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Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family

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ABSTRACT
Twenty independent, petal-specific chalcone synthase (CHS) cDNA clones have been isolated from *Petunia hybrida* variety Violet 30 (V30). Sequence analysis shows that the largest of these clones contains the entire coding sequence. Using this clone in Southern blot analysis reveals the presence of multiple CHS gene copies in the genome of *Petunia hybrida* V30. Hybridization and sequence analysis of the CHS cDNA clones shows that they are all copied from a single mRNA species. This indicates the presence of only one transcriptionally active CHS gene in petals. Finally we report the identification, cloning and partial characterization of this gene.

INTRODUCTION

Flavonoids represent a class of plant secondary metabolites, which play a central role in flower pigmentation and are thought to be involved in defence against environmental stress. The key-enzyme of flavonoid biosynthesis is chalcone-synthase (CHS). This enzyme condenses 3 molecules of malonyl-CoA and 1 molecule of 4 coumaroyl-Co A to yield naringenin chalcone (1). Isomerization and further substitution of this central intermediate leads to the synthesis of flavonols, flavonones, isoflavonoids and anthocyanins.

The enzymes of the flavonoid glycoside pathway share a number of interesting features: (I) their synthesis is coordinately regulated (2,3), (II) in most plant species the synthesis is tissue-specific (4), (III) environmental stress (UV light and phytopathogens) leads to a general induction of the pathway in otherwise non-expressing tissues (5-7).

The characterization of the regulation phenomena mentioned above at the molecular level has just begun. cDNA and chromosomal CHS-clones have been isolated from parsley (8) and *P. hybrida* (Roter traum) (9). Both systems, however, are genetically ill-defined, complicating analysis of the genomic constitution. We therefore chose to clone CHS genes from a genetically well-defined variety of *P.hybrida* In this paper we describe the isolation and sequence analysis of a full size CHS cDNA clone from *Petunia hybrida* V30. Using this clone we have established that CHS genes comprise a multigene family of which only one member is actively transcribed in floral tissue.

MATERIALS AND METHODS

Material

Plant variety, plasmids, phages and bacterial strains are listed in Table I.

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In vitro translation of mRNA

In vitro translation of poly A $^{+}$ mRNA from *Petunia hybrida* in rabbit reticulocyte lysates was carried out as described previously (17).

Analysis of CHS-protein by Western-blotting.

Protein extracts from various tissues were prepared as described (17). Protein concentrations were determined using the Biorad Protein assay with bovine serum albumin as a standard. Analysis of CHS-protein by Westernblotting was carried out as described previously (17), using an antiserum raised against parsley CHS and ^{125}I-protein-A.

Construction of a petal-specific cDNA-library from P.hybrida (V30).

PolyA $^{+}$RNA isolated from petals of *P. hybrida* (V30) (17) was converted to ds-cDNA (18). After homopolymeric tailing with dC residues, the cDNA was annealed to Pst I cut, dG-tailed pUC 9 DNA (PL-Biochemicals) and transformed to *E. coli* RR1 (19). Transformants were plated and screened by hybridization as described (20).

Isolation of DNA

Nuclear DNA from *P. hybrida* (V30) was isolated from the top four leafs of 3 months old plants or from 3 weeks old seedlings by a published procedure (21). Large and small scale DNA isolations of λ-phage were conducted as described (19). Plasmid DNA was isolated by the alkaline lysis method (19)

Southern-blots and preparation of probes

Ten micrograms of *P. hybrida* DNA was digested to completion with EcoRI and electrophoresed on 0.8% agarose gels. The DNA was transferred to Gene-screen plus membranes and hybridized as recommended by the supplier (New England Nuclear). Copy numbers were estimated by running samples of cloned DNA in parallel and comparing autoradiographic intensities of hybridized DNA. Copynumber calculations were based on a haploid genome size of 1.5 pg for *P. hybrida*. Southern-blotting and hybridization analysis of cloned DNA fragments was carried out using Hybond-N membranes as recommended by the supplier (Amersham). Washing conditions of the blots are specified in the figure legends. ^{32}P-labelled hybridization probes were prepared by nick-translation of restrictionfragments purified from (sub)clones in M13 mp vectors. We preferred subcloning in M13 because of the absence of cross-hybridization of this vector to *P. hybrida* DNA (unpublished data).

DNA-sequence analysis

For a complete sequence analysis of cDNA clones, series of overlapping subclones in M13mp phage were constructed using nuclease Bal 31 (22). These were sequenced using the dideoxy-chaintermination method (23) with ^{35}S-ATP. For sequence analysis of 3' and 5' ends, cDNA inserts were recloned in both orientations in M13mp10 and mp11. The synthetic oligo-nucleotide 5' dTTGAGACTGTTGTCCTCCA 3' was a gift of Dr. J.H. van Boom, State University of Leiden.

Isolation and analysis of genomic CHS-clones

P. hybrida (V30) nuclear DNA was digested to completion with EcoRI and ligated into λgtWES arms. Ligated DNA was packaged in vitro using the Hohn and Murray procedure (19). A second library of P. hybrida (V30) DNA, partially digested with Sau3AI, in the phage vector EMBL3 was generated according to Dean et. al. (24). Both libraries, containing $5x10^5$ (λgtWES) and $2x10^5$ (λEMBL3) plaques

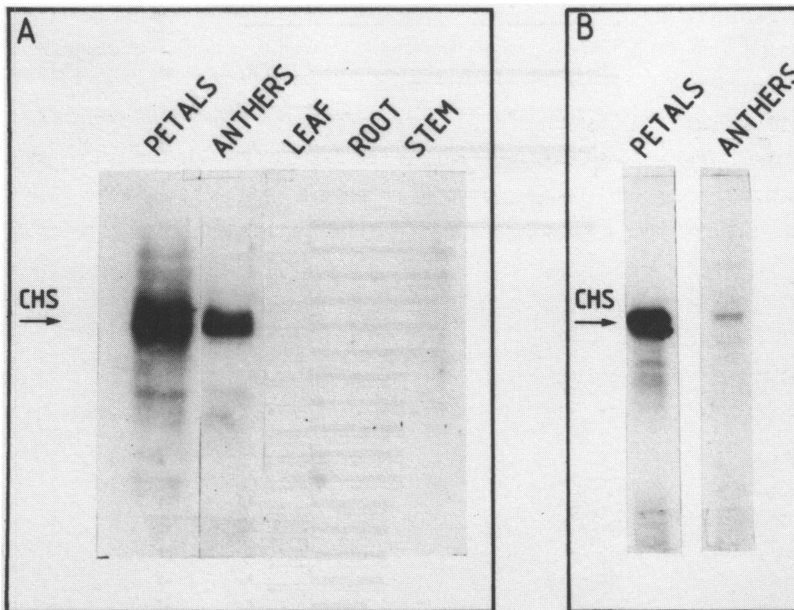

Figure 1: Analysis of CHS protein (A) and CHS translatable mRNA (B) levels in various tissues of *P. hybrida* (V30). A: Samples of 50 µg of protein, isolated from different tissues were analyzed for CHS content by SDS-gel electrophoresis followed by protein blotting as described (17). B: polyA$^+$mRNA was translated in a rabbit reticulocyte lysate and labelled CHS immunoprecipatated with a parsley anti-CHS serum. The product was electrophoresed on an SDS-acrylamide gel and visualized by autoradiography as described previously (17).

respectively, were screened by hybridization as described (19). Restriction-endonuclease maps of λgtWES inserts were generated by recloning of the inserts in pUC18 and subsequent analysis of single and multiple restriction-enzyme digests. Restriction endonuclease maps of inserts in λEMBL3 were generated by analysis of partial restriction-enzyme digests specifically labelled at the cosR site (25).

RESULTS

CHS expression: choice of tissue

Previous studies from other laboratories have indicated that the chalcone synthase gene (CHS) occurs in at least two copies in the genome of several higher plants (9,26). The plant systems used in these studies are however genetically ill-defined and heterozygosity of the CHS locus cannot be completely excluded. To circumvent these problems we chose to study an inbred variety of *Petunia hybrida* called Violet-30 (V30) which is homozygous for all 91 markers tested including 28 flavonoid-specific loci. Southern blots of V30 nuclear DNA restricted with EcoRI and probed with an incomplete CHScDNA from the Petunia variety Roter Traum (9) reveals at least 8 hybridizing fragments ranging in size from 1.5 to 25 kb. (cf. Fig. 4). This suggests that CHS genes occur in multiple copies in the genome of *Petunia hybrida* and raises the question which of these genes are actively transcribed.

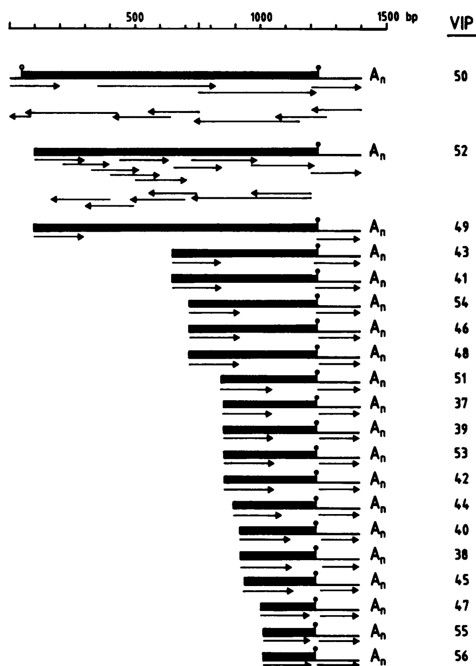

Figure 2: CHS cDNA sequencing strategy. Horizontal arrows indicate direction and extent of sequence determinations. Thick bars: protein coding regions. Thin bars: untranslated regions, ♀ translation start codon (ATG), ♦ translation stop codon (TAA). For details: see Materials and Methods.

As a first approach to analyze the structure and expression of individual CHS genes we decided to compare cDNA sequences with genomic clones. To help selecting the proper tissue from which to clone CHS-specific mRNA sequences, we examined the expression of CHS in different tissues of line V30 by in vitro translation and protein blotting. We used a monospecific anti-Parsley CHS serum, previously shown to specifically recognize *P. hybrida* CHS (17). Fig. 1 shows that flower petals contain the highest level of translatable CHSmRNA and of CHS protein. This tissue was therefore selected as a starting material for CHScDNA cloning.

Expression of CHS genes.

Isolation of CHScDNA clones. One way to get insight into the level of expression of individual members of a multigene family is to analyze a number of independently isolated cDNA clones. Assuming an equal conversion of each mRNA into cDNA, the percentage of cDNA clones found to be related to a particular gene is a reflection of the transcriptional activity of that gene. For this purpose a cDNA library based on *P. hybrida* (V30) petal mRNA containing 120,000 clones was constructed as outlined in Material and Methods. The library was screened for the presence of CHS-sequences by hybridization with a heterologous incomplete *P. hybrida* CHScDNA (pcP7, (9)). Under low stringency conditions of

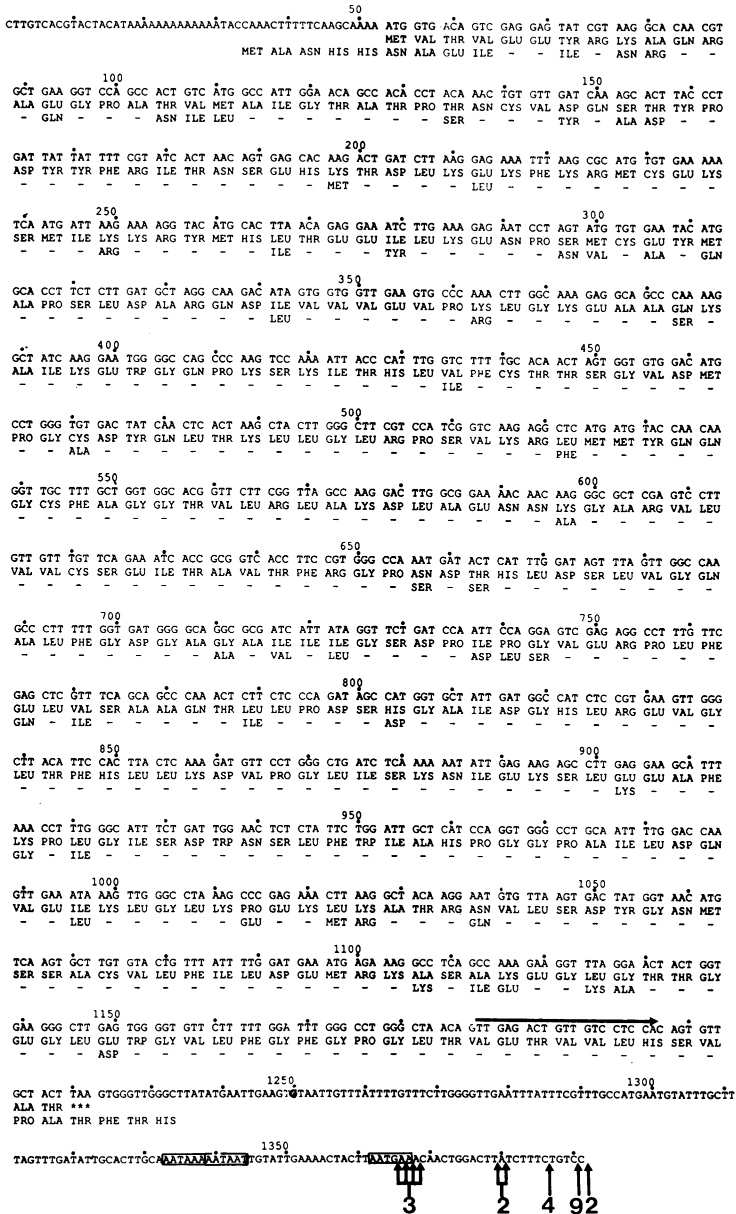

Figure 3: Combined sequence data on 20 independently isolated CHS cDNA clones. The strand homologous to the mRNA and its derived aminoacid sequence are shown. Furthermore the aminoacid sequence of parsley CHS as published previously (8) is shown; bars represent aminoacids identical to the *P. hybrida* (V30) enzyme. Numbers printed bold indicate the number of cDNA clones that have the polyA tail attached at that position. Three potential polyadenylation signals are boxed. ⟶ denotes tail sequence primer.

hybridization (2xSSC, 65°C) a total of 20 CHS-clones was identified and subsequently isolated. Insert analysis showed that three of them (VIP49, VIP50 and VIP52) contained inserts varying from 1450-1500bp. corresponding to the length of mature CHS-mRNA (cf. Fig. 2). The other 17 cDNA clones contained inserts varying in size from 800 to 550 bp. and are therefore partial cDNA clones. From sequence analysis (presented furtheron; fig. 4) and comparison with the sequence of a previously published parsley CHS-cDNA clone (8), we infer that clone VIP50 is indeed a full size CHS-cDNA clone. Finally we observe that VIP50 and VIP52 express in E. coli an in-frame lacZ-CHS fusion product which is recognized by an antiserum raised against parsley CHS (van Tunen, A. and Mol, J. pers. comm.) strengthening the view that authentic CHS sequences have been cloned.

Relationship between the CHScDNA clones. To determine the nucleotide sequence relationship between the twenty CHScDNA clones isolated, we hybridized the full size cDNA clone to a blot containing all 20 CHScDNA clones. All hybrids formed melted simultaneously upon stringent washing of the filters (not shown). Since sequence heterogeneity of the cDNA clones should have been detected by differential melting of the hybrids (cf. Figs. 4 & 6) we conclude that the cDNA sequences are highly similar if not identical. In order to obtain more conclusive data on this point, we subjected the CHS cDNA clones to a partial sequence analysis. The sequence strategy used is outlined in Fig. 2. The objective was to fully sequence the two largest cDNA clones (VIP50 and VIP52) and the termini of the remaining clones. The rationale for this is the observation that in a number of cases the different members of multigene families show maximum sequence divergence in their 3' untranslated regions of the mRNA (cf. 27, 28, Koes, R.E. and Spelt, C.E. unpublished). Fig. 3 shows the sequence of the longest CHScDNA clone as determined from sequence analysis of overlapping subfragments constructed by nuclease Bal 31 deletion. The sequence analysis of the 3' termini of most of the cDNA clones, using the universal M13 sequence primer, was complicated by the presence of long dT tracts in the cDNA clones. We therefore used a synthetic 19-mer complementary to the very end of the protein-coding region, as depicted in fig. 3. All 20 cDNA clones primed efficiently using this 19-mer and we could not detect a single base change in the 20 tails sequenced. The same holds for the 5' ends of the cDNA clones all containing protein-coding information.

Based on the number of identical cDNA clones we conclude that petal tissue of *P. hybrida* (V30) contains only one major species of CHSmRNA.

Features of the CHScDNA sequence. The combined sequence data obtained from 20 independently isolated CHScDNA clones are summarized in Fig. 3. The coding region contains 1266 nucl., which specifies a protein of 42,500 dalton. This is in close agreement with the size of *P. hybrida* CHS as published previously (42.000 dalton, (17)). Comparison of the coding sequence with the sequence of a parsley CHS cDNA clone (8;) reveals a homology of 72% at the nucleotide level. At the aminoacid level however a much higher homology exists (86%) indicating that a significant number of base changes are silent. Furthermore the CHS protein of *P. hybrida* (V30) lacks 5 aminoacids from the NH_2-terminus and 3 aminoacids from the COOH-terminus compared to the parsley enzyme. No significant homology to the parsley sequence could be detected in the 3' untranslated region.

During the analysis of the 3 ends we noticed that the polyA tails were attached at different sites

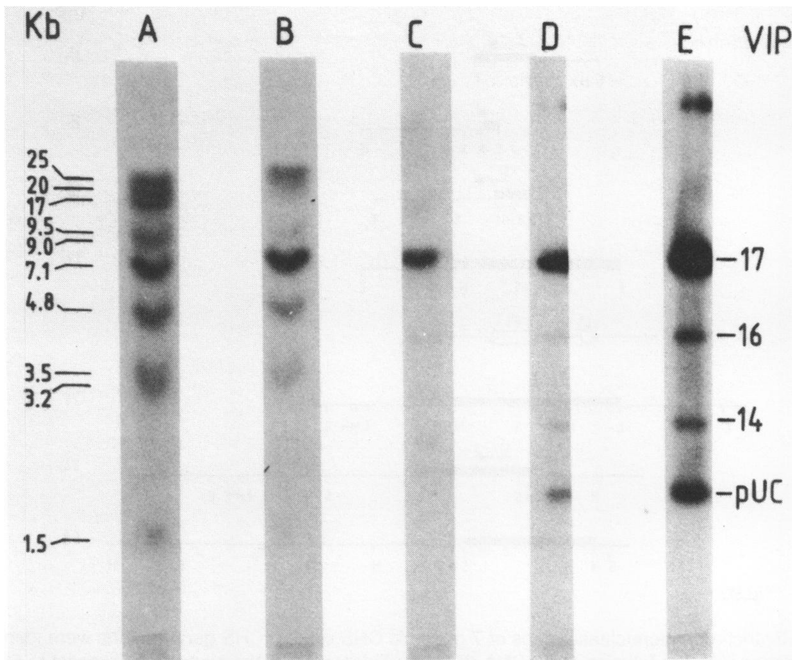

Figure 4: Identification of the transcriptionally active CHS gene. *P. hybrida* (V30) nuclear DNA was digested with EcoRI, electrophoresed on a 0.8% agarose gel, transferred to Genescreen Plus and hybridized with full size CHS cDNA (VIP 50). The filter was initially washed at low stringency (2xSSC, 65°C) and autoradiographed (panel A). Then the washing was continued under progressively more stringent conditions (0.5xSSC, 65°C; panel B and 0.1 x SSC 65°C panel C). Lanes D and E show hybridization signals from a mixture of cloned genomic CHS fragments (lane D: 2 x 10^{-5} pmoles of each corresponding to 1 gene copy; lane E: 10^{-4} pmoles of each corresponding to 5 gene copies). Washing conditions were as in panel C.

in the different cDNA clones. The number of clones with a polyA tail at a particular site is indicated in fig. 3'. Polyadenylation of viral and animal mRNA's has been shown to occur at a single site 10-30 nucl. downstream from a highly conserved polyadenylationsignal (consensus AATAAA). A perfect polyadenylation signal is present at nucl, 1336 of the CHScDNA sequence, which may account for the 3 polyA tails attached around nucl. 1367, but seems to be too far upstream to account for the 17 polyA tails attached between nucl. 1380 and 1392. At nucl.1365 however an imperfect polyadenylation-signal (AATGAA) is present which may account for polyadenylation in this region. In a recent study, Dean et al (29) describe a similar situation for SSU and Cab genes in Petunia. The plant polyadenylation sequence is highly variable but the most frequently used sequence is AATAAT. Such a sequence is also present in the CHS sequence around nucleotide 1341 (Fig. 3). These polyadenylation signals may account for the variable nature of poly A addition at CHSmRNA of Petunia.

Identification and cloning of the transcriptionally active CHS gene from Petunia hybrida V30. In one of the previous sections we showed that petal tissue of *P. hybrida* (V30) contains one major species of

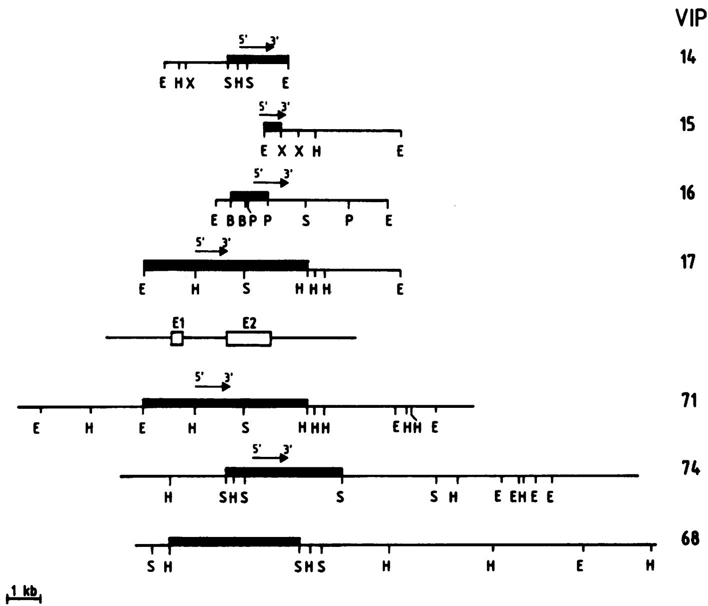

Figure 5: Restriction endonuclease maps of 7 genomic CHS clones. CHS gene regions were identified by hybridization with the full-size CHScDNA (VIP 50). Orientation of the clones with respect to 5' → 3' direction was determined by a second hybridization with a partial CHS cDNA clone (VIP46, cf. Fig. 2) E=EcoRI, H=HindIII, S=Sst I, P=Pst I, X=XbaI, B=Bam HI, E_1=Exon 1, E_2=Exon 2.

CHSmRNA. To find out if this mRNA is transcribed from a single gene we hybridized the full size CHScDNA clone to genomic blots containing EcoRI restricted V30 DNA and analyzed the stability of the hybrids at increasing stringency. Fig. 4 shows the result of this analysis. At low stringency the cDNA lights up at least 10 fragments ranging in size from 1.5 to 25 kb. At higher stringencies melting of all the hybrids except for the hybrid with the 7 kb EcoRI fragment is observed. From a parallel single-copy reconstruction experiment using cloned DNA (see below) we infer that the 7 kb fragment occurs once only in the genome. This implies that the 7 kb fragment contains a single transcriptionally active CHS gene. To isolate this gene genomic libraries were constructed in the vectors λgtWES and λEMBL3. Both libraries were screened by hybridization with CHScDNA and 7 positive plaques were isolated. Subsequent insert analysis showed that insert sizes ranged from 3.7 to 15 kb. Only two of the clones (VIP 17 and VIP71) show restriction maps which match perfectly (Fig. 5) and are therefore clones of the same gene. These two clones both contain a 7 kb EcoRI fragment and are therefore candidates to carry the active CHS gene. This was confirmed by thermal melting analysis (Fig. 6). Finally a sequence analysis of the VIP17/VIP71. gene shows that this gene matches the cDNA perfectly and that it contains two exons (278 and 988 bp respectively) split by an intron (1348bp) (manuscript in preparation). This implies that we have cloned the transcriptionally active CHS gene from *Petunia hybrida* (V30). Experiments are in progress to analyze the other genomic CHS clones. Sequence analysis carried out so far shows that all λ clones isolated contain a distinct 3' CHS gene terminus.

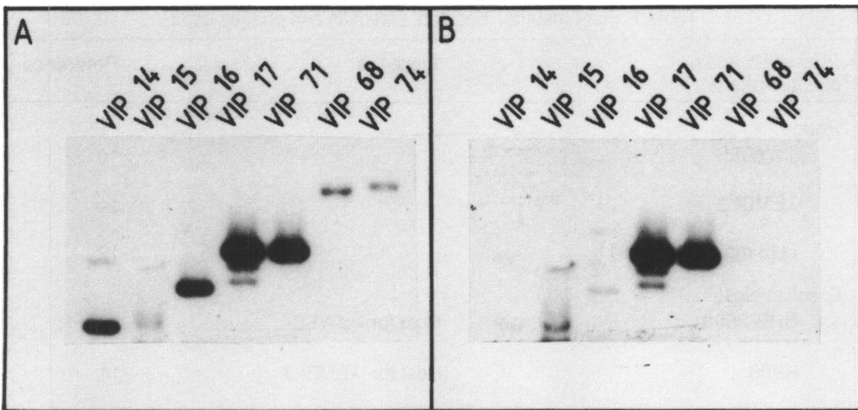

Figure 6: Identification of CHS genomic clones carrying the transcriptionally active CHS gene. DNA of different genomic clones was digested with EcoRI, electrophoresed on a 1% agarose gel, transferred to Hybond-N and hybridized with the full-size CHS cDNA. The filter was washed at low stringency (2xSSC, 65°C) and autoradiographed (panelA). Then the washing was continued at high stringency (0.1xSSC, 65°C; panel B).

DISCUSSION

Many plant genes isolated so far are members of a multigene family (21, 24, 30, 31, 32). In this paper we show that *Petunia hybrida* V30 contains 10 EcoRI fragments hybridizing to chalcone synthase (CHS) cDNA. (Fig.. 4). From cloning experiments so far we infer the presence of at least 6 CHS genes per haploid genome. Allelism of the genes is highly unlikely in view of the inbred nature of the variety V30 (Table I). In a recent paper Reif et al. (9) claim the presence of only 2 distinct CHS gene copies in three different varieties of *P. hybrida*. This apparent discrepancy could be due to the fact that these authors used more stringent hybridization conditions. Indeed, when Southern-blot experiments are conducted using nuclear DNA from different *P. hybrida* varieties, CHScDNA detects multiple (8-11) EcoRI restriction fragments at low stringency (2xSSC, 65°C) in all cases tested (unpublished data). At high stringency (0.1xSSC, 65°C) however the CHS probe detects 2 genes in some P. hybrida varieties (including the ones in (9)), whereas in other varieties V30 (this paper) and Mitchell (24) only one gene is detected. We conclude therefore that the discrepancy is due to both differences in hybridization stringencies and *P. hybrida* varieties used. The lower hybridization stringencies used in this study are justified by the observation that the different CHS genes are all equally homologous (75-80%) to the parsley CHS cDNA clone pLF15 (8, and our own data). This datum strongly suggests that the CHS-related fragments indeed carry a CHS multigene family although we cannot formally exclude the possibility that some of the fragments carry genes for related condensing enzymes of fatty acid or xanthone metabolism.

From hybridization and sequence analysis of 20 independently isolated CHS cDNA clones, we conclude that they are all copied from the same mRNA. The independence of the CHS cDNA clones is confirmed by the observation that all clones vary in at least two of the following features: (i) 5'end of the cDNA, (ii) site of polyadenylation, (iii) length of the dA.dT tail, (iv) length of the dG.dC tails. Based on

Table 1: Plant material, bacteria, plasmids and phages used.

Plant variety/strain plasmid/phage	Properties	Reference
Phage:		
λgtWES		10
λEMBL3		11
M13 mp 10 and mp 11		12
E. coli strains:		
BHB 2600	Host for λgtWES	13
K803	Host for λEMBL3	14
RR1	cDNA cloning	15
JM103	Host for M13	12
plasmids:		
pUC9 and pUC18		12
pcP7	partial CHS cDNA from P. hybrida (Roter traum)	9
P. hybrida variety:		
V30	purple flowers, homozygous for An1, An2, An3, An4, An6, An8, An9, An10, An11, An12, Ht1, Ht2, Hf1, Hf2, Rt, Gf, Mt, mf1, Ac, fl, Ph1-6, Fa, po.	16

these findings we conclude that only one CHS gene is transcriptionally active in petals of *P. hybrida* (V30). The presence of multiple CHS genes however poses the intruiging question whether the others are inactive pseudogenes or genes active in other tissues or under different environmental or developmental conditions. Sequence analysis so far has shown that the other cloned CHS genes lack the typical characteristics of pseudogenes. (manuscript in preparation) and may therefore be functional. It is known that in other plant and plantcell culture systems (5-7) flavonoid biosynthesis can be induced by environmental stress (UV-light, fungal elicitors). We have therefore initiated a similar stress programme for *P. hybrida* in order to find out if other CHS genes can become active under such conditions.

We report to have cloned from *P. hybrida* (V30) a 7 kb EcoRI fragment carrying a complete transcriptionally active CHS gene plus flanking sequences. Tissue-specificity, coordination and inducibility by light and environmental stress may be specified by 5' upstream and 3' downstream regulatory elements (cf. 33-35). We have started to test this hypothesis by mutagenizing these regions and analyzing their biological function after reintroduction in homologous (*P. hybrida* Red Star) and heterologous (tobacco) plant systems.

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