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Promoter analysis of the chalcone synthase (*chs*A) gene of *Petunia hybrida*: a 67 bp promoter region directs flower-specific expression

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Abstract

In order to scan the 5′ flanking region of the chalcone synthase (*chs* A) gene for regulatory sequences involved in directing flower-specific and UV-inducible expression, a chimaeric gene was constructed containing the *chs* A promoter of *Petunia hybrida* (V30), the chloramphenicol acetyl transferase (*cat*) structural sequence as a reporter gene and the *chs* A terminator region of *Petunia hybrida* (V30). This chimaeric gene and 5′ end deletions thereof were introduced into *Petunia* plants with the help of Ti plasmid-derived plant vectors and CAT activity was measured. A 220 bp *chs* A promoter fragment contains *cis*-acting elements conferring flower-specific and UV-inducible expression. A promoter fragment from − 67 to + 1, although at a low level, was still able to direct flower-specific expression but could not drive UV-inducible expression in transgenic *Petunia* seedlings. Molecular analysis of binding of flower nuclear proteins to *chs* A promoter fragments by gel retardation assays showed strong specific binding to the sequences from − 142 to + 81. Promoter sequence comparison of *chs* genes from other plant species, combined with the deletion analysis and gel retardation assays, strongly suggests the involvement of the TACPyAT repeats (− 59 and − 52) in the regulation of organ-specificity of the *chs* A gene in *Petunia hybrida*. We also describe an *in vitro* organ-specific transient expression system, in which flower or purple callus protoplasts are used, that enables us to pre-screen organ-specific expression of a chimaeric reporter gene.

Introduction

Many groups attempt to elucidate the molecular mechanisms underlying regulation of gene expression in higher plants. Few gene systems however allow a thorough analysis of regulatory processes at multiple levels. A powerful model system is provided by the flavonoid biosynthesis route. This pathway results in the synthesis of flower pigments and has been studied in depth at the biochemical, enzymatic and molecular levels in *Petunia hybrida, Zea mays* and *Antirrhinum majus* [24, 33]. Besides their role in floral pigmentation, certain classes of flavonoids function as phytoalexines [21], nodulation signals [22] or protective agents against harmful UV light [5]. Synthesis of the enzymes of the flavonoid biosynthesis pathway has been shown to be coordinate, developmentally and spatially regulated and inducible by a variety of internal and external

factors [32]. Therefore, a complex combination of *cis*-acting elements must be involved in the control of the expression of these flavonoid genes. Chalcone synthase (CHS) represents the key enzyme of flavonoid biosynthesis. In *Petunia hybrida* (line V30) *chs* genes comprise a small multigene family of which one member (*chs* A) is transcribed to a major extent in floral tissue and in UV-irradiated seedlings [18].

In this paper we report the analysis of the *chs* A promoter for the presence of regulatory sequences on three different levels. First, we constructed a chimaeric gene consisting of the 5′ *chs* A flanking region, the structural chloramphenicol acetyl transferase (*cat*) sequence as a reporter gene and the *chs* A terminator sequence, and created 5′ upstream deletion mutants thereof. Prior to plant transformation flower-specific expression of these constructs was pre-screened in an *in vitro* organ-specific transient expression system in which flower or purple callus protoplasts versus leaf protoplasts were used. After testing the chimaeric genes they were introduced into *Petunia* and their expression was analysed. Second, we compared promoter sequences of the *chs* genes of *Petunia hybrida* [18] with those of other plants [10, 14, 29] and with promoter sequences of other flavonoid genes of *P. hybrida* such as chalcone flavonone isomerase (*chi*) [32] and dihydroflavonol reductase (*dfr*) [2]. This comparison revealed stretches of homology that may be involved in regulation of gene expression. Third, we analysed the binding of nuclear protein(s) to *chs* A promoter fragments by gel retardation assays.

Materials and methods

DNA technology

For the introduction and removal of restriction sites in the *chs* and *cat* sequences the M13 oligonucleotide-directed *in vitro* mutagenesis method was used [19]. Standard techniques were used for recombinant DNA work [23]. Deletions of the chimaeric construct on the 5′ end were made by using existing restriction sites in the *chs* A promoter and by Exonuclease III digestion [13].

Transformation and growth of plants

Chimaeric constructs were cloned in the binary vector Bin 19 [3] and *Petunia hybrida* (line W115) plants were transformed as described by van der Krol *et al.* [20]. Primary transformants were self-fertilized and seeds were collected.

Growth of seedlings and UV induction

Seeds of self-fertilized transgenic plants were sterilized and germinated on solidified (0.8% agar) Murashige and Skoog medium [25] supplemented with 3% (w/v) sucrose. The seedlings were maintained at 26 °C in a cycle of 16 h light, 8 h dark. After 7–9 days half of the seedlings per Petri dish were collected and frozen in liquid nitrogen and the remaining seedlings were irradiated for 16 h with light obtained from TL tubes (1 × TL 40W 27, 1 × TL 40W 36, 2 × TL 40W 33, Philips) at 20 cm distance. CAT assay was performed afterwards on irradiated and non-irradiated seedlings. UV induction of transgene expression is defined as an enhancement of at least ten times background CAT activity after UV irradiation.

Nucleic acid extractions and Southern blot analysis

All plasmid DNA isolations were performed as described earlier [23]. Plant DNA was isolated from leaf tissue as described [8]. RNA was extracted from flower buds, stage 4 as defined by Koes *et al.* [18], as described before [16]. To average out small differences in developmental stage of the flower buds, five flower buds taken from one plant were pooled. Southern blot analyses were performed as described by Koes *et al.* [16].

Primer extension analysis

Primer extension experiments using oligomer EL-4 (5′-d GATCAACAGTTGTAGG-3′) or

TS-10 (5′-d TACGATGCCATTGGG-3′) and 5 μg floral RNA (stage 3 as defined by Koes *et al.* [18]) were performed essentially as described previously [17]. Oligomer EL-4 anneals to the 5′ region of the *chs* A gene, position 157 [18] and oligomer TS-10 anneals to the 5′ region of the structural *cat* gene, position 264 [1]. Primers used in the extension experiment were adjusted to a specific activity of 2000 Ci/mmol. Annealing conditions of primer to template mRNA were determined empirically (42 °C for both EL-4 and TS-10). To be able to relate primer extension signals to mRNA steady-state level, an excess of radio-labelled primer was used in the hybridization procedures.

Sequence analysis

DNA sequences were analysed by a combination of dot-matrix, sequence alignment and best-fit methods of the GCG program package [9].

CAT assay

CAT assays were performed according to Gorman *et al.* [11] using limbs of three flower buds (stage 4) per transformant or an equivalent of other plant tissues, like anthers, flower stem, stem, leaves and seedlings. The CAT activity was expressed as the percentage chloramphenicol acetylated/mg protein/h under linear assay conditions. Protein concentrations were determined using the Bio-Rad Protein Assay.

Transient flower-specific expression system

Protoplast were isolated from limbs of flower buds (stage 4 and 5 as described by Koes *et al.* [18]), purple callus [6] or leaf tissue essentially as described by Pröls *et al.* [26]. After sterilization unfolded flower limbs and leaves were cut feather-like with a scalpel and purple callus was mashed for optimal digestion. Flower and purple callus tissue was digested with 1% cellulase R10 (Onozuka) and 0.7% macerozyme R10 (Onozuka), leaf tissue was digested with 1% cellulase and 0.1% macerozyme.

Supercoiled plasmid DNA (20 μg/2 × 10^6 protoplasts) was transferred into freshly prepared protoplasts using the polyethylene glycol (PEG4000) method [26]. After DNA introduction the protoplasts were incubated for 16 h in the dark at 26 °C in 3 cm Petri dishes in 3 ml K3G medium [26], CAT assay was performed and protein concentration was determined with the Bio-Rad Protein Assay.

Preparation of crude nuclear extracts

One gramme of fresh or frozen flower tissue (limbs or anthers) was ground in a mortar with liquid nitrogen and dissolved in 3 ml buffer A [10 mM NaCl, 10 mM MES pH 6.0, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 20 mM β-mercaptoethanol, 0.2 mM PMSF, 0.6% (v/v) Triton X-100 and 0.25 M sucrose]. The solution was filtered through two layers of cheesecloth and centrifuged twice for 10 min at 2000 rpm. The pellet was washed again in buffer A and after centrifugation the pellet was dissolved in 6 ml 5 × buffer A and 45 g Percoll was added. This solution was centrifuged for 5 min at 4000 rpm. The fraction on top of the Percoll gradient was washed twice with buffer A, the pellet resuspended in 2 ml buffer C [20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT] and the nuclei were disrupted by ultrasonic treatment. After 2 h on ice, the sonicated extract was centrifuged for 25 min at 15000 rpm. The supernatant represents the crude nuclear extract and was stored in aliquots at −70 °C.

Gel retardation experiments

Gel retardation experiments were carried out according to Schneider *et al.* [28]. *Chs* A promoter fragments from 170 to 220 bp in length

were end-labelled by filling in the sticky ends with the large fragment of *E. coli* DNA polymerase I and [α-^{32}P] dATP. The radio-labelled fragment was isolated by polyacrylamide gel electrophoresis, and incubated (10–20 fmol DNA, equivalent to 20–40 cps) with 1 μg nuclear extract and 1 μg poly (dI-dC) : (poly dI-dC) at 25 °C for 30 min in binding buffer [25 mM Hepes, pH 7.9, 10 mM $CaCl_2$ 1 mM DTT and 10% (v/v) glycerol]. As non-homologous and homologous competitors we used respectively linear pTZ18 vector DNA and pTZ18 in which a *chs* promoter fragment was cloned. Reaction mixtures were loaded on 5% polyacrylamide gels (4.94 : 0.06% acrylamide : bisacrylamide) and run in TBE buffer [100 mM Tris, 100 mM boric acid and 2 mM EDTA]. The gel was pre-electrophoresed for 2 h at 11 V/cm. Electrophoresis was carried out at the same voltage gradient at room temperature. The gel was dried and autoradiography was performed at − 70 °C using an intensifying screen.

Results

Construction of the chs *A*-cat *fusion genes*

New restriction sites were introduced in the *chs* and *cat* gene to allow a transcriptional gene fusion. Therefore the *chs* A gene was cloned as a *Sph* I-*Hin*d III fragment from the genomic clone VIP 71 [17] in M13mp19 and by the M13 oligonucleotide-directed mutagenesis method a *Nco* I site was introduced at the translation start site (Fig. 1). The *cat* gene [1] was cloned as a *Sal* I fragment in M13mp19 and an *Nco* I site was introduced at the translation start codon, a *Bgl* II site was introduced downstream of the translation stop codon at position 899 [1] while an *Eco* RI site, in the middle of the structural *cat* sequence, was removed (Fig. 1, VIP 116). The mutated *cat* fragment was digested with *Eco* RI and *Bgl* II, the ends filled in and ligated to the 1.2 kbp *chs* A trailer sequence (Fig. 1, VIP 122) cut with *Sma* I, resulting in VIP 123. The mutated 2.4 kbp *chs* A promoter sequence (VIP 119) was digested with *Sal* I and *Nco* I and ligated in front of the *cat* gene in VIP 123 digested with *Sal* I and *Nco* I, resulting in the chimaeric *chs* A-*cat* gene construct VIP 124 (Fig. 1). It has to be noted that this chimaeric gene also contains the *chs* A untranslated leader and tail sequences besides the authentic *chs* A gene promoter and trailer sequences. Several promoter deletion mutants were constructed. The chimaeric gene that contains an 800 bp *chs* A promoter region (see Fig. 3, HIP 125) was obtained by deleting the upstream *Eco* RI promoter fragment from VIP 124. The construct with a 530 bp *chs* A promoter region (Fig. 3, HIP 126) was obtained by deleting the *Hinc* II fragment of VIP 124. The remaining deletion mutants (HIP 127–129) were constructed by using the Exonuclease III/S1 digestion method [13]. The exact promoter length was determined by DNA sequencing. The control construct with a constitutive promoter was made by ligating the cauliflower mosaic virus (CaMV) 35S *Eco* RI-*Bam* HI promoter fragment [20] in VIP 123, digested with *Eco* RI-*Bam* HI (see also Figs. 4 and 6). All constructs were subcloned in the *A. tumefaciens* vector Bin 19 [3] as *Eco* RI and *Hin*d III fragments. Transgenic plants were obtained using the leaf disc method and 20–40 independent transformants per construct were analysed. The number of T-DNA insertions varied from 1–5 copies per genome as determined by Southern blot hybridization (data not shown).

Organ-specific expression of the chimaeric cat *gene in transgenic plants*

The expression of the introduced chimaeric *cat* gene was analysed at the enzyme level in transgenic plants. CAT activity was measured in different tissues of transgenic plants, such as leaf, stem, anthers and corolla of a flower bud and flower stem and in UV-treated seedlings. Of each construct at least 20 independent transformants were analysed for the organ specificity of the expression. Although for some constructs (see also Fig. 3) only a few transgenic plants were obtained that actually showed expression of the transgene, all expressors still showed the same organ specifi-

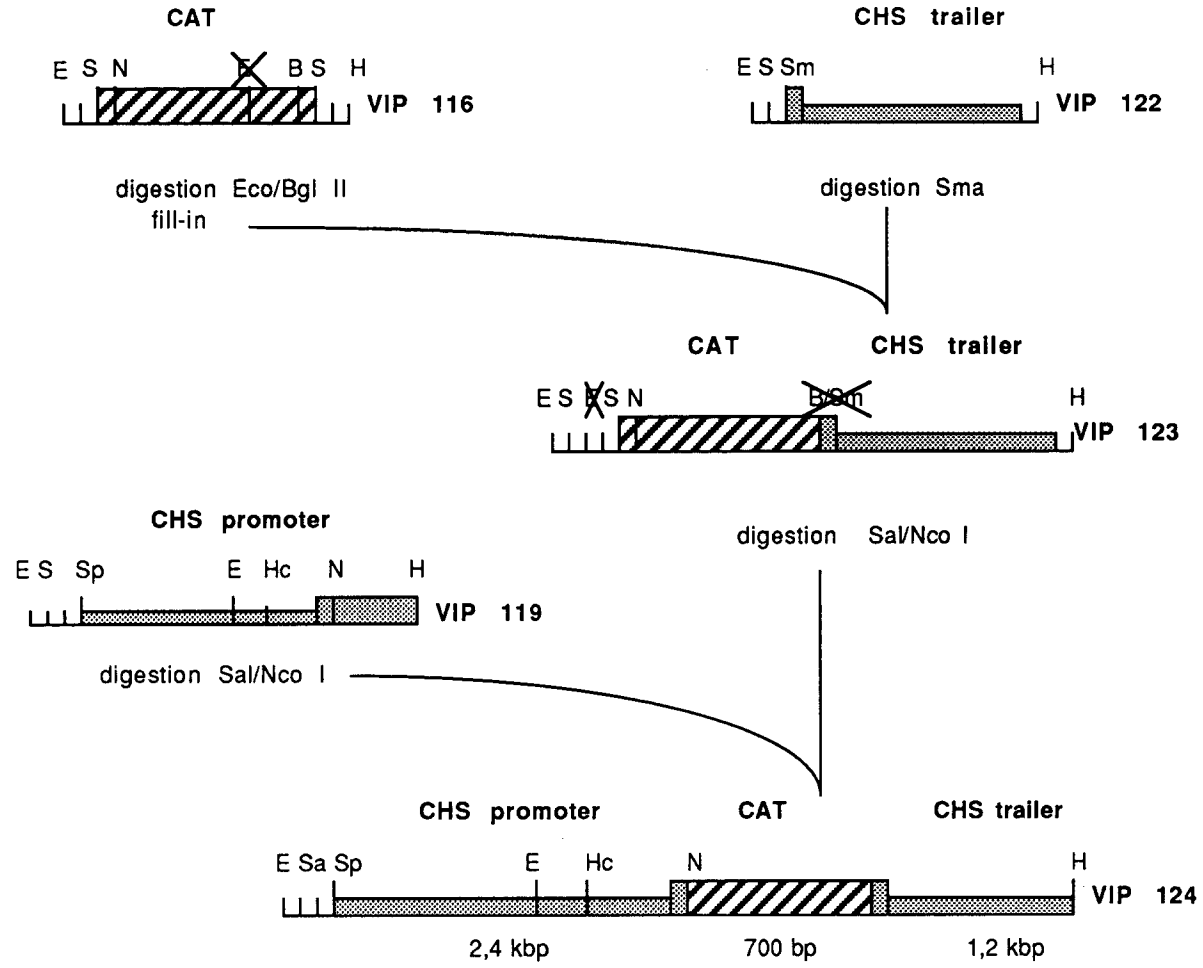

Fig. 1. Construction of the chimaeric *chs*A-*cat* gene (VIP 124).
A *Sal* I fragment of the structural *cat* gene cloned in M13 mp19 (VIP 116) was used for site-directed mutagenesis. At the translation start site an *Nco* I restriction site was introduced and downstream of the translation stop codon a *Bgl* II site. In addition, an *Eco* RI site in the structural gene was removed. The *chs* A trailer was subcloned from the genomic *chs* A clone VIP 71 [17] in pUC 18 (VIP 122). The mutagenized *cat* fragment was digested with *Eco* RI and *Bgl* II, filled in and cloned in front of the *chs* A trailer (VIP 122) that was cut with *Sma* I, resulting in VIP 123. In this way the *Eco* RI site in the polylinker of VIP 116 was destroyed, as well as the *Bgl* II and *Sma* I sites. The *chs* A promoter was subcloned from the genomic *chs* A clone VIP 71 [17] as a *Sph* I-*Hin*d III fragment in M13 mp19. By site-directed mutagenesis an *Nco* I restriction site was introduced at the translation start codon (VIP 119). VIP 119 was digested with *Sal* I and *Nco* I and ligated in VIP 123 digested with *Sal* I and *Nco* I (partially), resulting in the chimaeric gene VIP 124. Non-shaded regions represent pUC 18 polylinker sequences. Restriction enzyme sites are abbreviated as follows: B = *Bgl* II, E = *Eco* RI, H = *Hin*d III, Hc = *Hin*c II, N = *Nco* I, S = *Sal* I, Sm = *Sma* I, Sp = *Sph* I.

city as the endogenous *chs* A gene (Fig. 2, panel A), since CAT activity could be found in pigmented floral organs like corolla and anthers and in the flower stem. Furthermore, the expression of the introduced gene could also be induced in UV-irradiated transgenic seedlings (Fig. 2, panel B). This is consistent with the UV inducibility of the endogenous *chs* gene(s) in seedlings as was found by Koes *et al.* [18]. As can be seen, uninduced seedlings also exhibit a low CAT activity. This is not surprising, since seedlings are often slightly coloured due to stress when

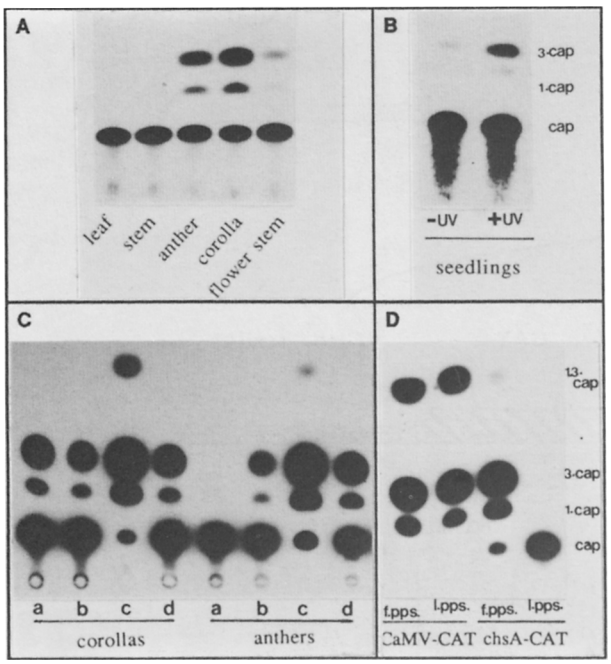

Fig. 2. Tissue-specific and developmentally specific expression of the chimaeric *chs* A-*cat* gene.
Panel A: CAT activity measured in different tissues of a transgenic plant (HIP 125.1). Panel B: UV induction of CAT activity in transgenic seedlings (HIP 125.1). Panel C: CAT activity measured at different stages of flower development in corollas and anthers (HIP 125.1). Developmental stages are defined by bud sizes: a = 0–2 cm, b = 2–4 cm, c = 4–6 cm, d = open flower with open anthers. Panel D: transient CAT activity in flower protoplasts (f.pps.) and leaf protoplasts (l.pps.) after introduction of different chimaeric constructs (HIP 125 and HIP 130). Positions of the chloramphenicol and its acetylated derivatives are indicated, abbreviated as: cap, chloramphenicol; 1-cap, 1-acetate chloramphenicol; 3-cap, 3-acetate chloramphenicol; 1,3-cap, 1,3-diacetate chloramphenicol.

germinated in a Petri dish. CAT activity was also measured in corollas and anthers of flower buds of different stages (Fig. 2, panel C). The introduced gene showed an expression pattern during flower development in corollas and anthers similar to that of the resident *chs* A gene, as was reported by Koes *et al.* [18]. In contrast, the CaMV-*cat* construct was constitutively expressed in all tissues measured, and CAT activity in seedlings could not be enhanced by UV irradiation (data not shown).

A transient organ-specific expression system

Since it takes a long time to obtain and analyse transgenic plants we have developed a transient flower-specific expression system in order to prescreen chimaeric gene constructs. Introduction of the chimaeric *cat* genes by a PEG-mediated transfer procedure (see Materials and methods) in protoplasts isolated from flower and leaf tissue, resulted in a organ-specific transient expression pattern (Fig. 2, panel D). The constitutive CaMV-*cat* gene (HIP 130) was transiently expressed in both types of protoplasts; the *chs* A-*cat* gene, however, was only expressed in flower protoplasts (Fig. 2, panel D). Because of the difficulty in obtaining a large amount of protoplasts from flower tissue, purple callus protoplasts were tried as an alternative. Purple callus (AK5000) is a mutant line of the undifferentiated white Petunia callus AK1000 [6]. Organ-specific transient expression of the *chs* A-*cat* construct was also found in purple callus protoplasts (data not shown). All 5′-deletion mutants of the chimaeric construct were used in the transient flower-specific expression assay, and their flower specificity was analysed before introduction in the plant. The results of these *in vitro* experiments (as shown in Fig. 4, last row) are in agreement with the *in vivo* results qualitatively. However, we did not attempt to quantitate the levels of expression since these *in vitro* experiments may not reflect the expression levels *in planta*.

Comparing expression levels of the different deletion mutants: a silencer is present between − 2.4 kbp and − 800 bp, whereas a 67 bp chs *A promoter fragment still directs expression in flowers*

For each construct a large variation in CAT activity between different independent transformants was found (Fig. 3). The difference in expression level is not correlated to the number of inserted transgenes, but is probably caused by plant sequences that surround the insertion site. To be able to compare the expression levels of the different deletion mutants, the CAT activities of at

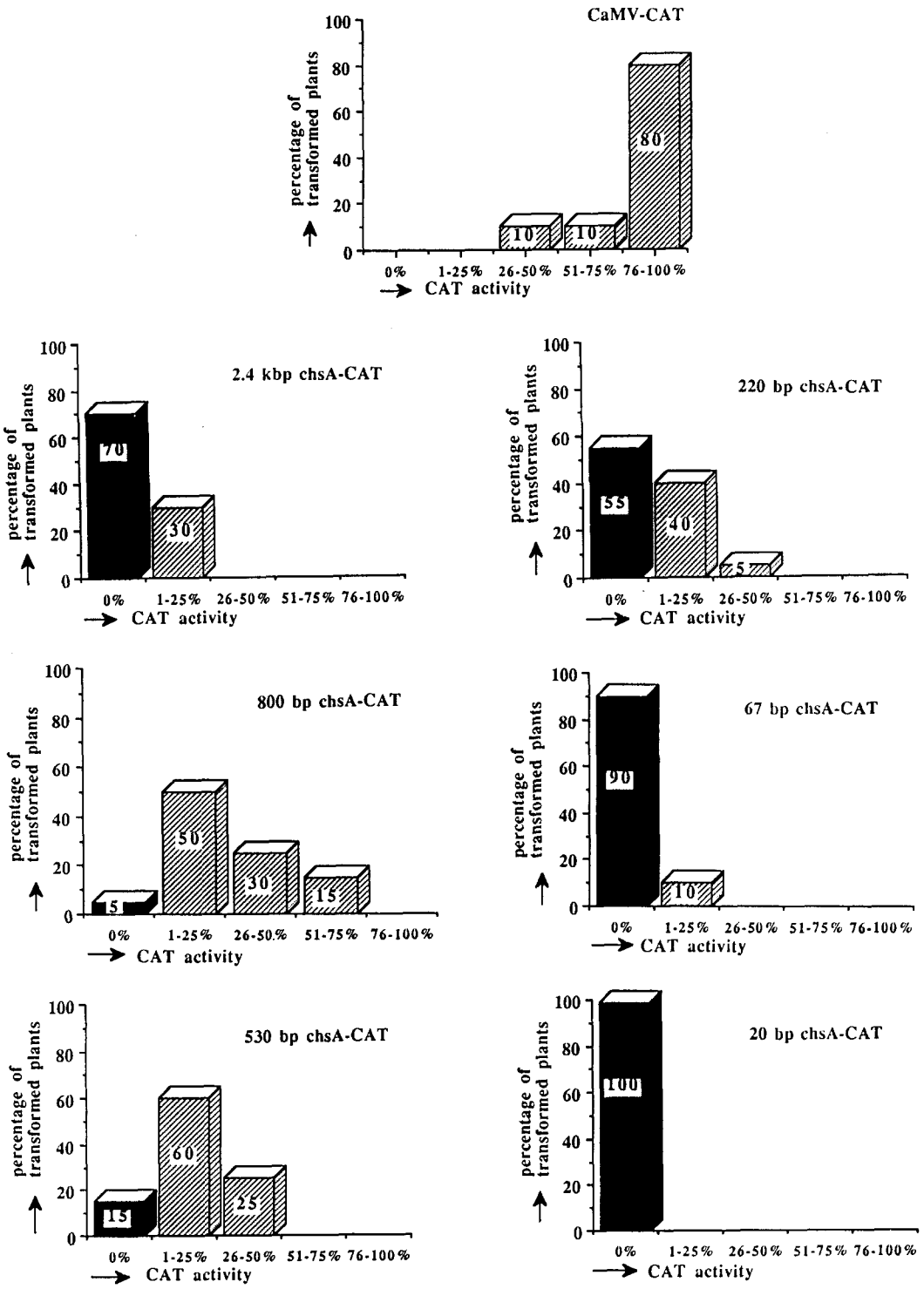

Fig. 3. Histograms showing the variation of the CAT activity found per construct.
Of each independent transformant the CAT activity in corollas of flower buds was determined and the results were plotted per construct. In a histogram the CAT activities are divided into groups with different levels of activity (X-axis) and plotted against the percentage of transformed plants that exhibit this activity (Y-axis). The CAT activity is expressed as the percentage chloramphenicol acetylated/mg protein/h under linear conditions. Per construct 20–40 independent transformants were used.

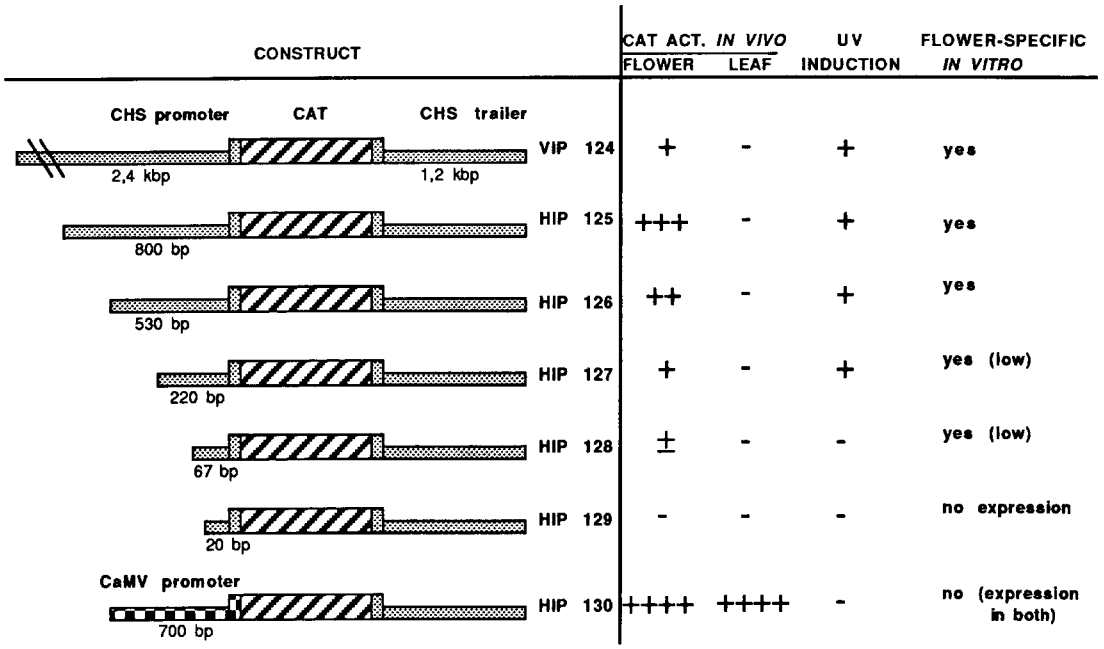

Fig. 4. Overview of the *in vivo* and *in vitro* expression of the chimaeric constructs.
Schematic representation of the organ specificity (CAT activity measured in corollas, anthers and stem of flowers versus leaves) and UV inducibility (CAT activity measured in transgenic seedlings after UV irradiation) of the expression of each construct. Their ability to give rise to organ-specific transient expression in flower protoplasts versus leaf protoplasts is presented in the last column. The CaMV-*cat* gene shows transient expression in leaf protoplasts as well as flower protoplasts. The CAT activity *in vivo* is presented schematically as follows: no activity (−), average activity 1–5% (±), average activity 5–15% (+), average activity 16–30% (++), average activity 31–45% (+++) and an average activity higher than 46% (++++). UV induction is defined as described in Materials and methods.

least 20 independent transformants were measured in corollas of flower buds and plotted per construct. In Fig. 3 expression levels are represented by block diagrams, visualizing the variation of CAT activity found for the individual transformants per construct. The CaMV-*cat* gene was expressed in all transformed plants and the expression level was relatively high. In contrast to this, 70% of the transformants containing the 2.4 kbp *chs* A-*cat* construct were devoid of CAT activity (presented as a black bar in Fig. 3), and the remaining transformants showed a very low CAT activity. Deletion of a large part of the upstream promoter region of the chimaeric construct (leaving an 800 bp *chs* A promoter) resulted in an increase of *cat* expression. Not only the percentage of expressors was highly increased (from 30% to 95%), but also higher CAT activities were obtained (Fig. 3). These results point to the presence of a silencer [15] between positions −2400 and −800. Further deletions of the *chs* A promoter region reduced not only the percentage of CAT expressors (85% for 530 bp *chs* A-*cat* to 45% for 220 bp *chs* A-*cat*) but also the levels of CAT activity. Ten percent of the transformants (2 out of 20) containing the 67 bp *chs* A-*cat* construct still exhibited flower-specific CAT activity. As expected, further deletion of the promoter fragment including the TATA box completely abolished the expression of the *cat* gene (Fig. 3).

Expression pattern of the different deletion mutants: expression driven by the 67 bp chs *promoter is still flower-specific but not UV-inducible*

To determine whether deletion within the *chs* A promoter region might change the organ specificity of the transgene expression, CAT activity was

also measured in other parts of the transgenic plants transformed with the different constructs, like in different parts of the flower (anthers, flower stem) and in leaves. Furthermore, seedlings of self-fertilized transformed plants were UV irradiated and induction of CAT activity was measured. In Fig. 4 the organ-specificity and UV inducibility of the expression of each construct is shown. The constitutive CaMV-*cat* gene is expressed in both flower and leaf tissue, and is not UV-inducible in transgenic seedlings. Chimaeric *chs* A-*cat* genes retain their organ specificity as well as their UV inducibility with promoters as short as 220 bp. Further deletion of the 5′ flanking region to 67 bp does not affect the organ specificity. In fact, the expressors exhibited the same developmental expression pattern in flower organs as the other *chs-cat* transgenes, whereas no expression was found in other non-pigmented organs. However, no detectable UV induction was found in seedlings of these plants.

Chimaeric chs *A*-cat *RNA steady-state level is less than 1% of the endogenous* chs *mRNA steady-state level*

To be able to relate the expression of the introduced *chs* A-*cat* and CaMV-*cat* genes to that of the endogenous *chs* gene, primer extension experiments were performed (Fig. 5). RNA was isolated from pooled flower buds of expressors of the different constructs (VIP 124 and HIP 130) and primer extension experiments were done as described in Materials and methods, using two primers; one specific for the introduced *cat* genes and one specific for the endogenous *chs* gene. Because the radio-labelled primers had the same specific activity and the annealing was performed under saturation conditions, the primer extension signals reflect the mRNA steady-state levels of the exogenous *cat* and endogenous *chs* genes. The steady-state CaMV 35S-*cat* mRNA level is comparable to that of the endogenous *chs* mRNA (Fig. 5, lane 2 versus lane 1). The level of the introduced *chs* A-*cat* mRNA however was less than 1% of the endogenous mRNA *chs* level (Fig. 5, lane 4 versus lane 3). By running the products of a sequence reaction primed by TS10 on the chimaeric *chs* A-*cat* construct on the same gel (Fig. 5), it could be concluded that the proper transcription start site was used *in planta*.

chs *A promoter sequence analysis: putative* cis-*acting elements involved in regulation of gene expression*

Comparison of the 5′ flanking regions of *chs* A and *chs*J of *Petunia hybrida* showed significant stretches of homology [18]. The major region of homology is observed between −188 of *chs* A down to the CAP site and includes a two-fold repetition of the sequence TACPyAT (around −56), also found in the *Antirrhinum majur chs* gene [29]. In this plant species this sequence has been shown to be of crucial importance for expression of the *chs* gene [30]. Further homology with the *A. majus chs* promoter region is found between position −97 to −83 (box I) and −129 to −112 (box II) (Fig. 6). Box I also contains a CACGTG motif, a sequence common to the promoters of a number of light-inducible genes [12]. Previously, the CACGTG motif in the parsley *chs* promoter was shown to be necessary for UV-inducible transient expression in protoplasts [27]. Comparison of the *chs* promoters with other flavonoid gene promoters that were cloned from petunia (*chi* genes by van Tunen *et al.* [32] and *dfr* by Beld *et al.* [2]) revealed a strong homology in the promoter sequence of all genes that are active in immature anther tissue. Therefore this sequence was designated as the 'anther box' [34]. All these boxes can be regarded as putative *cis*-acting elements that could be involved in regulation of *chs* gene expression. With regard to the elements mentioned above, it should be noticed that the 220 bp *chs* A-*cat* construct does not contain the 'anther box', and the 67 bp *chs* A-*cat* construct lacks all the boxes except for the TACPyAT sequence, and that these constructs are still expressed in both corolla and anther tissue of the flowers (see also Fig. 4 and Fig. 6; the promoter lengths of these constructs are marked with arrows in the sequence).

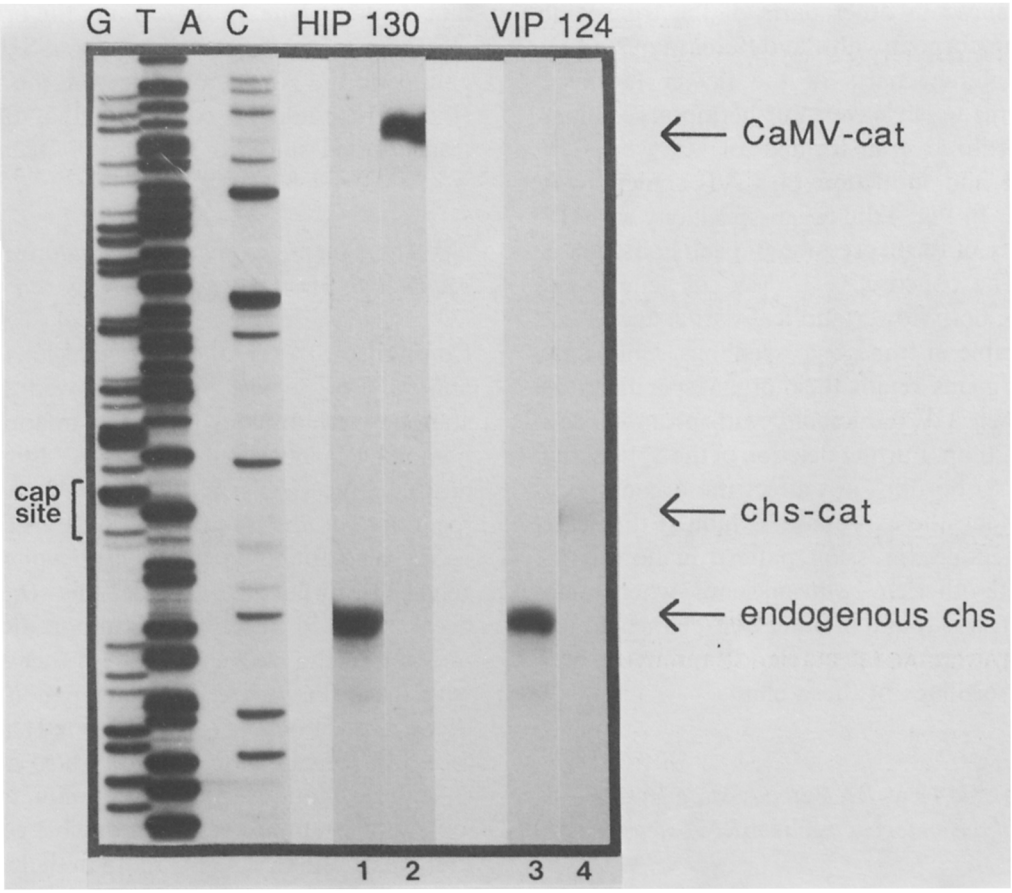

Fig. 5. Primer extension analysis of RNA isolated from transgenic plants.
Total RNA was isolated from flower buds of transformants containing the CaMV-*cat* construct (HIP 130) or the *chs* A-*cat* construct (VIP 124), and analysed for the endogenous *chs* mRNA level (lane 1: HIP 130; lane 3: VIP 124) and the exogenous *cat* mRNA level (lane 2: HIP 130; and lane 4: VIP 124). To determine if the right transcription start site was used a sequence ladder prepared with TS10 on VIP 124 was run on the same gel.

Specific binding of flower nuclear protein(s) to chs *A promoter fragments, as revealed by gel retardation*

Using the appropriate binding conditions (see Materials and methods) it was possible to obtain a flower nuclear protein-DNA complex with *chs* A promoter fragments, which could be visualized by gel retardation experiments (Fig. 7 panel A, lanes 2–4). Binding was flower-specific (leaf nuclear extracts did not bind) and could be competed for by twice the amount of unlabelled *chs* A promoter fragment, but not by excess of aspecific DNA (lanes 5–10). Also other flavonoid gene promoters (*chs*J, *chi* A and *dfr* A) could compete for the binding (data not shown). Several *chs* A promoter fragments were used. The strongest signal of retention was found with the − 142 to + 81 fragment (lanes 2–10), weaker retention with the − 362 to − 142 region (lanes 11 and 12) and the weakest signal of bound DNA was found with the − 245 to − 72 region (lanes 13 and 14). In panel B (Fig. 7) the *chs* A promoter and leader sequence, in which the putative *cis*-acting elements are marked, is drawn schematically. The fragments that were used in the retardation assays are shown below this sequence, as well as their relative binding strengths. The results presented in Fig. 7 suggest at least that the strong binding of

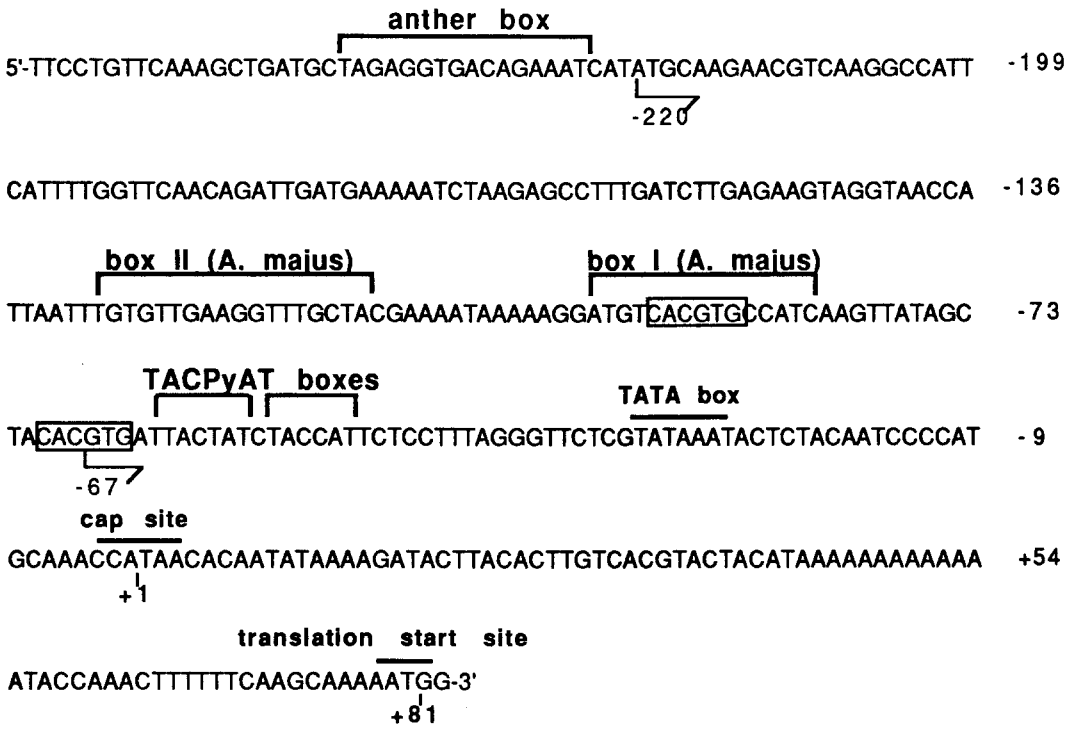

Fig. 6. Putative regulatory sequences in the promoter of the chs (A) gene.
Numbering of the nucleotides is relative to the CAP site. Putative regulatory sequences are marked, the CACGTG motifs are marked with rectangles. The promoter fragments that were present in 5′-deletion mutants of interest are marked with arrows.

the − 142 to + 81 fragment is caused by either the presence of the putative *cis*-acting elements within the − 72 to + 81 region or by a combination of elements within this fragment and sequences present in the upstream region (− 142 to − 72). Furthermore, the data show that neither box I, box II, a single CACGTG motif or a combination of these boxes significantly binds to nuclear proteins. The results also point to the presence of additional protein binding sequences in the region between − 362 and − 245.

Discussion

In this paper we show that the *cat* reporter gene, driven by the petunia *chs* A promoter is expressed organ-specifically in transgenic petunia plants, and exhibits the same developmental regulation as the endogenous *chs* gene. The expression of the chimaeric gene is also inducible by UV light in seedlings of transformants. In order to delimit the *cis*-acting elements involved in the regulation of *chs* gene expression several strategies were followed. A series of 5′ end deletions of the *chs* A-*cat* chimaeric gene were introduced into petunia, and their expression was analysed in at least 20 independent transformants. Only 30% of the plants transformed with the construct containing the largest 5′ (2.4 kbp) *chs* A flanking region shows CAT activity that is still organ-specific (Fig. 3). Deletion of the *chs* A region from − 2400 to − 800 leads to an increase of the percentage of expressors (95%) as well as to an increase of the CAT activity. This result indicates the presence of a silencer in the *chs* A promoter between − 2400 and − 800. A similar silencer has been reported by Kaulen *et al.* for the parsley *chs* promoter [15]. We cannot exclude however that this increase of activity is due to enhancement by the T-DNA sequences that are closer to the transcription initiation site in this construct. Further deletion of

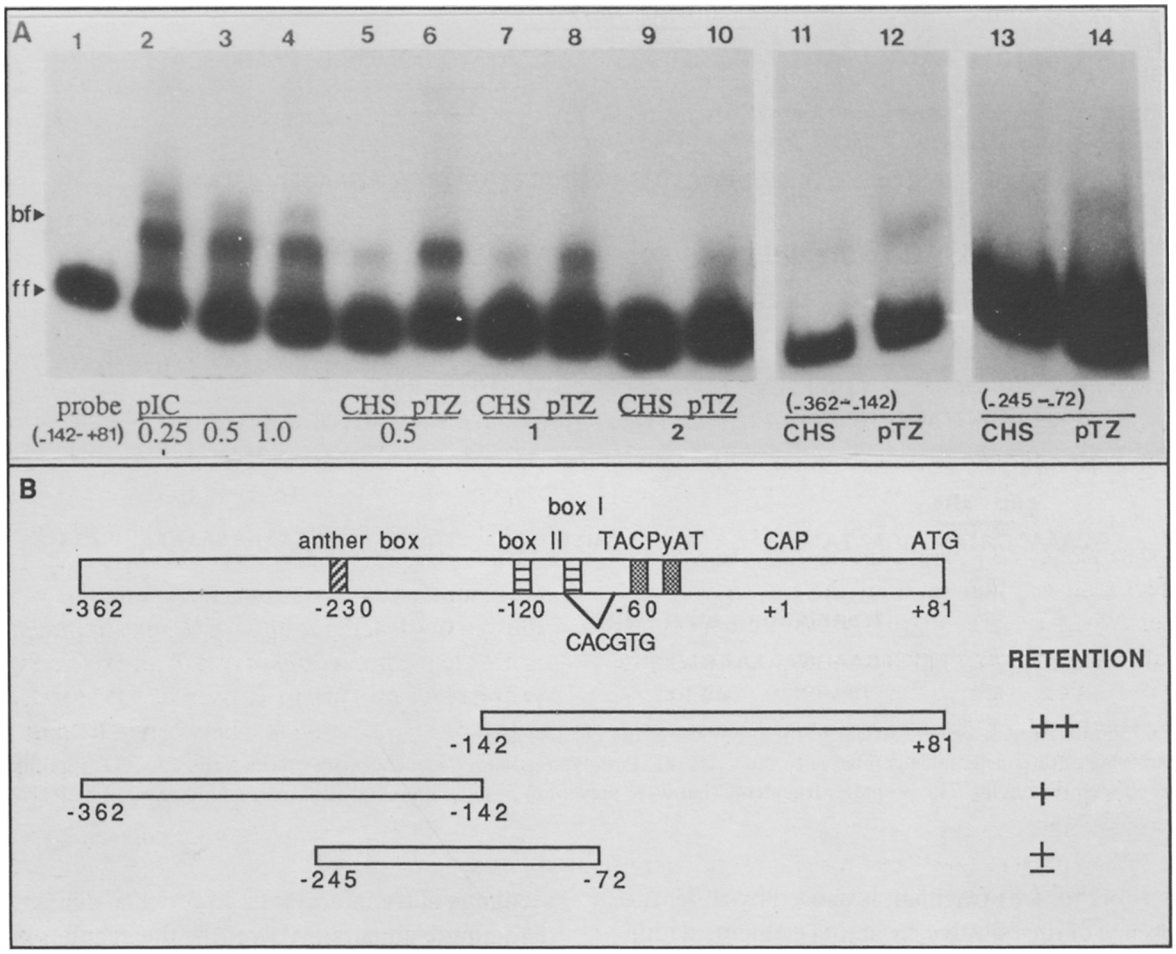

Fig. 7. Gel retardation assay of chs promoter fragments.
Panel A: *Bst* EII-*Nco* I fragment (– 142 to + 81) was used as a probe (lane 1) in binding experiments with flower nuclear extracts. Increasing amounts of poly dI-dC were added, resulting in a band shift (lanes 2–4). Competition with the same chs fragment (specific competitor) and pTZ vector DNA (aspecific competitor) was performed with 0.5 μg (lanes 5 and 6), 1 μg (lanes 7 and 8) and 2 μg (lanes 9 and 10). The *Apa* LI-*Bst* EII fragment of the chs promoter (– 362 to – 142) was also used as a probe and competed for binding with flower nuclear extract with 2 μg chs fragment (lane 11) and 2 μg pTZ DNA (lane 12). The same was done using the *Alu* I chs promoter fragment (– 245 to – 72) (lanes 13 and 14). Free fragment (ff) and bound fragment (bf) are indicated by arrows. Panel B: Schematic outline of the chs promoter and its putative regulatory sequences (see also Fig. 3). The fragments used in the retardation assays are shown below as well as their relative binding strength.

the *chs* A promoter reduces the CAT activity while the expression remains flower-specific. We found that a *chs* A promoter region as short as 67 nucleotides still exhibits the same organ- and temporal-specific expression pattern as the other *chs-cat* transgenes. However, UV-induced expression of this chimaeric gene in transgenic seedlings was lost, indicating that this region may lack the *cis* -acting sequences necessary for UV inducibility (see Fig. 4). This result does not distinguish between the absence of UV module(s) in the construct or lack of enhancer sequences allowing detection of the relatively weak UV induction. UV irradiation of seedlings transformed with constructs in which the CaMV enhancer sequence is cloned upstream of the 67 bp *chs* A promoter may answer this question. The chimaeric construct that lacked the TATA box (20 bp promoter) was

not able to drive expression to a detectable level (Fig. 3).

The steady-state *chs* A-*cat* mRNA level was compared to the endogenous *chs* mRNA level by primer extension assays. Both RNA molecules contain the same leader and tail sequences, and expression is driven by the same promoter. Surprisingly, the chimaeric mRNA steady-state level amounts to less than 1% of the endogenous *chs* mRNA level. This could be due to a difference in mRNA stability (the structural sequences of the messengers differ) or to a difference in transcription rate. Moreover, the chimaeric *chs* A-*cat* gene lacks the *chs* intron sequence, that could contain enhancers, as was shown for the intron in the maize *adh* gene [4]. Dean *et al.* also showed that sequences 3′ to the translation start codon affect transcription rates of the *rbc*S genes [7]. Presently, we are trying to assess the hypothesis that structural *chs* sequences might influence the transcription rate by constructing chimaeric *chs* A-*cat* genes that contain the *chs* A structural sequence (exons and/or intron).

A second strategy we followed to identify putative *cis*-acting elements was to compare *chs* A promoter sequences derived from several plant species with those of other flavonoid-specific genes of *P. hybrida*. This revealed several conserved sequence motifs (Fig. 6). A stretch of homology, designated 'anther box' (−239 to −224), was found to be present in flavonoid-specific promoters that drive expression in young anther tissue. Box I (−97 to −83) and box II (−129 to −112) represent striking homologies between the *chs* promoters of *P. hybrida* and *A. majus*. A two-fold repetition of a CACGTG motif is present in the *chs* A promoter, at −90 (in box I) and at −67. The CACGTG motif was reported previously to be a protein binding sequence in the *chs* promoter sequence of parsley [27] and *A. majus* [31], and is present in the promoters of a number of light-inducible genes [12]. Finally, at TACPyAT sequence, which has been implicated by transposon deletion analysis to be of crucial importance for expression of the *chs* gene of *A. majus* [30], is present two-fold in the *chs* A promoter (around −56).

The *in vivo* expression sutdies with chimaeric genes and the *in vitro* DNA-protein binding studies allow us to speculate on the actual function of the conserved sequences in regulation of flavonoid gene expression. Since the 220 bp *chs* A-*cat* construct is expressed in anther tissue (Fig. 4) and does not contain the 'anther box', it can be concluded that the 'anther box' is not exclusively responsible for regulating expression in anther tissue. The smallest construct that shows organ-specific expression contains a 67 bp *chs* A promoter region. Since this fragment lacks box I, box II (stretches of homology with *A. majus*) and both CACGTG motifs (one is partly disrupted), it is evident that these boxes are not of crucial importance for rendering the promoter organ-specific. Transgenic seedlings containing this construct were unable to synthesize CAT activity upon UV light treatment. It is not clear whether this is due to an extremely low expression level or to a lack of UV module(s) in the promoter sequence between −67 and +1. The gel retardation studies (Fig. 7) support the results from the promoter deletion series (Fig. 3 and 4). The *A. majus* homologues (box I and box II), one CACGTG motif and the 'anther box' did not bind strongly to proteins in flower nuclear extracts. Furthermore, the results of these studies revealed the involvement of sequences within the −72 to +81 region in the binding of flower nuclear proteins. This fragment contains one CACGTG motif, the TACPyAT sequence and the TATA box. The CACGTG motif (G box) is reported to be a more common protein binding *cis*-acting element [12]. This leaves the TACPyAT sequences in the *chs* A promoter region, extending from −67 to +1, to be the most likely candidates responsible for driving flower-specific expression of the reporter gene. This conclusion is evidently supported by the *in vivo* results whereas gel retardation experiments alone, in which promoter fragments or oligonucleotides bind to nuclear proteins, cannot prove the involvement of such sequences in regulation of gene expression.

The results described in this paper do not rule out a possible involvement of the sequences 3′ to the *chs* structural gene. However, the involvement

of such sequences in conferring temporally and organ-specific expression seems unlikely since we have found recently that there is no obvious difference in the expression pattern between a chimaeric gene in which the 3′ part of the *chs* gene was replaced by a 3′ region of the nopaline synthase gene (unpublished data).

Future studies are focused on the isolation of the (regulatory) gene(s) whose products bind to the *cis*-acting sequences implicated in organ-specific expression of the *chs* A gene.

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