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RESEARCH ARTICLE

Prediction of C-glycosylated apigenin (vitexin) biosynthesis in *Ficus deltoidea* based on plant proteins identified by LC-MS/MS

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BACKGROUND: Plant secondary metabolites act as defence molecules to protect plants from biotic and abiotic stresses. In particular, C-glycosylated flavonoids are more stable and reactive than their O-glycosylated counterparts. Therefore, vitexin (apigenin 8-C glucoside) present in *Ficus deltoidea* is well-known for its antioxidant, anti-inflammatory, and antidiabetic properties.
METHODS: Phenol based extraction was used to extract proteins (0.05% yield) with less plant pigments. This can be seen from clear protein bands in gel electrophoresis. In-gel trypsin digestion was subsequently carried out and analysed for the presence of peptides by LC-MS/MS.
RESULTS: Thirteen intact proteins are identified on a 12% polyacrylamide gel. The mass spectra matching was found to have 229 proteins, and 11.4% of these were involved in secondary metabolism. Proteins closely related to vitexin biosynthesis are listed and their functions are explained mechanistically. Vitexin synthesis is predicted to involve plant polyketide chalcone synthase, isomerization by chalcone isomerase, oxidation by cytochrome P450 to convert flavanone to flavone, and transfer of sugar moiety by C-glycosyltransferase, followed by dehydration to produce flavone-8-C-glucosides.
CONCLUSIONS: Phenol based extraction, followed by gel electrophoresis and LC-MS/MS could identify proteome explaining vitexin biosynthesis in *F. deltoidea.* Many transferases including β-1,3-galactosyltransferase 2 and glycosyl hydrolase family 10 protein were detected in this study. This explains the importance of transferase family proteins in C-glycosylated apigenin biosynthesis in medicinal plant.

Keywords C-glycosylation, vitexin, apigenin 8-C glucoside, proteins, peptides, LC-MS/MS

Introduction

Plants produce a broad spectrum of secondary metabolites which play critical roles in plant-environment interactions and provide protection against biotic and abiotic stresses such as pathogens, herbivores, drought, and ultraviolet light (Yang et al., 2012; Afrin et al., 2015). Secondary metabolites are produced along with the primary metabolite pathway during plant growth and development. Although plant secondary metabolites are not involved in energy production, growth, reproduction, or other plant primary functions, they perform important functions such as protection, attraction, and signaling (Bernhoft et al., 2010). These plant bioactive compounds exhibit pharmacological and/or toxicological effects that could be further developed into drugs for a wide range of diseases such as cancer, malaria, and schizophrenia (Hicks et al., 2011; Afrin et al., 2015).

Recent studies have also shown that many bioactive compounds from natural products contain sugar moiety in their molecular structures. Glycosylation could enhance the physiological, selectivity, stability, solubility, and pharmacological properties of compounds acting as functional food additives and cosmetic ingredients (Luley-Goedl and Nidetzky, 2011; Xiao et al., 2015, 2016). C-glycosylated flavonoids have received less attention compared to O-

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glycosides (Xiao et al., 2016). However, C-glycosylated flavonoids are more stable than O-glycosidic bonds because of their high resistance to chemical or enzyme-catalyzed hydrolysis (Bungaruang et al., 2013).

Vitexin (apigenin 8-C-glycoside) is one of best-known C-glycosides because of its remarkable pharmacological activities, which include anti-inflammatory (Zunoliza et al., 2009) and antioxidant properties (Farsi et al., 2011) and α-amylase and α-glucosidase inhibition that can reduce postprandial hyperglycemia and diabetic complications (Farsi et al., 2011; Choo et al., 2012). It is also regarded as a marker compound for *F. deltoidea*, locally known as Mas Cotek (Shafaei et al., 2012; Azemin et al., 2014; Mohd et al., 2016). *F. deltoidea*, which belongs to the family Moraceae, is a popular medicinal herb in Malaysia. It has traditionally been used by the locals to treat illnesses, including fever and headache, to regulate blood sugar and blood pressure, and to control cholesterol levels (Misbah et al. 2013). Therefore, the objective of this study was to predict the secondary mechanism of vitexin production in *F. deltoidea* based on the identified proteins as primary building blocks. Understanding this mechanism will enhance the production of vitexin as a potential lead compound in drug discovery.

Material and methods

Plant material

F. deltoidea was obtained from Nursery Herba Pak Ali (Skudai, Johor, Malaysia) and authenticated by the herbarium of Universiti Kebangsaan Malaysia (Bangi, Selangor, Malaysia) under specimen number 40213. The sample was confirmed to be *F. deltoidea* Jack var. *trengganuensis* Corner. The plant was cultivated in a mixture of sand and compost soil (1:1) and allowed to grow in natural glasshouse at the Plant Biotechnology Laboratory, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor Darul Takzim, Malaysia.

Protein extraction

Plant proteins were extracted from the leaves of *F. deltoidea* according to the method described by Isaacson et al. (2006). One gram of frozen leaf tissues was homogenized in liquid nitrogen and extracted by 10 mL cold extraction buffer consisting of 0.7 M sucrose, 0.1 M KCl, 0.5 M Tris-HCl (pH 7.5), 50 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride (PMSF), and 2% β-mercaptoethanol. An equal volume of Tris-buffered phenol was added to the mixture and then the mixture was incubated on a shaker for 30 min at 4°C. After incubation, the mixture was centrifuged for 30 min at 5000 × *g* at 4°C. The phenol phase, which was located at the top of the tube, was carefully harvested and the remaining mixture was re-extracted by the extraction buffer in an equal volume ratio. The mixture was incubated and centrifuged again to collect the phenol phase. Five volumes of cold ammonium acetate (0.1 M) in methanol was added into the recovered phenol solution and stored at −20°C overnight. The precipitate was obtained after centrifugation for 30 min at 5000 × *g* at 4°C. The protein pellet was gently mixed and rinsed twice with ice-cold methanol prior to centrifugation for 10 min at 5000 × *g* at 4°C. Acetone was used to perform the final wash for the pellet, and then the pellet was air-dried under a vacuum for 3 min. Protein concentration was estimated by Bradford assay (Bradford, 1976), using a serial concentration of bovine serum albumin (0–15 μg/mL) as the standard chemical for the calibration curve.

SDS gel electrophoresis (SDS-PAGE)

One-dimensional SDS–PAGE was performed to separate the extracted proteins according to the method described by Laemmli (1970). Samples were treated with rehydration buffer (8 M urea, 20 mM DTT, 4% CHAPS, and 5 mM Tris-Base), followed by boiling for 5 min. After centrifugation, 25 μL supernatant was loaded onto a 12% (w/v) polyacrylamide running gel. Electrophoresis was conducted at a constant current (20 mA) in 50 mM Tris–glycine–SDS (pH 8.3) running buffer for 2 h. A mixture of protein marker (Precision Plus Protein TM Prestained Standard Dual Xtra marker, BIO-RAD) was used to determine the molecular masses of the detected proteins, and Coomassie Brilliant Blue G-250 was used to stain for protein visualization.

In-gel digestion

The protein bands on the electrophoretic gel were cut and divided into three sections according to their molecular sizes (1–20, 21–50, and 51–250 kDa). They were then diced into smaller pieces of approximately 1–2 mm^2 in size and transferred into 1.5 mL centrifuge tubes. In-gel digestion was performed following the method explained by Shevchenko et al. (2006). The Coomassie Brilliant Blue G-250 dye on the excised protein bands was removed with three cycles of dehydration and hydration steps using acetonitrile and 100 mM ammonium bicarbonate, respectively. The proteins in the gel were then subjected to in situ reduction, alkylation, and finally digestion by trypsin overnight. The peptides eluted from the gel were concentrated and followed by purification using C18 Zip-Tips (Merck, Millipore, USA).

LC-MS/MS

The tryptic peptides were re-suspended in 0.1% (v/v) formic acid, and then analyzed by a micro-capillary UltiMate 3000 (Sunnyvale, CA) system integrated with a quadrupole-time-of-flight (QTOF) mass spectrometer (AB SCIEX QSTAR Elite; Foster City, CA) with a turbo spray ionization (TIS) source. A C18 reversed phase Zorbax 300SB column (150 × 0.3 mm, 5 μm) with a flow rate of 5 μL/min was used for

separation. A binary gradient system consisting of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) was used. The LC gradient was: 0–5 min, 2% B; 5–15 min, 2%–45% B; 15–16 min, 45%–80% B; 80% B hold for 2 min; 18–19 min, 80– 2% B; and 2% B hold for 10 min. The injection volume was 5 μL.

Protein identification

The mass spectrometric data was searched for protein matches using MS-Fit (University of California, 2017). The parameters were: database, NCBInr.2013.6.17 against *Arabidopsis thaliana*; digest used, trypsin; maximum number of missed cleavages, 2; constant modification, carbamidomethyl; minimum matches, 2; sort type, score sort; minimum parent ion matches, 1; MOWSE On, 1; MOWSE P-factor, 0.4 (Jiménez et al., 2001).

Results and discussion

Plant protein extraction

Phenol-based extraction was carried out to extract the plant proteins, which were then recovered by phenol. The extraction buffer was composed of PMSF to inhibit proteases that might be released upon cell rupture during extraction. EDTA was added into the buffer to hinder the activities of metalloproteases and oxidases by chelating metal ions. β-mercaptoethanol, a reducing agent, was used to protect proteins from oxidation, while potassium chloride was used to facilitate extraction via its salting effect. The addition of sucrose assisted phase separation between the extraction buffer and phenol phases so that phenol could be harvested for high recovery of the proteins.

In the present study, phenol-based extraction recovered 459.24 μg/g plant proteins from the leaves of *F. deltoidea*. The protein pellet was white in color, indicating that few plant contaminants such as chlorophylls and pigments were co-extracted by this method. The electrophoretic gel of the extracted protein mixture is presented in Fig. 1. Thirteen intact proteins were separated on the 12% polyacrylamide gel. The molecular size of the intact proteins ranged from 10 to 245 kDa, with most of them being in the intermediate range of 20–135 kDa. The protein bands were excised and digested with trypsin into peptides for mass spectrometric analysis.

Plant protein identification

The mass spectra were analyzed and matched to the protein database of the National Center for Biotechnology Information (NCBI) using MS-Fit. A total of 229 proteins were found. The identified proteins were categorized into several classes, such as proteins involved in secondary metabolism, hormone metabolism, and signaling, according to their functional groups as stated in the gene ontology of the GoMapMan database (http://www.gomapman.org/ontology). Only proteins related to secondary metabolite production were selected, and these are listed together with their matched peptide sequences in Table 1. Some of the proteins perform more than one function, and therefore are grouped into the miscellaneous category.

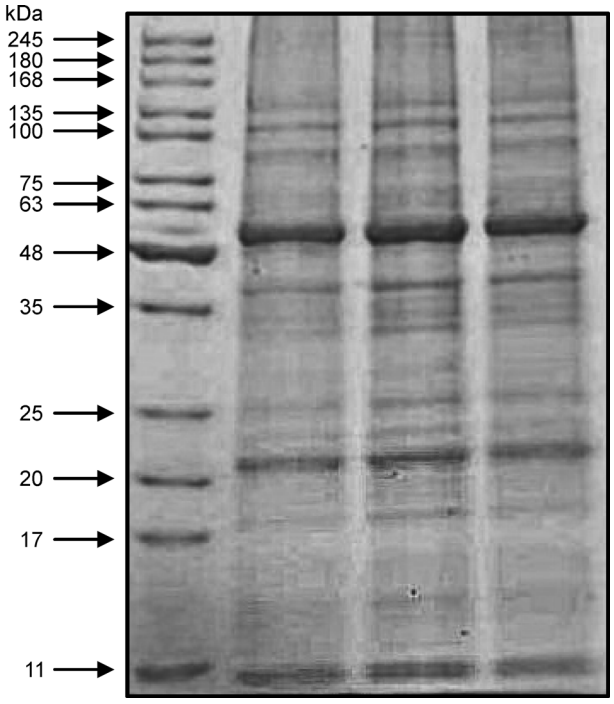

Figure 1 Electrophoretic gel image of proteins extracted from *Ficus deltoidea* leaves.

Biosynthesis of plant secondary metabolites

The biosynthesis of secondary metabolites is controlled by a complex network of many regulatory proteins known as transcription factors (TFs). The transcription factors usually bind to specific regions of promoters at the target genes, followed by activation or repression of their expressions to regulate secondary metabolism. Members of the TF family, including putative WRKY transcription factor 2, putative c-myb-like transcription factor MYB3R-4, ethylene-responsive factor 2 (ERF2) and 10 (ERF 10), auxin response factor 10 (ARF 10), and ABRE binding factor 4, were identified in the present study. TFs such as MYC, MYB, WRKY, and APETALA2/ethylene-responsive factor (AP2/ERF) have been shown to be involved in the regulation of secondary metabolism in medicinal plants (Afrin et al., 2015; Dey and Corina, 2015). The expression of MYB-like gene encoding enzyme, which is involved in the biosynthesis of secondary metabolites, is controlled by TFs. For example, PbMYB9 is an activator of the proanthocyanidin, anthocyanin, and flavonol pathways, and its function is essential for flavonoid biosynthesis in pear fruits (Zhai et al., 2016).

Table 1 Identified proteins related to secondary metabolites production

Category	Protein name	Accession number	Mass (Da)	MOWSE score	Sequence
Transcription factors	Putative WRKY transcription factor 2	NP_200438.1	74562	155	CTAPGCTVR; DSSQSNVFR; TNTDFSIDSNLR; FPFLPGVNGNALSSEK
	Putative c-myb-like transcription factor MYB3R-4	BAD94312.1	90420	71.6	MPEETMHIR; VDQICMEANVR; TDVQCLHRWQK; NDPLHDYSPLGIR
	Ethylene-responsive factor 2 (ERF2)	NP_193820.1	45668	211	EMQTIRDVMFK; NNKNNKAQTETR; VLSPPPPHMSLQR; KEMQTIRDVMFK; RNNVYFEIIEEPR; VFAIGLTLSWCLILR
	Ethylene-responsive factor 10 (ERF 10)	NP_171876.1	26393	123	MEPDLDLNASP; LRMEPDLDLNASP; ENVTTAVAVKDGGEK
	Auxin response factor 10 (ARF 10)	AAF04627.1	77795	103	WPNSPWR; HHHYQAR; IGDEPFSDFMK; VEAVAGSGGACSXVDK
	ABRE binding factor 4	NP_001154626.1	44845	22.6	DGNMEGSSGGGGESNVPPGR
Jasmonic acid	12-oxophytodienoate reductase 1 (OPR1)	NP_177794.1	41168	26.7	TMGEVHACPHTLMPMR; SYGNVPQ-PHAAIYYSQR
Cysteine	Serine O-acetyltransferase (Transferase)	NP_175988.1	34251	108	TGNTQDDDSR; VSESFAVDIHPGAK; DVPARTTAVGNPARLIGGK
Transferase	O-methyltransferase family protein	NP_177877.1	42327	169	ENGQTGK; AMSESSTMVM; AMSESSTMVMK; AMSESSTMVMKK
	Putative protein kinase	NP_001154651.1	47141	23.3	SDVYSFGVVLLEILTGR; DTGCAESGSSTPLMSGELK
	Putative β-1,3-Galactosyltransferase 2 (glycosyltransferase)	Q8L7F9	45758	548	YHEPEYWK; GEYSSRSFVSR; GMSHPSVTEAER; SVQESLQN-GAPLSDDMGK
	Putative glycosyl hydrolase family 10 protein	NP_192556.2	74402	9383	IVPLSGKVFAAATQR; NTNTNHTSDDNNDK; TQTWMGPAQ-MITDK; QTYNSFPVGTCINR; QLNKTDLMNAVQKR; IVPLSGKV-FAAATQRK; REQYIVIANVQATDK; TQTWMGPAQMITDKIK; TDLMNAVQKRLTDLLTR
	β-galactosidase 5	NP_175127.1	81444	3404	GLGPAGHSYVNWAAK; LGNYEEAHVF-TAGK; FSFSSQVNLRGGANK; GNLLVLFEELGGDISK; ENRKFSFSSQVNLR; QPLTWYKAYFDAPR; GSLAKQNKQPLTWYK; SAMQGFTE-KIVQMMK; DTTDYLWYTTSVDIK; GSCVAFLTNYHMNAPAK
	O-fucosyltransferase family protein	NP_176423.3	73297	31.8	AELDAYR; MPTIRPNK; EAKPGFYMK; IQETGAILVR; MLNATLVIPK
	Sucrose synthase 6 (glycosyltransferase)	NP_177480.1	106877	69.9	QSRYHMK; DGQEQHDVK; EHLMNEIEK; MSSSSQAMLQK; DNAEHMGYLADR
Polyketide	Polyketide cyclase/dehydrase and lipid transport superfamily protein	NP_850574.1	46265	217	MENALR; GIWSYVCK; FYLCDGSPMK; GAQLSQTSQNAGI; DFYMDNEYRK
Flavonol	Flavonol synthase 5	NP_201165.1	36964	32.2	ETFTQSIGGDTAEYVL; NYLGGINNW-DEHLFHR
Miscellaneous	Putative cytochrome P450 71A28	P58047.2	58217	133	FNWR; TNEFELER; VVQEHVDEGENK; FPLIAFPSLASFTI
	cytochrome P450, family 87, subfamily A, polypeptide 2	NP_001184974.1	55058	131	NDVDMCR; HFMAFGGGMR; LQMAAFLHSLVTK; LQMAAFLHSLVTK; RLTEEHETILRNR
	Cytochrome P450, family 706, subfamily A, polypeptide 7	NP_192970.1	58774	144	DEDENMSMNHVK;
	LACERATA	ACO87292.1	7506	126	MHDWISDNLR;
	Cytochrome b5 isoform B	NP_180831.1	15016	88.3	MGDEAKIFTL; SEVSEHNQAHD; CWI-VINGKVYN;VTKFLEDHPGGD; DVLLSSTGKD; ATDDFEDVG; MMEQYYV-GEI; TPPKQPHYNQ; DKTSEFIIK; LLQFLVPLAI; LGLAVGIR

In this study, 12-oxophytodienoate reductase 1 (OPR1) was identified. This protein is involved in the final step of the β-oxidation cycle to yield the end product of jasmonic acid (Schaller, 2001). The oxylipin-type molecule of jasmonic acid is synthesized from α-linolenic acid via the octadecanoid pathway (Schaller, 2001; Pauwels et al., 2009). Jasmonic acid and its derivatives are important signaling molecules for the production of secondary metabolites in the plant kingdom (Zhao et al., 2005; Pauwels et al., 2009). Jasmonic acid, which also acts as a phytohormone, can synthesize a wide variety of plant secondary metabolites, primarily terpenoids, flavonoids, alkaloids, glucosinolates, anthocyanins, and isoprenoids (Pauwels et al., 2009). Jasmonic acid and jasmonates (methyl jasmonate) are key signaling compounds that control the expression of specific genes such as jasmonate-responsive gene 1 (*jrg1*), and are followed by the synthesis of jasmonate-induced proteins (JIPs) whenever they are exposed to external stimuli such as biotic and abiotic stress (Kramell et al., 2000). Jasmonates induce transcription of the gene encoding phenylalanine ammonia lyase (PAL) as the key enzyme of the phenylpropanoid pathway in flavonoid synthesis (Kašparová and Siatka, 2014). The accumulation of secondary metabolites is considered the final consequence of the biochemical changes induced by jasmonates (Ishihara et al., 2002). The combination of the jasmonic acid and ethylene signaling pathways is essential for plant defense responses to stresses. Ethylene may not be a common signal for plant induction, and the effect of ethylene on secondary metabolite production is dependent on ethylene concentration (Zhao et al., 2005).

Post-translational modification by transferases

Some of the identified proteins are categorized as transferases. Transferases are involved in post-translational modification. For example, glycosyltransferases catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules through the formation of O-, N-, S-, and C-glycosidic bonds with acceptors of small molecules such as sugars, lipids, proteins, or small molecules including phenylpropanoids. The glycosylation is regulated by the combination of regioselective glycosyltransferases (GTs) and glycoside hydrolases (GHs) (Le Roy et al., 2016). In the present study, the proteins detected in this category included putative beta-1,3-galactosyltransferase 2, putative glycosyl hydrolase family 10, putative beta-galactosidase, beta-galactosidase 5, and O-fucosyltransferase family protein. GTs that catalyze sugar conjugation of secondary metabolites belonging to the GT1 family are known as uridine diphosphate-glycosyltransferases (UGTs). UGTs are used by plants to synthesize flavonoids. UGTs facilitate glycosylation from a donor called uridine diphosphate glucose (UDPglucose). Sucrose synthase was also identified in this study. Sucrose synthase is an enzyme (SuSy) used to catalyze a reversible sucrose conversion to fructose and UDPglucose in the presence of UDP (Bungaruang et al., 2013). In plants, the sugar donor is usually glucose, but it can also be galactose, xylose, rhamnose, arabinose, or glucuronic acid (Yonekura-Sakakibara et al., 2008). Flavonoid glycosylation typically occurs at ring positions bearing hydroxyl groups. When an acceptor has multiple binding sites for a sugar, UGTs exhibit regioselectivity by transferring the sugar to a specific position. Glycosylation is also regulated by glycoside hydrolases (GHs) in the hydrolysis and/or rearrangement of glycosidic bonds (Le Roy et al., 2016). Therefore, transferases are very important for vitexin synthesis in *F. deltoidea*. They are involved in the later stage of translation after flavanone synthesis.

Many phenylpropanoid pathway-derived products are toxic and unstable molecules, and therefore they seldom accumulate as their aglycones in plants (Alejandro et al., 2012; Väisänen et al., 2015). Glycosylation can therefore reduce phenylpropanoid toxicity and increase stability and solubility and can also influence compartmentalization and biological activity (Le Roy et al., 2016). Among the diverse phenylpropanoids, flavonoids are probably the best-characterized molecules in terms of glycosylation owing to their broad medical and commercial benefits. Natural flavonoids are present in the form of O-glycosides or C-glycosides in plants (Xiao et al., 2015).

C-glycosylated flavonoids have been found to possess better therapeutic properties than O-glycosylated flavonoids. C-glycosides can act as siderophores, antibiotics, antioxidants, attractants, and feeding deterrents (Brazier-Hicks et al., 2009). C-glycosylated flavonoids are also more stable against the activity of glycosidases under hydrolytic conditions (Rawat et al., 2009). Among the reported C-glycosides, flavone C-glycosides, especially vitexin, isoorientin, orientin, isovitexin, and their multi-glycosides have been frequently mentioned in the literature. O-glycosides are likely to have lower in vivo lifetimes (Bungaruang et al., 2013).

Vitexin (apigenin-8-C-glucoside) and isovitexin (apigenin-6-C-glucoside) are the main C-glycosylated flavonoid constituents of *F. deltoidea*. Isovitexin is an isomer of vitexin, and its C-glycosylation happens at C-6. They are considered the chemical markers for this herb (Azemin et al., 2014). Often, C-glycosyl flavonoids are produced in greater amounts than O-glycosyl flavonoids by weight (Courts and Williamson, 2015). The level of vitexin in the leaf extracts of *F. deltoidea* was found to be higher than that of isovitexin. For instance, 12.31 mg/g vitexin and 4.81 mg/g isovitexin were reported in methanol extract, and 6.20 mg/g vitexin and 0.81 mg/g isovitexin were found in water extract (Shafaei et al., 2012). Another study that assessed the content of vitexin and isovitexin in *F. deltoidea* varieties using the high-performance liquid chromatographic method found that vitexin and isovitexin comprised 1.53% and 0.9% of a 10 mg leaf sample (Mohd et al., 2016).

The chemical name of vitexin is 8-D-glucosyl-4′,5,7-trihydroxy-flavone, and is known as ‘Mujingsu’ in Chinese. It

is an active component in many traditional Chinese medicines. To our knowledge, no different pharmacological properties of vitexin and isovitexin have been reported to date. Praveena et al. (2013) reported that C-8 glycosylation decreased the negative charge on the oxygen atom at the C-3 position, leading to better antioxidant potency of vitexin as compared to apigenin. However, Brazier-Hicks and Edwards (2013) revealed that vitexin and isovitexin exhibited similar pharmacological effects, partly owing to their similarity in chemical structures. Both C-glycosides were found to possess antioxidant (Farsi et al., 2011) and anti-inflammatory properties (Zunoliza et al., 2009), as well as α–amylase and α-glucosidase inhibition (Farsi et al., 2011; Choo et al., 2012), which may reduce postprandial hyperglycemia and diabetic complications.

Secondary mechanism of vitexin synthesis

A large number of plant secondary metabolites derive from phenylalanine and tyrosine as precursors via the phenylpropanoid pathway (Le Roy et al., 2016). Hence, flavonoids are considered to be phenylpropanoid-derived compounds (Stafford, 1991). Flavonoids are synthesized from the condensation of p-coumaroyl-CoA with three malonyl-CoA molecules by chalcone synthase (CHS), which in turn produces a flavanone containing a 2-phenylchroman backbone (Le Roy et al., 2016). The 2-phenylchroman backbone is the basic structure of flavanols, isoflavonoids, flavonols, flavones, and anthocyanidins. In the present study, flavonol synthase (FLS) was detected; FLS is used to synthesize flavonols through dihydroflavonols as intermediates. FLS expression can be induced by several abiotic stresses including UV-B, abscisic acid, cold, sucrose, salicylic acid, and ethephon. Previous studies indicate that detection of FLS also explains the presence of flavonols such as myricetin and kaempferol (Dzolin et al., 2015). Xu et al. (2012) revealed that FLS could also catalyze the formation of dihydrokaempferol to kaempferol (flavonol) and the conversion of kaempferol from naringenin (flavanone).

Biosynthesis of vitexin in *F. deltoidea* follows four major steps in a mechanistic pathway: condensation by plant polyketide chalcone synthase (CHS), isomerization either spontaneously or catalyzed by chalcone isomerase (CHI), oxidation by cytochrome P450 (CYP P450) to convert flavanone to flavone, and finally the transfer of sugar moiety by C-glycosyl transferase (CGT), followed by a dehydration step to produce flavone-6-C-glucosides, as illustrated in Fig. 2.

CHS is one of the plant type III polyketide synthases (PKS). CHS is the first committed enzyme in the biosynthesis of flavonoids and directs carbon flux from the general phenylpropanoid metabolism to the flavonoid pathway (Saito

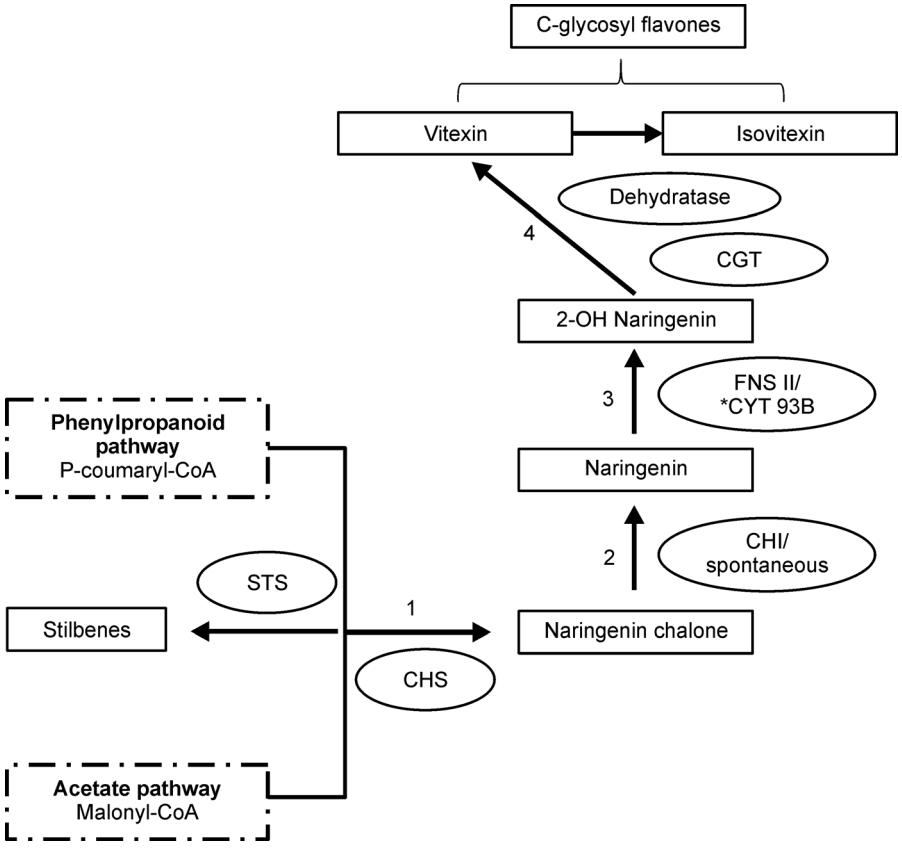

Figure 2 Flavone biosynthesis pathway CHS: chalcone synthase; STS: stilbene synthase; CHI: chalcone isomerase; FNS: flavone synthase; CGT: C-glycosyltransferase.

et al., 2013; Zhang et al., 2017). It initiates the loading of p-coumaroyl-CoA to its active site, followed by two malonyl-CoA units, until a linear tetraketide chain is created, which is then circularized to form the chalcone product through an aromatase-like mechanism (Austin and Noel, 2003). The spontaneous cyclization of the triketide intermediate results in the formation of naringenin chalcone. Lussier et al. (2013) suggested that naringenin chalcone was produced via a Claisen cyclization reaction. CHS initiates the elongation of p-coumaroyl-CoA to a C15 skeleton resulting branch of the phenylpropanoid pathway, and produces a variety of stress-induced compounds and pigments. Light is one of the most important factors triggering flavonoid biosynthesis and induction of light-responsive gene expression. This may explain why flavonoids are scarcely produced in plants grown in the dark, since there is a lack of genes encoding expression for CHS (Kaltenbach et al., 1999).

The second step of flavone synthesis is the isomerization of chalcone to flavanone by chalcone isomerase. CHI catalyzes the stereospecific cyclization of naringenin chalcone to (2S)-naringenin (Saito et al., 2013). Although this step can occur spontaneously, CHI-catalyzed isomerization is approximately 107-fold more efficient than spontaneous isomerization (Bednar and Hadcock, 1988). The non-enzymatic conversion of chalcones yields racemic (2R/S)-flavanones. Since only (2S)-flavanones are intermediates of the subsequent flavonoid pathway, CHI specificity guarantees efficient formation of biologically active (2S)-flavanone (Bednar and Hadcock, 1988; Cheng et al., 2011). The expression of genes encoding for CHI is also upregulated by UV-B irradiation (Cheng et al., 2011).

After isomerization of chalcone to flavanone by chalcone isomerase (CHI), the subsequent pathway branches to several different flavonoid classes, including aurones, dihydrochalcones, flavanonols (dihydroflavonols), isoflavones, flavones, flavonols, leucoanthocyanidins, anthocyanins, and proanthocyanidins (Mierziak et al., 2014). Flavones are synthesized from flavanones by the introduction of a double bond between the C-2 and C-3 positions by flavone synthase (FNS) (Martens and Mithofer, 2005). FNS converts naringenin either directly to apigenin (flavones) or 2-hydroxyflavanones by FNS II (Fig. 2). The conversion of naringenin to 2-hydroxyflavanones is catalyzed by flavanone-2-hydroxylase (F2H) (Yonekura-Sakakibara and Hanada, 2011). It is noteworthy that FNS II can also have F2H or FNS activity. FNS II functions as oxygen- and NADPH-dependent CYP P450 membrane-bound monooxygenases, which are widespread among higher plants. FNS II belongs to the CYP 93B subfamily for dicots and the CYP 93G subfamily for monocots (Martens and Mithofer, 2005). CYP P450-linked enzymes are implicated in the biosynthesis of various structural, growth regulatory, and protective substances in plant cells via numerous metabolic pathways. They contribute to the stable equilibrium of phytohormones and signaling molecules by controlling their biosynthesis and catabolism. They are involved in the biosynthesis of pigments, volatiles, antioxidants, allelochemicals, and defense compounds, including phenolics and their conjugates, flavonoids, coumarins, lignans, glucosinolates, cyanogenic glucosides, benzoxazinones, isoprenoids, and alkaloids (Morant et al., 2003; Mizutani and Sato, 2011).

In this study, five CYP P450s-based proteins were detected (Table 1). CYP 706 is involved in terpenoid metabolism. CYP 71A28 was previously known as CYP 713A2, belonging to the subfamily of CYP 71 A, which has been shown to have monoterpene hydroxylase activity. CYP 87 is involved in plant hormone metabolism, and LACERATA (CYP 86A8) is involved in fatty acid metabolism (Bak et al., 2011). Plant CYP P450s are bound to membranes, usually anchored on the cytoplasmic surface of the endoplasmic reticulum. They need to be coupled to electron-donating proteins such as CYP P450 reductases or CYP b5 for activation (Bak et al., 2011). Even though all CYP P450s found in this study are related to terpenoid, hormone, and fatty acid biosynthesis, proteins of CYP P450s are also known to have multi-catalyzing functions. Therefore, the identified CYP P450s might also be involved in the introduction of double bond to the C-2 and C-3 positions in order to convert naringenin to 2-hydroxyflavanones in third step of vitexin biosynthesis.

However, little is known about flavone-C-glycoside biosynthesis. Flavanone, which is the core intermediate of the flavone pathway, is most likely to be a precursor. This reaction is mediated by C-glycosyltransferase (CGT), which catalyzes the formation of flavone-C-glycosides from flavanone precursors (Brazier-Hicks et al., 2009). Figure 2 shows that flavanones are hydroxylated by F2H/FNS II to form 2-hyroxylflavanones, and then glycosylated to 2-hydroxylflavanone C-glycosides by CGT. This C-glycosylated 2-hydroxyflavanone is consequently dehydrated to produce flavone C-glycosides.

Kerscher and Franz (1987) reported that CGT from *Fagopyrum esculentum* seedlings catalyzed the transfer of glucose to 2-hydroxyflavanones. Naringenin, naringenin-chalcone, and flavones such as apigenin and chrysin cannot act as glucosyl acceptors in C-glycosyl-flavonoid biosynthesis. C-glycosylation takes place after dehydration of 2-hydroxynaringenin. This finding was also supported by another study on cereal crops where C-glycosylated flavones were synthesized through the action of CGT and dehydratase on 2-hydroxyflavanones (Brazier-Hicks et al., 2009). The dehydration step from unstable 2-hydroxyflavanones can occur spontaneously or can be catalyzed by an enzyme (Akashi et al., 2005). However, flavone-6-C-glucosides are preferentially formed in plants. 2-hydroxyflavanone conjugates undergo spontaneous dehydration to yield a mixture of flavone-6-C- and flavone-8-C-glucosides (Brazier-Hicks et al., 2009). Isoflavone is formed by 1,2-elimination of water from 2-hydroxyisoflavanone catalyzed by 2-hydroxyisoflavanone dehydratase (HID). HID displays clear substrate specificity and is distinguishable from differently substituted

2-hydroxyisoflavanone. Histidine and aspartic acid are critical residues for HID catalysis (Hakamatsuka et al., 1998; Akashi et al., 2005). The spontaneous dehydration to form isoflavones is slow compared to the enzyme-catalyzed reaction. This suggests that production of flavones in plants primarily depends on enzymes. The non-enzymatic slow production of isoflavone in plants becomes an alternative process (Akashi et al., 2005).

CGT from *Zea mays* displays O-glycosylation activity toward naringenin, but C-glycosylation activity toward 2-hydroxynaringenin (Falcone Ferreyra et al., 2013). This indicates that the activity of CGT is highly specific to 2-hydroxynaringenin and its derivatives, such as 2,4′,5,7-tetrahydroxyflavanone and 2,5,7-trihydroxyflavanone. CGT does not accept flavanones, flavones, or flavonols as glucose acceptors (Kerscher and Franz, 1987; Nagatomo et al., 2014). Vitexin and isovitexin have been found to be present only in certain plants, especially in plants with medicinal values (He et al., 2016). Medicinal plants such as pearl millet (El Amrani et al., 2004), hawthorn (Akashi et al., 1999), pigeon pea (Brazier-Hicks et al., 2008; Crosby et al., 2011), mung bean (Crozier et al., 2009), mosses (Day et al., 2003; Dixon et al., 2009), Passiflora (Du et al., 2010a, 2010b), bamboo (Dürr et al., 2004; François et al., 2004), mimosa (Ha et al., 2010), wheat leaves (Halpin et al., 1999), and chaste tree or chaste berry (Hasegawa et al., 2007) have been previously reported to contain flavones.

PKS has three processing domains; namely, the ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains (Li et al., 2015). The dehydratase (DH) domain of PKS was identified in this study. Dehydratase domains of PKSs function to generate an α,β-unsaturated bond through a dehydration reaction in a *cis* or *trans* configuration (Akey et al., 2010). This catalyzes the formation of the unsaturated triketide intermediate using malonyl-CoA as the chain extension substrate (Wu et al., 2005). The double bonds are formed by DH domains through abstraction of the α-proton and concomitant protonation of the β-hydroxyl group of the nascent β-hydroxyacyl-ACP polyketide intermediate, resulting in loss of one water molecule (Li et al., 2015). The active site of DH contains a histidine residue from the N-terminal and an aspartate residue from the C-terminal. Histidine and aspartic are conserved across DH-containing enzymes (Wu et al., 2005; Akey et al., 2010; Ishikawa et al., 2012; Li et al., 2015). The products of all DH reactions contain α, β-double bonds conjugated with the thioester carbonyl (acetyl-CoA) (Akey et al., 2010). Several similarities have been observed between DH domain of PKS and HID. Both proteins possess conserved histidine and aspartic acid residues at their active sites for catalytic activity. They catalyze formation of an α, β-double bond by the loss of a water molecule (Li et al., 2015). They also display high substrate specificity, where double bond confirmation depends on the chirality of the β− OH substrate. Thus, the DH domain of PKS identified from this study might be responsible for the dehydration step to produce corresponding vitexin in *F. deltoidea* leaves.

Conclusions

Vitexin biosynthesis in *F. deltoidea* was predicted to follow a four-step mechanism involving plant polyketide chalcone synthase (CHS); isomerization, either spontaneous or catalyzed by chalcone isomerase (CHI); oxidation by CYP P450 to convert flavanone to flavone; and the transfer of sugar moiety by C-glycosyltransferase (CGT), followed by dehydration to produce flavone-8-C-glucosides. The detection of the proteins also supports previous findings that vitexin is present in medicinal plants. *F. deltoidea* is a traditional medicinal plant that is widely used by indigenous peoples in South-east Asian countries. Further studies are required to elucidate the related biochemical pathways in order to trigger vitexin production.

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Compliance with ethics guidelines

Farah Izana Abdullah, Lee Suan Chua, and Zaidah Rahmat declare that they have no conflicts of interest.

References

Adam Z, Khamis S, Ismail A, Hamid M (2012). *Ficus deltoidea*: A potential alternative medicine for diabetes mellitus. Evid Based Complement Alternat Med, 2012: 632763

Afrin S, Huang J J, Luo Z Y (2015). JA-mediated transcriptional regulation of secondary metabolism in medicinal plants. Sci Bull, 60 (12): 1062–1072

Akashi T, Aoki T, Ayabe S (2005). Molecular and biochemical characterization of 2-hydroxyisoflavanone dehydratase. Involvement of carboxylesterase-like proteins in leguminous isoflavone biosynthesis. Plant Physiol, 137(3): 882–891

Akashi T, Fukuchi-Mizutani M, Aoki T, Ueyama Y, Yonekura-Sakakibara K, Tanaka Y, Kusumi T, Ayabe S (1999). Molecular cloning and biochemical characterization of a novel cytochrome P450, flavone synthase II, that catalyzes direct conversion of flavanones to flavones. Plant Cell Physiol, 40(11): 1182–1186

Akey D L, Razelun J R, Tehranisa J, Sherman D H, Gerwick W H, Smith J L (2010). Crystal structures of dehydratase domains from the curacin polyketide biosynthetic pathway. Structure, 18(1): 94–105

Alejandro S, Lee Y, Tohge T, Sudre D, Osorio S, Park J, Bovet L, Lee Y, Geldner N, Fernie A R, Martinoia E (2012). AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr Biol, 22 (13): 1207–1212

Austin M B, Noel J P (2003). The chalcone synthase superfamily of type

III polyketide synthases. Nat Prod Rep, 20(1): 79–110

Azemin A, Dharmaraj S, Hamdan M R, Mat N, Ismail Z, Khamsah S M (2014). Discriminating *Ficus deltoidea* var. bornensis from Different Localities by HPTLC and FTIR Fingerprinting. J Appl Pharm Sci, 4: 69–75

Bak S, Beisson F, Bishop G, Hamberger B, Höfer R, Paquette S (2011). Cytochromes P450. Arab B, e0144

Bednar R A, Hadcock J R (1988). Purification and characterization of chalcone isomerase from soybeans. J Biol Chem, 263(20): 9582–9588

Bernhoft A, Siem H, Bjertness E, Meltzer M, Flaten T, Holmsen E (2010). Bioactive compounds in plants–benefits and risks for man and animals. in Proceedings from a Symposium Held at The Norwegian Academy of Science and Letters, Novus forlag, Oslo

Bradford M M (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72(1-2): 248–254

Brazier-Hicks M, Edwards R (2013). Metabolic engineering of the flavone-C-glycoside pathway using polyprotein technology. Metab Eng, 16: 11–20

Brazier-Hicks M, Evans K M, Cunningham O D, Hodgson D R W, Steel P G, Edwards R (2008). Catabolism of glutathione conjugates in *Arabidopsis thaliana*. Role in metabolic reactivation of the herbicide safener fenclorim. J Biol Chem, 283(30): 21102–21112

Brazier-Hicks M, Evans K M, Gershater M C, Puschmann H, Steel P G, Edwards R (2009). The C-glycosylation of flavonoids in cereals. J Biol Chem, 284(27): 17926–17934

Bungaruang L, Gutmann A, Nidetzky B (2013). Leloir glycosyltransferases and natural product glycosylation: Biocatalytic synthesis of the C-glucoside nothofagin, a major antioxidant of redbush herbal tea. Adv Synth Catal, 355(14-15): 2757–2763

Cheng H, Li L, Cheng S, Cao F, Wang Y, Yuan H (2011). Molecular cloning and function assay of a chalcone isomerase gene (GbCHI) from Ginkgo biloba. Plant Cell Rep, 30(1): 49–62

Choo C Y, Sulong N Y, Man F, Wong T W (2012). Vitexin and isovitexin from the Leaves of *Ficus deltoidea* with *in-vivo* α-glucosidase inhibition. J Ethnopharmacol, 142(3): 776–781

Courts F L, Williamson G (2015). Critical Reviews in Food Science and Nutrition The occurrence, fate and biological activities of C- glycosyl flavonoids in the human diet. Crit Rev Food Sci Nutr, 55(10): 1352–1367

Crosby K C, Pietraszewska-Bogiel A, Gadella T W J Jr, Winkel B S J (2011). Förster resonance energy transfer demonstrates a flavonoid metabolon in living plant cells that displays competitive interactions between enzymes. FEBS Lett, 585(14): 2193–2198

Crozier A, Jaganath I B, Clifford M N (2009). Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep, 26(8): 1001–1043

Day A J, Gee J M, DuPont M S, Johnson I T, Williamson G (2003). Absorption of quercetin-3-glucoside and quercetin-4′-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. Biochem Pharmacol, 65(7): 1199–1206

Dey S, Corina Vlot A (2015). Ethylene responsive factors in the orchestration of stress responses in monocotyledonous plants. Front Plant Sci, 6: 640

Dixon D P, Hawkins T, Hussey P J, Edwards R (2009). Enzyme activities and subcellular localization of members of the Arabidopsis glutathione transferase superfamily. J Exp Bot, 60(4): 1207–1218

Du Y, Chu H, Chu I K, Lo C (2010a). CYP93G2 is a flavanone 2-hydroxylase required for C-glycosyl-flavone biosynthesis in rice. Plant Physiol, 154(1):324–33

Du Y, Chu H, Wang M, Chu I K, Lo C (2010b). Identification of flavone phytoalexins and a pathogen-inducible flavone synthase II gene (SbFNSII) in sorghum. J Exp Bot, 61(4): 983–994

Dürr C, Hoffmeister D, Wohlert S E, Ichinose K, Weber M, Von Mulert U, Thorson J S, Bechthold A (2004). The glycosyltransferase UrdGT2 catalyzes both C- and O-glycosidic sugar transfers. Angew Chem Int Ed Engl, 43(22): 2962–2965

Dzolin S, Ahmad R, Zain M M, Ismail M I (2015). Flavonoid distribution in four varieties of *Ficus deltoidea* (Jack). J Med Plant Herb Ther Res, 3: 1–9

El Amrani A, Barakate A, Askari B M, Li X, Roberts A G, Ryan M D, Halpin C (2004). Coordinate expression and independent subcellular targeting of multiple proteins from a single transgene. Plant Physiol, 135(1): 16–24

Falcone Ferreyra M L, Rodriguez E, Casas M I, Labadie G, Grotewold E, Casati P (2013). Identification of a bifunctional maize C- and O-glucosyltransferase. J Biol Chem, 288(44): 31678–31688

Farsi E, Shafaei A, Hor S Y, Ahamed M B K, Fei M, Attitalla I H (2011). Correlation between enzymes inhibitory effects and antioxidant activities of standardized fractions of methanolic extract obtained from *Ficus deltoidea* leaves. Afr J Biotechnol, 10(67): 15184–15194

François I E J A, Van Hemelrijck W, Aerts A M, Wouters P F J, Proost P, Broekaert W F, Cammue B P A (2004). Processing in Arabidopsis thaliana of a heterologous polyprotein resulting in differential targeting of the individual plant defensins. Plant Sci, 166(1): 113–121

Ha S H, Liang Y S, Jung H, Ahn M J, Suh S C, Kweon S J, Kim D H, Kim Y M, Kim J K (2010). Application of two bicistronic systems involving 2A and IRES sequences to the biosynthesis of carotenoids in rice endosperm. Plant Biotechnol J, 8(8): 928–938

Hakamatsuka T, Mori K, Ishida S, Ebizuka Y, Sankawa U (1998). Purification of 2-hydroxyisoflavanone dehydratase from the Cell Cultures of *Pueraria lobata.* Phytochemistry, 49(2): 497–505

Halpin C, Cooke S E, Barakate A, El Amrani A, Ryan M D (1999). Self-processing 2A-polyproteins–a system for co-ordinate expression of multiple proteins in transgenic plants. Plant J, 17(4): 453–459

Hasegawa K, Cowan A B, Nakatsuji N, Suemori H (2007). Efficient multicistronic expression of a transgene in human embryonic stem cells. Stem Cells, 25(7): 1707–1712

He M, Min J W, Kong W L, He X H, Li J X, Peng B W (2016). A review on the pharmacological effects of vitexin and isovitexin. Fitoterapia, 115: 74–85

Hicks M A, Barber A E 2nd, Giddings L A, Caldwell J, O'Connor S E, Babbitt P C (2011). The evolution of function in strictosidine synthase-like proteins. Proteins, 79(11): 3082–3098

Isaacson T, Damasceno C M B, Saravanan R S, He Y, Catalá C, Saladié M, Rose J K (2006). Sample extraction techniques for enhanced proteomic analysis of plant tissues. Nat Protoc, 1(2): 769–774

Ishihara A, Ogura Y, Tebayashi S, Iwamura H (2002). Jasmonate-

induced changes in flavonoid metabolism in barley (Hordeum vulgare) leaves. Biosci Biotechnol Biochem, 66(10): 2176–2182

Ishikawa F, Haushalter R W, Burkart M D (2012). Dehydratase-specific probes for fatty acid and polyketide synthases. J Am Chem Soc, 134 (2): 769–772

Jiménez C R, Huang L, Qiu Y, Burlingame A L (2001). Searching sequence databases over the internet: protein identification using MS-Fit. Curr Protoc Protein Sci, Chapter 16: 5

Kaltenbach M, Schröder G, Schmelzer E, Lutz V, Schröder J (1999). Flavonoid hydroxylase from Catharanthus roseus: cDNA, heterologous expression, enzyme properties and cell-type specific expression in plants. Plant J, 19(2): 183–193

Kašparová M, Siatka T (2014). Production of flavonoids and isoflavonoids in jasmonic acid-induced red clover suspension cultures. Ceska Slov Farm, 63(1): 17–21

Kerscher F, Franz G (1987). Biosynthesis of Vitexin and Isovitexin : Enzymatic Synthesis of the C-Glucosylflavones Vitexin and Isovitexin with an Enzyme Preparation from *Fagopyrum esculentum* M. Seedlings. Z Naturforsch C, 42: 519–524

Kramell R, Miersch O, Atzorn R, Parthier B, Wasternack C (2000). Octadecanoid-derived alteration of gene expression and the "oxylipin signature" in stressed barley leaves. Implications for different signaling pathways. Plant Physiol, 123(1): 177–188

Laemmli U K (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227(5259): 680–685

Le Roy J, Huss B, Creach A, Hawkins S, Neutelings G (2016). Glycosylation Is a Major Regulator of Phenylpropanoid Availability and Biological Activity in Plants. Front Plant Sci, 7: 735

Li Y, Dodge G J, Fiers W D, Fecik R A, Smith J L, Aldrich C C (2015). Functional Characterization of a Dehydratase Domain from the Pikromycin Polyketide Synthase. J Am Chem Soc, 137(22): 7003–7006

Luley-Goedl C, Nidetzky B (2011). Glycosides as compatible solutes: biosynthesis and applications. Nat Prod Rep, 28(5): 875–896

Lussier F X, Colatriano D, Wiltshire Z, Page J E, Martin V J J (2013). Engineering microbes for plant polyketide biosynthesis. Comput Struct Biotechnol J, 3(4): e201210020

Martens S, Mithöfer A (2005). Flavones and flavone synthases. Phytochemistry, 66(20): 2399–2407

Mierziak J, Kostyn K, Kulma A (2014). Flavonoids as important molecules of plant interactions with the environment. Molecules, 19 (10): 16240–16265

Misbah H, Aziz A A, Aminudin N (2013). Antidiabetic and antioxidant properties of *Ficus deltoidea* fruit extracts and fractions. BMC Complement Altern Med, 13(1): 118

Mizutani M, Sato F (2011). Unusual P450 reactions in plant secondary metabolism. Arch Biochem Biophys, 507(1): 194–203

Mohd K S, Rosli A S, Azemin A, Mat N, Zakaria A J (2016). Comprehensive Biological Activities Evaluation and Quantification of Marker Compounds of *Ficus deltoiea* Jack Varieties. Int J Pharmacogn Phytochem Res, 8: 1698–1708

Morant M, Bak S, Møller B L, Werck-Reichhart D (2003). Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Curr Opin Biotechnol, 14(2): 151–162

Nagatomo Y, Usui S, Ito T, Kato A, Shimosaka M, Taguchi G (2014). Purification, molecular cloning and functional characterization of flavonoid C-glucosyltransferases from *Fagopyrum esculentum* M. (buckwheat) cotyledon. Plant J, 80(3): 437–448

Pauwels L, Inzé D, Goossens A (2009). Jasmonate-inducible gene: What does it mean? Trends Plant Sci, 14(2): 87–91

Praveena R, Sadasivam K, Kumaresan R, Deepha V, Sivakumar R (2013). Experimental and DFT studies on the antioxidant activity of a C-glycoside from Rhynchosia capitata. Spectrochim Acta A Mol Biomol Spectrosc, 103: 442–452

Rawat P, Kumar M, Sharan K, Chattopadhyay N, Maurya R (2009). Ulmosides A and B: flavonoid 6-C-glycosides from Ulmus wallichiana, stimulating osteoblast differentiation assessed by alkaline phosphatase. Bioorg Med Chem Lett, 19(16): 4684–4687

Saito K, Yonekura-Sakakibara K, Nakabayashi R, Higashi Y, Yamazaki M, Tohge T, Fernie A R (2013). The flavonoid biosynthetic pathway in Arabidopsis: structural and genetic diversity. Plant Physiol Biochem, 72: 21–34

Schaller F (2001). Enzymes of the biosynthesis of octadecanoid-derived signalling molecules. J Exp Bot, 52(354): 11–23

Shafaei A, Farsi E, Ismail Z, Asmawi M Z (2012). Quantitative High Performance Thin-Layer Chromatography Method for Analysis of Vitexin and Isovitexin in Extracts of Leaves of *Ficus deltoidea.* Asian J Chem, 24: 2286

Shevchenko A, Tomas H, Havlis J, Olsen J V, Mann M (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc, 1(6): 2856–2860

Stafford H A (1991). Flavonoid evolution: an enzymic approach. Plant Physiol, 96(3): 680–685

University of California (2017). Ms-Fit. Available at: http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form = msfitstandard.

Väisänen E E, Smeds A I, Fagerstedt K V, Teeri T H, Willför S M, Kärkönen A (2015). Coniferyl alcohol hinders the growth of tobacco BY-2 cells and Nicotiana benthamiana seedlings. Planta, 242(3): 747–760

Wu J, Zaleski T J, Valenzano C, Khosla C, Cane D E (2005). Polyketide double bond biosynthesis. Mechanistic analysis of the dehydratase-containing module 2 of the picromycin/methymycin polyketide synthase. J Am Chem Soc, 127(49): 17393–17404

Xiao J, Capanoglu E, Jassbi A R, Miron A (2016). Advance on the flavonoid *C*-glycosides and health benefits. Crit Rev Food Sci Nutr, 56(sup1 Suppl 1): S29–S45

Xiao J, Chen T, Cao H (2015). Flavonoid glycosylation and biological benefits. Biotechnol Adv,

Xu F, Li L, Zhang W, Cheng H, Sun N, Cheng S, Wang Y (2012). Isolation, characterization, and function analysis of a flavonol synthase gene from Ginkgo biloba. Mol Biol Rep, 39(3): 2285–2296

Yang C Q, Fang X, Wu X M, Mao Y B, Wang L J, Chen X Y (2012). Transcriptional regulation of plant secondary metabolism. J Integr Plant Biol, 54(10): 703–712

Yonekura-Sakakibara K, Hanada K (2011). An evolutionary view of functional diversity in family 1 glycosyltransferases. Plant J, 66(1): 182–193

Yonekura-Sakakibara K, Tohge T, Matsuda F, Nakabayashi R, Takayama H, Niida R, Watanabe-Takahashi A, Inoue E, Saito K

(2008). Comprehensive flavonol profiling and transcriptome coexpression analysis leading to decoding gene-metabolite correlations in Arabidopsis. Plant Cell, 20(8): 2160–2176

Zhai R, Wang Z, Zhang S, Meng G, Song L, Wang Z, Li P, Ma F, Xu L (2016). Two MYB transcription factors regulate flavonoid biosynthesis in pear fruit (*Pyrus bretschneideri* Rehd.). J Exp Bot, 67(5): 1275–1284

Zhang X, Abrahan C, Colquhoun T A, Liu C and Sciences A (2017). A proteolytic regulator controlling chalcone synthase stability and flavonoid biosynthesis in *Arabidopsis*. Plant Cell Online, tpc-00855

Zhao J, Davis L C, Verpoorte R (2005). Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv, 23(4): 283–333

Zunoliza A, Khalid H, Zhari I, Rasadah M A (2009). Anti-inflammatory activity of standardised extracts of leaves of three varieties of *Ficus deltoidea.* Int J Pharm Clin Res, 1: 100–105