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Molecular cloning of the flavanone 3β-hydroxylase gene (FHT) from carnation (*Dianthus caryophyllus*) and analysis of stable and unstable FHT mutants

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Abstract Using a cDNA encoding the flavanone 3β-hydroxylase (FHT) from *Dianthus caryophyllus* (carnation) as a probe, we isolated the FHT gene from a genomic library. Sequence analysis revealed that the FHT gene consists of three exons and two introns. Two putative light-regulated elements were identified in the promoter region by sequence comparison. Southern blot analysis indicated that a single copy of the FHT gene is in the plant genome. Furthermore, a stable and an unstable FHT mutant of *D. caryophyllus*, both showing almost no FHT activity, were analyzed by Southern, Northern and Western blotting. It turned out that the FHT gene is present in both mutants, but no protein was detectable in the mutant flowers. FHT mRNA in amounts comparable to that found in the wild-type is present in flowers of the stable mutant, indicating a block in translation, but not in flowers of the unstable mutant, indicating a block in transcription. The translational block of the FHT mRNA of the stable mutant was demonstrated by in vitro translation of total flower mRNA followed by the specific measurement of FHT activity.

Key words Carnation · *Dianthus caryophyllus* Flavanone 3β-hydroxylase gene · 2-oxoglutarate-dependent dioxygenase · Translational block

Introduction

Flavanone 3β-hydroxylase (FHT, EC 1.14.11.9), an enzyme of the flavonoid pathway in plants, catalyzes the conversion of (*2S*)-flavanones to (*2R,3R*)-dihydroflavonols by 3β-hydroxylation (Fig. 1). Dihydroflavonols serve as precursors for various classes of flavonoids, such as flavonols, catechins, proanthocyanidins and anthocyanidins. FHT activity was first demonstrated in crude extracts of flower buds of *Matthiola incana*, but subsequently, the enzyme has also been detected in parsley cell cultures as well as in flower extracts of *Antirrhinum*, *Dahlia*, *Zinnia*, *Verbena*, *Streptocarpus*, *Petunia* and, recently, in *Dianthus caryophyllus* (Forkmann 1991). According to its co-factor requirements, the soluble enzyme belongs to the 2-oxoglutarate-dependent dioxygenases. Enzymes of this class are involved in various catalytic steps of primary metabolism and plant, fungal and bacterial secondary metabolism (Britsch et al. 1993).

Computer analysis has revealed significant sequence homology of the FHT to other known 2-oxoglutarate-dependent dioxygenases, such as hyoscyamine 6β-hydroxylase, deacetoxycephalosporin C synthase, or deacetylcephalosporin C synthase and to related non-heme iron-(II) enzymes as aminocyclopropanecarboxylic acid oxidase (ethylene-forming enzyme, EFE), isopenicillin N synthase or putative anthocyanidin synthase (ANS) (Matsuda et al. 1991).

FHT from *Petunia hybrida* has been purified to apparent homogeneity, antibodies raised and a FHT cDNA cloned from a cDNA library of *P. hybrida* (Britsch et al. 1992). Moreover, by heterologous screening FHT cDNA clones were isolated from flower-specific libraries of *D. caryophyllus*, *Callistephus chinensis* and *Matthiola incana* (Britsch et al. 1993).

In the present paper, we report the cloning of the flavanone 3β-hydroxylase gene from *D. caryophyllus*. Data obtained by sequence analysis and Southern blotting are discussed. Furthermore, a stable and an unstable mutant of *D. caryophyllus* were analyzed, both of which have been shown to be blocked at the FHT step. With a FHT-specific enzyme test following in vitro translation of total flower mRNA, we demonstrate that translation of the FHT mRNA is blocked in the stable FHT mutant.

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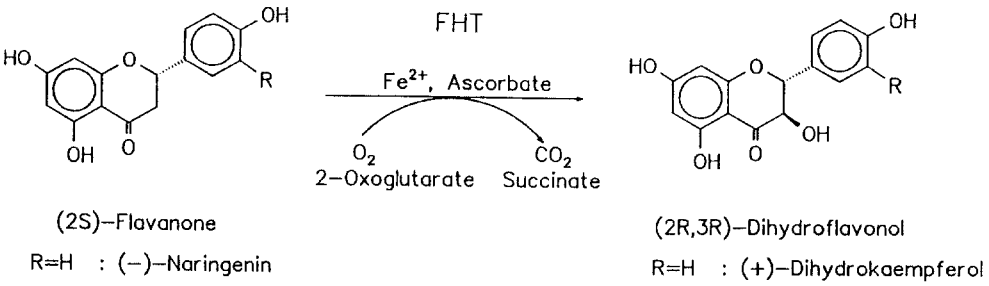


Fig. 1 Stereospecific hydroxylation of (*2S*)-flavanones resulting in the formation of (*2R,3R*)-dihydroflavonols catalyzed by flavanone 3β-hydroxylase. The (*2S*)-configuration of the substrate changes to the (*2R*)-conformation in the product as a consequence of the introduction of the hydroxyl group at C-3. The optical activity is usually levorotatory for the (*2S*)-flavavones and dextrorotatory for the (*2R,3R*)-dihydroflavonols

Materials and methods

Plant material

Dianthus caryophyllus vars 'Tanga', 'Nora' and 'Aladin' were obtained from Barbaret & Blanc (Antibes, France). 'Tanga' is a red-flowering wild-type of the pelargonidin type. 'Nora' is a spontaneous stable mutant that is almost totally blocked at the FHT step and 'Aladin' is a spontaneous unstable FHT mutant with flowers showing thin red stripes on a white background. The plants were cultivated under standard conditions in a greenhouse.

Isolation of plant DNA and RNA

Plant genomic DNA was isolated from 10 g young leaves as described by Schwarz-Sommer et al. (1984). Total RNA was prepared from 5 g young flower buds according to the method of Logemann et al. (1987). Subsequently, poly(A^+)RNA was isolated from total RNA by affinity chromatography on oligo(dT) cellulose (Sambrook et al. 1989). Either fresh material or plants frozen at –70°C were used.

Construction and screening of a genomic library

The genomic library was constructed using the EMBL4 vector (Frischauf et al. 1983). The phage DNA was digested with *Bam*HI and *Sal*I, and the vector arms were purified in a sucrose gradient. CsCl-purified genomic DNA was partially digested with *Mbo*I and size-fractioned by gel electrophoresis in 0.7% agarose. Fragments of 16–23 kb in size were electroeluted and ligated to the EMBL4 arms (Sambrook et al. 1989). The ligation mix was packaged in vitro, and 1 million recombinant phages were screened with the full-length FHT cDNA from carnation.

Blotting techniques

For Southern blotting, 10 µg genomic DNA was digested with restriction enzyme, fractioned in 1% agarose gels and transferred to nylon filters (Hybond N^+, Amersham) by standard techniques (Sambrook et al. 1989). Hybridizations were performed overnight at 68°C in 3×SSPE. Filters were washed twice in 2×SSPE/0.1% SDS at 68°C for 30 min. For Northern blotting, 4 µg poly(A^+)RNA was electrophoresed in 1.2% agarose gels and transferred to Hybond N filters (Amersham) (Sambrook et al. 1989). Hybridizations were carried out overnight at 42°C in 5×SSPE/50% formamide, and the filters were washed twice in 2×SSPE/0.1% SDS at 25°C for 15 min. Probes for hybridizations were prepared by the random priming method following the procedure of Feinberg and Vogelstein (1983).

DNA sequence analysis

Genomic inserts were subcloned into the pUC19 vector (Yanisch-Perron et al. 1985). DNA sequencing of both strands was performed by constructing a nested set of deletions using exonuclease III (Henikoff 1984) in the pUC19 plasmid and subsequent sequencing of the deletion plasmids by the dideoxy chain-termination method (Sanger et al. 1977).

Nuclease S1 mapping and primer extension analysis

Nuclease S1 mapping and primer extension analysis to determine the transcription start site of the FHT gene were performed according to Sambrook et al. (1989).

Western blotting

Proteins were isolated from 1 g young flower buds as described by Britsch and Grisebach (1986). For Western blotting, electrophoresis was done on 12% polyacrylamide gels (Laemmli 1970). The separated proteins were transferred to nitrocellulose membranes (Schleicher & Schüll). Polyclonal antibodies raised against petunia FHT (Britsch et al. 1992) were diluted 1:1000. Detection was performed using the Western blotting detection reagents according to the supplier's protocol (Amersham).

In vitro translation

In vitro translation was performed by adding 2 µl of a 1 m*M* amino acid mixture (Promega) and 5 µg of poly(A^+)RNA to 40 µl reticulocyte lysate (Promega). After incubation for 1 h at 30°C the translation mixture was purified using a Sephadex G50 (Pharmacia) column with 1 ml bed volume, equilibrated and eluted with enzyme buffer (0.1 *M* TRIS/HCl, pH 7.5, 20 m*M* sodium ascorbate).

Assay of FHT activity

The FHT assay was performed as described previously (Britsch and Grisebach 1986) using (±)-[^{14}C]naringenin as substrate. For thin-layer chromatography (TLC) on cellulose plates (Merck), the solvent system chloroform/acetic acid/water (10:9:1, v/v/v) was used. Radioactivity on the TLC plates was determined with a TLC analyzer (Berthold, Wildbad, FRG).

Results and discussion

Molecular characterization of the genomic locus of the FHT gene

To isolate the FHT locus of *D. caryophyllus*, a genomic library consisting of 16–23 kb *Mbo*I fragments of carnation wild-type DNA was constructed. The library was screened with carnation FHT cDNA as a probe, and several independent recombinant clones were isolated. Southern blot analyses using the full-length FHT cDNA revealed that the restriction pattern of one of the phages corresponds to the genomic blot (data not shown). The clone was sequenced, and the structure of the gene was determined by comparing the DNA sequence of the cDNA and that of the genomic clone (Fig. 2). No nucleotide exchanges were found to have occured in the cDNA sequence when it was compared to the genomic DNA sequence.

The FHT gene of *D. caryophyllus* consists of three exons (508 bp, 429 bp and 579 bp) interrupted by two introns of 2103 bp and 673 bp, respectively. Since this is the first time a complete sequence of a FHT gene is described, no comparison can be drawn with homologues of other plant species. However, the comparison of the carnation FHT gene with gene sequences of other previously characterized 2-oxoglutarate-dependent and related dioxygenases reveals that, despite their similarity in amino acid sequence, the gene structures seem to be totally different. Tomato ethylene-forming enzyme genes *eth1* and *eth2*, for example, consist of four exons and three introns each (Köck et al. 1991; Holdsworth et al. 1987). The maize anthocyanidin synthase gene *A2*, however, contains no introns (Menssen et al. 1990).

The nucleotide sequence around the ATG translation start codon is in agreement with the consensus proposed for plant genes (Joshi 1987a). At the 3′ end of the gene a putative polyadenylation signal AATATA was found 180 bp downstream of the TAG translation stop codon and 90 bp before the polyadenylation site (Joshi 1987b).

The transcription start site was determined by nuclease S1 mapping and primer extension analysis (data not shown). A putative TATA box is located upstream of the transcription start site at position –20 to –30, and a putative CAAT box is found at an appropriate distance from the cap site at position –73 to –81. Both boxes perfectly match the consensus sequences for eukaryotic promoters (Joshi 1987a).

The promoter region contains two putative light-responsive elements, at positions –191 to –199 and –369 to –377, which resemble the consensus sequence GGTTAA determined for the light-responsive *Pisum sativum rbcS-3A* gene (Green et al. 1988). Although these elements have to be tested for functionality, light-regulation of the FHT gene would be consistent with the observation that the FHT enzyme of carnation shows a first activity peak in not yet opened flower buds in order to provide dihydroflavonols for the synthesis of flavonols. A second FHT activity peak in newly opening buds, probably due to light-induction of the FHT gene, provides dihydroflavonols for anthocyanin biosynthesis (Stich et al. 1992). Definitive proof of light-regulation due to these sequence elements will come from deletion analyses and footprinting experiments.

The CACGTG motif which mediates light responsiveness of the CHS gene of *Antirrhinum majus* (Staiger et al. 1989) was not found in the FHT promoter.

Copy number of FHT genes in the genome of *D. caryophyllus*

To determine the number of FHT genes in the genome of *D. caryophyllus*, wild-type genomic DNA was digested with *Eco*RI, *Eco*RV and *Hin*dIII, blotted and hybridized at high stringencies. A 400-bp *Eco*RI fragment comprising the 3′ terminal part of the carnation FHT cDNA was used as a probe (Fig. 3).

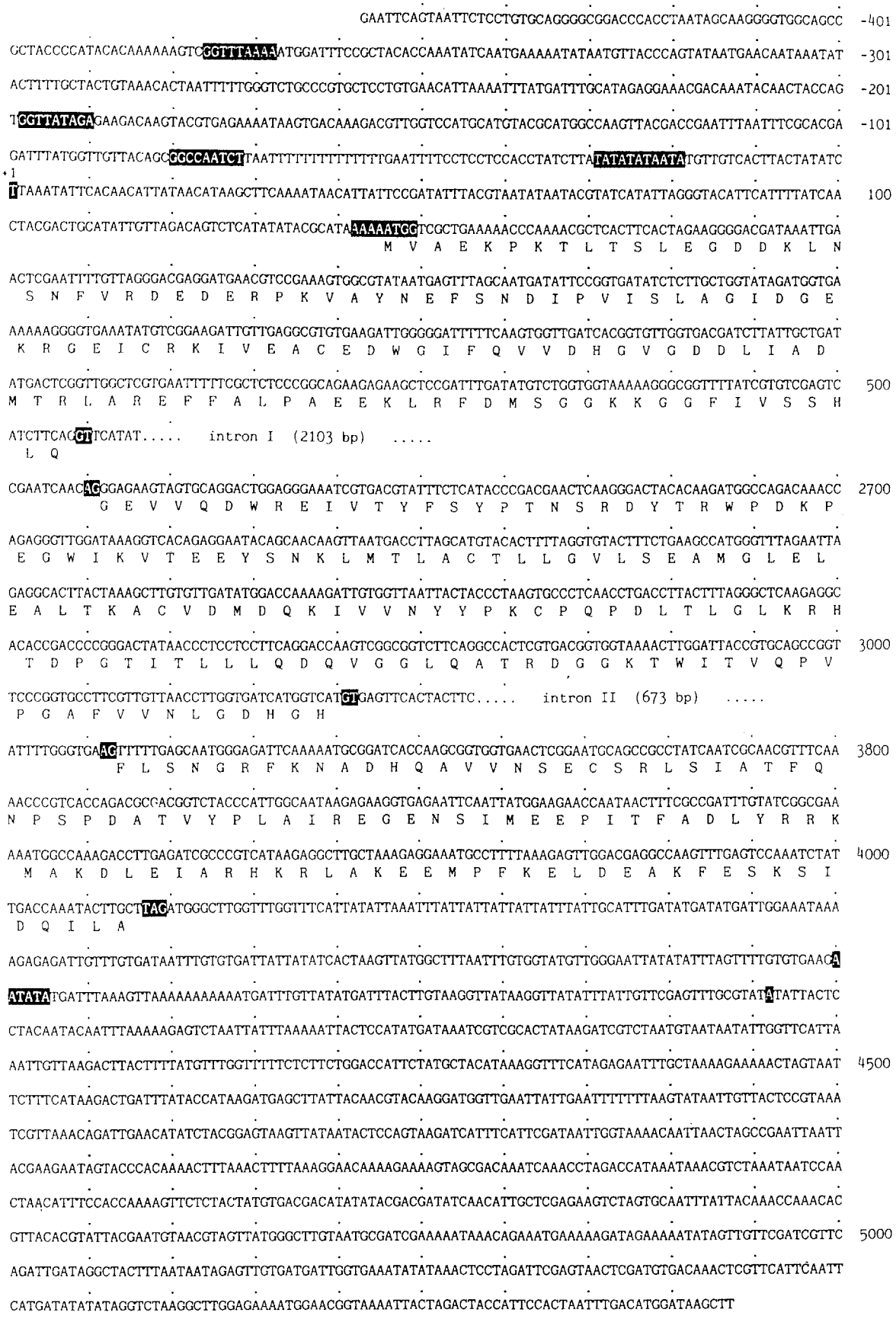
The presence of only one band in each lane after hybridization with the 3′ probe indicates that the FHT gene occurs as a single-copy gene per haploid genome of *D. caryophyllus*. Analogous results were obtained for *Petunia hybrida* (Britsch et al. 1992), *Callistephus chinensis*, *Rosa hybrida* and *Matthiola incana*, where only one FHT gene is present in the genomes as well (data not shown). Therefore, the FHT gene does not seem to belong to a gene family as has been found for other flavonoid genes. In *Petunia*, for example, at least seven genes have been identified encoding the chalcone synthase (Koes et al. 1987), two encoding the chalcone isomerase (Van Tunen et al. 1988) and three encoding the dihydroflavonol 4-reductase (Beld et al. 1989).

Analysis of FHT mutants of D. caryophyllus

In order to analyze the defects of the stable FHT mutant 'Nora' and the unstable FHT mutant 'Aladin', genomic DNA was prepared and digested with *Eco*RI, *Eco*RV and *Hin*dIII. A Southern blot analysis was carried out using the FHT cDNA of *D. caryophyllus* as a probe for hybridization (Fig. 4).

The hybridization pattern revealed no difference between the wild-type, stable and unstable mutant. So, the stable defect of the carnation variety 'Nora' can not be due to a larger deletion in the FHT gene. For the same reason, the unstable mutation of 'Aladin' is not the result of the insertion of a transposable element into the FHT gene. Although Southern blot analysis with the methylation sensitive restriction enzyme *Hpa*II exhibited no difference between wild-type and FHT mutants, it can not be excluded that the unstability of the carnation variety 'Aladin' is caused by spontaneous demethylation of a specific GC-pair in the FHT promoter.

In Western blot analyses in which 50 µg of total protein from the wild-type was used, a 41 kDa FHT protein was detectable with a polyclonal antibody raised against petunia FHT protein. This antibody exhibits some unspecific-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+1
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M V A E K P K T L T S L E G D D K L N
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S N F V R D E D E R P K V A Y N E F S N D I P V I S L A G I D G E
AAAAAGGGGTGAAATATGTCGGAAGATTGTTGAGGCGTGTGAAGATTGGGGGATTTTTCAAGTGGTTGATCACGGTGTTGGTGACGATCTTATTGCTGAT
K R G E I C R K I V E A C E D W G I F Q V V D H G V G D D L I A D
ATGACTCGGTTGGCTCGTGAATTTTTCGCTCTCCCGGCAGAAGAGAAGCTCCGATTTGATATGTCTGGTGGTAAAAAGGGCGGTTTTATCGTGTCGAGTC 500
M T R L A R E F F A L P A E E K L R F D M S G G K K G G F I V S S H
ATCTTCAGGTTCATAT..... intron I (2103 bp) .....
L Q
CGAATCAACAGGGAGAAGTAGTGCAGGACTGGAGGGAAATCGTGACGTATTTCTCATACCCGACGAACTCAAGGGACTACACAAGATGGCCAGACAAACC 2700
G E V V Q D W R E I V T Y F S Y P T N S R D Y T R W P D K P
AGAGGGTTGGATAAAGGTCACAGAGGAATACAGCAACAAGTTAATGACCTTAGCATGTACACTTTTAGGTGTACTTTCTGAAGCCATGGGTTTAGAATTA
E G W I K V T E E Y S N K L M T L A C T L L G V L S E A M G L E L
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E A L T K A C V D M D Q K I V V N Y Y P K C P Q P D L T L G L K R H
ACACCGACCCCGGGACTATAACCCTCCTCCTTCAGGACCAAGTCGGCGGTCTTCAGGCCACTCGTGACGGTGGTAAAACTTGGATTACCGTGCAGCCGGT 3000
T D P G T I T L L L Q D Q V G G L Q A T R D G G K T W I T V Q P V
TCCCGGTGCCTTCGTTGTTAACCTTGGTGATCATGGTCATGTGAGTTCACTACTTC..... intron II (673 bp) .....
P G A F V V N L G D H G H
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F L S N G R F K N A D H Q A V V N S E C S R L S I A T F Q
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M A K D L E I A R H K R L A K E E M P F K E L D E A K F E S K S I
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D Q I L A
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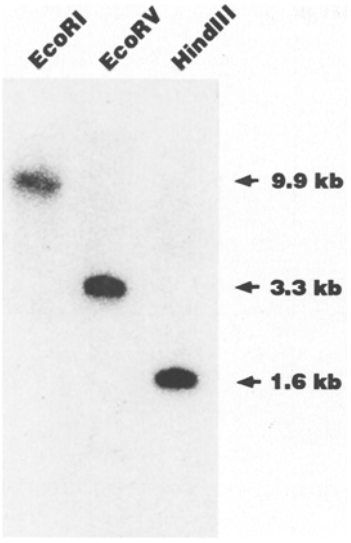

Fig. 3 Southern blot analysis of DNA from wild-type carnation. Genomic DNA (10 µg per lane) was hybridized to a [^{32}P]-labelled 400-bp *Eco*RI fragment containing only the 3′ region of the carnation FHT cDNA

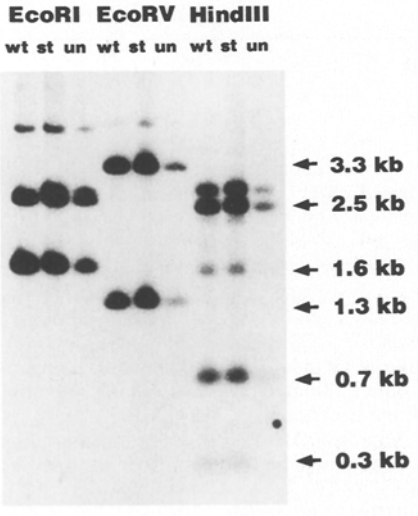

Fig. 4 Southern blot analysis of genomic DNA from the wild-type (*wt*), stable FHT mutant (*st*) and unstable FHT mutant (*un*) of *D. caryophyllus*. Ten micrograms genomic DNA per lane was hybridized to the [^{32}P]-labelled full-length carnation FHT cDNA

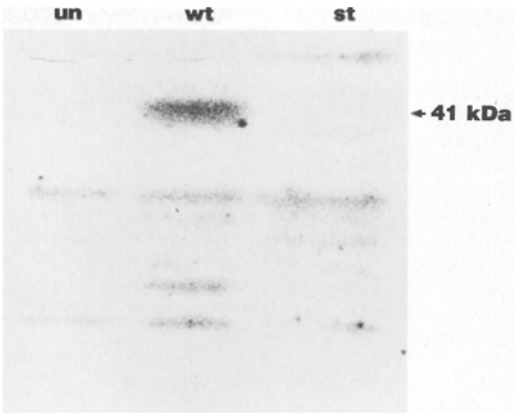

Fig. 5 Western blot analysis of 50 µg of total protein from flowers of the wild-type (*wt*), stable FHT mutant (*st*) and unstable FHT mutant (*un*) of *D. caryophyllus*. The filter was incubated with a polyclonal antibody raised against petunia FHT protein

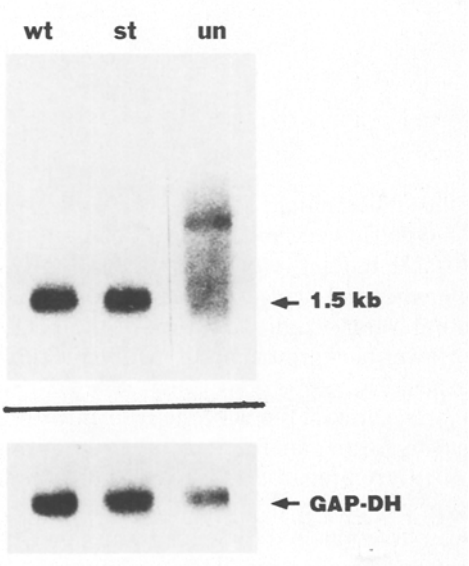

Fig. 6 Northern blot analysis of poly(A$^+$)RNA from the wild-type (*wt*), stable FHT mutant (*st*) and unstable FHT mutant (*un*) of *D. caryophyllus*. Four micrograms poly(A$^+$)RNA per lane was hybridized to the [^{32}P]-labelled full-length carnation FHT cDNA. The wild-type and stable mutant lanes were exposed overnight; the unstable mutant lane, 1 week. Hybridization of the same blot with a GAP-DH probe was performed to test equal loading. All lanes were exposed overnight

Fig. 2 DNA sequence and derived protein sequence of the FHT gene from *D. caryophyllus*. The following sequence motives are *boxed* (*top to bottom*): two putative light-responsive elements, CAAT box, TATA box, transcription and translation start sites, exon-intron borders, translation stop codon, putative polyadenylation signal and polyadenylation site. The entire nucleotide sequence including the introns has been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X70378

ities in all of the carnation varieties analyzed so far (Koch 1992). However, compared to the wild-type, in the stable and unstable mutants no FHT protein is present (Fig. 5).

Therefore, a Northern blot analysis was performed with 4 µg poly(A$^+$)RNA isolated from young flower buds of the wild-type, stable and unstable mutant. Hybridization was done with carnation FHT cDNA. It turned out that in flow-

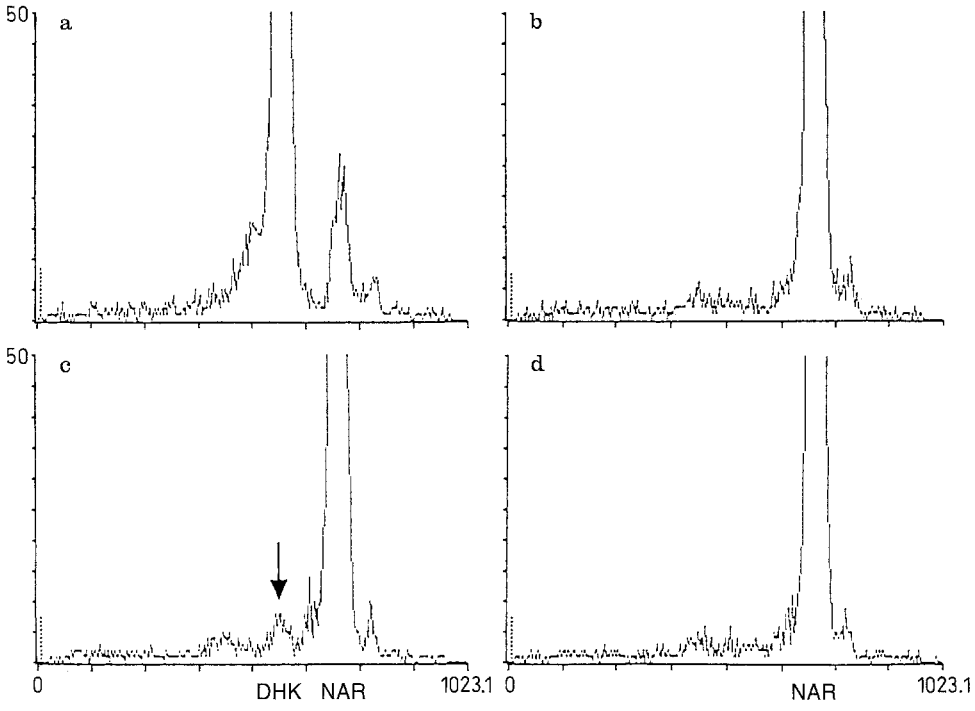

Fig. 7a–d Radioscan of TLC on cellulose from FHT assays using (±)-[^{14}C] naringenin as substrate. **a** Assay with crude extract of wild-type carnation, **b** with reticulocyte lysate, **c** with in vitro synthesized FHT using poly(A$^+$)RNA from flower buds of wild-type carnation, **d** with in vitro synthesized FHT using poly(A$^+$)RNA from flower buds of the stable FHT mutant. *NAR* naringenin, *DHK* dihydrokaempferol

ers of the stable mutant as much FHT mRNA is present as in wild-type flowers, indicating a defect in translation. In the unstable mutant only traces of FHT mRNA were detectable when the exposure time was extended to 1 week, indicating a transcriptional block of the FHT gene in the white flower background and functional expression in the red stripes (Fig. 6).

The translational block of the FHT mRNA of the stable mutant was further analyzed by in vitro translation of 5 µg poly(A$^+$)RNA prepared from young flower buds of the wild-type and mutant using reticulocyte lysate. Translation mixtures were purified by gel filtration on Sephadex G50 columns and assayed for FHT activity with a specific enzyme test (Britsch and Grisebach 1986).

The result is shown in Fig. 7. As a positive control for assaying FHT activity a crude extract of wild-type carnation was used (Fig. 7a), demonstrating the functionality of the test system. The reticulocyte lysate itself showed, as expected, no FHT activity (Fig. 7b). The in vitro translation products of wild-type poly(A$^+$)RNA exhibited FHT enzyme activity, as indicated by the small dihydrokaempferol peak (Fig. 7c). The translation mixture derived from poly(A$^+$)RNA of the stable mutant, however, revealed no FHT activity, as expected for a blocked translation (Fig. 7d). Since in Western blot analyses no FHT protein fragments specific for the stable mutant were detected, the translational defect of the FHT mRNA of this mutant is probably due to a non-functional leader that inhibits translation initiation.

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