidA* coding region of pBI221 (Clontech).

RESULTS

The *PI* gene has two transcriptional initiation sites

In order to determine the transcriptional initiation site of the *PI* gene, we performed primer-extension and S1 nuclease-mapping experiments, the results of which demonstrate the existence of two putative transcriptional initiation sites for the *PI* gene (Fig. 1A,B). We defined ‘C’ at the –83 position (the translational initiation is numbered as +1) and ‘T’ at –51 as the two putative transcriptional initiation sites of the *PI* gene, since they were the longest products among the two clusters seen in common with both experiments. Both presumptive transcriptional initiation sites are located 30 bases downstream from the putative TATA boxes and putative CAAT boxes located 106 bases and 71, 55 bases upstream from each transcriptional initiation, respectively (Fig. 1C). This DNA sequence structure also supports the existence of two transcriptional start sites for the *PI* gene.

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-300
   TATCTTCTTGGTACTGTAAAAAAAGGAGAGAAAATAGAGTTGGCTATGTG

-250
   TAATAAGCGAACCAAAAGCAAGCCTTCCATGACTGTGCCCTCAAGAAAGT

-200
   AGCTTTGTTTTCAATCCCAAACTGTCAAAGTCTCTCTTCACCTCAAGATT

-150                                              P
   AATCAAAACATTTCTCTCTCTATCTCATCAATGTTACTTTAAAACCAATG

-100                t1                            t2
   CTCCTCTTCTTGTTCTTCATATAAACCACATATCCTCTCCTCCATATCTT

-50
   AACAATTTCATAGCAAACCCTAAAATTGAGAAAGAGATAGAGAGAGAAAG

  +1
   ATG
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Fig. 1. Transcriptional initiation sites of the *PI* gene. (A) Primer extension and (B) S1 nuclease mapping analysis. Possible transcriptional start sites are indicated by asterisks on the DNA sequence. S1 nuclease digestion was performed at concentrations of 100, 200, and 500 units/ml, lanes 1 to 3 respectively. Vegetative RNA was used for lane 4 (digested with 100 Units/ml S1 nuclease). The arrowhead indicates the position of the probe. (C) DNA sequence of the *PI* promoter region (the sequence data of the *PI* genomic DNA is in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB035137). Putative CAAT and TATA boxes are indicated by double underlines and boxes, respectively. Arrows and dotted arrows indicate inverted repeats. t1 and t2 indicate putative transcriptional start points. The initiation codon is in bold; P indicates the 5′ end of the S1 mapping probe.

Deletion from the 5′ end revealed that the *PI* promoter consists of discrete elements responsive to induction and maintenance signals

In order to define the regulatory elements in the *PI* promoter, deletion derivatives of the 1.5 kb region of the *PI* promoter were generated, and in-frame translational fusions to the *E. coli uidA* gene which encodes the β-glucuronidase (GUS) enzyme (Jefferson et al., 1987) were made (15G, Fig. 2). We regard this promoter region of the *PI* gene to be sufficient since a 4.8 kb genomic fragment having the same 5′ end complements the *pi-2* mutant allele (data not shown).

Fig. 2 summarizes the constructs of the promoter deletion series. We introduced these constructs (PI::GUS series) into the *Arabidopsis* genome by vacuum infiltration (Bechtold et al., 1993). At least ten independent stable transformants of each construct were isolated, and flowers of each line were stained for GUS enzyme activity. Whole mounts of plant tissues were observed, and we found that some transgenic lines carrying the

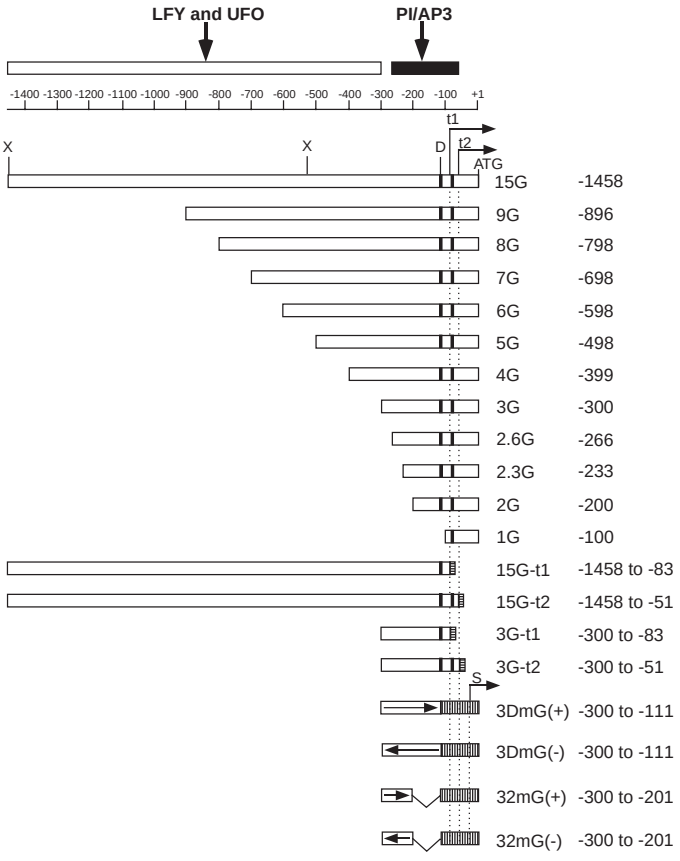

Fig. 2. The elements and constructs of the *PI* promoter. Top: The distal region of the *PI* promoter (white box) interacts with *LFY* and *UFO* either individually or cooperatively. The proximal region (black box) is required for *PI* autoregulation by the PI/AP3 complex. Bottom: A schematic diagram of the PI::GUS constructs analyzed. The endpoints of the promoter elements are listed. ATG and the right ends of boxes indicate translational initiation. Arrows labelled t1, t2, and s indicate transcriptional start sites of the *PI* and 35S promoters. Black, vertical striped, and horizontal striped boxes indicate TATA boxes of *PI*, the –90 35S minimal promoter, and poly-linker, respectively. Arrows in the box indicate the orientations of the *PI* promoter fragments. X, *Xba*I; D, *Dra*I.

Table 1. Summary of the expression patterns of PI::GUSs in the wild type[a]

			Stage 3, 4		Stage 5, 6			Stage 7-9		Stage 10[b]		
Construct	IM	Stage 1, 2	Whorl 1	Whorl 2-4	se	pe, st	ca	se, ca	pe, st	se, ca	pe, st	Total number[c]
RNA[d]	–	–	–	+++	–	+++	–	–	+++	–	+++	
15G	++	++	++	++	++	+++	+++	+	+++	–	+++	10/11
9G	++	++	++	++	++	+++	+++	+	+++	–	+++	12/15
8G	++	++	++	++	++	+++	+++	+	+++	–	+++	26/27
7G	++	++	++	++	++	+++	+++	+	+++	–	+++	20/21
6G	–	–	+	++	++	+++	+++	+	+++	–	+++	20/20
5G	–	–	–	+	–[e]	+++	++	–	+++	–	+++	17/18
4G	–	–	–	–	–[e]	+++	–[e]	–	+++	–	+++	14/18
3G	–	–	–	–	–[e]	+++	–[e]	–	+++	–	+++	12/13
2.6G	–	–	–	–	–	++[f]	–	–	++[f]	–	+++	10/11
2.3G	–	–	–	–	–	–	–	–	–	–	–	7/10
2G	–	–	–	–	–	–	–	–	–	–	–	18/26
1G	–	–	–	–	–	–	–	–	–	–	–	13/17
15G-t1	–	–	–[e]	++	–	++	+	–	+	–	+++	11/11
15G-t2	–	–	+	++	++	+++	+++	+	+++	–	+++	10/10
3G-t1	–	–	–	–	–	–	–	–	–	–	–	9/10
3G-t2	–	–	–	–	–	++[f]	–	–	++[f]	–	+++	20/20
3DmG(+)	–	–	–	–	–	–	–	–	+/–[g]	–	+/–[g]	6/11
3DmG(–)	–	–	–	–	–	–	–	–	+[h]	–	++[h]	10/10
32mG(+)	–	–	–	–	–	–	–	–	–	–	–	20/20
32mG(–)	–	–	–	–	–	–	–	–	–	–	–	10/10

[a]Relative levels of GUS staining denoted by: +++, high; ++, moderate; +, low; –, not detectable.
Abbreviations: IM, inflorescence meristems; se, sepals; pe, petals; st, stamens; ca, carpels including their primordia.
[b]Observations were continued until anthesis.
[c](the number of lines that show the indicated pattern)/(the total number of transgenic lines obtained).
[d]Endogenous *PI* RNA was detected by in situ hybridization.
[e]Some transgenic lines occasionally showed very weak GUS staining.
[f]GUS staining was observed only in the tips of organ primordia.
[g]Half of the transgenic lines showed GUS staining in the bases of petals and stamens, and the others were under detectable levels.
[h]GUS staining was observed in the base of petals and filamentous connectives.

same construct show variability in their GUS staining patterns. Table 1 presents the typical pattern for the localization of GUS enzyme activity that has been observed in more than 70% of independent transgenic lines (unless indicated otherwise) derived from each construct.

15G, which contains 1.5 kb of the *PI* promoter, shows petal- and stamen-specific GUS expression in stage 10 and older flowers (Fig. 3A,B). PI-GUS transgenic lines with a 698 bp or longer promoter region (9G, 8G and 7G) show an identical GUS expression pattern to 15G (Fig. 3C). However, GUS activity was also observed throughout the inflorescence meristems (IM) and the stage 1 and 2 flowers, in the first whorl of the stage 3 and 4 flowers, and in the sepals and carpel primordia of the stage 5

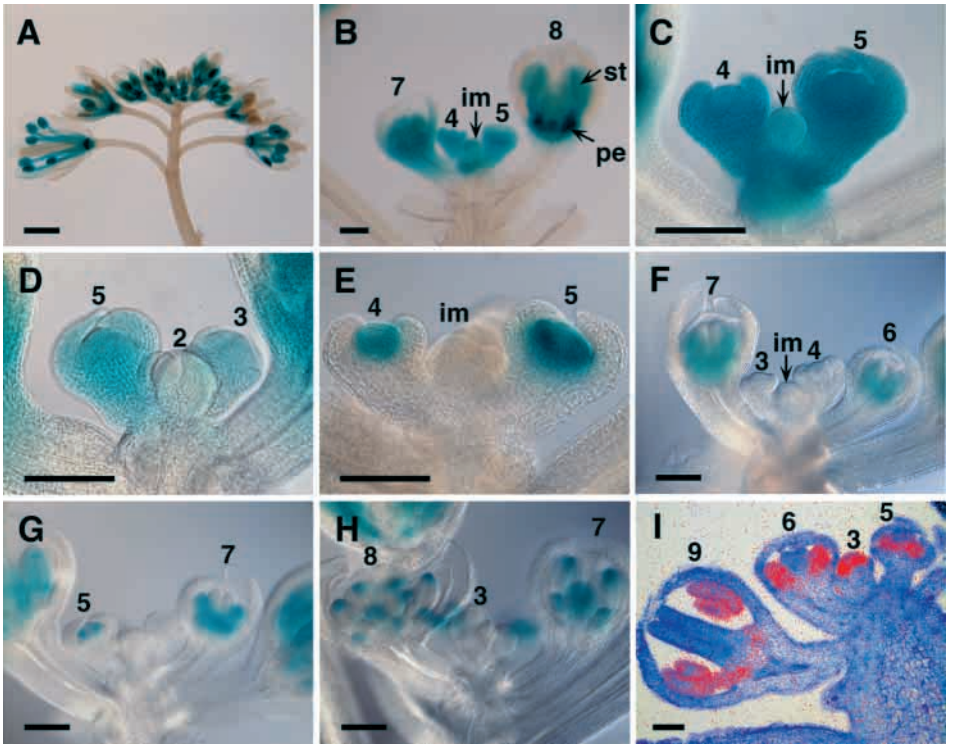

Fig. 3. GUS expression patterns conferred by deletions of the *PI* promoter. (A, B) GUS expression conferred by the 15G construct. (C) GUS expression in transgenic 7G flowers. This pattern is representative of 9G, 8G and 7G constructs. GUS staining conferred by (D) 6G, (E) 5G, (F) 4G, (G) 3G and (H) 2.6G. (I) The localization of endogenous *PI* transcripts was detected by in situ hybridization (red dots indicate hybridization signal). Numbers indicate the floral stage. im, inflorescence meristem; pe, petal; st, stamen. Scale bars, (A) 1 mm; (B-I) 100 μm.

to 9 flowers, even though endogenous *PI* transcripts have not been detected in these regions by in situ RNA hybridization (Fig. 3I) (Goto and Meyerowitz, 1994). Since further deletion either from 5′ to −598 or from 3′ to the second transcriptional initiation site at −51 eliminates the ectopic expression and restores *PI*-specific expression (6G, 15G-t2; Fig. 3D, Table 1), we are confident that promoter-deletion experiments will reveal the *cis*-elements essential to *PI* gene expression.

5′ deletions to −498 (5G) result in GUS activity in a spatial and temporal pattern that is most similar to the endogenous *PI* RNA localization detected by in situ RNA hybridization (Fig. 3E,I). These results suggest that the region from −498 to +1 includes a sufficient number of *cis* elements for *PI* transcription. Further deletion to −399 (4G) extinguished GUS expression in the stage 3 and 4 flowers (early expression) (Fig. 3F), suggesting that deletion to -399 disrupts the sequence required for early expression occurring in response to induction signals. Deletion to −300 (3G) restored GUS activity to a level indistinguishable from that of the longer constructs in the stage 5 and older flowers, whereas no expression was detected in the earlier stages (Fig. 3G). In constructs containing 5′ deletions to −266, the level of late expression was reduced, and the region of GUS expression was exclusively in the tips of organ primordia (2.6G, Fig. 3H). Further deletions to −233 (2.3G), −200 (2G), and −100 (1G) totally abolished the GUS expression. These results suggest that deletion to -266 partially removes the elements and deletion to −233 totally disrupts the essential sequence required for the late expression.

To define a minimal region required for the late expression, 3′ deletions based on the 3G construct were made. 3′ deletions to −51, the second transcriptional initiation site (t2), and to −83, the first transcriptional initiation site (t1)resulted in a GUS expression pattern identical to 2.6G (Table 1) and a total loss of GUS activity, respectively. Since any further 3′ deletion abolished the transcriptional initiation, we used a minimal promoter from the −90 region of the 35S promoter of Cauliflower Mosaic Virus (−90 CaMV)(Benfey et al., 1989). The −300 to −111 region of the *PI* promoter was fused to −90 CaMV in both orientations (3DmG+ and −). As a result, 50% of the forward-construct transgenic lines (3DmG+) and 100% of the reverse-construct lines (3DmG−) showed weak GUS activity in the base or connectives of the petals and the stamens of the stage 7 and older flowers. GUS activity was not observed in the flowers carrying the constructs of the −300 to −201 region fused to -90 CaMV in either orientation (32mG+ and −). Taken together, the 5′ and 3′ deletion results suggest that the core element essential for late expression lies between −266 and −111, and that the −300 to −51 region is required to elicit the full spectrum of late expression.

Late expression of *PI* mediated by a proximal promoter is dependent on both *PI* and *AP3*

In the mutant alleles of *pi* and *ap3*, the late expression of *PI* is reduced, but its early expression is not affected (Goto and Meyerowitz, 1994), suggesting that the autoregulation of *PI* works only during late expression when its expression domain is coincident with that of *AP3*. In addition, biochemical studies have revealed that PI and AP3 form a heterodimer and bind to a specific DNA sequence (Goto and Meyerowitz, 1994; Riechmann et al., 1996). In order to define the elements responsive to the PI/AP3 complex in the *PI* promoter region, deletion derivatives of PI::GUS were introduced into the loss- and gain-of-function alleles of *PI* and *AP3*. We crossed PI-GUS plants with the strong mutant alleles, *pi-1* and *ap3-3*, and with 35S-PI and 35S-AP3, which express *PI* and *AP3* constitutively.

Mutations in *PI* result in the homeotic transformation of

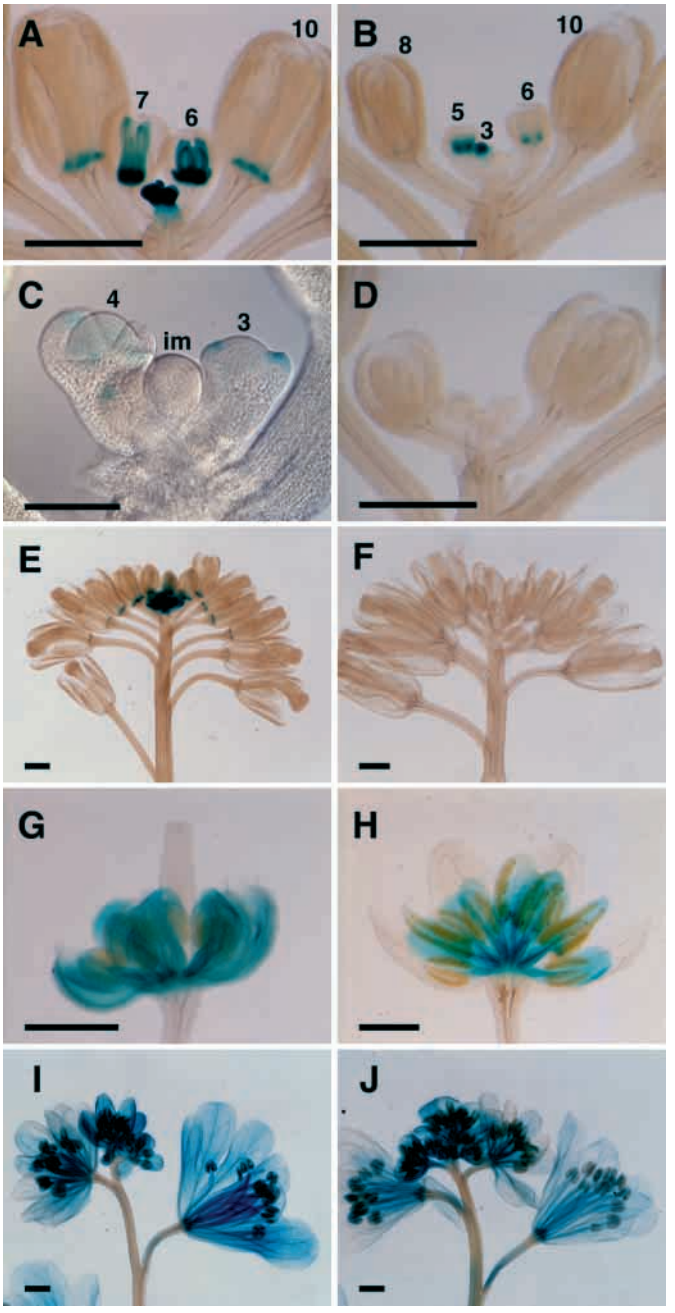

Fig. 4. PI::GUS expression patterns in the loss- and gain-of-function alleles of *PI* and *AP3*. (A-D) GUS expression conferred by deletions of the *PI* promoter in *pi-1*. (A) 15G construct representative of 9G, 8G and 7G constructs, (B) 6G, (C) 5G, (D) 4G, which is representative of 3G, 2G and 1G. (E) 15G and (F) 3G construct in *ap3-3*. (G,H) Mature flowers of 35S-PI (G) and 35S-AP3 (H), which carry the 3G construct. (I,J) Double transgenic plants of 35S-PI;35S-AP3 carrying 3G (I) and 15G (J) constructs. Scale bars, (C) 100 μm; others 500 μm.

petals to sepals and stamens to carpels (Bowman et al., 1989; Hill and Lord, 1989). 9G, 8G, and 7G constructs show a GUS staining pattern indistinguishable from that of 15G in *pi-1* mutant flowers (Fig. 4A). GUS activity observed in the filamentous organs of the second and the third whorls of stage 6 and 7 flowers is likely due to the long lifetime of GUS protein expressed in the early stages. In the present study, most of the GUS activity disappeared in flowers older than stage 10, although it could still be detected in the basal regions of the second and third whorl organs, a region of high cell proliferation in which, in *pi-1*, endogenous *PI* is also expressed (Goto and Meyerowitz, 1994). This expression is independent of *PI* autoregulation because active PI protein is not present in *pi-1* flowers. 6G does not show GUS activity in the IM or in stage 1 and 2 flowers, and shows basically the same pattern as 15G in the later stages (Fig. 4B). The 5G construct shows only faint GUS expression in the tips of floral organ primordia of *pi-1* mutants (Fig. 4C). The 4G and shorter constructs, which as wild types exhibit only late expression, exhibited no detectable GUS activity in the *pi-1* mutants (Fig. 4D). These results demonstrate that early *PI* expression is *PI*-independent and that late expression requires a functional *PI* gene product.

ap3 mutant flowers show a similar phenotype to *pi* mutants (Bowman et al., 1989; Jack et al., 1992). The GUS expression patterns produced by both the 15G and 3G constructs in the strong mutant allele, *ap3-3*, were identical to those in *pi-1* (Fig. 4E,F) in that the early expression mediated by 15G remained and the late expression in 3G disappeared. These data indicate that *AP3* as well as *PI* are required for the late expression of *PI*.

To test whether the 300 bp of the *PI* promoter region is sufficient for *PI* autoregulation, we crossed a 3G construct into two gain-of-function alleles, producing 35S-PI and 35S-AP3 plants. The sepals of 35S-PI flowers are partially transformed to petals (petalloid sepals), but the other organs are not affected (Krizek and Meyerowitz, 1996), and the carpels of 35S-AP3 plants are transformed to stamens or stamenoid carpels (Jack et al., 1994). With the 3G construct, however, 35S-PI showed GUS expression in the petalloid sepals in addition to the petals and stamens (Fig. 4G), and, in the 35S-AP3 plants, GUS activity was observed in the transformed stamens or stamenoid carpels as well as in the normal stamens (Fig. 4H). These transgenic lines were crossed to obtain the double transgenic line (35S-PI;35S-AP3), in which strong GUS activity was observed throughout the flower (Fig. 4I). The localization as well as the strength of the GUS activity was identical to that of 15G in 35S-PI;35S-AP3 (Fig. 4J). These results clearly demonstrate that the 3G construct contains the *cis*-elements that respond to the autoregulatory signals of PI/AP3 and activate the transcription of *PI* in the floral organs. The fact that the GUS activity was observed in the floral organs but not in other tissues suggests the other factor(s) expressed only in the flower itself is required for *PI* autoregulation, as previously noted by Krizek and Meyerowitz, (1996).

Interactions between the PI/AP3 complex and the *PI* promoter are indirect

To investigate *PI* autoregulation at the molecular level, we tested whether the PI/AP3 complex binds to the 300 bp region of the *PI* promoter that is required for autoregulation. Electrophoretic mobility shift assays (EMSA) were used to test both the 300 bp whole region and its overlapping divided fragments for their ability to bind in vitro-translated PI/AP3 protein. None of the fragment could be bound by the PI/AP3 complex (data not shown), which is consistent with there being no CArG box-like sequence in this region. The CArG box is the consensus sequence to which MADS domain-containing proteins such as PI and AP3 can bind in vitro (reviewed by Riechmann and Meyerowitz, 1997). This result suggests the possibility that the *PI* promoter is indirectly regulated by the PI/AP3 complex in the autoregulatory circuit.

To determine whether the modification of existing proteins or de novo gene expression are required for *PI* autoregulation, we assayed the transcriptional activity of the *PI* promoter with and without cycloheximide treatment using the AP3-GR induction system (Sablowski and Meyerowitz, 1998). AP3-GR is a fusion protein of AP3 and the glucocorticoid receptor, and it is activated to functional AP3 under dexamethasone treatment. 35S-PI;35S-AP3-GR;3G triple transgenic flowers were treated for 24 hours with dexamethasone alone or with dexamethasone combined with cycloheximide, and transcripts derived from the *PI* -300 promoter were detected by in situ RNA hybridization with an anti-*uidA* probe. For comparison with the promoter of the putative direct target, AP3::GUS containing 600 bp of 5′ sequences with three putative CArG box (Hill et al., 1998; Tilly et al., 1998) was used (Fig. 5).

When treated with dexamethasone alone, PI::GUS (3G) RNA accumulated in the highly proliferating cells in the second and the third whorls of the young floral meristem (Fig. 5C,D), while this construct was not expressed in early-stage wild-type or untreated flowers (Figs 3G, 5A,B). *uidA* RNA was not detected in the 3G lines treated with cycloheximide combined with dexamethasone (Fig. 5E,F). It did, however, accumulate throughout the floral meristem in an AP3-GUS background when treated with either dexamethasone alone or with dexamethasone combined with cycloheximide (Fig. 5I,J and K,L), whereas the *uidA* RNA was detected only in the second and third whorls of untreated flowers (Fig. 5G,H). These results suggest that *PI* autoregulation requires de novo protein synthesis in addition to PI and AP3 proteins.

LFY and *UFO* affect early expression of *PI*

Mutations in *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*) genes result in a dramatic reduction of *PI* expression (Weigel and Meyerowitz, 1993; Levin and Meyerowitz, 1995). To test whether the early or late expression of *PI* are influenced by *LFY* and *UFO*, we introduced the 15G and 3G constructs into the loss- and gain-of-function alleles of *LFY* and *UFO*. Mutations in *LFY* result in more inflorescence-like flowers that occasionally produce floral organs (Weigel et al., 1992). The 15G transgene is expressed in some of the petalloid and stamenoid organs of *lfy-6* flowers, but not in stage 3 and 4 flowers (Fig. 6A). A similar expression pattern is observed with the 3G construct, although the expression level is rather low (Fig. 6B). These results suggest that *LFY* affects the early but not the late expression of *PI*. In contrast to *lfy* mutants, mutations in *UFO* do not affect the expression patterns of either the 15G or 3G construct (Fig. 6C,D). *ufo* mutants also produce more inflorescence-like flowers with variable homeotic transformations of the floral organs (Ingram et al., 1995; Levin and Meyerowitz, 1995; Wilkinson and Haughn, 1995). We observed GUS expression not only in petalloid and

stamenoid organs but also in filamentous organs of the second and third whorls. Early expression of the 15G construct was also unaffected (data not shown). These results suggest that *PI* expression is not highly influenced by mutations in the *UFO* gene.

If the 300 bp *PI* promoter region contains the *cis* element responsive to *LFY*, the gain-of-function allele of *LFY* (35S-LFY) (Weigel and Nilsson, 1995) should induce the ectopic expression of GUS in non-floral tissues. Ectopic GUS expression has been observed with 15G in the malformed terminal flower at the apex of 35S-LFY's primary shoot (Fig. 6F). GUS activity has also been observed in sectors of cauline leaves (Fig. 6F) and in the inflorescence and floral meristem of young plants (Fig. 6E). In contrast to 15G, the 3G construct showed GUS activity only in the petals and stamens, even in the terminal flower (Fig. 6H), although activity was also observed in the inflorescence of young plants (Fig. 6G, arrowhead). These results suggest that *LFY* affects the early expression of *PI* through the distal region of the *PI* promoter (–1458 to –301). The gain-of-function allele of *UFO* (35S-UFO) causes morphological changes in *Arabidopsis* pleiotropically; flowers have extra stamens and stamenoid carpels in the fourth whorl and sometimes the sepals change to petals/petalloid-sepals and the leaves are progressively lobed (Lee et al., 1997). Both 15G and 3G constructs show GUS activity in the petals and stamens of this variant, including those formed ectopically in the first and fourth whorls and in the lobed edges of young leaves (Fig. 6I-L). These results suggest that *cis*-acting elements responsive to *UFO* lie within the –300 to +1 proximal region of the *PI* promoter or, alternatively, that *UFO* induces a factor that mediates the transcription of *PI* through the PI/AP3 autoregulatory circuit.

LFY and UFO are known to work in combination with each other and with another floral homeotic protein to induce organ identity genes (Lee et al., 1997; Parcy et al., 1998). To see the resulting combinatorial effects on *PI* transcriptional regulation, we crossed 35S-LFY or 35S-UFO with 35S-PI or 35S-AP3. The 35S-LFY;35S-PI plants have first-whorl petals and the 35S-LFY;35S-AP3 plants have fourth-whorl stamens in addition to malformed terminal flowers, i.e., they have simply additive phenotype. The GUS activity of 15G as well as that of 3G was localized in an organ-specific manner, that is, in the petals and stamens, including ones developed ectopically (Fig. 6M and not shown). In 35S-UFO;35S-AP3, no obvious difference in GUS expression was observed between 15G and 3G (data not shown). 35S-UFO;35S-PI has a phenotype similar to that of 35S-AP3;35S-PI, and GUS expression was observed in the whole flowers with either 15G or 3G constructs (Fig. 6N). These results suggest that *AP3* and *UFO* are interchangeable in their role in mediating late *PI* expression. The seedlings of 35S-LFY;35S-UFO are growth-arrested and have no mature leaves, but the shoot meristem of these plants can develop a floral bud-like meristem over time (Parcy et al., 1998). The 3G construct was expressed mainly in this flower-like meristem (Fig. 6P, arrowhead), whereas GUS activity derived from 15G was observed throughout the plant (Fig. 6O). These results indicate that the coexistence of LFY and UFO is sufficient to induce *PI* expression in a flower-independent manner and that the *cis*-acting elements responsive to LFY/UFO lie in the distal region of the *PI* promoter.

DISCUSSION

Early and late expression of *PI* is conferred by different promoter regions

Both primer-extension and S1 nuclease-mapping experiments suggest that the *PI* gene has two transcriptional initiation sites, an observation further supported by the positions of two putative TATA boxes (Fig. 1C). GUS fusion experiments demonstrate that either transcriptional initiation site is functional. With the distal promoter region, both of the transcriptional fusion constructs (15G-t1, 15G-t2) can express GUS in a pattern identical to that of the endogenous *PI* gene (Table 1). These two transcriptional initiation sites, however, are not equivalent since t1 transcriptional fusion without the distal promoter (3G-t1) fails to express GUS, though the t2 transcriptional fusion construct (3G-t2) retains late expression. These results raise the possibility that these two transcriptional initiations are used differentially during development, as has been observed in *Xenopus c-myc* genes (Vriz et al., 1989). That is, t1 may be primarily used for early expression in the context of the distal promoter, whereas t2 may be required for late expression. Further analysis is necessary to confirm this hypothesis.

In order to simultaneously observe the effects of the differential usage of two transcriptional initiation sites, we combined *PI* promoter with the *uidA* gene translationally. However, translational fusion constructs with 698 bp and longer regions of the *PI* promoter have shown unexpected GUS expression in inflorescence meristems (IM) and stage 1 and 2 flowers where endogenous *PI* RNA is not detected. This early mis-expression is reduced in 6G and 15G-t2 (transcriptional fusion) flowers and is almost eliminated in 5G and 15G-t1 flowers (Table 1). These results suggest that translational fusion constructs longer than 6G contain elements leading to early mis-expression of the *uidA* gene. We must consider, however, that the long lifetime of the GUS protein enables GUS activity to reach visible levels even when the transcriptional expression levels are too low to detect. In addition, there is leakage of X-glucuronide products in the lines having strong GUS activity. Another reason for the difference in expression patterns between endogenous *PI* transcripts detected by in situ hybridization and the GUS activity of PI-GUS is that another part of the gene, i.e. introns and 3′ UTR, etc. may act to repress the *PI* expression in the IM. The intron's contribution to the enhanced gene expression of *AGAMOUS* (*AG*) of *Arabidopsis* and *PLENA* of *Antirrhinum*, the C-class floral organ identity genes, has been demonstrated (Sieburth and Meyerowitz, 1997; Bradley et al., 1993).

Our promoter deletion experiments show, however, that major regulatory elements for the spatial and temporal expression of the *PI* gene lie within the 1.5 kb region of the 5′ sequence. Our observations suggest that the *PI* promoter can be split into two regions: the distal region (–1458 to –301), which promotes the initial expression of *PI* in response to induction signals (establishment), and the proximal region (–300 to +1), which promotes the late expression maintained by the PI/AP3 autoregulatory circuit.

Homeotic genes affecting *PI* expression

The distal promoter region bears *cis*-acting elements

responsive to *LFY* and *LFY/UFO* combinatorial regulation. Parcy et al. (1998) have shown that *LFY* together with *UFO* induce *AP3* expression in a flower-independent manner. Our data show that *PI* expression is also induced in seedlings when both *LFY* and *UFO* are expressed constitutively (Fig. 6O). This flower-independent *PI* induction is conferred by the distal promoter because the proximal promoter cannot mediate activation by *LFY/UFO* (Fig. 6P). If *LFY/UFO* activates *PI* expression via *AP3* expression, *LFY/UFO* should activate the transcription from the proximal promoter (3G), but our data suggest that *LFY/UFO* directly activates *PI* via the distal promoter region. The effects of *UFO* alone on the transcriptional regulation of *PI* have been obscure; while Levin and Meyerowitz (1995) have observed a reduction in *PI* transcripts in the *ufo* mutant, Wilkinson and Haughn (1995) have not. We observed no significant difference in PI::GUS expression between the wild type and *ufo-2*, the strong mutant allele (Figs 3A, 6C). However, we found that *PI* expression is activated in the gain-of-function allele of *UFO*. The morphological changes in the flower in 35S-UFO are very similar to those in 35S-AP3; i.e. carpels are transformed to stamens. PI::GUS, either 3G or 15G, is expressed in the fourth whorl stamens as well as in the second whorl petals and third whorl stamens (Fig. 6J,L). These results lead us to speculate that *UFO* affects *PI* expression through *AP3*. Both 3G and 15G were also expressed, however, in the leaf primordia and the lobed regions of leaves of 35S-UFO (Fig. 5I,K), which is not seen in 35S-AP3, suggesting that *UFO* alone, independent of *AP3* expression, is involved in the *PI* regulatory cascades. Taken together, these results suggest that *LFY* and *UFO* either individually or cooperatively affect *PI* expression mediated by the distal promoter.

To clarify the effects of A- and C-class floral organ identity genes on the transcriptional regulation of the B-class gene, we investigated the effects by crossing the PI::GUS construct into gain-of-function alleles of the A- and C-class genes. The GUS expression patterns of 15G and 3G constructs in the 35S-AP1 and 35-AG flowers were organ-specific, suggesting that *AP1* and *AG* may not affect *PI* expression directly but through a change in meristem or organ identity.

Indirect interactions between PI/AP3 and the *PI* promoter

In this study, we have shown that the 250 bp upstream region of the transcriptional initiation site is sufficient for *PI* autoregulatory expression. However, in contrast to both *AP3* and *GLOBOSA* (*GLO*) and *DEFICIENS* (*DEF*), the *Antirrhinum* B-class genes, there is no CArG box-like sequence in this promoter region. In addition to deletion analysis, we used a biochemical approach to define the *cis*-acting elements mediating the PI/AP3 autoregulation, making

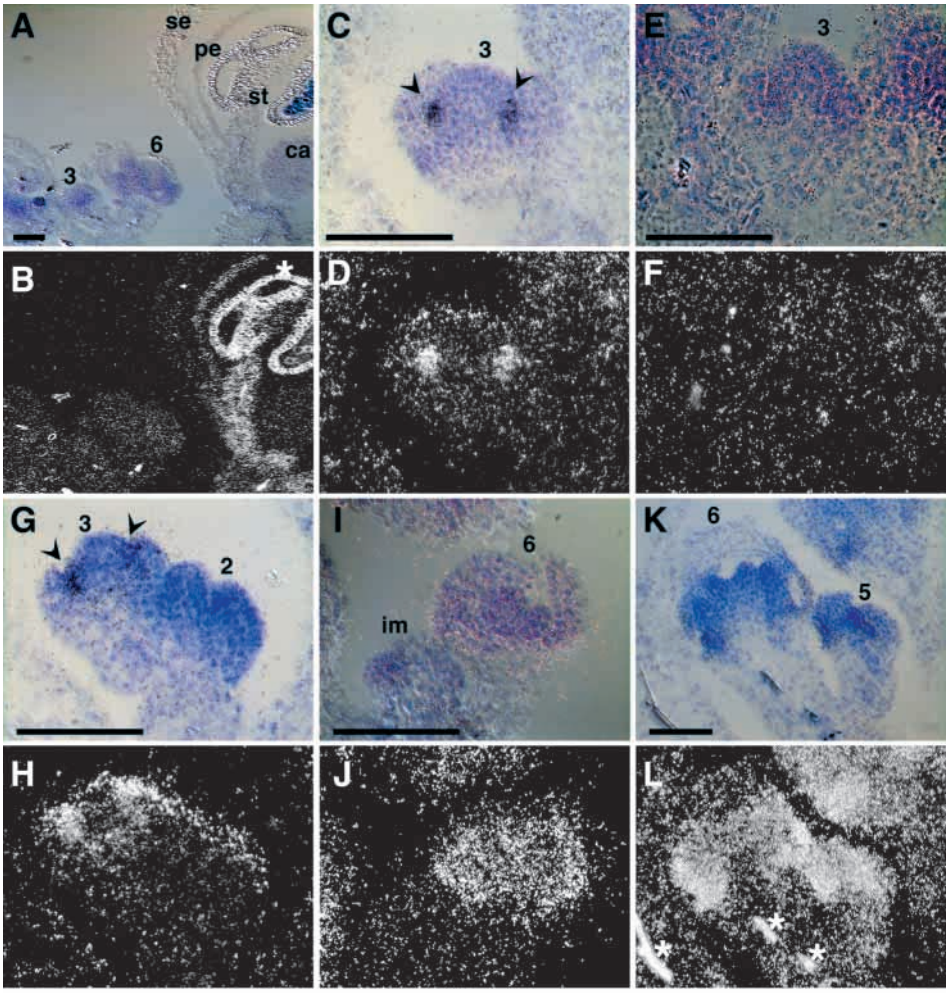

Fig. 5. In situ hybridization of *uidA* mRNA driven by the *PI* or *AP3* promoter in flowers induced by steroid-activated AP3-GR. (A-F) Longitudinal sections of 35S-PI;35S-AP3-GR;PI-GUS (3G) flowers. (A,B) Bright-field (A) and dark-field (B) images of untreated flowers showing hybridization in the petal and stamen of the later-stage flower. (C,D) A stage 3 flower treated with dexamethasone alone showing a hybridization signal in the second whorl (arrowheads). (E,F) A stage 3 flower treated with both dexamethasone and cycloheximide showing no hybridization signal. (G-L) Longitudinal sections of 35S-PI;35S-AP3-GR;AP3-GUS flowers. (G,H) Untreated flower showing hybridization in the second and third whorls of stage 3 (arrowheads) but not stage 2 flowers. (I,J) A stage 6 flower treated with dexamethasone alone showing hybridization in all whorls. (K,L) Stage 5 and 6 flowers treated with both dexamethasone and cycloheximide showing hybridization in all whorls. Note that the cell walls of tapetum and vascular cells show high intensity in the dark-field images (asterisks). Numbers indicate the floral stage. im, inflorescence meristem; se, sepal; pe, petal; st, stamen; ca, carpel. Scale bars, 100 μm.

it possible to locate PI/AP3 binding sites other than the CArG box. The EMSA experiments however, showed no PI/AP3 binding sequence in the 250 bp sequence upstream from the transcriptional initiation site or in the 50 bp untranslated region.

The above results led us to consider whether the post-translational modification of the PI, AP3 protein, or the expression of another protein encoded by a gene downstream from *PI* and *AP3* that acts as a cofactor of the PI/AP3 complex could be required for PI/AP3 and *PI* promoter interactions. SRF, a mammalian MADS-domain protein, changes its DNA binding affinity with protein phosphorylation (Manak et al., 1990; Marais et al., 1992). This phosphorylation site is not conserved in plant MADS proteins, but there is a potential calmodulin-dependent phosphorylation site in the MADS domain (Schwarz-Sommer et al., 1990). The extradenticle protein of *Drosophila* is a cofactor of Hox proteins and increases DNA binding affinity by interactions with these proteins (Chan et al., 1994). We observed that AP3-GR, which is activated by glucocorticoid hormone, can induce PI::GUS (3G) expression without but not with cycloheximide, whereas AP3::GUS is induced regardless of the presence of cycloheximide (Fig. 5). These data show that the PI/AP3 complex requires de novo protein synthesis to upregulate *PI* expression. *uidA* RNA was detected in a small region of the dexamethasone-treated flower of 3G;35S-PI;35S-AP3 (Fig. 5C,D), which can possibly be explained by the expression domain of the new protein being restricted and the 24-hour treatment being insufficient to activate the transcription of PI-GUS in the whole flower. We do not yet know whether the new protein is a cofactor that interacts with PI/AP3 or a transcription factor functioning independently of PI/AP3. To

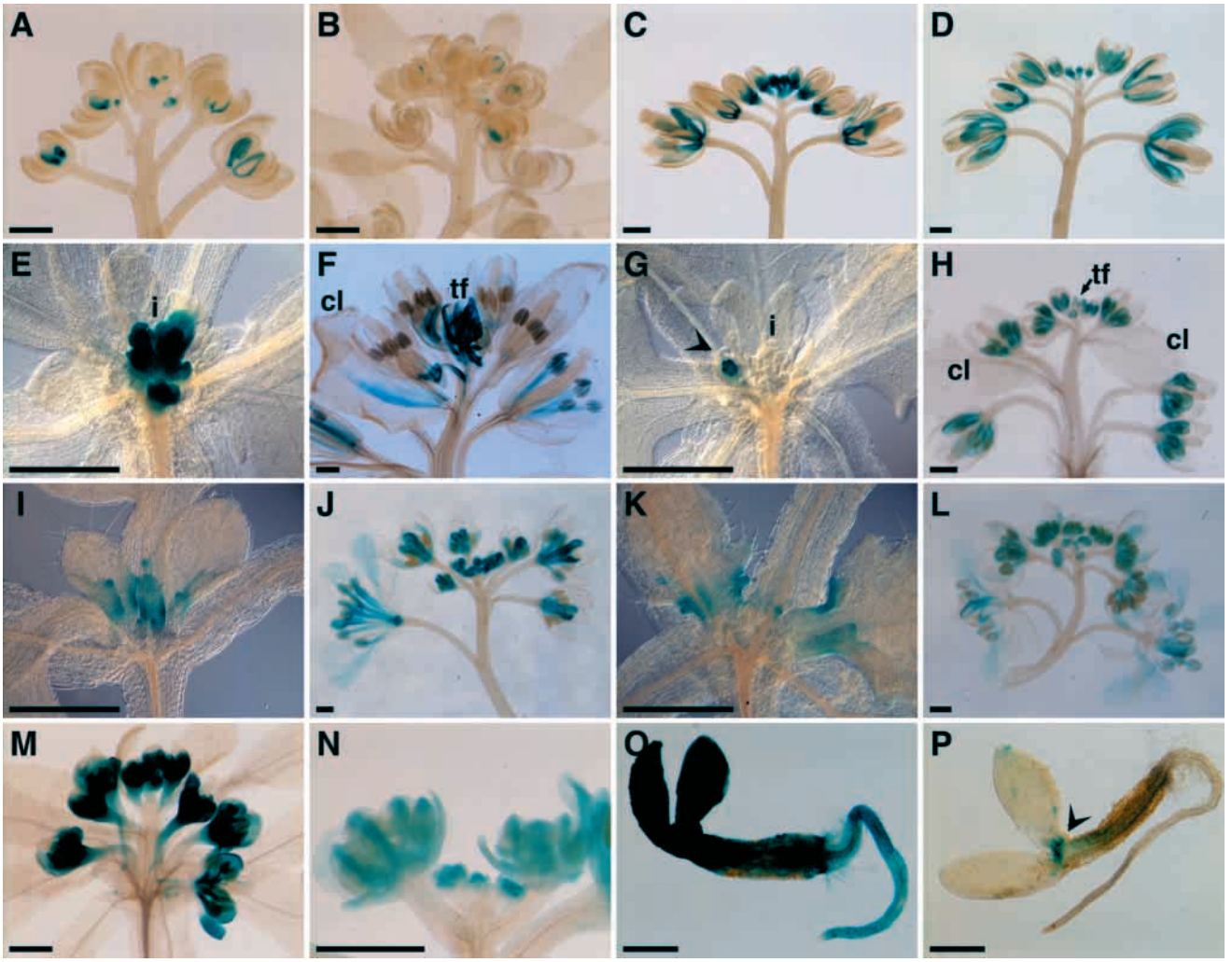

Fig. 6. PI::GUS expression patterns in the loss- and gain-of-function alleles of *LFY* and *UFO*. (A) 15G and (B) 3G expression patterns in the inflorescence of *lfy-6*. (C) 15G and (D) 3G expression patterns in the inflorescence of *ufo-2*. (E,F) 15G expression pattern in 35S-LFY, the gain-of-function allele 13 days and 3 weeks after germination, respectively. (G,H) 3G expression pattern in 35S-LFY 13 days and 3 weeks after germination, respectively. (I,J) 15G expression patterns in 35S-UFO 14 days after germination in the vegetative growth and mature inflorescence, respectively. (K,L) 3G expression patterns in 35S-UFO at 14 days after germination and in mature inflorescence, respectively. (M) 3G expression pattern in the inflorescence of the double transgenic line of 35S-PI;35S-LFY. (N) 3G expression pattern in the inflorescence of the 35S-PI;35S-UFO. 15G (O) and 3G (P) expression patterns in the 35S-LFY;35S-UFO 17 days after germination. These plants have only cotyledons and no mature leaves at the age when normal plants are starting to bolt. i, inflorescence; tf; terminal flower; cl; cauline leaf. Arrowhead in G indicates activity in inflorescence of young plant; in P indicates flower-like meristem. Scale bars, 500 μm.

address this question, cloning of the gene whose product interacts with PI/AP3 is underway.

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