Tree Genetics & Genomes (2013) 9:85–92
DOI 10.1007/s11295-012-0536-1

ORIGINAL PAPER

Molecular cloning and characterization of flavonol synthase in *Acacia confusa*

Hwei-Choong Toh · Sheng-Yang Wang · Shang-Tzen Chang · Fang-Hua Chu

Received: 8 March 2012 / Revised: 25 May 2012 / Accepted: 29 May 2012 / Published online: 17 June 2012

Abstract Flavonoids are abundant in the leaves, flowers, branches, bark, and heartwood of *Acacia confusa* Merr. (Leguminosae), and the extracts have been found to have strong antioxidant activities. Flavonols are the major class of flavonoids found in *A. confusa*. The full-length gene (designated as *AcFLS*) contains an open reading frame of 996 nucleotides encoding 331 amino acid residues with a predicted molecular weight of 38 kDa and pI of 5.7. *AcFLS* mRNAs were abundant in the seedlings leaves and the flowers. Wounding by cutting leaves induced maximum *AcFLS* mRNA accumulation 6-12 h after treatment. AcFLS could enzymatically transform dihydromyricetin, dihydroquercetin, and naringenin into the flavonols myricetin, quercetin, and kaempferol, respectively. AcFLS could bind these three substrates with similar affinity.

Keywords *Acacia confusa* · Biosynthesis · Flavonoids · Flavonols · Kaempferol · Myricetin · Quercetin · Protein structure modeling

Introduction

Flavonoids are a well-known class of polyphenols widely distributed in plants and characterized by their C6-C3-C6 skeleton. Flavonoids can be classified into eight subgroups: flavanones, dihydroflavonols, flavones, flavonols, flavan-3,4-diols, flavan-3-ols, anthocyanidins, and proanthocyanidins, depending on the oxidation state and substitution pattern of their C-ring structure. The isoflavonoids are also considered to be a class of flavonoids (Hegnauer and Gpayer-Barkmeijer 1993). Flavonols are the most abundant of all the flavonoids in plants. Many physiological functions, including regulation of auxin transport, modulation of flower color, protection against ultraviolet radiation, prevention against microorganism and pest invasion, and signaling interactions with insects and microbes have been attributed to flavonols (Bohm et al. 1998; Harborne and Williams 2000; Winkel-Shirley 2001). Some pharmacological attributes of flavonols, such as anti-angiogenic, anti-inflammatory, antioxidative, cardioprotective, or neuroprotective properties and even chemoprevention activity have been studied for possible benefits to human health (Harborne and Williams 2000; Ross and Kasum 2002; Havsteen 2002). In plants, glycosylated flavonols and methylated flavonols are commonly stored in vacuoles and in the cell wall, respectively (Grotewold 2005; Yazaki 2005).

There are three common structures of flavonols, including myricetin, quercetin, and kaempferol, which can be converted from the dihydroflavonols dihydrokaempferol, dihydroquercetin or dihydromyricetin, respectively, by flavonol synthase (FLS). FLS is a non-heme ferrous enzyme that belongs to the 2-oxoglutarate-dependent dioxygenase (2-ODD) superfamily and requires ascorbic acid for full activity (Holton et al. 1993; Prescott and John 1996). FLS can determine the flower color in soybean (*Glycine max*), a single-base deletion in the FLS gene was found to change the flower color from purple to magenta (Takahashi et al. 2007). The induction of

Communicated by R. Sederoff

H.-C. Toh · S.-T. Chang · F.-H. Chu (✉)
School of Forestry and Resource Conservation,
National Taiwan University,
Taipei 106, Taiwan
e-mail: fhchu@ntu.edu.tw

S.-Y. Wang
Department of Forestry, National Chung-Hsing University,
Taichung 402, Taiwan

FLS by ultraviolet-B irradiation is also associated with the accumulation of flavonols in soybean (Kim et al. 2008). As FLS converts both flavanones and dihydroflavonols to the related flavonols, it is usually classified as a bifunctional dioxygenase and is defined as having hydroxylation and desaturation activities (Lukačin et al. 2003; Prescott et al. 2002).

Acacia confusa belongs to the family Leguminosae. The leaves, flowers, branches, bark, and heartwood of *A. confusa* are all rich in flavonoids, and extracts possess strong antioxidant activities (Hsieh and Chang 2010). The major flavonoid subgroup found in *A. confusa* is the flavonols, including kaempferol, quercetin, myricetin, and melanoxetin (Wu et al. 2008). Melanoxetin is hydroxylated at the C7 and C8 positions of the A-ring rather than at the C5 and C7 positions of others (Wu et al. 2005, 2008). As a result, the study of the flavonoid biosynthetic pathway, especially flavonol biosynthesis in *A. confusa*, becomes important and valuable. However, research related to the flavonoids biosynthetic pathway in this species, and even in the genus *Acacia*, is incomplete.

To elucidate the functions of the flavonol synthase that catalyzes the biosynthesis of flavonols in *A. confusa*, we cloned and characterized a putative flavonol synthase gene, designated as *AcFLS*. We have characterized this flavonol synthase through sequence analysis, phylogenetic analysis, and protein structure modeling. Using high-performance liquid chromatography (HPLC), we have identified the bifunctional activity of this flavonol synthase, which converts the flavanone (naringenin) and the dihydroflavonols (dihydromyricetin and dihydroquercetin) into the corresponding flavonol products. AcFLS binds the three substrates with similar affinity. *AcFLS* is expressed in all tissues, with highest expression in the leaves of seedlings and flowers of mature trees.

Materials and methods

Plant materials and wounding treatment

Seedlings (1.5- and 3-year-old) were grown in the nursery of the School of Forestry and Resource Conservation, National Taiwan University. Multiple collections of leaves, flowers, branches, bark, and sapwood were collected from 30-year-old trees in June 2011, frozen on dry ice, and then stored at −80°C. For local wounding treatment, 3-year-old seedlings were used—the first 1 cm of the leaf vein from each leaf tip was measured, and then the leaf tip was trimmed off perpendicular to the vein. All seedlings were treated under normal greenhouse conditions, and all experiments were performed in triplicate.

Cloning of *AcFLS* gene from *A. confusa*

Total RNA was extracted from the leaves of *A. confusa*, which were frozen in liquid nitrogen and stored at −80°C. cDNA was synthesized from 2 μg of total RNA using Superscript™ III Reverse Transcriptase (Invitrogen) with oligo-dT as a primer. Full-length *AcFLS* was cloned in three steps. First, the cDNA products were used as a template for quantitative RT-PCR amplification. Degenerate primers were designed to generate a unique 377-bp fragment (forward primer: 5'-TGGCCYAAKAACCCYYCTTCWTAC-3', reverse primer: 5'-TTGTACTTRCCATTGCTHAGDAYCTC-3'). The amplification conditions were as follows—1 min denaturation at 94°C; 30 cycles at 94°C for 30 s, 42°C for 40 s, and 72°C for 1 min, followed by 10 min at 72°C. Next, the 5'-end and 3'-end of *AcFLS* cDNA were synthesized by the BD SRMAT™ RACE cDNA amplification kit (Clontech, CA 94043). The putative full-length sequences were assembled using BLAST (bl2seq) (http://www.ncbi.nlm.nih.gov) from the sequence of these DNA fragments. Finally, the full-length DNA fragment of this gene was amplified by p*fu* DNA polymerase using a pair of primers (forward primer: 5'-CATACATGGAGGT TGAAAGAGTGCAGTGCTTG-3'; reverse primer: 5'-TCACTGAGGAAGCTTATTGAATTTGCGGTG-3') with start and stop codons. PCR products were separated on 1.2% (*w/v*) agarose gels, cloned into pGEM T–Easy vectors, and then sequenced.

Protein sequence comparison and phylogenetic analysis

For protein sequence comparison and analysis, the deduced amino acid sequence and open reading frame (ORF) encoded by *AcFLS* were analyzed, and a sequence comparison was conducted with BLAST. From among the best BLAST hits, the published genes were selected for comparison. A phylogenetic tree was constructed by aligning the protein sequences of 2-ODD superfamily genes, including flavonol synthase (FLS), anthocyanidin synthase (AS), and flavanone 3-hydroxylase (F3H) using ClustalW2. Analysis was conducted using the neighbor-joining method and visualized with the Tree View program. The 2-ODD superfamily sequences were FLS: *Solanum tuberosum* (ACN81826), *Camellia sinensis* (ABA54917), *Ricinus communis* (XP_002522207), *Allium cepa* (AAO63023), *Rudbeckia hirta* (ABN79672), *Nicotiana tabacum* (ABE28017), *Vitis vinifera* (BAE75806); AS: *Eustoma grandiflorum* (BAJ08932), *Solenostemon scutellarioides* (ABP57081), *Theobroma cacao* (ADD51355), *Brassica oleracea var. capitata* (AAO73440), *Matthiola incana* (AAB82287); F3H: *S. tuberosum* (AAM48289), *Malus x domestica* (AAX89397), *Arabidopsis thaliana* (CAD37988), *G. max* (AAU06217), *Petroselinum crispum* (AAP57394), *Oryza sativa japonica* (AAL58118),

Arabidopsis lyrata subsp. lyrata (XP_002871353) and *A. thaliana* (CAP09035).

Expression of AcFLS in *E. coli*

To clone *AcFLS* into an expression vector, its ORF was amplified with primers containing the *Nde*I and *Xho*I sites (upstream primer: 5'-GGAATTCCATATGGAGGTTGAAAGAGTGCAGTGC-3', downstream primer: 5'-CCGCTCGAGCTGAGGAAGCTTATTGAATTTGCG-3') and cloned into pET-21a(+), yielding His-tagged AcFLS (pET-AcFLS) protein. The recombinant plasmid was introduced into *E. coli* (BL21-CodonPlus-RIL) using heat shock. The transformants were grown at 37°C in 250 ml Luria Bertani medium (100 ppm ampicillin) until the optical density (OD600) reached 0.4–0.7. For induction, isopropyl-β-D-thio-galactoside was added to a final concentration of 0.1 mM, and the cells were further cultured at 20°C for 20 h. Then, the cells were harvested and resuspended in 15 mL 20 mM imidazole sodium phosphate buffer (0.02 M Na_2HPO_4, 0.02 M NaH_2PO_4, 0.5 M NaCl). The cells were lysed by sonication with an ultrasonic processor at 4°C, and centrifuged at 8,500 rpm for 20 min. His-tagged *AcFLS* was purified by using Ni Sepharose 6 Fast Flow with 100 mM imidazole sodium phosphate buffer. In addition, AcFLS protein was concentrated by Amicon Ultra and resuspended in storage buffer [0.1 M NaH_2PO_4 (pH 6.8), 10% glycerol]. The final concentration of purified AcFLS was measured using the Bradford assay.

Assay of AcFLS

The FLS activity assay was based on that described in Ferreyra et al. (2010). Each 250 μl reaction buffer contained 10 mM α-ketoglutaric acid, 10 mM ascorbic acid, 0.25 mM ferrous sulfate, 100 mM NaH_2PO_4 (pH 6.8), and 0.1 mM substrate. The reaction mixtures containing 75 μg AcFLS were incubated at 25°C in open vials with gentle shaking. After 60 min, the mixtures were extracted with ethyl acetate

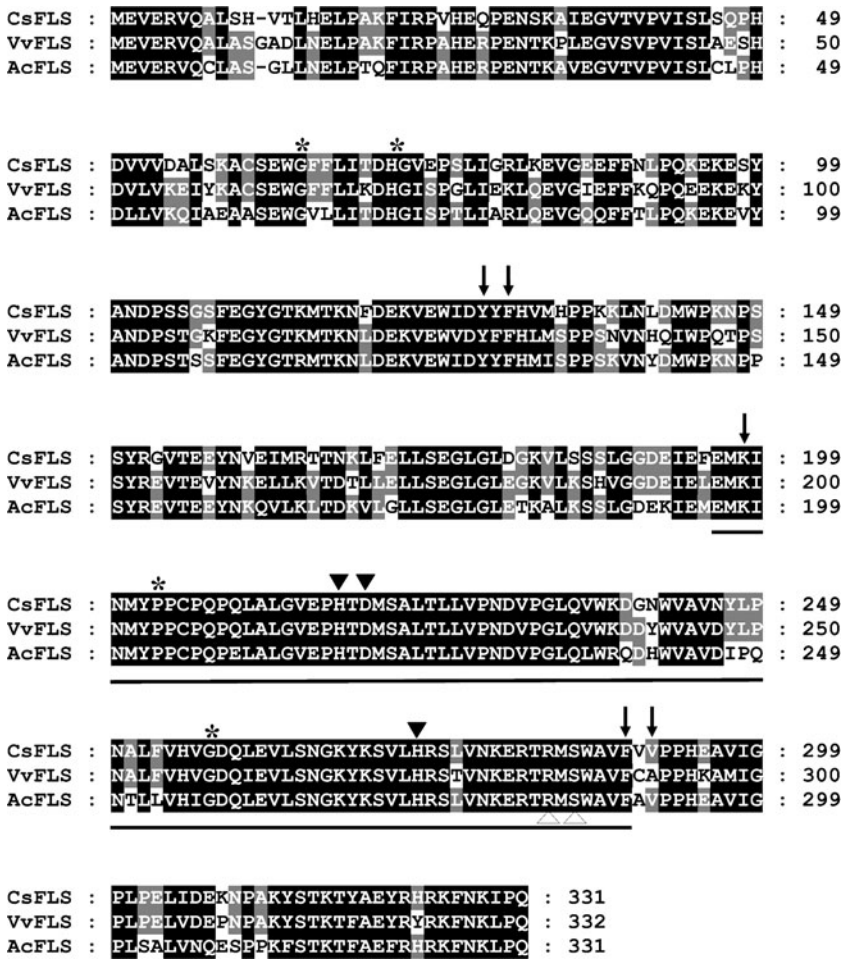

Fig. 1 Amino acid sequence alignment of FLSs between *A. confusa*, *V. vinifera,* and *C. sinensis*. The sequence alignment indicates the amino acid residues involved in iron binding (*filled inverted triangle*), 2-oxoglutarate binding (△) and 2-ODD superfamily conserved residues (*asterisk*). The superimposition of AcFLS tertiary structures upon the template AtANS identified five potential substrate binding residues (*arrows*). The 2-ODD superfamily gene conserved region is *underlined*

and dried with nitrogen gas. The flavonoids were resuspended in 50 μl of 100% methanol, which were then analyzed by HPLC (Agilent Technologies 1200 Series) with a Phenomenex Luna 5 μC18 reverse phase column (250×4.60 mm). The injection volume was 10 μl. The binary mobile phase consisted of methanol (A) and dH_2O (B) (0–5 min, A/ B 20%:80%; 5–30 min, A/B 20%:80%; 30–50 min, A/B 100%:0%). The flow rate was 1 mL/min. The detector was initially set at 280 nm and changed to 368 nm after 20 min. Standard dihydromyricetin, dihydroquercetin, naringenin, myricetin, quercetin, and kaempferol were purchased from Sigma-Aldrich.

Protein structure modeling

Homology models were generated for AcFLS based on the crystal structure of *Arabidopsis* anthocyanidin synthase (AtANS) (Wilmouth et al. 2002). The models were generated using the Web-based SWISS-MODEL server homology modeling pipeline (Schwede et al. 2003). The crystal structure of AtANS (PDB ID: 1GP5) in complex with Fe^{2+}, 2-oxoglutarate, and dihydroquercetin served as the template structure. The AcFLS model was visualized and analyzed using Swiss-PDB Viewer DeepView software v.4.0.1 and rendered in solid 3D. The DeepView iterative magic fit function was used in structural comparisons by aligning the isoform homology models.

Results

Protein sequence analysis of AcFLS

The full-length AcFLS (GenBank accession number: JN812062) contains an ORF of 996 nucleotides encoding 331 amino acid residues with a predicted molecular weight of 38 kDa and pI of 5.7. The putative amino acid sequences deduced from the ORF show 74% and 73% sequence identity with the flavonol synthases from *V. vinifera* (BAE75810) and *C. sinensis* (AMB88786), respectively. Furthermore, AcFLS contains several consensus elements, including a conserved domain typical of the 2OG-Fe(II) dioxygenase superfamily, a ferrous iron (II) binding residue motif (the $HxDx_nH$ motif consisting of His_{217}, Asp_{219}, and His_{273}), and the putative 2-oxoglutarate binding residue motif (the RxS motif consisting of Arg_{283} and Ser_{285}). Several amino acid residues conserved in the 2-ODD superfamily (Gly_{64}, His_{71}, Pro_{203}, and Gly_{257}) and the residues corresponding to substrate binding (Tyr_{128}, Phe_{130}, Lys_{198}, Phe_{289}, and Val_{291}) are also present (Fig. 1). Phylogenetic analysis of AcFLS with other 2-ODD superfamily genes indicated that FLS of *A. confusa* is closely related to FLS from *V. vinifera* (Fig. 2). Among the 2-ODD superfamily genes, AcFLS was found to have sequence similarity of 49%–74% with other FLSs and to have 44%–45% and 28%–33% sequence identity to ANSs and F3Hs, respectively.

Protein structure modeling of AcFLS

Modeling of AcFLS was based on the crystal structure of AtANS (Wilmouth et al. 2002) and built with the Web-based SWISS-MODEL server (Schwede et al. 2003). The degree of similarity of the overall structures of AcFLS and AtANS was 43% (*E*-value<1). Noticeably, the AcFLS model exhibits a 2-ODD superfamily conserved domain. The substrate dihydroquercetin (DHQ) binding site (Tyr_{128}, Phe_{130}, Lys_{198}, Phe_{289}, Val_{291}), the 2-OG binding RxS motif, and the Fe^{2+} binding $HxDx_nH$ motif are found in the conserved region, which constitutes the active site of AcFLS (Fig. 3).

Functional analysis of AcFLS

After purification, about 625 μg of AcFLS protein was obtained from 250 ml of bacterial culture. Each 250 μl reaction mixture (dihydromyricetin, dihydroquercetin, or naringenin was used as substrate) contained 75 μg AcFLS and was incubated at 25°C for 60 min. An equal volume of storage buffer replaced the protein in the control groups. HPLC showed that AcFLS has the ability to convert substrates dihydromyricetin, dihydroquercetin, and naringenin to the corresponding flavonols 0.154 μM/μg protein/min of myrice-

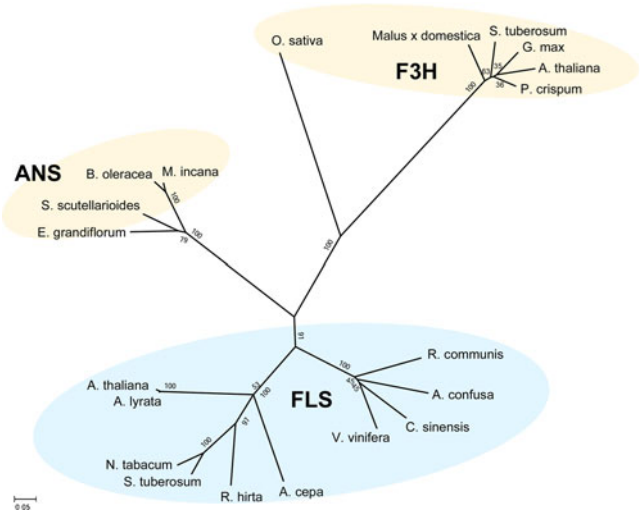

Fig. 2 Phylogenetic tree analysis of AcFLS and other 2-ODD superfamily genes. The analysis was performed with ClustalW based on the neighbor-joining method

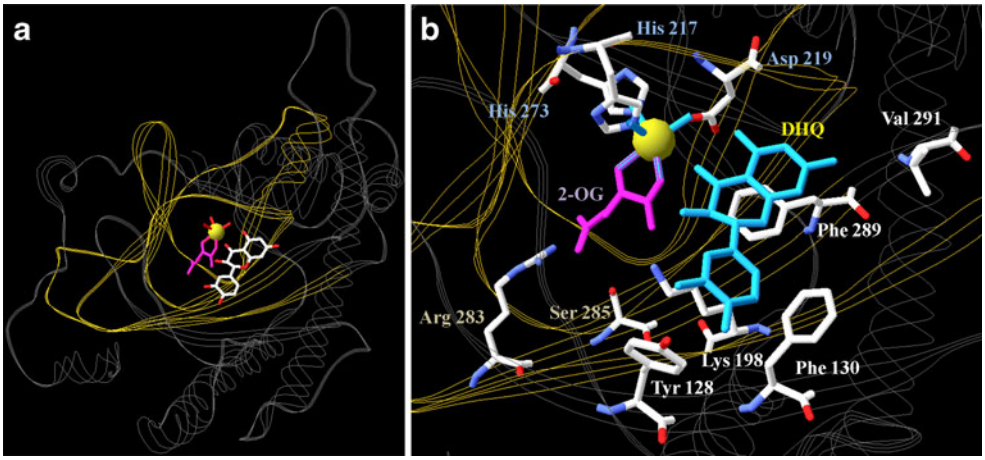

Fig. 3 Homology model of AcFLS protein structure (**a**) and active site prediction (**b**) based on AtANS (Wilmouth et al. 2002). The conserved domain of the 2-ODD superfamily is *highlighted* in *yellow*. Furthermore, important active site residues are represented as *bonds*. The substrate dihydroquercetin (DHQ, *light blue bond*), cofactor 2-OG (*purple bond*), and Fe^{2+} (*yellow sphere*) are also shown in the active site

tin, 0.150 μM/μg protein/min of quercetin, and 0.163 μM/μg protein/min of kaempferol, respectively (Fig. 4).

Transcript abundance of *AcFLS* in various tissues and following wounding treatment

To obtain the general pattern of *AcFLS* mRNA expressed in *A. confusa*, transcript abundance was estimated in tissues harvested from 1.5-year-old seedlings and 30-year-old trees using RT-PCR. *AcFLS* shows the highest relative transcript abundance in leaves of seedlings and flowers of mature trees (Fig. 5). Wounding experiments on leaves of 3-year-old seedlings indicated maximum *AcFLS* mRNA accumulation approximately 6–12 h after treatment (Fig. 6).

Discussion

Protein sequence analysis of AcFLS

This study has identified *A. confusa* flavonol synthase (AcFLS) as a 2-ODD superfamily gene, which contains a conserved domain of the 2-ODD superfamily, ferrous iron(II) binding residues (the $HxDx_nH$ motif), and 2-OG binding residues (the

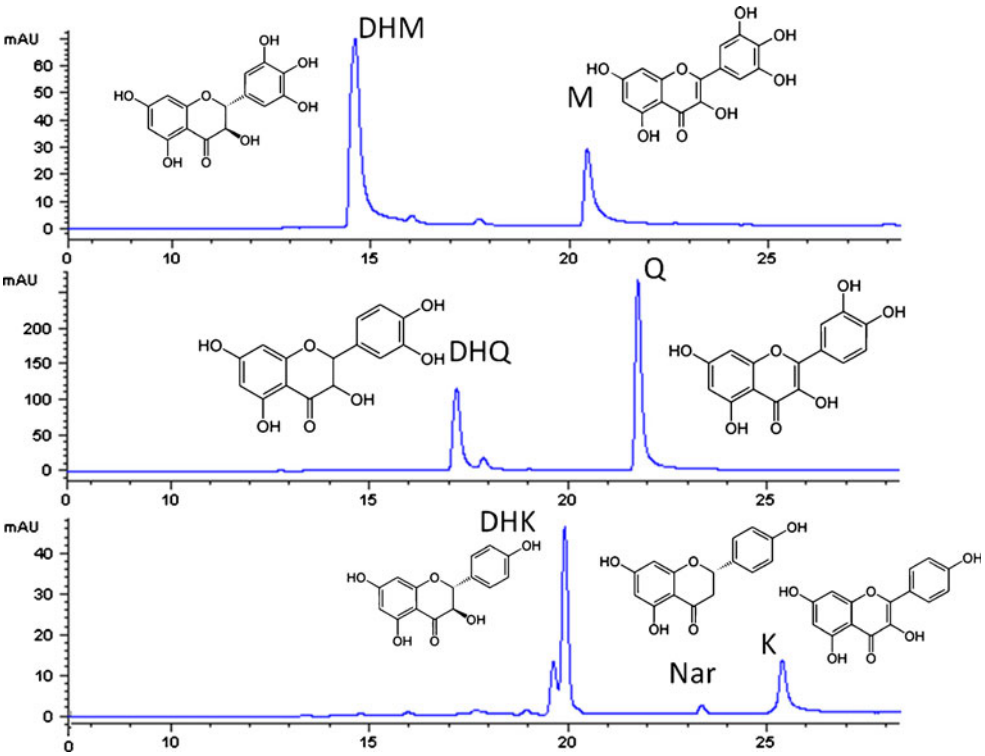

Fig. 4 AcFLS converts dihydroflavonols (*DHM*, *DHQ*) and flavanone (*Nar*) substrates to flavonols. *DHM*, *DHQ* was detected by UV absorbance at 280 nm, whereas *Nar*, *DHK*, *M*, and *Q* were detected at 368 nm. *DHM*: dihydromyricetin; *M*: myricetin; *DHQ*: dihydroquercetin; *Q*: quercetin; *DHK*: dihyrokaempferol; *N*: naringenin; *K*: kaempferol. Standard and blank data were not shown

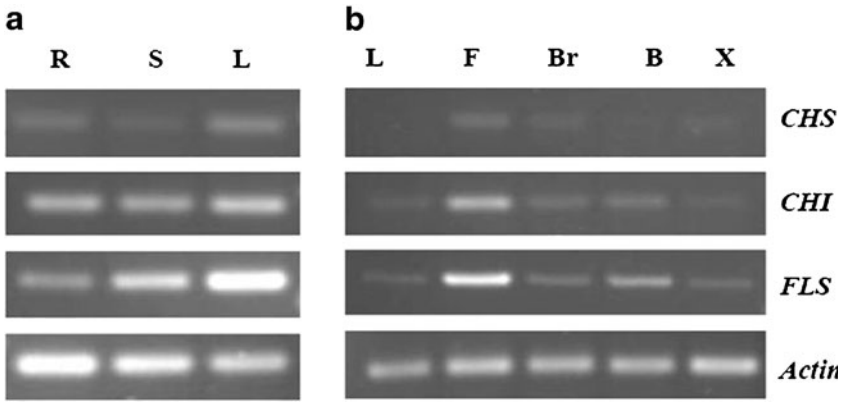

Fig. 5 RT-PCR analysis of *AcFLS* gene expression in various parts of *A. confusa*. **a** 1.5-year-old seedling, **b** 35-year-old tree. Actin was used as internal control. *R*: root; *S*: stem; *L*: leaf, *F*: flower; *Br*: branch; *B*: bark; *X*: xylem

RxS motif). The four residues (Gly_{64}, His_{71}, Pro_{203}, Gly_{257}) required for the proper folding of the flavonol synthase polypeptide were also found. In addition, five amino acid residues in *Arabidopsis*, His_{132}, Phe_{134}, Lys_{202}, Phe_{293}, and Glu_{295} were required for substrate binding (Chua et al. 2008). The corresponding residues found in AcFLS were Tyr_{128}, Phe_{130}, Lys_{198}, Phe_{289}, and Val_{291}, respectively. According to the results of Chua et al. (2008), the $H_{132}F$ mutant exhibited approximately 20% higher activity than wild-type *Arabidopsis*. The position corresponding to His_{132} in AcFLS is Tyr_{128}, which could be regarded as a similar replacement to Phe that may increase the activity of the enzyme. The other residues changed in the substrate binding sites cause catalytic activity reduction (Chua et al. 2008). Furthermore, in the phylogenetic tree analysis, AcFLS is located in the flavonol synthase group, in a subgroup with *R. communis*, *C. sinensis*, and *Vitis vinefera*.

Protein structure modeling of AcFLS

FLS and ANS both belong to the 2-ODD superfamily and are similar in sequence and function. ANS has been proven to react with the same substrates as FLS in vitro and to produce the same corresponding products. The crystal structure of AtANS was studied by Wilmouth et al. (2002). As no protein crystal structure has yet been obtained for FLS, to identify the amino acids responsible for the catalytic behavior of AcFLS, models were generated for AcFLS based on the crystal structure of AtANS (Wilmouth et al. 2002). The main chain of AcFLS contains eight β strands, which form a hydrophobic cavity, and a jellyroll fold. The Val_{291} is one of the substrate DHQ binding residues of AcFLS, and the corresponding residue in AtFLS is Glu_{295} (corresponding position in AtANS is Glu_{306}). AtFLS exhibited drastically reduced (less than 10% of wild-type activity) when Glu_{295} was replaced with the hydrophobic amino acid, leucine. Athough Val_{291} is also a hydrophobic amino acid, our study shows that AcFLS still retained the ability to transform 67.0–72.8% substrates to corresponding flavonols. Protein crystal structure analysis showed that Glu at position 196 could replace the function of Val_{291}, which could not interact with the substrate DHQ. A hydrogen bond interaction between the A ring C-7 hydroxyl group and the side chain carboxyl of Glu_{196} (3.30 and 3.52 Å) in DHQ allows AcFLS to maintain its activity.

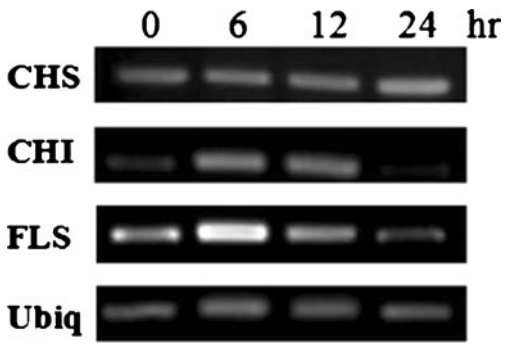

Fig. 6 *AcFLS* mRNA accumulation following wound treatment. Changes in AcFLS mRNA level within the leaves were determined over time following wounding at 0, 6, 12, and 24 h

The structure of two major flavonols (melanoxetin or transilitin) found in *A. confusa* heartwood are different from common flavonoids. Normally, the hydroxyl group is located at C-5 and C-7 position on the A-ring of flavonoids; however, in melanoxetin and transilitin, the A ring hydroxyl group is positioned at C-7 and C-8. Substrates of this kind of flavonol are bound via a hydrogen bond interaction between the A ring C-7, or a closer bond distance, C-8 hydroxyl group, and the side chain carboxyl of Glu_{196}. The range of donor–receptor distance for the hydrogen bonds we propose are smaller than 3.5 Å (Fig. 7) (Baker and Hubbard 1984).

Expression pattern of *AcFLS* in various tissues and after wounding treatment

In 1.5-year-old seedlings and 30-year-old trees, the general pattern of *AcFLS* mRNA was most abundant in the leaves and the flowers, consistent with results in *A. thaliana* (Owens et al. 2008). Transcript abundance of FLS in *V. vinifera* was found to vary with developmental stage with the highest level found in the leaves, flowers, and fruit skin (Fujita et al. 2006).

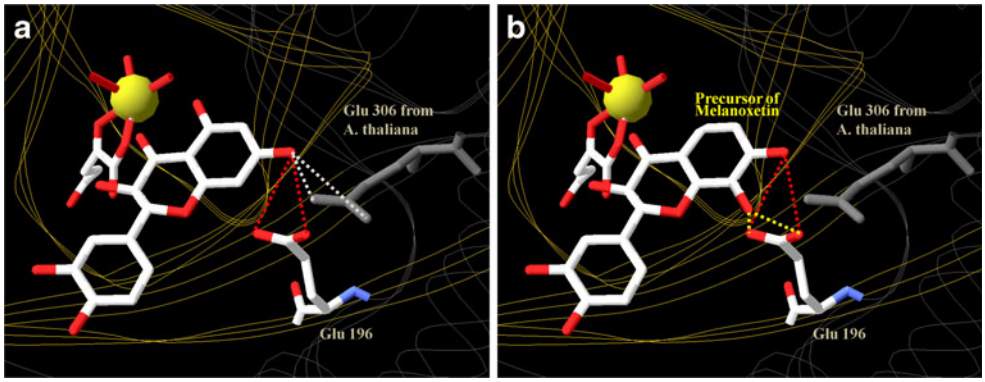

Fig. 7 Active site of AcFLS. **a** Glu_{196} might replace Val_{219} to interact with C-7 of substrate DHQ via a hydrogen bond (showed as *red dashed lines*). The *grey bond* indicates Glu_{306} from AtANS. (Glu_{306} is related to substrate binding; the corresponding residue in AtFLS is Glu_{295}). Val_{219} is the related residue in AcFLS. The *grey dashed line* shows a hydrogen bond between Glu_{306} of AtANS and DHQ. **b** As the substrate is changed to the precursor of melanoxetin ((−)−2,3-*trans*-3',4',7,8-tetrahydroxydihydroflavonol), Glu_{196} could also interact with C-7 or C-8 of this substrate (*red* and *yellow dashed lines*). DHQ, 2-OG, and important active site residues are showed as *bonds*, and Fe^{2+} ion is presented as a *yellow sphere*. *DHQ*: dihydroquercetin, *2-OG*: 2-oxologlutarate

Flavonols exhibiting remarkably high antioxidant activity are known to be abundant in the leaves, flowers, branches, and heartwood of *A. confusa* (Hsieh and Chang 2010). We found that *AcFLS* is expressed in the leaves, flowers, branches, bark, and sapwood. A previous study reported that the antioxidants extracted from *A. confusa* bark are not flavonoids, but hydroxybenzoic acid; however, the bark contains tannin, a flavonol polymer (Tung et al. 2009). Our bark sample contained some vascular cambium that was too thin to split, so we assume that the flavonoids in *A. confusa* heartwood could be produced in vascular cambium and stored in sapwood. Flavonols regulate flower color or fruit skin color in most of the plants (Takahashi et al. 2007; Fujita et al. 2006). *AcFLS* showed its highest abundance in *A. confusa* flowers. The deep yellow color in the *A. confusa* flower may be controlled at least in part by *AcFLS*.

Many reports have indicated that FLS responds to various light intensities, and certain chemicals, and can be rapidly activated by stresses such as UV-B radiation, pathogen infection, and herbivory (Ferreyra et al. 2010; Owens et al. 2008). In the wounding treatment, the maximum *AcFLS* mRNA level occurred approximately 6 h after wounding, and the normal transcript abundance was restored at 12 h. In poplar (*Populus* spp.), however, *FLS* did not respond to wounding treatment, but did respond to high light intensity (Mellway et al. 2009). We therefore conclude that *FLS* exhibits different expression patterns under stress in different plants.

Acknowledgments We thank the Council of Agriculture Executive Yuan of Taiwan for financial support.

References

Baker EN, Hubbard RE (1984) Hydrogen bonding in globular proteins. Prog Biophys Mol Biol 44:97–139

Bohm H, Boeing H, Hempel J, Raab B, Kroke A (1998) Flavonols, flavones and anthocyanins as native antioxidants of food and their possible role in the prevention of chronic diseases. Z Ernahrungswiss 37:147–163

Chua CS, Biermann D, Goo KS, Sim TS (2008) Elucidation of active site residues of *Arabidopsis thaliana* flavonol synthase provides a molecular platform for engineering flavonols. Phytochemistry 69:66–75

Ferreyra MLF, Rius S, Emiliani J, Pourcel L, Feller A, Morohashi K, Casati P, Grotewold E (2010) Cloning and characterization of a UV-B-inducible maize flavonol synthase. Plant J 62:77–91

Fujita A, Goto-Yamamoto N, Aramaki I, Hashizume K (2006) Organ-specific transcription of putative flavonol synthase genes of grapevine and effects of plant hormones and shading on flavonol biosynthesis in grape berry skins. Biosci Biotech Biochem 70:632–638

Grotewold E (2005) Plant metabolic diversity: a regulatory perspective. Trends Plant Sci 10:57–62

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992. Phytochemistry 55:481–504

Havsteen BH (2002) The biochemistry and medical significance of the flavonoids. Pharmacol Therapeut 96:67–202

Hegnauer R, Gpayer-Barkmeijer RJ (1993) Relevance of seed polysaccharides and flavonoids for the classification of the Leguminosae: a chemotaxonomic approach. Phytochemistry 34:3–16

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu CY, Farcy E, Stevenson TW, Cornish EC (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature 366:276–279

Hsieh CY, Chang ST (2010) Antioxidant activities and xanthine oxidase inhibitory effects of phenolic phytochemicals from *Acacia confusa* twigs and branches. J Agric Food Chem 58:1578–1583

Kim BG, Kim JH, Kim J, Lee C, Ahn JH (2008) Accumulation of flavonols in response to ultraviolet-B irradiation in soybean is related to induction of flavanone 3-beta-hydroxylase and flavonol synthase. Mol Cells 25:247–252

Lukačin R, Wellmann F, Britsch L, Martens S, Matern U (2003) Flavonol synthase from *Citrus unshiu* is a bifunctional dioxygenase. Phytochemistry 62:287–292

Mellway RD, Tran LT, Prouse MB, Campbell MM, Constabel CP (2009) The Wound-, pathogen-, and ultraviolet B-responsive MYB134 gene encodes an R2R3 MYB transcription factor that regulates proanthocyanidin synthesis in poplar. Plant Physiol 150:924–941

Owens DK, Alerding AB, Crosby KC, Bandara AB, Westwood JH, Winkel BSJ (2008) Functional analysis of a predicted flavonol synthase gene family in *Arabidopsis*. Plant Physiol 147:1046–1061

Prescott AG, John P (1996) Dioxygenases: molecular structure and role in plant metabolism. Annu Rev Plant Biol 47:245–271

Prescott AG, Stamford NPJ, Wheelera G, Firmina JL (2002) In vitro properties of a recombinant flavonol synthase from *Arabidopsis thaliana*. Phytochemistry 60:589–593

Ross JA, Kasum CM (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. Annu Rev Nutr 22:19–34

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res 31:3381–3385

Takahashi R, Githiri SM, Hatayama K, Dubouzet EG, Shimada N, Aoki T, Ayabe S, Iwashina T, Toda K, Matsumura H (2007) A single-base deletion in soybean flavonol synthase gene is associated with magenta flower color. Plant Mol Biol 63:125–135

Tung YT, Wu JH, Huang CY, Kuo YH, Chang ST (2009) Antioxidant activities and phytochemical characteristics of extracts from *Acacia confusa* bark. Bioresour Technol 100:509–514

Wilmouth RC, Turnbull JJ, Welford RWD, Clifton IJ, Prescott AG, Schofield CJ (2002) Structure and mechanism of anthocyanidin synthase from *Arabidopsis thaliana*. Structure 10:93–103

Winkel-Shirley B (2001) Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126:485–493

Wu JH, Tung YT, Wang SY, Shyur LF, Kuo YH, Chang ST (2005) Phenolic antioxidants from the heartwood of *Acacia confusa*. J Agric Food Chem 53:5917–5921

Wu JH, Huang CY, Tung YT, Chang ST (2008) Online RP-HPLC-DPPH screening method for detection of radical-scavenging phytochemicals from flowers of *Acacia confusa*. J Agric Food Chem 56:328–332

Yazaki K (2005) Transporters of secondary metabolites. Curr Opin Plant Biol 8:301–307