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# Expression of a putative *flavonoid 3′-hydroxylase* in sorghum mesocotyls synthesizing 3-deoxyanthocyanidin phytoalexins

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**Abstract**

In sorghum, ingress of *Cochliobolus heterostrophus* stimulates the synthesis of 3-deoxyanthocyanidins that act as phytoalexins. Apigeninidin and luteolinidin are two major phytoalexins induced in the first 24 h after infection. In an attempt to understand genetic regulation of the biosynthesis of sorghum phytoalexins, we isolated a differentially expressed partial cDNA. Characterization and comparison showed that this cDNA sequence corresponds to a putative *flavonoid 3′-hydroxylase*. Full length sequence characterization allowed us to establish that the sorghum putative *f3′h* cDNA encodes a peptide of 517 amino acids that has domains conserved among cytochrome P450 proteins functioning in the flavonoid biosynthetic pathway. Heterologous expression of the putative *f3′h* cDNA in *Escherichia coli* yielded a membrane preparation that catalyzed the hydroxylation of naringenin. We show here that transcription of the flavonoid 3′-hydroxylase was coordinately regulated with that of chalcone synthase and dihydroflavonol reductase, and expression of these genes was induced within the first 24 h of fungal challenge. Synthesis of apigeninidin and luteolinidin followed the induced expression of the *f3′h* gene, implicating its role in fungal induced expression of sorghum phytolaexins.


*Keywords:* Apigeninidin; *Cochliobolus heterostrophus*; Cytochrome P450; Eriodictyol; Flavonoid 3′-hydroxylase; Luteolinidin; Naringenin; Phytoalexin; *Sorghum bicolor*; 3-Deoxyanthocyanidins

## 1. Introduction

The flavonoid biosynthetic pathway in plants has been an extensively characterized system for genetic and biochemical studies of secondary metabolites. Ever since the isolation of chalcone synthase, which controls the first committed step of this pathway [18], efforts have been made to isolate mutants and clone genes that are required at different steps [10]. Apart from their importance as a molecular genetic system, flavonoids are involved in various biological functions during the growth and development of a plant [33]. Some of these functions include attraction of pollinating agents via pigmentation of floral organs [17], pollen tube germination [24,43], protection from UV exposure [3,20,21], and defense against insects [41] and pathogens by acting as phytoalexins [7,25]. The role of flavonoids as phytoalexins has been very well exemplified in the studies of sorghum–*Colletotrichum* interactions [27]. Sorghum plants challenged with conidia of *Colletotrichum sublineolum* produce reddish-brown pigments in the leaf epidermal cells at the site of fungal infection [32]. Mass spectrometry of these pigments allowed the identification of 3-deoxyanthocyanidins, which

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include luteolinidin, 5-methoxy-luteolinidin, apigeninidin, and the caffeic acid ester of arabinosyl 5-*O*-apigeninidin. These compounds accumulate in intracellular inclusion bodies, which migrate towards the site of fungal penetration and then release their contents, killing both the fungus and the cells that synthesize them [23,31,32]. Through the use of micro spectrometry, Snyder et al. [31] quantified phytoalexin levels in infected host cells and found these to be 150 μM, which exceeds the amount required for toxicity to the fungus in vitro. A similar response can be observed when sorghum plants are challenged with the fungus *Cochliobolus heterostrophus* that is not pathogenic to sorghum [1,26].

The antimicrobial activity of 3-deoxyanthocyanidins in sorghum has been established, but their biosynthetic route is not very well elucidated. It has been proposed that these compounds arise from the phenylpropanoid pathway [11,23]. In the first committed step, the action of chalcone synthase (CHS) leads to the synthesis of chalcone, which isomerizes to naringenin by the activity of chalcone isomerase (CHI). Depending upon the genetics and the species studied, this pathway can take different routes leading to the synthesis of various derivatives of naringenin [8,39,40]. In maize and sorghum, some of the major end products of this pathway include anthocyanins and phlobaphenes (Fig. 1). Luteoferol and apiferol are two flavan-4-ols

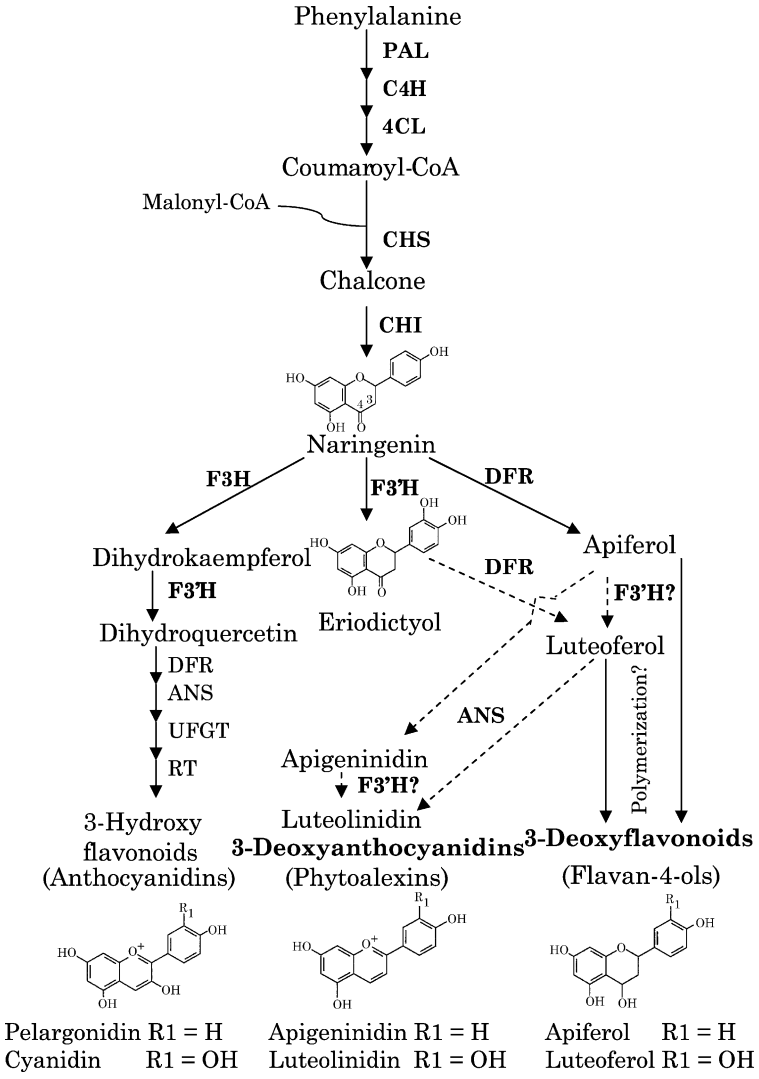


Fig. 1. Schematic representation of the flavonoid biosynthesis pathway in maize and sorghum. Shown are three branches diverging from naringenin and leading to the synthesis of 3-hydroxyflavonoids (anthocyanins), 3-deoxyflavonoids (flavan-4-ols/phlobaphenes) and 3-deoxyanthocyanidins (phytoalexins). Solid arrows indicate the steps where the respective gene has been isolated and characterized. Dotted arrows indicate the proposed or uncharacterized steps. Enzymes shown are: PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, coumarate 4-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol reductase; UFGT, UDPG-flavonoid glucosyl transferase; RT, rhamnosyl transferase, and ANS, anthocyanidin synthase; Pathway modeled after Lo and Nicholson [23] and Styles and Ceska [34].

that are precursors of condensed phlobaphene pigments [34]. These flavan-4-ols have also been suggested to be the precursors for the 3-deoxyanthocyanidin phytoalexins, luteolinidin and apigeninidin [6,38]. When infected with *C. sublineolum*, a resistant sorghum cultivar had a greater and faster accumulation of phytoalexins than a susceptible cultivar [22]. In addition to the speed and timing of induction, this study showed that the resistant cultivar produces luteolinidin and 5-methoxy luteolinidin, while these compounds were not detected in the susceptible cultivar. Luteolinidin differs from apigeninidin by *o*-dihydroxylation of the B ring and as proposed previously, this reaction can be catalyzed by a flavonoid 3′-hydroxylase (F3′H) [22,38]. To date, the functionally characterized *f3′h*s of plants are from petunia, Arabidopsis and *Perilla* [4,16,30] while in soybean, a point mutation has been used to genetically establish the *f3′h* function in pubescence color [36]. Using enzyme activity assays in petunia [4], *Perilla* [16], maize [19] and Arabidopsis [30], the F3′H has been shown to hydroxylate naringenin into eriodictyol. In sorghum, F3′H has been proposed to function in the synthesis of eriodictyol which may serve as the precursor for 3-deoxyanthocyanidin phytoalexins [22]. Eriodictyol would thus be a substrate for a dihydroflavonol reductase (DFR) to produce luteoferol. However, in the absence of F3′H activity, naringenin may be converted into apiferol by the action of DFR as has been previously demonstrated in sorghum [14]. In this study, we isolated a putative *f3′h* from sorghum to study its expression properties and any coordinated regulation with other flavonoid structural genes that have previously been implicated in the biosynthesis of 3-deoxyanthocyanidin phytoalexins.

## 2. Materials and methods

### 2.1. Genetic stocks, plant growth conditions, and fungal inoculation

The sorghum genetic stock RR-30 used in this study carries a functional *y1* gene (*Y1-rr-30* allele) which conditions red pericarp and red glume pigmentation [5]. Other genes required for 3-deoxyflavonoid pigmentation biosynthesis are also functional in this genetic stock as indicated by the red pigmentation of the seeds. This stock originated from a candystripe line CS8110419 (*Y1-cs* allele) by germinal excision of the *Candystripe1* transposon from *y1* as described previously [5,6]. Seeds were surface sterilized using 10% bleach and then imbibed in water for 12 h before planting in rolls of germination paper and incubated in the dark for 7 days at a constant temperature (26 °C) in a growth chamber. This treatment produces seedlings with mesocotyls that are uniformly etiolated. Fungal cultures of *C. heterostrophus* were maintained on potato dextrose agar plates under constant illumination at room temperature. This fungus was chosen because previous studies have shown that its attempted penetration elicits an extremely rapid phytoalexin response [1,22]. To challenge plants with the fungus, a conidial suspension was misted onto 20 etiolated seedlings that were then incubated in the dark at 26 °C in a plastic bag to maintain high relative humidity. For controls, 20 etiolated seedlings were sprayed with mock inoculation medium. Samples of 20 mesocotyls were collected for each time point and these were 0, 3, 6, 9, 12, 24 and 36 h postinoculation (hpi). At the time of tissue collection, mesocotyls were excised 5 mm above the point of attachment to the seed and 5 mm below the coleoptile. Tissue samples were collected and divided into two equal halves for biochemical characterization of compounds and steady state transcript analysis as explained below.

### 2.2. DNA and RNA gel blot hybridizations

Plant genomic DNA was isolated from seedling leaves using the CTAB method [28]. Restriction enzyme digestions were performed using enzymes, reagents and reaction conditions from Promega (Madison, WI) and fractionated on 0.8% agarose gels [29]. DNA gel blot hybridizations were performed using a hybridization mixture containing dextran sulfate (10%), NaCl (1 M), SDS (1%), Tris–HCl (10 mM) and 0.25 mg/ml salmon sperm DNA. Blots were pre-hybridized at 65 °C for 6 h in the hybridization buffer without a labeled probe followed by hybridizations for about 20 h at 65 °C.

To isolate total RNA, tissues were ground in liquid nitrogen and then extracted using TriReagent (Molecular Research Center, Inc., Cincinnati, OH). The RNA was separated on a denaturing gel containing 5% (v/v) formaldehyde, 1.2% (w/v) agarose and 1×RNA buffer (0.4 M MOPS, 0.1 M anhydrous sodium acetate and 0.01 M sodium EDTA). The fractionated RNA was transferred onto a nylon membrane (Osmonics, Inc., Minnetonka, MN). RNA gel blot hybridizations were performed for 24 h at 43 °C in a hybridization mixture containing 50% formamide, 0.25 M sodium phosphate at pH 7.2, 0.25 M sodium chloride, 1 mM EDTA, 7% SDS, and 0.05 mg/ml sheared salmon sperm DNA. All filters were washed in a solution containing 0.1×SSC (1×SSC is 0.15 M sodium chloride and 0.015 M sodium citrate), and 0.5% SDS once at 50 °C for 15 min, and twice at 65 °C for 15–30 min. Filters were exposed to X-OMAT film (KODAK, Rochester, NY) for 1–4 days before developing. Filters were stripped by washing for 15 min in a boiling solution of 0.1% SDS before re-hybridization.

### 2.3. Isolation of probes

Positions of all probe fragments and primers used in this study are shown in Fig. 2A. Sorghum probe fragment F387 was PCR amplified using primer sequences based on the maize partial EST sequence (accession no. BG873885): forward primer (F), 5′-AGTGCGAGGTGGACGGGTTC-3′, and reverse primer (R), 5′-GCAGACGGCAGCAGTC–

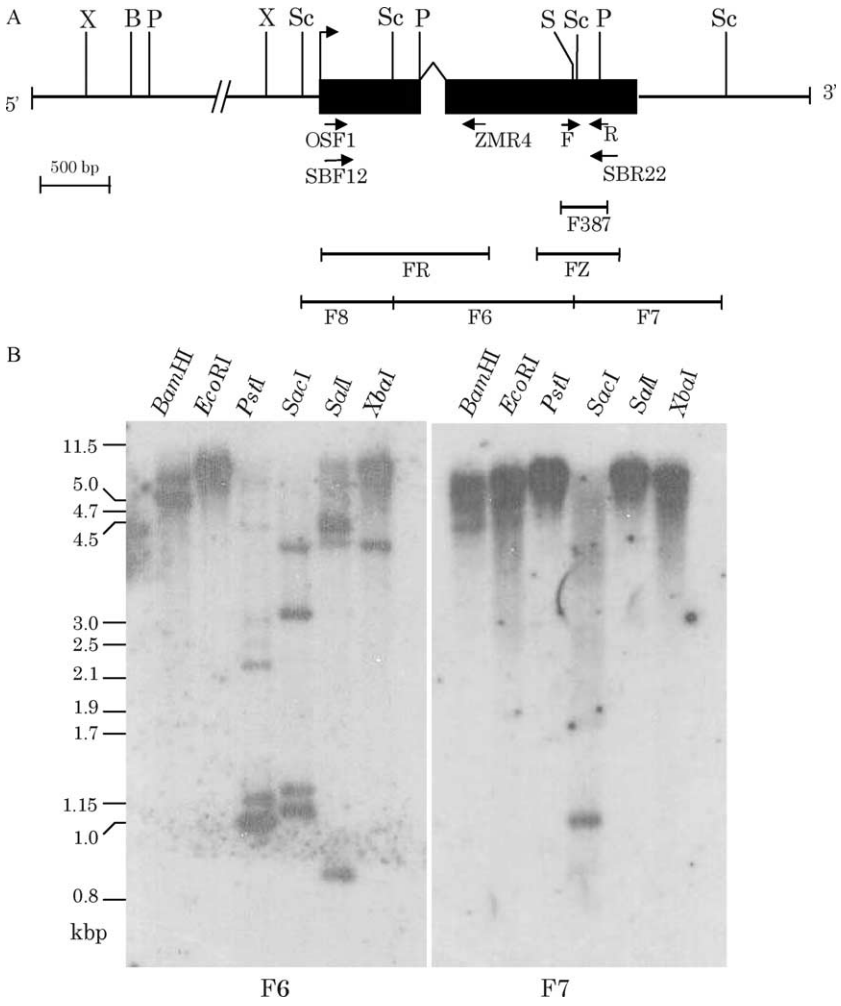


Fig. 2. Isolation and characterization of the sorghum *f3′h*. (A) Diagrammatic representation of the isolated sorghum *f3′h* genomic sequence (accession no. AY675076). Restriction enzyme sites shown are: B, *Bam*HI; P, *Pst*I; Sc, *Sac*I; S, *Sal*I; X, *Xba*I. Bent lines represent the 226 bp intron separating two exons. A bent arrow depicts the putative transcription start site. Horizontal arrows represent the primers used to amplify various probe fragments. Forward primer 'OSF1' and reverse primer 'ZMR4' were used to amplify the rice 5′ probe fragment (FR) using rice genomic DNA as template. Primers 'F' and 'R' were used to amplify a 387 bp fragment (F387) from sorghum RR-30 genomic DNA and cDNA from RNA isolated from 24 h postinoculated mesocotyls and primers 'SBF12' and 'SBR22' were used to amplify the full length sorghum cDNA. Horizontal lines below the gene show the positions of F387, FR and FZ probe fragments used to screen the λ genomic library and gene specific fragments F6, F7 and F8 used in DNA gel blot analysis. (B) Gel blot of the genomic DNA, extracted from sorghum RR-30 line and restricted with various enzymes, hybridized with 5′ or 3′ gene specific fragments as probes. Probe fragments are shown under each blot and their positions are indicated in Fig. 2A. Molecular weights in kilobase pair are shown on the left.

TCCCCT-3′. Probe FZ was isolated from the maize EST clone obtained from Dr Pat Schnable, Iowa State University, Ames, IA (www.plantgenomics.iastate.edu/maize). A partial genomic sequence from the 5′ half of the putative exon 1 of the rice gene (accession no. AC021892) [15] was PCR amplified using primers OSF1, 5′-CATACGGCCATGGACGTTGTGCCT-3′, and ZMR4, 5′-AAACGTCTCCTTGATCACCGC-3′ and this fragment was named as FR.

### 2.4. Isolation of genomic and cDNA sequences

A λ FIX II (Stratagene, La Jolla, CA) genomic library prepared from RR-30 seedling leaf DNA was screened to isolate full length sorghum putative *f3′h* gene. All screenings were performed using replicate filters hybridized either to the rice (FR) or maize gene (FZ) fragments as probes; details of these probe fragments are presented as follows. All DNA fragments used as probes were labeled with $^{32}$P-dCTP using a Prime-a-Gene labeling system (Promega, Madison, WI). For cDNA isolation, 10 μg of total RNA isolated from 0 to 24 h postinoculated mesocotyls was reverse transcribed using an oligo dT primer and Superscript II reverse transcriptase following the conditions from the supplier (Invitrogen, Carlsbad, CA). The 'F' and 'R' primer pair described above was used to amplify reverse transcribed products. Full length cDNA was amplified using gene specific primers SBF12, 5′-CTTCTAGAACCGAG-CAACTCAAACCATGGAC-3′, and SBR22, 5′-TTGGAT-CCCCTACTCCGCTGCGTAT-3′). Standard PCR buffer and reaction conditions were followed [29] with

the modified annealing temperature of 60 °C for 2 min, followed by polymerization at 72 °C for 2 min. All PCR products were cloned into the pGemT-Easy plasmid vector (Promega, Madison, WI) and sequenced from both ends using vector primers.

### 2.5. DNA sequencing and analysis

All DNA sequencings were done at the Pennsylvania State University's Nucleic Acid Facility using the method of dye primer cycle sequencing and reactions were run on a 3100 capillary machine (Applied Biosystems, Foster city, CA). Sequence assembling was done using the GCG® Wisconsin Package 1 (Accelrys, Inc., Burlington, MA). Multiple sequence alignment analysis and drawing the neighbor joining tree was done using the ClustalX program [35]. Homology searches were done using the BLAST program available from the NCBI web site http://www.ncbi.nlm.nih.gov/BLAST/ [2]. The conserved domain search was done using the NCBI tool CDSearch at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

### 2.6. Heterologous expression of sorghum putative *f3′h* cDNA

The coding sequence of sorghum *f3′h* cDNA was cloned into the expression vector pET41c (+) (EMD Biosciences, San Diego, CA) as an *Nco*I-*Eco*RI fragment. The in-frame position of the cloned fragment was confirmed by sequencing the plasmid sub-clone. A pilot reaction of IPTG induction and western blot analysis was performed to confirm the synthesis of the fusion protein. Western blotting was done using GST antibodies and a band shift from the GST peptide control was observed (data not shown). A 5 h induction using 1 mM IPTG was done when the bacterial culture $OD_{600}$ had reached 0.6. The culture was then centrifuged and the pellet was suspended in a solution containing 10 mM Tris (pH 7.8) and 0.75 M sucrose. Cells were lysed by sonication and the cell debris was removed by centrifuging at 10,000 × *g* for 10 min. Bacterial membranes were prepared by centrifuging the supernatant at 300,000 × *g* for 2 h. Cytosolic extract was retained and the membrane pellet was suspended in a solution containing 25% sucrose and 5 mM EDTA (pH 7.8). The enzyme assay was done by incubating the bacterial membrane extract with naringenin (Sigma–Aldrich, St Louis, MO) as the substrate. The assay mixture contained 0.372 mM NADPH, 0.05 M bicine buffer (pH 8.5), 2.0 mM DTT and 0.25 mM naringenin. The membrane extract was added such that the quantity of total protein was 100 μg in the reaction mixture. The total volume was made to 200 μl and incubated at 30 °C for 45 min. The reaction mixture was centrifuged at 15,000 × *g* for 1 min, filtered using 0.45 μm Acrodisc LC 13 mm syringe filters (Gelman Laboratory, Ann Arbor, MI) and was used for LC/MS analyses.

LC/MS analyses of the products of naringenin incubations were performed on a Quattro II mass spectrometer (Micromass, Beverly, MA) interfaced to a Shimadzu LC10ADvp pump. Separations were performed on a 1× 150 mm BetaBasic C18 column, with a solvent gradient based on solvent A = water + 0.15% formic acid and solvent B = methanol and a flow rate of 0.05 ml/min delivered using a pre-injection split. Solvent composition was programmed as follows: 0–5 min (99%A, 1%B), followed by a linear gradient to 50%A/50%B at 12 min, another linear gradient to 100%B at 20 min, with a hold at 100%B until 25 min. Electrospray ionization was performed in the negative ion mode.

### 2.7. Analysis of compounds accumulating in sorghum mesocotyls

One hundred milligram of tissue was placed in 0.5 ml of HPLC grade methanol and compounds were allowed to leach from the tissue at 4 °C for 24 h in the dark [22]. The extract was then filtered through 0.45 μm Acrodisc LC 13 mm syringe filters (Gelman Laboratory, Ann Arbor, MI) and analyzed by HPLC following the conditions described previously [23]. To separate compounds, two reversed-phase C18 columns connected in tandem were used and compounds were detected at 480 nm. Purified HPLC grade luteolinidin and apigeninidin (Extrasynthese, Genay Cedex, France) were used for measuring concentrations of compounds observed through HPLC as described previously [23]. To identify major compounds, PDMS (plasma desorption mass spectrometry) was used [42]. Total flavan-4-ols present in the mesocotyl extracts collected at different time intervals were determined by measuring flavylium ions released by incubating the extracts with acidic butanol [9]. Flavylium ions concentration was determined by spectrophotometer using absorbance at 550 nm ($\lambda_{max}$).

## 3. Results

### 3.1. Isolation of genomic and cDNA sequences corresponding to a putative *f3′h* of sorghum

During the onset of this experiment, information on *f3′h* gene sequences from monocots was very limited in GenBank and other plant genome databases. An unannotated EST sequence of maize (see Section 2) was considered to be a putative *f3′h*. Positions of primers 'F' and 'R' that successfully amplified the putative *f3′h* sequence are shown in Fig. 2A. This primer pair generated a 387 bp sorghum DNA sequence, whose translated product showed high sequence similarity with available F3′Hs in the GenBank establishing that we had a partial sequence of the sorghum putative *f3′h*. Further sequence searches with putative maize and sorghum partial sequences allowed us to identify a putative *f3′h* rice genomic sequence (accession AC021892). With these different sequences in hand, we

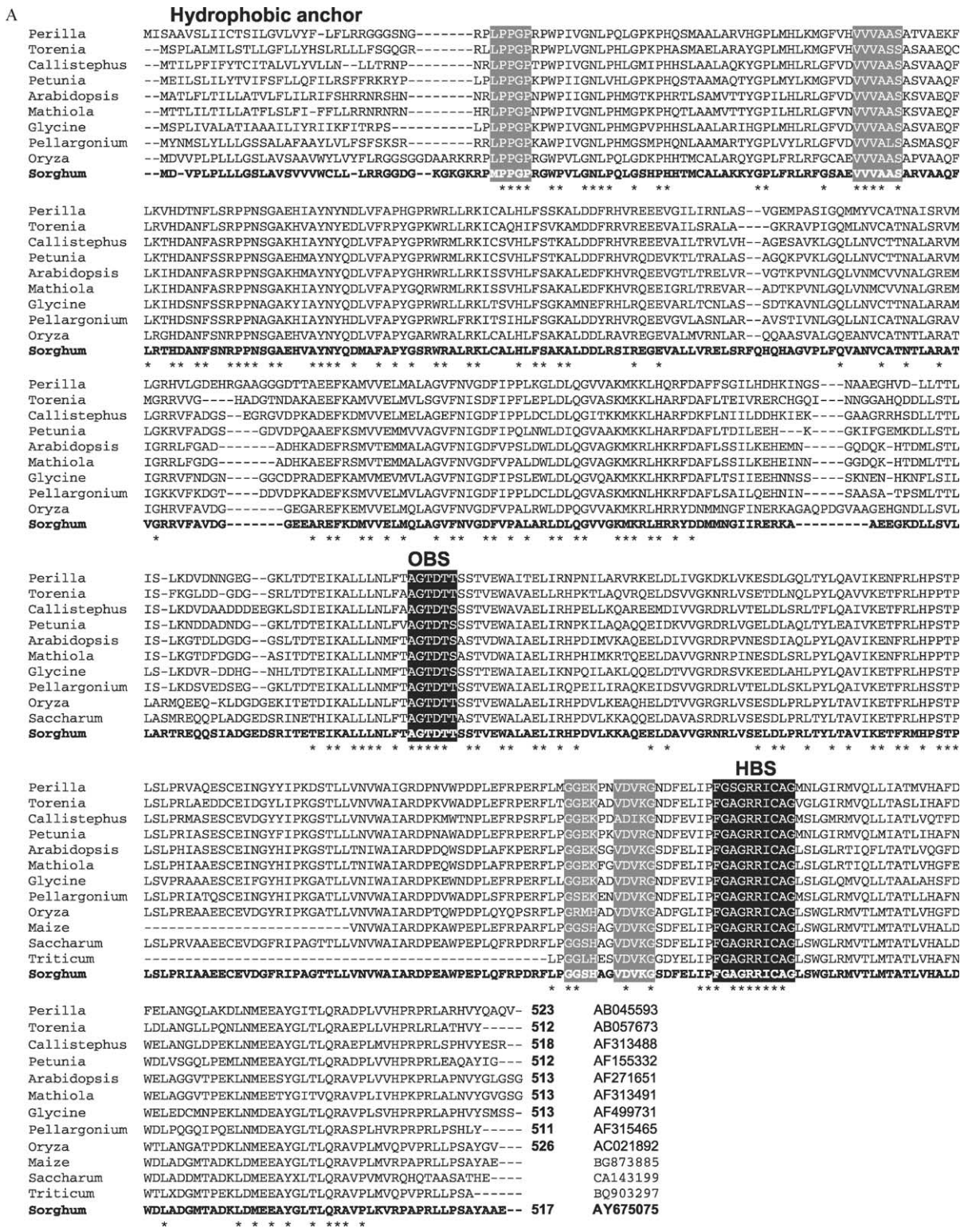


Fig. 3. Sequence characterization and phylogenetic analysis. (A) Multiple sequence alignment of deduced amino acid sequences of F3′H from sorghum cDNA (accession no. AY675075), and 12 other plant species using ClustalX program. Sequences used are from *Arabidopsis thaliana* (AF271651), *Callistephus chinensis* (AF313488), *Glycine max* (AF499731), *Mathiola incana* (AF313491), *Pellargonium hortorum* (AF315465), *Perilla frutescens* (AB045593), *Petunia hybrida* (AF155332), *Saccharum officinarum* (CA143199), *Triticum aestivum* (BQ903257), *Torenia hybrida* (AB057673), *Oryza sativa* (AC021892), and *Zea mays* (BG873885). Lightly shaded areas are F3′H specific sequences and regions shaded dark are conserved domains found in the CYP450 family of proteins. Specific regions indicated above the first sequence are a hydrophobic anchor, oxygen binding site (OBS), and heme binding site (HBS). Asterisks indicate conserved amino acids. The last amino acid is not numbered in partial sequences. Only the F3′Hs of Arabidopsis, petunia, *Perilla*, and sorghum have been tested functionally using enzyme assays [4,16,30,this study]. (B) Phylogenetic tree showing the evolutionary relatedness of sorghum F3′H with that of other plant cytochrome P450s (CYPs). Selection of CYPs used in this tree was based on their functional roles in the flavonoid pathway or on their activities as

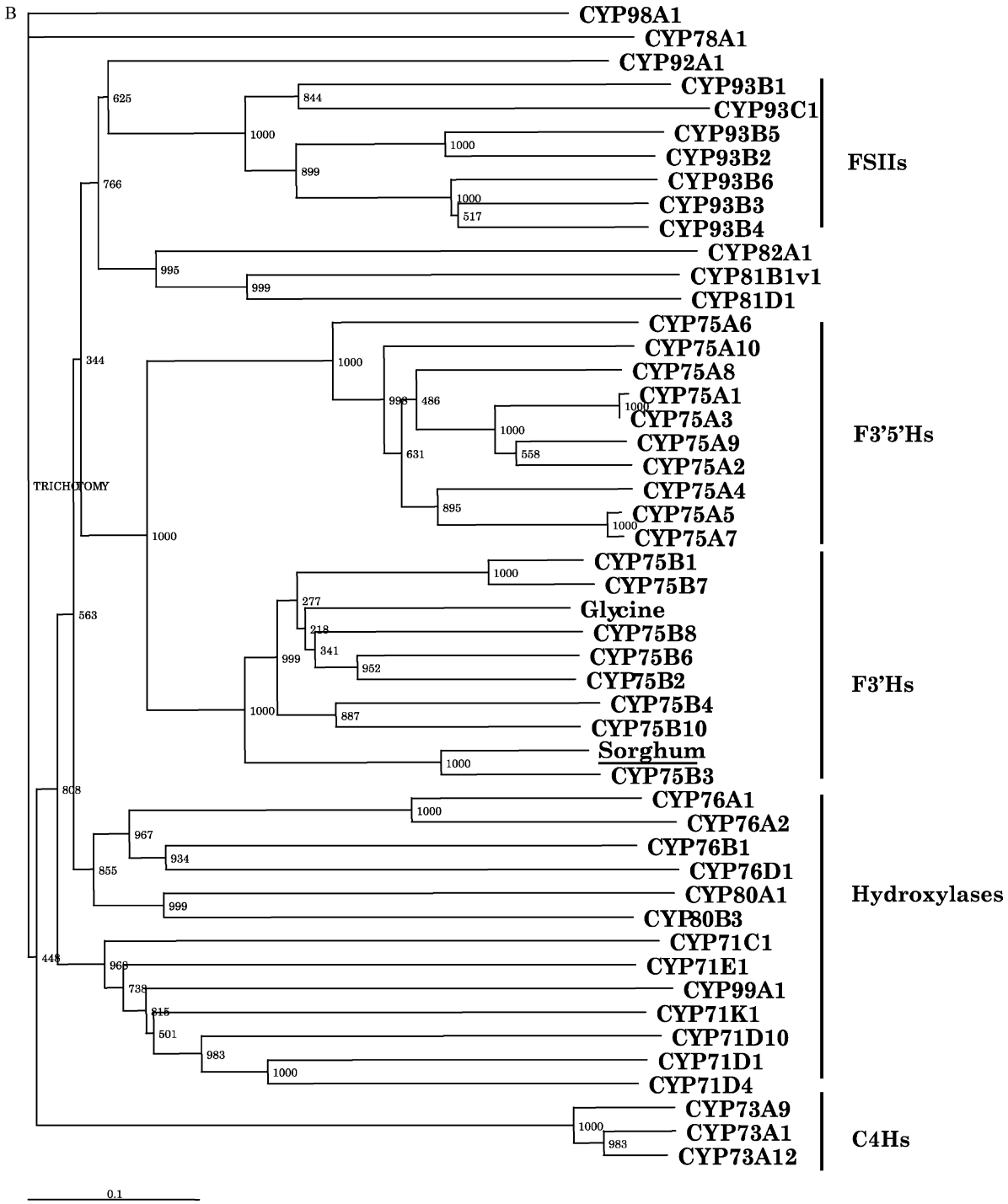


Fig. 3 (*continued*)

hydroxylases. These proteins are listed on http://www.icgeb.org/~p450srv/biblioD.html Specific groups of well characterized hydroxylases are shown on the right side of the tree. Different CYPs and their accession numbers (in parentheses) are: CYP71C1 (X81827); CYP71D1 (AY192573); CYP71D4 (AJ296346); CYP71D10 (AF022459); CYP71E1 (AF029858); CYP71K1 (AP002968); CYP73A1 (Z17369); CYP73A9 (U29243); CYP73A12 (U19922); CYP75A1 (X71130); CYP75A2 (X70824); CYP75A3 (Z22545); CYP75A4 (D85184); CYP75A5 (U72654); CYP75A6 (D14590); CYP75A7 (D14589); CYP75A8 (AJ011862); CYP75A9 (AF313490); CYP75A10 (AB012925); CYP75B1 (AL133421); CYP75B2 (AF155332); CYP75B3 (AC021892); CYP75B4 (AB045593); CYP75B6 (AF313488); CYP75B7 (AF313491); CYP75B8 (AF315465); CYP75B10 (AB057672); CYP76A1 (X71658); CYP76A2 (X71657); CYP76B1 (Y09920); CYP76D1 (AB032833); CYP78A1 (L23209); CYP80A1 (U09610); CYP80B3 (AF191772); CYP81B1v1 (AJ000477); CYP81D1 (D78606); CYP82A1 (U29333); CYP92A1 (AY072297); CYP93B1 (AB001380); CYP93B2 (AF156976); CYP93B3 (AB028151); CYP93B4 (AB028152); CYP93B5 (AF188612) CYP93B6 (AB045592); CYP93C1 (A023636); CYP98A1 (AF029856), and CYP99A1 (AF029857). *Glycine max* and *Sorghum bicolor* *f3′h*s have not yet been assigned a CYP number. Bootstrap values (1000 replicates) are placed on nodes.

synthesized probe fragments in the 5′ (probe FR) and 3′ (probe FZ) regions for screening filters of a sorghum genomic library. From a total of 500,000 plaques screened, three positive λ clones that hybridized to both the probes on replica filters were identified. Further restriction mapping allowed us to select the largest clone, which was then used to sub-clone and sequence internal restriction fragments carrying the gene sequences. A partial restriction map of the positive λ-Sb-411 clone and the putative *f3′h* gene is presented in Fig. 2A.

Gel blot hybridization analysis of sorghum genomic DNA showing hybridization patterns with two of the fragments of the putative *f3′h* gene used as probes is shown in Fig. 2B. An internal fragment (F6) corresponding to the intron and major region of the exon 2, hybridizes to 1–4 bands in different digests. Restriction bands in lanes carrying DNA digests of *Pst*I, and *Sac*I show expected sized bands of approximately 1.0 and 1.1 kbp, respectively. However, other bands observed with varying intensities in *Pst*I, *Sac*I and *Sal*I digestions may represent additional homologs to the F6 probe fragment. Some of the high molecular weight bands may either represent undigested or partially digested genomic DNA. Hybridization with a 3′*Sac*I fragment (F7) shows a single band of 1.0 kbp in the *Sac*I digested genomic DNA lane. Digestion with *Eco*RI shows a single band of higher molecular weight with both the probes. Overall these results indicate that the probe fragments hybridize to the cloned putative *f3′h* gene sequence, while other genomic bands hybridizing to these probes may belong to sequences that encode another F3′H or closely related F3′5′Hs as has been observed in petunia [4].

Pair wise alignment of the genomic and cDNA sequences allowed us to identify a 226 bp intron and a deduced peptide sequence of 517 amino acids. A putative start transcription site was identified 55 bp 5′ to the first ATG using the BDGP software at http://www.fruitfly.org/seq_tools/promoter.html. Alignment of the deduced amino acid sequence of sorghum putative F3′H with available sequences of the functionally identified and putative plant F3′Hs from GenBank is shown in Fig. 3A. Overall, the sorghum sequence shows a 55–57% similarity with F3′Hs from dicots and higher similarity to F3′Hs from monocots; 78% with rice, 90% with sugarcane and 65% with barley. Domains conserved among many cytochrome P450s are also found in the sorghum sequence and these regions include heme- (HBS) and oxygen-binding sites (OBS). In addition, the characteristic hydrophobic membrane anchor is present at the amino-terminus. A landmark sequence GGEK has previously been reported to be present in petunia and other dicot F3′Hs [4,36]. We find that a slight modification of this conserved sequence represented by GGSH is present in sorghum and other monocots (with an exception of rice). Based on alignments of several F3′Hs and F3′5′Hs (not shown), other landmark sequences (VVVAAS and VDVKG; Fig. 3A) were identified to be present in F3′Hs only. A phylogenetic tree based on the deduced amino acid sequences of F3′Hs, F3′5′Hs and several other cytochrome P450s that have diverse functions in plants was drawn using the ClustalX program [35] (Fig. 3B). This phylogenetic tree clearly suggests that our putative F3′H sequence from sorghum falls within the CYP75B family which contains F3′Hs from dicots and monocots. Some of these proteins have been shown to be involved in hydroxylation reactions in natural product biosynthesis [4,16,30]. As expected, this phylogenetic tree shows that families of F3′5′Hs (CYP75A) and F3′Hs (CYP75B) may possibly have evolved from a common protein with a function of B ring hydroxylation.

### 3.2. Functional characterization of the putative *f3′h* cDNA

To test the functionality of the putative *f3′h* cDNA, an enzyme assay was performed. Incubations of naringenin with recombinant bacterial membrane extract yielded small amounts of an intermediate product that appeared in negative ion LC/MS analysis and whose apparent molecular ion had an *m/z* 16 atomic mass units higher than that of naringenin, as expected for a mono-hydroxylation product. The product, which we propose is eriodictyol, appears at a retention time of 14.6 min, and naringenin elutes at 16.1 min, as evident in reconstructed ion chromatograms for *m/z* 287 and 271, respectively (Fig. 4). The earlier retention time of the product is consistent with the greater polarity of the metabolite that is conferred by the additional hydroxyl group. Signal/noise ratio of the product peak in the enzyme-treated sample was calculated to exceed three. Control extracts (no enzyme) did not yield a peak at 14.59 min in the reconstructed ion chromatogram that had a signal/noise ratio greater than 1, therefore the metabolite can be considered to be of too low abundance to be detected in the control sample. This result indicates that the putative cDNA clone encodes a functional hydroxylase activity.

### 3.3. Sorghum putative *f3′h* is induced by fungal ingress

We tested the hypothesis that the sorghum *f3′h* gene is induced by fungal inoculation and participates in the biosynthesis of 3-deoxyanthocyanidin phytoalexins. We first performed RT-PCR (reverse transcription PCR) experiments using ‘F’ and ‘R’ primers. No amplification was observed in the 0 hpi sample, while the 24 hpi sample generated a 387 bp fragment whose sequence had a 100% match with the genomic 387 bp fragment that we had amplified previously. Further, induction experiments using RNA gel blots hybridized to *f3′h* cDNA as a probe show that steady state transcript levels differ in tissues obtained at different time points after inoculation with *C. heterostrophus* (Fig. 5). No transcript for *f3′h* was detected between 0 and 3 hpi. From 6 hpi onwards, *f3′h* transcript levels begin to increase and reach a maximum at 12 hpi. A similar pattern of transcript accumulation was observed for *chs* and induction of this gene in *C. heterostrophus* inoculated

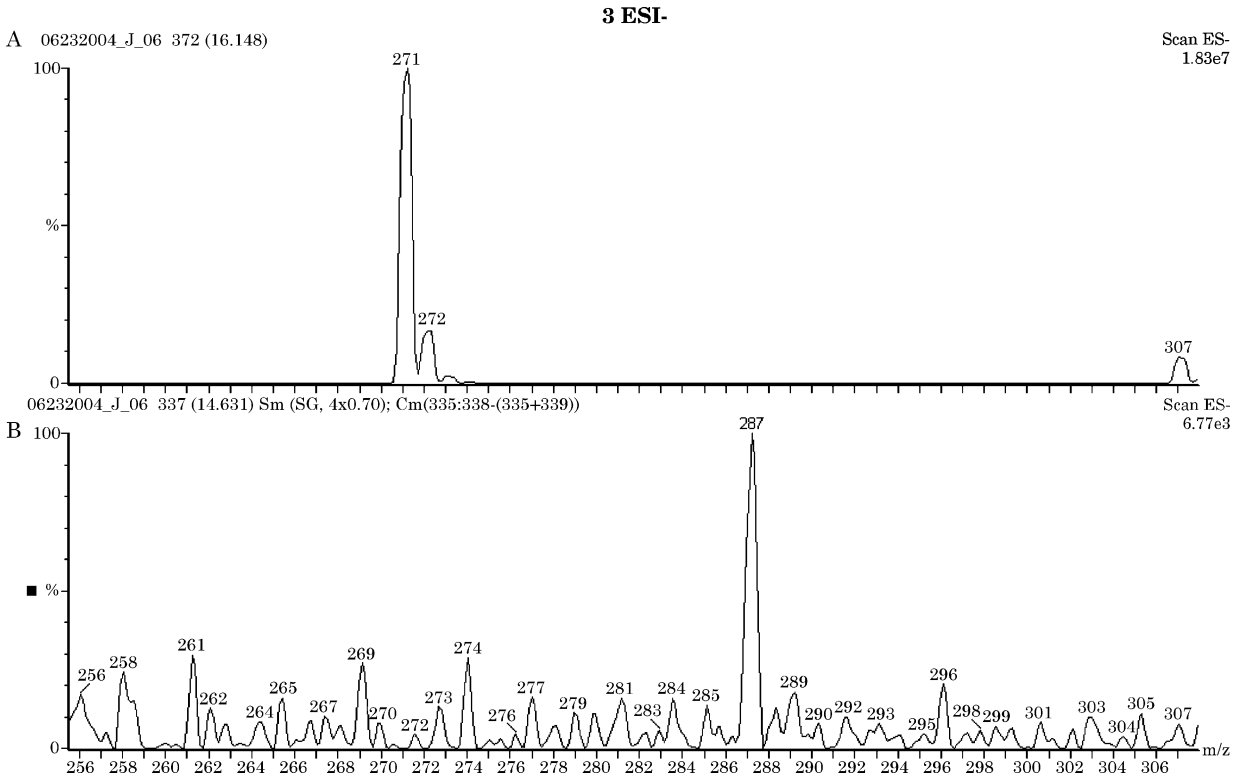


Fig. 4. LC/MS analysis of products of naringenin incubated with *E. coli* membrane extract containing recombinant sorghum putative F3′H fusion protein. Electrospray ionization was performed in negative ion mode. Panels A and B are from the same LC/MS analysis. Panel A shows major ions corresponding to the unreacted substrate, naringenin (271 *m/z*, retention time 16.15 min). Panel B shows major ions corresponding to the newly synthesized product, which we infer is eriodictyol (287 *m/z*, retention time 14.63 min). The 287 *m/z* product was seen in four independent samples. Duplicate negative controls in which naringenin was incubated with non-recombinant *E. coli* extract yielded no peak of 287 *m/z* with signal/noise greater than 1.0 at retention times characteristic of this product.

mesocotyls has been demonstrated previously [23]. Compared to *chs* and *f3′h*, *dfr* transcript accumulated at a much lower level in early (3–12 hpi) time points but showed a sharp increase of its expression at 24 hpi. These expression studies were repeated four times and establish that sorghum *f3′h* and two other genes of the sorghum flavonoid biosynthetic pathway show induction of their steady state transcripts in *C. heterostrophus* challenged mesocotyl tissues.

### 3.4. Temporal synthesis of 3-deoxyanthocyanidins in inoculated tissues

Etiolated seedlings of sorghum line RR-30 carrying the *Y1-rr* (red perciarp/red glume allele) [6] were either inoculated with conidia of *C. heterostrophus* or treated with mock buffer and incubated in the dark. Very light brown lesions were barely visible in mesocotyls collected from 9 hpi. At 12 hpi, mesocotyls of fungal challenged plants produced visible red–brown pigments indicating the presence of phytoalexin compounds (not shown). Pigments were more intense in 24 hpi mesocotyls than those produced in the mesocotyls of 12 hpi. It has been shown previously that reddish-brown lesion development does not reflect

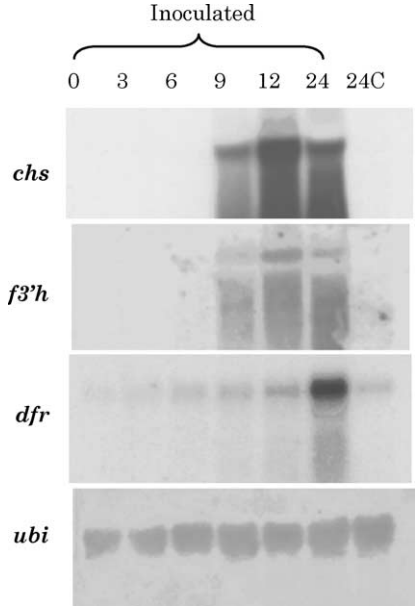


Fig. 5. Fungal induced expression of *chs*, *f3′h*, and *dfr* genes in sorghum. RNA gel blots were prepared from total RNA from mesocotyls taken at different time intervals after inoculation of plants with the fungus *C. heterostrophus*. Lanes shown are: inoculated samples at 0, 3, 6, 9, 12 and 24 hpi and an uninoculated control at 24 h. The panel with *ubiquitin* gene hybridization is shown for RNA loading accuracy.

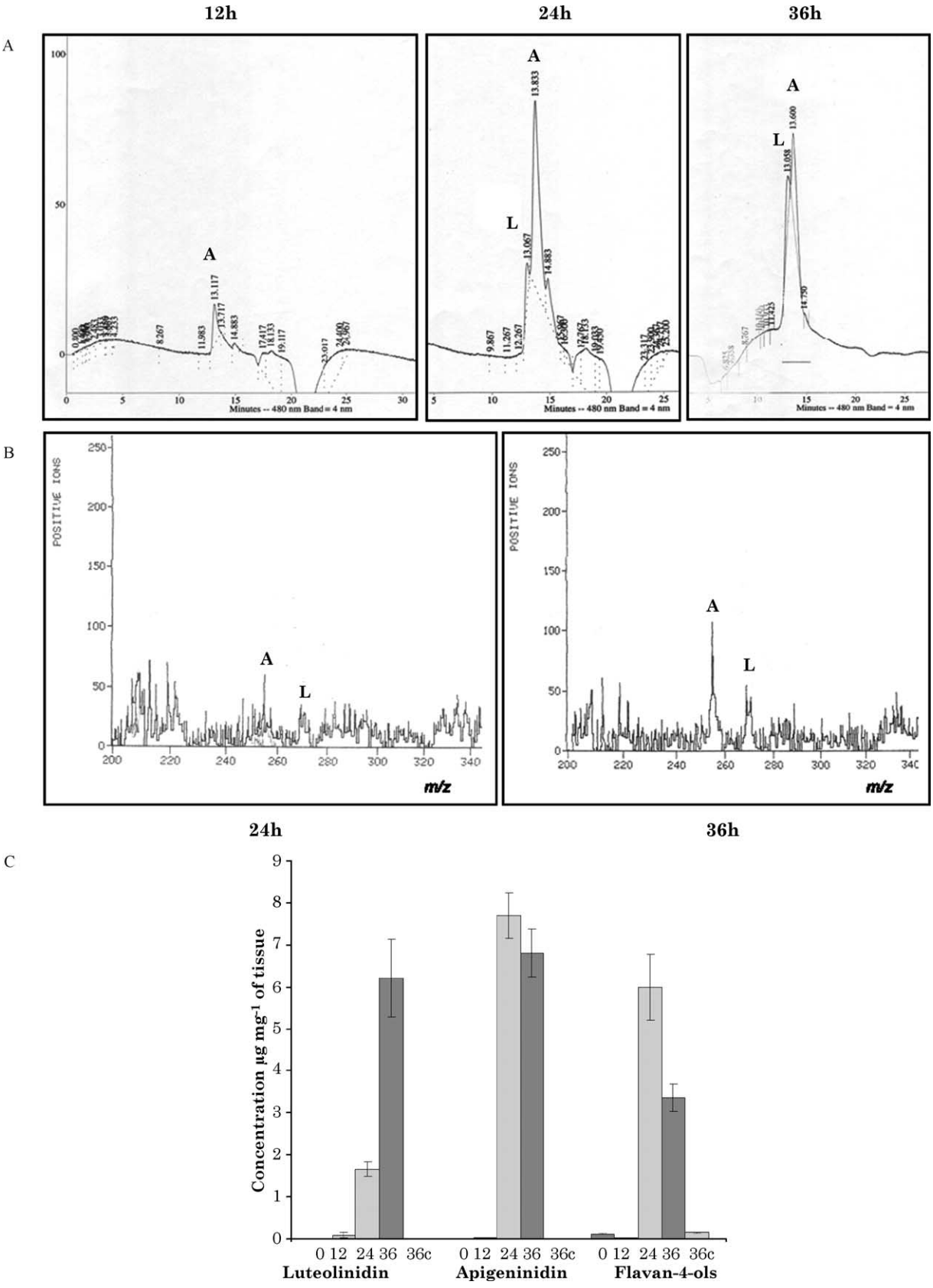


Fig. 6. Detection and temporal synthesis of 3-deoxyanthocyandin phytoalexins and flavan-4-ols. (A) HPLC analysis of 3-deoxyanthocyanidin phytoalexins. Mesocotyls were processed for HPLC analysis and major pigments detected at 480 nm were luteolinidin (L) and apigeninidin (A). Compounds were detected by their retention times in minutes (*x*-axis) and their relative absorbance at 480 nm (*y*-axis). (B) Plasma Desorption Mass Spectrometry (PDMS) confirmation

the extent of fungal growth and rather it shows the speed of a sorghum genotype in synthesizing 3-deoxyanthocyanidin phytoalexins around the sites of fungal infection [22,23]. Pigments were extracted in HPLC grade methanol, measured by HPLC at 480 nm and identified based on their retention times compared to standards (Fig. 6A). Major compounds identified were luteolinidin (~13.0 min peak) and apigeninidin (~13.8 min peak). At 12 hpi, apigeninidin was detectable at very low concentrations, which increased almost six fold after 24 h of incubation following inoculation. At 36 hpi, appreciable amounts of apigeninidin could be detected. Luteolinidin was clearly detectable at 24 hpi and its concentration increased 2.5 times at 36 hpi. Additionally, peaks representing 5-methoxyluteolinidin (14.8 min), and 7-methoxyapigeninidin (16.2 min) were also detected at 480 nm. None of the compounds were detected in the mock inoculation controls.

To confirm the identity of different types of 3-deoxyanthocyanidins produced in inoculated samples, mesocotyl extracts were further subjected to PDMS and results are presented in Fig. 6B for 24 and 36 hpi extracts. Peaks corresponding to specific mass/charge for apigeninidin (A, 255 *m/z*), and luteolinidin (L, 271 *m/z*) are shown. In five independent experiments, 36 hpi samples produced approximately 30% more of luteolinidin relative to 12 hpi extracts. Amounts of luteolinidin and apigeninidin were calculated from five HPLC measurements and total flavan-4-ols were analyzed by the spectrophotometer at 550 nm. Results are presented in Fig. 6C. These results indicate that, during the time course of synthesis of apigeninidin and luteolinidin, the amount of total flavan-4-ols shows a relative reduction at 36 hpi as compared to 24 hpi.

## 4. Discussion

### *4.1. Sorghum putative f3′h encodes a functional protein homologous to CYP450s*

Genetics of loci responsible for the activity of F3′H has been extensively studied in several dicots to understand its function in the anthocyanin biosynthetic pathway [4,30,44]. To test the hypothesis that F3′H has a role in sorghum phytoalexin biosynthesis we cloned and characterized a genomic sequence and its corresponding cDNA. A 3′ end fragment of the isolated gene showed that this sequence was unique in the genome of sorghum. A previous study in maize measured the enzyme activity of F3′H and suspected the presence of two *f3′h* genes, one of which encodes the function of the *pr1* locus [19]. Our DNA gel blot hybridized with fragments from the 5′ end did show additional homologous sequences, suggesting the existence of another isoform of *f3′h* in sorghum. Alternatively, additional bands could belong to sequences encoding F3′5′Hs which have been shown to share 60–70% homology at the DNA level [4]. Our multiple sequence alignment and phylogenetic analysis showed that the sorghum putative flavonoid 3′-hydroxylase belongs to the family of cytochrome P450 proteins (CYPs). Moreover, this analysis also revealed that F3′5′Hs form a different but closely related cluster to that of the F3′Hs. Flavonoid 3′-hydroxylases have been shown to contain a characteristic short sequence motif of GGEK, that distinguishes them from their nearest relatives F3′5′Hs [4]. Our sequence alignment shows that GGEK motif may be conserved only among dicots, and this motif is modified to GGSH in several monocot F3′Hs. In addition, we identified two other short sequences, VVVAAS and VDVKG (see Fig. 3A), that are found in all F3′Hs. Functional significance of these motifs with respect to their activities is yet to be elucidated.

We further used *Escherichia coli* as a host to express the sorghum fusion protein and assayed its activity using naringenin as a substrate. These assays indicated that F3′H fusion protein from *E. coli* membrane extracts was active to hydroxylate naringenin, validating the functional nature of the isolated cDNA. Given that production of 3′-hydroxylated phytoalexins begins as the transcript for this hydroxylase gene appears (Figs. 4, 5 and 6), it seems most likely that the hydroxylation position on naringenin is 3′, which would yield eriodictyol. Based on the enzyme assays for a hydroxylation reaction and multiple sequence alignment results, we conclude that we have isolated a cDNA sequence corresponding to an F3′H.

### *4.2. Coordinated expression of f3′h, chs, dfr, and synthesis of phytoalexins*

In sorghum, fungal ingress induces the production of phytoalexins at the site of infection [32]. It has been shown that expression of the sorghum *chs* gene increases after fungal inoculation and thus this gene in sorghum can be used as a reliable marker during induction of phytoalexins [23]. Sorghum *f3′h* showed an induction pattern which was very similar to that of *chs* and peaks at 12 hpi. However, the amount of induced *f3′h* transcript was much lower than *chs*, which also showed a rapid turnover of its transcript. Interestingly, expression of *chs*, *f3′h*, and *dfr* showed accumulation of their transcripts with a gradual progression over time. Both *chs* and *f3′h* seem to show similar RNA turnover as observed by the appearance of smear in those lanes. Thus, the temporal induction coincides with the position of these three genes at their proposed steps in

of 3-deoxyanthocyanidins in total extract. Major ions at 24 and 36 hpi were identified as apigeninidin (255 *m/z*) and luteolinidin (271 *m/z*). (C) Bar diagram showing quantitative changes in the concentrations of apigeninidin, luteolinidin, and flavan-4-ols. Amounts of apigeninidin and luteolinidin were obtained from HPLC measurements. Total flavan-4-ols were obtained by spectrophotometer (see Section 2). The error bars correspond to standard deviation of measurements obtained from five replications.

the flavonoid or phytoalexins biosynthesis. Genetic studies in the anthocyanin pathway have demonstrated that mutations in *f3′h*s lead to accumulation of pelargonidin through the metabolism of dihydrokaempherol which is one of the substrates for the F3′H enzyme [12–13,19]. Apart from the anthocyanin pathway, 3-deoxyanthocyanidins and flavan-4-ols originate from naringenin (see Fig. 1) [34]. Our recent studies on the expression of the maize *f3′h* gene indicate that it may also have an essential role in flavan-4-ol synthesis in maize (C. Svabek and S. Chopra, unpublished). It has also been shown that light induced accumulation of anthocyanins can be suppressed by the induction of 3-deoxyanthocyanidins in response to fungal stress [23]. In our study, we have performed pathogen stress in the dark, thereby eliminating the interference caused by the light induced accumulation of structural genes' transcripts required for anthocyanin biosynthesis. However, it will be interesting to further study the light induced expression pattern of *f3′h* to understand the regulation of biosynthesis of light induced anthocyanins and fungus induced anthocyanidins.

We have thus established a direct correlation between the transcriptional induction of three of the flavonoid biosynthetic genes and accumulation of 3-deoxyanthocyanidin phytoalexins. Naringenin and eriodictyol may be the two substrates used by DFR to synthesize apiferol and luteoferol, respectively. Apiferol and luteoferol are also the proposed precursors for apigeninidin and luteolinidin, respectively [9,14,34]. In *Perilla*, a related flavonoid called apigenin is synthesized from naringenin by the action of flavone synthase 2 (FS2) and is then further modified to luteolin by F3′H activity [16]. Results reported here suggest that in sorghum, catalysis of naringenin to eriodictyol may be carried out by an F3′H induced 9-12 hpi. Further accumulation of apigeninidin observed at 24 hpi may be brought about by the activity of DFR. We do not yet have direct biochemical evidence, but the induction of *f3′h* transcript at 12–24 hpi may also be responsible for catalysis of apiferol to luteoferol.

Luteolinidin and the 5-methoxy derivative of luteolinidin are known to be more toxic to the infecting fungal pathogen, when compared to the other phytoalexins [26]. Increased accumulation of these compounds between 24 and 48 hpi has thus been implicated to be an effective defensive strategy against the penetrating fungus. Studies have shown variable timings of accumulation of luteolinidin, apigeninidin and their methoxy-derivatives in different sorghum cultivars [1,23,38]. These variations could be dependent upon the activities of *f3′h* and other genes introgressed in different genetic stocks of sorghum used in those studies. Our results indicate a correlation between transcriptional induction of *f3′h* and temporal accumulation of luteolinidin, which perhaps is a critical step that defines the speed of accumulation of phytoalexin compounds. For example, as compared to a resistant cultivar, a susceptible sorghum line had a much delayed phytoalexin accumulation [37]. In addition to a delayed response phenotype, certain susceptible cultivars have been shown to accumulate only apigeninidin, which may indicate a block at the F3′H step [22]. To further establish the functional importance of F3′H in phytoalexin biosynthesis in sorghum, gene knockouts or near-isogenic lines differing in the *f3′h* gene activity will be useful.

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## References

[1] Aguero ME, Gevens A, Nicholson RL. Interaction of *Cochliobolus heterostrophus* with phytoalexin inclusions in *Sorghum bicolor*. Physiol Mol Plant Pathol 2002;61:267–71.
[2] Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 1997;25:3389–402.
[3] Bieza K, Lois R. An Arabidopsis mutant tolerant to lethal ultraviolet-B levels shows constitutively elevated accumulation of flavonoids and other phenolics. Plant Physiol 2001;126:1105–15.
[4] Brugliera F, Barri-Rewell G, Holton TA, Mason JG. Isolation and characterization of a flavonoid 3′-hydroxylase cDNA clone corresponding to the *Ht1* locus of *Petunia hybrida*. Plant J 1999;19:441–51.
[5] Chopra S, Brendel V, Zhang J, Axtell JD, Peterson T. Molecular characterization of a mutable pigmentation phenotype and isolation of the first active transposable element from *Sorghum bicolor*. Proc Natl Acad Sci USA 1999;96:15330–5.
[6] Chopra S, Gevens A, Svabek C, Wood KV, Peterson T, Nicholson RL. Excision of the *Candystripe1* transposon from a hyper-mutable *Y1-cs* allele shows that the sorghum *y1* gene control the biosynthesis of both 3-deoxyanthocyanidin phytoalexins and phlobaphene pigments. Physiol Mol Plant Pathol 2002;60:321–30.
[7] Dixon RA, Steele LC. Flavonoids and isoflavonoids—a gold mine for metabolic engineering. Trends Plant Sci 1999;4:394–400.
[8] Dong X, Braun E, Grotewold E. Functional conservation of plant secondary metabolic enzymes revealed by complementation of Arabidopsis flavonoid mutants with maize genes. Plant Physiol 2001;127:46–57.
[9] Grotewold E, Chamberlin M, Snook M, Siame B, Butler L, Swenson J, et al. Engineering secondary metabolism in maize cells by ectopic expression of a transcription factor. Plant Cell 1998;10:721–40.
[10] Harborne JB, Williams CA. Advances in flavonoid research since 1992. Phytochemistry 2000;55:481–504.

[11] Hipskind J, Hanau R, Leite B, Nicholson RL. Phytoalexin synthesis in sorghum: identification of an apigeninidin acyl ester. Physiol Mol Plant Pathol 1990;36:381–96.
[12] Hoshino A, Morita Y, Jeong-Doo C, Saito N, Toki K, Tanaka Y, et al. Spontaneous mutations of the *flavonoid 3′-hydroxylase* gene conferring reddish flowers in the three morning glory species. Plant Cell Physiol 2003;44:990–1001.
[13] Kaltenbach M, Schroder G, Schmelzer E, Lutz V, Schroder J. Flavonoid hydroxylase from *Catharanthus roseus*: cDNA, heterologous expression, enzyme properties and cell type specific expression in plants. Plant J 1999;19:183–93.
[14] Kambal AE, Bate-Smith EC. A genetic and biochemical study on pericarp pigmentation in a cross between two cultivars of grain sorghum, *Sorghum bicolor*. Heredity 1976;37:417–21.
[15] Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, et al. Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science 2003;301:376–9.
[16] Kitada C, Gong Z, Tanaka Y, Yamazaki M, Saito K. Differential expression of two cytochrome P450s involved in the biosynthesis of flavones and anthocyanins in chemo-varietal forms of *Perilla futescens*. Plant Cell Physiol 2001;42:1338–44.
[17] Koes RE, Quattrocchio F, Mol JNM. The flavonoid biosynthetic pathway in plants: function and evolution. BioEssays 1994;16: 123–32.
[18] Kreuzaler F, Ragg H, Fautz E, Kuhn DN, Hahlbrock K. UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc Natl Acad Sci USA 1983;80:2591–3.
[19] Larson R, Bussard JB, Coe Jr EH. Gene-dependent flavonoid 3′ hydroxylation in maize. Biochem Genet 1986;24:615–24.
[20] Lavola A. Accumulation of flavonoids and related compounds in birch induced by UV-B irradiance. Tree Physiol 1998;18:53–8.
[21] Li J, Ou-Lee TM, Raba R, Amundson RG, Last RL. Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell 1993;5:171–9.
[22] Lo S-C, de Verdier K, Nicholson RL. Accumulation of 3-deoxyanthocyanidin phytoalexins and resistance to *Colletotrichum sublineolum* in sorghum. Physiol Mol Plant Pathol 1999;55:263–73.
[23] Lo S-C, Nicholson RL. Reduction of light induced anthocyanin accumulation in inoculated sorghum mesocotyls. Plant Physiol 1998; 116:979–89.
[24] Mo Y, Nagel C, Taylor LP. Biochemical complementation of chalcone synthase mutants defines a role for flavanols in functional pollen. Proc Natl Acad Sci USA 1992;89:7213–7.
[25] Nicholson RL, Hammerschmidt R. Phenolic compounds and their role in disease resistance. Ann Rev Phytopathol 1992;30:369–89.
[26] Nicholson RL, Kollipara SS, Vincent JR, Lyons PC, Cadena-Gomez G. Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. Proc Natl Acad Sci USA 1987;84:5520–4.
[27] Nicholson RL, Wood KV. Phytoalexins: what are they and how can we measure them? Physiol Mol Plant Pathol 2001;59:63–9.
[28] Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 1984;81:8014–8.
[29] Sambrook J, Russell DW. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 2001.
[30] Schoenbohm C, Martens S, Eder C, Forkmann G, Weisshaar B. Identification of the *Arabidopsis thaliana* flavonoid 3′-hydroxylase gene and functional expression of the encoded P450 enzyme. Biol Chem 2000;381:749–53.
[31] Snyder BA, Leite B, Hipskind J, Butler LG, Nicholson RL. Accumulation of sorghum phytoalexins induced by *Colletotrichum graminicola* at the infection site. Physiol Mol Plant Pathol 1991;39: 463–70.
[32] Snyder BA, Nicholson RL. Synthesis of phytoalexins in sorghum as a site specific response to fungal ingress. Science 1990;248:1637–9.
[33] Stafford HA. Flavonoid metabolism. Boca Raton: CRC Press; 1990, p. 298.
[34] Styles ED, Ceska O. Pericarp flavonoids in genetic strains of *Zea mays*. Maydica 1989;34:227–37.
[35] Thompson JD, Higgins DJ, Gibson TJ. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;2:4673–80.
[36] Toda K, Yang D, Naoki Y, Watanbe S, Harada K, Takahashi R. A single-base deletion in soybean flavonoid 3′-hydoxylase gene is associated with gray pubescence color. Plant Mol Biol 2002;50: 187–96.
[37] Wharton PS, Julian AM. A cytological study of compatible and incompatible interactions between *Soghum bicolor* and *Colletotrichum sublineolum*. New Phytol 1996;134:25–34.
[38] Wharton PS, Nicholson RL. Temporal synthesis and radiolabelling of the sorghum 3-deoxyanthocyanidin phytoalexins and the anthocyanin, cyanidin 3-dimalonyl glucoside. New Phytol 2000;145:457–69.
[39] Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology and biotechnology. Plant Physiol 2001;127:485–93.
[40] Winkel-Shirley B. It takes a garden. How work on diverse plant species has contributed to an understanding of flavonoid metabolism. Plant Physiol 2001;127:1399–404.
[41] Wiseman BR, Widstrom NW. Resistance of corn populations to larvae of the corn earworm (Lepidoptera:Noctuidae). J Econ Entomol 1992;85:601–5.
[42] Wood KV, Bonham CC, Hipskind J, Nicholson RL. Analysis of anthocyanins and 3-deoxyanthocyanidins by plasma desorption mass spectrometery. Phytochemistry 1994;37:557–60.
[43] Ylstra B, Touraev A, Moreno RMB, Stoger E, Van Tunen AJ, Vicentie O, et al. Flavonols stimulate development, germination, and tube growth of tobacco pollen. Plant Physiol 1992;100:902–7.
[44] Zabala G, Vodikin L. Cloning of the pleiotropic *T* locus in soybean and two recessive alleles that differentially affect structure and expression of the encoded flavonoid 3′-hydroxylase. Genetics 2003; 163:295–309.