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A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes

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Synthesis of flavonoid pigments in flowers requires the co-ordinated expression of genes encoding enzymes in the phenylpropanoid biosynthetic pathway. Some *cis*-elements involved in the transcriptional control of these genes have been defined. We report binding of petal-specific activities from tobacco and *Antirrhinum majus* (snapdragon) to an element conserved in promoters of phenylpropanoid biosynthetic genes and implicated in expression in flowers. These binding activities were inhibited by antibodies raised against Myb305, a flower-specific Myb protein previously cloned from *Antirrhinum* by sequence homology. Myb305 bound to the same element and formed a DNA-protein complex with the same mobility as the *Antirrhinum* petal protein in electrophoretic mobility shift experiments. Myb305 activated expression from its binding site in yeast and in tobacco protoplasts. In protoplasts, activation also required a G-box-like element, suggesting co-operation with other elements and factors. The results strongly suggest a role for Myb305-related proteins in the activation of phenylpropanoid biosynthetic genes in flowers. This is consistent with the genetically demonstrated role of plant Myb proteins in the regulation of genes involved in flavonoid synthesis.

Key words: flower-specific gene expression/H-box/Myb transcription factor/phenylpropanoid biosynthetic genes

Introduction

Genes involved in the synthesis of flavonoid pigments represent a convenient model for the study of transcriptional control in plants. Pigment production is not essential for plant viability and provides a readily visible marker for gene expression, allowing the isolation of many mutants. These advantages have been used mostly in maize and extensively in snapdragon (*Antirrhinum majus*), where the availability of well-characterized transposons has allowed tagging and cloning of some regulatory genes. This genetical approach has shown that genes encoding flavonoid biosynthetic genes are transcriptionally regulated in maize by genes related to *myb* and *myc* proto-oncogenes (Paz-Ares *et al.*, 1987; Ludwig *et al.*, 1989; Grotewold *et al.*, 1991), and by a *myc*-related gene in *Antirrhinum* (Goodrich *et al.*, 1992).

The developmentally programmed synthesis of pigments is not the only role of flavonoid biosynthetic genes. Flavonoids also participate in responses to stresses such as UV irradiation, wounding and pathogen attack (Hahlbrock and Scheel, 1989) and may be regulators of hormone transport (Jacobs and Rubery, 1988). The flavonoid biosynthetic pathway itself is a branch of the more general phenylpropanoid biosynthetic pathway, which also provides precursors for the production of antimicrobial substances and cell wall components such as lignin and suberin (Hahlbrock and Scheel, 1989). Reflecting this diversity of functions, the promoters of genes involved in flavonoid synthesis direct complex patterns of reporter gene expression in transgenic plants, during development and in response to environmental signals (Bevan *et al.*, 1989; Liang *et al.*, 1989; Ohl *et al.*, 1990; Schmid *et al.*, 1990; Fritze *et al.*, 1991; Hauffe *et al.*, 1991). Biochemical approaches have been used to study how a variety of developmental and environmental signals are combined in the regulation of phenylpropanoid and flavonoid biosynthetic genes.

One of the genes involved in flavonoid synthesis encodes phenylalanine ammonia lyase (PAL), which is the first enzyme in the phenylpropanoid pathway. The promoter of a PAL gene (*gPAL2*) from bean (*Phaseolus vulgaris*) directs expression of the *GUS* reporter gene in tobacco petals (where PAL is needed for flavonoid pigment synthesis), in vascular tissue (where PAL participates in lignin synthesis) and in the response to wounding in potato (Bevan *et al.*, 1989, Liang *et al.*, 1989). The correlation between *gPAL2* promoter expression and PAL function in heterologous systems suggests that the mechanisms controlling its expression are conserved in plants. Similar results have been obtained with the promoters of genes encoding other enzymes involved in flavonoid synthesis, such as chalcone synthase (CHS) and 4-coumarate-CoA-ligase (4-CL) (Schmid *et al.*, 1990; Fritze *et al.*, 1991; Hauffe *et al.*, 1991).

Cis-elements in the promoters of these genes have been defined by *in vivo* footprinting, deletion and mutation analyses (Lois *et al.*, 1989; Schulze-Lefert *et al.*, 1989a,b; Loake *et al.*, 1992; D.Hatton *et al.*, unpublished data). The best characterized elements are related to the sequences described as the G-box and the H-box. G-boxes contain the core sequence CACGTG and are important for the expression of a variety of plant promoters in response to stimuli such as light, anaerobiosis and the hormone abscisic acid (Marcotte *et al.*, 1989; Schulze-Lefert *et al.*, 1989a,b; DeLisle and Ferl, 1990; Donald and Cashmore, 1990). The H-box (CCTACC) (Loake *et al.*, 1992; Yu *et al.*, 1993) is related to a consensus sequence found in several genes encoding enzymes involved in phenylpropanoid biosynthesis (Lois *et al.*, 1989). Elements conforming to this consensus sequence have been implicated in the response to UV light (Schulze-Lefert *et al.*, 1989b) and to other environmental

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stresses (Yu *et al.*, 1993) and in expression in flowers (Sommer *et al.*, 1988; van der Meer *et al.*, 1990).

In the present work, we show that tobacco and *Antirrhinum* petal-specific proteins bind to H-box-like sequences within the promoters of phenylpropanoid biosynthetic genes. The petal proteins are shown to be antigenically related to Myb305, a flower-specific Myb protein, which had been previously cloned from *Antirrhinum* by homology to the conserved Myb DNA binding domain (Jackson *et al.*, 1991). This Myb protein binds to the same element as the petal proteins and activates expression from it in yeast and tobacco cells. The data show that H-box-related sequences in the promoters of genes encoding enzymes involved in phenylpropanoid and flavonoid synthesis in flowers are targets for a flower-specific Myb transcriptional activator. These results, from a biochemical approach, are consistent with the genetically demonstrated function of Myb proteins in the control of flavonoid biosynthetic genes in maize.

Results

A gPAL2 promoter fragment expressed in tobacco petals is bound by a petal-specific protein

Previous work (D.Hatton, M.-H.Yung, R.Sablowski and M.Bevan, in preparation) has shown that a fragment of the *gPAL2* promoter from *Phaseolus vulgaris* (fragment A, from −251 to −150 relative to the transcriptional start) directs expression in xylem and petals of transgenic tobacco (*Nicotiana tabacum* Samsun) when fused to a cauliflower mosaic virus (CaMV) minimal promoter and the *GUS* reporter gene.

As a first step towards understanding the tissue-specific expression from fragment A, the activity of proteins binding to it was examined in nuclear extracts from different tobacco tissues. A binding activity was detected specifically in petal extracts (Figure 1b), more abundantly in petal collars than in petal tubes (Figure 1a). This distribution correlated with the accumulation of pigment in petals and with the higher expression from fragment A in petal collars than in petal tubes (D.Hatton *et al.*, in preparation). Protease digestion confirmed that the petal-specific binding activity depended on a protein component (data not shown).

Figure 1c shows that the petal binding activity appears in late stages of petal development (stages defined by Drews *et al.*, 1992; morphology of flowers in these stages shown in Figure 1a), when pink flavonoid pigment starts to accumulate (Drews *et al.*, 1992). GUS activity in the petal collars of transgenic tobacco was detected after the appearance of the binding activity (Figure 1d). The levels of expression of the *GUS* reporter gene directed by fragment A in mature petals varied between independent transgenic plants, but the profile of expression during petal development was reproducible. Plants transformed with the pBIN421.9 vector alone did not express GUS in petals (not shown).

The results above correlated pigment accumulation and the activity of the *gPAL2* fragment A, during petal development and in different petal regions, with the detection of a protein binding to this promoter fragment.

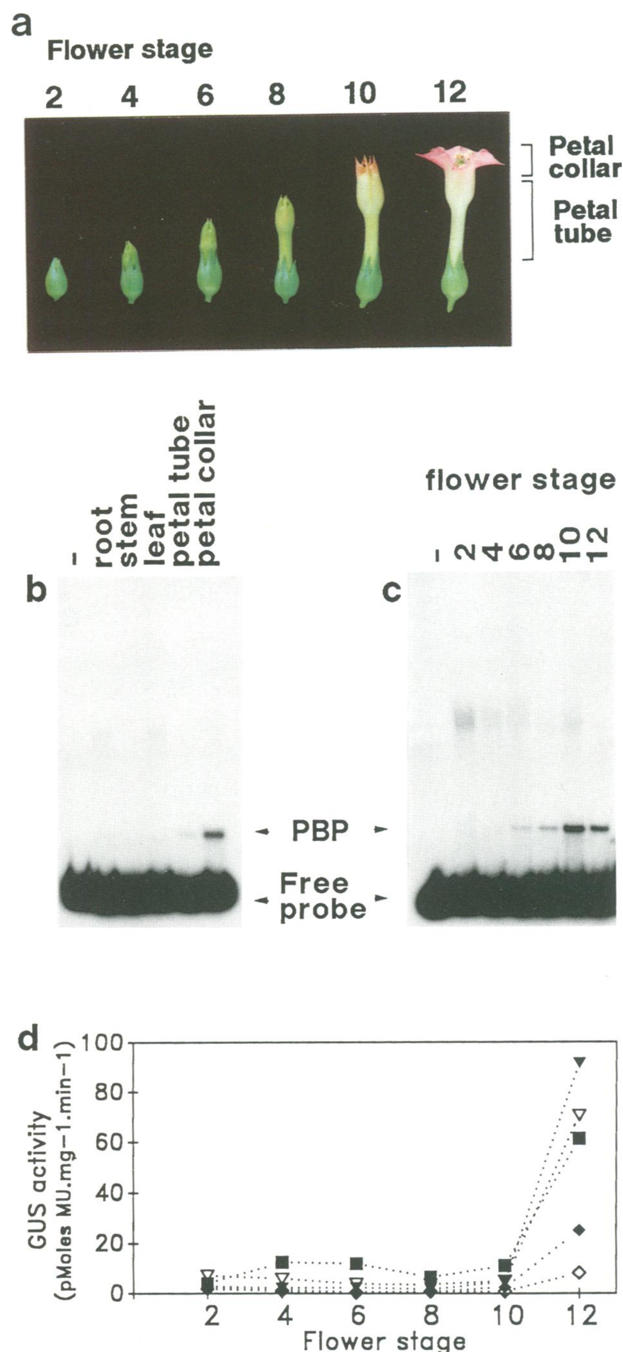


Fig. 1. (**a**) Morphology of tobacco flowers at different stages of development (defined by Drews *et al.*, 1992). The pigmented petal collar and pale petal tube are indicated in the mature flower (stage 12). (**b**) Binding to the *gPAL2* promoter fragment A (see probe A in Figure 2c) by proteins (7 μg per sample) in nuclear extracts from tobacco organs (indicated above each lane), detected by EMSA. Arrows point to the petal-specific binding activity (PBP, see below) and the free probe band. (**c**) Appearance of the petal-specific binding activity during tobacco petal development. EMSA was performed with probe A (see Figure 2c) and 10 μg protein from nuclear extracts from petal collars in different stages of development. The arrows points the petal-specific binding activity (PBP) and the free probe band. (**d**) GUS activity directed in tobacco petal collars by the *gPAL2* promoter fragment (fragment A, see Figure 3). Each symbol represents one independent representative transgenic plant.

The petal-specific activity binds to an element conserved in phenylpropanoid biosynthetic genes

The site bound by the petal-specific activity on fragment A was determined by methylation interference (Figure 2a). The binding site (box P, Figure 2b) is similar to H-box-like elements described in the promoters of several other plant genes involved in flavonoid pigment synthesis (Figure 9). Some of these elements have been implicated in the expression of these promoters in flowers (see Discussion).

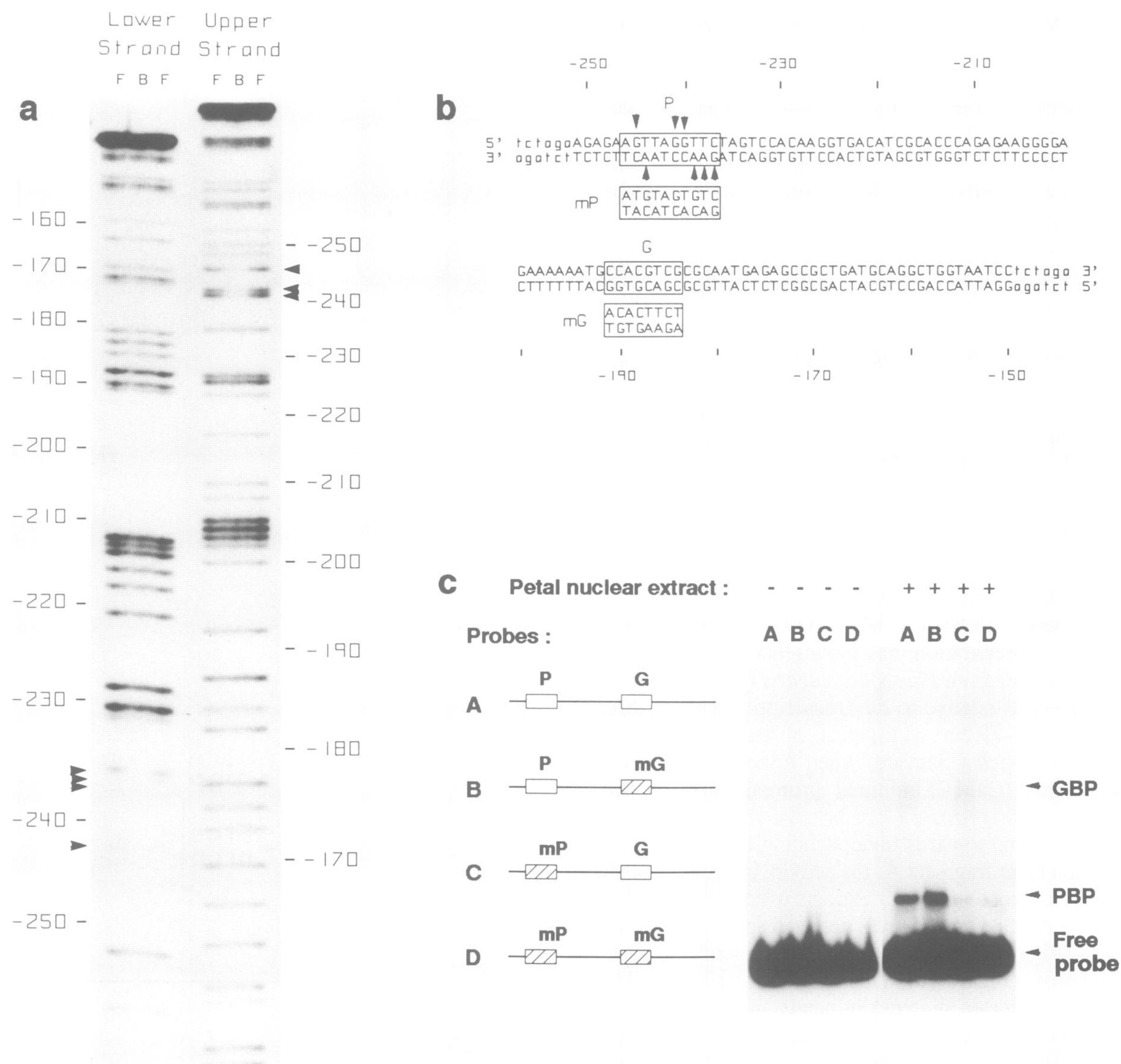


Fig. 2. (a) Binding site of the petal-specific activity in fragment A, determined by methylation interference. Each group of three lanes corresponds to the cleavage products of one of the strands of the methylated DNA probe; F indicates free probe and B indicates probe bound by the petal-specific activity. Numbers indicate positions of nucleotides in the *gPAL2* promoter sequence, relative to the transcriptional start. Arrows point to the positions of nucleotides whose methylation decreases binding by the petal protein. (b) Sequence of the probe used in panel a. Lower case nucleotides correspond to vector sequences and upper case ones to *gPAL2* promoter sequences (numbers show nucleotide positions relative to the transcriptional start). Boxes P (the target site for the petal binding activity) and G (the G-box-like sequence) are indicated. Mutations introduced in these elements are also indicated (mP and mG). Arrows point to the nucleotides whose methylation decreases binding by the petal protein. (c) Effect of mutations in boxes P and G on binding by proteins from tobacco petal nuclear extracts, determined by EMSA. Probe A corresponds to the sequence above; other probes contain the mutated versions of boxes P and G, as indicated (sequences also shown above). Arrows point to the bands corresponding to the GBP, PBP and free probe.

Figure 2b also shows a G-box-like sequence (box G) previously identified in the *gPAL2* promoter (D.Hatton *et al.*, in preparation). Mutations in boxes P and G were introduced in fragment A, which altered G residues identified by the methylation interference, and their effect on the binding to petal proteins was tested. Mutations in box P dramatically reduced binding by the petal-specific protein (P-box-binding protein, PBP; Figure 2c), confirming the result from methylation interference. Figure 2c also shows that box G was the target site of an additional binding activity detected in the petal nuclear extracts (G-box-binding protein, GBP).

The tobacco petal-specific binding activity is recognized by antibodies against a plant Myb protein

Box P contains the sequence CTAACT, which is also part of an element shown to mediate the activation of the maize *Bz1* promoter by the maize Myb protein, C1 (Roth *et al.*, 1991). This element and box P are similar to the vertebrate Myb consensus binding site PyAAC(T/G)G (Biedenkapp *et al.*, 1988; see Figure 9). This, and the involvement of *myb* genes in the control of genes in the maize flavonoid pathway (Paz-Ares *et al.*, 1987; Grotewold *et al.*, 1991), suggested that the petal protein might be a Myb-related transcription factor. To test this hypothesis, several

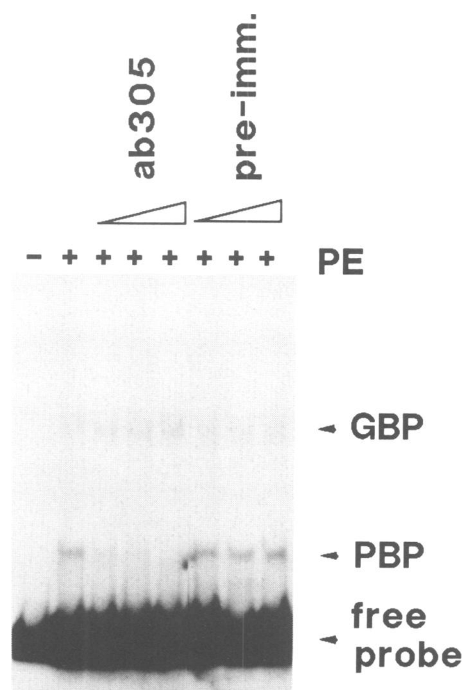


Fig. 3. EMSA showing the inhibition of binding of fragment A by tobacco PBP, caused by pre-incubation with anti-Myb305 antiserum. Samples contained probe A (Figure 2), 3.6 μg protein petal nuclear extract (PE) and increasing amounts (final dilutions 1:2000, 1:1000 or 1:400) of anti-Myb305 antiserum (ab305) or the corresponding pre-immune serum, as indicated. Arrows point to the bands corresponding to the GBP, PBP and free probe.

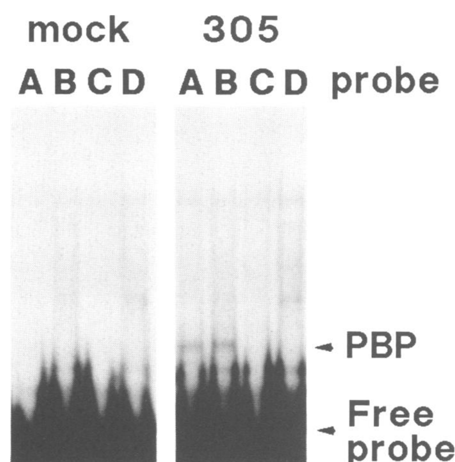


Fig. 4. Binding of *in vitro* synthesized Myb305 to box P in fragment A, shown by EMSA. Probes A, B, C and D are described in Figure 2. Mock-translated extract or Myb305-translated extract was added as indicated. Arrows point to the bands corresponding to PBP and free probe.

antibodies raised against Myb proteins were tested against PBP. Three anti-C1 sera and the BP-52 antiserum, which recognizes the conserved amino-terminal region of v-Myb (Boyle *et al.*, 1986) had no effect on PBP (data not shown), but antibodies raised against the *Antirrhinum* flower-specific Myb305 protein inhibited binding of the tobacco petal protein to fragment A, even at 1:2000 dilution (Figure 3). In the same samples, the anti-Myb305 serum had no effect on the G-box binding activity (GBP), confirming specificity for PBP.

The anti-Myb305 serum did not cross-react in Western blots with the closely related Myb340 protein or any other Myb proteins identified from *Antirrhinum* (V.Hocher, F.A.Culianez-Macia and C.Martin, unpublished data). This suggests that the antiserum is highly specific for Myb305, and that the tobacco petal protein is closely related to Myb305.

Myb305 made in vitro binds to the P-box

If Myb305 is related to the tobacco PBP, the *Antirrhinum* Myb protein should bind to the same DNA sequences as the tobacco protein. To verify this, Myb305 was transcribed and translated *in vitro*, and binding to fragment A was tested in comparison with mock-transcribed/translated extract. A binding activity was detected specifically in the Myb305-mRNA programmed extract (Figure 4). This binding activity was abolished by mutations of box P, indicating that box P is a binding site for Myb305.

Among several other *Antirrhinum* Myb proteins tested, at least two more (Myb315 and Myb340) bound less strongly but detectably to fragment A and shifted the probe to different positions, relative to Myb305 (data not shown). Thus the *gPAL2* promoter can be bound by additional Myb proteins, possibly also binding to box P.

A binding activity from Antirrhinum petal extracts is likely to correspond to Myb305.

A binding activity similar to tobacco PBP was detected in *Antirrhinum* petal extracts (Figure 5), but not in other *Antirrhinum* tissues (data not shown). The time-course of appearance of this binding activity during *Antirrhinum* flower development (Figure 5a) resembled the time-course observed for PBP in tobacco. Binding was also inhibited by mutations in box P (Figure 5b) and by the anti-Myb305 serum (Figure 5c). Retarded bands migrating faster than PBP were also detected (Figure 5b and c). Since these bands resulted from binding to the same site as PBP and were also inhibited by anti-Myb305, they may correspond to proteolysis products of PBP or to other closely related proteins in *Antirrhinum* flowers.

The binding activity identified in *Antirrhinum* petal extracts shifted the fragment A probe to the same position as the *in vitro* synthesized Myb305 (Figure 5d). Binding to the same sequences, the same mobility shift in EMSA and recognition by antibodies against a cloned DNA binding protein can be used as criteria for identifying a cloned factor as a binding activity detected in nuclear extracts (Katagiri and Chua, 1992). Based on these criteria and on the similarity between the time-courses of *Antirrhinum* PBP appearance and Myb305 expression during flower development (Jackson *et al.*, 1991), it seems most likely that the *Antirrhinum* PBP corresponds to Myb305.

Northern analysis detected mRNA from tobacco petals with homology and similar size to Myb305 mRNA (data not shown). The similarities between *Antirrhinum* and tobacco PBPs suggest that the latter could be the tobacco homologue of Myb305.

Antirrhinum PBP binds to an H-box-like element involved in the expression of the Antirrhinum CHS promoter in flowers.

Of all the potential PBP binding sites depicted in Figure 9, the element in the *Antirrhinum CHS* promoter is of particular

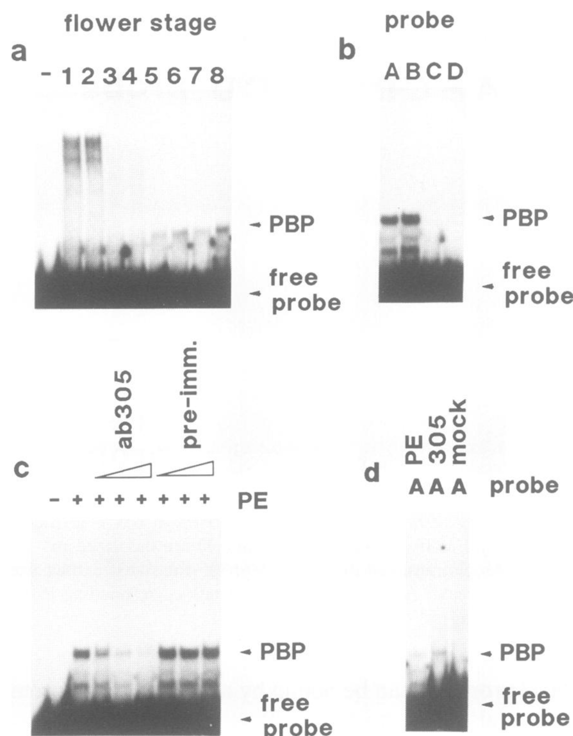


Fig. 5. (a) Appearance of an activity binding to probe A during *Antirrhinum* flower development. EMSA was performed with probe A (see Figure 2c) and 5 μg protein from nuclear extracts from flowers in different stages of development (stages described by Jackson *et al.*, 1991). Arrows point to the bands corresponding to PBP and to free probe. (b) Effect of mutations in boxes P and G on the binding of proteins from *Antirrhinum* petals to fragment A, determined by EMSA. Probes A, B, C and D are described in Figure 2. Arrows point to the bands corresponding to PBP and free probe. (c) EMSA showing the inhibition of the *Antirrhinum* PBP by pre-incubation with anti-Myb305 antiserum. Samples contained probe A (Figure 2), 3.6 μg protein petal nuclear extract (PE) and increasing ammounts (final dilutions 1:2000, 1:1000 or 1:400) of anti-Myb305 antiserum (ab305) or the corresponding pre-immune serum, as indicated. Arrows point to the bands corresponding to PBP and free probe. (d) EMSA comparing *Antirrhinum* PBP and *in vitro* synthesized Myb305. Samples contained *Antirrhinum* petal nuclear extract (PE), Myb305- and mock-translated extracts, as indicated. Arrows point to the bands corresponding to PBP and free probe.

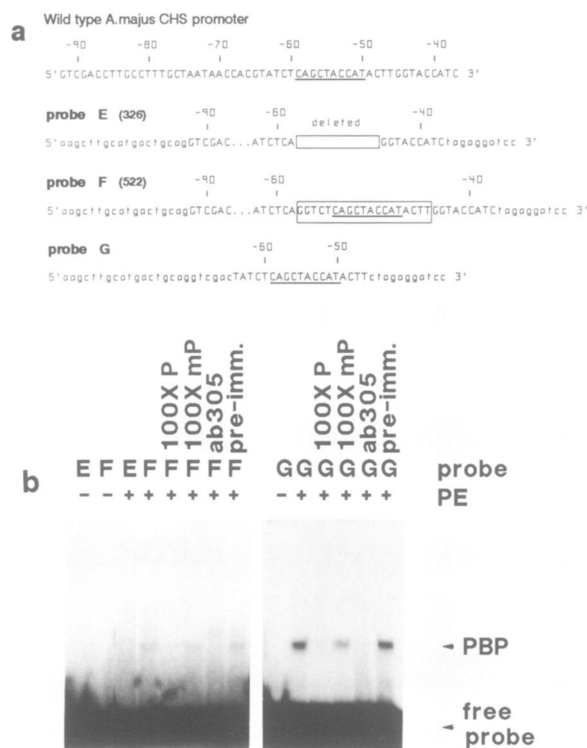


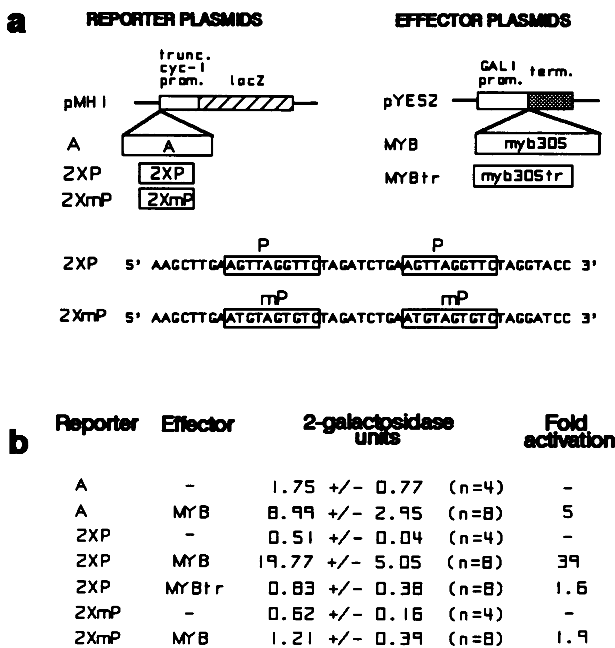
Fig. 6. (a) Sequences of probes derived from the *Antirrhinum CHS* promoter. Nucleotides in lower case correspond to vector sequences and those in upper case to *CHS* promoter sequences. The wild-type *CHS* promoter sequence from nucleotides −92 to −37 relative to the transcriptional start is shown. In probe E (derived from stock JII 326), sequences from −57 to −46 have been deleted; the deleted region is replaced by the boxed sequence in probe F (derived from stock JII 522). Probe G contains only the region of the *CHS* promoter containing the H-box-like sequence flanked by the same vector sequences from probes E and F. The H-box-like sequence is underlined. (b) Binding of *Antirrhinum* PBP to probes E, F and G, determined by EMSA. Samples contained the indicated probes and *Antirrhinum* petal nuclear extract (PE). Indicated samples contained unlabelled oligonucleotides corresponding to a 100-fold molar excess of P-box or its mutated version (sequences in Figure 7), or were pre-incubated with 1:400 final dilution of anti-Myb305 (ab305) or pre-immune serum. Arrows point to the bands corresponding to PBP and free probe.

interest. Binding of PBP to this sequence would link our biochemical data to existing genetic evidence that this element is important for CHS expression in *Antirrhinum* flowers (Sommer *et al.*, 1988; Y.Kishima and C.Martin, unpublished results).

The sequences in Figure 6a correspond to the TATA-proximal region of the *CHS* promoter in two *Antirrhinum* stocks derived from the same original stock which contained a Tam3 insertion in the promoter (Sommer *et al.*, 1985; Y.Kishima and C.Martin, unpublished data). Stock 522 suffered transposon excision, but retained the H-box-like sequence and has wild-type levels of CHS expression. Imprecise transposon excision in stock 36 deleted the H-box-like element and resulted in a 50% reduction of CHS expression. The difference between the two promoters is a 19 bp insertion in 522, containing the H-box-like element. Figure 6b shows that two activities from *Antirrhinum* petals bind to the probe derived from stock 522, but not to the one from stock 326. The faster migrating band corresponded to PBP, since it could be abolished by pre-incubation with anti-Myb305 serum and by competition with excess unlabelled P-box, but not by the mutated P-box. PBP also bound to a probe containing only the H-box-like sequence and a few flanking base pairs from the *Antirrhinum CHS* promoter (Figure 6b). Thus the *Antirrhinum* flower-specific PBP binds to a promoter element known to be involved in the expression of the *CHS* gene in *Antirrhinum* flowers.

Myb305 activates expression from box P in yeast

The results above correlate the binding of Myb305-related proteins with expression activated from H-box-like sequences in flowers. Thus these proteins could function as transcriptional activators. In addition, structural features of the carboxy-terminal region of Myb305 suggest a role in transcriptional activation (Jackson, 1991; Jackson *et al.*, 1991). To test this hypothesis, Myb305 was introduced in yeast in the presence of a reporter construct containing the *gPAL2* fragment A (Figure 7). The reporter construct alone directed some expression in yeast, perhaps reflecting the presence of binding sites for yeast factors. Introduction of Myb305, however, stimulated expression of the reporter gene 5-fold. Mutations in fragment A resulted in high



Reporter	Effector	2-galactosidase units		Fold activation
A	-	1.75 +/- 0.77	(n=4)	-
A	MYB	8.99 +/- 2.95	(n=8)	5
2XP	-	0.51 +/- 0.04	(n=4)	-
2XP	MYB	19.77 +/- 5.05	(n=8)	39
2XP	MYBtr	0.83 +/- 0.38	(n=8)	1.6
2XmP	-	0.62 +/- 0.16	(n=4)	-
2XmP	MYB	1.21 +/- 0.39	(n=8)	1.9

Fig. 7. (a) Constructs used for yeast transformation. Reporter plasmids were derived from the integrating plasmid pMH1 (Materials and methods). Fragments A (Figure 2), 2XP or 2XmP (sequences indicated) were inserted upstream of the truncated *cyc-1* promoter fused to *lacZ*. Effector plasmids contained the complete coding sequence of Myb305 or a truncated version of it (MYBtr, with a carboxy-terminal deletion from Ile174), directed by the *GAL1* promoter in the expression vector pYES2. (b) Transcriptional activation by Myb305 in yeast. Four independent transformants were chosen for each reporter plasmid. Eight transformants containing the effector plasmid corresponded to two colonies chosen after a second transformation of each original reporter transformant. 2-galactosidase assay and enzyme units are described in Materials and methods; each value is the mean for the number of transformants indicated, with the corresponding standard deviation. Fold activation is relative to the corresponding activity in the absence of effector plasmid.

background activities in yeast, which could again reflect interaction with endogenous factors (data not shown). For this reason, reporters with only P-box and its mutated version were tested. Introduction of Myb305 resulted in a 39-fold, P-box-dependent stimulation of expression. Deletion of 34 carboxy-terminal amino acid residues of Myb305 (from Ile174) dramatically reduced this stimulation to 1.6-fold. These results show that Myb305 can activate transcription from its binding site in yeast, and that this function depends on its negatively charged carboxy-terminal region, which is a conserved feature of activation domains of eukaryotic transcription factors (Ptashne, 1988).

Myb305 activates expression from the gPAL2 fragment in tobacco protoplasts

Protoplast transient assays were used to test if Myb305 could also activate expression from its target site in plant cells. Reporter constructs were based on a plasmid containing the TATA sequence of the CaMV 35S promoter (from nucleotide −60 relative to the transcriptional start), upstream of the *GUS* reporter gene (Figure 8a). The *gPAL2* promoter fragment, and its versions with mutated P-box and G-box, were inserted upstream of the CaMV TATA sequence. A plasmid containing the CaMV promoter sequence from nucleotide −90 was used as a control with a sequence also driving expression in protoplasts, but unrelated to fragment A. This sequence includes the well-characterized AS-1 element (Lam *et al.*, 1989) upstream of the TATA sequence.

Figure 8b shows that Myb305 activated expression of the *GUS* reporter in protoplasts, and that this activation was largely mediated by fragment A. The −60 construct directed low levels of expression in tobacco leaf protoplasts. Insertion of fragment A upstream of the CaMV TATA sequence increased expression, suggesting that this *gPAL2* fragment was a target for endogenous transcriptional activators in tobacco protoplasts. Nevertheless, expression was further stimulated 2.5- to 3-fold by co-transformation with a plasmid expressing Myb305, relative to co-transformation with the effector plasmid without Myb305 sequences (this control plasmid and water control yielded comparable results; data not shown). In contrast to the reporter plasmid containing fragment A, expression from constructs −60 and −90 was only slightly stimulated by Myb305. The levels of expression driven by construct −90 were still far from saturating in the transient expression system used, since the complete CaMV 35S promoter could drive GUS expression up to 4-fold higher, in similar experiments (not shown).

Activation from construct −60 by Myb305 varied between experiments, ranging from 1.2- to almost 2-fold. The stimulation from fragment A, however, was consistently higher. The reason for the stimulation from the −60 construct is unclear, but may reflect the accidental presence of a P-box-like sequence in pUC119 upstream of the CaMV promoter insert (not shown).

To investigate in more detail which sequences within fragment A mediate the activation of expression by Myb305 in tobacco protoplasts, the mutated versions of fragment A depicted in Figure 2 were used (Figure 8d). Expression from fragment A in the absence of Myb305 was abolished by mutations in the G-box-like element and reduced by mutations in the P-box element. Therefore these sequences were involved in the activation by endogenous transcription factors in protoplasts. The Myb305-dependent activation was reduced by mutations in the P-box (this is more clearly seen if GUS activities are expressed relative to the levels of the −60 CaMV control, as shown above the bars in Figure 8d). The decreased effect of Myb305 as a result of mutations that abolish its binding site *in vitro* suggests that Myb305 activated transcription by binding directly to the P-box in the promoter fragment. Surprisingly, activation by Myb305 also required the G-box-like sequence, which was not necessary for binding to the promoter fragment *in vitro*. This suggests that Myb305 needs at least one additional factor binding to fragment A, in order to form a functional transcriptional activation complex. Alternatively, another transcription factor could act on the G-box-like sequence independently of Myb305, but the expression of this additional factor could be controlled by Myb305.

Discussion

Myb305-related proteins are involved in the activation of phenylpropanoid biosynthetic genes in flowers

We have shown that sequences found in the promoters of phenylpropanoid and flavonoid biosynthetic genes are bound by Myb-related proteins from tobacco and *Antirrhinum* petals. One of the binding sites described in this work (box P) is similar to the sequence bound by vertebrate Myb proteins, PyAAC(G/T)G (Biedenkapp *et al.*, 1988). Other

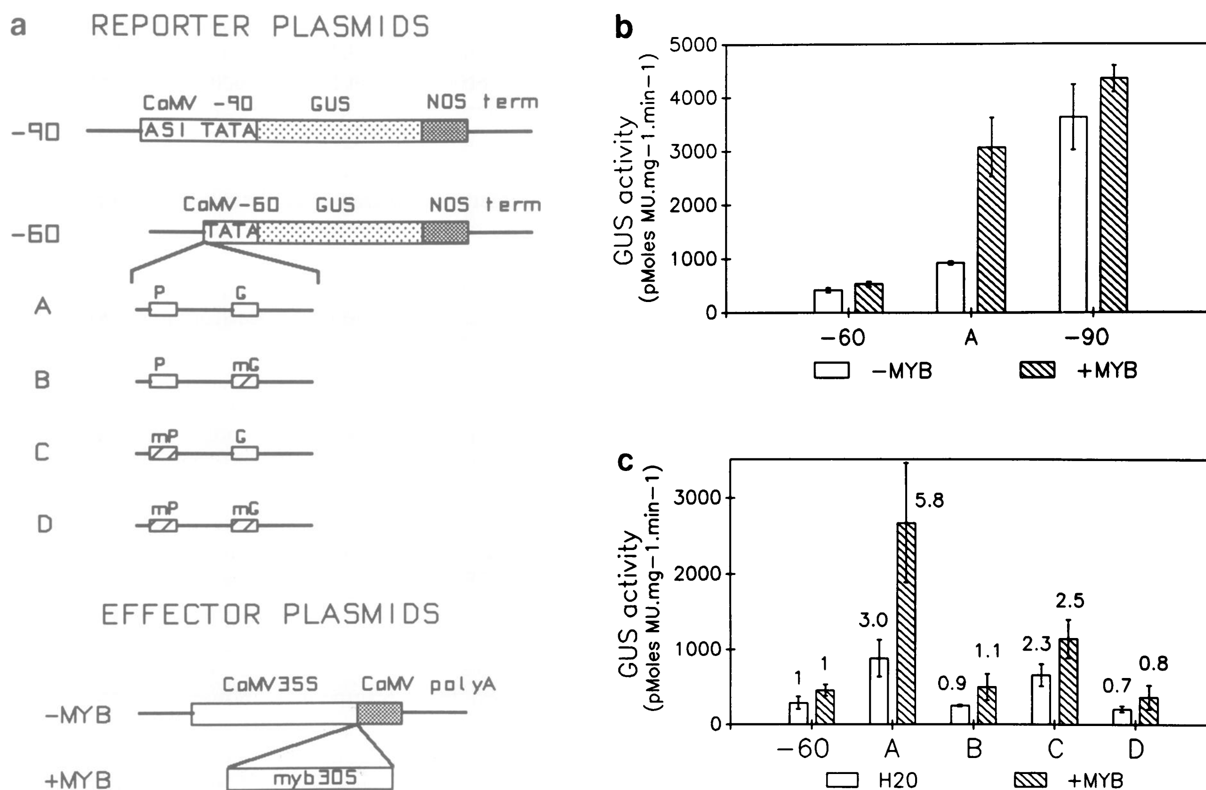


Fig. 8. (**a**) Constructs used for protoplast transformation. Constructs −90 and −60 are pBI221.9 and pBI221.8, respectively (see Materials and methods). −60 contains the TATA region of the CaMV 35S promoter up to nucleotide −60 relative to the transcriptional start; −90 contains the sequence from the same promoter up to nucleotide −90, which also includes the AS-1 element (see text). Constructs A, B, C and D contain the corresponding sequences from Figure 2 inserted upstream of the CaMV TATA region in construct −60. Plasmid −MYB is pJIT60 (Materials and methods); +MYB contains the complete coding sequence of *myb305* inserted in pJIT60. (**b**) Activity of reporter plasmids −60, A or −90 in protoplasts after co-transformation with effector plasmids −MYB or +MYB. Bars indicate mean GUS activities and standard deviation for four separate transformations. (**c**) Effect of mutations in elements P and G on the transactivation by Myb305. The reporter plasmids indicated were transformed into protoplasts, combined with the effector plasmid +MYB or with the same volume of water (H2O). Bars indicate mean GUS activities and standard deviation for four separate transformations. The number above each bar represents the fold activation relative to the −60 construct.

sequences bound specifically by the petal proteins are less similar to the vertebrate Myb target site. These include the binding site in the *Antirrhinum CHS* promoter and two other elements present in the *gPAL2* promoter (R.Sablowski and M.-H.Yung, unpublished data). These sequences suggest a consensus binding site for the petal proteins, which is conserved in the promoters of several phenylpropanoid biosynthetic genes (Lois *et al.*, 1989; Figure 9).

In several cases, the sequences listed in Figure 9 have been implicated in gene expression in flowers. Genetic evidence obtained by *in vivo* mutagenesis resulting from imprecise transposon excision has shown that the PBP binding site in the *Antirrhinum CHS* promoter is important for CHS expression in *Antirrhinum* flowers (Sommer *et al.*, 1988; Y.Kishima and C.Martin, unpublished data). In addition, box P is present within fragment A of the *gPAL2* promoter which drives expression in tobacco petals. The *gPAL2* promoter contains two other binding sites for the tobacco and *Antirrhinum* petal proteins, one on an adjacent 103 bp promoter fragment, which also drives expression in tobacco petals (D.Hatton *et al.*, in preparation) and another identical to the H-box described by Loake *et al.* (1992) in the *CHS15* promoter from bean. This H-box is involved in expression in tobacco petal epidermis (Loake *et al.*, 1992; Yu *et al.*, 1993). In petunia, a 67 bp fragment from the *chsA* promoter which conferred flower-specific expression on a reporter gene contains a sequence related to the binding site in the *Antirrhinum CHS* promoter (van der Meer *et al.*, 1990).

The present work provides strong evidence that a PBP from *Antirrhinum* petals corresponds to the flower-specific Myb305, which has been cloned from *Antirrhinum* by sequence homology to the conserved Myb DNA binding domain (Jackson *et al.*, 1991). Furthermore, a direct interaction has been demonstrated between this cloned plant Myb protein and a sequence from a plant promoter. Transient assays in maize aleurone cells have previously provided evidence that a sequence similar to box P, in the maize *Bz1* promoter (Figure 9), is a target site *in vivo* for the maize Myb protein, C1 (Roth *et al.*, 1991). Nevertheless, a direct interaction between C1 and its target sequence has not yet been demonstrated.

Myb305 not only bound to box P, but also activated expression from promoters containing this sequence in yeast and in tobacco protoplasts. This confirmed the previous suggestion that Myb305 was a transcriptional activator, based on its carboxy-terminal region with overall negative charge and α-helical tendencies (Jackson, 1991).

Taken together, the data imply that proteins related to Myb305 and their binding sites are part of the mechanism which activates expression of phenylpropanoid biosynthetic genes in flowers, although direct genetic evidence for the role of Myb305 *in planta* is presently lacking.

SEQUENCE	SPECIES	GENE	NOTE	REFERENCE
-235 AGAACCTAACTT -246	*Phaseolus vulgaris*	gPAL2	a, b	This work
-131 TCCACCAACCCC -120	*Phaseolus vulgaris*	gPAL2	a, b	Hatton et al., in prep.
- 78 CCCACCTACCAG - 67	*Phaseolus vulgaris*	gPAL2	a	Hatton et al., in prep.
-159 AACTCCTACCTC -148	*Phaseolus vulgaris*	CHS15		Loake et al., 1992
- 66 CTCACCTACCCT - 55	*Phaseolus vulgaris*	CHS15	b	Loake et al., 1992
- 61 CTCAGCTACCAT - 50	*Antirrhinum majus*	CHS	a, b	Sommer et al., 1988
-171 GGTACCTAACCT -160	*Antirrhinum majus*	CHI		Martin et al., 1991
-148 TTCTCCTAACTT -137	*Antirrhinum majus*	DFR		Martin et al., 1991
- 42 ACCTCCTACCAA - 31	*Antirrhinum majus*	Candi		Martin et al., 1991
- 61 ACTATCTACCAT - 50	*Petunia hybrida*	chsA	b	Van der Meer et al., 1990
-141 CCAACCTAACCT -130	*Petroselinum crispum*	CHS	c	Schulze-Lefert et al., 1989b
-115 CTCACCTACCAA -104	*Petroselinum crispum*	PAL-1	c	Lois et al., 1989
-161 CTCACCAACCCC -150	*Petroselinum crispum*	4-CL-1/-2	c	Lois et al., 1989
- 74 CTAACCTACCAC - 63	*Arabidopsis thaliana*	CHS		Ohl et al., 1990
- 77 CACGTCTAACTG - 66	*Zea mays*	Bz1	d	Roth et al., 1991
${}^{A}_{C}ACC^{T}_{A}A^{A}_{C}C$	Consensus			
${}^{T}_{C}AAC^{T}_{G}G$	myb consensus binding site			Biedenkapp et al., 1988

Fig. 9. Sequences related to box P identified in promoters of phenylpropanoid biosynthetic genes. Notes: a, binding site for PBP; b, lies within sequences involved in expression in flowers; c, UV-inducible *in vivo* footprint; d, mediates activation by the myb protein, C1.

The regulation of phenylpropanoid biosynthetic genes in flowers by Myb proteins would be consistent with the genetically demonstrated role of *myb* genes (*C1, Pl*) in the control of flavonoid biosynthetic genes in maize (Paz-Ares *et al.*, 1987; Grotewold *et al.*, 1991). There is evidence for the conservation of factors regulating transcription of flavonoid biosynthetic genes in plants (Goodrich *et al.*, 1992). Some comes from expression of maize regulatory genes in dicotyledonous plants. In *Arabidopsis*, constitutive expression of both *Lc* and *C1* leads to pigment production in tissues where it does not normally occur, showing that these maize genes can also activate flavonoid biosynthesis in an evolutionarily distant plant (Lloyd *et al.*, 1992). The need for both *Lc* (which is *myc*-related) and *C1* (*myb*-related) to activate flavonoid synthesis in *Arabidopsis* is consistent with evidence that Myc and Myb factors act together to activate expression of the *Bz1* target gene in maize (Goff *et al.*, 1990, 1992; Roth *et al.*, 1991). Constitutive expression of *Lc* alone in tobacco leads to increased pigmentation only in flowers (Lloyd *et al.*, 1992). One possible explanation for this flower-specific effect is that Lc may need an endogenous Myb partner to form a functional transcriptional activator. The flower-specific Myb-related tobacco protein described in this work is an obvious candidate.

Myb305 is unlikely to be functionally equivalent to C1/Pl from maize, however, because the homology between Myb305 and C1/Pl does not extend beyound the amino-terminal DNA binding domain (Jackson *et al.*, 1991). In addition, the regulation of flavonoid biosynthetic genes in *Antirrhinum* flowers appears to be more complex than in the maize aleurone. In *Antirrhinum*, genes encoding proteins in the initial steps of the pathway (PAL, CHS and CHI) are regulated differently from those in later steps (F3H, DFR and UFGT) (Martin *et al.*, 1991; Jackson *et al.*, 1992). Also, regulation of the same genes varies in different parts of petals. For example, the regulatory gene *Del* is necessary for the expression of genes in the later steps of flavonoid synthesis in petal tubes, but not in petal lobes (Almeida *et al.*, 1989; Goodrich *et al.*, 1992; Jackson *et al.*, 1992). This example shows that specific *trans*-acting factors can take part in the regulation of a particular segment of the developmental programme of flavonoid synthesis in flowers. The same may apply to Myb305. Pigment production early in petal development precedes accumulation of Myb305 mRNA (Jackson *et al.*, 1991), therefore Myb305 may regulate pigment synthesis only in later stages of petal development. The finding that additional *Antirrhinum* Myb proteins bind to the *gPAL2* fragment bound by Myb305 (R.Sablowski, unpublished data) suggests that other Myb proteins could also regulate pigment synthesis in *Antirrhinum* petals.

Myb305 works in combination with other transcription factors to activate expression

The activation from the *gPAL2* promoter fragment by Myb305 in tobacco protoplasts depended not only on the Myb305 binding site, but also on a G-box-like sequence. This is consistent with several observations that H-box- and G-box-like elements act together to activate expression (Schulze-Lefert *et al.*, 1989b; Loake *et al.*, 1992; Arias *et al.*, 1993). Myb proteins are known to activate expression in combination with other transcription factors, as in the case of C1 and Myc factors from maize, mentioned above. Other examples are the yeast Myb protein, BAS1, which needs a homeodomain protein partner (BAS2) to activate the HIS4 promoter (Tice-Baldwin *et al.*, 1989), and the chicken c-Myb and v-Myb, which activate the *mim-1* promoter in combination with bZIP factors (Burk *et al.*, 1993; Ness *et al.*, 1993). Combined with these data, our results suggest that Myb305 activates the *gPAL2* promoter fragment in combination with an endogenous factor binding to the G-box-like sequence.

A tobacco bZIP protein similar to TAF-1 (Oeda *et al.*, 1991) binds to the G-box-like element in the *gPAL2* promoter

fragment (R.Sablowski and M.Bevan, unpublished data). Therefore a bZIP protein might be the partner of Myb305 in the activation from the *gPAL2* fragment A. Interestingly, however, the G-box core sequence (CACGTG) also matches the Myc consensus binding site (CANNTG; Murre *et al.*, 1989). In addition, the sequence which mediates activation of the maize *Bz1* promoter by the Myc protein, B (Roth *et al.*, 1991), is similar to the G-box-like element in the *gPAL2* promoter fragment. Thus the G-box-like sequence in the *gPAL2* promoter could be the target site for a Myc protein. Interactions between Myb305 and cloned bZIP and Myc proteins are being assessed *in vitro* and in transient assays, to test these hypotheses.

Sequences related to the Myb305 binding site are also involved in responses to UV and pathogens

H-box-like sequences have also been implicated in the response to UV light and other environmental stresses, and in the response of cultured cells to *p*-coumaric acid (Schulze-Lefert *et al.*, 1989b; Loake *et al.*, 1992; Yu *et al.*, 1993). To understand these transcriptional responses, *trans*-acting factors binding to the relevant *cis*-elements have to be identified. Proteins binding to the H-box have been purified and partially characterized biochemically (Yu *et al.*, 1993), but no data are available on their relationship to known transcription factors.

The presence of families of Myb-related proteins in plants, with different patterns of expression (Marocco *et al.*, 1989; Jackson *et al.*, 1991; Avila *et al.*, 1993) implies that these proteins might perform a number of roles during plant development or in responses to the environment. The fact that elements related to the Myb binding site described here are involved in the response to UV light and other stresses raises the question whether Myb proteins may also play a role in those responses.

Materials and methods

General techniques

Standard cloning techniques were used (Sambrook *et al.*, 1989). Mutations in probe sequences were created by PCR (Vallette *et al.*, 1989). DNA sequencing was based on Sanger *et al.* (1977), using a modified T7 polymerase (Pharmacia) and templates prepared according to Murphy and Ward (1990). Protein concentration was measured by the method of Bradford (1976).

Nuclear protein minipreps

The method was modified from M.Hammond-Kosack (in preparation), with buffers described by Holdsworth and Laties (1989). Tissue from the collar region of a single tobacco flower, or comparable amounts from other sources, was ground to powder in liquid nitrogen inside an Eppendorf tube. Nuclei were released in 0.5 ml extraction buffer with 1 mM PMSF and 5 μg/ml of antipain and leupeptin. The slurry was centrifuged at 700 *g* through one layer of Miracloth into a tube containing 0.5 ml of 30% Percoll (Sigma) in extraction buffer. Pelleted nuclei were resuspended in 100 μl dialysis buffer containing 5 mM dithiothreitol (DTT), lysed for 30 min on ice after addition of 10 μl saturated ammonium sulfate, and centrifuged (12 000 *g* for 10 min). Proteins were precipitated from the supernatant with 0.5 vol saturated ammonium sulfate, resuspended in 20 μl dialysis buffer with 5 mM DTT, desalted by spin-dialysis through Bio-Gel 6PDG (Bio-Rad, equilibrated in the same buffer) and kept frozen in liquid nitrogen.

Electrophoretic mobility shift assay (EMSA)

Probes were labelled by filling in 3′ recessed termini with labelled dNTPs, using Klenow polymerase fragment (Sambrook *et al.*, 1989). EMSA was based on Holdsworth and Laties (1989), except that samples (10 μl final volume) contained 50 ng/μl poly(dIdC) and were incubated for 90 min at 4°C, before electrophoresis. Pre-incubation with antibodies was on ice for 30 min before addition of probe.

Methylation interference assay

Methylation interference was performed according to Baldwin (1988).

In vitro transcription and translation

Myb305 mRNA was translated from the cDNA sequence cloned in pBluescript (Stratagene), using an mRNA capping kit (Stratagene) according to the manufacturer's instructions. The mRNA was translated *in vitro* using a rabbit reticulocyte lysate (Amersham) according to the manufacturer's instructions. A mock-translated negative control was prepared in parallel. SDS–PAGE of control translations containing [^{35}S]methionine confirmed the production of a polypeptide of the size expected for Myb305.

Plant transformation

The *gPAL2* promoter fragment was inserted upstream of a fusion of the TATA region (up to nucleotide −60) of the CaMV 35S promoter, with the *GUS* reporter gene, in the vector pBIN421.9 (a derivative of pBIN19; Bevan, 1984). *Nicotiana tabacum* Samsun was transformed by agroinfection (Horsch *et al.*, 1985) with this construct or the vector only. Kanamycin-resistant transformants were grown to maturity in a glasshouse. GUS activity was measured fluorimetrically in petal extracts as described by Jefferson (1987).

Protoplast transformation

Reporter constructs for protoplast transformation (see Figure 8) were based on pBI221.9, which contains a fusion of the CaMV 35S promoter TATA region (up to −60), the *GUS* reporter gene and the nopaline synthase terminator, downstream of the polylinker in pUC19. pBI221.8 corresponds to pBI221.9, with CaMV sequences up to nucleotide −90. The effector plasmid contained the complete Myb305 coding sequence inserted in the polylinker of pJIT60 (J.F.Guerineau, unpublished), which is a pUC-derived vector containing sequences from the CaMV 35S promoter upstream of a polylinker fused to the CaMV polyadenylation signal. All plasmids were purified by banding in CsCl (Sambrook *et al.*, 1989).

Preparation and transformation of tobacco leaf protoplasts was based on Negrutiu *et al.* (1987) (see this reference for composition of solutions). Aliquots of $0.5-1.0 \times 10^5$ protoplasts in 50 μl MaMg were mixed with 5 μg reporter plasmid and 0.5 μg effector plasmid in 10 μl. 50 μl PEG.CMS were added and gently mixed, followed after 20 min by 870 μl 0.2 M $CaCl_2$. After a further 20 min, protoplasts were collected by centrifugation and resuspended in 250 μl MSP.9M (Draper *et al.*, 1988). Transformed protoplasts were incubated for 48 h at 25°C, 16 h photoperiod (170 $\mu E/m^2/s$), then lysed by addition of 50 μl of 5 × concentrated GUS extraction buffer (Jefferson, 1987) and centrifuged; GUS activity was assayed fluorimetrically in the supernatant as described by Jefferson (1987).

Yeast transformation and lacZ assays

Reporter plasmids described in Figure 7 were derived from the integrating plasmid pMH1 (M.Holdsworth, in preparation), which contains truncated *cyc-1* promoter fused to the *lacZ* reporter gene. Effector plasmids were derived from pYES2 (Invitrogen Corp.), wich contains the GAL1-inducible promoter for expression. Yeast cells (*Saccharomyces cerevisiae* strain JB811: *ura3-52*, *leu2*, *trp1*) were transformed with linearized reporter plasmids according to Gietz *et al.* (1992). Plasmid integration was confirmed by Southern analysis. Four transformants for each reporter plasmid were used for a second transformation with effector plasmids. Transformants were grown in liquid medium to an A_{600} of 1.0−1.4 and 2-galactosidase activity was assayed and enzyme units calculated according to Miller (1972).

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