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Anthocyanin accumulation and differential gene expression in wild-type and mutant *Syzygium malaccense* fruits during their growth and ripening

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Abstract

Ripe wild-type Malay apple (*Syzygium malaccense*) cv. Saraek fruit epidermis is red, whereas a mutant has a white skin. Wild-type and mutant fruit were used as a fruit model to study the regulation and gene expression patterns of anthocyanin biosynthesis and a myeloblastosis (MYB) transcription factor during growth and development. Fruit size, color, anthocyanin composition, and the expression of genes involved in anthocyanin biosyntheses were determined during fruit growth and ripening. Wild-type ripe fruit had a greater diameter and fruit mass than the mutant. The total anthocyanin content was approximately nine-fold higher in wild-type red fruit than in mutant white fruit. The major anthocyanin in the fruit skin of the wild-type was cyanidin-3-*O*-glucoside with minor amounts of pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and cyanidin-3,5-*O*-diglucoside. No anthocyanin was found in mutant fruit. The accumulation of cyanidin-3-*O*-glucoside during fruit growth and ripening was correlated with red color development and activities of phenylalanine ammonia lyase (PAL) and UDP-glucose:flavonoid-3-*O*-glucosyltransferase (UFGT). We cloned fragments and characterized seven genes involved in anthocyanin biosynthesis pathway namely *phenylalanine ammonia lyase* (*SmPAL*), *chalcone synthase* (*SmCHS*), *chalcone isomerase* (*SmCHI*), *flavanone-3-hydroxylase* (*SmF3H*), *dihydroflavonol 4-reductase* (*SmDFR*), *leucoanthocyanidin dioxygenase* (*SmLDOX*), and *UDP glucose-flavonoid-3-O-glucosyl transferase* (*SmUFGT*), as well as a *MYB transcription factor* (*SmMYB*). The expressions of all the genes were determined by semi-quantitative reverse transcription (RT)-PCR and quantitative real-time RT-PCR. The skin of wild-type fruit contained transcripts of all above mentioned genes, whereas the mutant fruit showed no *SmUFGT* and *SmMYB* expressions, which correlated with the absence of anthocyanin accumulation during fruit growth and ripening. These results suggest that lack of anthocyanin biosynthesis in mutant fruit may be *via* the regulation of *UFGT* and *MYB* transcription factor expressions.

Additional key words: chalcone synthase, cyanidin-3-*O*-glucoside, Malay apple, MYB transcription factor, phenylalanine ammonia lyase, UDP glucose-flavonoid-3-O-glucosyl transferase.

Introduction

The genus *Syzygium* from the family *Myrtaceae* contains several species producing edible fruits. Their fruits are named 'apples' and indeed the crispiness of the fruit flesh is reminiscent of apples. The fruits of some commercially important species of *S.* are rather similar in form, but differ in taste. Depending on the cultivar, *S. samarangense* produces a green or pink skinned fruits, both called Java apple or Java rose apple whereas *S. malaccense* produces fruits called Malay apple, which has a dark red/purple skin. Synonyms used for Malay apple are Malacca apple or Malay rose apple. The Malay apple has an edible aril, which is white, juicy, sweet, and slightly-acid with a pleasant flavor, located inside a dark red to purple pericarp that is rich in bioactive secondary metabolites including

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Abbreviations: CHI - chalcone isomerase; CHS - chalcone synthase; DFR dihydroflavonol-4-reductase; F3GT - flavonol-3-O-glucosyltransferase; F3H - flavanone-3-hydroxylase; LDOX - leucoanthocyanidin dioxygenase; MS - mass spectrometry; MYB - myeloblastosis; PAL - phenylalanine ammonia lyase; RT - reverse transcription; UFGT - UDP-glucose:flavonoid-3-*O*-glucosyltransferase.
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anthocyanins, oligomeric proanthocyanins, and xanthones (Fu *et al*. 2007, Ji *et al*. 2007).

There are at least two cultivars of Malay apple grown in Thailand, called locally Mamieo and Saraek. Both cultivars are different in shape and color. 'Mamieo' is more ovoid and has a dark purple skin, whereas 'Saraek' has a stripe red skin. White mutants of both 'Mamieo' and 'Saraek' have been found in Thailand. Kotepong *et al*. (2011) reported that wild-type fruit of 'Mamieo' has a total anthocyanin content 30-times higher than white mutant fruit. Cyanidin-3-*O*-glucoside accounted for a large proportion of the total anthocyanin content.

Anthocyanins are water-soluble flavonoid pigments, which are found in higher plants and are responsible for red, blue, and purple color of many fruits, vegetables, flowers, and seeds. Anthocyanin biosynthesis involves a common branch of the flavonoid pathway and has been intensively investigated in many plants including *Arabidopsis*, petunia, maize, and grape. The regulation of anthocyanin biosynthesis is mainly at the level of transcription of the structural genes by transcription factors. The structural genes of the anthocyanin biosynthetic pathway have been isolated from many fruit and include *phenylalanine ammonia lyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone-3-hydroxylase (F3H*), *flavonoid-3'-hydroxylase* (*F3'H*), *dihydroflavonol-4-reductase* (*DFR*), *leucoanthocyanidin reductase* (*LDOX*), and *UDP-glucose:flavonoid-3-O-glucosyltransferase* (*UFGT*) (Winkel-Shirley 2002). In apple, analysis of transcript abundance of these genes during fruit development indicates that the expressions of most of the genes in the pathway (*CHS, F3H, DFR, LDOX*, and *UFGT*) are coordinated during anthocyanin accumulation in apple skin (Honda *et al*. 2002). The expression pattern in apple differs from that in grape (*Vitis vinifera* and *V. labruscana*), in which *UFGT* induction during anthocyanin accumulation is a key regulatory step in coloration (Kobayashi *et al*. 2001, Zhang *et al*. 2013). In mangosteen fruit, *GmMYB10* appears to play a role in the specific regulation of anthocyanin biosynthesis both on-tree and off-tree. The *GmUFGT* may be a key biosynthetic gene in mangosteen pigmentation (Palapol *et al*. 2009a). The transcription factors which control anthocyanin biosynthesis include myeloblasosis (MYB) transcription factors, basic helix-loop-helix transcription factors, and a repeat-containing protein WD40 (Broun 2005, Koes *et al.* 2005). Plant MYB transcription factors play important roles in many secondary metabolic pathways including anthocyanin and flavonol. Myeloblastosis transcription factors are classified by conserved imperfect repeats in the DNA-binding domain, termed R1, or R2R3. There are 126 R2R3 MYB genes in *Arabidopsis* (Stracke *et al.* 2001) and 108 in grape (Matus *et al.* 2008). In grape, mutation were found in two genes encoding MYB transcription factors that regulate *flavonol-3-O-glucosyltransferase* (*F3GT*) expression (Kobayashi *et al*. 2004, Walker *et al*. 2007).

The aim of this work was to compare content of anthocyanins, which are associated with color development, and their composition in fruit skin of wild-type and mutant Malay apple. We tried to show how anthocyanins accumulation at different fruit stages is related to enzyme activities and expressions of anthocyanin biosynthetic genes and the MYB transcription factor. We tested the hypothesis that lack of red color in the mutant is due to lack of a regulatory gene in anthocyanin biosynthetic pathway.

Materials and methods

Fruit collection: The white mutant tree of Malay apple (*Syzygium malaccense* (L.) Merr. & L.M. Perry) 'Saraek' was found naturally by a grower who planted the wild type of Malay apple, which were propagated by layering. Malay apple with red (wild-type) and white (mutant) skin were obtained from the same orchard in the Nonthaburi province of central Thailand. Trees of both wild type and mutant of Malay apple were about 20 years old and were approximately 3 m high. Flowers were tagged at full bloom. Fruits were bagged at stage 2 (Fig. 1 Suppl.). Samples of 30 fruits were collected randomly from several trees throughout the growing season. Developmental stages were divided into 8 stages: stage 1 - 21 d after full bloom (DAFB); stage 2 - 28 DAFB; stage 3 - 35 DAFB; stage 4 - 42 DAFB; stage 5 - 49 DAFB; stage 6 - 52 DAFB; stage 7 - 55 DAFB, and stage 8 - 58 DAFB which is a fully ripe stage (Fig. 1 Suppl.). Stage 7 is the normal picking stage for export markets whilst fruit is picked at stage 8 if sold at local markets. After harvest, fruits were transported in an air-conditioned car (25 °C) to the laboratory within 2 h.

Fruit growth was determined at each fruit developmental stage. An individual fresh mass was measured using a digital balance (*AND*, Tokyo, Japan). Fruit width and fruit diameter were measured using a digital vernier caliper (*Insize*, GA, USA). Fruit skin color was measured using a *CR-300 Chromameter* (*Minolta*, Osaka, Japan) as L^*, a^*, and b^* values (the *CIE* color system, Selahle *et al.* 2015) and converted to a hue angle (a color wheel with red-purple at an angle of 0^o, yellow at 90^o, and bluish-green at 180^o). Both high a^* and high b^* values and low hue angles describe the intensity of red and yellow colors of the material whereas L^* values describe its lightness (Selahle *et al.* 2015). The color reading was taken twice at the top, middle, and bottom regions of each fruit and then averaged to estimate a value for each fruit. After fruit skin color measurement, the fruit skin was peeled off carefully with a knife. The samples of the fruit skin were frozen immediately in liquid nitrogen and stored at -86 C until used for further experiments.

Anthocyanin analysis: Total anthocyanins were extracted from fruit skin according to the method described by Piccaglia *et al.* (2002). One gram of each sample was homogenized with 20 cm^3 of methanol (99.9 %) plus HCl (37 %) (99:1, v/v) using a *Polytron PT 2100* (*Kinematica*, Luzern, Switzerland) with a 1.2 mm diameter head at a speed of 12 for 1 min and then shaken at 4 °C in darkness for 6 h. The aqueous phase was removed and the pellet was re-extracted four times with 20 cm^3 of methanol (99.9 %) plus HCl (37 %) (99:1, v/v) within 24 h and then adjusted

to a final volume of 100 cm^3 with the same solution. The combined aqueous extracts were centrifuged at 8 000 *g* and 4 °C for 10 min to remove the pellet. The absorbance of the supernatant was measured at 530 nm using a *Genesys 10UV* spectrophotometer (*Thermo Spectronic*, Rochester, NY, USA). Total anthocyanin content was expressed as cyanidin equivalents (a molar absorbance of 34.3). All measurements were made in triplicate. The samples were kept in an amber bottle at -86 °C until individual anthocyanins were quantitified.

Individual anthocyanins were analyzed by HPLC and LC tandem mass spectrometry (MS/MS), using a modified method described by Stevenson *et al.* (2006). The samples were filtered through a syringe filter, a 0.45 µm nylon membrane (*Chromtech*, Apple Valley, MN, USA) before injection. Individual samples were analyzed using a *Shimadzu* analytical HPLC with a column oven, auto-sampler injector, vacuum solvent degas module, and *LC-10AD VP* detector (*Shimadzu*, Kyoto, Japan). Separation was performed on two connected columns. The first was a guard column to filter out any contamination (using a 3 mm diameter security guard cartridge; *Phenomenex*, Torrance, CA, USA), the second was a *Synergi* 250 × 4.6 mm column filled with *Polar RP* 4 µm diameter particles, 8 nm pore size (*Phenomenex*). A gradient of two mobile phases was used: A: acetonitrile (99.9 %) + formic acid (98 %) (99:1, v/v) and B: acetonitrile (99.9 %) + water + formic acid (98 %) (5:92:3, v/v/v). A flow rate was 1 $cm^3\ min^{-1}$ at a column temperature of 45 °C. The gradient consisting of the mobile phase A was 0 % at 0 min and ramped linearly to 20 % at 20 min, to 30 % at 26 min, to 50 % at 28.5 min, to 95 % between 32 and 35 min, and back to 0 % between 36 and 42 min. The individual anthocyanins were identified at 520 nm using the retention times and by comparison with standard solutions of known concentrations.

The LC-MS/MS analysis of samples at stage 8 (Fig. 1 Suppl.) was carried out using a mass spectrometer (*Bruker*, Bellerica, MA, USA) equipped with an electrospray ionization source. Separation was performed on a 150 × 4.6 mm column, *2XHypersil Gold*, 3 µm particle diameter. The anthocyanin fractions were analyzed in the positive ion mode using both a molecular ion mass (M+), and a tandem mass spectrogram (MS/MS) of the fractions at 530 nm, and comparison of retention times and fragmentations with an authentic commercial standard of cyanidin-3-*O*-glucoside (*Polyphenols*, Sandnes, Norway).

Activities of PAL and UFGT: Enzymes were extracted from fruit skin according to Lister *et al*. (1996a) with a slight modification. The frozen fruit skin (3 g) at each stage was homogenized in 15 cm^3 of 50 mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing 50 mM sodium ascorbate, 18 mM 2-mercaptoethanol, 2 g of polyvinylpyrrolidone, and 0.1 % (v/v) *Triton X-100*. The sample was homogenized using a *Polytron PT 2100* (*Kinematica*, Luzern, Switzerland) with a 1.2 mm diameter head at a speed of 12 for 1 min. The sample was incubated on ice for 10 min, then centrifuged at 20 000 *g* for 20 min to remove particulate matter. Ammonium sulphate was added to the supernatant at 35 % saturation followed by centrifugation at 20 000 *g* for 20 min to remove polyvinylpyrrolidone, and more ammonium sulfate was added to reach a final saturation of 80 %. This fraction was centrifuged at 20 000 *g* for 20 min. Afterwards, the pellet was re-suspended in 3 cm^3 of the homogenization buffer without polyvinylpyrrolidone and *Triton X-100* and dialyzed with a dialysis buffer (the homogenization buffer without polyvinylpyrrolidone and *Triton X-100*) overnight to give a partially purified extract,which was used for PAL and UFGT enzyme activities. All enzyme extractions steps were carried out at 4 °C. The enzyme extract was frozen in liquid nitrogen and stored at -86 °C until enzyme activity analyses.

Activity of PAL was measured using the method of Lister *et al*. (1996a) with a modification. A reaction mixture comprised 0.2 cm^3 of the enzyme extract and 0.8 cm^3 of 60 mM borate buffer (pH 8.8). The reaction was initiated by addition of 0.2 cm^3 of L-phenylalanine solution (10 mg dm^{-3}) and incubated at 30 °C in a water bath for 1 h. The reaction was stopped by adding 125 mm^3 of 40 % (m/v) trifluoroacetic acid and then centrifuged at 12 000 *g* and 4 °C for 5 min to pellet the denatured protein. Activity of PAL was assesed by measuring absorbance at 290 nm using a *Genesys 10UV* spectrophotometer (*Thermo Spectronic*, Rochester, NY, USA). Triplicate assays were performed for each enzyme extract. One unit of PAL was defined as the yield of 1 µmol of *trans*-cinnamic acid per mg of protein.

Activity of UFGT was measured using the method of Lister *et al*. (1996b) with a modification. A reaction mixture comprised 0.2 cm^3 of the enzyme extract, 0.2 cm^3 of 50 mM bicine buffer (pH 8.5), 30 mm^3 of 1 mM quercetin, and 20 mm^3 of 2.5 mM UDP-glucose. The reaction mixture was incubated in a water bath at 30 °C for 30 min. The reaction was halted by addition of 0.15 cm^3 of 20 % (m/v) trichloroacetic acid in methanol (100 %) and then centrifuged at 12 000 *g* and 4 °C for 5 min. The supernatant was stored at -86 °C until quantification by HPLC. The samples were filtered through a syringe filter, a 0.45 µm nylon membrane (*Chromtech*, Apple Valley, MN, USA) before injections. Analysis of quercetin-3-*O*-glucose was performed using a 50 mm NH_2 column *Inersil®*, 5 mµ particle diameter, with a 4.0 × 10 mm HPLC guard column, *Inersil® ODS-3*, 5 µm particle diameter. A flow rate was 1 $cm^3\ min^{-1}$ at a column temperature of 30 °C. An injection volume was 20 mm^3. Mobile phase were: A: acidified water (0.66 M formic acid) and B: acidified methanol (0.66 M formic acid in 100 % methanol) in a linear gradient, where A:B ratio changed from 50:50 to 15:85 during the first 5 min followed by an isocratic mixture for 1 min and back to the initial conditions. The product was detected at 350 nm and compared with a standard (quercetin-3-*O*-glucoside). One unit of UFGT was defined as the production of 1 mol quercetin-3-*O*-glucoside per second. Protein content in the enzyme extract was measured using the method described by Bradford (1976) with bovine serum albumin as a standard.

Extraction of RNA and cDNA synthesis: Approximately 5 g of frozen samples were ground in a *Retsch MM 301* mixing mill (*Retsch*, Newtown, PA, USA). Total RNA was isolated from 2 g of fruit skin tissue as described by Lopez-Gomez and Gomez-Lim (1992). The total RNA was treated with *DNase I* (RNase free, *Fermentas*, Burlington, Canada). The first strand cDNA was synthesized from 4 µg of total RNA following the protocol of an *Omniscript* RT kit (*Qiagen*, Hilden, Germany) as a template for semi-quantitative RT-PCR and quantitative real-time RT-PCR.

Isolation of anthocyanin biosynthesis genes and a MYB transcription factor: Athocyanin biosynthesis genes and a MYB transcription factor were isolated from stage 8 of wild-type fruit skin using forward and reverse degenerate primers (Table. 1 Suppl.) based on conserved regions of similar genes in the *NCBI* database. The amplification reactions of anthocyanin biosynthesis genes were as follows: 94 °C for 30 s and 5 min and then 54 °C for 30 s (*SmPAL*, *SmCHI*) or 56 °C for 30 s (*SmDFR, SmCHS, SmF3H, SmLDOX,* and *SmUFGT*) with a final period at 72 °C for 30 s and 10 min. For the isolation of a MYB transcription factor, degenerate primers and amplification reactions were carried out as described by Takos *et al*. (2006).

The amplified PCR fragments from each gene were purified using a gel extraction kit (*QIAquick®* gel extraction, *Qiagen*, Hilden, Germany) and cloned into a pGEM-T vector (*pGEM®-T Easy*, *Promega*, Fitchburg, WI, USA). Transformation of the vector with the inserted DNA was conducted using *Escherichia coli* strain DH5α cells. Plasmid DNA containing the vector and inserted DNA was extracted using a miniprep kit (*QIAprep® Spin Miniprep* kit, *Qiagen*). The sequence analysis of the clone was conducted by automatic sequencing using an *ABI PRISM®377* DNA sequencer (*Applied Biosystems*, Foster City, CA, USA). The sequences of all amplified fragments were compared with the genes in the *Genbank* database using the *BLAST* program from *NCBI*. A new pair of specific primers for the gene were encoded for all genes (Table 1 Suppl.) and used for semi-quantitative RT-PCR and real-time quantitative PCR. Specific primers were tested for specificity using plasmid amplification and the product analyzed on a 1.2 % (m/v) agarose gel stained with ethidium bromide. All primers were designed with *Primer3* (*v. 0.4.0*; http://frodo.wi.mit.edu/primer3). The size of quantitative PCR products ranged from 121 to 181 bp (Table 2 Suppl.).

Gene expressions: Gene expressions were analyzed using real time quantitative PCR. Fifty-fold diluted cDNA was used. Quantitattive PCR was performed using an *ABI Prism® 7000* real-time PCR machine (*Applied Biosystems*, Foster City, CA, USA) using *SYBR green* (*QuantiTectTM SYBR®Green* PCR kit, *Qiagen*, Hilden, Germany) following the manufacturer's instructions. All reactions were performed in triplicate using 3 mm^3 of diluted template (50 ×), 1 mm^3 of each forward and reverse primers (2.5 µM), and 12.5 mm^3 of 2× *Master mix* to a final volume of 25 mm^3. Reactions were initiated at 95 °C for 15 min followed by 40 cycles of 95 °C for 5 s, 60 °C for 5 s, 72 °C for 30 s and completed by a melting curve analysis. A negative water control and a melting curve were included in every run. A melting peak, a dissociation curve, and sequencing were analyzed to confirm the expected product rather than the primer dimmer. The data were normalized to *actin* transcription (*SmACT*) to minimize variation in cDNA template levels.

Statistical analysis: Experimental data were analyzed statistically by *ANOVA*, and significance of the differences between means was estimated by the Duncan's new multiple range test (DMRT) at $\alpha = 0.05$. Experimental data are averages of three replications ± standard errors. All experiments were repeated at least twice.

Results

All Malay apple fruits were separated into eight stages of development. Wild-type fruits at stage 4 of development had a red striped skin at the proximal part of the fruit. The streaked red color subsequently covered the remainder of the fruit. The color became darker over time, and fruits were dark red at stage 8 (Fig. 1 Suppl.). Fruit of the mutant had a light green color from stage 1 to stage 4. The green changed to white or creamy with a very light pink during stages 5 to 8 (Fig. 1 Suppl.). Both wild-type and mutant fruits had characteristic shapes. Mutant fruit was more ovoid than the wild-type fruit. The fruits of the wild-type tended to have a greater diameter (Fig. 1*A-D*), height (Fig. 1*B-E*), and mass (Fig. 1*C-F*) than those of the mutant.

The L* values of wild-type fruits remained stable from stage 1 to stage 3 and then decreased sharply during maturation and ripening (stages 6 to 8), whereas L* values of mutant fruits slightly increased from stage 1 to stage 8 (Fig. 2*A-E*). The a* values of both wild-type fruits and mutant fruits did not change during stages 1 to 3 and then increased rapidly in wild-type fruits and increased steadily in mutant fruits from stage 4 to stage 8 (Fig. 2*B-F*). Mutant fruits had higher b* values than wild-type fruits from stage 1 to stage 7, but no significant difference at stage 8 (Fig. 2*C-G*). Mutant fruits also had higher hue values than wild-type fruits from stage 5 to stage 8 (Fig. 2*D-H*).

A considerably higher total anthocyanin content was observed in wild-type fruits than in mutant fruits at all stages of growth except stage 1 (Fig. 3*A-B*). When fruits were ready to harvest (stage 7), the total anthocyanin content of wild-type fruits was 5-times higher than that of mutant fruits, whereas at the ripe stage (stage 8), wild-type fruits had a 9-times higher total anthocyanin content (Fig. 3*A-B*).

Four anthocyanins were detected in the fruit skin with similar retention times to the standards (Fig. 2*A* Suppl., Table 3 Suppl.). The major anthocyanin in the skin of ripe fruits was cyanidin-3-*O*-glucoside with the minor content of pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and cyanidin-3,5-*O*-diglucoside (Fig. 2*A* Suppl., Fig. 3 Suppl. and Table 3 Suppl.). No anthocyanins were found in the epidermis of mutant fruits (Fig. 2*B* Suppl.). The

major anthocyanin cyanidin-3-*O*-glucoside content in the epidermis of wild-type fruits increased rapidly during growth and ripening (Fig. 4).

Activities of PAL in the fruit skin of both wild-type fruits and mutant fruits were comparable and increased continuously from stage 1 to 8 (Fig. 5*A-C*). Activity of UFGT in the skin of wild-type fruits increased steadily from stage 1 to stage 8 whereas UFGT activity in the skin of mutant fruits was below the detection limit at all stages of fruit growth (Fig. 5*B-D*).

From the skin of wild-type fruits at stage 8, we isolated seven partial coding sequences of genes involved in the anthocyanin biosynthetic pathway: *SmPAL* (accession No. GU233756; 381 bp; 126 amino acids), *SmCHS* (accession No. GU233757; 550 bp; 182 amino acids), *SmCHI* (accession No. GU233758; 484 bp; 161 amino acids), *SmF3H* (accession No. GU233759; 794 bp; 264 amino acids), *SmDFR* (accession No. 233760; 574 bp; 191 amino acids), *SmLDOX* (accession No. GU233761; 580 bp; 192 amino acids), and *SmUFGT* (accession No. GU233762; 947 bp; 351 amino acids) and a MYB transcription factor: *SmMYB* (accession No. HM773023; 249 bp; 83 amino acids).The possibility that differences in anthocyanin biosynthesis during growth and ripening in Malay apple were related to gene expression patterns was investigated. Seven anthocyanin-related genes were isolated by PCR from *S. malaccense* cDNA templates. All genes showed a high homology to other plant anthocyanin biosynthetic genes (Table 4 Suppl.). The Malay apple SmPAL, SmCHS, and SmCHI translated protein sequences showed the highest homology with *Pyrus pyrifolia*: a 72 - 97 % amino acid identity. The Malay apple SmF3H, SmDFR, SmLDOX, and SmUFGT showed a 55 - 91 % identity to *Pyrus cerasifera* (Table 4 Suppl.). The SmUFGT involved in the last step of glucose-based anthocyanin biosynthetic pathway showed a somewhat lower identity (55 - 63 %) at the amino acid level in comparison with other species (Table 4 Suppl.). Myeloblastosis transcription factors showed a 85 - 88 % identity with red fruits of cherry plum, grape, litchi, mangosteen, and strawberry (Table 4 Suppl.).

Transcriptions of all seven genes were detectable in the skin of both wild-type fruits and mutant fruits (Fig. 6) with the exception of *SmUFGT* in the mutant, which was below the detection limit (Fig. 6*N*). In the wild-type, the transcript abundance of *SmPAL* increased slightly during early fruit growth, and a high transcript abundance was found during ripening (Fig. 6*A*). In mutant fruits, the transcript abundance of *SmPAL* remained stable during fruit growth and ripening (Fig. 6*H*). The transcript abundances of *SmCHS*, *SmCHI*, *SmF3H*, *SmDFR*, and *SmLDOX* were similar in wild-type and mutant fruits (Fig. 6*B-F*, *I-M*). In wild-type fruits, *SmUFGT* transcription gradually increased from stage 3 to stage 7 and then increased rapidly at stage 8 with a more than an 80-fold increase (Fig. 6*G*). In the wild-type, the transcription of

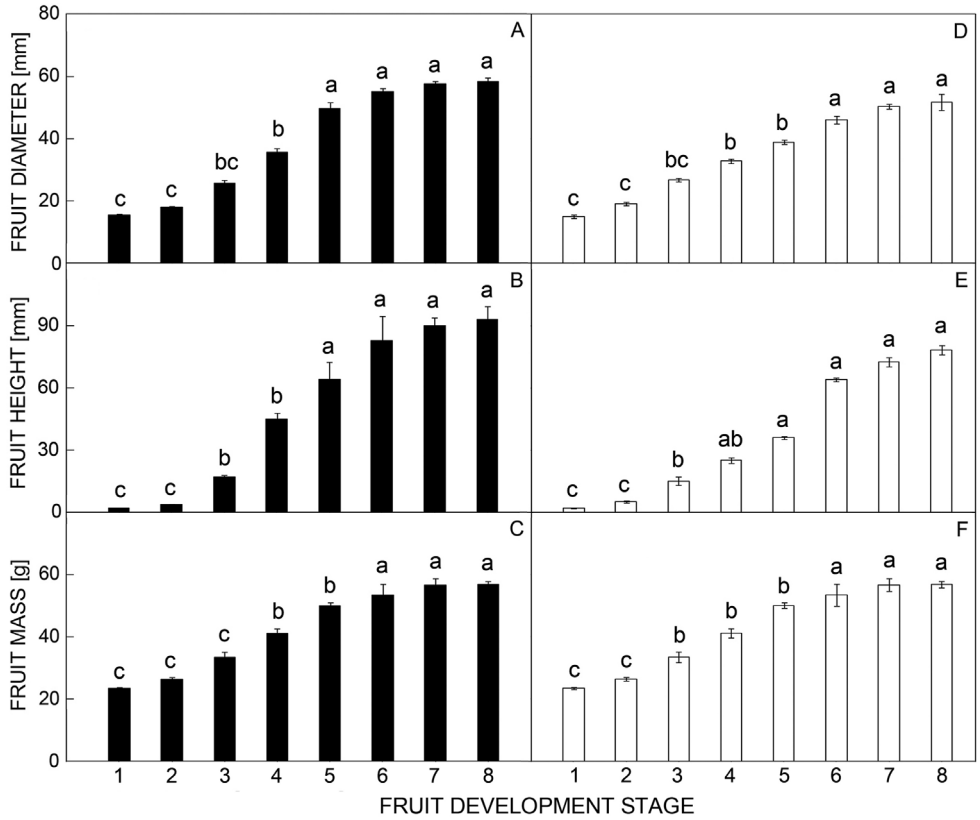

Fig. 1. Changes in diameter (*A*,*D*), height (*B*,*E*), and mass (*C*,*F*) of wild-type (■) and mutant (□) Malay apple fruits during their growth and ripening. Means ± SEs, $n = 3$. Different letters indicate significant differences according to the Duncan's new multiple range test ($P < 0.05$).

the *SmMYB* showed a more than 28-fold gradual increase from stage 3 to stage 7 and then a decrease at stage 8 (Fig. 7*A*). In mutant fruits, the transcript abundance of *SmMYB* remained very low (Fig. 7*B*).

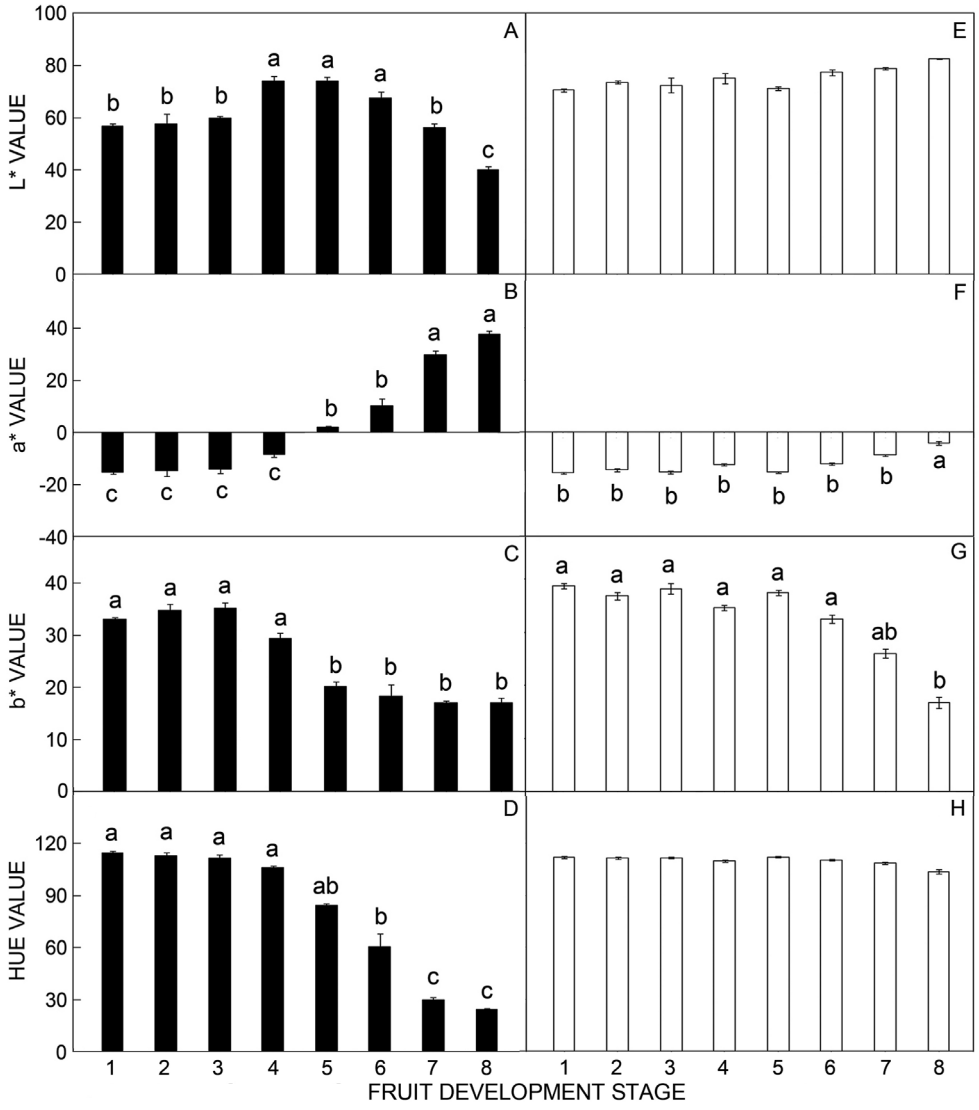

Fig. 2. Changes in L*(*A*,*E),* a*(*B*,*F*), b* (*C*,*G*), and hue (*D*,*H*) values of wild-type (■) and mutant (□) Malay apple fruits during their growth and ripening. Means ± SEs, $n = 3$. Different letters indicate significant ($P < 0.05$) differences according to the Duncan's new multiple range test.

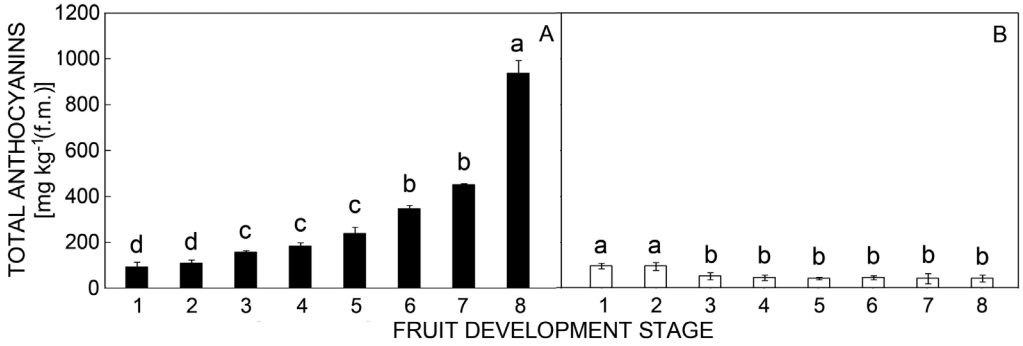

Fig. 3. Changes in total anthocyanin content in the skin (*A*,*B*) of wild-type (■) and mutant (□) Malay apple fruits during their growth and ripening. Means ± SEs, $n = 3$. Different letters indicate significant differences according to the Duncan's new multiple range test ($P < 0.05$).

Discussion

During fruit growth, we observed a large increase in skin anthocyanin content in wild-type fruits from the early stage to the ripe stage (stages 1 - 8). Ripe wild-type fruits had a much higher skin anthocyanin content than ripe mutant fruits. Higher skin anthocyanin content of ripe wild-type fruits was related to the deepness of the red color. This was reflected in the measured lower b*, L* and hue values and higher a* values in the wild-type compared to the mutant. A similar increase of anthocyanin content during ripening has also been reported in many other fruits such as avocado (Ashton *et al*. 2006), lychee (Cronje 2008), apple (Awad and De Jager 2002), blueberry (Kalt *et al*. 2003), muscadine grape (Lee and Talcott 2004), grape (Segade *et al*. 2008), mangosteen (Palapol *et al*. 2009b), and mango (Sivankalyani *et al*. 2016). Ripe wild-type fruits of Malay apple 'Mamieo' with a dark purple skin contain total anthocyanin content about 3 times higher than 'Saraek' (Kotepong *et al.* 2011). This suggests that anthocyanin accumulation in wild-type fruits correlates well with red color development. Although the fruits of the white mutant at stage 1 contained the amount of anthocyanins similar to the wild-type, a very low anthocyanin content was found at further stages of growth and ripening.

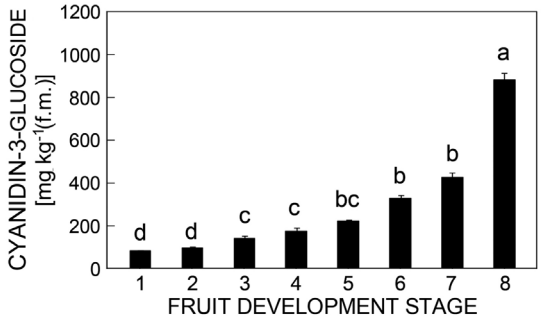

Fig. 4. Changes in cyanidin-3-*O*-glucoside content of the skin of wild-type Malay apple fruits during their growth and ripening. Means ± SEs, n = 3. Different letters indicate significant differences according to the Duncan's new multiple range test ($P < 0.05$).

The major anthocyanin found in the skin of wild-type fruits was cyanidin-3-*O*-glucoside confirming that already reported (Kotepong *et al*. 2011). Its increase accounted for most of the increase in the total anthocyanin content during the late stages of fruit growth and ripening. Red color development in the wild-type fruit skin was closely correlated with the strongly increasing content of cyanidin-3-*O*-glucoside. Cox *et al.* (2004) reported a similar increase in cyanidin-3-*O*-glucoside content during avocado ripening, which correlates with red coloration of the skin. The major anthocyanins in avocado also include cyanidin-3-*O*-glucoside (Ashton *et al.* 2006). In apple, the content of cyanidin-3-galactoside increases during maturation and ripening coinciding with an increase in red blush (Whale and Singh 2007). Small amounts of the other anthocyanins including pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, and cyanidin-3,5-*O*-diglucoside were also detected in the fruit skin of wild-type Malay apple. No anthocyanin was found in the mutant fruit skin. In the absence of other pigments, the dramatic increase in content of cyanidin-3-*O*-glucoside alone explains the final red appearance of the wild-type fruits. The PAL and UFGT are important enzymes in anthocyanin biosynthesis (Xie *et al*. 2011). The activities of PAL and UFGT in the wild-type fruit skin continuously increased during fruit growth and ripening. Both PAL and UFGT activities showed positive correlations with the total anthocyanin content of red wild-type fruit. Activity of PAL was also found in the white mutant skin. This indicates that PAL may not be a key enzyme regulating anthocyanin synthesis in white mutant fruit of Malay apple, which is similar to that reported for apple (Liu *et al*. 2013). The increase in PAL activity in red skin fruit during fruit growth and ripening was involved in an increase of anthocyanin biosynthesis whereas PAL

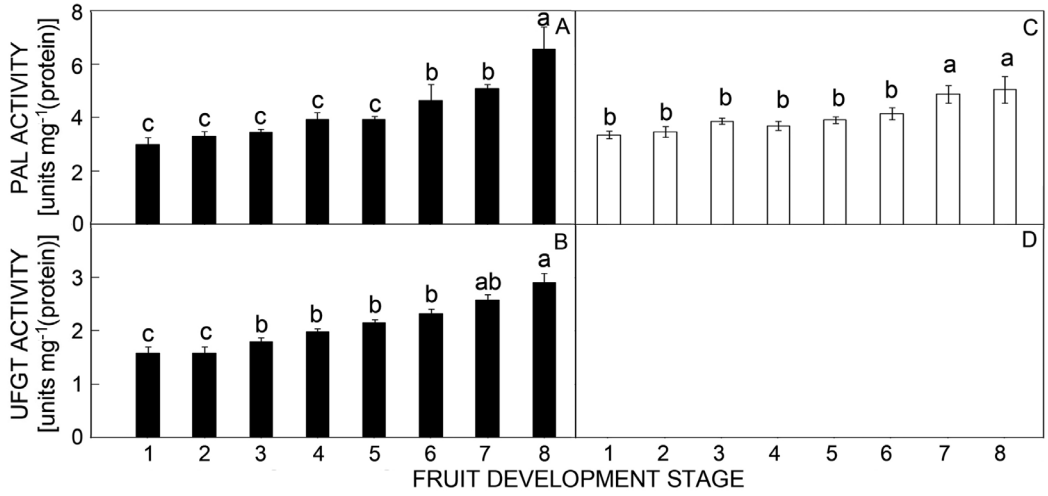

Fig. 5 Phenylalanine ammonia lyase (PAL) (*A,C*) and UDP-glucose:flavonoid-3-*O*-glucosyltransferase (UFGT) (*B*,D) activities in the skin of wild-type (■) and mutant(□) of Malay apple fruits during their growth and ripening. Means ± SEs, n = 3. Different letters indicate significant differences according to the Duncan's new multiple range test ($P < 0.05$).

activity showed in white skin fruit may be involved in biosynthesis of other phenolic compounds. The PAL is the first enzyme in the phenylpropanoid pathway leading to a wide range of compounds, such as phenols, anthocyanins, flavonoids, and lignins, which are abundant in apple skin (Vogt 2010). Nevertheless, anthocyanins are probably the major phenolic compounds being synthesized in red skin of many fruits during ripening, and the total anthocyanin

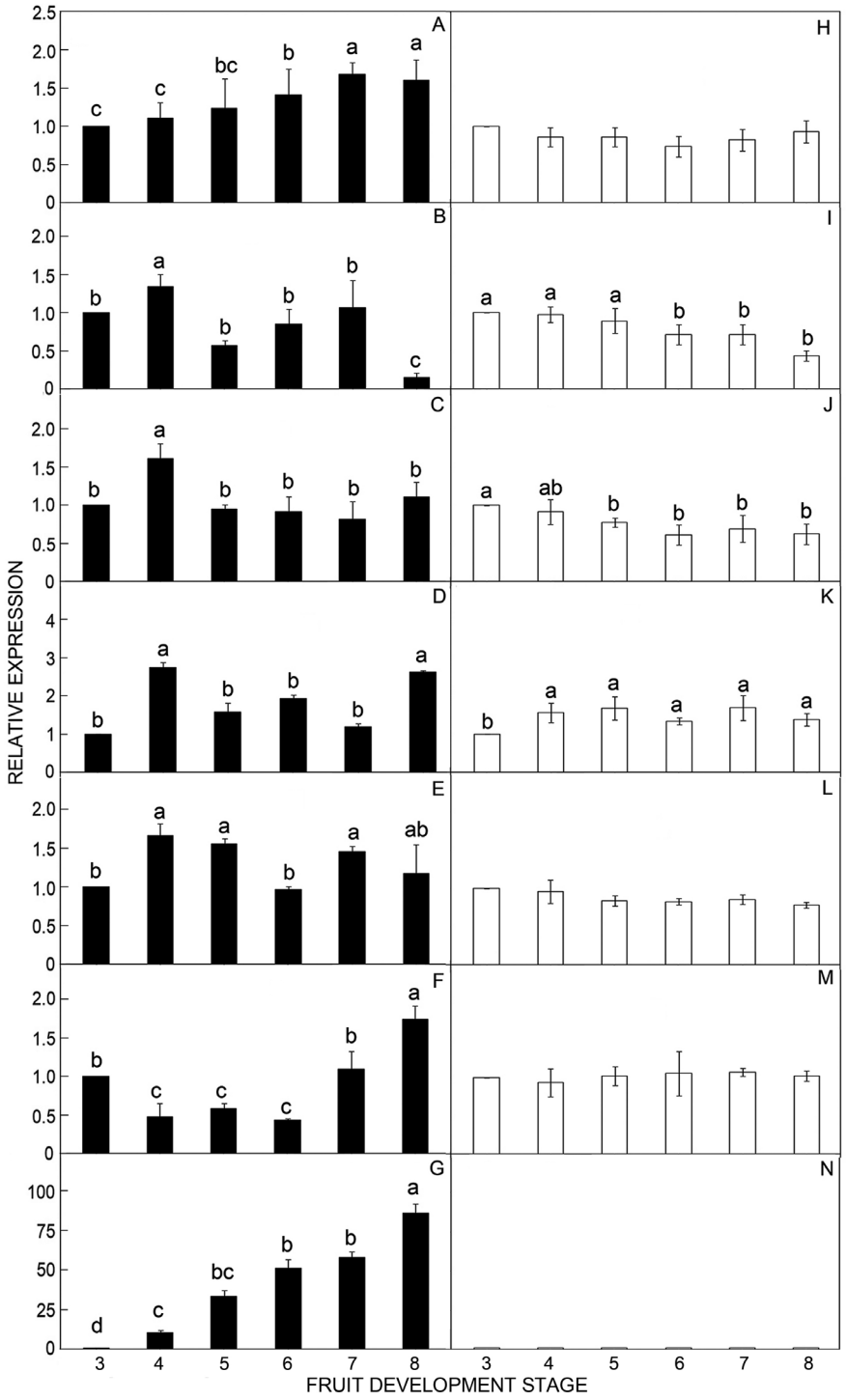

Fig. 6. Relative expressions of anthocyanin biosynthesis genes in Malay apple wild-type (■) and mutant (□) fruits during their growth and ripening. Real-time PCR was used to analyze *phenylalanine ammonia lyase* (*A*,*H*), *chalcone synthase* (*B*,*I*), *chalcone isomerase* (*C*,*J*), *flavanone-3-hydroxylase* (*D*,*K*), *dihydroflavonol-4-reductase* (*E*,*L*), *leucoanthocyanidin dioxygenase* (*F*,*M*), and *UDP-glucose:flavonoid-3-O-glucosyltransferase* (*G*,*N*) expression patterns. The column heights indicate relative mRNA abundances relative to stage 3, which was set to 1.0. All real time-PCR reactions were normalized using the *actin* gene. Means ± SEs, $n = 3$. Different letters indicate significant differences according to the Duncan's new multiple range test ($P < 0.05$).

content correlated well with PAL activity. According to Ju *et al.* (1995b), PAL catalyzes a reaction producing precursors of anthocyanin synthesis under conditions of sufficient resources, but changes in anthocyanin accumulation can occur independently of changes in PAL activity. In ripe apples, anthocyanin accumulation decreased even though PAL activity was relatively high. Therefore, the maximum PAL activity is not the regulating factor for anthocyanin accumulation in ripe apple (Wang *et al.* 2000).

Activity of UFGT was also positively correlated with anthocyanin accumulation during fruit growth and ripening in wild-type Malay apple fruit. Similarly, changes in UFGT activity parallel those in PAL activity in ripening grape (Mori *et al*. 2004). These data suggested a parallel upregulation of the activities of several enzymes involved in anthocyanin biosynthesis. Our data furthermore suggest that the absence of UFGT activity in the mutant fruit skin did not block PAL activity. Although a correlation is often found between PAL activity and anthocyanin synthesis in fruit skin, PAL activity is not the regulating factor for anthocyanin accumulation in the fruit (Wang *et al.* 2000). Our data also suggest independence in the regulation of PAL and UFGT activities.

Ju *et al.* (1995a) reported that the importance of UDP-galactose:flavonoid-3-*O*-glucosyltransferase in regulating anthocyanin biosynthesis in apples probably depends on the availability of its precursor, namely cyanidin. In strawberry fruit, changes in UFGT activity has been shown to parallel with PAL activity (Xu *et al.* 2014). This suggests that anthocyanin biosynthesis is closely associated with developmental stages of the fruit. In strawberry, flavonoid-3-*O*-glucosyltransferase activity is not essential for redirection from flavonol to anthocyanin formation during fruit ripening (Halbwirth *et al*. 2006). It has been shown that flavonoid enzyme activity peaks during fruit ripening at early and late developmental stages (Halbwirth *et al*. 2006). In grape, an increase in UFGT activity is concomitant with a rise in anthocyanin biosynthesis (Mori *et al*. 2005). This indicates that UFGT is required for anthocyanin biosynthesis in red coloration of many fruits. This close correlation between UFGT activity and anthocyanin content suggests a causal relation. Therefore, a key step for regulation of anthocyanin biosynthesis in Malay apple was likely to be at the final step of the pathway controlled by UFGT rather than a step controlled by PAL.

The transcripts of *SmPAL*, *SmCHS*, *SmCHI*, *SmF3H, SmDFR,* and *SmLDOX* in both the wild-type and the mutant of Malay apple 'Saraek', were comparatively abundant throughout the fruit growth and ripening period. This suggests *SmPAL*, *SmCHS*, *SmCHI*, *SmF3H, SmDFR*, and *SmLDOX* are not key genes regulating anthocyanin biosynthesis in Malay apple. It was reported that expression of *FlPAL6* during fruit ripening correlates with the rapid accumulation of anthocyanin in strawberry (Pombo *et al.* 2011). In contrast, transcript abundances of *CHI* and *DFR* genes were high during the early stage of fruit development whereas there was no anthocyanin accumulation in strawberry (Li *et al*. 2001).

The transcription of *SmUFGT* increased markedly with red coloration during fruit growth and ripening. In the wild-type Malay apple 'Mamieo', the largest increase (more than 160-fold) was found in the transcription of the *SmUFGT* from stage 3 to stage 7 and a decline at the final stage (stage 8) whereas the largest increase (more than 80-fold) from stage 3 to stage 8 was found in the wild-type Malay apple 'Saraek'. Those increases in *SmUFGT* expressions were correlated with an increase in fruit skin color, the total anthocyanin content, cyanidin-3-*O*-glucoside content, and UFGT activity. The expression pattern of the *SmUFGT* was similar to the expression of an *UFGT* in strawberry fruit (Almeida *et al.* 2007). The *UFGT* catalayzes anthocyanidin glycosylation at a final step of the anthocyanin biosynthetic pathway in grape fruit skin, which contains a high anthocyanin content. The role of *UFGT* in regulating anthocyanin accumulation in apple skin has been previously reported (Honda *et al.* 2002, Kondo *et al.* 2002, Zhang *et al*. 2013). Some studies reported a specific correlation between anthocyanin biosynthesis and *UFGT* expression (Kondo *et al*. 2002), other studies also reported the correlation of *UFGT* expression with other anthocyanin biosynthetic genes (Honda *et al*. 2002). Most of the anthocyanins in apple skin are galactosylated. It has been suggested that the *UFGT* gene isolated from apples encodes a galactosyl-transferase (Honda *et al.* 2002, Kondo *et al*. 2002). Walker *et al.* (2007) found that white grapes show no UFGT activity. They found that the white grapes have a detectable but

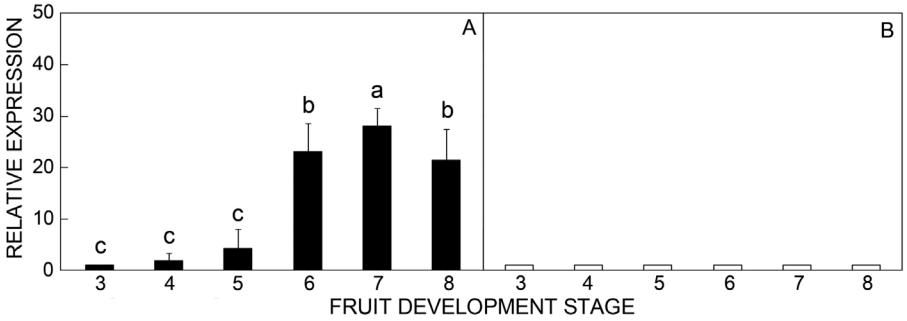

Fig. 7. Relative expression of the myeloblastosis transcription factor (*A*,*B*) in Malay apple wild-type (■) and mutant (□) fruits during their growth and ripening. Real-time PCR was used to analyze expression patterns. The column heights indicate relative mRNA abundances relative to stage 3, which was set to 1.0. All real time-PCR reactions were normalized using the *actin* gene. Means ± SEs, $n = 3$. Different letters indicate significant differences according to the Duncan's new multiple range test ($P < 0.05$).

very low amounts of *UFGT* transcripts. The sequence of the transcripts in the red and white grapes is the same showing that the mutation is not in the *UFGT* gene. It was established that a mutation in a MYB transcription factor is the cause of a low anthocyanin content. In contrast, we found no detectable *UFGT* transcription in the Malay apple mutant. This indicates that the mutation was either in the gene itself or in the transcription factor.

During red coloration, the transcription of the *Sm*Myb increased markedly with onset of red coloration during fruit development. This expression pattern is similar to that of *MdMYB10* in apple (Espley *et al*. 2007) and also similar to that of *VvMYBPA1* in grape berry skins (Bogs *et al*. 2007). Expression patterns therefore suggest that *GmMYB* is a candidate to regulate anthocyanin biosynthesis of Malay apple fruits. The expression patterns of anthocyanin biosynthesis genes correlated with that of *GmMYB10,* which increases strongly with the onset of color development. In grape, *VvMYBA1* and *VvMYBA2* regulate specifically the expression of the *UFGT* gene (Kobayashi *et al.* 2002, Walker *et al.* 2007). In other species, regulation of anthocyanin biosynthesis has been shown to be at the level of transcription in pigmented organs other than fruits (Mol *et al.* 1998, Schwinn *et al*. 2006) and transcription indicates a final phenotype including color, and anthocyanin content and composition (Castellarin and Di Gaspero 2007).

In conclusion, the present work shows that cyanidin-3-*O*-glucoside was the major anthocyanin in the red/purple skin of the fruits of Malay apple. The cyanidin-3-*O*-glucoside content markedly increased during wild-type fruit growth and maturation. This increase was correlated with color development, the activity of UFGT (the enzyme that apparently catalyses the last step of anthocyanin synthesis), and the expression of *SmUFGT*. The mutant, with a white fruit epidermis, lacked detectable anthocyanins, had no detectable UFGT activity, and no detectable expressions of *SmUFGT* and *SmMyb*. The *SmMYB* appears to play a role in the specific regulation of anthocyanin biosynthesis in Malay apple fruits. The *SmUFGT* may be a key gene in Malay apple pigmentation.

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