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Expression of a putative flavonoid 3\(^{\prime}\)-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\(^{\prime}\)-hydroxylase. Full length sequence characterization allowed us to establish that the sorghum putative f3\(^{\prime}\)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\(^{\prime}\)h cDNA in Escherichia coli yielded a membrane preparation that catalyzed the hydroxylation of naringenin. We show here that transcription of the flavonoid 3\(^{\prime}\)-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\(^{\prime}\)h gene, implicating its role in fungal induced expression of sorghum phytolaexins.

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
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 microspectrometry, Snyder et al. [31] quantified phytoalexin levels in infected host cells and found these to be 150 \( \mu \)M, 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, flavanone 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 the presence of an additional methoxy group. These results suggest that the resistant cultivar is able to produce more of these compounds during the response to infection. The 3-deoxyanthocyanidins are a class of phytoalexins that are synthesized in response to fungal infection [1].

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 yl 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 yl 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′-AGTCGAGGTGACGTTCTC-3′, and reverse primer (R), 5′-GCAGACGCCGCACTGTC-
TCCCT-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 32P-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'-CTTCTAGAACCAGGCAACTCAAACCATGGAC-3', and SBR22, 5'-TTGGATCCCCTA CCTCGCTGAT-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 pGem-T-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 NcoI-EcoRI 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 was visualized. 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 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 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, Callistephus chinensis, Glycine max, Mathiola incana, Pellargonium hortorum, Petunia hybrida, Saccharum officinarum, Triticum aestivum, Torenia hybrida, Oryza sativa, and Zea mays. 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...
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 (AF296346); CYP71D10 (AF022459); CYP71E1 (AF029858); CYP71K1 (AP002968); CYP73A1 (Z17369); CYP73A9 (U9243); 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 (AF045593); CYP75B4 (AF313488); CYP75B7 (AF313491); CYP75B8 (AF315465); CYP75B10 (AB057672); CYP76A1 (X71658); CYP76A2 (X71657); CYP76B1 (Y09920); CYP76D1 (AB032833); CYP78A1 (L23209); CYP80A1 (U09610); CYP80B3 (AF191772); CYP81B1v1 (AJ00477); CYP81D1 (D78606); CYP82A1 (U29333); CYP93A1 (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 f3'5'hs 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 \( \lambda \) 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 \( \lambda \)-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 \( PstI \), \( SacI \) and \( SalI \) 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’\( SacI \) fragment (F7) shows a single band of 1.0 kbp in the \( SacI \) digested genomic DNA lane. Digestion with EcoRI 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 \( F3'H \) from dicots and higher similarity to \( F3'H \) 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 VDKKG; 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 \( m/z \) of 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 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
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. 6. Detection and temporal synthesis of 3-deoxyanthocyanidin 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-methoxy-luteolinidin (14.8 min), and 7-methoxy-apigeninidin (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 prl 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 GGGEK, that distinguishes them from their nearest relatives F3’5’Hs [4]. Our sequence alignment shows that GGGEK 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
the flavonoid or phytoalexins biosynthesis. Genetic studies in the anthocyanin pathway have demonstrated that mutations in $f3'h$ lead to accumulation of pelargonidin through the metabolism of dihydrokaempferol 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 apigenidin 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, apigeni- din 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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