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Identification of the *Arabidopsis thaliana* Flavonoid 3'-Hydroxylase Gene and Functional Expression of the Encoded P450 Enzyme

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The phenylpropanoid pathway results in the synthesis of thousands of compounds, including flavonoids like flavonols, anthocyanidins and tannins. In *Arabidopsis thaliana*, the lack of tannins in the seed coat (*testa*) causes the *transparent testa* (*tt*) phenotype. In the present study, we identified the gene responsible for the *tt*7 mutation. We show that *TT7* encodes the enzyme flavonoid 3'-hydroxylase (F3'H), and demonstrate that this P450-dependent monooxygenase has F3'H activity. The availability of the *AtF3'H* gene and promoter sequence will allow us to study the coregulation of a complete set of flavonol and anthocyanidin biosynthesis genes in *A. thaliana*, and makes *in vitro* synthesis of hydroxylated flavonoids more feasible.

Key words: *Arabidopsis* / Flavonoid biosynthesis / P450 monooxygenase / Transparent testa / tt7.

Introduction

The phenylpropanoid metabolism provides plants with various important compounds, including lignin, coumarins, and other phenolics (Hahlbrock and Scheel, 1989). One important branch leads to the production of flavonoids, and in particular to flavonols, anthocyanidins and tannins. This branch is well understood at the genetic and molecular level because mutations in flavonoid-related genes are non-lethal and cause easily recognisable phenotypes such as alterations in flower or seed colour (Holton and Cornish, 1995).

During maturation of *A. thaliana* seeds, dark-brown flavonoid pigments, mainly condensed tannins, accumulate in the seed coat (Chapple *et al.*, 1994). As a consequence of disordered or disrupted flavonoid biosynthesis, plants produce yellow or pale-brown seeds. More than 15 loci involved in flavonoid biosynthesis were identified on the basis of alterations in seed colour (Koornneef, 1990; Shirley *et al.*, 1995; Focks *et al.*, 1999). They were designated *transparent testa* (*tt*) because the phenotype is due to the reduction or absence of pigments in the seed coat (*testa*), revealing the yellow colour of the cotyledons. So far, five *TT*-loci have been studied at the molecular level and the functions of the respective gene products have been elucidated. Four loci encode enzymes of early steps in flavonoid biosynthesis (Weisshaar and Jenkins, 1998): *tt*4 mutants are deficient in chalcone synthase (CHS) (Chang *et al.*, 1988), *tt*5 in chalcone flavanone isomerase (CFI) (Shirley *et al.*, 1992), *tt*6 in flavanone 3-hydroxylase (F3H) (Wisman *et al.*, 1998), and *tt*3 in dihydroflavonol 4-reductase (DFR) (Shirley *et al.*, 1992). The fifth locus is *TTG1* (*Transparent Testa Glabra1*), which encodes a regulatory protein similar to the WD40 factor AN11 from *Petunia hybrida* (Walker *et al.*, 1999).

Another locus involved in flavonoid biosynthesis in *A. thaliana* is *tt7*. The *tt7-1* allele has been isolated from an EMS (methanesulfonic ethyl ester) – mutagenised population of the Landsberg *erecta* (L*er*) ecotype (Koornneef *et al.*, 1982). Seeds from *tt*7 plants are pale-brown, and the anthocyanin content of the whole plant body is reduced. *tt*7 plants accumulate the 4'-hydroxylated flavonoids pelargonidin (anthocyanidin) and kaempferol (flavonol), while wild-type plants contain mainly the corresponding 3',4'-hydroxylated products cyanidin and quercetin. The data indicated that *TT7* is required for flavonoid 3'-hydroxylase (F3'H) activity, and it has been suggested that *TT7* encodes F3'H (Koornneef *et al.*, 1982; Sheahan and Rechnitz, 1993). However, this assumption had not yet been proven.

The F3'H enzyme is a cytochrome P450-dependent monooxygenase that requires NADPH as a co-factor (Forkmann, 1991). This enzymatic activity was first demonstrated in microsomal preparations from cultured *Happloppapus gracilis* cells (Fritsch and Grisebach, 1975). A cDNA encoding a F3'H has recently been isolated from *Petunia hybrida* (Brugliera *et al.*, 1999). No other *F3'H* sequences have been reported so far, a fact that might be explained by the sequence conservation of the many P450 genes existing in plants (Schuler, 1996) which makes identification of a specific gene difficult. Here we report the identification of the *F3'H* gene from *A. thaliana*, show that the *TT7* locus codes for *AtF3'H*, and demonstrate that the encoded enzyme has F3'H activity. The *AtF3'H* expression pattern correlates well with the accumulation of 3',4'-hydroxylated flavonoids during development and after stress.

Results

tt7 Plants Carry an Internal STOP Codon in the Putative *AtF3'H* Gene

A putative P450 gene was annotated by the Arabidopsis Genome Initiative (AGI; BAC F13G24, GenBank accession number: AL133421; protein ID: CAB62611.1) that displayed 77% overall amino acid (aa) sequence similarity (68 % identity) to the known *PhF3'H* sequence (Figure 1A). BAC F13G24 maps to the upper arm of chromosome V at about 20 cM (closest RI marker: mi97), and *tt7* maps to a similar position on the classical genetic map. The postulated open reading frame (ORF) of 513 codons was amplified by RT-PCR. Comparison of the resulting cDNA sequence with the predicted gene confirmed the proposed exon/intron structure. DNA gel-blot analysis showed (i) that the restriction pattern of genomic DNA detected with an *AtF3'H* fragment is in agreement with the restriction map deduced from BAC F13G24, and (ii) that the gene is present in single copy in the *A. thaliana* genome. We hypothesised that the putative P450 gene encodes AtF3'H.

DNA sequences corresponding to the putative *AtF3'H* gene were amplified from the *A. thaliana* ecotypes Columbia (Col) and L*er*, and from *tt*7 plants. Sequencing of the resulting fragments revealed that the *tt*7 mutant carries a point mutation that leads to an internal ochre stop codon in the first exon of the putative *AtF3'H* gene (Figure 1). The mutation terminates the ORF after 113 aa, most probably resulting in a non-functional peptide. The DNA sequences are available from GenBank: *AtF3'H* cDNA Col, AF271651; *AtF3'H* cDNA L*er*, AF271650; *AtF3'H* cDNA *tt7*, AF271649. Comparison of the Col and L*er* sequences revealed a *Bst*XI restriction polymorphism which was used to generate a CAPS marker. This marker, designated F3prH, was placed on the Lister and Dean RI map where it mapped to the top of chromosome V (20 cM).

Prior to this work, a plant line with pale-brown seeds was isolated from an *En-1*-mutagenised *A. thaliana* population. In contrast to the wild type, plants of this line (R22) contain only derivatives of pelargonidin and kaempferol which are hydroxylated at the 4' and not at the 3' position. The mutation in R22 was shown to be allelic to the *tt7* mutation (M. Sagasser and B. Weisshaar, unpublished results). Sequencing of the putative *AtF3'H* gene from R22 revealed a frameshift mutation in the ORF. The data from the two independent *tt7* alleles and the corresponding biochemical phenotypes show (i) that the putative gene indeed encodes F3'H, and (ii) that in *tt7* the *AtF3'H* gene is mutated.

AtF3'H Expressed in Yeast Shows Flavonoid 3'-Hydroxylase Activity

To prove that the newly isolated gene actually encodes an enzyme with F3'H activity, the enzyme was expressed in yeast. Microsomal fractions from expression vector-containing yeast cells were subjected to a hydroxylation assay using [^{14}C]-labelled flavonoids as substrates. Analyses of the reaction products by TLC (Figure 2A) showed that naringenin was converted to eriodictyol, and dihydrokaempferol to dihydroquercetin (see Figure 2B). The F3'H activity was dependent on the presence of the recombinant protein, and on NADPH as a cofactor. Product

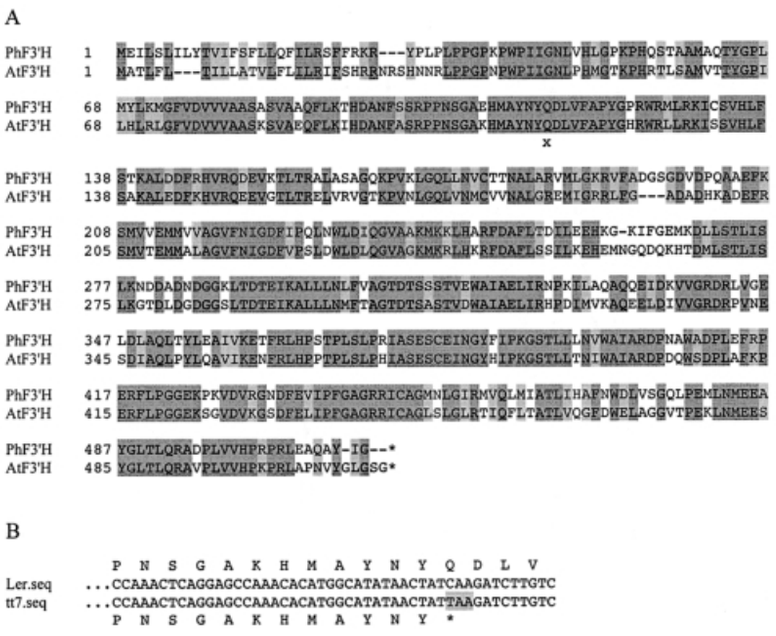

Fig. 1 Comparison of F3'H Amino Acid and *AtF3'H* DNA Sequences.
(A) Comparison of deduced amino acid sequences from AtF3'H and PhF3'H. Identical amino acid positions are highlighted in dark grey, similar positions are marked in light grey. Dashes represent gaps introduced for optimal alignment. The x marks the position of the internal stop codon in the *tt7* sequence.
(B) Comparison of L*er* wild-type and *tt7* mutant *AtF3'H* sequences. The stop codon introduced by the mutation is highlighted.

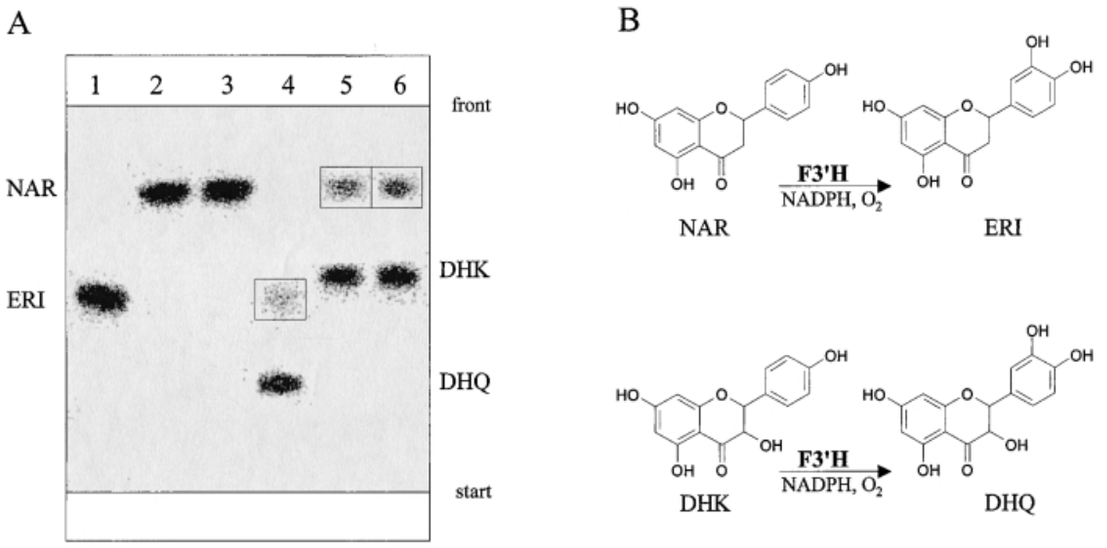

Fig. 2 Activity Assay of AtF3'H Expressed in Yeast.
(A) Autoradiograph of the TLC resolving the products of the assay. [^{14}C]-labelled flavonoids extracted from the six samples were loaded, and the plate was developed in CAW. Samples 1 to 3 contained naringenin (NAR) as substrate, samples 4 to 6 contained dihydrokaempferol (DHK). Samples 1 and 4 contained microsomes from F3'H-expressing yeast cells and show full conversion of the substrates. Samples 2 and 5 are controls without NADPH. In case of samples 3 and 6, the microsomes were derived from yeast cells transformed with pYES2 without the *AtF3'H* ORF. Samples 5 and 6 contain traces of NAR (boxed) present in the DHK substrate (DHK was synthesised from NAR), in sample 4 this NAR was converted to eriodictyol (ERI; boxed). DHQ: dihydroquercetin.
(B) Chemical structures of educts and products, and scheme of the hydroxylation reaction.

identity was confirmed in three different solvent systems by co-chromatography with authentic standards. Taken together, the data clearly demonstrate that the *AtF3'H* gene was isolated.

The *AtF3'H* Gene Is Expressed in Siliques

The expression of the *AtF3'H* gene was studied in various plant organs including siliques, and upon irradiation with UV light. RNA gel-blot analysis revealed high expression of the *AtF3'H* in siliques and to an lower extent in stems, flowers, and senescing leaves, and showed *AtF3'H* transcript accumulation after UV light treatment of cultured *A. thaliana* cells (Figure 3). The expression pattern observed for the *AtF3'H* gene correlates well with the distribution of 3',4'-hydroxylated flavonoids in *A. thaliana* plants (see Discussion) and UV-treated cultured cells which accumulate 3',4'-hydroxylated flavonols in the vacuole.

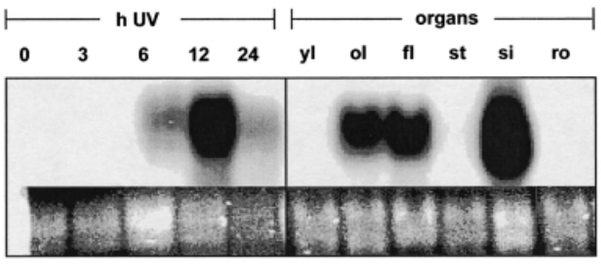

Fig. 3 RNA Gel-Blot Analysis of *AtF3'H* Expression.
Total RNA (10 μg) of various *A. thaliana* (Col) organs and of UV-treated cultured cells were loaded, the *AtF3'H* ORF fragment was used as probe. As loading control, the ethidium bromide-stained 18S rRNA band is shown. The duration of the UV-treatment prior to harvesting (in hours) and the organs are indicated above the lanes. yl, young leaf; ol, senescing leaf; fl, flower; st, stem; si, silique; ro, roots.

Discussion

The aim of this work was to identify and isolate the *A. thaliana* gene for F3'H, the P450 monooxygenase of flavonoid biosynthesis catalysing the 3'-hydroxylation of the flavonoid B-ring to the 3',4'-hydroxylated state. Sequences provided by the *A. thaliana* genome sequencing project (BAC F13G24; see http://www.mips.biochem.mpg.de/proj/thal/) allowed the identification of a candidate gene for *AtF3'H*. This assignment was based on sequence similarity to *PhF3'H* (Brugliera *et al.*, 1999) and the known map position of the biochemically characterised *tt7* mutation (Koornneef *et al.*, 1982). The initial hypothesis was confirmed by the detection of null mutations in two different *TT7* alleles (*tt7-1* and the *f3'h-2f* allele from R22) affecting the *AtF3'H* gene. *AtF3'H* is, like the genes for *CHS*, *CFI*, *DFR*, and *F3H*, a single copy gene in *A. thaliana*. This lack of redundancy explains why mutations in these genes are easily obtained in *A. thaliana*. The single point mutation in the original *tt7-1* allele fits the expectation for a mutation caused by EMS. A difference in the map position of about 14 cM was observed between the classic genetic map and the RI marker next to BAC F13G24. This difference exemplifies the difficulties in integrating genetic and

physical maps, but at the same time illustrates the usefullness of such information in selecting candidate genes.

Although line R22, which was shown to contain a mutation allelic to *tt7*, was isolated from an *En-1* mutagenised population (Wisman *et al.*, 1998), the recessive *tt* phenotype was not linked to an *En-1* insertion. This prevented the identification of *TT7* by transposon tagging. However, the line provided an additional *tt7* allele designated *f3'h-2f* which was useful for gene identification. Allele *f3'h-2f* contains a mutation resembling an *En-1* footprint, indicating that non-precise transposon excision has caused the mutation.

Final proof that *AtF3'H* indeed encodes the expected enzyme was provided by demonstrating enzymatic activity *in vitro*. Cytochrome P450-dependent monooxygenases are membrane-bound heme proteins which interact with NADPH-cytochrome P450-reductase (CPR). AtF3'H was expressed using yeast cells, and microsomal fractions were used in the assay. The yeast cells used provided the required CPR activity (Urban *et al.*, 1994). AtF3'H converted both, naringenin and dihydrokaempferol, into the corresponding 3',4'-hydroxylated products. PhF3'H accepted a similar range of educts (Brugliera *et al.*, 1999). Taken together, AtF3'H has been conclusively identified as a functional P450 monooxygenase catalysing the hydroxylation of flavonoids. In the future, the expression system established in this work will be used to determine the exact substrate specificity of AtF3'H. The recombinant enzyme will also be very useful for the synthesis of different 3',4'-hydroxylated flavonoids which will allow *e.g.* biochemical assays with downstream enzymes.

Up to now, no ESTs for *AtF3'H* have been submitted to GenBank. Based on our studies, the expression level of the gene can be fairly high, but requires specific environmental or developmental conditions for activity. Thus, it is reasonable to assume that genes that are expressed only in response to specific triggers are underrepresented in the EST collections, and this might also be the case for *AtF3'H*. The observation that there are 12 ESTs for *AtCHS* in the database, and 4 ESTs for *AtCFI*, might indicate that *AtF3'H* is regulated in a way different to *AtCHS* and *AtCFI*. The expression pattern for *AtF3'H* shows a good correlation to the accumulation pattern of 3',4'-hydroxylated flavonoids. Expression in siliques provides precursors for tannin biosynthesis in the seed coat. In senescing leaves, which show under certain growth conditions accumulation of red anthocyanin pigments (cyanidin derivatives) during senescence, *AtF3'H* expression is required for anthocyanidin biosynthesis. The observation that the *AtF3'H* expression is strongest in siliques matches previous studies which showed that quercetin is the predominant flavonol in seeds (Shirley *et al.*, 1995). The availability of the *AtF3'H* promoter and cDNA will allow further experiments to examine the expression pattern of the gene and its correlation with the distribution of 3',4'-hydroxylated flavonoids.

Materials and Methods

Plant Material and Standards

Seeds of *tt7* were obtained from the Nottingham Arabidopsis Stock Centre (NASC line N88), line R22 was isolated from the *En-1*-mutagenised *A. thaliana* population (Wisman *et al.*, 1998). TLC standards for NAR, ERI, and DHQ were obtained from Roth (Karlsruhe, Germany); DHK was from the Forkmann laboratory collection.

Standard Molecular Biology Techniques

Basic molecular biology techniques were applied as described (Sambrook *et al.*, 1989). Where required, PCR fragments were inserted into pCR2.1-TOPO (Invitrogen, Groningen, The Netherlands) prior to sequencing. Sequences of plasmid DNA or PCR fragments were determined on PE Biosystems (Weiterstadt, Germany) 377 sequencers using BigDye-terminator chemistry. Oligonucleotides were purchased from metabion (München, Germany). Total RNA was isolated from various organs of six week old plants, from senescent leaves of eight week old plants, and from UV-treated cultured *A. thaliana* cells (At7; Trezzini *et al.*, 1993). RNA isolation, RNA gel-blot analysis and probe labelling was performed as described (Feldbrügge *et al.*, 1994). DNA fragments for labelling were generated by PCR and gel-purified. Fragments were labelled by random priming using premixed reagents (Roche, Mannheim, Germany). Genomic DNA was isolated from *A. thaliana* leaves using the Nucleon PhytoPure kit (apbiotech, Freiburg, Germany).

cDNA Synthesis

The *AtF3'H* cDNA fragment representing the complete ORF was amplified from a cDNA-pool generated using the SMART™ PCR cDNA Synthesis Kit (Clontech, Palo Alto, USA) with total RNA from plants exposed for 24h to UV-containing white light. Primer sequences were: 5'-GCCGGATCCATGGCAACTCTATTTCTCAC-3' and 5'-GCAGAATTCAATTTTAACCCGACCCGAG-3'.

Heterologous Expression in Yeast

The complete *AtF3'H* ORF was inserted into the vector pYES2 (Invitrogen). Yeast strain INVSc1 (Invitrogen) was transformed as described (Gietz *et al.*, 1992). Cells were cultured in YPGA, SGI and YPGE medium (Urban *et al.*, 1997). Microsome preparation has also been described earlier (Urban *et al.*, 1994).

Assay of AtF3'H Enzyme Activity and Product Analysis by TLC

Flavanones and dihydroflavonols labelled with [^{14}C] were prepared using CHS, CFI, and F3H enzymes as described (Britsch *et al.*, 1981, 1992). The incubation mixture (final volume 200 µl) contained: 165 µl 0.1 M Tris-HCl pH 7.5, 0.3 nmol radioactive substrate (67 Bq; naringenin or dihydrokaempferol, respectively), 10 µl 20 mM NADPH and 25 µl microsomal fraction. After incubation for 15 min at 25 °C the reaction was stopped by adding 20 µl MeOH containing a mixture of the respective unlabelled flavonoids. The reaction mixture was extracted twice with EtOAc (100 and 50 µl). Material from the pooled upper phase was co-chromatographed with authentic flavonoid samples on precoated cellulose plates (Merck, Darmstadt) with the following solvent systems: (1) $CHCl_3$-HOAc-H_2O (10:9:1; CAW); (2) 30% HOAc; (3) HOAc-HCl-H_2O (30:3:10). Plates were analysed using Fuji BAS 1000 Bio-Imaging Analyzer, and flavonoids were detected as described (Eigen *et al.*, 1957; Barton, 1986).

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