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Chalcone synthase-like genes active during corolla development are differentially expressed and encode enzymes with different catalytic properties in *Gerbera hybrida* (Asteraceae)

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Abstract

Recent studies on chalcone synthase (CHS) and the related stilbene synthase (STS) suggest that the structure of *chs*-like genes in plants has evolved into different forms, whose members have both different regulation and capacity to code for different but related enzymatic activities. We have studied the diversity of *chs*-like genes by analysing the structure, expression patterns and catalytic properties of the corresponding enzymes of three genes that are active during corolla development in *Gerbera hybrida*. The expression patterns demonstrate that *chs*-like genes are representatives of three distinct genetic programmes that are active during organ differentiation in gerbera. *Gchs1* and *gchs3* code for typical CHS enzymes, and their gene expression pattern temporally correlates with flavonol (*gchs1, gchs3*) and anthocyanin (*gchs1*) synthesis during corolla development. *Gchs2* is different. The expression pattern does not correlate with the pigmentation pattern, the amino acid sequence deviates considerably from the consensus of typical CHSs, and the catalytic properties are different. The data indicate that it represents a new member in the large superfamily of *chs* and *chs*-related genes.

Introduction

To understand the regulation and diversity of plant secondary metabolism at a genetic level, chalcone synthase genes (*chs*) have been recently studied in different contexts (reviewed in [28]). Chalcone synthase is a polyketide synthase that catalyses the first dedicated reaction of the flavonoid pathway, the stepwise addition of three malonyl-CoA molecules to 4-coumaroyl-CoA to synthesize naringenin chalcone (Fig. 1).

The first *chs* gene was isolated from UV-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z38096 (*gchs1*), Z38097 (*gchs2*), Z38098 (*gchs3*), Z38099 (*gpal1*) and Z17221 (*gdfr1*).

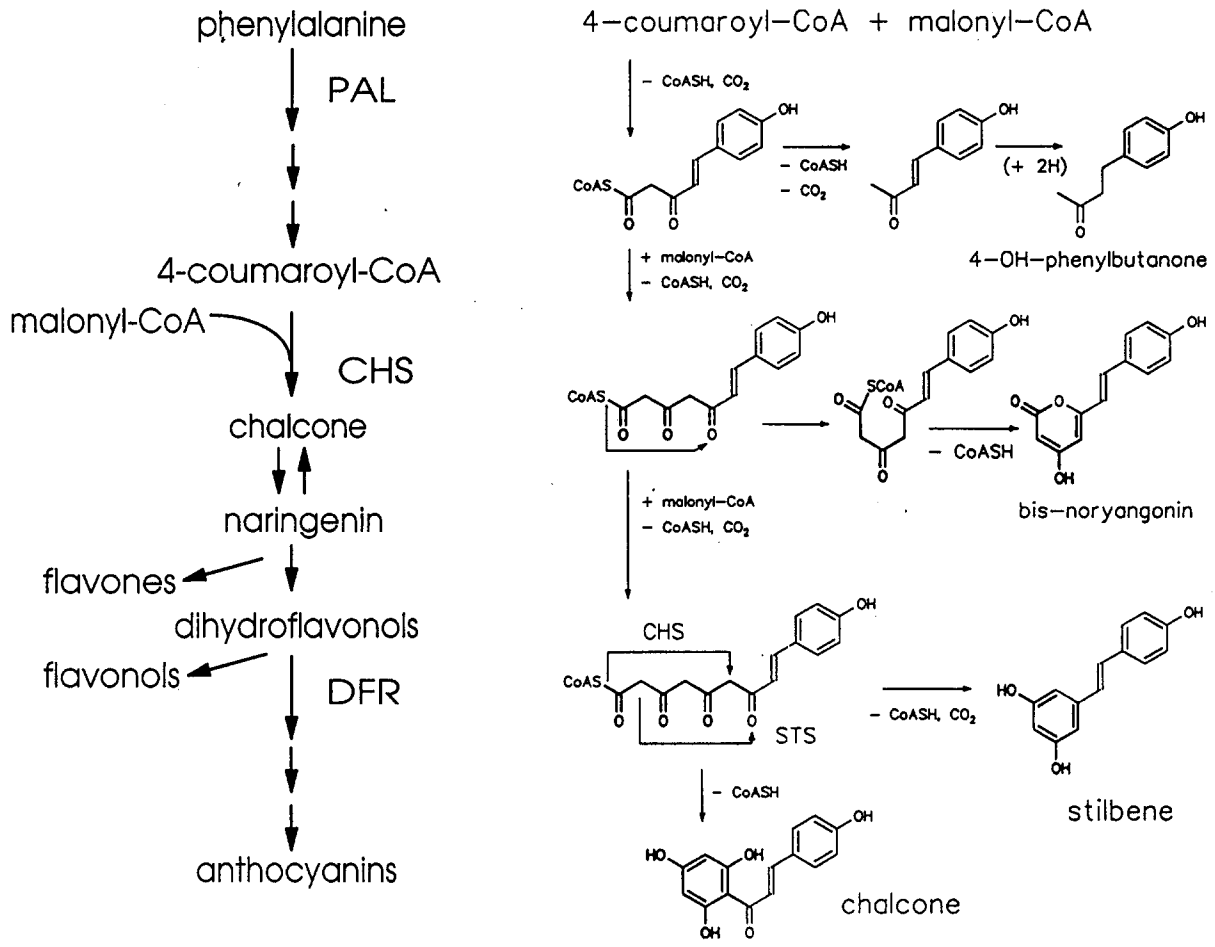

Fig. 1. Left: the position of CHS, PAL and DFR in the flavonoid pathway. Flavones, flavonols and anthocyanins accumulate during corolla development in gerbera [1]. *Right*: CHS and STS reactions. *In vivo* three malonyl-CoA are sequentially added to 4-coumaroyl-CoA. The STS reaction differs from the CHS reaction in the last step of ring closure [46]. *In vitro* several other products accumulate as a result of premature release of the intermediate from the enzyme [22, 29, 47]. The reaction resulting in the accumulation of 4-OH-phenylbutanone has been observed *in vivo* as well [5].

irradiated parsley suspension cultures as a cDNA related to flavonol accumulation [40]. Other external stimuli, like pathogen attack-related elicitors (reviewed in [10]), wounding [61], symbiotic Rhizobia [39, 57] and different light incitement [2, 36] have been shown to induce *chs* transcription in parsley and other plants. The developmental regulation of *chs* expression related to anthocyanin pigmentation has been best characterized during flower development in petunia [25, 27] and snapdragon [31], and during kernel development in maize [17, 60]. Recently, involvement of flavonoids in gametophyte development in maize and petunia has been demonstrated [7, 55, 58]. It is evident that plants differ in their capacity to regulate *chs* expression and parts of the flavonoid pathway with respect to various stimuli. Furthermore, plants can induce the same *chs* gene in different contexts and obviously by different stimuli [20, 26, 44, 54].

The primary structure of CHS protein is highly conserved and the amino acid sequences display 80–90% identity among angiosperms and conifers [16, 35]. Several CHS-related proteins and functions have been described and some of the corresponding genes have been cloned. All cloned examples are stilbene synthases [16, 33, 45, 51] that probably evolved from CHS [56] and use the

same substrates, but form a different ring system (Fig. 1). Acridone synthases perform a CHS-type reaction with N-methylanthraniloyl-CoA, and partial protein sequences indicate that the protein is closely related to the typical CHSs [3]. Some molecules identified as side-products of CHS reactions (4-OH-phenylbutanone and bis-noryangonin) [22, 29] occur *in vivo*, and an enzyme synthesizing 4-hydroxyphenylbutanone has been demonstrated in plants [5].

These data suggest that the structure of *chs*-like genes in plants has evolved into different forms and diversified into a super-gene family, whose members have both different regulation and capacity to code for different but related enzymatic activities. We have studied *chs*-like genes in *Gerbera* by analysing the structure, expression patterns and catalytic properties of the corresponding enzymes of three *chs*-like genes that are active during corolla development in *Gerbera hybrida*. The three genes code for two different enzymatic activities. *Gchs1* and *3* encode a typical CHS enzyme, whereas *gchs2* codes for an enzyme that has different catalytic properties compared to CHS or STS, and represents a novel CHS-like activity. The expression pattern of each gene differs from the others, suggesting three distinct roles for them during corolla development.

Materials and methods

Plant material

Gerbera hybrida var. Regina was obtained from Terra Nigra BV and was grown under standard greenhouse conditions. Developmental stages of the inflorescence have been described before [21].

Isolation of plant DNA and RNA

Total DNA was extracted by the method of Dellaporta *et al.* [12]. Total RNA was isolated essentially as described by Jones *et al.* [24]. Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose affinity chromatography [41].

PCR and cDNA cloning

The PCR cloning strategy was as described by Helariutta *et al.* [21]. Partially degenerate primers including *Bam* H1 sites 5′-AGCAGGATCC AA(A/G) GC(C/T) AT(C/T) AA(A/G) GA(G/A) TGG GG-3′ and 5′-AGGAGGATCC AA GCA ACC (T/C)TG (T/C)TG (A/G)TA CAT CAT-3′, corresponding to peptides KAIKEWG and MMYQQGCF for *chs*, and inosine-containing partially degenerate primers, 5′-ACGTGGATCC CA(T/C)GGI GGI AA(T/C) TT(T/C) CA(A/G) GG-3′ (HGGNFQG) and 5′-ACGTGGATCC AC (A/G)TC (T/C)TG (A/G)TT (A/G)TG (T/C)TG (T/C)TC (EQHNQDV), for *pal* were used. The major PCR product was cloned into pSP73 plasmid (Promega) and sequenced as described [21].

Construction and screening of the cDNA library, and sequence analysis of the cDNA clones was performed as earlier described [21].

RNA and DNA blot analyses

Nick-translated fragments from the 3′ end were used as probes for gene specific detection [41]. For *gchs1*, the gene-specific probe represented the last 307 bp of the cDNA, for *gchs2* 281 bp and for *gchs3* 291 bp. For *dfr* mRNA detection, a full-length cDNA [21] was used as a probe and for *pal*, a PCR fragment from the conserved area.

10 μg total RNA or digested DNA was loaded per lane. The electrophoresis and hybridizations were done as described by Sambrook *et al.* [41]. The washing stringencies were 0.2× SSC at 59 °C for *gchs3*, at 62 °C for *gchs2*, at 65 °C for *gchs1*, 68 °C for *dfr* and 2× SSC at 68 °C for *pal*.

In situ *hybridization*

In situ hybridization was performed as described [9]. Cross-sections from the central region of the ligule were analysed. For synthesis of the RNA probes using the SP6 and T7 promoters [41], the

gene-specific fragments were cloned into the pSP73 vector (Promega).

Expression in Escherichia coli

The plasmids pHTT401 (containing *gchs1*), pHTT402 (*gchs2*), pHTT407 (*gchs3*) and pHTT406 (*chs* from *Petroselinum hortense*) that express the various CHS-like enzymes in *E. coli* were constructed by inserting the coding sequence under the *tac* promoter in pKKtac, a derivative of the vector pKK223–3 (Pharmacia) that contains the *lacI* gene in the plasmid [53]. In these expression constructs, the initiation of translation takes place at the plant gene's ATG. The vector without an insert served as the control. For enzyme production, *E. coli* DH5α [19] harbouring the expression constructs were grown to A_{600} = 0.8–1.0, induced for 1.5 h with 1 mM IPTG at 28 °C, pelleted and stored at −70 °C.

Enzymatic reaction

Preparation of *E. coli* and plant extracts was performed as described by Fliegmann *et al.* [16]. For enzyme assays, 20 μg protein extracted from *E. coli* or plant were used. The enzymatic reaction was performed as described by Fliegmann *et al.* [16], with the exception that 100 μM starter-CoA was used. After the reaction, either 20 μl glacial acetic acid (low-pH extraction) or 100 μl 1 M Tris-HCl pH 8.8 (high-pH extraction) was added. The ethyl acetate-extractable material was evaporated to dryness, dissolved in 10 μl of ethyl acetate and analysed by thin-layer chromatography (cellulose, Merck Art. 5716, 15% acetic acid in water) followed by autoradiography.

Flavonol analyses

Flavonol pigments were isolated from freeze dried corollas and characterized by HPLC as previously described [1]. Quantitative measurements were obtained by averaging the data from 9 flowers harvested from three plants in the afternoon on three separate occasions.

Results

Isolation of the gchs1–3 *cDNAs*

PCR from reverse-transcribed ray floret corolla RNA with the degenerate *chs* primers yielded a fragment of expected size (181 bp) and identity as confirmed by sequencing (data not shown). The PCR fragment was used as a probe in screening a cDNA library constructed in pUEX1 (Amersham), prepared from the developing ray floret corollas of var. Regina. Sixteen independent clones hybridizing to the probe were isolated from a library of 25 000 cfu. Restriction and partial sequencing analyses indicated that the clones represent three related sequences: 11 *gchs2*-like, 3 *gchs1*-like and 2 *gchs3*-like.

The longest isolate of each group (1485 bp for *gchs1*, 1600 bp for *gchs2*, 1431 for *gchs3*) was subcloned into pSP73 (Promega) and subsequently sequenced. The putative translational start and stop codons and the amino acid sequence were deduced from the cDNA sequence. The start codon for each clone is in the region that is in consensus with all other *chs*-like genes characterized so far. Also, in the *gchs1* and *gchs2* sequences, this first methionine codon is preceded by one or more stop codons in the same reading frame. The analysis shows that we have determined the sequence of the entire protein coding region for three members of the *chs* gene family that are active during corolla development. By Southern blotting with the 181 bp PCR fragment as a probe, about 10 bands were observed indicating the presence of *chs* gene family in the gerbera genome (data not shown).

Comparison of the primary structure of GCHS1–3 to other CHS-like proteins

We compared the identity of the GCHS1–3 amino acid sequences to each other and to several CHS and STS sequences. Whereas the iden-

Table 1. Identity (%) of *gchs1–3* genes between each other and several *chs* and *sts* genes at the level of deduced amino acid sequence. CHS of *Petroselinum hortense* (V01538 [40]); C2 of *Zea mays* (X60205 [60]); CHS of *Pinus sylvestris* (X60754 [16]); STS of *Vitis* [33]; STS of *Arachis hypogaea* (A00769 [45]); STS of *Pinus sylvestris* (X60753 [16]).

	GCHS2	GCHS3	CHS *P. hortense*	CHS *Z. mays*	CHS *P. sylvestris*	STS *Vitis*	STS *A. hypogaea*	STS *P. sylvestris*
GCHS1	74.1	88.9	86.4	83.7	79.0	74.5	71.2	65.4
GCHS2		73.4	69.9	68.3	67.9	65.1	65.3	61.3
GCHS3			84.0	83.3	77.0	74.5	70.2	64.1
CHS *P. hortense*				83.0	81.3	75.5	70.7	65.6
CHS *Z. mays* (C2)					78.5	74.7	68.6	65.6
CHS *P. sylvestris*						71.7	70.2	73.8
STS *Vitis*							67.4	63.8
STS *A. hypogaea*								66.1

tity of GCHS1 to GCHS3 and to other CHS enzymes was 77–89%, the identity of GCHS2 to GCHS1 or GCHS3 or to other CHS sequences was only 68–74%. The identity of the GCHS2 amino acid sequence to STS of *Arachis hypogaea* [45], *Vitis* [33] and *Pinus sylvestris* [16] was 65% or less (Table 1).

We also compared the deduced amino acid sequence of GCHS1–3 to the CHS strict consensus sequence derived from the comparison of 9 CHS sequences (of 8 different plant species, Fig. 2). The functionality of these sequences has been demonstrated either based on genetics, or by biochemical characterization of the corresponding enzyme. GCHS1 differed from the consensus at 5 sites, GCHS3 at 10 sites and GCHS2 at 49 sites. For comparison, STS from *A. hypogaea* [45] deviates from the CHS consensus at 38 sites. The comparative analyses of the primary structure of the various gerbera CHS proteins indicates that

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1     MASSVDMKAIRDAQRAEGPATILAIGTATPANCVYQADYPDYYFRITKSEHMVDLKEKFKRMCDKSM   67
3   MATSPAVIDVETIRKAQRAEGPATILAIGTATPANCVYQADYPDYYFRVTESEHMVDLKEKFQRMCDKSM   70
2    MGSYSSDDVEVIREAGRAQGLATILAIGTATPPNCVAQADYADYYFRVTKSEHMVDLKEKFKRICEKTA   69
co              R  QRA GPA   AIGTA P N V Q  YPDYYF IT S H T LK KF RMC KS

1   IRKRYMHITEEYLKQNPNMCAYMAPSLDVRQDLVVVEVPKLGKEAAMKAIKEWGHPKSKITHLIFCTTSG  137
3   IRKRYMHITEEFLKENPSMCKFMAPSLDARQDLVVVEVPKLGKEAATKAIKEWGFPKSKITHLVFCTTSG  140
2   IKKRYLALTEDYLQENPTMCEFMAPSLNARQDLVVTGVPMLGKEAAVKAIDEWGLPKSKITHLIFCTTAG  139
co  I  R M  TEE L  NP  C Y APSLD RQD VV EVP LGK AA  AIKEWGQPKS ITH  FCTTSG

1   VDMPGADYQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPNDT  207
3   VDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPNEG  210
2   VDMPGADYQLVKLLGLSPSVKRYMLYQQGCAAGGTVLRLAKDLAENNKGSRVLIVCSEITAILFHGPNEN  209
co  VDMPG DYQLTK LGLR  V R MMYQQGCFAGGTVLR AKD AENN GARV VVCSEITAVTFRGP

1   HLDSLVGQALFGDGAAAVIVGSDPDLTTERPLFEMVSAAQTILPDSEGAIDGHLREVGLTFHLLKDVPGL  277
3   HLDSLVGQALFGDGAAAVIIGSDPDLSVERPLFEMVSAAQTILPDSEGAIDGHLKEVGLTFHLLKDVPAL  280
2   HLDSLVAQALFGDGAAALIVGSGPHLAVERPIFEIVSTDQTILPDTEKAVKLHLREGGLTFQLHRDVPLM  279
co  H DS VGQALF DGA A   G DP   VE P       AQT  PDS GAIDGHLREVGL FHLLKDVPG

1   ISKNIEKALTTAFSPLGINDWNSIFWIAHPGGPAILDQVELKLGLKEEKLRATRHVLSEYGNMSSACVLF  347
3   IAKNIEKALIQAFSPLNINDWNSIFWIAHPGGPAILDQVEFKLGLREEKLRASRHVLSEYGNMSSACVLF  350
2   VAKNIENAAEKALSPLGITDWNSVFWMVHPGGRAILDQVERKLNLKEDKLRASRHVLSEYGNLISACVLF  349
co   SKNI   L  AF    ISD N  FW AHPGGPAILD VE K  L       TR VLS YGNMSSACV F

1   IIDEMRKKSSENGAGTTGEGLEWGVLFGFGPGLTVETVVLHSVPTTVTVAV                    398
3   ILDEMRKKSIKDGKTTTGEGLEWGVLFGFGPGLTVETVVLHSLPATISVATQN                   403
2   IIDEVRKRSMAEGKSTTGEGLDCGVLFGFGPGMTVETVVLRSVRVTAAVANGN                   402
co  ILDEMR      G  TTGEGL  GVLFGFGPGLT ETVVL SV
```

Fig. 2. Alignment of the deduced amino acid sequences for GCHS1–3. co, strict consensus of CHS sequences based on 9 molecules that have been tested functionally: *A. majus* (X03710 [50]); *A. thaliana* (M20308 [15]); *P. hybrida chsA* (X14591 [18, 26, 38]); *Z. mays C2* (X60205 [60]; *WHP* (X60204 [17]); *P. hortense* (V01538 [40]); *P. lobata* (D10223 [34]); *P. sylvestris* (X60754 [16]); *S. alba* (X14314 [13]). The amino acid residues in GCHS1–3 deviating from the consensus have been marked in bold.

GCHS1 and 3 are relatively homologous to the CHS proteins, whereas the structure of GCHS2 is more deviant. The sequence of GCHS2 deviates from the STS sequences, as well. We have also demonstrated (see below) that the GCHS2 enzyme differs from chalcone synthases in its substrate specificity and reaction.

Organ-specific expression of the gerbera chs *genes*

The expression patterns of the *gchs1–3* genes were analysed using probes derived from the 3′ ends of the clones. Under the washing stringencies used, the probes do not cross-hybridize. In the Southern analyses *gchs2* and *3* probes hybridize to a single target sequence, and *gchs1* to two sequences (Fig. 3). In the latter case, it is possible that the probe measures the expression pattern corresponding to two very homologous genes. During the screening of the cDNA library representing ray floret corolla we could, however, find only *gchs1*-like cDNA species, indicating that *gchs1* is most abundantly expressed during corolla development.

To determine the organ specificity of the *gchs1–3* genes, organs covering several developmental stages and floret types were separately pooled for northern analysis (Fig. 4). *Gchs1* and *gchs3* expression are detected only in pappus and the ligular part of corolla, the tissues which both have the potential to accumulate anthocyanins in different gerbera varieties. *Gchs3* expression is particularly strong in pappus. In contrast, *gchs2* expression is detected in both vegetative and reproductive organs. The expression is strong in the leaf, scape (the inflorescence stem) and corolla (both in the ligule and the unpigmented tube), moderate in the bract and carpel, detectable in the root and pappus but not detectable in the stamen. This demonstrates that *gchs2* expression has a developmental pattern that is strikingly different from those of *gchs1* and *gchs3*.

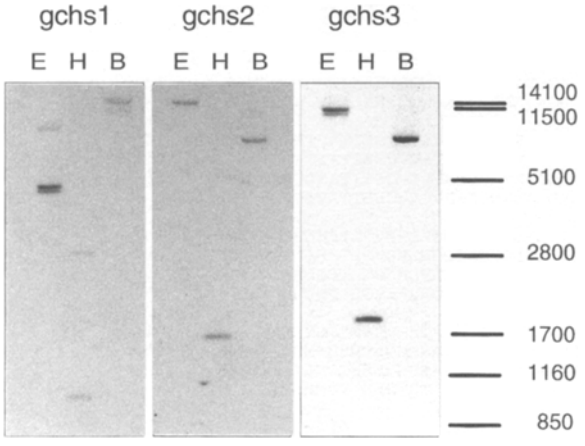

Fig. 3. Southern analyses of *chs*-like genes in gerbera with gene-specific probes hybridizing to *gchs1*, *gchs2* and *gchs3*, respectively. *Gchs1* probe recognizes two fragments, *gchs2* and *gchs3* one fragment with various enzymes. B, *Bam* HI; E, *Eco* RI; H, *Hind* III. The positions of lambda DNA marker fragments and their sizes in bp are indicated.

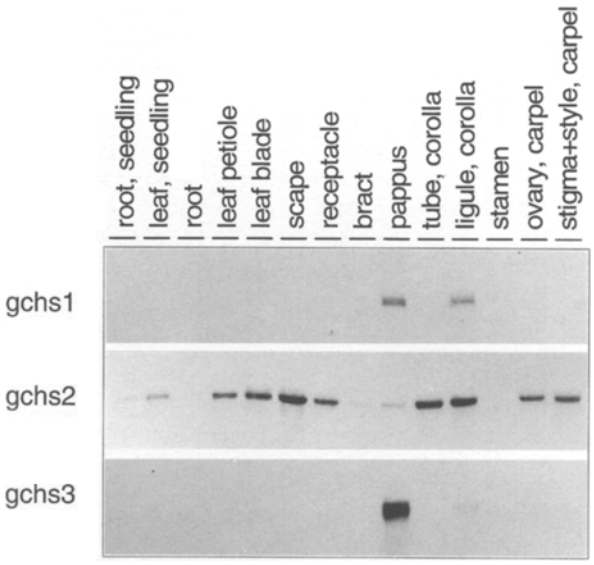

Fig. 4. Organ specificity of *gchs1–3* expression. Organs from several developmental stages and floret types were pooled for the northern analysis. Scape, the inflorescence stem; pappus, the specialized calyx of Asteraceae; tube/ligule, the unpigmented/pigmented region of corolla.

Temporal and spatial analysis of gchs1–3 *expression during corolla development*

To analyse the expression patterns of the isolated gerbera *chs*-like genes, we focused on corolla development and compared their expression pat-

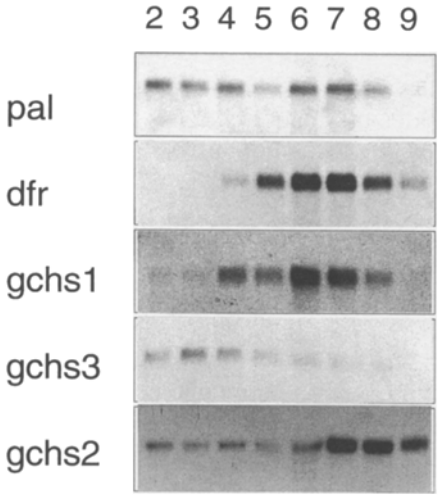

Fig. 5. Temporal expression patterns of *gchs1–3*, *pal* and *dfr* during corolla development. The developmental stages are described in [21]. The *pal* expression pattern with early (stage 3–4) and late (stage 7) peaks, separated by a period of low expression (stage 5) is reproducible in independent experiments.

terns to those coding for phenylalanine-ammonia lyase (*pal*) and dihydroflavonol-4-reductase (*dfr*, Fig. 1). The three gerbera *chs*-like genes differ in their temporal patterns of expression (Fig. 5). *Gchs3* expression peaks early during corolla development (around stage 3) and this peak coincides with the early period of strong *pal* expression. *Gchs1* expression peaks at stages 6–7, in coordination to the late *pal* expression (strengthening simultaneously) and *dfr* expression (that peaks slightly later), and is temporally related to anthocyanin accumulation. *Gchs2* expression peaks also at stage 7, but the gene is expressed throughout corolla development.

We also determined *in situ* the expression patterns during corolla development for the genes at the time of their highest level of expression (Fig. 6a). *Gchs3* expression is epidermis-specific. The expression is equally strong in both epidermal cell layers. The same pattern is observed for *pal* expression at the early stage. *Gchs1* expres-

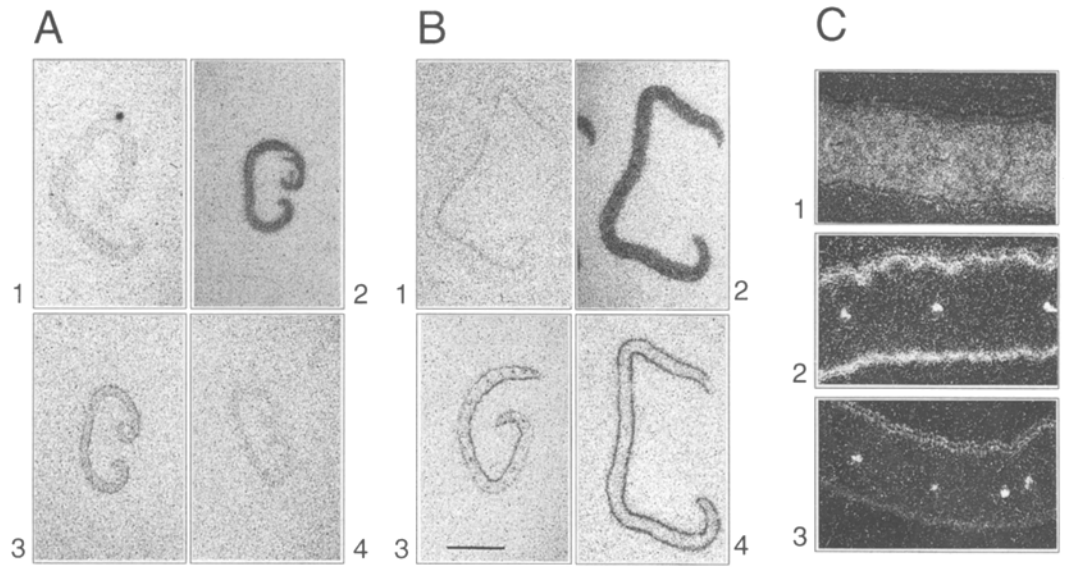

Fig. 6. *Gchs1–3*, *pal* and *dfr* expression during corolla development *in situ* (cross-sections). A. Early expression patterns: 1, *gchs3* (stage 5); 2, *gchs2* (stage 3); 3, *pal* (stage 3); 1–3, antisense probe; 4, the sense control for *pal*. No signal over background was observed with *gchs3* and *gchs2* sense control probes. X-ray film exposure. B. Late expression patterns: 1, *gchs1* (stage 7); 2, *gchs2* (stage 7); 3, *pal* (stage 6); 4, *dfr* (stage 7). No signal over background was observed with *gchs1*, *gchs2* and *dfr* sense control probes. With *pal* sense probe similar unspecific distribution of grains was observed as in the early stage. X-ray film exposure. Bar: 1 mm. C. 1, the mesophyll specific expression of *gchs2* (antisense probe); 2, the epidermis specific *dfr* expression (antisense probe); 3, sense control for *gchs2* expression: epidermis and vascular bundles reflect light, no grains are observed over background. Film emulsion exposure.

sion (at stage 6–7) is also epidermis-specific, but the adaxial expression is stronger than abaxial (Fig. 6b). The late epidermal *pal* expression shares this pattern, which is also related to the anthocyanin pigmentation pattern of var. Regina. At this stage *pal* expression is also observed in the vascular bundles. *Dfr* expression is equally strong in both epidermal cell layers. In contrast, *gchs2* expression is detected only in the mesophyll of the ligule (Fig. 6c).

The results indicate that the three gerbera *chs*-like genes exhibit a distinct genetic regulation during corolla development. *Gchs1* expression is temporally and spatially related to *pal* and *dfr* expression, and anthocyanin accumulation. The developmental pattern of *gchs3* expression coincides with the early *pal* expression. The same early nature of *gchs3* expression pattern is observed during pappus development (peak at stage 2), whereas *gchs1* is strongly expressed at later stages as well (stage 4 to 6; data not shown). The constitutive, mesophyll specific *gchs2* expression pattern is in contrast to that of the other genes and to the anthocyanin accumulation pattern.

Analysis of flavonols during corolla development

The gerbera corollas contain anthocyanin, flavonol and flavone classes of flavonoids [1]. To study the role of the early *pal* and *gchs3* expression, flavonol profiles of the stage 3+ (ray florets slightly longer than bracts, length of the ray floret corollas < 15 mm, corolla dry weight about 1 mg) and 7 (inflorescence half-opened, length of the ray floret corollas 35–40 mm, corolla dry weight about 7 mg) were compared. At stage 3+ the corollas are green and no anthocyanins are visible to the eye. However, they contain a trace of kaempferol-3-glucosides and a significant quantity of kaempferol-3-malonylglucosides (8.8 $\pm$ 1.1 μg per mg dry weight). At stage 7 the epidermal cell layers of the ligular parts of the corollas are anthocyanin pigmented, and a greater concentration of flavonols (3.6 $\pm$ 1.3 μg/mg kaempferol-3-glucosides; 7.1 $\pm$ 1.2 μg/mg kaempferol-3-malonylglucosides) are detected. Thus, the total amount of kaempferol-3-malonylglucosides at stage 3+ is about 9 μg and at stage 7 about 50 μg, and the amount of kaempferol-3-glucosides at stage 7 is about 30 μg. In addition to flavonols, an unidentified flavonoid was observed at both stages.

The early detection of both *pal* and *gchs3* expression and flavonols suggests that *gchs3* could be specifically involved in the production of flavonols at the early phase of corolla development. However, it may act together with *gchs1* that is also expressed to some extent at the early stage. On the other hand, flavonol production continues until the late stages of corolla development, suggesting that *gchs1* takes over the function. Its expression is temporally related to both flavonol and anthocyanin accumulation during corolla differentiation.

Analysis of the enzymatic activity of GCHS1–3

In order to investigate the catalytic properties of GCHS1–3 we cloned the cDNAs into the expression vector pKKtac and produced the enzymes in *E. coli*. Production of the proteins in the recombinant strains was verified by an experiment based on the method of Sancar *et al.* [42] in which a plasmid encoded polypeptide of the expected size was detected for each construct (data not shown). A parsley *chs* cDNA was used as a reference for the CHS function, and for the *E. coli* background, the vector with no insert was used.

The initial product of typical CHS reactions with 4-coumaroyl-CoA is naringenin chalcone, but *in vitro* most of it is converted non-enzymatically to naringenin in the course of the reactions, and therefore this is the main product observed in chromatograms. The radioactivity at the front detected with low-pH extractions represents malonic acid liberated from malonyl-CoA by thioesterases in the extracts (unpublished results). Figure 7A shows that CHS activity with 4-coumaroyl-CoA was absent in leaf and the *E. coli* control, and present in corolla and in *E. coli* extracts with all cloned CHSs, except for GCHS2.

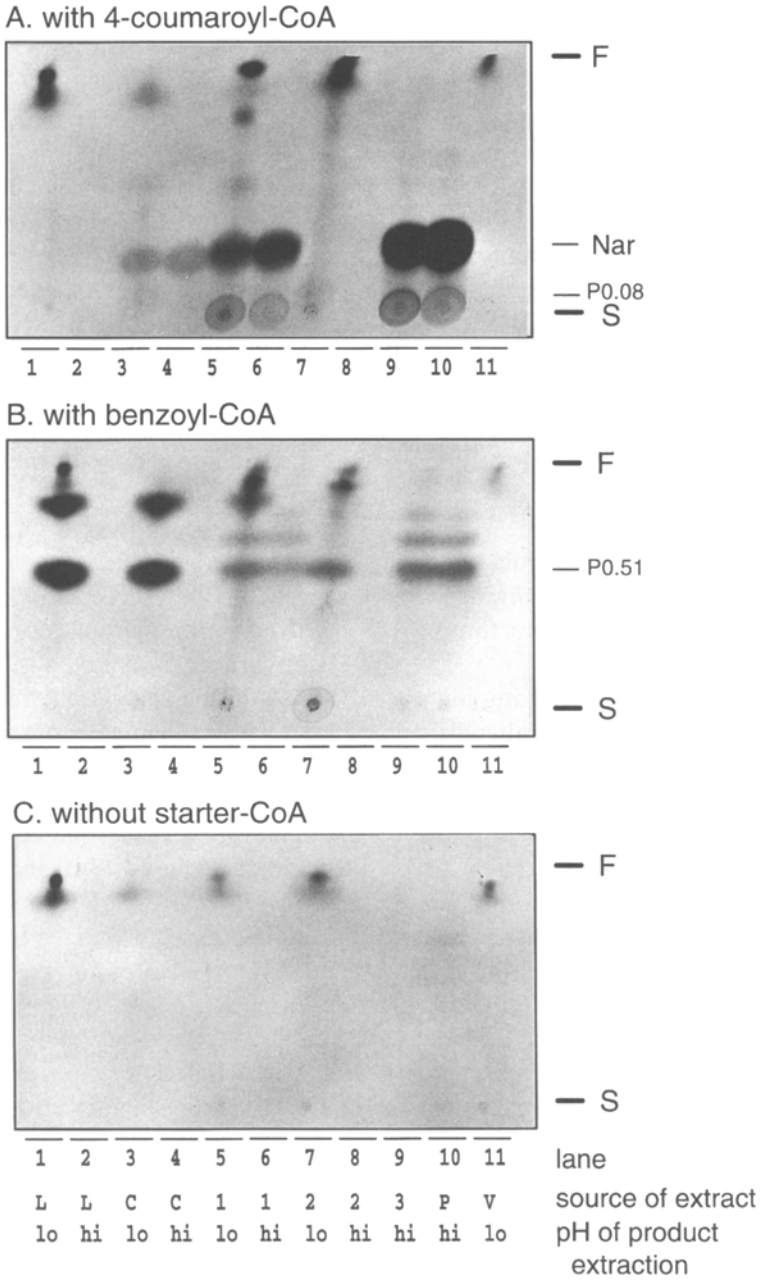

Fig. 7. Thin-layer chromatographic analysis of *in vitro* CHS reaction products. A, B, C: starter CoA esters used in the reactions. L, leaf; C, corolla; 1,2,3, GCHS1 to 3 expressed in *E. coli*; P, parsley CHS expressed in *E. coli*; V, *E. coli* control (vector). The pH at extraction of the products is labelled lo (pH 4) or hi (pH 8.8). S and F, start and front of the chromatograms. Nar, position of naringenin; P0.08 and P0.51, position of the unknown products.

This protein reproducibly reveals no significant formation of naringenin, but produces with 4-coumaroyl-CoA a very faint signal close to the start (P0.08).

This result does not correspond to the typical properties of CHS or STS [30, 33, 43]. It suggests that GCHS2 is either an inactive protein or is atypical in requiring a different starter-CoA

ester. We therefore tested several others (aliphatic: acetyl-CoA, propionyl-CoA, butanoyl-CoA, hexanoyl-CoA; aromatic: caffeoyl-CoA, benzoyl-CoA). The typical CHSs (parsley CHS, GCHS1, GCHS3) accept the linear substrates with the same specificity as described before for the CHS purified from parsley [47] (not shown), but GCHS2 reveals no significant amount of radioactive product, and the same result is obtained with caffeoyl-CoA (not shown). Of the tested substrates, only benzoyl-CoA leads to a product with GCHS2 (Fig. 7B, P0.51). The typical CHSs synthesize, apart from additional products, a substance migrating to roughly the same position. However, the P0.51 of GCHS2 is not extracted to the organic phase in high pH while at least part of the CHS derived signal is. The product accumulation pattern of STS from *A. hypogaea* with benzoyl-CoA is also different from that of GCHS2 (data not shown).

These data indicate that GCHS2 deviates in its properties from the typical CHSs. We therefore tried to identify such an activity in plant extracts from gerbera. Both leaf and corolla extracts exhibit high activity with benzoyl-CoA in the formation of the P0.51 product (Fig. 7B). The most important result of these experiments is that leaf extracts are active with benzoyl-CoA, but not with 4-coumaroyl-CoA (Fig. 7A), in correlation with the mRNA accumulation pattern of GCHS2 (Fig. 4). This provides strong evidence that the activity discovered with GCHS2 expressed in *E. coli* reflects an activity present in the plant tissues.

Discussion

We have cloned the cDNA and characterized the expression pattern of three *chs*-like genes that are active during corolla development in *Gerbera hybrida*. Furthermore, we have analysed the primary structure and enzymatic properties of the corresponding enzymes. Based on data from various experiments, three distinct roles can be defined for these genes. *Gchs1* and *gchs3* code for CHS enzymes. Temporally, *gchs3* expression correlates with early synthesis of flavonols and *gchs1* with late synthesis of both flavonols and anthocyanins. *Gchs2* codes for an enzyme with different catalytic properties than CHS.

The expression patterns of the *gchs1–3* demonstrate that CHS-like genes are representatives of distinct genetic programmes active during organ differentiation in *Gerbera*. *Gchs1* and *3* are both representatives of specific floral organ differentiation patterns, which ultimately are regulated through the interactions of the plant homeotic genes [8]. Still, the expression pattern of these genes differ, *gchs3* expression being highly abundant, specifically during pappus (sepal) differentiation. *Gchs2* is active both during the vegetative and reproductive phases of development, and its organ specificity is variable in both phases, suggesting that there are organ-specific factors involved in the modulation of *gchs2* mRNA levels in various tissues.

Also during corolla differentiation, *gchs1–3* reflect different genetic programmes. *Gchs1* and *3* belong to two different epidermis-specific regulation patterns, early-uniform (expression equally strong in both epidermal cell layers) (*gchs3*) and late-polar (expression stronger in the adaxial epidermis) (*gchs1*). *Pal* is involved in both programmes, as evidenced by the identical spatial and temporal expression patterns with both *gchs1* and *3* peaks, respectively. Since the *pal* probe is from a conserved region of the protein, it is possible that different genes are involved. Furthermore, *dfr* expression is under another (very late, uniform) regulation programme. The temporal difference between *chs* and *dfr* gene expression during corolla development has also been observed in other species [32]. In contrast, *gchs2* belongs to a temporally constitutive, mesophyll-specific regulation pathway. The role for the late epidermis-specific (*pal* + *gchs1*) programme is obviously anthocyanin and co-pigment synthesis. During corolla development, a temporal *gchs1* programme that overlaps with *dfr* expression, is observed also in snapdragon and petunia [23, 59]. In contrast, in carnation and lisianthus the flavonol production related early biosynthetic gene expression (*pal* and *chs*) completely precedes

the late biosynthetic genes (*dfr*) involved in anthocyanin production [11, 52]. The involvement of both an early (carnation-lisianthus-like) and a late (petunia-snapdragon-like) biosynthetic gene programme during corolla development is exceptional. The role for the early epidermis-specific (*pal* + *gchs3*) programme, in addition to give extra copigment supply, may be to give flavonol based protection for the young, developing florets.

The unusual features of *gchs2* gene expression and GCHS2 enzyme *in vitro* suggest a role that differs from typical CHS. The most important result is the altered substrate specificity of GCHS2 compared to CHS. With the *in vivo* substrate for CHS, 4-coumaroyl-CoA, GCHS2 is strikingly inactive, whereas GCHS2 has strong activity with benzoyl-CoA in a variety of different assay conditions (data not shown). The same activity is also found in plant extracts. To understand the catalytic potential of GCHS2 *in vitro*, experiments for solving the structure of the benzoyl-CoA-derived product (P0.51) are underway.

The structural and enzymological results suggest that GCHS2 is a novel enzyme of plant secondary metabolism, which probably shares a common evolutionary origin with CHS in a way analogous to STS [45]. Tropf *et al.* [56] have shown that STS probably has evolved from CHS polyphyletically, several times during evolution. To study the evolution of *gchs2*, we have recently isolated two other clones very homologous to *gchs2* from a genomic library (M. Kotilainen and Y. Helariutta, unpublished) indicating the presence of a *gchs2*-like subfamily in the gerbera genome. Studies on the substrate specificity suggest that GCHS2 is involved in the biosynthesis of phenolic compounds, as CHS and STS. The hypothesis is also supported by reports of Asteraceae-specific phenolic compounds which have accumulation patterns nearly identical to *gchs2* expression in gerbera. In *Encelia californica* chromenes accumulate at lower levels in roots and fruits, at higher levels in leaves, stems and capitula [37]. In the capitula of *Ageratum houstonianum* the largest amounts of chromenes are found in florets (mainly corolla tissue), but low amounts are also found in bracts and receptacle. In leaf, chromenes accumulate only in mesophyll [49]. Chromenes, which (based on feeding experiments) biogenetically originate from the phenylpropanoid pathway, are phenolic compounds typical for the Asteraceae (isolated, for example, from various *Gerbera* species [4]) and many of them are biologically active as insecticides and repellants [6, 37]. For relating GCHS2 activity to the accumulation of certain secondary metabolites, we have produced transgenic gerberas lacking detectable *gchs2* mRNA and corresponding enzyme activity, by antisense strategy [14] with anti-*gchs2* (P. Elomaa and Y. Helariutta, unpublished).

The study of *chs*-like genes active during corolla development in gerbera further extends the dynamic picture of the *chs* super-gene family in plants. Besides the well characterized *chs* and *sts* genes (reviewed by Schröder and Schröder [46]), there are several other recent reports of novel members in the family. Acridone synthase, which catalyses a CHS-like reaction with a N-methylanthraniloyl-CoA has been recently purified from *Ruta graveolens* and the tryptic fragments have been shown to display homology to CHS [3]. In petunia two UV-inducible, structurally atypical *chs* genes have been isolated [26], and in *Brassica* unusual anther-specific CHS genes have been found [48]. The genetic programmes behind the gerbera *chs* genes, on the other hand, are examples of the regulatory diversification of the *chs*-like genes. Future work on the structure-function relationships and molecular evolution with different members of the family will reveal how the structure of a *chs*-like gene (both in its coding and regulatory regions) has been modified for several functions and what are the evolutionary relationships of the various members of the super-gene family.

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