

RESEARCH PAPER

Identification of flavone phytoalexins and a pathogen-inducible flavone synthase II gene (*SbFNSII*) in sorghum

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Abstract

Following inoculation with the anthracnose pathogen *Colletotrichum sublineolum*, seedlings of the sorghum resistant cultivar SC748-5 showed more rapid and elevated accumulation of luteolin than the susceptible cultivar BTx623. On the other hand, apigenin was the major flavone detected in infected BTx623 seedlings. Luteolin was demonstrated to show stronger inhibition of spore germination of *C. sublineolum* than apigenin. Because of their pathogen-inducible and antifungal nature, both flavone aglycones are considered sorghum phytoalexins. The key enzyme responsible for flavone biosynthesis has not been characterized in monocots. A sorghum pathogen-inducible gene encoding a cytochrome P450 protein (CYP93G3) in the uncharacterized CYP93G subfamily was identified. Transgenic expression of the P450 gene in *Arabidopsis* demonstrated that the encoded protein is a functional flavone synthase (FNS) II *in planta*. The sorghum gene was then termed *SbFNSII*. It is a single-copy gene located on chromosome 2 and the first FNSII gene characterized in a monocot. Metabolite analysis by liquid chromatography–tandem mass spectrometry (LC-MS/MS) in precursor ion scan mode revealed the accumulation of 2-hydroxynaringenin and 2-hydroxyeriodictyol hexosides in the transgenic *Arabidopsis* plants. Hence, *SbFNSII* appears to share a similar catalytic mechanism with the licorice and *Medicago truncatula* FNSIIs (CYP93B subfamily) by converting flavanones to flavone through the formation of 2-hydroxyflavanones.

Key words: *Colletotrichum sublineolum*, CYP93G3, flavones, flavone synthase II, sorghum.

Introduction

Flavones are a large group of flavonoid metabolites with different physiological functions in higher plants. Although flavones are generally colourless, they function to alter coloration in flowers by complexing with anthocyanin pigments (Goto and Kondo, 1991). Flavones also serve as antioxidants to protect plants from UV irradiation. In leguminous plants, flavones are important signal molecules for establishing symbiotic relationships with root nodulation bacteria. Luteolin was the first flavone known to induce *nod* gene expression in the diazotrophic bacterium *Sinorhizobium meliloti* (Peters *et al.*, 1986), leading to the production of Nod factors which are essential for the root nodulation process. Silencing of flavone synthases in *Medicago truncatula* resulted in flavone-deficient roots with reduced nodulation after inoculation with the bacterium

(J Zhang *et al.*, 2007, 2009). In maize, the *C*-glycosyl flavone maysin is responsible for the insecticidal activity in silk tissues toward corn earworm (Rector *et al.*, 2002). Several flavones were also shown to have activities against a variety of organisms, including plants, nematodes, molluscs, fungi, oomycetes, and bacteria (Martens and Mithöfer, 2005), and some of them may be considered as phytoalexins.

In addition to their important physiological roles in plants, flavones have been demonstrated to show significant pharmacological activities, elevating their importance as nutritional components in our diet. For example, luteolin is considered to have the potential for cancer prevention and therapy due to its ability to induce apoptosis, inhibit cell proliferation, metastasis, and angiogenesis, and sensitize tumour cells to therapeutic-induced cytotoxicity (Lin *et al.*,

2008). Besides, luteolin is well characterized for its antioxidant and anti-inflammatory activities both *in vitro* and *in vivo* (Seelinger et al., 2008). Possible mechanisms for its different pharmacological properties include scavenging of reactive oxygen species (Lien et al., 1999), enhancement of endogenous antioxidants (Leung et al., 2006), reduction of nuclear factor- κ B activities (Shi et al., 2004), inhibition of DNA topoisomerases I (Chowdhury et al., 2002) and II (Cantero et al., 2006), phosphatidylinositol 3-kinase (Bagli et al., 2004), insulin-like growth factor 1 receptor (Fang et al., 2007), and fatty acid synthase (Brusselmans et al., 2005), promotion of the degradation of a transcription factor (STAT3) involved in inflammation (Selvendrian et al., 2006), as well as stabilization of the tumour suppressor p53 (Shi et al., 2007).

Biosynthesis of flavones begins with flavanones which are the precursors for all the major flavonoid classes (Fig. 1). In dicots, two distinct flavone synthase (FNS) enzymes, FNSI and FNSII, have been described. FNSI, a soluble Fe²⁺/2-oxoglutarate-dependent dioxygenase (DOX) restricted to members of Apiaceae, directly introduces a double bond between C2 and C3 in flavanones (Martens and Mithöfer, 2005). FNSI shows high sequence identity to flavanone 3-hydroxylase (F3H), another DOX enzyme which uses the same flavanone substrates. Results from site-directed mutagenesis strongly suggested that parsley FNSI was evolved from gene duplication of F3H, followed by functional diversification (Gebhardt et al., 2007). On the other hand, FNSII is a cytochrome P450, oxygen- and NADPH-dependent, and membrane-bound monooxygenase found in other flavone-accumulating dicot families including Leguminosae, Asteraceae, Plantaginaceae, and Lamiaceae (Martens and Mithöfer, 2005). All the characterized FNSII

enzymes belong to the P450 CYP93B subfamily, and most of the recombinant proteins converted flavanone to flavone directly in enzyme assays. However, FNSII genes from the legumes *Glycyrrhiza echinata* (licorice) and *M. truncatula* were found to encode enzymes with flavanone 2-hydroxylase activities, leading to the formation 2-hydroxyflavanones *in vitro* (Akashi et al., 1998; Zhang et al., 2007), and flavones were formed only after acid treatments. In addition to maysin in maize, flavone glycosides are constitutively accumulated in cereal crops such as wheat (Cavaliere et al., 2005) and rice (Shih et al., 2008). However, the enzymatic steps responsible for flavone biosynthesis are still not fully understood in monocots.

Sorghum, *Sorghum bicolor* (L.) Moench, is the fifth-ranking cereal in the world and it is a close relative of maize (*Zea mays*). The plant is a rich source of distinct phytochemicals such as dhurrin (Busk and Moller, 2002), sorgoleone (Czarnota et al., 2001), and 3-deoxyanthocyanidins (Snyder and Nicholson, 1990). The 3-deoxyanthocyanidins are a unique class of flavonoid phytoalexins synthesized by sorghum as an essential component of active defence mechanisms (Nicholson et al., 1987; Snyder and Nicholson, 1990; Lo et al., 1999). Recently a pathogen-inducibile stilbene synthase gene was functionally characterized in sorghum (Yu et al., 2005). Subsequently, *trans*-piceid was detected in infected sorghum seedlings, but its role in defence appeared to be secondary toward the anthracnose pathogen *Colletotrichum sublineolum* (Yu et al., 2008). In continuous attempts to investigate the roles of secondary metabolites in sorghum disease resistance, the flavone aglycones luteolin and apigenin have been identified as new phytoalexin compounds in the present study. Subsequently, a sorghum pathogen-inducible P450 gene belonging to the

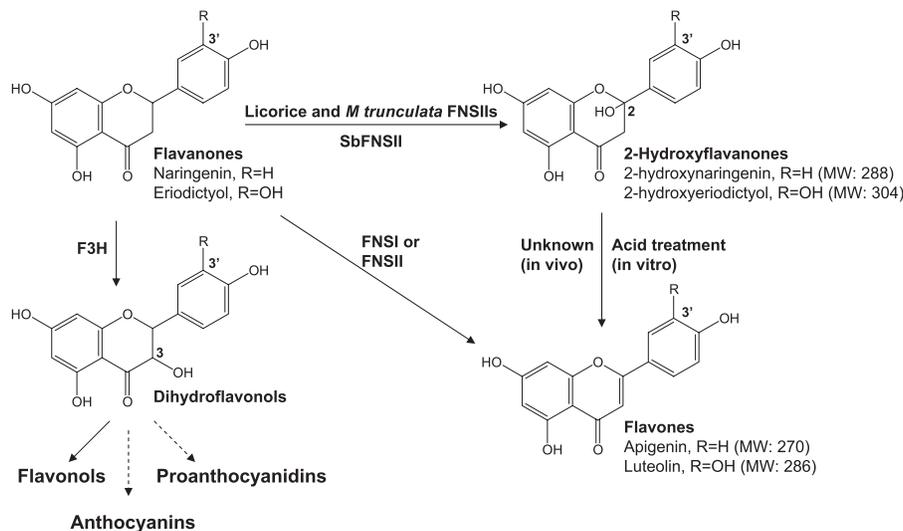


Fig. 1. Biosynthesis of flavones from flavanones. Two FNS enzyme systems have been described in dicots for flavone biosynthesis. FNSI is a soluble enzyme while FNSII is a membrane-bound P450 enzyme. FNSI and most FNSII enzymes convert flavanones to flavones directly. The licorice and *M. truncatula* FNSII enzymes catalyse the conversion of flavanones into 2-hydroxyflavanones which can be converted to flavones on acid treatment *in vitro*. SbFNSII was found to have a similar catalytic mechanism in this study. Flavanone 3-hydroxylase (F3H) also uses flavanones as substrates, producing dihydroflavonols which are the precursors for flavonols, anthocyanins, and proanthocyanidins. Dotted lines indicate multiple reaction steps.

uncharacterized CYP93G subfamily first identified in the rice genome (Nelson *et al.*, 2004) was isolated and characterized. Metabolite analysis in transgenic *Arabidopsis* overexpressing the sorghum gene demonstrated that the encoded protein is an FNSII enzyme having a catalytic mechanism similar to that of the licorice and *M. truncatula* enzymes.

Materials and methods

Sorghum growth conditions and fungal inoculation

Seeds of the sorghum cultivars (SC748-5 and BTx623) used in this study were former collections of the late Professor Ralph L. Nicholson of Purdue University (West Lafayette, IN, USA). Sorghum seeds were planted in rolls of germination paper and kept in the dark for 4 d as described previously (Lo *et al.*, 1996). Etiolated seedlings with elongated mesocotyls were then inoculated with conidial suspensions of *C. sublineolum* at 3.0×10^6 conidia ml^{-1} with Tween-20 as a wetting agent (100 μl 100 ml^{-1}). The inoculated plants were kept at 100% relative humidity at room temperature for 24 h.

RNA experiments

Total RNA was extracted from mesocotyl tissues (1.0 g) using the Trizol method (Invitrogen). DNase I-treated RNA samples (3 μg) were reversed transcribed by M-MLV reverse transcriptase (Promega). Primers CL590 (5'-CGCAAGACCACCGTCTTCTT-3') and CL592 (5'-GGTAGCTTTTCCTGTTGCCG-3') were used for amplification of *SbFNSII*, while primers HEL794 (5'-GCGACCTTACCGACTACCTC-3') and HEL795 (5'-AATATCCACGTCGCACTTCA-3') were used for amplification of a sorghum actin gene. The 5' and 3' ends of the *SbFNSII* transcript were mapped based on the sorghum expressed sequence tag (EST) contig TC105061 using a rapid amplification of cDNA ends (RACE) procedure as described by the manufacturer (Roche Molecular Biochemicals). PCR amplifications were programmed as follows: pre-incubation (95 °C for 10 min), followed by 30 cycles of denaturation (95 °C for 30 s), annealing (55–58 °C for 30 s), and extension (72 °C for 1 min), and finalized by an extension step at 72 °C for 7 min.

Sequence analysis

Sequences of the CYP93 family were retrieved from GenBank and aligned by the ClustalW method (www.ebi.ac.uk/clustalw). The alignment was used to perform phylogenetic analysis by the Neighbor-Joining method in the MEGA 4 program using default parameters (Kumar *et al.*, 2004). The bootstrap values for nodes in the phylogeny tree are from 1000 replications.

Generation of transgenic *Arabidopsis* plants

The *SbFNSII* coding region was amplified from sorghum cDNA by the above PCR protocol using the primers CL659 (5'-GATGGATGCATCCGTGTTAC-3') and CL660 (5'-CTATGCTATGGGTGAGAATC-3'). The PCR product was inserted between the cauliflower mosaic virus (CaMV) 35S promoter and nopaline 3'-terminator in the plasmid vector 103c-SK (E. Lam, Rutgers University, NJ, USA). The resulting overexpression cassette was cloned into the binary vector pCAMBIA 1300 (CAMBIA, Canberra, Australia) which harbours the hygromycin resistance gene for selection of plant transformants. *Agrobacterium*-mediated transformation of the final construct into *Arabidopsis* (Col-0) wild type and *tt6* mutants was performed by the floral dip method (Clough and Bent, 1998). The *tt6* line (SALK_113904) was obtained from the *Arabidopsis* Biological Resource Center

(Columbus, OH, USA). Harvested seeds were surface sterilized and germinated on Murashige and Skoog (MS) (Sigma) agar containing 3% (v/v) sucrose and $25 \mu\text{g ml}^{-1}$ hygromycin (Sigma). Resistant seedlings were transplanted and placed in a growth chamber (22 °C; 16 h light, 8 h dark). To induce the endogenous flavonoid pathway, T₁ seeds were germinated on MS plates without nitrogen sources. Tissues (0.5–1 g) were collected for metabolite analysis after 7 d.

HPLC–MS analysis of plant metabolites

Plant tissues (0.5 g) ground to fine powder in liquid nitrogen were extracted in 100% HPLC-grade methanol (500 μl). For acid hydrolysis, an equal volume of 2 N HCl was added to the samples for incubation at 90 °C for 1 h. Filtered samples (10 μl) were injected onto a Agilent 1100 series HPLC system (Agilent Technologies, CA, USA) connected with an Eclipse XDB-C18 column (5 μm , $2.1 \times 150 \text{ mm}$, Agilent Technologies). Separation was performed using a solvent system of 0.5% formic acid/water (v/v) (A) and 0.5% formic acid/methanol (B) with a linear gradient of 15–60% B over 20 min, then held at 60% B for 5 min and equilibrated at 15% B for 10 min. The flow rate was maintained at 0.2 ml min^{-1} and the elution monitored by a diode-array detector (200–600 nm) in tandem with an API2000-QTRAP[®] quadrupole-linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) operating in positive ionization mode. Enhanced full scan and product ion spectra in the mass range of m/z 150–1000 were acquired using parameters optimized for maximum sensitivities: curtain gas, 20 (arbitrary unit); collisionally activated dissociation gas, medium; ion spray voltage, 5300 V; capillary temperature, 350 °C; nebulizing gas (GS1), 60 (arbitrary unit); heating gas (GS2), 60 (arbitrary unit); declustering potential, 75 V; entrance potential, 10 V; collision energy, 40 V; collision energy spread, 5 V. For precursor ion scan (PIS) analysis of flavone and 2-hydroxyflavanone *O*-glycosides, Q1 was operated at unit resolution and Q3 was operated at low resolution, and the mass spectra were obtained over a scan range of 150–1000 amu. MS³ experiments were accomplished by raising the orifice bias potential to induce fragmentation in the lens region, mass-selecting the appropriate product ion in Q1, inducing its fragmentation in Q2, and mass-analysing the second-generation product ions in Q3 via a linear ion trap. To scan for the known cereal flavone compounds in infected sorghum samples, an information-dependent data acquisition (IDA) protocol was performed. Quantification of flavones in the plant samples was achieved by liquid chromatography–mass spectrometry (LC-MS) operating in multiple reaction monitoring (MRM) mode. The transition reactions of apigenin (m/z 271.2 → 153.1) and luteolin (m/z 287.2 → 153.1) were monitored after LC separation, and the LC-MRM peak areas were used for concentration determination. Authentic standards of luteolin and apigenin (Sigma) were used for metabolite identification and quantification. Data acquisition, peak integration, and calculations were all interfaced to a computer workstation running the Analyst 1.4.2 software (Applied Biosystems/MDS Sciex).

In vitro spore germination assays

For germination assays, conidia were dislodged with water from the surface of *C. sublineolum* cultures growing on oatmeal agar, and filtered through a layer of cheesecloth to remove hyphae and debris. Spore suspensions (20 μl ; $5.0 \times 10^4 \text{ ml}^{-1}$) containing different concentrations (5–50 μM) of either apigenin or luteolin (Sigma) dissolved in 3% ethanol (for improved solubility) were inoculated on the surface of plastic Petri plates. Control experiments were prepared by incubating the spores in 3% ethanol without the test compounds. After dark incubation at room temperature for 9–12 h, germination and appressorium formation was examined and counted under a dissecting microscope.

Results

Accumulation of flavone aglycones in infected sorghum seedlings

LC-MS/MS analysis was performed to investigate the differentially accumulated metabolites in the sorghum cultivar SC748-5 after inoculation with *C. sublineolum*. SC748-5 has been demonstrated to show stable resistance to the anthracnose pathogen across different geographical locations (Mehta *et al.*, 2005; Perumal *et al.*, 2009). The metabolite profile of methanol extracts prepared from infected seedlings revealed the accumulation of a flavone aglycone (Fig. 2A) which was not detected in uninoculated samples (data not shown). Under positive ionization mode, the compound eluted at 27.7 min generated an $[M + H]^+$ ion at m/z 287, which is consistent with the molecular weight of a [luteolin + H]⁺ ion. Identification of the m/z 287 ion was confirmed by its MS/MS fragmentation pattern in comparison with that obtained for an authentic luteolin standard (Fig. 2B). The time-course accumulation of pathogen-inducible flavones in SC748-5 was then compared with that of a highly susceptible cultivar BTx623 (Fig. 2C). In SC748-5 seedlings, luteolin was first detected at 48 h after inoculation and continued to accumulate up to $\sim 2 \mu\text{g g}^{-1}$ tissue during the course of the experiment. In BTx623 seedlings, luteolin were detected at 72 h after inoculation and the level was considerably (~ 3 -fold) lower compared with the SC748-5 seedlings. On the other hand, appreciable amounts of apigenin up to $\sim 6 \mu\text{g g}^{-1}$ tissue were found to accumulate in the BTx623 seedlings starting from 72 h after inoculation. Apigenin was identified by LC-MS/MS analysis in comparison with an authentic standard (data not shown).

Following the identification of luteolin and apigenin as pathogen-inducible metabolites in sorghum seedlings, we addressed the question of whether they may function as phytoalexins. *In vitro* bioassays were therefore performed to investigate their effects on spore germination of *C. sublineolum*. On hydrophobic surfaces, the conidia of *C. sublineolum* germinate with short germ tubes, and melanized appressoria are formed within 9–12 h. Luteolin was found to exhibit strong inhibitory effects on the fungal pathogen in a dose-dependent manner (Fig. 3). Thus, spore germination was reduced by almost 60% at 20 μM and by >80% at 50 μM luteolin. On the other hand, apigenin was considerably less effective than luteolin at all the concentrations examined (Fig. 3). Spore germination was reduced by <50% at 50 μM apigenin.

Identification of CYP93G3 as a pathogen-inducible gene in sorghum

To understand the enzymatic mechanism of flavone synthesis in sorghum, an attempt was made to identify putative FNS-encoding sequences in the public databases. Using the known FNSII sequences to blast search against the DCFI Plant Gene Index (<http://compbio.dfci.harvard.edu/tgi/plant.html>), several sorghum contiguous sequences annotated to encode cytochrome P450 enzymes were retrieved. Among them, the expression of TC105061 (831 bp) was found to be up-regulated in infected sorghum seedlings. RT-PCR analysis revealed that TC105061 transcripts were detected starting at 24 h after inoculation in the SC748-5 seedlings (Fig. 4). On the other hand, the expression was only detected at 48 h after inoculation in the BTx623 seedlings. In both cultivars, no basal levels of

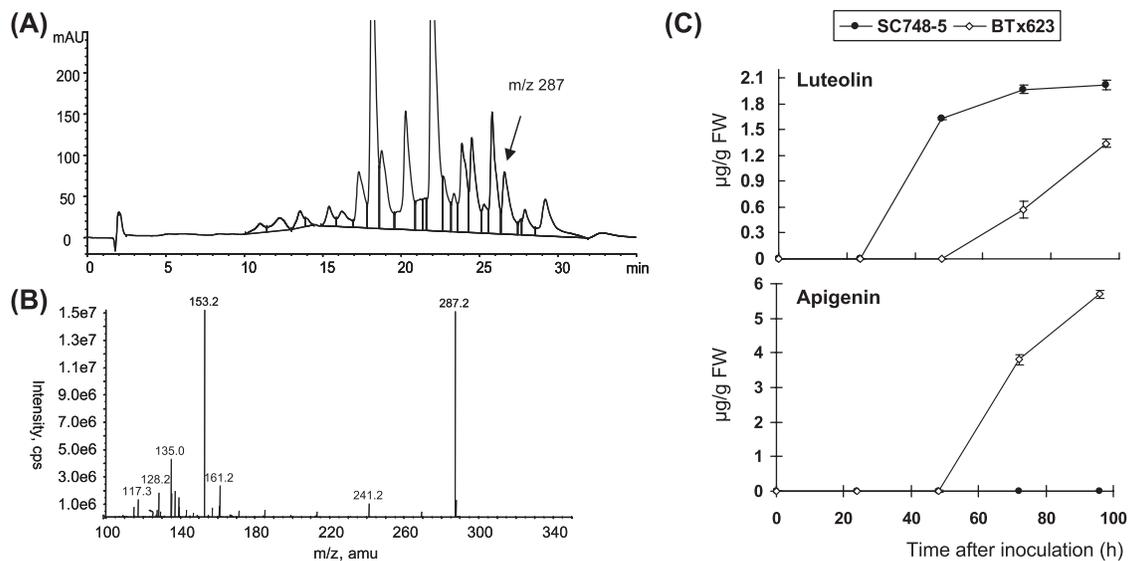


Fig. 2. Accumulation of pathogen-inducible flavones in sorghum seedlings. (A) LC-MS/MS detection of luteolin in methanol extracts prepared from SC748-5 seedlings after inoculation with *C. sublineolum*. The indicated peak with an m/z 287 ion was not detected in control plant samples (not shown). (B) The MS/MS spectrum of the m/z 287 ion is identical to that obtained for an authentic luteolin standard (not shown). (C) Time-course accumulation of luteolin and apigenin in infected SC748-5 and BTx623 seedlings. FW, fresh weight.

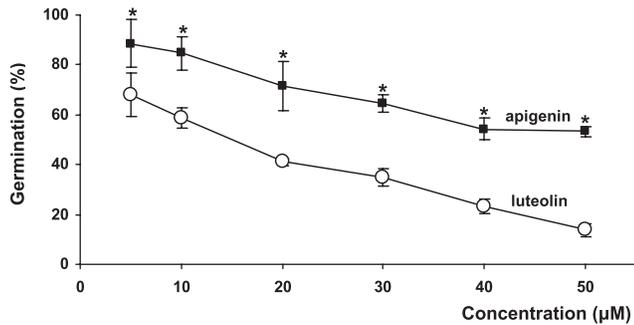


Fig. 3. Effects of luteolin and apigenin on spore germination of *C. sublineolum*. Percentages of spore germination after the flavone treatments at the indicated concentrations are shown with control ethanol-treated spores defined as 100%. Data represent average values obtained from at least three replications and error bars represent standard deviations. *These values are deemed significantly higher (Student's *t*-test, $P < 0.05$) than those obtained for luteolin at the same concentration.

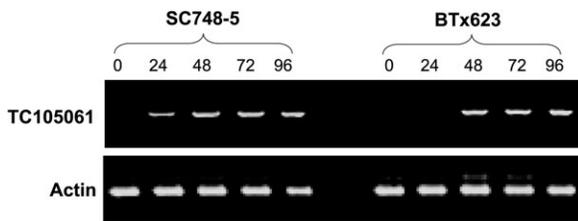


Fig. 4. RT-PCR expression analysis of the TC105061 sequence in infected SC748-5 and BTx623 plants. Four-day-old seedlings were inoculated with *C. sublineolum*, and RNA samples were collected at the indicated time points (h) after inoculation.

expression were recorded in uninfected seedlings (data not shown).

The full-length cDNA clone was then recovered by RACE experiments using RNA samples prepared from infected sorghum seedlings. The sorghum P450 sequence encodes a protein of 541 amino acid residues with a predicted molecular mass of 59.280 kDa and a calculated pI of 7.987. The encoded protein contains features that are highly conserved among cytochrome P450 enzymes, including the proline hinge region, the oxygen-binding pocket, and the most diagnostic haem-binding signature motif near the C-terminus (Supplementary Fig. S1 available at *JXB* online). The sequence was found to be located on chromosome 2 in the sorghum BTx623 genome (<http://www.phytozome.net/cgi-bin/gbrowse/sorghum/>). The assigned gene identifier is Sb02g000220 and there are no introns present. Sequence analysis of the encoded protein (NCBI accession XP_002461286) revealed 69.7% and 53.1% identities with the rice sequences Os06g01250 and Os04g01140 (TIGR rice genome annotation), respectively. Both rice sequences are uncharacterized P450 proteins designated as members of the CYP93G subfamily (Nelson *et al.*, 2004; <http://drnelson.utmem.edu/rice.html>). On the other hand, the sorghum protein shows moderate sequence identity to the characterized

FNSII sequences (members of the CYP93B subfamily) from *Gerbera hybrida* (CYP93B2, 39.3%), *Antirrhinum majus* (CYP93B3, 40.5%), *Perilla frutescens* (CYP93B6, 37.5%), *M. truncatula* (CYP93B10, 38.8%), and *G. echinata* (CYP93B1, 38.0%). A phylogeny tree was then constructed using the Neighbor-Joining method for the different CYP93 subfamily proteins. As shown in Fig. 5, the sorghum protein is closely related to the two rice (*Oryza sativa*) CYP93G sequences. Hence, the sorghum sequence was designated as CYP93G3, a new member in this subfamily (David Nelson, P450 Nomenclature Committee, personal communication). The soybean (*Glycine max*) CYP93A subfamily, which includes a pterocarpan 6a-hydroxylase involved in the later stage of isoflavonoid biosynthesis (Tian *et al.*, 2008), is located on the same branch with the CYP93B sequences. On the other hand, the CYP93B subfamily proteins appear to be more distantly related to the sorghum and rice sequences.

Characterization of *SbFNSII* biochemical functions in transgenic *Arabidopsis*

To examine whether CYP93G3 is a functional FNS enzyme *in planta*, the sorghum coding sequence was overexpressed in transgenic *Arabidopsis* under the control of the CaMV 35S promoter. The *Arabidopsis tt6* mutant, which is defective in the single-copy F3H gene, was chosen as the host for *Agrobacterium*-mediated transformation. F3H and FNS are competing enzymes using the same flavanone substrates (Fig. 1). A total of 13 primary transgenic lines (confirmed by PCR genotyping) were obtained and constitutive expression of the sorghum transgene was confirmed by RT-PCR analysis (data not shown). T₁ seeds of more than five independent lines were germinated on MS medium deficient in nitrogen to induce the endogenous flavonoid pathway. As constitutively expressed secondary metabolites are often modified by *O*-glycosylation in plants, 10-day-old *Arabidopsis* seedlings were extracted in methanol followed by acid hydrolysis to release the flavonoid aglycones for LC-MS/MS analysis. As shown in Fig. 6A and B, two distinct peaks corresponding to luteolin and apigenin were detected in all the transgenic samples analysed. Their identities were confirmed by LC-MS/MS analysis as described above. The sorghum gene was also transformed into *Arabidopsis* wild-type (Col-0) plants. Quantitative analysis revealed that lower amounts of both flavone aglycones were detected in the transgenic plant samples in the Col-0 background compared with those in the *tt6* background (Fig. 6C).

The above results clearly demonstrated that CYP93G3 functions as an FNSII enzyme *in planta*, and thus the sorghum gene was named *SbFNSII*. In the legume species licorice and *M. truncatula*, FNSII enzymes were found to function through the formation of 2-hydroxyflavone intermediates (Fig. 1; Akashi *et al.*, 1998; Zhang *et al.*, 2007). As the acid treatment would convert 2-hydroxyflavones to the corresponding flavones (Akashi *et al.*, 1998; Zhang *et al.*, 2007), an attempt was made to detect the presence of their *O*-glycosylated derivatives in non-hydrolysed

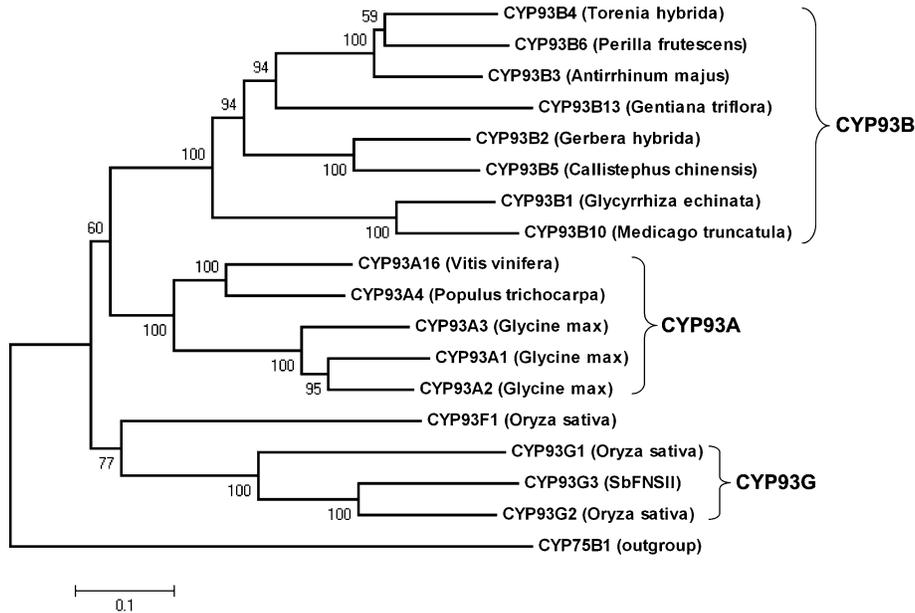


Fig. 5. Sequence analysis of CYP93G3 (SbFNSII) and other CYP93 family proteins. An unrooted phylogenetic tree of CYP93 proteins was constructed by MEGA4.0. Numbers at the nodes represent bootstrap values from 1000 replications.

samples prepared from the transgenic *Arabidopsis* lines. LC-MS/MS analyses were then performed to scan for precursor ions of m/z 289 and m/z 305, which are consistent with the molecular weight of the protonated ions of 2-hydroxynaringenin and 2-hydroxyeriodictyol, respectively. As shown in Fig. 7A, the total ion chromatogram of the PIS of m/z 289 revealed the most distinct peak (M1, m/z 451) eluted at 18.6 min in the transgenic plant sample. The MS/MS spectrum of the m/z 451 ion showed the diagnostic m/z 289 ion, indicating the loss of a hexosyl unit (162 Da) from the parent ion (Fig. 7B). An MS³ experiment of the m/z 289 daughter ion produced a fragmentation pattern (Fig. 7C, D) which was essentially identical to that reported previously for a [2-hydroxynaringenin + H]⁺ ion (Zhang et al., 2007). Similarly, the total ion chromatogram of the PIS of m/z 305 revealed the most distinct peak (M2, m/z 467) eluted at 16.87 min (Fig. 8A). The MS/MS spectrum of m/z 467 showed the m/z 305 daughter ion, also indicating the loss of a hexosyl unit (162 Da) (Fig. 8B). An MS³ experiment of the m/z 305 daughter ion generated a fragmentation pattern that is consistent with that predicted for a [2-hydroxyeriodictyol + H]⁺ ion (Fig. 8C, D). Hence, M1 and M2 could be conclusively identified as 2-hydroxynaringenin and 2-hydroxyeriodictyol *O*-hexosides, respectively.

LC-PIS analyses were also performed to detect the presence of flavone *O*-glycosides in the non-hydrolysed transgenic plant samples. Table 1 summarizes the different *O*-glycosides of apigenin and luteolin identified. In all cases, MS³ experiments were conducted for structural confirmation of the protonated flavone daughter ions. Taken together, the *SbFNSII*-overexpressing transgenic *Arabidopsis* lines were demonstrated to accumulate *O*-glycosylated derivatives of both 2-hydroxyflavanones and flavones. On the other hand, none of these flavonoid metabolites was

detected in the non-transformed plant samples (data not shown).

Discussion

Phytoalexins are low molecular weight antimicrobial compounds produced by plants in response to infection or stress (Paxton, 1981; Nicholson and Hammerschmidt, 1992). In sorghum, the 3-deoxyanthocyanidins represent a unique group of flavonoid phytoalexins important for defence against *C. sublineolum* (Snyder and Nicholson, 1990; Lo et al., 1999). The anthracnose pathogen can cause severe foliar damages and result in substantial yield loss (Thomas et al., 1996). Over the years, host plant resistance has remained the most effective strategy for control of this sorghum disease (Perumal et al., 2009). Frequently plants produce multiple structurally related defence metabolites during host–pathogen interaction (Kuc, 1995; Smith, 1996). As part of ongoing efforts to understand the biochemical basis of sorghum anthracnose resistance, luteolin and apigenin have been identified as new flavonoid phytoalexins in sorghum in this study.

The antimicrobial activities and health-beneficial properties of flavones are well documented (Martens and Mithöfer, 2005). In contrast, only a few examples of pathogen- or stress-inducible flavones in plants have been described. Both luteolin and apigenin were identified in soybean cotyledons following treatment with an elicitor prepared from *Diaporthe phaseolorum* f. sp. *meridionalis* (Modolo et al., 2002). In addition, a complex mixture of *C*-glycosylated flavone derivatives (cucumerins A and B, vitexin, orientin, isoorientin) were detected in cucumber leaves expressing induced resistance against the powdery

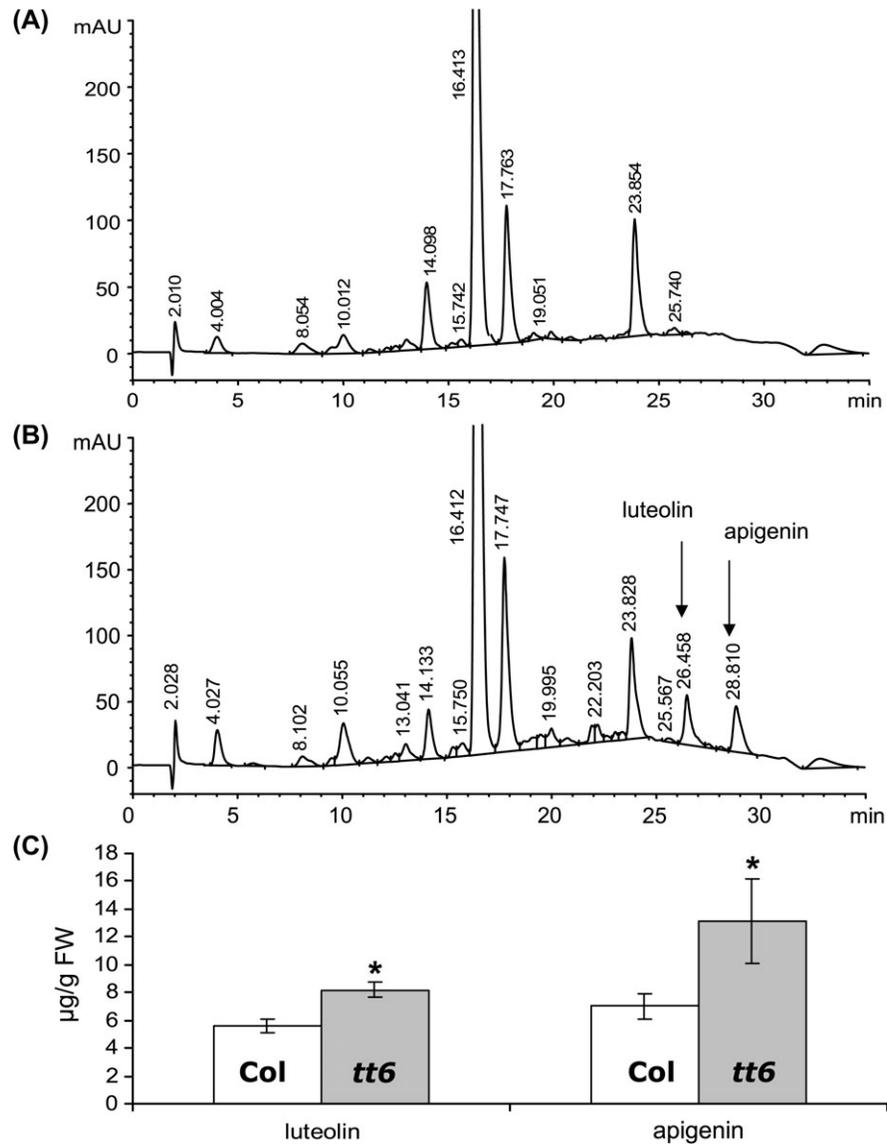


Fig. 6. LC-MS/MS detection of flavones in acid-hydrolysed extracts of (A) untransformed *tt6* plants and (B) transgenic *Arabidopsis tt6* plants overexpressing SbFNSII. The identification was confirmed by their MS/MS spectra (not shown). (C) Quantitative analysis of luteolin and apigenin in transgenic *Arabidopsis Col-0* and *tt6* plants. Data are expressed as average values of three transgenic lines in each genetic background and error bars represent the standard deviation. The flavones were not detected in untransformed *Col-0* and *tt6* plants (not shown). *These values are deemed significantly higher (Student's *t*-test, $P < 0.05$) than those obtained for the transgenic lines in the *Col-0* background. FW, fresh weight.

mildew fungus *Podosphaera xanthii* (McNally *et al.*, 2003). Using LC-MS/MS analysis with an IDA protocol, the various flavone *C*-glycosides known to accumulate in cereal crops, such as maysin, isovitexin, isoorientin, and isoscaparin (Grotewold *et al.*, 1998), were not detected in the present infected sorghum samples (data not shown). Instead, luteolin and apigenin aglycones were identified as the major flavone metabolites in the resistant (SC748-5) and susceptible (BTx623) cultivars, respectively, following inoculation with *C. sublineolum*. The elevated and more rapid accumulation of luteolin in the resistant cultivar strongly suggested that this compound plays an important role in defence against the anthracnose pathogen. In fact, luteolin showed dose-dependent inhibition on *C. sublineolum* spore

germination while apigenin was considerably less effective over the same concentration range (Fig. 3). Consistent with this, luteolin has been demonstrated to show higher cytotoxicity than apigenin on a human oesophageal adenocarcinoma cell line (Q Zhang *et al.*, 2009).

Previously, SC748-5 and BTx623 were found to show different 3-deoxyanthocyanidin phytoalexin responses (Lo *et al.*, 1999). Thus, the resistant cultivar accumulated luteolinidin which was not detected in the susceptible cultivar after inoculation with *C. sublineolum*. Luteolinidin was shown to be more fungitoxic than apigeninidin (Nicholson *et al.*, 1987) which was produced by infected BTx623 seedlings as the major 3-deoxyanthocyanidin. Luteolin and luteolinidin are 3'-hydroxylated flavonoids,

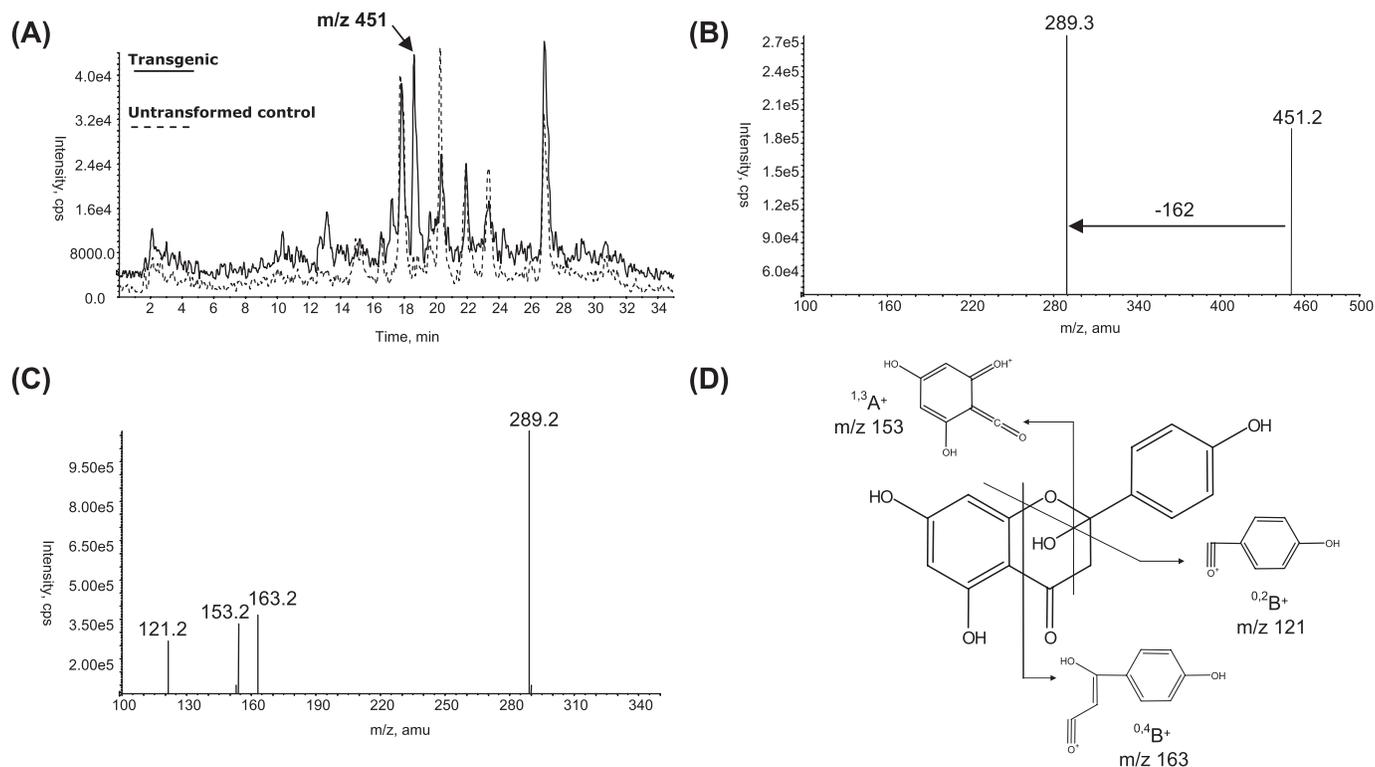


Fig. 7. LC-PIS detection of a 2-hydroxynaringenin O-hexoside in methanol extracts of transgenic *Arabidopsis tt6* plants overexpressing SbFNSII (A) Precursor ions of m/z 289 were scanned following LC separation. The most distinct peak (arrow) identified in the transgenic plant sample produced a major ion at m/z 451 following MS analysis (B) MS/MS spectrum of the m/z 451 ion indicating the loss of a hexosyl unit (162 Da) (C) M3⁺ analysis for the m/z 289 daughter ion generating a fragmentation pattern consistent with that for 2-hydroxynaringenin (D).

and the synthesis of both flavonoid compounds requires the enzyme activities of flavonoid 3'-hydroxylase (F3'H). A family of F3'H genes have recently been characterized in sorghum, and the pathogen-inducible F3'H gene was expressed at minimal levels in infected BTx623 plants (Shih *et al.*, 2006). Hence, F3'H may represent a molecular component essential for anthracnose resistance in sorghum by generating those 3'-hydroxylated flavonoids that showed stronger *in vitro* inhibitory effects. Recent improvements in sorghum transformation efficiencies (Gao *et al.*, 2005; Wang *et al.*, 2007; Gurel *et al.*, 2009) may allow routine transgenic analysis of candidate genes (e.g. F3'H) for disease resistance and other useful agronomical traits in the near future.

Following the identification of flavones in infected sorghum seedlings, it was demonstrated that *SbFNSII* is a pathogen-inducible gene encoding a functional FNSII enzyme *in planta*. Although many cereal crops are known to accumulate flavone metabolites, *SbFNSII* is the first example of a functional FNSII gene reported in a monocot. Expression of *SbFNSII* was induced earlier in SC748-5 than in BTx623 following inoculation (Fig. 4), consistent with the more rapid accumulation of luteolin in the resistant plants (Fig. 2). In addition, *SbFNSII* is a new P450 member (CYP93G3) belonging to the CYP93G subfamily. Cytochrome P450 enzymes are known to participate in a diverse array of biosynthetic and degrada-

tive pathways in plants, animals, and fungi (Schuler and Werck-Reichhart, 2003). In flavonoid metabolism, they are involved in the biosynthesis of anthocyanins and tannins, flavones, and the leguminous isoflavonoid phytoalexins (Ayabe and Akashi, 2006), and members of the same subfamily usually catalyse the same metabolic reaction. For example, cinnamate 4-hydroxylase (CYP73A) provides *p*-coumaroyl-CoA which is a key substrate required for the formation of all flavonoids. In addition, the flavonoid B-ring hydroxylation patterns are determined by the activities of flavonoid 3',5'-hydroxylases (CYP75A) and F3'Hs (CYP75B). In the CYP93 family, isoflavone synthase (CYP93C) and dihydroxypterocarpan 6 α -hydroxylase (CYP93A) are legume-specific enzymes involved in the biosynthesis of isoflavonoids (Ayabe and Akashi, 2006). Furthermore, the CYP93B subfamily consists of the FNSII enzymes from different dicot families. Hence, the CYP93G subfamily potentially represents the FNSII enzymes in monocots or at least in Poaceae. In addition to *SbFNSII* (CYP93G3) and the rice members CYP93G1 and CYP93G2, a number of EST sequences (400–900 bp) in maize (FL159302), barley (BE215500, GH208016), wheat (BE426428, CV763338), and sugarcane (CA141841) with >70% sequence identity to *SbFNSII* could be retrieved from the NCBI databases by tblastn searches. It remains to be elucidated whether these P450 homologous sequences also encode proteins with FNSII activities.

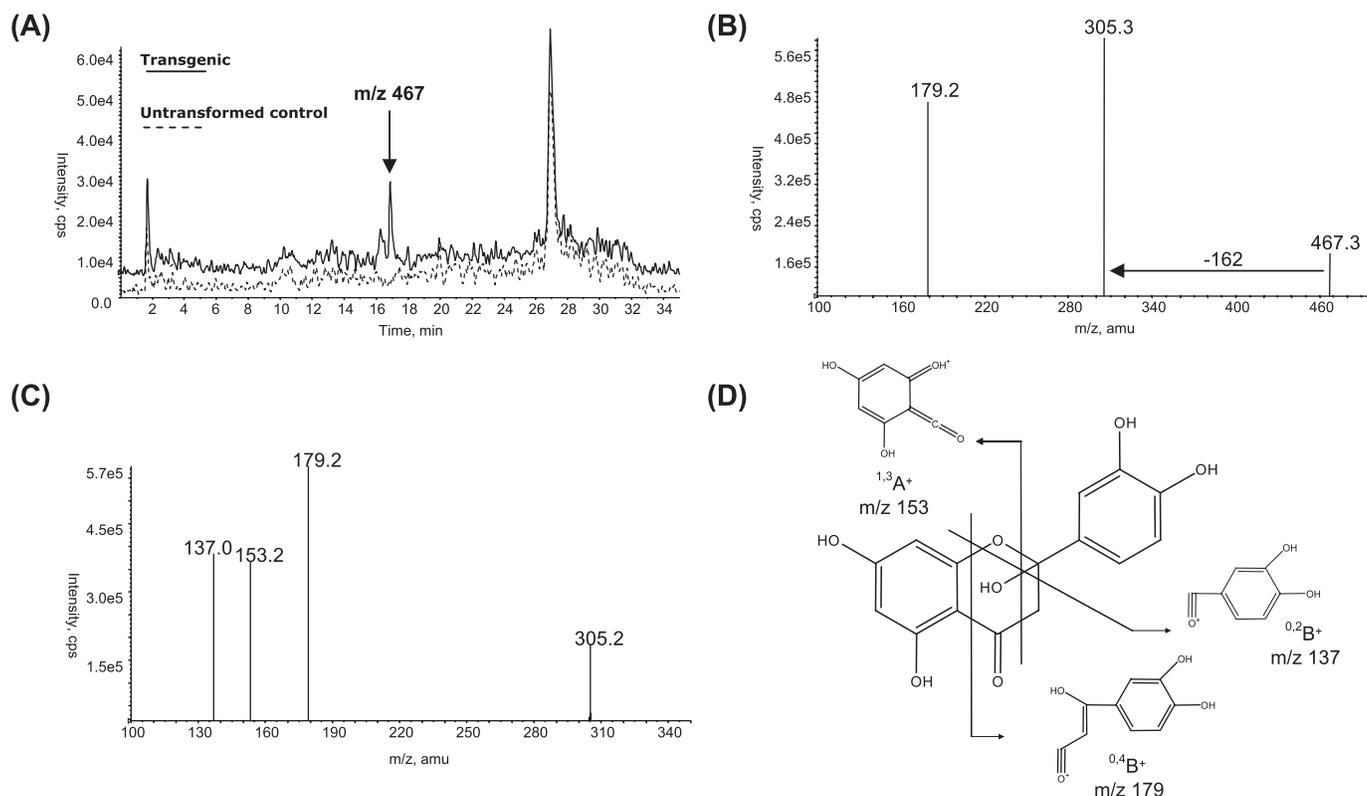


Fig. 8. LC-PIS detection of a 2-hydroxyeriodictyol *O*-hexoside in methanol extracts of transgenic *Arabidopsis tt6* plants overexpressing SbFNSII (A) Precursor ions of *m/z* 305 were scanned following LC separation. The most distinct peak (arrow) identified in the transgenic plant sample produced a major ion at *m/z* 467 following MS analysis (B) MS/MS spectrum of the *m/z* 467 ion indicating the loss of a hexosyl unit (162 Da) (C) M3³ analysis for the *m/z* 305 daughter ion generating a fragmentation pattern consistent with that for 2-hydroxyeriodictyol (D).

Table 1. List of flavone *O*-glycosides accumulated in transgenic *Arabidopsis*

The metabolites were identified in non-hydrolysed extracts by LC-PIS analysis in positive ionization mode. The identities of the [flavone + H]⁺ ions (in bold) had been confirmed by their MS³ spectra.

Precursor ion	Retention time (min)	MS/MS ions	Compound assignment
611	13.0, 16.9	449, 287	Luteolin di- <i>O</i> , <i>O</i> -hexosides
449	20.2, 22.9	287	Luteolin hexosides
595	13.2, 16.3	433, 271	Apigenin di- <i>O</i> , <i>O</i> -hexosides
433	17.2, 18.6, 22.3	271	Apigenin hexosides

In this study, the biochemical function of SbFNSII was established by transgenic analysis in *Arabidopsis*. Different flavonoid metabolites are known to accumulate in this model plant, including flavonols, proanthocyanidins, and anthocyanins (Winkel-Shirley, 2001). However, flavones are not present in almost all cruciferous plants (including *Arabidopsis*), consistent with the absence of FNSI- or FNSII-encoding sequences in the *Arabidopsis* genome (Martens and Mithöfer, 2005). Thus, *Arabidopsis* represents a useful system for *in planta* analysis of the FNS transgene without any background flavone accumulation. Recently the parsley FNSI gene was engineered in *Arabidopsis*, but flavone

accumulation occurred only after exogenous application of naringenin (Yun *et al.*, 2008). In contrast, all of the transgenic *Arabidopsis* lines were found to accumulate apigenin and luteolin *O*-glycosides constitutively (Table 1). The flavonoid pathway in *Arabidopsis* can be activated under nitrogen stress (Dong *et al.*, 2001), thus providing a range of substrates for the introduced flavonoid enzymes in the transgenic plants. Apparently the endogenous flavanones (naringenin and eriodictyol) were used as the physiological substrates for SbFNSII. This was more evident by the higher levels of flavone accumulation in the transgenic *tt6* plants (Fig. 6C) in which the competing enzyme F3H is defective.

While most FNSIIs converted flavanones to flavones directly, the licorice and *M. truncatula* FNSIIs instead produced 2-hydroxyflavanones which could be converted to flavones after acid treatments (Akashi *et al.*, 1998; Zhang *et al.*, 2007). The detection of 2-hydroxyflavanone *O*-glycosides in the present transgenic *Arabidopsis* lines strongly suggested that SbFNSII shares a similar catalytic mechanism with the two legume FNSII enzymes. In the transgenic *Arabidopsis* plants, an unknown endogenous enzyme may cause the dehydration of some 2-hydroxyflavanones to flavones. Such conversion was observed in transformed yeast cells expressing an FNSII from *M. truncatula* (Zhang *et al.*, 2007). The different flavones and 2-hydroxyflavanones accumulated as a result of the SbFNSII activities in

the transgenic lines were apparently modified by the endogenous flavonoid *O*-glycosyltransferases. On the other hand, since no 2-hydroxyflavanones or their derivatives were detected in the infected sorghum samples by LC-MS/MS analysis using the IDA protocol (data not shown), an effective enzyme that allowed the complete conversion of 2-hydroxyflavanones to flavone is likely to be present in sorghum. In fact, 2-hydroxyflavanones may serve as a common intermediate for the formation of flavone aglycones and flavone *C*-glycosides in cereal plants. Recently, a rice recombinant enzyme was demonstrated to *C*-glucosylate 2-hydroxyflavanones preferentially, but with negligible activities toward flavones (Brazier-Hicks et al., 2009). In addition, dehydratase activities converting 2-hydroxyflavanone *C*-glycosides to flavone *C*-glucosides were identified in both rice and wheat protein extracts (Brazier-Hicks et al., 2009). Apparently a dehydratase enzyme specific for the 2-hydroxyflavanone aglycones is instead required for the inducible flavone aglycone formation in infected sorghum seedlings. Further investigations to understand the complete enzymology of flavone biosynthesis in sorghum will be largely facilitated by the recent completion of the sorghum genome sequence (Sasaki and Antonio, 2009).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Sequence alignment of CYP93G and selected CYP93B proteins by the ClustalW method. The putative proline hinge region (P), oxygen-binding pocket (O), and haem-binding motif (H) are boxed (SbFNSII = CYP93G3).

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