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Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: coordinate, light-regulated and differential expression of flavonoid genes

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In this paper we report the isolation of cDNA clones encoding the flavonoid-biosynthetic enzyme chalcone flavanone isomerase (CHI) from *Petunia hybrida*. A nearly full size cDNA clone, isolated from a corolla-specific expression library, was characterized by sequence analysis. Using this CHI cDNA and the previously cloned flavonoid-specific chalcone synthase (CHS) cDNA we show that CHI and CHS genes are coordinately and tissue-specifically expressed in a developmental and light-regulated manner. Furthermore, coordinate induction of both mRNAs is observed after continuous irradiation of Petunia plantlets with UV light, probably as part of the plants UV defence mechanism. The two CHI genes, denoted A and B, were isolated from a genomic library of the Petunia inbred line V30. Both genes are transcriptionally active: gene A is transcribed in corolla, tube and UV-irradiated plantlets (1.0 kb mRNA), whereas gene B is only transcribed in immature anthers (1.0 kb mRNA). In combination with Southern blot analysis these data implicate the presence of two distinct non-allelic CHI genes in the genome of the *P.hybrida* line V30. Unexpectedly, mature anthers accumulate a 0.3 kb larger CHI RNA. This RNA is transcribed from CHI gene A and has a 0.3 kb 5′ extension relative to the gene A transcript found in corolla tissue. Futhermore it is neither coordinately expressed with CHS mRNA nor UV inducible. Its biological function is still obscure, since no active CHI enzyme could be demonstrated in the same tissue.

Key words: chalcone flavanone isomerase/chalcone synthase/UV-induction/coordinate expression/*Petunia hybrida*

Introduction

Flavonoids represent a class of secondary metabolites which is widespread in the plant kingdom and may serve specific functions in flower pigmentation, UV protection, plant defence against pathogens or in legume nodulation (Chappel and Hahlbrock, 1984; Dixon, 1986; Peters *et al.*, 1986).

The flavonoid biosynthesis pathway branches off from the more general phenyl-propanoid pathway (Figure 1) and has the following interesting properties: (i) the biosynthesis of the enzymes involved is regulated in a coordinate way (Ebel and Hahlbrock, 1982); (ii) the synthesis of these enzymes is under developmental control and tissue-specific in most plant species (Ebel and Hahlbrock, 1982; Harborne, 1986); (iii) the pathway is inducible in a number of plant species both *in vivo* or in cell cultures by environmental stress factors such as UV light or phytopathogens (Dixon, 1986; Douglas *et al.*, 1987; Kaulen *et al.*, 1986; Kuhn *et al.*, 1984). In *Petunia hybrida* this route has been characterized extensively at the genetic, biochemical and enzymatic level (Schram *et al.*, 1984; Wiering and de Vlaming, 1984) and therefore provides a powerful system to study regulation of gene expression in higher plants.

To investigate the regulatory aspects of the phenomena mentioned above at the molecular level, a number of genes encoding the enzymes of this biosynthesis route were cloned and characterized in different laboratories. Recently, several genes from the general phenylpropanoid pathway were cloned [phenylalanine ammonia lyase, PAL (Edwards *et al.*, 1985), 4-Coumarate:CoA ligase, 4CL (Douglas *et al.*, 1987)]. Genes encoding the first enzyme in the flavonoid biosynthesis branch, chalcone synthase (CHS), were cloned from a number of plant species including *Antirrhinum majus* (Sommer and Saedler, 1986), *Zea mays* (Wienand *et al.*, 1986), *Phaseolus vulgaris* (Ryder *et al.*, 1984), *Petroselinum hortense* (Reimold *et al.*, 1983) and *P.hybrida* (Koes *et al.*, 1987; Reif *et al.*, 1985). Recently, the isolation of cDNA clones specific for the second enzyme in flavonoid biosynthesis, chalcone flavanone isomerase (CHI) from *P.vulgaris* was reported (Mehdy and Lamb, 1987).

In order to expand current knowledge about structure, genetic organization, tissue-specific and coordinated expression of flavonoid genes we decided to clone additional genes (gene families) from the same biosynthesis route. The availability in *P.hybrida* of mutants affecting the expression of the consecutive enzyme in the pathway, chalcone flavanone isomerase [CHI, (van Weeley *et al.*, 1983)] makes the corresponding CHI genes prime candidates for cloning. The CHI enzyme catalyses the stereo-specific isomerization of naringenin chalcones into the corresponding flavanones (Figure 1). The Petunia CHI enzyme has an M_r of ~29 000 and multiple forms are present in corolla and anther tissues (van Tunen and Mol, 1987).

In this paper we report the cloning of CHI cDNAs using a highly specific antiserum described previously (van Tunen and Mol, 1987). We isolated genomic clones and show that the *P.hybrida* cultivar studied contains two distinct non-allelic CHI genes which are differentially expressed in flower tissue. Finally, we present evidence for co-expression of CHS and one of the CHI genes in different parts of the flower and under UV stress conditions.

Results

Cloning of CHI cDNA from P.hybrida

In *P.hybrida*, the enzymes involved in flavonoid biosynthesis are found predominantly in maturing flowerbuds. In fact, CHI is present at relatively high levels in corollas of fully developed flowerbuds (van Weely *et al.*, 1983). For this

reason the poly(A)$^+$ mRNA used for cDNA cloning was isolated from corolla tissue of mature flowerbuds. *P.hybrida* line R27 was selected for mRNA extraction because of the availability of a potent antiserum raised against CHI isolated from this source (van Tunen and Mol, 1987). The presence of CHI mRNA within this preparation was verified by *in vitro* translation and immunoprecipitation experiments. SDS−PAGE of the immunoprecipitated translation products reveals a protein band with an M_r corresponding to that of purified CHI protein (M_r = 29 000, Figure 2, lane 1). The nature of the polypeptide migrating at M_r = 51 000 is unknown at present. We think that it is unrelated to CHI because the M_r = 51 000 protein is not detected after hybrid selection (Figure 2, lane 2). Moreover, the CHI mRNA detectable in corolla tissue (see further on) contains insufficient coding capacity for an M_r = 51 000 protein.

Using standard procedures (see Materials and methods) a cDNA expression library based on the λgt 11 vector (Young and Davis, 1983) was constructed. After screening the library (which contained ~175 000 recombinants) with the CHI antibody, three positive clones were detected and purified. These clones contained cDNA inserts of 753, 680 and 200 bp. The λ clone containing the 753 bp insert was designated λCHI-A. Using this cDNA insert as a probe in hybridization experiments it was shown that the other two clones had a melting behaviour indistinguishable from that of λCHI-A (data not shown) implying that the cDNAs are derived from the same gene. This was confirmed by subsequent sequence analysis.

In order to identify λCHI-A as CHI-specific, two types of experiments were carried out. First, R27 corolla mRNA was hybrid selected with immobilized λCHI-A DNA. The hybrid selected RNA encodes a single immuno-precipitable protein with M_r = 29 000 which is identical to that of purified CHI protein (Figure 2, lane 2). Because hybrid selection alone does not unequivocally prove the identity of an isolated cDNA clone, a second type of experiment was conducted: protein from plaques resulting from a λCHI-A (containing the largest, 753 bp insert) infection was extracted and tested for CHI enzyme activity. Such an extract shows a CHI-specific conversion of naringenin chalcone into flavanone which can be blocked completely with CHI antiserum. Neither protein extracts from λ phages containing the 680 bp inset nor those from control λ phages lacking an insert show CHI enzyme activity. The CHI activity in λCHI-A extracts is surprising in view of the fact that the CHI polypeptide is part of the large M_r = 140 000 CHI−*lac* Z fusion protein. It suggests that CHI does not require complex folding or extensive post-translational modification in order to be enzymatically active.

The DNA sequence of the λCHI-A clone was determined (Figure 3). It contains an open reading frame (ORF) with a calculated capacity of 25 kd. This figure correlates well with the M_r of the CHI enzyme from flower tissue [M_r = 29 000 for corolla CHI (van Tunen and Mol,

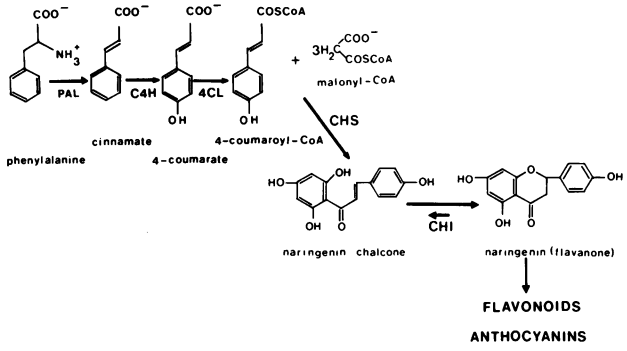

Fig. 1. Metabolic role of CHI and CHS in the phenylpropanoid pathway.

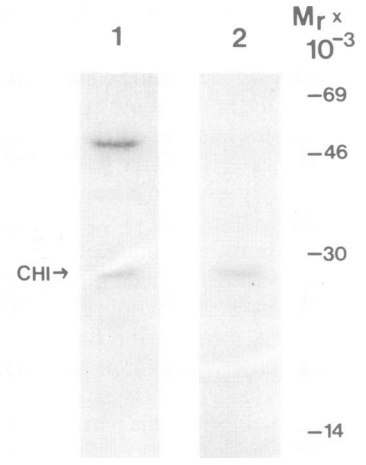

Fig. 2. Immunoprecipitation of *in vitro* translation products of mRNAs selected by hybridization to λCHI-A. RNA samples were translated in a rabbit reticulocyte lysate using [^{35}S]methionine as a radioactive label. After translation, CHI antiserum was added and immunocomplexes were precipitated with protein A-Sepharose. Translation products were analyzed after electrophoresis on a 12.5% SDS−polyacrylamide gel. **Lane 1:** poly(A)$^+$ RNA from R27 corollas, **lane 2:** poly(A)$^+$ RNA from R27 corollas after hybrid selection to λCHI-A. ^{14}C-labelled mol. wt markers are in kd.

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 ** * *** **                                                    50
 CAAAA ATG TCT CCT CCA GTG TCC GTT ACT AAA ATG CAG GTT GAG AAT TAC GCT TTC GCA
       met ser pro pro val ser val thr lys met gln val glu asn tyr ala phe ala
                                                   100
 CCG ACC GTG AAC CCT GCT GGT TCC ACC AAT ACC TTG TTC CTT GCT GGT GCT GGG CAT AGA
 pro thr val asn pro ala gly ser thr asn thr leu phe leu ala gly ala gly his arg
                                              150
 GGT CTG GAG ATA GAA GGG AAG TTT GTT AAG TTT ACG GCG ATA GGT GTG TAT CTA GAA GAG
 gly leu glu ile glu gly lys phe val lys phe thr ala ile gly val tyr leu glu glu
                                         200
 AGT GCT ATT CCT TTT CTG GCC GAA AAA TGG AAA GGC AAA ACC CCC CAG GAG TTG ACT GAC
 ser ala ile pro phe leu ala glu lys trp lys gly lys thr pro gln glu leu thr asp
                                    250
 TCG GTC GAG TTC TTT AGG GAT GTT GTT ACA GGT CCA TTT GAG AAA TTT ACT CGA GTT ACT
 ser val glu phe phe arg asp val val thr gly pro phe glu lys phe thr arg val thr
          300                                                   350
 ATG ATC TTG CCC TTG ACG GGC AAG CAG TAC TCG GAG AAG GTG GCG GAG AAT TGT GTT GCG
 met ile leu pro leu thr gly lys gln tyr ser glu lys val ala glu asn cys val ala
                                                   400
 CAT TGG AAG GGG ATA GGA ACG TAT ACT GAT GAT GAG GGT CGT GCC ATT GAG AAG TTT CTA
 his trp lys gly ile gly thr tyr thr asp asp glu gly arg ala ile glu lys phe leu
                                              450
 GAT GTT TTC CGG AGT GAA ACT TTT CCA CCT GGT GCT TCC ATC ATG TTT ACT CAA TCA CCC
 asp val phe arg ser glu thr phe pro pro gly ala ser ile met phe thr gln ser pro
                                         500
 CTA GGG TTG TTG ACG ATT AGC TTC GCT AAA GAT GAT TCA GTA ACT GGC ACT GCG AAT GCT
 leu gly leu leu thr ile ser phe ala lys asp asp ser val thr gly thr ala asn ala
                                    550
 GTT ATA GAG AAC AAG CAG TTG TCT GAA GCA GTG CTG GAA TCA ATA ATT GGG AAG CAT GGA
 val ile glu asn lys gln leu ser glu ala val leu glu ser ile ile gly lys his gly
          600                                                   650
 GTT TCT CCT GCG GCA AAG TGT AGT GTC GCT GAA AGA GTA GCG GAA CTG CTC AAA AAG AGC
 val ser pro ala ala lys cys ser val ala glu arg val ala glu leu leu lys lys ser
                                              700
 TAT GCT GAA GAG GCA TCT GTT TTT GGA AAA CCG GAG ACC GAG AAA TCT ACT ATT CCA GTG
 tyr ala glu glu ala ser val phe gly lys pro glu thr glu lys ser thr ile pro val
                                         750
 ATT GGA GTC TAG TTTAGGACAAGTAAAATGTTTTAGTGCT
 ile gly val STOP
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Fig. 3. Nucleotide sequence of λCHI-A cDNA and deduced aminoacid sequence. The 3′ *Eco*RI site is an internal restriction enzyme site of the λCHI A insert and does not originate from the *Eco*RI linkers used in the cDNA cloning procedure. *** denotes homology with Joshi box (see text).

Fig. 4. Southern blot analysis of total V30 plant DNA (genom. DNA) and genomic CHI clone A (CHI clone A) and B (CHI clone B) digested with *Hin*dIII. After blotting and hybridization with the λCHI-A probe the filter was sequentially washed under low (L) stringency conditions (2 × SSC/1%SDS/60°C) and under high stringency (H) conditions (0.1 × SSC/1%SDS/60°C).

1987)] and with that of the protein immuno-precipitated after hybrid-select translation. The sequence around the first ATG codon found contains a relatively high degree of homology to the plant consensus ATG start-codon region as published recently by Joshi (1987) providing additional indications that the cDNA clone contains the entire CHI-coding sequence. In summary these experiments show that λCHI-A is a CHI-specific, nearly full length cDNA clone.

The P.hybrida genome contains two distinct CHI genes

CHS genes in *P.hybrida* comprise a multigene family containing at least eight members (Koes *et al.*, 1987). To find out whether CHI genes have been amplified to a similar extent the number of CHI genes in *P.hybrida* was estimated by Southern blot analysis. The *P.hybrida* line V30 was chosen for further study because it is a genetically well-characterized inbred line from which we previously cloned the CHS multigene family (Koes *et al.*, 1987). Genomic DNA from the line V30 was digested with restriction enzyme *Hin*dIII which does not cleave within the cDNA insert. Hybridization with the cDNA at low and high stringency reveals only one strongly hybridizing band (2.0 kb, Figure 4) which is present at a single copy (data not shown). These results therefore suggest the presence of a single CHI gene in *P.hybrida* V30. To document this point further, *Sau*3A partially digested V30 DNA was cloned in λEMBL-3 as described previously (Koes *et al.*, 1986). However, screening with λCHI-A yielded two classes of clones with completely different restriction maps. The first class contains a

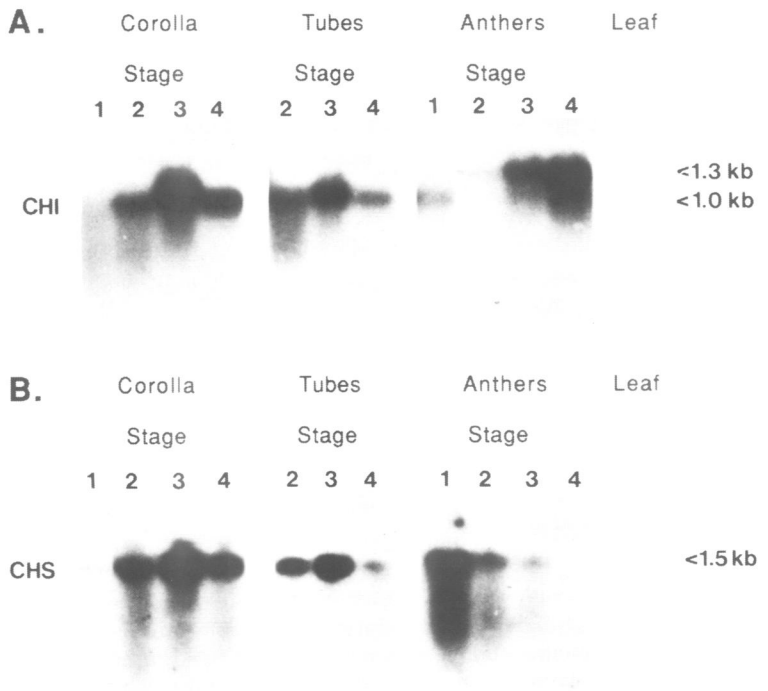

Fig. 5. Northern blot analysis of mRNAs extracted from various V30 plant tissues in different development stages. The length of flower buds which are fully developed but still closed is defined as 100%. The figures above each lane correspond with the developmental stage of the buds: **stage 1**, 30–45%; **stage 2**, 45–60%; **stage 3**, 60–75% and **stage 4**, 75–90%. After blotting the filters were hybridized with either a CHI cDNA (panel A) or a CHS cDNA (panel B) probe. The length of the hybridizing transcripts is indicated.

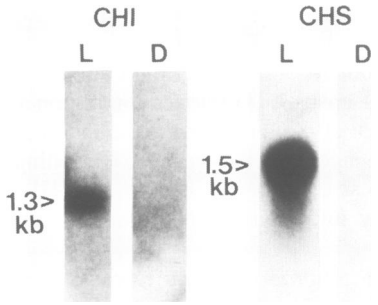

Fig. 6. Northern blot analysis of mRNA extracted from V30 flower tissues of plants grown under normal greenhouse conditions (light = L) or plants placed in a dark room for 4 days (dark = D). Flowerbuds of stage 2 and 3 were mixed (45–75% of maximal length) and mRNA was extracted. The blot was sequentially hybridized with a CHI (left) and a CHS cDNA (right) probe.

2.0 kb *Hin*dIII restriction fragment which hybridizes to the cDNA even under high stringent conditions. It corresponds with the 2.0 kb fragment present in *Hin*dIII digested V30 DNA (Figure 4) and therefore contains a CHI gene designated as gene A. The second class contains a 0.9 kb *Hin*dIII fragment which also hybridizes to λCHI-A but only at low stringency. Critical re-examination of the Southern blot depicted in Figure 4 shows a weakly hybridizing fragment of the same size present only at low stringency. Therefore, a second CHI gene (designated as gene B) must be present in the genome of *P.hybrida* V30.

The structure of both CHI genes was determined by restriction mapping, RNase protection analysis (see further on) and DNA sequence analysis (to be presented elsewhere) and is depicted in Figure 8. CHI gene A, which is homologous to the cDNA, contains no introns, whereas CHI gene B contains three small introns.

Developmental co-expression of CHI and CHS mRNA in floral tissue of P.hybrida

During floral development, the enzymes of the flavonoid biosynthetic pathway accumulate and disappear in a coordinate way (Danglmayer *et al.*, 1983; Gerats *et al.*, 1984). To determine whether accumulation of the relevant mRNA sequences also proceeds in a coordinate fashion, mRNA extracted from floral buds differing in their developmental stage was subjected to Northern blot analysis using CHI- and CHS-specific probes. First, ^{32}P-labelled λCHI-A insert DNA detects an mRNA of about 1.0 kb in both corolla and flower tube extracts, but not in leaf tissue. This mRNA species has the proper length to encode CHI with M_r = 29 000. The intensity of the hybridization signal peaks at developmental stage 3 in both corolla and tube (Figure 5A). This accumulation of CHI mRNA in corolla tissue correlates well with the appearance of CHI enzyme activity which is at a maximum at stage 3 and 4 (data not shown). Second, a similar blot was hybridized with a CHS cDNA probe (Koes *et al.*, 1986). The pattern obtained (Figure 5B) parallels that of CHI mRNA accumulation and from this we infer a close coordination in the expression of CHI and CHS genes in corolla and tube tissue.

While in corolla and tube tissue CHI mRNA accumulation peaks just before CHI enzyme activity, this clear, straightforward correlation is not seen in anthers. Here the situation is more complex; two types of CHI mRNAs accumulate. First, a CHI mRNA of 1.0 kb is expressed early

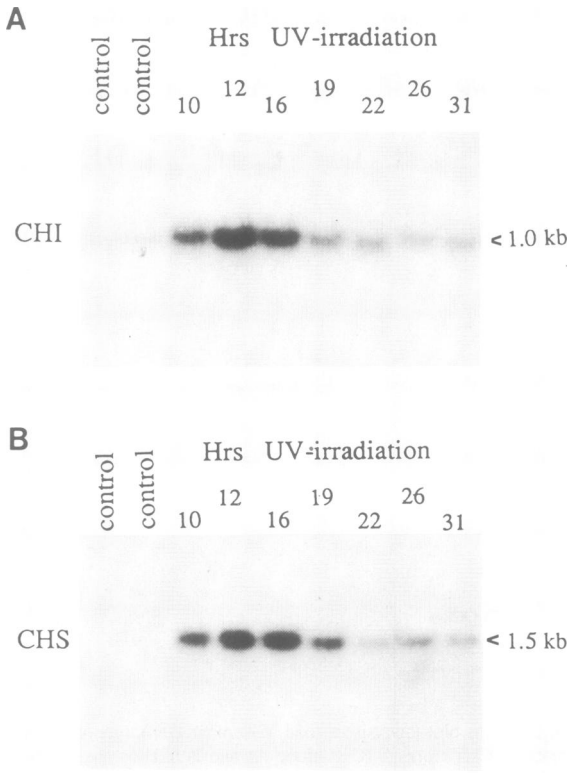

Fig. 7. Northern blot analysis of mRNA extracted from 8 day old Petunia hybrida V30 seedlings irradiated with continuous UV light. The length of the UV irradiation is indicated above each lane. As a control mRNA was extracted from seedlings grown under normal light. The blot was sequentially hybridized with a CHI (**panel A**) and a CHS cDNA (**panel B**) probe.

in anther development in a coordinate way with CHS mRNA (Figure 5). In anther tissue extracts of this developmental stage CHI enzyme activity could be detected. Second, surprisingly, an additional CHI RNA of 1.3 kb accumulates in mature anthers. The biological function of this CHI RNA species remains unclear since no CHI enzyme activity could be detected in extracts of stage 4 anthers (data not shown). Moreover, no CHS mRNA is detected in anther tissue extracts of the same developmental stage (Figure 5B).

Light regulation of CHI and CHS expression in flower tissue

Light is an important factor involved in the induction and regulation of flavonoid biosynthesis (Hrazdina, 1982). In order to investigate the regulatory effect of light at CHI and CHS mRNA levels, total RNA was extracted from flower tissue of plants grown under normal greenhouse conditions or from plants which were placed in darkness for 4 days. Both the CHI 1.0 kb mRNA and the CHS mRNA are present in corollas of light-grown plants but absent in dark treated plants (Figure 6). A 12 h light treatment after the dark period results in the reappearance of both mRNAs (data not shown). These results show that the expression of CHI and CHS genes in corollas is regulated by light.

Co-induction of CHI and CHS mRNA in seedlings of P.hybrida after UV light stress

In a number of plants and plant cell culture systems UV light has been shown to be a potent inducer of the flavonoid

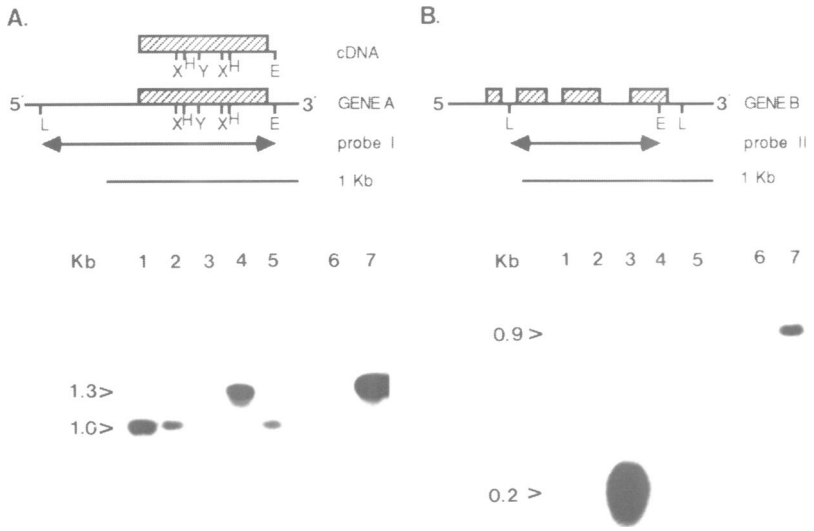

Fig. 8. Structure of the two CHI genes and specific CHI gene expression by RNase protection analysis. H = *Hin*cII, E = *Eco*RI, L = *Hin*dIII, X = *Xba*I and Y = *Xho*I. Only one *Hin*dIII site upstream of gene A is drawn; the second one is located 2.0 kb downstream. The filled-in boxes represent coding regions. **Panel A**, expression of CHI gene A; **panel B**, expression of CHI B. Lane 1, corolla (mix of all stages); lane 2, tube (mix of all stages); lane 3, anthers stage 1; lane 4, anthers stage 4; lane 5, UV-treated seedlings (mix of all timepoints); lane 6, no plant RNA added; lane 7, probe control (no RNase treatment).

biosynthesis system which is thought to play an important role in plant defence (Bufler and Bangerth, 1982; Chappell and Hahlbrock, 1984; Douglas *et al.*, 1987; Kaulen *et al.*, 1986; Kuhn *et al.*, 1984; Moehle *et al.*, 1985). To study this phenomenon in petunia, 8 day old V30 seedlings were subjected to continuous UV-irradiation and induction was monitored by Northern blot analysis. Figure 7 shows that nonirradiated seedlings contain very low levels of CHS and CHI mRNA. Continuous UV irradiaton leads to an enhanced transient accumulation of both the 1.0 kb CHI and CHS mRNA. The maximum level of induction is reached after 12 h of continuous UV irradiation. The 1.3 kb CHI RNA however, is not induced under these UV stress conditions. As in the previous paragraph we observe a close coordination of CHI and CHS expression.

Differential expression of CHI genes in P.hybrida

In the previous paragraphs it is shown by Northern blotting that CHI mRNA is only found in flower tissue and in UV irradiated plantlets. In order to analyse which specific CHI gene is expressed and to determine the nature of the 1.3 kb CHI RNA, RNase protection experiments were performed. CHI gene A was monitored using an *in vitro* synthesized RNA probe which spans the entire coding region and 0.5 kb upstream sequence of CHI gene A (probe I, Figure 8A). The CHI gene B specific RNA probe spans an internal *Hin*dIII–*Eco*RI region (probe II, Figure 8B).

CHI gene A expression is observed in corolla and tube tissue and seedlings illuminated with UV light as shown by the generation of a 1.0 kb protected fragment (Figure 8A, lanes 1, 2 and 5). This is expected from an intronless CHI A gene. CHI gene B expression is only observed in immature anthers (Figure 8B, lane 3). The 0.2 kb signal observed is resolved into three protected fragments on high-resolution gels (not shown) as expected on the basis of gene B structure (three introns). CHI gene B is therefore an anther-specific gene. In mature anthers probe I protects a 1.3 kb RNA (Figure 8A, lane 4). Since the probe extends into the promoter region of gene A, the 1.3 kb RNA observed previously (Figure 5, anthers stage 4) represents a 0.3 kb 5′ extension of the 1.0 kb CHI gene A transcript.

Discussion

In this paper we report the isolation of both cDNA and genomic clones encoding the flavonoid-specific enzyme CHI from *P.hybrida*. The structure and expression pattern of the two CHI genes present in *P.hybrida* line V30 was studied. Furthermore, we were able to demonstrate light-regulated and coordinate accumulation of CHI and CHS mRNAs.

Three distinct CHI specific cDNA clones were isolated from a λgt 11 expression library using a previously described antiserum (van Tunen and Mol, 1987). Definitive proof for the identity of the cDNA clones came from hybrid select translation and enzyme activity measurements of the λCHI–*lac*Z fusion protein.

Coordinate induction of mRNAs and proteins of the general phenylpropanoid, flavonoid and related phytoalexin pathways has been reported in plants and tissue cultures of Umbelliferae in response to phytopathogen and UV light stress (Chappel and Hahlbrock, 1984; Kuhn *et al.*, 1984; Tietjen and Matern, 1983) and Leguminosae (Cramer *et al.*, 1985; Dixon, 1986; Medhy and Lamb, 1987). In this paper it is demonstrated for the first time that mRNAs coding for two consecutive enzymes (CHS and CHI) from the flavonoid biosynthesis pathway accumulate coordinately during corolla, tube and anther development (Figure 5). In addition, coordinate induction is light-regulated; CHI and CHS mRNAs disappear when plants are placed in darkness for 4 days (Figure 6) and reappear in plants subsequently transferred to normal light. Whether *de novo* synthesis and/or degradation are affected by the dark/light regime cannot be concluded from these experiments and will require run-off transcription assays in isolated nuclei (Chappell and Hahlbrock, 1984; Douglas *et al.*, 1987). A similar light/dark regulation has been observed for photosynthetically active genes (Kuhlemeier *et al.*, 1987). Flavonoids have been suggested to play a role as UV protectants in young plantlets

(Chappell and Hahlbrock, 1984). It has been shown that UV induction of flavonoids is regulated at the mRNA level in young plantlets (Kaulen *et al.*, 1986) and plant cell cultures (Kuhn *et al.*, 1984). Our UV induction experiments confirm and expand these observations: *Petunia* V30 seedlings respond to continuous UV irradiation with a coordinate accumulation of CHI and CHS mRNA (Figure 7) and the subsequent synthesis of flavonoid pigments (E.Wellmann, personal communication).

Genomic cloning experiments, Southern hybridization and RNase protection experiments have revealed the presence of two distinct CHI genes denoted as CHI-A and CHI-B. In view of the inbred nature of *P.hybrida* V30 both CHI genes are expected to be non-allelic. This is confirmed by segregation analysis of both genes in genetic crosses (to be published elsewhere). The absence of introns in CHI-A is suggested by similarities in restriction maps of the genomic CHI-A clone and the CHI cDNA and sequence data and is confirmed by RNase protection experiments. In contrast, three small introns in the coding region of CHI-B are revealed (Figure 8). The nucleotide sequence homology of both CHI genes is ~70–80% (exons) based on hybridization kinetics and initial sequence analysis. This explains the weak hybridization signals of gene B restriction fragments on Southern blots (Figure 4) or gene B mRNA on Northern blots (Figure 5). At this stage it remains puzzling why so many more copies of the CHS gene (8–10, Koes *et al.*, 1986) are present in the same *P.hybrida* cultivar (V30). Likewise, the *P.vulgaris* genome contains a similar-sized CHS gene family (Ryder *et al.*, 1988) whereas the presence of only a single CHI gene is reported recently (Mehdy and Lamb, 1987). We did not detect common restriction-enzyme cleavage-sites in the cDNA clones from *Petunia* and *Phaseolus* indicating a substantial divergence. This is in line with the low immunological cross-reactivity of CHI enzymes of both plants (Dixon,R.A. *et al.*, submitted).

The two CHI genes not only show considerable differences in structure but also exhibit tissue-specific and differential expression. CHI gene A is expressed in corolla, tubes and UV treated seedlings, whereas gene B is expressed only in immature anthers and is therefore an anther-specific gene. The differential expression of CHI-A and B predicts the presence of different CHI-enzymes in corolla and anthers. Indeed, as reported previously (van Tunen and Mol, 1987) anther and corolla CHI iso-enzymes show considerable differences with respect to M_r, isoelectric point and antibody inhibition kinetics. Experiments are in progress to establish the relationship between CHI gene B and the classically defined gene Po which has been previously shown to affect CHI expression in anthers (van Weeley *et al.*, 1983; van Tunen and Mol, 1987).

Although the 1.0 kb CHI transcript from gene A is coordinately regulated with CHS mRNA (Figure 5) a 1.3 kb CHI RNA resulting from a 5′ extension of the 1.0 kb gene A transcript accumulates in mature anthers. This is surprising because CHI enzyme activity is absent in the late stages of anther development. Furthermore, the 1.3 kb CHI RNA is neither coordinately regulated with CHS mRNA (Figure 5) nor UV induced (Figure 7). For these reasons it is unlikely that the 1.3 kb CHI RNA is involved in flavonoid metabolism. The experiments mentioned above further indicate that separate promoters are involved rather than alternative splicing mechanisms as has been shown for the gene encoding the stress-inducible hydroxy-proline-rich glycoprotein extension (Chen and Varner, 1985), the pallida gene in *A.majus* (Coen *et al.*, 1986) and its homologue in *Z.mays* (Schwarz-Sommer *et al.*, 1987).

The molecular basis for coordinate light-induction and tissue-specific developmental control is still poorly understood and will be subject for future studies. Comparison of flanking regions from active CHS, CHI and the recently cloned dihydroflavonol reductase (DFR) genes (A.G.M.Gerats and M.G.Beld, unpublished) in combination with site-directed mutagenesis experiments will facilitate the identification of *cis*-acting sequences involved in regulation of flavonoid gene expression.

Materials and methods

Materials

λgt 11 phage DNA and the relevant host (Y1090) were obtained from Promega Biotec. λEMBL-3 phage DNA was isolated according to the protocol described by Maniatis *et al.* (1982). *P.hybrida* varieties R27 and V30 were grown under greenhouse conditions. The line V30 is homozygous for at least 91 markers (Koes *et al.*, 1986).

λgt 11 cDNA library construction and screening

Double stranded, blunt end cDNA was prepared starting with 2 μg R27 poly(A)$^+$ mRNA using the Amersham cDNA synthesis system which is based on the Gubler and Hoffman method (Gubler and Hoffmann, 1983). After synthesis the cDNA was methylated with *Eco*RI methylase according to the protocol of the supplier (Biolabs). Subsequently, chromatography over Sephadex G 100 was performed. Phosphorylated *Eco*RI linkers were ligated to the cDNA. After *Eco*RI digestion, the products were size-fractionated on a Biogel A50 column in order to eliminate cleaved linkers. Appropriate fractions were precipitated and ligated at a 1:1 molar ratio to dephosphorylated λgt 11 arms. *In vitro* packaging (Promega Biotec) was performed and the resulting library was titrated on *Escherichia coli* Y1090. Plating and screening of the library was performed following the Promega Biotec protocol and using a preabsorbed rabbit antiserum against petunia CHI (van Tunen and Mol, 1987). Positive plaques were identified in the screening procedure using goat anti-rabbit IgG conjugated with alkaline phosphatase as a second antiserum (Promega Biotec) and BCIP and NBT as substrates (Promega Biotec).

Hybrid-select translation and CHI enzyme measurement

Hybrid selection of poly(A)$^+$ RNA was performed following the protocol of Maniatis *et al.* (1982) with λCHI-A DNA immobilized on nitrocellulose filters (Schleicher and Schuell). Released RNA was translated *in vitro* using a rabbit reticulocyte translation system (New England Nuclear). Immunoprecipitation and analysis of translation products by SDS–PAGE was performed as reported previously (Mol *et al.*, 1983) using 10 μl of CHI-antiserum.

CHI enzyme activity was measured as described previously (Mol *et al.*, 1985).

λEMBL-3 library construction and screening

A λEMBL-3 library containing ~250 000 recombinants was constructed as reported previously (Koes *et al.*, 1986) using *P.hybrida* V30 nuclear DNA partially digested with *Sau*3AI. The resulting library was screened by hybridization with a ^{32}P-labelled DNA probe which was prepared by nick translation of CHI-cDNA. The positive clones were isolated and purified. Physical maps of λEMBL-3 inserts were made by analysis of partial restriction-enzyme digests specifically labelled at the cos R site (Rackwitz *et al.*, 1984). Subsequently, more precise maps were established after subcloning the inserts in M13 mp18 or pUC19 and followed by analysis of single and multiple restriction digests.

UV induction and RNA and DNA extractions

Eight-day old Petunia seedlings were irradiated with a Philips TL 40W/12 UV lamp placed at a distance of 20 cm. A homemade perspex UV filter (cut off <320 mm) was placed in between to remove light of harmful wavelength. RNA was extracted from plant tissue by grinding in liquid N_2 using pestle and mortar. Extraction buffer (0.75 ml per 1–5 g plant tissue, 100 mM Tris–HCl, pH 8.5, 100 mM NaCl, 20 mM EDTA and 1% w/v sarkosyl) was added. Subsequently this solution was phenolized twice, ethanol

precipitated and the resulting pellet dissolved in 0.5 ml H_2O. An equal volume of 4 M LiAc was added, incubated at 4°C for 3 h and centrifuged. The RNA pellet was dissolved in H_2O, ethanol precipitated, washed with 70% ethanol, dried and dissolved in 200–100 µl H_2O. Poly(A)$^+$ mRNA was prepared from total mRNA using Messenger Activated Paper™ (Amersham).

Total plant DNA, phage λ DNA and plasmid DNA were isolated as described previously (Koes *et al.*, 1986).

DNA sequence analysis

The dideoxy chain termination method was used for sequencing M13 clones (Koes *et al.*, 1986). Overlapping clones were prepared by cloning different restriction enzyme fragments.

Southern and Northern blot analysis

^{32}P-labelled CHI cDNA was prepared by nick translation as described (Koes *et al.*, 1986). For Southern blot analysis 10 µg of *P.hybrida* DNA was digested with *Hin*dIII and electrophoresed on 0.8% agarose gels. The DNA was transferred to Gene-screen plus membranes and hybridized according to the protocol of the supplier (New England Nuclear). Copy number calculations were carried out according to Koes *et al.* (1986). Southern blotting and hybridization of cloned DNA fragments was performed according to the supplier, using Hybond-N membranes (Amersham). For Northern blot analysis 10 µg total mRNA was loaded on formaldehyde gels (Maniatis *et al.*, 1982) and after electrophoresis blotted onto Gene-screen plus membranes according to the suppliers protocol (New England Nuclear). The formaldehyde gels were briefly stained with ethidium bromide and photographed before blotting. After hybridization, Northern and Southern blots were washed in 0.1 × SSC/1% SDS/60°C unless stated otherwise.

RNase protection analysis

^{32}P-labeled RNA was synthesized *in vitro* using T7-polymerase (Promega Biotec), [^{32}P]UTP (Amersham) and the GeneScribe-Z™ (US Biochemical Corporation) vector pTZ18U containing an *Eco*RI–*Hin*dIII CHI gene A restriction fragment (probe I, Figure 8) or pTZ19U containing a *Eco*RI–*Hin*dIII CHI gene B restriction fragment (probe II, Figure 8). After a DNase treatment the RNA probe was hybridized with 10 µg total mRNA extracted from different V30 plant tissues in 20 µl 40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl and 50% (v/v) formamide for 16 h at 45°C. Then 0.3 ml 10 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.3 M NaCl, 5 µg/ml RNase A (Boehringer) and 2 U/ml RNase T1 (BRL) was added and the solution was incubated at 30°C for 60 min. Under these conditions perfect hybrids are protected from RNase degradation whereas hybrids containing mismatches are digested to oligonucleotides. After addition of 20 µl 10% SDS and 5 µl proteinase K (10 mg/ml) incubation was continued for 15 min at 37°C. Protected RNA fragments were recovered by extraction with phenol/chloroform (1:1), ethanol precipitation and visualized on formaldehyde/agarose gels (Maniatis *et al.*, 1982). Prior to autoradiography gels were fixed in 10% TCA and dried.

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Note added in proof

These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under the accession number Y00852.