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Isolation and expression analysis of anthocyanin biosynthetic genes in *Morus alba* L.

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Abstract

Anthocyanins from mulberry fruits are used in medicine. However, little anthocyanin can be detected in other tissues and sometimes also mulberry fruits are colorless. The aim of this study was to investigate which gene or genes have the strongest correlation with the anthocyanin biosynthesis. The expression of several anthocyanin synthesis genes were determined in different tissues of two white and two purple fruit cultivars. Genes encoding dihydroflavonol reductase (*MaDFR*) and anthocyanidin synthase (*MaANS*) showed a high expression only in fruit tissue of purple-fruit cultivars. During the development of mulberry fruits, the anthocyanin content was well correlated with the transcripts abundance of *MaDFR, MaANS,* and *MaCHS* (encoding chalcone synthase). The skin of female mulberry flowers turns red under irradiance because of up-regulated expressions of *MaCHS, MaDFR*, and *MaANS*. These three genes may control the anthocyanin biosynthesis in mulberry and up-regulation of them may greatly increase the anthocyanin content.

Additional key words: anthocyanidin synthase, chalcone isomerase, chalcone synthase, dihydroflavonol reductase, flavanone-3-hydroxylase, phenylalanine ammonia lyase.

Introduction

Mulberry fruits are formed from a cluster of flowers. Each flower after fertilization develops into a drupe, and as the drupes expand, they merge forming a multiple fleshy fruit called a syncarp. It takes about 35 - 40 d from fertilization to maturity. During growth, mulberry fruit changes colour from green through red to black-purple. The pigment from the mulberry fruit is used as authorised food colorant and the fruit has long been used as medicine (Chan *et al*. 2009). The berries of *Morus nigra* L. and *M. alba* L. are consumed as fresh fruit or as jam, marmalade, frozen desserts, ice cream, and wine (Pawlowska *et al*. 2008). The red coloration of flowers, fruits, and vegetables depends mainly on the composition and concentration of anthocyanins, and their biosynthesis have been thoroughly studied (Macheix *et al*. 1990). A variety of anthocyanin functions have been reported, including protection against high irradiance or as attractants for animals (Grayer *et al*. 1994, Koes *et al*. 1994) Anthocyanins have been extracted from grape skins (Jayaprakasam *et al*. 2005) or blueberries (Nicoue *et al*. 2007). However, the content of anthocyanins in mulberry fruit is 64.70 ± 0.45 mg g^{-1}(d.m.) which is about twice of that in blueberries (Qin *et al.* 2010).

The pathway of the anthocyanin biosynthesis usually contains two sections: early and late (Deroles 2009). The early section comprising phenylalanine ammonia lyase (*PAL*), cinnamate-4-hydroxylase (*C4H*), 4-coumarate*: CoA ligase* (*4CL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and flavanone 3′-hydroxylase (*F3′H*) results in the formation of dihydroflavonols. Genes expressing the three enzymes in the late section, dihydroflavonol reductase (*DFR*), anthocyanidin synthase

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Abbreviations: ANS - anthocyanidin synthase; C4H - cinnamate-4-hydroxylase; CHI - chalcone isomerase; CHS - chalcone synthase; 4CL - 4-coumarate: CoA ligase; DFR - dihydroflavonol reductase; F3′H - flavanone-3′-hydroxylase; PAL - phenylalanine ammonia lyase; UFGT - UDP-glucose: flavonoid 3-glucosyltransferase.
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(*ANS*), and UDP-glucose: flavonoid 3-glucosyltransferase (*UFGT*), lead to the generation of anthocyanins. Many genes in the anthocyanin synthesis pathway have been isolated from plants, including structural genes and several transcription factors (Holton and Cornish 1995). The abundant anthocyanins in mulberry are cyanidin-3-rutinoside and cyanidin-3-glucoside (Zou *et al*. 2011). However, there have been only few molecular studies of the anthocyanin biosynthesis pathway in mulberry. Members of *MaPAL* (GenBank accession No. HM064433) and *MaDFR* (acc. No. AY700051) families have been reported; and *MaCHI* and *MaANS* genes were obtained by our group.

Mulberry (*Morus alba* L.) fruit is generally purple, but some cultivars are pale. There are generally mutants with *1*) blockage of structural genes encoding enzymes of the anthocyanin biosynthesis; *2*) down-regulation of the expression of transcription factors which control the transcription of structural genes; and *3*) lack of the expression of other genes including those encoding proteins involved in the vacuolar sequestration of anthocyanins (Kotepong *et al*. 2011). Mutations in *CHS* (Sommer and Saedler 1986, O'Neill *et al*. 1990, Kubo *et al*. 2007), *CHI* (Martin *et al*. 1991, Holton and Cornish 1992), *F3H* (Martin *et al*. 1985), and *DFR* (Wang *et al*. 1993, Goldsbrough *et al*. 1994) were confirmed. Furthermore, a white-fruited phenotype is also due to the impairment or down-regulation of the *ANS* gene in *Duchesnea indica* (Debes *et al*. 2011). Similar results were reported by Kim (2004, 2005) when analyzing participation of the *ANS* gene in anthocyanin accumulation of *Allium cepa*. The expression of biosynthetic genes in anthocyanin accumulation is regulated by MYB transcription factors in the fruits of grapes (Grotewold *et al*. 2006), apples (Kobayashi *et al.* 2001, Schwinn *et al.* 2006), Chinese bayberries (Debes *et al*. 2011), and red pear (Jin 1997).

Isolation of anthocyanin biosynthetic genes using sequence homology is a widely performed approach in apple (Honda *et al*. 2002, Kim *et al*. 2003) and grape (Boss *et al.* 2003). In this study, we isolated four structural genes of anthocyanin biosynthetic enzymes (*MaCHS*, *MaF3H*, *MaF3'H*, and *MaDFR*). The expression of these genes together with *MaPAL*, *MaCHI*, and *MaANS* were determined in different tissues in two white and two purple fruit cultivars. The aim was to further elucidate the molecular mechanism of the anthocyanin synthesis and to develop valid methods to manipulate coloration in mulberry fruits.

Materials and methods

Plants: Two purple fruit cultivars *Morus atropurpurea* cvs. Jialing No. 30 (JL30) and Dashi (DS), and two white fruit cultivars *Morus alba* cvs. Zhenzhubai (ZZB) and Baiyuwang (BYW) were selected. They were vegetatively propagated from winter bud in a nursery under controlled conditions (an irradiance of 50 μmol m^{-2} s^{-1}, 16-h photoperiod, day/night temperatures of 30/20 °C, and a relative humidity of 75/80 %). Fruits (20 for each cultivar) were picked at 0, 20, and 40 d after full bloom (DAFB) and marked as stages S1, S2, and S3, respectively. The fruits were frozen in liquid N_2 and stored at -80 °C for RNA extraction and other analyses.

Extraction and identification of anthocyanins: Total anthocyanins extraction was performed according to the method of Zou *et al*. (2011). Dried powder of mulberry fruits (about 0.5 g) was placed in a capped tube, then mixed in 23.8 liquid-to-solid ratio with an extraction solution [63.8 % (v/v) methanol with 1 % (m/v) trifluoroacetic acid (TFA)]. The tube with the suspension was immersed in water in an ultrasonic device and treated at 42 °C for 40 min. Then, the samples were centrifuged at 10 000 *g* for 10 min, the supernatant was collected and diluted with the extraction solution 1:10. All samples were filtered through a 0.45-μm syringe filter (*Pall Life Sciences*, Ann Arbor, MI, USA). Anthocyanins in the samples were analyzed using a *Waters 1525* system (Waters, Massachusetts, USA). The absorbance was monitored at 520 nm. An *Elite ® C18* column (250 mm × 4.6 mm, 5 μm) (*Amersham Biosciences*, Buckinghamshire, UK) and an auto-injector were used. The samples were separated and eluted using a mobile phase consisting of 15 % (v/v) acetonitrile (solvent A) and 85 % (v/v) formic acid (solvent B) at a flow rate of 1.0 cm^3 min^{-1}, a column temperature was 30 °C and an injection volume was 0.01 cm^3. Identification was based on comparison of retention times and fragmentation with authentic standards of cyanidin-3-O-glucoside and cyanidin-3-galactoside (*ChromaDex*, Irvine, CA, USA).

For determination of the individual anthocyanins, fruit extractions were fractionated using silica plates (20 × 20 cm, 1 mm height, *Merck*, Darmstadt, Germany) according to Santos *et al*. (2013). The mobile phase was composed of ethyl acetate, glacial acetic acid, formic acid, and distilled water (100:11:11:15). All anthocyanin standards were diluted in methanol.

Determination of flavonoid content: Total flavonoid content was analyzed by a colorimetric method (Chang *et al*. 2002). Lyophilized powder (0.5 g) produced from a fresh mulberry fruit by a vacuum evaporator (*Thermo*, Waltham, MA, USA), was dissolved in 30 cm^3 of 75 % (v/v) ethanol and maintained at about 65 °C for 2 h. A sample of this solution (5 cm^3) was mixed with 10 cm^3 of 95 % ethanol, 1 cm^3 of 10 % (m/v) $AlCl_3 . 6 H_2O$), 1 cm^3 of 1 M potassium acetate and 33 cm^3 of deionized water. After the reaction mixture was incubated at room temperature for 30 min, its absorbance was measured at 420 nm on a spectrophotometer (model *100-20*, *Hitachi*,

Table 1. Degenerated primers for cloning anthocyanin biosynthetic genes in mulberry.

Gene	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Product [bp]	GenBank ID
MaCHS	TCACHAACAGYGAGCAC	ACACAWGCACTTGACAT	864	JF499387
MaF3H	CAGAGAGTTCTTCGCTTTG	CAGAATTGGCTTCTCTCCT	370	KC521448
MaF3′H	ATGGTGGTGGAGATGATGGTG	GCTCKTTGTAGDGTGAGCCCA	857	KC521446, KC521447
MaDFR	AAAGATGACTGGTTGGATG	CCAAGCTGTACTTGAACTC	535	KC521445

Table 2 Primers for genome walking and full-length cDNA cloning. Adaptors 1 and 2 were used to ligate the genome which was digested by different restriction enzymes. Adaptor 2 was modified by $–NH_2$ and $–PO_4$. The upstream fragment of *MaDFR* was isolated by using the Nest-PCR method and the two pairs of primers were *MaDFR*upGSP1 and Ap1, and *MaDFR*upGSP2 and Ap2. The two pairs of primers used in cloning the *MaDFR* downstream fragment were *MaDFR*downGSP1 and Ap1, and *MaDFR*downGSP2 and Ap2. The two pairs of primers used in cloning the *MaF3′H1* upstream fragment were *MaF3′H1*upGSP1 and Ap1, and *MaF3′H1*upGSP2 and Ap2. The two pairs of primers used in cloning the *MaF3′H1* downstream fragment were *MaF3′H1*downGSP1 and Ap1, and *MaF3′H1*downGSP2 and Ap2. The two pairs of primers used in cloning the MaF3′H2 upstream fragment were *MaF3′H2*upGSP1 and Ap1, and *MaF3′H2*upGSP2 and Ap2. The two pairs of primers used in cloning the *MaF3′H2* downstream fragment were *MaF3′H2*-downGSP1 and Ap1, and *MaF3′H2*downGSP2 and Ap2.

Primer name	Sequences (5′ - 3′)
Adaptor1	GTAATACGACTCACTATAGGGCAC GCGTGGTCGACGGCCCGGGCTGGT
Adaptor2	NH_2-ACCAGCCC-PO_4
*MaDFR*upGSP1	GCTTTACCTTCTTCACGTT
*MaDFR*upGSP2	CGCAGGGTCGCGCACGGTGGCTC
Ap1	TCTGTGAGGTCACGTCCAGC
Ap2	TTGCCTTGGACTACGAGCAGG
*MaDFR*downGSP1	GTTCAAAGGCTTTGAAG
*MaDFR*downGSP2	GAAGAAGACATAGGAAATGTGG
MaDFR-Full-F	ATGGGATCGGTGAGTGAGAC
MaDFR-Full-R	TTAGGCCGCTCCATTAGGCT
*MaF3′H1*upGSP1	TGGGTCTTAAGGAACTGGGC
*MaF3′H1*upGSP2	ACCACGTCCACCTGGCCCAAAC
*MaF3′H1*downGSP1	CTCCAGGCCGTAGTGAAGG
*MaF3′H1*downGSP2	TCCGGCTTCATCCCTCCACCCCG
MaF3′H1-Full-F	ATGGCCTCTATCACCACCAT
MaF3′H1-Full-R	TCAATATAAGTGTTGGTCTA
*MaF3′H2*upGSP1	CTTAAGGAACTGGGCTGCCA
*MaF3′H2*upGSP2	CCGACGCCGCTGCCACCACGTC
*MaF3′H2*downGSP1	GAATAGCGGCCGAGAACTGC
*MaF3′H2*downGSP2	GTGTGGGCTATAGCACGTGACC

Tokyo, Japan), using rutin as standard. The total flavonoid content in the fruit was determined using three replicates and expressed as milligrams of rutin equivalents per dry mass unit.

Isolation of total RNA and cloning anthocyanin biosynthetic genes: Total RNA was prepared as described by Jaakola *et al*. (2001). The 1 % (m/v) ethidium bromide-stained agarose gel and absorbance spectra at wavelengths of 220 - 300 nm were used to detect the quality of the isolated RNA. Degenerate primers of *CHS*, *F3H*, *F3′H*, and *DFR* were designed based on the highly conserved peptide regions (Table 1 Suppl.). A cDNA was synthesized from JL30 mRNA which was reverse-transcribed by *M-MLV* reverse transcriptase (*TaKaRa*, Tokyo, Japan) from random primers using standard methods. Sequences of *MaCHS*, *MaF3H*, *MaF3′H*, and *MaDFR* were compared with known sequences using the *NCBI BLAST* server. The upstream and downstream sequences of *MaDFR* and two *MaF3′H*s were cloned using genome walking (Balzergue *et al*. 2001). The primers are shown in Table 2 Suppl.

Phylogenetic analysis: The protein sequence of *Vitis vinifera* F3′H (acc. No. ABH066.1) and F3′5′H (acc. No. ABH06585.1) were used initially as query sequences to search against 28 gene model predictions from *Phytozome* (www.phytozome.net) using the *BLASTp* algorithm (Altschul *et al*. 1990), phylogeny test, and 1 000 bootstrap replicates. The comparison of two MaF3′Hs amino acid sequences was carried out using *Clustal X*.

Gene expression profile: In semi-quantitative RT-PCR, gene-specific primers were designed using *Primer 5.0* program from identified genes for RT-PCR (Table 3). The *actin* gene (acc. No. HQ163775) was used as internal control. Conditions for PCR of *MaCHI*, *MaF3H*, *MaF3′H1*, and *MaF3′H2* fragments were: 25 cycles of 94 °C for 30 s, 49 °C for 1 min, and 72 °C for 1 min; for *MaPAL* (acc. No. HM064433), *MaCHS*, *MaDFR*, and *MaANS*: 25 cycles of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 1 min. Amplification products were visualized in a 2 % (m/v) agarose gel stained with ethidium bromide (0.5 μg cm^{-3}).

The real time quantitative PCR (RT-qPCR) was performed using a *StepOne*™ real-time PCR system (*Life Technologies*, Carlsbad, CA, USA) following the manufacturer's instructions. All reactions were performed with *SYBR® Premix Ex Taq™ II* (*Tli RNaseH Plus*) according to the procedure described by the manufacturer (*TaKaRa*, Tokyo, Japan). Reactions were performed in triplicate using 0.01cm^3 of *SYBR® Premix Ex Taq II*, 0.0004 cm^3 of each primer (10 μM), 0.0004 cm^3 of *ROX* reference dye, 0.002 cm^3 of cDNA, and nuclease-free water to a final volume of 0.02 cm^3. Reactions were incubated at 95 °C for 30 s, followed by 40 cycles of

amplification at 95 °C for 5 s and then 60 °C for 34 s, after which a final cycle was performed at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The raw data were analyzed with *StepOnePlus*™ software, and expression was normalized to an *actin* gene to minimize variation in cDNA template content. Primers are shown in Table 4.

Table 3. Primers for semi-quantitative PCR analysis.

Gene	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Product [bp]
MaPAL	ATTCTCAACCAAGTCCA	GTTCCTCAAGTTCTCCT	499
MaCHS	GCATGTGTGAGAAATCTCTG	AGCTTAGTGAGTTGGTAGTC	269
MaCHI	TTTACCAGGGTGACGATGA	GCCTCCGAAAGTAGTTTGT	284
MaF3H	AAAAGGCGGTTTCATTG	GATTGTTCCTGGATCTGTG	346
MaF3'H1	CCGTAATCGGGAACCTG	GCCACTACCGTCACCAAA	464
MaF3'H2	TGGCAATGGGAAGCACA	CGCTCACAAGCCTATCTCG	251
MaDFR	CCCATTTCTTAGTCCCA	CAACGAACATATCCTCCA	450
MaANS	CAGGGAAGATCCAAGGC	GAGGGCATCTCGGGTAG	292
MaACT3	CGAAGGCTATGCCCTTCCAC	GCTCATCCGATCAGCAATACCC	445

Table 4. Primers for RT-qPCR analysis.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
MaCHS	ACTGAGGCATTCAAGCCTTT	AGCCACACTTAGCCTCCACT
MaCHI	CGCCAGGATCCTCTATTCTC	CGCCTCCGAAAGTAGTTTGT
MaF3H	AACACTTGGATCACCGTTCA	CTTGAACCTTCCATTGCTCA
MaF3'H1	TCCAGCTACTCACTGCAACC	TGTGAGCCCATATGCTTCAT
MaDFR	GTCCCACTATGCCTCCAAGT	GCAGAGGTCATCCAAGTGAA
MaANS	CTATGAAGGCAAATGGGTGA	CACCTTCTCCTTGTTGACGA
MaACT3	GCATGAAGATCAAGGTGGTG	CATCTGCTGGAAGGTGCTAA

Results and discussion

The fragment of cDNA corresponding to four anthocyanin biosynthetic genes – *MaCHS* (864 bp; acc. No. JF499387), *MaF3H* (370 bp, KC521448), *MaF3'H1* (857 bp; KC521446), and *MaDFR* (535 bp; KC521445) – were isolated using degenerate primers. The partial amino acid sequence of *MaCHS* showed 93 % identity to that of *Gossypium hirsutum*. The protein sequences had three CHS-specific conserved motifs (marked Motif I, II, and III) (see Table 3 Suppl.). There was active site with a highly conserved Cys residue in motif I, as well as from at least seven other residues shown to be highly conserved among plant CHSs. In motif II, a Phe active residue and two other conserved residues, Asp and Gly, were included. Conserved motif III contained His and Asn active residues, one Ser which formed the coumaroyl-binding pocket and four other conserved residues (Trp and three Gly residues).

The partial sequence of *MaF3H* showed high similarity to that of *Juglans nigra* and contained three conserved motifs for 2-oxoglutarate-dependent dioxygenases (2-ODDs) (Table 4 Suppl.). It is noteworthy that the amino acid residues His46, Asp48, and His90 for ligating the ferrous ion, and Arg114 and Ser116 participating in 2-oxoglutarate binding (RXS motif) were at the similar positions to other plant F3Hs (Britsch *et al*. 1993, Lukacin *et al*. 1997).

The coding sequences of two *MaF3'H*s were 1 527 and 1 554 bp long, respectively. *MaF3'H1* encoded 508 amino acids, whereas MaF3′H2 had a full-length open reading frame (ORF) encoding 517 amino acids.

Table 5. The content of anthocyanins [mg g^{-1}(d.m.)] and flavonoids [relative units] in fruits of different mulberry cultivars. Means ± SE, $n = 3$.

Species	BYW	ZZB	JL30	DS
Cyanidin-3-*O*-glucoside	0.08 ± 0.02	0.05 ± 0.01	17.78 ± 1.58	7.01 ± 0.08
Cyanidin-3-*O*-rutinoside	0.09 ± 0.02	0.04 ± 0.01	19.92 ± 1.06	5.79 ± 0.06
Total flavonoids	200.48 ± 2.41	320.24 ± 11.15	371.65 ± 1.56	336.89 ± 9.94

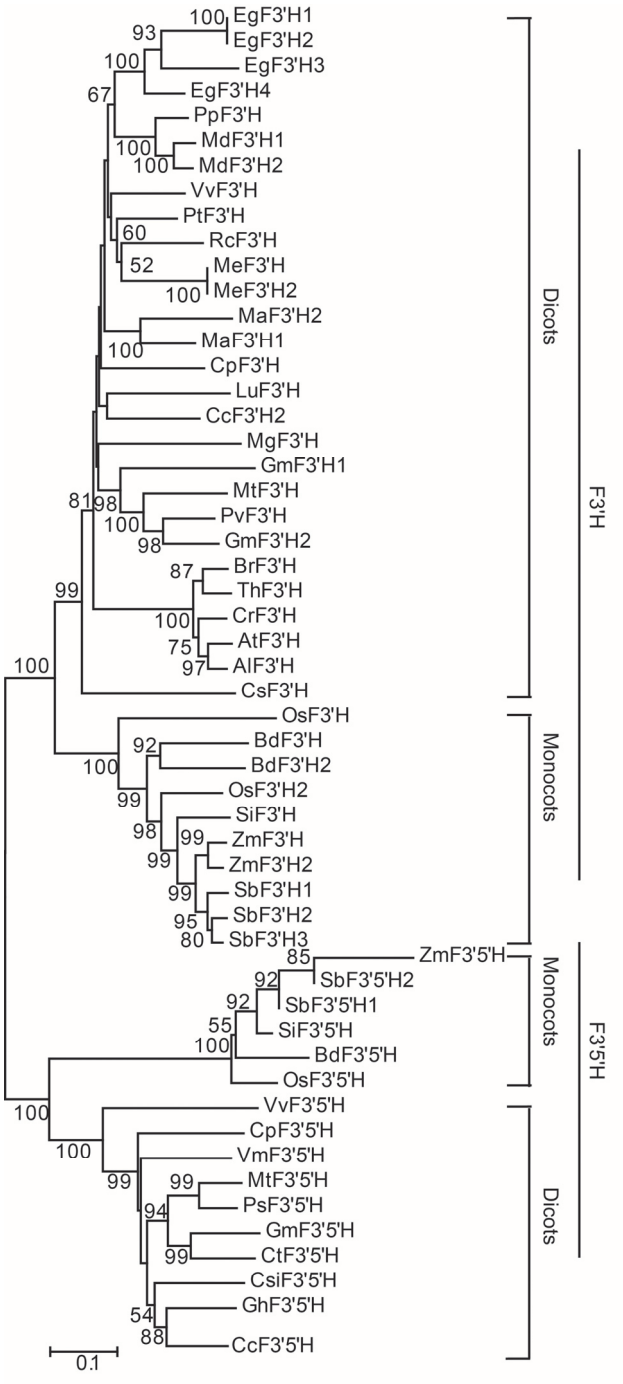

Fig. 1. The phylogenetic tree of two mulberry *MaF3'H*s genes in comparison with *F3'H*s and *F3'5'H*s in other plant species generated by the neighbor-joining method in *Mega 4.0*. Al - *Arabidopsis lyrata*, At - *Arabidopsis thaliana*, Bd - *Brachypodium distachyon*, Br - *Brassica rapa*, Cc - *Citrus clementine*, Cp - *Carica papaya*, Cr - *Capsella rubella*, Cs - *Cucumis sativus*, Eg - *Eucalyptus grandis*, Gh - *Gossypium hirsutum*, Gm - *Glycine max*, Lu - *Linum usitatissimum*, Md - *Malus domestica*, Me - *Manihot esculenta*, Mg - *Mimulus guttatus*, Mt - *Medicago truncatula*, Os - *Oryza sativa*, Pp - *Prunus persica*, Pv - *Panicum virgatum*, Rc - *Ricinus communis*, Sb - *Sorghum bicolor*, Si - *Setaria italic*, Th - *Thellungiella halophile*, Vv - *Vitis vinifera*, Zm - *Zea mays*.

NCBI BLASTp indicates that two *MaF3'H*s had broad similarities to *F3'H*s from other plants and a moderate to low similarity to other *P450*s, such as *F3'5'H* and *F5H*. When pairwise aligned on the whole-molecule scale, the two genes showed 76 and 72 % similarities to *Prunus avium*, respectively. A phylogenetic analysis shows that two *MaF3'H*s and *F3'H*s from *Vitis vinifera* (BAE47004.1) and *Malus* × *domestica* (ACR14867.1) form a closely related subgroup which further groups with monocot *F3'H* from *Sorghum bicolor* (ABG54321.1). This group was close to another *F3'H* group, but far from the *F3'5'H* group (Fig. 1). Both *MaF3'H*s bore all the conserved motifs featured by P450s: the proline rich 'hinge' region PPGPNPWP necessary for optimal orientation of the enzyme (Yamazaki *et al*. 1993, Murakami *et al.* 1994), the binding pocket motif AGTDTS, the heme domain FGAGRRICAG required for heme iron binding, as well as the pocket-locking motif E-R-R triad (Hasemann *et al*. 1995, Chapple 1998). Most of all, the *F3'H*-specific conserved motifs VDVKG, VVVAAS, and GGEK are exactly the same as those previously reported (Boddu *et al.* 2004) (see Fig. 3 Suppl.).

The full-length genome sequence of *MaDFR* was isolated using genome walking based on the partial sequence of cDNA obtained. It contained 6 exons and 5 introns including full-length ORF encoding 346 amino acids. The amino acid sequence alignment shows that *MaDFR* shared 75 and 73 % identity with *HlDFR* and *MdDFR*, respectively (see Fig. 5 Suppl.). In the flavonoid biosynthetic pathway, the DFR enzyme catalyzes the NADPH-dependent reduction of *2R*, *3R*-trans-dihydroflavonols to leucoanthocyanidins (Johnson *et al*. 1999). A putative NADP binding site (aa 10-30, VTGASGFIGSWLI/VMRLLEKGY) with a very high sequence similarity with other DFRs was also found in the same region (near the N-terminus) of the amino acid sequences (Lacombe *et al*. 1999).

Anthocyanins belong to a class of flavonoids derived ultimately from phenylalanine. A total of 19 types of anthocyanidins, aglycons, or chromophores of anthocyanins are known and can be divided into 6 major groups: pelargonidins, cyanidins, peonidins, delphinidins, petunidins, and malvidins (Jaakola 2013). Anthocyanins in mulberry were putatively identified as cyanidin-3-glucoside and 3-rutinoside (Fig. 2), this result was also proved by HPLC, and identified also in HPLC analyses of the same cultivar by Zou (2011). The elution profiles of four samples by HPLC were obtained, and showed very similar patterns (Fig. 5 Suppl.). The anthocyanin content was closely associated with the fruit colour. Based on their relative peak areas, the anthocyanin content in JL30 was 36.1 mg g^{-1}(d.m.) – three times of that in DS. There was 47 % of cyanidin-3-glucoside and 53 % of cyanidin-3-rutinoside in JL30, compared with 55 and 45 % in DS, respectively. The content of anthocyanins in ZZB and BYW were not significantly different. The total flavonoid content was highest in JL30 and lowest in BYW (Table 5). The

content in ZZB was not significantly different to that in DS, although there was an abundant accumulation of anthocyanins in the purple fruit cultivars. Anthocyanins from mulberry fruits have been also previously isolated and identified by Qin *et al*. 2010. Mulberry apparently produces only cyanidin-based anthocyanins (see Fig. 6 Suppl.). The major anthocyanin found in *Litchi chinensis* is cyanidin-3-rutinoside (91 %; Wei *et al*. 2011). In view of this, each plant may show a unique compositionand content of anthocyanins. Particularly, mulberry fruits have more anthocyanins than most plants and the mechanisms involved require careful investigation.

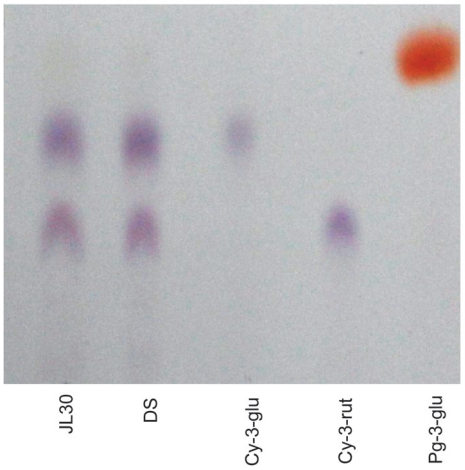

Fig. 2. The TLC plate of anthocyanins from fruits of two purple mulberry cultivars JL30 and DS. Cy-3-glu - cyanidin-3-glucoside, Cy-3-rut - cyanidin-3-rutinoside, Pg-3-glu - pelargonidin-3-glucoside.

To elucidate the molecular mechanisms of anthocyanin accumulation, the expression of anthocyanin biosynthetic genes in various organs or tissues was studied using semi-quantitative RT-PCR (Fig. 3). Two expression patterns of these genes could be distinguished: *1*) *MaPAL*, *MaCHI*, *MaCHS*, *MaF3H*, and *MaF3'H1* expressed at approximately equivalent levels in leaf, stem, root, petiole, bark, stipule, and fruit; and *2*) *MaDFR* and *MaANS* showed low expressions in various tissues except of fruit. The expressions of *MaDFR* and *MaANS* showed significant relationships to the anthocyanin content. In addition, the coding mRNA of *MaF3'H2* was not detected in any tissues (data not shown). Most genes were expressed more in young compared to mature leaves, especially *MaCHS*.

The fruit colour and content of anthocyanins depended on the expression of anthocyanin biosynthetic genes (Table 6). During fruit developmental stages of the purple fruit cultivars (Fig. 4), the expressions of the four

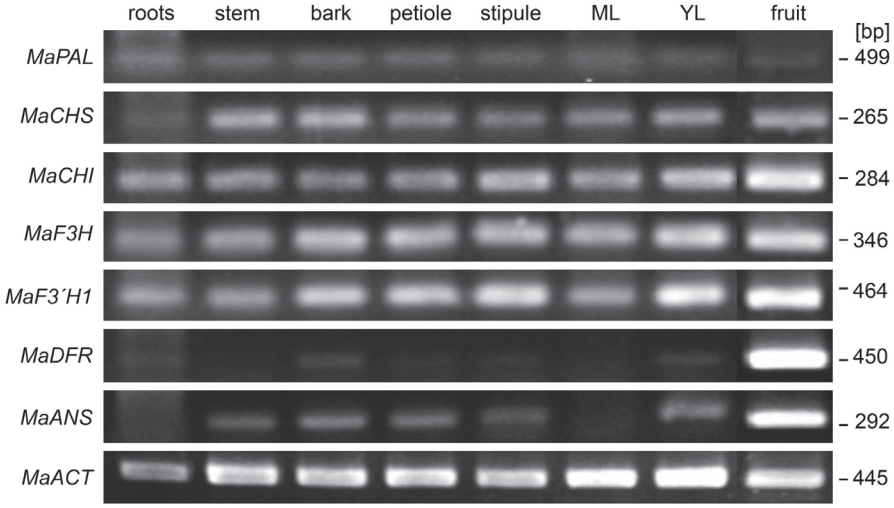

Fig. 3. Expression profiles of seven anthocyanin biosynthetic genes in various tissues of JL30. Semi-quantitative RT-PCR was used to analyze the expression of *MaPAL*, *MaCHS*, *MaCHI*, *MaF3H*, *MaF3'H*, *MaDFR*, and *MaANS* in root, stem, bark, petiole, stipule, mature leaf (ML), young leaf (YL), and fruit. The expression of *MaACT3* was used to normalize expression levels of the genes under identical conditions.

genes increased slightly, whereas low transcript abundances was found in the white fruit cultivars. In the purple fruit cultivars, the transcription of *MaCHS* gradually increased more than 130-fold in DS and 240-fold in JL30 from the stages S1 to S3. *MaDFR* and *MaANS* showed a more than 50-fold gradual increase both in DS and in JL30. Interestingly, *MaF3'H1* showed only a 10-fold gradual increase from the stages S1 to S3. These result suggests that *MaCHS*, *MaDFR*, and *MaANS* were important for the biosynthesis of anthocyanins.

Although the mutant ZZB lost colour during fruit development, the skin of flowers directed to the sun was partially red (Fig. 5). These pigments were extracted and proved to be anthocyanins – the HPLC elution profiles were similar to those in 40 DAFB fruits of JL30 and DS (Fig. 2). Furthermore, the third peak with a similar retention time to peak 1 was discovered. The red and green flowers were separated and expressions of seven anthocyanin biosynthetic genes were determined (Table 7). The result of RT-qPCR shows that the

expressions of *MaF3H* and *MaF3'H1* were less than 2-fold higher in red than in green flowers, whereas the expressions of *MaCHS* and *MaANS* showed a more than 4-fold increase in red flowers. Especially, the expression of *MaDFR* was more than 10-fold increased. It was indicated that *MaCHS*, *MaANS*, and *MaDFR* were induced by sunlight significantly.

In the present study, 4 genes were isolated from 40 DAFB fruits of JL30: *MaCHS*, *MaF3H*, *MaF3'Hs* (*MaF3'H1* and *MaF3'H2*), and *MaDFR*. There are two genes coding flavonoid-3'-hydroxylase in mulberry: *MaF3'H2*, which has not been detected in all tissues yet, and *MaF3'H1*, which had a high expression in the present study. This result indicates that *MaF3'H1* has important functions, although the two genes are very similar but may be regulated by different factors. The tissue-specific expression analysis in mulberry indicates that the late biosynthetic genes (LBGs) were well-correlated with the accumulation of anthocyanins in various tissues, especially the genes *MaDFR* and *MaANS*, and that the early biosynthetic genes (EBGs) were less associated

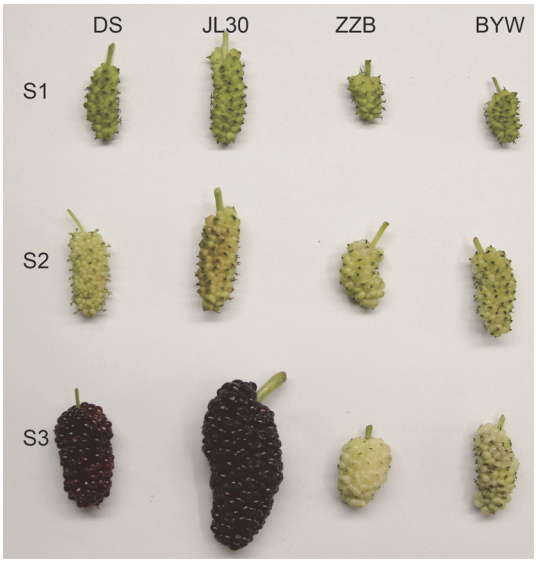

Fig. 4. Colours of fruits at different developmental stages (0, 20, and 40 d after full bloom, S1, S2, S3) of mulberry cultivars DS, JL30, ZZB, and BYW.

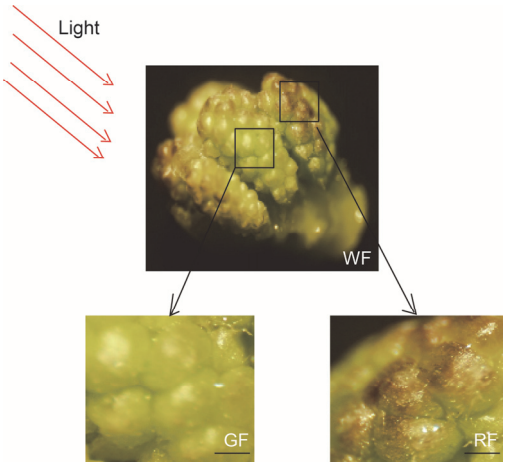

Fig. 5. Flowers buds of ZZB. WF - the whole flowers forming one syncarp; GF - the whole flower is green, RF - the flower with red parts; the *scale bar* = 0.1 mm.

Table 6. Expression profiles of four anthocyanin biosynthetic genes in fruit from white (ZZB and BWY) and purple fruit (JL30 and DS) cultivars at three stages (0, 20, and 40 DAFB). Means ± SE, $n = 3$.

	Stages	*CHS*	*F3'H1*	*DFR*	*ANS*
DS	S1	0.053 ± 0.001	0.220 ± 0.008	0.035 ± 0.001	1.225 ± 0.011
	S2	0.482 ± 0.022	1.032 ± 0.072	0.187 ± 0.035	2.221 ± 0.305
	S3	7.104 ± 0.150	3.304 ± 0.040	1.680 ± 0.074	25.629 ± 0.611
JL30	S1	0.073 ± 0.003	0.398 ± 0.003	0.055 ± 0.006	1.585 ± 0.203
	S2	2.923 ± 0.047	0.712 ± 0.006	1.099 ± 0.130	11.487 ± 2.037
	S3	18.005 ± 0.054	5.110 ± 0.039	3.729 ± 0.058	79.419 ± 22.530
ZZB	S1	0.271 ± 0.010	0.202 ± 0.005	0.021 ± 0.001	0.098 ± 0.001
	S2	0.803 ± 0.026	0.739 ± 0.133	0.069 ± 0.013	0.616 ± 0.053
	S3	0.798 ± 0.018	0.332 ± 0.024	0.007 ± 0.001	2.170 ± 0.030
BYW	S1	0.061 ± 0.001	0.060 ± 0.001	0.008 ± 0.000	0.066 ± 0.001
	S2	0.164 ± 0.003	0.150 ± 0.003	0.015 ± 0.001	0.409 ± 0.002
	S3	0.200 ± 0.011	0.091 ± 0.002	0.013 ± 0.001	0.428 ± 0.041

Table 7 Expression profile of five anthocyanin biosynthetic genes in different parts of ZZB female flowers. Means ± SE, $n = 3$.

Flower parts	*CHS*	*F3H*	*F3'H1*	*DFR*	*ANS*
Green	0.030 ± 0.002	0.116 ± 0.007	0.097 ± 0.003	0.008 ± 0.000	0.068 ± 0.001
Red	0.111 ± 0.012	0.210 ± 0.020	0.257 ± 0.015	0.073 ± 0.008	0.304 ± 0.043

with the anthocyanin content. Similar results have been discovered in many plants including grape (Boss *et al.* 1996), Chinese bayberry (Niu *et al.* 2010), cauliflower (Chiu *et al*. 2010), and kiwifruit (Montefiori *et al*. 2011).

Gene mutants of *MaF3'H1*, *MaDFR*, *MaANS*, and regulating genes occurring in white fruit cultivars prevented anthocyanin accumulation. The content of total flavonoids in ZZB, accompanied by the expression of *MaCHS*, showed no significant difference to that in JL30 and DS, although there were trace anthocyanins. Therefore, the transcription of *MaCHS* might affect the accumulation of flavonoids but not the colour of mulberry fruits. Major chalcones were used to produce anthocyanins, and a minor proportion of chalcones could be converted into other flavonoids in purple fruits. This may be why such high contents of anthocyanins occurred in fruits.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J.: Basic local alignment search tool. - J. mol. Biol. **215**: 403-410, 1990.

Balzergue, S., Dubreucq, B., Chauvin, S., Le-Clainche, I., Le Boulaire, F., De Rose, R., Lepiniec, L.: Improved PCR-walking for large-scale isolation of plant T-DNA borders. - Biotechniques **30**: 496-498, 502, 504, 2001.

Boddu, J., Svabek, C., Sekhon, R., Gevens, A., Nicholson, R.L., Jones, A.D., Chopra, S.: Expression of a putative flavonoid 3 '-hydroxylase in *Sorghum* mesocotyls synthesizing 3-deoxyanthocyanidin phytoalexins. - Physiol. mol. Plant Pathol. **65**: 101-113, 2004.

Boss, P.K., Davies, C., Robinson, S.P.: Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. - Plant Physiol. **111**: 1059-1066, 1996.

Britsch, L., Dedio, J., Saedler, H., Forkmann, G.: Molecular characterization of flavanone 3-beta-hydroxylases. Consensus sequence, comparison with related enzymes and the role of conserved histidine residues. - Eur. J. Biochem. **217**: 745-754, 1993.

Chan, K.C., Ho, H.H., Huang, C.N., Lin, M.C., Chen, H.M., Wang, C.J.: Mulberry leaf extract inhibits vascular smooth muscle cell migration involving a block of small GTPase and Akt/NF-kappaB signals. - J. Agr. Food Chem. **57**: 9147-9153, 2009.

Chang, C.C., Yang, M.H., Wen, H.M., Chern, J.C.: Estimation of total flavonoid content in propolis by two complementary colorimetric methods. - J. Food Drug Anal. **10**: 178-182, 2002.

Chapple, C.: Molecular-genetic analysis of plant cytochrome P_{450}-dependent monooxygenases. - Ann. Rev. Plant Physiol. Plant mol. Biol. **49**: 311-343, 1998.

Chiu, L.W., Zhou, X., Burke, S., Wu, X., Prior, R.L., Li, L.: The purple cauliflower arises from activation of a MYB transcription factor. - Plant Physiol. **154**: 1470-1480, 2010.

Debes, M.A., Arias, M.E., Grellet-Bournonville, C.F., Wulff, A.F., Martinez-Zamora, M.G,, Castagnaro, A.P., Diaz-Ricci, J.C.: White-fruited *Duchesnea indica* (Rosaceae) is impaired in *ANS* gene expression. - Amer. J. Bot. **98**: 2077-2083, 2011.

Deroles, S.: Anthocyanin biosynthesis in plant cell cultures: a potential source of natural colourants. - In: Kevin, G., Kevin, D., Chris, W. (ed.): Anthocyanins: Biosynthesis, Functions and Applications. Pp. 107-117, Springer, New York 2009.

Goldsbrough, A., Belzile, F., Yoder, J.I.: Complementation of the tomato anthocyanin without (aw) mutant using the *dihydroflavonol 4-reductase* gene. - Plant Physiol. **105**: 491-496, 1994.

Grayer, R.J., Harborne, J.B.: A survey of antifungal compounds from higher plants 1982-1993. - Phytochemistry **37**: 19-42, 1994.

Grotewold, E.: The genetics and biochemistry of floral pigments. - Annu. Rev. Plant Biol. **57**: 761-780, 2006.

Hasemann, C.A., Kurumbail, R.G., Boddupalli, S.S., Peterson, J.A., Deisenhofer, J.: Structure and function of cytochromes P_{450}: a comparative analysis of three crystal structures. - Structure **3**: 41-62, 1995.

Holton, T.A., Cornish, E.C.: Genetics and biochemistry of anthocyanin biosynthesis. - Plant Cell **7**: 1071-1083, 1995.

Honda, C., Kotoda, N., Wada, M., Kondo, S., Kobayashi, S., Soejima, J., Moriguchi, T.: Anthocyanin biosynthetic genes are coordinately expressed during red coloration in apple skin. - Plant Physiol. Biochem. **40**: 955-962, 2002.

Jaakola, L.: New insights into the regulation of anthocyanin biosynthesis in fruits. - Trends Plant Sci. **18**: 477-83, 2013.

Jaakola, L., Pirttila, A.M., Halonen, M., Hohtola, A.: Isolation of high quality RNA from bilberry *Vaccinium myrtillus* L. fruit. - Mol. Biotechnol. **19**: 201-203, 2001.

Jayaprakasam, B., Vareed, S.K., Olson, L.K., Nair, M.G.: Insulin secretion by bioactive anthocyanins and anthocyanidins present in fruits. - J. Agr Food Chem. **53**: 28-31, 2005.

Jin, H.: Multi-functionality and diversity within the plant MYB-gene family. - Plant mol. Biol. **41**: 577-585, 1997.

Johnson, E.T., Yi, H.K., Shin, B.C., Oh, B.J., Cheong, H.S., Choi, G.: *Cymbidium hybrida* dihydroflavonol 4-reductase does not efficiently reduce dihydrokaempferol to produce orange pelargonidin-type anthocyanins. - Plant J. **19**: 81-85, 1999.

Kim, S., Binzel, M.L., Yoo, K.S., Park, S., Pike, L.M.: Pink P, a new locus responsible for a pink trait in onions *Allium cepa* resulting from natural mutations of anthocyanidin synthase. - Mol. Genet. Genomics **272**: 18-27, 2004.

Kim, S., Yoo, K.S., Pike, L.M.: Development of a codominant PCR-based marker for allelic selection of the pink trait in onion *Allium cepa*, based on the insertion mutation in the promoter of the *anthocyanidin synthase* gene. - Theor. appl. Genet. **110**: 573-578, 2005.

Kim, S.H., Lee, J.R., Hong, S.T., Yoo, Y.K., An, G., Kim, S.R.: Molecular cloning and analysis of anthocyanin biosynthesis genes preferentially expressed in apple skin. - Plant Sci. **165**: 403-413, 2003.

Kobayashi, S., Ishimaru, M., Ding, C.K., Yakushiji, H., Goto, N.: Comparison of UDP-glucose : flavonoid 3-*O*-glucosyltransferase *UFGT* gene sequences between white grapes *Vitis vinifera* and their spots with red skin. - Plant Sci. **160**: 543-550, 2001.

Koes, R.E.F., Quattrocchio, M., Mol, J.N.: The flavonoid biosynthetic pathway in plants: function and evolution. - Bioessays **16**: 123-132, 1994.

Kotepong, P., Ketsa, S., Van Doorn, W.G.: A white mutant of

Malay apple fruit *Syzygium malaccense* lacks transcript expression and activity for the last enzyme of anthocyanin synthesis, and the normal expression of a MYB transcription factor. - Funct. Plant Biol. **38**: 75-86, 2011.

Kubo, H., Nawa, N., Lupsea, S.A.: *Anthocyaninless1* gene of *Arabidopsis thaliana* encodes a UDP-glucose:flavonoid-3-O-glucosyltransferase. - J. Plant Res. **120**: 445-449, 2007.

Lacombe, E., Hawkins, S., Van Doorsselaere, J., Piquemal, J., Goffner, D., Poeydomenge, O., Grima Pettenati, J.: Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. - Plant J. **11**: 429-441, 1997.

Lukacin, R., Britsch, L.: Identification of strictly conserved histidine and arginine residues as part of the active site in *Petunia hybrida* flavanone 3-beta-hydroxylase. - Eur. J. Biochem. **249**: 748-757, 1997.

Macheix, J.J., Fleuriet, A., Billot, J.: Fruit Phenolics. - CRC Press, Boca Raton 1990.

Martin, C., Carpenter, R., Sommer, H., Saedler, H., Coen, E.S,: Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the pallida locus by transposon tagging. - EMBO J. **4**: 1625-1630, 1985.

Martin, C., Prescott, A., Mackay, S., Bartlett, J., Vrijlandt, E.: Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. - Plant J. **1**: 37-49, 1991.

Montefiori, M., Espley, R.V., Stevenson, D., Cooney, J., Datson, P.M., Saiz, A., Allan, A.C.: Identification and characterisation of F3GT1 and F3GGT1, two lycosyltransferases responsible for anthocyanin biosynthesis in red-fleshed kiwifruit *Actinidia chinensis*. - Plant J. **65**: 106-118, 2011.

Murakami, K., Mihara, K., Omura, T.: The transmembrane region of microsomal cytochrome-P_{450} identified as the endoplasmic-reticulum retention signal. - J. Biochem. **116**: 164-175, 1994.

Nicoue, E.E., Savard, S., Belkacemi, K.: Anthocyanins in wild blueberries of Quebec: extraction and identification. - J. Agr. Food Chem. **55**: 5626-5635, 2007.

Niu, S.S., Xu, C.J., Zhang, W.S., Zhang, B., Li, X., Lin-Wang, K., Chen, K.S.: Coordinated regulation of anthocyanin biosynthesis in Chinese bayberry *Myrica rubra* fruit by a R2R3 MYB transcription factor. - Planta **231**: 887-899, 2010.

O'Neill, S.D., Tong, Y., Sporlein, B., Forkmann, G., Yoder, J.I.: Molecular genetic analysis of chalcone synthase in *Lycopersicon esculentum* and an anthocyanin-deficient mutant. - Mol. gen. Genet. **224**: 279-288, 1990.

Pawlowska, A.M., Oleszek, W., Braca, A. Quali-quantitative analyses of flavonoids of *Morus nigra* L. and *Morus alba* L., *Moraceae* fruits. - J. Agr. Food Chem. **56**: 3377-3380, 2008.

Qin, C.G., Li, N., Niu, W.Y., Ding, J., Zhang, R., Shang, X.Y.: Analysis and characterisation of anthocyanins in mulberry fruit. - Czech J. Food Sci. **2**: 117-126, 2010.

Santos, D.T., Cavalcanti, R.N., Rostagno, M.A., Queiroga, C.L., Eberlin, M.N., M. Meireles, A.A.: Extraction of polyphenols and anthocyanins from the jambul (*Syzygium cumini*) fruit jeels. - J. Food Public Health **3**: 12-20, 2013.

Schwinn, K., Venail, J., Shang, Y.J., Mackay, S., Alm, V., Butelli, E., Martin, C.: A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. - Plant Cell **18**: 831-851, 2006.

Sommer, H., Saedler, H.: Structure of the *chalcone synthase* gene of *Antirrhinum majus*. - Mol. gen. Genet. **202**: 429-434, 1986.

Wang, X., Olsen, O., Knudsen, S.: Expression of the *dihydroflavonol reductase* gene in an anthocyanin-free barley mutant. - Hereditas **119**: 67-75, 1993.

Wei, Y.Z., Hu, F.C., Hu, G.B., Li, X.J., Huang, X.M., Wang, H.C.: Differential expression of anthocyanin biosynthetic genes in relation to anthocyanin accumulation in the pericarp of *Litchi chinensis* Sonn. - PLoS One **6**: e19455, 2011.

Yamazaki, S., Sato, K., Suhara, K., Sakaguchi, M., Mihara, K., Omura, T.: Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. - J. Biochem. **114**: 652-657, 1993.

Zou, T.B., Wang, M., Gan, R.Y., Ling, W.H.: Optimization of ultrasound-assisted extraction of anthocyanins from mulberry, using response surface methodology. - Int. J. mol. Sci. **12**: 3006-3017, 2011.