
Analysis of Flavanone 3-Hydroxylase in Arabidopsis Seedlings

Coordinate Regulation with Chalcone Synthase and Chalcone Isomerase

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A genomic clone encoding flavanone 3-hydroxylase (F3H) was isolated from Arabidopsis thaliana. The deduced amino acid sequence is 72 to 94% identical to all previously reported F3H proteins. Low-stringency DNA blot analysis indicated that F3H is encoded by a single gene in Arabidopsis. The F3H locus was mapped to the bottom of chromosome 3 and therefore does not correspond to any of the 13 flavonoid-deficient transparent testa mutants for which a map position is known. Analysis of gene expression in etiolated seedlings exposed to white light and in two putative regulatory mutants, ttg and tt8, demonstrated that the Arabidopsis F3H gene is coordinately expressed with chalcone synthase and chalcone isomerase in seedlings, whereas dihydroflavonol reductase expression is controlled by distinct regulatory mechanisms. The F3H gene may represent a pivotal point in the regulation of flavonoid biosynthesis because its expression is coordinated with different subsets of genes in different plant species.

Flavonoids are secondary metabolites found in all higher plants that have numerous important functions. It has been known for some time that flavonoids act as signaling molecules in plant-microbe interactions, provide pigmentation to attract pollinators, and act as phytoalexins (reviewed in Hahlbrock and Scheel, 1989; Dooner et al., 1991; Koes et al., 1992; van der Meer et al., 1992; Li et al., 1993; Stapleton and Walbot, 1994). At the biochemical level, the flavonoid biosynthetic pathway is one of the best characterized of all plant secondary pathways (Fig. 1), and genes encoding flavonoid biosynthetic enzymes have been cloned in a variety of plant species.

Arabidopsis thaliana provides a simple model for studying flavonoid biosynthesis because CHS, CHI, and DFR appear to be encoded by single copy genes (Feinbaum and Ausubel, 1988; Shirley et al., 1992). These genes are expressed at specific developmental times, in response to cytokinins, and in response to environmental stresses such as high-intensity white light, UV light, and heat or cold (Feinbaum and Ausubel, 1988; Feinbaum et al., 1991; Kubasek et al., 1992; Shirley and Goodman, 1993; Deikman and Hammer, 1995; Leyva et al., 1995). Thirteen loci required for flavonoid biosynthesis have been identified in Arabidopsis (Shirley et al., 1995; M. Koornneef, personal communication). Mutations at these loci interfere with the synthesis of flavonoid pigments in the seed coat (the tt phenotype) and in some cases also disrupt the synthesis of pigments in vegetative and floral tissues. Seven of these loci have been correlated with specific steps of the flavonoid biosynthetic pathway. Of particular interest are two putative regulatory loci, TTG and TT8. Plants carrying mutations at these loci express wild-type levels of CHS and CHI mRNA but little or no mRNA for DFR (Shirley et al., 1995). In addition, in seedlings grown in darkness and then exposed to UV-B or blue light, CHS and CHI transcripts are coordinately induced, whereas DFR mRNA levels increase slightly later (Kubasek et al., 1992). This indicates that, as in snapdragon and petunia, CHS and CHI expression is controlled by mechanisms that are, at least in part, distinct from those that control DFR expression (Martin et al., 1991; Jackson et al., 1992; Quattrocchio et al., 1993).

F3H catalyzes an early step in flavonoid metabolism, the formation of dihydroflavonols from flavanones, and therefore provides precursors for many classes of flavonoid compounds (Fig. 1). In addition, F3H gene expression appears to be pivotal in the regulation of the flavonoid pathway; it is coordinately regulated with CHS and CHI in petunia but with downstream genes in snapdragon. Here we report the characterization of a genomic clone encoding F3H in Arabidopsis. DNA blot analysis suggests that F3H is encoded by a single gene in this species. The F3H locus was mapped to the bottom of chromosome 3. RNA blot analysis indicates that in Arabidopsis seedlings, flavonoid gene expression is controlled in a manner analogous to what has been described in petunia flowers.

Abbreviations: CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone-3-hydroxylase; RI, recombinant inbred; RT-PCR, reverse transcription-PCR; tt, transparent testa.

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**Figure 1.** Schematic of the flavonoid biosynthetic pathway. The genes shown in bold have been cloned in Arabidopsis. Abbreviations: flavan-3' hydroxylase (F3'H), flavonol synthase (FS), anthocyanidin synthase (AS), glucosyl transferase (GT), rhamnosyl transferase (RT), and O-methyl transferase (OMT).

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

RI lines constructed by Lister and Dean (1993) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). RI lines and wild-type *Arabidopsis thaliana* plants were grown in soil in a growth chamber at 21°C with a 16-h light/8-h dark cycle. For RNA expression studies, Arabidopsis wild-type (Landsberg), *tt4* and *tt8* seedlings were grown under 120 μE m⁻² s⁻¹ continuous white light on Murashige and Skoog Suc plates (Kubasek et al., 1992).

**PCR Cloning of an F3H Gene Fragment**

Degenerate oligonucleotide primers were designed based on alignments of the deduced amino acid sequences of F3H from alfalfa, apple, barley, grape, maize, Matthiola, carnation, and petunia (Meldgaard, 1992; Britsch et al., 1993; Davies, 1993; Sparvoli et al., 1994; Deboo et al., 1995). Primers (Amitoff, Boston, MA) with the following sequences were used in these experiments: 5'-GA(CT)GA(GA)(AC)(GATC)CC(GATC)AA(GA)GT-3' (F3HSEN1), 5'-GT(GATC)-CA(GA)GA(CT)TG(GA)(AC)(GATC)GA-3' (F3HSEN2), 5'-GG(GA)TT(CT)TG(GA)AA(GA)GT(GATC)GA(GA)-(GATC)GC-3' (F3HAN1), and 5'-GT(GATC)GC(CT)TG(GATC)AA(GA)GATC(CC(GATC)-CC-3' (F3HAN2). PCR reactions were performed using 100 ng of Landsberg genomic DNA as template according to the method of Gould (1989), with the exception that 100-μM primer concentrations were used. Optimal annealing temperatures were determined empirically (60°C for F3HSEN1/F3HAN1 and 54°C for F3HSEN2/F3HAN2). Amplification products from a reaction using the F3HSEN1/F3HAN1 primers were fractionated in a 0.8% low-melting-point agarose/TAE gel, and a band of the expected size was excised and purified using a Wizard PCR Prep column (Promega). This product was reamplified with the F3HSEN2/F3HAN2 primers to produce a 350-bp product, purified as above, and sequenced directly using Sequenase version 2 (United States Biochemical) according to the manufacturer's protocol.

**Probe Synthesis**

Probes for library screening and DNA blot analysis were synthesized by PCR using digoxigenin-labeled dUTP (Boehringer Mannheim). The ratio of digoxigenin-dUTP: dTTP in all reactions was between 1:2 and 1:6. The F3HSEN2/F3HAN2 primer combination was used on the 350-bp product described above, whereas pH3H0.7 was labeled using T3 and T7 primers. The products of these reactions were purified through Wizard PCR Prep columns to remove unincorporated nucleotides.

To generate probes for RNA blot analysis, cDNA corresponding to the coding regions of CHS, CHI, F3H, and DFR was synthesized by RT-PCR (Shirley and Hwang, 1995). The CHS, CHI, and DFR products were cloned into the EcoRI and SalI sites of pBluescript KS+ (Stratagene), and the F3H product was cloned into the Smal site. Inserts were labeled by PCR using T3 and T7 primers and 50 μCi of [α-32P]dATP (Dupont-NEN) in a 20-μL reaction. CHS and actin (AAt2) probes were synthesized in the same way except that CHS-specific or SP6 and T7 primers were used. Probes were separated from unincorporated nucleotides using 1 mL of Sephadex G-50 (Sigma) spin columns.

**F3H Cloning and Sequencing**

Genomic clones for the F3H gene were isolated from a λ FIX library constructed of Landsberg genomic DNA (Voytas et al., 1990). Approximately 30,000 plaques were screened as described previously (Shirley et al., 1992) using the 350-bp F3H fragment as a probe. Prehybridization (1 h) and hybridization (overnight) were at 65°C in the hybridization buffer of Church and Gilbert (1984). Filters were washed two times for 20 min in 500 mL of 40 mM NaHPO₄, pH 7.2, 1 mM EDTA, and 1% (w/v) SDS at the temperature of hybridization. The filters were then processed for digoxigenin probe detection according to the manufacturer's protocol (Boehringer Mannheim). Phage DNA was prepared from liquid lysates according to the method of Grossberger (1987). DNA fragments were subcloned into pBluescript KS+ vectors (Stratagene) and used to transform Escherichia coli strain JM109. The plasmid subclones were as follows: pH3H 4.9, pH3H1.2, pH3H0.6, and pH3H0.7.

Plasmid DNA was isolated for sequencing according to the method of Ish-Horowicz and Burke (1981). Sequencing was performed on both strands using T3 and T7 primers and primers designed from this primary sequence using [α-35S]dATP and Sequenase version 2 (United States Biochemical).
chemical) as described previously (Shirley et al., 1992). DNA sequence analysis was carried out with the DNA STAR software package (Madison, WI).

DNA and RNA Gel Blot Analysis

Genomic DNA was isolated from 3-week-old Landsberg and Columbia plants as described by Watson and Thompson (1986). This DNA was digested, fractionated in a 0.8% agarose gel with Tris-borate-EDTA buffer, and transferred to 0.2 µm Biotrans nylon membranes (ICN) in 25 mM sodium phosphate (pH 6.5) by capillary action. Filters were irradiated using a UV Crosslinker (Fisher Biotech, Pittsburgh, PA) and then processed as described for plaque lifts, except that the prehybridization, hybridization, and washes were at 58°C.

Total RNA was isolated from 3-d-old seedlings according to the method of Simon et al. (1992) and was fractionated in formaldehyde gels (Gerard and Miller, 1986). Gels were stained with ethidium bromide after fractionation to ensure that all lanes were loaded equally. Transfer, prehybridization, hybridization, and posthybridization washes were as described for DNA blots but at a temperature of 65°C. The damp filters were exposed to x-ray film at -80°C using intensifying screens.

F3H Mapping

DNA was isolated from 24 of the RI lines (Lister and Dean, 1993), and DNA blot analysis was performed as described above. The lines used for this analysis were CL4, CL13, CL33, CL37, CL115, CL190, CL191, CL194, CL217, CL231, CL238, CL242, CL245, CL263, CL267, CL283, CL284, CL288, CL295, CL302, CL303, CL332, CL358, and CL370. Segregation data were analyzed using MapMaker (Lister and Dean, 1993).

RESULTS

Isolation of the Arabidopsis F3H Gene

To isolate F3H sequences from Arabidopsis, two nested pairs of degenerate oligonucleotide primers were designed that corresponded to identical amino acids in F3H sequences from eight plant species (Fig. 2). These primers amplified a 350-bp product from the Arabidopsis genome that was sequenced and found to have high homology to other F3 sequences (see "Materials and Methods"). This fragment was used to screen an Arabidopsis (ecotype Landsberg) genomic library (Voytas et al., 1990), and two λ clones were purified to homogeneity. Restriction enzyme digestion and DNA blot analysis indicated that the two clones were overlapping (data not shown). A 4.9-kb EcoRI restriction fragment from one of the clones (λF3H1), which appeared to contain the entire coding region of F3H as well as approximately 1 kb of promoter sequence, was subcloned into pBluescript, as were subclones of this EcoRI fragment (Fig. 2).

The sequence of 2319 bp encompassing the Arabidopsis F3H gene was determined (Pelletier and Shirley, 1995). The deduced amino acid sequence showed high homology with all previously reported F3H sequences, with barley (72% amino acid identity) being the least similar and Matthiola (94% amino acid identity) being the most similar (Table I). Furthermore, amino acids that are conserved in these proteins were also present in the deduced amino acid sequence of the Arabidopsis gene, including three His residues that have been implicated in the binding of iron at the active site (His 75, His 217, and His 275) (Britsch et al., 1993). This idea is supported by the recently reported crystal structure of isopenicillin N synthase from Aspergillus nidulans (Roach et al., 1995). In this enzyme, which is closely related to F3H, two analogous His residues and an Asp residue have been shown to bind Mn at the active site.

DNA Blot Analysis and Mapping of the Arabidopsis F3H Gene

To determine the copy number of F3H in Arabidopsis, genomic DNA from ecotypes Landsberg and Columbia was digested with EcoRI, HindIII, and BamHI, and low

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<th>Plant</th>
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<td>Arabidopsis</td>
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<td>Matthiola</td>
<td>95</td>
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<td>Carnation</td>
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<td>Grape</td>
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stringency DNA blot analysis was performed. A single band was detected in all samples when a probe that did not contain recognition sites for these enzymes was used (Fig. 3). Identical results were obtained using a hybridization temperature of 55°C with a probe to the most conserved region of F3H (data not shown). This indicates that F3H is encoded by a single gene in Arabidopsis. In addition, polymorphisms were detected between the two ecotypes using EcoRI (Fig. 3) and DraI (data not shown).

To determine if the F3H locus maps near any of the known tt loci, genomic DNA from 24 RI lines was digested with EcoRI, and DNA blot analysis was performed. The segregation data (not shown) were used to map the F3H locus to the lower arm of chromosome 3, 2.4 cm from m457 and 3.7 cm from g2778 (C. Lister, personal communication). Of the 11 previously mapped tt loci (Shirley et al., 1995) and two identified recently by M. Koornneef (personal communication), only tt5 and tt6 were reported to be located in this region. The tt5 mutation has been shown to correspond to an allele of the CHI gene (Shirley et al., 1992). Recent evidence indicates that the correct map location for tt6 is actually chromosome 5 (J. Campanella and C. Town, personal communication). Thus, a mutation in the F3H structural gene has not yet been identified in Arabidopsis.

**F3H Gene Expression in Response to White Light and in Two Regulatory Mutants**

Arabidopsis seedlings grown in darkness for 3 d and then exposed to UV-B or blue light accumulate CHS and CHI mRNA coordinately, whereas DFR mRNA accumulates slightly later (Kubasek et al., 1992). To determine if F3H is coordinately regulated with CHS and CHI or with DFR in response to light, wild-type seedlings were grown in darkness for 72 h and exposed to white light (which included wave lengths in the blue region). RNA was extracted after 0, 1, 2.5, 5, and 8 h of light exposure, and the pattern of mRNA accumulation for CHS, CHI, F3H, and DFR was determined by RNA blot analysis. This experiment demonstrated that CHS, CHI, and F3H steady-state mRNA levels were highest approximately 2.5 h after induction with light, and DFR levels were highest approximately 5 h after induction (Fig. 4). Although the kinetics of induction differed slightly from those reported earlier (Kubasek et al., 1992), possibly because white rather than blue light was used in this experiment, it was clear that the F3H gene was coordinately expressed with CHS and CHI and preceded expression of the DFR gene.

The results of this experiment suggested that a similar pattern of regulation would be observed in the two putative regulatory mutants, ttg and tt8, which contain reduced steady-state levels of mRNA for DFR but wild-type levels of CHS and CHI (Shirley et al., 1995). This hypothesis was tested by performing RNA blot analysis on 3-d-old Landsberg, ttg, and tt8 seedlings grown in continuous white light because flavonoid genes are expressed at high levels in wild-type plants at this stage of development (Kubasek et al., 1992). As expected, CHS and CHI mRNAs were detected at high levels in wild-type seedlings (Fig. 5). The slightly lower levels of CHS and CHI in tt8 appeared to be due to unequal loading of the samples because mRNA levels for an actin gene (AAc2) were also reduced. F3H expression was similar to that observed for CHS and CHI among all three seedling types, indicating that neither TTG nor TT8 is necessary for F3H mRNA accumulation in seedlings.

**DISCUSSION**

**Isolation and Characterization of the Arabidopsis F3H Gene**

We have isolated a genomic clone from Arabidopsis that has high homology to all previously reported F3H sequences. Furthermore, based on the predicted protein sequences from these genes, all amino acids that are absolutely conserved in other F3H proteins are found in the Arabidopsis protein as well, including three His residues that have been implicated in the binding of iron at the active site (Britsch et al., 1993). Low-stringency DNA blot analysis suggests that F3H is present as a single copy gene in Arabidopsis (Fig. 3). This conclusion is strengthened by

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Figure 3. DNA blot analysis of F3H gene copy number in Arabidopsis. Each lane contained 5 μg of DNA from Arabidopsis ecotypes Columbia (C) and Landsberg (L) digested with HindIII (H), EcoRI (E), or BamHI (B). The filter was hybridized with pF3H0.7 at a temperature of 58°C. The positions of molecular weight markers are shown.
Analysis of Flavanone 3-Hydroxylase in Arabidopsis Seedlings

Figure 4. RNA blot analysis of flavonoid gene expression in etiolated seedlings exposed to white light. Arabidopsis seedlings were grown in darkness for 3 d and then shifted to white light. Total RNA was extracted after 0, 1, 2.5, 5, and 8 h of light exposure and fractionated in formaldehyde gels. Ethidium bromide staining of rRNA was used to confirm equivalent loading. RNA blots containing 10 μg of RNA per lane were hybridized with CHS, CHI, F3H, and DFR probes.

The fact that the three expressed sequence tag sequences with high homology to F3H (GenBank accession nos. T44308, F14453, and F14454) appear to be identical to the sequence reported here. This is not surprising because the other three cloned flavonoid genes from Arabidopsis also appear to be single copy (Feinbaum and Ausubel, 1988; Shirley et al., 1992). In addition, F3H is believed to be encoded by a single gene in maize and petunia, even though other flavonoid enzymes are encoded by small gene families in these species (Britsch et al., 1992; Deboo et al., 1995). The F3H gene was mapped to the lower arm of chromosome 3. None of the mapped tt loci are located in this region of the genome, so a mutation in the Arabidopsis F3H structural gene remains to be identified.

Regulation of F3H in Developing Seedlings

Two lines of evidence presented in this paper indicate that CHS, CHI, and F3H are coordinately regulated in 3-d-old Arabidopsis seedlings, and DFR expression is controlled, at least in part, by distinct regulatory mechanisms. First, seedlings that were grown in darkness and then exposed to white light accumulated the highest steady-state levels of mRNA for CHS, CHI, and F3H at about 2.5 h, whereas mRNA levels for DFR were highest after approximately 5 h of light exposure (Fig. 4). Second, RNA blot analysis of Landsberg, ttg, and tt8 seedlings grown in continuous white light demonstrated that CHS, CHI, and F3H steady-state mRNA levels were unaffected in either mutant, whereas DFR mRNA was not detectable in ttg and was reduced in tt8 (Fig. 5).

Analysis of transgenic plants containing regulatory genes from other species has provided additional insights into the regulation of flavonoid gene expression. It has been shown that the maize R gene can complement the ttg mutation in Arabidopsis and that the maize Lc gene (an R homolog) can complement the petunia an2 mutation (Martin et al., 1991; Jackson et al., 1992). In contrast, studies on the petunia regulatory mutants an1, 2, and 11 indicate that the division of control is different in flowers of this species; F3H is coordinately regulated with CHS and CHI rather than with downstream genes (Quattrocchio et al., 1993). This is identical to what we have observed in Arabidopsis seedlings.
expression of genes downstream of F3H in Arabidopsis and petunia. Less is known about ttt because complementation studies have not been performed, although it has been speculated that T78 may be an Arabidopsis homolog of CI (Shirley et al., 1995). In contrast to the results obtained using the maize R gene, the snapdragon homolog DEL, which coordinately regulates F3H with downstream genes, cannot complement ttt (Mooney et al., 1995). Overexpression of DEL does, however, induce flavonoid synthesis in tobacco flowers and vegetative tissues of tomato. Therefore, although there appear to be significant differences in the mechanisms controlling flavonoid gene expression in these diverse plant species, there are also striking similarities in the regulation of the flavonoid pathway in Arabidopsis and petunia.

The biological significance of the differential control of flavonoid gene expression remains a mystery. Why is F3H coordinately expressed with CHS and CHI in Arabidopsis seedlings and petunia flowers while it is expressed with DFR in maize seedlings and snapdragon flowers? Flavonoids serve a wide range of functions, many of which are specific to particular tissues or plant species (reviewed in Hahlbrock and Scheel, 1989; Dooner et al., 1991; Stafford, 1991; Koes et al., 1993). Arabidopsis flavonoid-deficient mutants are more sensitive to UV-B light (Li et al., 1993), so it is possible that the pattern of induction by white light described in this paper may reflect the need for specific flavonoids that provide protection from UV damage. Specifically, it may be advantageous for seedlings to accumulate dihydroflavonols or flavonols (Fig. 1) before allowing these intermediates to be converted into compounds that may not afford as much UV protection. The differences in the regulation of flavonoid genes in Arabidopsis and maize seedlings may point to alternative or additional mechanisms of UV protection.

It is clear that much more work is required before the regulation of the flavonoid biosynthetic pathway and its biological significance are fully understood. Antibodies against the Arabidopsis CHS, CHI, F3H, and DFR enzymes are currently being developed to examine induction patterns at the protein level (M.K. Pelletier, C.C. Cain, and B.W. Shirley, unpublished results). Methods are also becoming available for accurate identification and quantification of flavonoid end products in Arabidopsis (Graham, 1991; Li et al., 1993). These tools, together with the well-characterized flavonoid mutants available in a variety of plant species, offer promise for answering these fundamental questions in plant metabolism.

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