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THESIS

ANTHOCYANIN BIOSYNTHESIS AND GENE EXPRESSION DURING FRUIT GROWTH AND RIPENING OF MALAY APPLE (*Syzygium malaccense*) CULTIVARS

PANUMAS KOTEPONG

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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In Thailand, Malay apple (*Syzygium malaccense*) has two cultivars, namely 'Mamieo' and 'Saraek'. The fruit skin of mature Malay apple is initially glossy red, then changes to purple in cv. Mamieo and red streaks in cv. Saraek. Other group has been identified having mature fruits with white skin in both cultivars. Therefore, both cultivars of red and white fruit were used as a fruit model to study the regulatory mechanisms and gene expression of anthocyanin biosynthesis. The results showed that the fruit growth pattern showed a single sigmoidal curve in all Malay apple cultivars. During ripening, firmness and titratable acidity (TA) decreased significantly, while soluble solids content (SSC) and SSC/TA ratio increased rapidly in all Malay apple cultivars. Anthocyanin pigments were also extracted from fruit skin, and identified by HPLC and LC-MS. The skin of red fruit of both Malay apples contained five glucose-based anthocyanins namely cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, cyanidin-3,5-*O*-diglucoside and peonidin-3,5-*O*-diglucoside but peonidin-3,5-*O*-diglucoside was not found in cv. Saraek. Cyanidin-3-*O*-glucoside accounted for a large proportion of the total anthocyanin content which markedly increases during fruit growth and ripening in both red fruits. The cyanidin-3-*O*-glucoside content in the skin of red Malay apple fruit cv. Mamieo was higher than red Malay apple fruit cv. Saraek at the red maturity stage. No anthocyanins were found in both white fruits. The accumulation of cyanidin-3-*O*-glucoside during fruit growth and ripening was correlated with colour development, increased activities of phenylalanine ammonia lyase (PAL) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UF3GlucT; here abbreviated to UFGT). The partial-length of 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*) was characterized. The expression of all genes was determined by semi-quantitative RT-PCR and quantitative real-time RT-PCR. In the red fruit skin, transcripts were detected of seven genes that encode enzymes in the anthocyanin biosynthetic pathway. The expression patterns of *SmUFGT* correlated with anthocyanin accumulation. The skin of both white fruits contained transcripts of all seven genes identified, except *SmUFGT*. The skin of both white fruits have no UFGT activity. The data indicated that the lack of anthocyanins in the white fruits is due to lack of *SmUFGT* expression. *SmUFGT* is a key biosynthetic gene in Malay apple pigmentation.

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Student's signature Thesis Advisor's signature

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LIST OF ABBREVIATIONS

1-MCP	=	1-methylcyclopropene
ANS	=	anthocyanidin synthase
bHLH	=	Basic helix-loop-helix
BLAST	=	basic local alignment search tool
bp	=	base pairs
BSA	=	bovine serum albumin
cDNA	=	complementary deoxyribonucleic acid
CHI	=	chalcone isomerase
CHS	=	chalcone synthase
*C*p	=	crossing point
C_T	=	threshold cycle
DEPC	=	diethyl pyrocarbonate
DFR	=	dihydroflavonol 4-reductase
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
dNTPs	=	deoxynucleotide triphosphate (s)
EDTA	=	ethylene diamine tetraacetic acid
F3H	=	flavanone 3-hydroxylase
F3'H	=	flavanoid 3'-hydroxylase
F3'5'H	=	flavanoid 3',5'-hydroxylase
F3GT/UFGT	=	UDP glucose:flavonoid 3-*O*-glucosyltransferase
HPLC	=	high performance liquid chromatography
LB medium	=	Luria-Bertani medium
LC-MS	=	liquid chromatography mass spectrometry
LDOX	=	leucoanthocyanidin dioxygenase
NCBI	=	National Center for Biotechnological Information
PAL	=	phenylalanine ammonia lyase
PCR	=	polymerase chain reaction
PVP	=	polyvinylpyrrolidone
PVPP	=	polyvinyl polypyrolidone

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LIST OF ABBREVIATIONS (Continued)

RNA	=	ribonucleic acid
RT-PCR	=	reverse transcriptase polymerase chain reaction
SDS	=	sodium dodecyl sulfate
SSC	=	soluble solids content
TA	=	titratable acidity
X-Gal	=	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

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ANTHOCYANIN BIOSYNTHESIS AND GENE EXPRESSION DURING FRUIT GROWTH AND RIPENING OF MALAY APPLE *(Syzygium malaccense)* CULTIVARS

INTRODUCTION

The red colour of fruits is usually determined by the quantity and composition of anthocyanins which are synthesized at an increasing rate during maturation and reach a maximum in the fully ripe fruit (Gross, 1987). Anthocyanins are a subclass of the flavonoids. The recent heightened interest in these compounds is mainly due to their possible health benefits. Red cabbage and purple sweet potato, for example, have been found to protect against tumor development (Hagiwara *et al.*, 2002). The presence of anthocyanins is one of the arguments for regular fruit consumption.

Anthocyanins are produced from an anthocyanidin, which becomes conjugated to one or more sugar moieties. The anthocyanins in many fruits are based on six common anthocyanidins: pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin. The sugars commonly linked to anthocyanidins are glucose, galactose, rhamnose, and arabinose. Combinations of these sugars can also be attached to one C atom of the phenyl rings. Other compounds (methyl groups, acetic acid, propionic acid, malonic acid, caffeic acid, sinapic acid, and ferulic acid, for example) can further be attached to the sugar moieties (Andersen and Jordheim, 2006; Grotewold, 2006).

Just as other flavonoids, anthocyanins are synthesized by the phenylpropanoid pathway. The conversion of phenylalanine to *trans*-cinnamate, mediated by phenylalanine ammonia lyase (PAL), is the first committed step. This leads to the production of 4-coumaroyl-CoA. After the further combination with malonyl-CoA the chalcone backbone of flavonoids is formed, consisting of two phenyl rings. Chalcone synthase (CHS) catalyses the formation of an intermediate chalcone. This product is then isomerized by chalcone isomerase (CHI) to form naringenin. The latter is oxidized by enzymes such as flavanone 3-hydroxylase (F3H), flavonoid 3'-

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hydroxylase (F3'H) and flavonoid 3'5'-hydroxylase (F3'5'H). The products become initially reduced by the enzyme dihydroflavonol 4-reductase (DFR) to the corresponding colourless leucoanthocyanidins. Leucoanthocyanidin dioxygenase (LDOX, also called anthocyanidin syntase, ANS) produces anthocyanidins from leucoanthocyanidins. The anthocyanidins are subsequently coupled to sugar molecules by enzymes like UDP-glucose:flavonoid 3-*O*-glucosyltransferase (F3GT, sometime also called UFGT although this name could include other flavonoid glycosyltransferase activities) or the corresponding enzymes that attach sugars other than glucose at position 3 and/or at C atoms other than 3 (Winkel-Shirley, 2002; Xie and Dixon, 2005).

Many genes have been isolated that encode the enzymes involved in the synthesis of anthocyanins, or encode proteins that regulate their presence in the vacuole. Several transcription factors have been isolated as well. Some of these coordinate the expression of all genes encoding the enzymes of the biosynthetic pathway, whereas others regulate the expression of one or more of these enzymes (Koes *et al*., 2005; Grotewold, 2006; Schwinn *et al*. 2006). Difference in colour intensity can be due to differences in expression of genes encoding enzymes of the biosynthetic pathway, or in different expression of regulatory genes. For example, the difference between red and white grapes was found to be due to much lower transcription levels of *UFGT* in the white grapes. The gene sequence was nonetheless the same in both red and white grapes, hence the mutation was not in *UFGT*. The difference was due to a mutation in a gene encoding a MYB transcription factor, which was found to regulate *UFGT* expression (Walker *et al.*, 2007).

The genus *Syzygium* in the Myrtaceae contains several species that produce edible fruit. The fruit of a number of these species are indicated by the word apple and indeed the crispiness of the fruit flesh is reminiscent of that of apples. The fruits of some commercially important species of *Syzygium* are rather similar in form, but differ in taste. Depending on the cultivar, *Syzygium samarangense* for example produces a green or pink-skinned fruit, both called Java apple or Java rose apple, whilst *Syzygium malaccense* (Malay apple), locally named ‘Mamieo’ and ‘Saraek’

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cultivars, whose fruit skin colours are dark red/purple and red streaks, respectively. Some synonyms used for Malay apple are Malacca apple or Malay rose apple. However, there are new cultivars of both Malay apples whose the skin colour of mature fruit exhibits white. The difference in fruit skin colouration between two cultivars of Malay apple is yet unclear. Therefore, both cultivars of Mamieo with red and white skin and Saraek with red and white skin were used as a fruit model to characterize the anthocyanin synthesis both in the red and white fruits.

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OBJECTIVES

1. To study fruit growth and quality changes of red and white Malay apple cultivars.

2. To identify individual anthocyanins of red and white Malay apple cultivars.

3. To study enzyme activities involved in anthocyanin biosynthesis of red and white Malay apple cultivars during fruit growth and ripening.

4. To clone and characterize the anthocyanin biosynthesis genes of red and white Malay apple cultivars during fruit growth and ripening.

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LITERATURE REVIEW

1. Anthocyanins

Anthocyanins are the largest group of naturally occurring phytochemical, and impart the rich colors ranging from red to violet and blue to fruits, vegetables, and other plants. The interest in anthocyanins has increased immensely during the past decade due to their array of healthily beneficial phytochemicals. Anthocyanins can protect against health damage by some types of harmful oxidants through different mechanisms (Jaldappagari *et al.*, 2008). The anthocyanins (in Greek: *anthos* means flower, and *kyanos* means blue) belong to the widespread class of phenolic compounds, which are collectively named flavonoids (Mazza and Miniati, 1993). These are glycosides of polyhydroxy and polymethoxy derivative of 2-phenylbenzopyrylium or flavylium cation. Anthocyanins have a 15-carbon (C_{15}) base structure comprised of two phenyl rings (called the A- and B-rings) connected by a three-carbon bridge that usually forms a third ring (called the C-ring). The degree of oxygenation of the B-ring has the greatest impact on the colour of anthocyanin pigments (Figure 1) (Davies, 2004).

The differences between individual anthocyanins relate to the number of hydroxyl groups, the nature and number of sugars attached to the molecule, the position of this attachment, and the nature and number of aliphatic or aromatic acids attached to sugars in the molecule. Presently, there are 17 known naturally occurring anthocyanidins or aglycone which are listed in Table 1. Only six anthocyanidins are common in higher plants especially in fruits: pelargonidin (Pg), peonidin (Pn), cyanidin (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp) (Table 2). The glycosides of the three non-methylated anthocyanidins (Cy, Dp and Pg) are the most widespread in nature, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers. The distribution of the six most common anthocyanidins in the edible parts of plants is cyanidin (50%), pelargonidin (12%), peonidin (12%), delphinidin (12%), petunidin (7%) and malvidin (7%). The following four classes of anthocyanidin glycosides are common: 3-monosides, 3-biosides, 3, 5-diglycosides

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and 3,7-diglycosides. The 3-glycosides occur about two and half times more frequently than 3, 5-diglycosides. Therefore, the most widespread anthocyanin is cyanidin 3-glucoside (Kong *et al.*, 2003; Jaldappagari *et al.*, 2008).

The most significant function of anthocyanins is their ability to impart color to the plants which they occur. They play a definite role in the attraction of animals for pollination and seed dispersal, and thus they are of considerable value in the co-evolution of these plant-animal interactions. Anthocyanins can act as antioxidants, phytoalexins or antibacterial agents (Kong *et al.*, 2003). Anthocyanins are soluble in polar solvents, and can normally be extracted from common plants materials by methanol containing small amounts of HCl or formic acid. Addition of the acid lowers the pH value of the solution and safely protects against degradation of their structures with the non-acylated anthocyanin pigments (Revilla *et al.*, 1998). The stability of anthocyanins is increased considerably by the addition of a glycoside. Anthocyanins with a β-glucosidic linkage are mostly formed in fruit. Each anthocyanidin can vary in the number and type of sugar substitution. In monoglycosides, glycosylation generally occurs at the 3 position on the C-ring, and the most common substitutes are glucose, arabinose or galactose. In diglycosides, either disaccharide can attach at position 3 e.g. 3-glucoside, or two monosaccharides can be linked to different hydroxyls e.g. 3, 5-diglucosides. Triglycosides are rare in fruit. Anthocyanins can be acylated with either organic or phenolic acids, which often contribute to their stability (Macheix *et al.*, 1990).

Individual and content of anthocyanins depend on fruit species which varies greatly. The most of anthocyanins found in red fruit such as apple, mangosteen, strawberry, lychee and grape. The main cyanidin pigment which accounts for the red color in apple fruit skin is cyanidin-3-galactoside while cyanidin-3-arabinoside, cyanidin-3-rutinoside, cyanidin-3-xyloside, and cyanidin-3-glucoside exist only in minor contents in some apple cultivars (Lancaster, 1992; Gómez-Cordovés *et al.*, 1996). Cyanidin-3-sophoroside and cyanidin-3-glucoside were the major compounds and the only ones that increase with fruit colour development (Palapol *et al.*, 2009a). Anthocyanin content in raspberries increased as berries matured, and total phenolic

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content decreased from the green to pink stage followed by a significant increase in total phenolics from the pink stage to the ripe stage (Wang and Lin, 2000). The anthocyanin content in raspberries ranged between 145-607 mg/100g (Tian *et al.*, 2006). In strawberry, total anthocyanin content ranged between 200 and 600 mg/kg, with pelargonidin-3-glucoside constituting 77-90% followed by pelargonidin-3-rutinoside (6-11%) and cyanidin-3-glucoside (3-10%) (da Silva *et al.*, 2007). Anthocyanins showed mainly cyanidin as aglycone (99-85%), although one peonidin and some pelargonidin (1-15%) derivatives were also detected in fig (*Ficus carica* L.) from five different varieties. Rutinose was the most usual substituting sugar, but glucose was also found (Dueñas *et al.*, 2008). Lee and Wicker (1991) and Rivera-López *et al.* (1999) also reported that cyanidin-3-rutinoside was the major anthocyanin found and cyanidin-3-glucoside and malvidin-3-acetylglucoside were also identified. In plum, the major anthocyanin was cyanidin-3-rutinoside in ripe fruits and followed by peonidin-3-rutinoside, cyanidin-3-glucoside, cyanidin-3-xyloside and peonidin-3-glucoside, respectively (Usenik *et al.*, 2009). The kokum fruit was found to contain a very high concentration of anthocyanins (2.4 g/100 g), which were identified as cyanidin 3-glucoside and cyanidin 3-sambubioside by HPLC, mass and NMR spectroscopy (Nayak *et al.*, 2010).

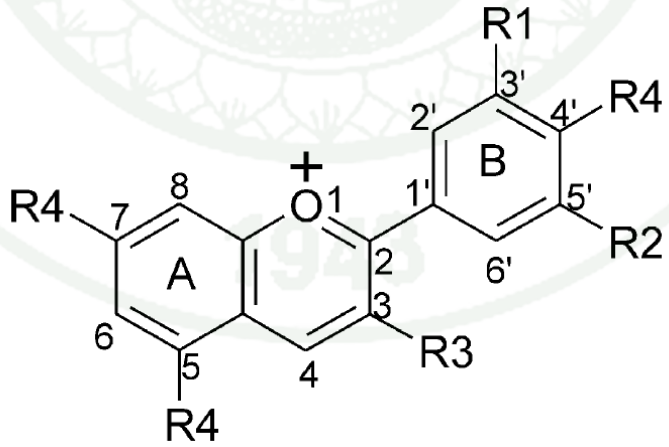


Figure 1 The flavalium cation. R_1 and R_2 are H, OH, or OCH_3; R_3 is a glycosyl or H; and R_4 is OH or a glycosyl.

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Table 1 Naturally occurring anthocyanidins.

Name	Substitution pattern							Color
	3	5	6	7	3'	4'	5'	
Apigeninidin	H	OH	H	OH	H	OH	H	Orange
Aurantinidin	OH	OH	OH	OH	H	OH	H	Orange
Capensinidin	OH	OMe	H	OH	OMe	OH	OMe	Bluish-red
Cyanidin	OH	OH	H	OH	OH	OH	H	Orange-red
Delphinidin	OH	OH	H	OH	OH	OH	OH	Bluish-red
Europinidin	OH	OMe	H	OH	OMe	OH	OH	Bluish-red
Hirsutidin	OH	OH	H	OMe	OMe	OH	OMe	Bluish-red
6-Hydroxycyanidin	OH	OH	OH	OH	OH	OH	H	Red
Luteolinidin	H	OH	H	OH	OH	OH	H	Orange
Malvidin	OH	OH	H	OH	OMe	OH	OMe	Bluish-red
5-Methylcyanidin	OH	OMe	H	OH	OH	OH	H	Orange-red
Pelargonidin	OH	OH	H	OH	H	OH	H	Orange
Peonidin	OH	OH	H	OH	OMe	OH	H	Orange-red
Petunidin	OH	OH	H	OH	OMe	OH	OH	Bluish-red
Pulchellidin	OH	OMe	H	OH	OH	OH	OH	Bluish-red
Rosinidin	OH	OH	H	OMe	OMe	OH	H	Red
Tricetinidin	H	OH	H	OH	OH	OH	OH	Red

Source: Kong *et al.* (2003)

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Table 2 Anthocyanidin identified in fruits.

Types of fruit	Anthocyanidin
Apple	Cyanidin
Cranberry	Peonidin
Blueberry	Cyanidin, delphinidin, malvidin, petunidin, and peonidin
Raspberry	Cyanidin
Blackcurrent	Cyanidin and delphinidin
Cherry	Cyanidin and peonidin
Orange	Cyanidin and delphinidin
Peach	Cyanidin
Plum	Cyanidin and peonidin
Strawberry	Pelargonidin
Grape	Malvidin, delphinidin, cyanidin, peonidin, petunidin, and pelargonidin

Source: Counsell (1981)

2. Anthocyanin biosynthesis

Anthocyanins are synthesized in the cytosol and those involved in pigmentation are generally transported into the vacuole via the flavonoid biosynthesis pathway. The precursors of anthocyanins are produced by the glycolytic pathway (phosphoenolpyruvate) and the pentose-phosphate pathway/Calvin cycle. The anthocyanin biosynthesis pathway can be divided into two main parts: (1) precursors of general phenylpropanoid metabolism (Figure 2A and 2B) and (2) specific steps toward flavonoid biosynthesis (Figure 2B). Phenylalanine is converted to *p*-coumaroyl-CoA, a key substrate that feeds into the flavonoid pathway, through a stepwise series of three enzymatic conversions catalysed by phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL). *p*-Coumaryl-CoA is the main precursor of flavonoids, lignin and other phenylpropanoids (Figure 2A). *p*-Coumaric acid is used to construct the C-6-C-3 (B aromatic rings and

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carbons corresponding to the C-ring) portion of the basic flavonoid structure (Figure 2B) (Tanaka *et al.*, 1998; Delgado-Vargas and Paredes-López, 2002).

PAL has been studied extensively as it is the between primary metabolism and the phenylpropanoid pathway. PAL activity increases concomitantly with accumulation of anthocyanin in apples (Macheix *et al.*, 1990; Lister *et al*., 1996b). Cheng and Breen (1991) also reported that enzymatic activity of PAL had a peak during fruit ripening, which correlated with the rapid accumulation of anthocyanin in strawberry. However, PAL is the key enzyme for the phenylpropanoid pathway and not specific to anthocyanin biosynthesis (Lancaster *et al.*, 1994). PAL catalyzes a reaction to produce precurcors of anthocyanin synthesis, under conditions of sufficient precursors, changes in anthocyanin accumulation can occur independently of changes in PAL activity (Ju *et al.*1995b). In ripened 'Jonathan' apples, an anthocyanin accumulation decreased, even though PAL activity was relatively high. Therefore, maximum PAL activity is not the regulating factor for anthocyanin accumulation in ripe apple (Wang *et al.*, 2000). The second part of the pathway flavonoids is involved in generating (Figure 2B).

1. Chalcone synthase (CHS)

The entry point into the flavonoid pathway is the formation of chalcone, which establishes the flavonoid C_{15} backbone. The initial chalcone formed in most species is naringenin chalcone. It is synthesized from one molecule of *p*-coumaroyl-CoA and three acetate units derived from three molecules of malonyl-CoA through the action of the chalcone synthase, one of the best characterized enzymes in secondary metabolism (Davies, 2004). The activity of chalcone synthase has been related to anthocyanin biosynthesis in many plants. Chalcone synthase activity and flavonoid content were relatively high and constant from fruitlet to maturation stage but it does not play a regulatory role in anthocyanin synthesis in apples exposed to light (Ju *et al.*, 1995a). Three genomic clones of the chalcone synthase gene, *Chs1*, *Chs2*, and *Chs3* were obtained from *Vitis vinifera* cv. Cabernet Sauvignon, and their structure and mRNA accumulation in grape berry (Goto-Yamamoto *et al.*, 2002).

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Tsuda *et al.* (2004) examined the gene expression of six genes in the anthocyanin biosynthesis pathway of white and red peaches and a deep-red nectarine. The results suggest that *CHS* and *DFR* are the key regulatory genes in mature red peach and nectarine.

2. Chalcone isomerase (CHI)

The accumulation of naringenin chalcone in plant tissues is rare. Naringenin chalcone is rapidly isomerized by chalcone isomerase to form naringenin via an acid base catalysis mechanism. Even in the absence of chalcone isomerase, chalcone can spontaneously isomerize to form naringenin chalcone, albeit at a slower rate (Holton and Cornish, 1995). In grape, phenylalanine ammonia lyase and chalcone isomerase activities were high during early development of the young berry and rapidly decreased thereafter, but were activated again when rapid anthocyanin synthesis subsequently occurred (Hiratsuka *et al.*, 2001). The *FaCHI* transcript level of strawberry cv. Queen Elisa during fruit development was highest in the turning stage and decreased at the red stage (Almeida *et al.*, 2007).

3. Flavanone 3-hydroxylase (F3H/F3'H/F3'5'H)

Flavonone 3-hydroxylase (F3H) catalyzes an early step in flavonoid pathway, the formation of dihydroflavonols from flavonones, and therefore provides precursors for many class of flavonoid compound including anthocyanins. F3H is a member of the 2-oxoglutarate-dependent dioxygenase protein family. Flavoniod 3'-hydroxylase (F3'H) and the closely related enzyme, flavonoid 3',5'-hydroxylase (F3'5'H) introduces 3'- or 3'- and 5'-hydroxyl groups respectively into the B-ring of a number of flavonoids (Davies, 2004). Naringenin chalcone is converted to dihydrokaempferol by F3H. Dihydrokaempferol can subsequently be hydroxylated by F3'H to produce dihydroquercetin or by F3'5'H to produce dihydromyricetin. F3'5'H can also convert dihydroquercetin to dihydromyricetin. At least three enzymes are required for converting the colorless dihydroflavonols (dihydrokaempferol, dihydroquercetin, and dihydromyricetin) to anthocyanins (Holton and Cornish, 1995).

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Several studies had been reported on different transcriptional regulations of *F3H* in many plants. cDNA clones of the F3'H and F3'5'H genes were first isolated from petunia. A mutation of F3H causes loss of activity and prevents the progression along the anthocyanin pathway. The mutants of petunia and snapdragon have white flowers (Martin *et al.*, 1991; Britsch *et al.*, 1992). In flowers, stems, tendrils and seeds of grape, they accumulated at a higher level of mRNA for *F3'H* than *F3'5'H*. The berry skin at the harvest stage accumulated a high transcript level of *F3'5'H* and a high level of delphinidin-based anthocyanins but small leaf accumulated prodelphinidin (Jeong *et al.*, 2006). The *Delila* gene of snapdragon coordinately controls *F3H* expression with downstream genes such as dihydroflavonol reductase (Martin *et al.*, 1991), while in Arabidopsis F3H gene was coordinately controlled with the upstream genes for chalcone isomerase and chalcone synthesis by regulatory genes (Pelletier and Winkel-Shirley, 1996).

4. Dihydroflavonol 4-reductase (DFR)

Dihydroflavonol 4-reductase catalyzes the reduction of dihydroflavonols (dihydrokaempferol, dihydroquercetin, and dihydromyricetin) to leucoanthocyanidins using a NADPH as a co-factor, a key late step in the biosynthesis of anthocyanins. These leucoanthocyanidins are the immediate precursors of anthocyanidins (Delgado-Vargas and Paredes-López, 2002; Lo Piero *et al.*, 2006). DFR genes have been isolated from several plants. Northern analysis revealed in apple (*Malus sylvestris* L. Mill. var. *domestica*) and grape (*Vitis vinifera* L.) that in non-red cultivars the level of DFR expression was slower than in red cultivar (Boss *et al.*, 1996; Honda *et al.*, 2002). In contrast, in some white grape cultivars such as Riesling, Semillon, and Chardonnay, the content of DFR transcripts was only slightly lower than the one shown by the red-skined grapes (Boss *et al.*, 1996). In red and non-red orange cultivars exhibit DFR genes characterized by identical encoding region. Their expression level is quite different, being about 250-fold less in the non-red orange than in the red orange cultivars (Lo Piero *et al.*, 2006). In strawberry, the DFR gene is a mainly involved in anthocyanin biosynthesis during colour development at the late stages of fruit ripening (Moyano *et al.*, 1998; Almieda *et al*., 2007).

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Tsuda *et al.* (2004) reported that *DFR* is the key regulatory gene of anthocyanin biosynthesis in mature red peach and nectarine.

5. Anthocyanidin synthase (ANS)/ Leucoanthocyanidin dioxygenase (LDOX)

The leucoanthocyanidins are converted to anthocyanidins by anthocyanidin synthase, also referred to as the leucoanthocyanidin dioxygenase in the anthocyanin biosynthesis pathway (Davies, 2004). The leucoanthocyanidin dioxygenase gene encodes an enzyme belonging to the class of 2-oxoglutarate-dependent dioxygenases (Holton and Cornish, 1995). The *ldox* gene has been shown to be expressed in Shiraz grapevine in all the plant organs: leaf, tendril, green cane, root, seed, flower, berry skin and berry flesh (Boss *et al.*, 1996). Almieda *et al*. (2007) found that the transcript levels in 'Queen Elisa' strawberry of *ANS* increased during fruit development and were highest at the red stage.

6. UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT, 3GT, UFGluT)

The initial 3-*O*-glycosylation of 3-hydroxyanthocyanidins is an essential step in formation of anthocyanins. However, 3-glycosylation is often only the first of multiple sugar additions that may occur onto the anthocyanin. These include further addition at the 3-position and also glycosylation at hydroxyls of A- and B-rings (Davies, 2004). The UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) is responsible for the transfer of the glucose from UDP-glucose to the hydroxyl group in position 3 of the C-ring. Since this is an essential final step required to stabilize anthocyanidins so that they can accumulate as water soluble pigments in the vacuoles. Depending on the B-ring hydroxylation pattern, three major types of anthocyanins can finally be distinguished. Each type has a characteristic colour, since the visible absorption maximum becomes longer as the number of hydroxyl groups in the B-ring increases: pelargonidin-derived pigments are responsible for orange, pink or red colours, cyanidin-derived pigments for red or magenta, and delphinidin-derived pigments for purple or blue (Holton and Cornish, 1995).

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Several studies had been reported on different transcriptional regulations of UFGT gene in many plants. Boss *et al*. (1996) reported that the expression of the UFGT gene as the key regulatory gene to control red colour skin in grape. This activity is also strongly correlated with anthocyanin accumulation in apple. Kobayashi *et al*. (2001) found that there were no differences in either coding or promoter sequences of *UFGluT* between white and red skin grape cultivars. They concluded that the mutation of the regulatory gene controlling UFGluT gene expression causes the change from white to red skin. Almieda *et al*. (2007) found that the transcript level of *FaFGT* was expressed increasingly during fruit development in strawberry cv. Queen Elisa. Griesser *et al.* (2008) also reports the function of *UFGT* in strawberry fruit in down-regulating an anthocyanidin-3-*O*-glycosyltransferase gene by RNA interference. The average *FaGT1* transcript levels of transgenic fruit were about 15% of the levels in control fruit and the pelargonidin-3-*O*-glucoside content in the fruit was reduced to 7.5% of control levels.

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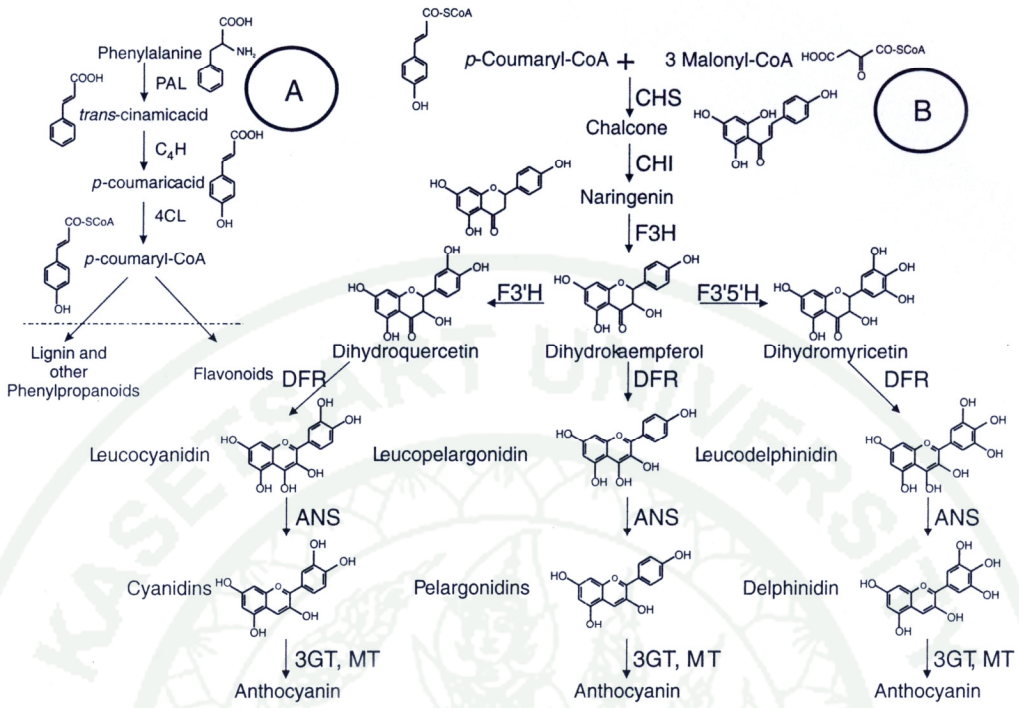


Figure 2 Anthocyanin biosynthesis pathway. (A) General phenylpropanoid metabolism. Enzymes involved: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaryl:CoA ligase. (B) Specific steps of anthocyanin biosynthesis. Enzymes involved: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, F3'H, F3'5'H, flavanol hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; 3GT, glucosyl transferase; MT, methyl transferase.

Source: Delgado-Vargas and Paredes-lópez (2002)

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3. Factors controlling anthocyanin biosynthesis

The factors controlling anthocyanin biosynthesis can be divided into 2 factors. The first factor is genetics which depends on enzymes involved in anthocyanin biosynthesis mentioned above and the second factor is environments such as light, temperature, nutrients, pH, plant growth regulators, and storage conditions.

3.1 Light

Light has been shown to be the most important environment factor affecting anthocyanin biosynthesis in many plants (Gross, 1987; Lancaster, 1992). In red-fruited cultivars, anthocyanin accumulation is induced by light in the UV-B region when applied in combination with infrared (Arakawa, 1988). Ju *et al.* (1999) have shown in 'Fuji' apples that light-reflecting mulches increase anthocyanin concentration and UDP-galactose:flavonoid-3-*O*-glucosyl transferase (UFGalT) enzyme activity in anthocyanin biosynthetic pathway, and that effect is greater for those mulches with a higher reflectivity in ultraviolet and infrared. Exposure to sunlight increased total anthocyanin concentrations in grape cv. Merlot (Spayd *et al.*, 2002). The high light intensity is imperative for coloring in red Chinese sand pears (Huang *et al.*, 2009). In contrast, Ratanamano *et al.* (2005) reported that the sunlight had no significant effect on both anthocyanin content and PAL activity in mangosteen. The anthocyanin accumulation and gene expression of anthocyanin biosynthesis were suppressed by shading during fruit growth and ripening in grape (Jeong *et al.*, 2004). Wang *et al.* (2000) also reported that PAL is not the only regulating factor for anthocyanin accumulation in bagged mature and ripe Jonathan apples.

3.2 Temperature

Temperature is a major factor in anthocyanin accumulation in many plants. In apple, low temperatures have long been considered to promote anthocyanin synthesis; and high temperatures in autumn, to inhibit it (Mazza and Miniati, 1993). The inhibition of anthocyanin biosynthesis in grape berries grown under high night

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temperature condition could be caused by lower expression levels of anthocyanin biosynthetic genes at an early stage of ripening and lower activities of anthocyanin biosynthetic enzymes, particularly UFGT (Mori *et al.*, 2005). The anthocyanins accumulation and expression of PAL, CHS, DFR, and UFGT were strongly induced during low temperature in red orange fruit (Lo Piero *et al.*, 2005).

3.3 Nutrients

The relationships between fruit nutrient concentrations during the season and flavonoid compounds have been studied in many plants. Application of nitrogen decreased the concentration of cyanidin-3-galactoside, catechins and total flavoniods and the percentage of blush in 'Elstar' apples fruit skin (Awad and de Jager, 2002). Phosphite, salts of phosphorus acid fertilizer solution was effective in activating plant defence mechanisms, since anthocyanin content increased in strawberry (Moor *et al.*, 2009). The phosphorus-calcium mixed compounds, caused an enhancement of red peel color which increased in concentration of flavonoid compounds and increased PAL and UFGalT activities greatly and CHI activity moderately (Li *et al.*, 2002). Vitrac *et al*. (2000) reported that the involvement of calcium involved in the induction of anthocyanin biosynthesis by sugar in grape cells.

3.4 pH

Anthocyanins behave like indicators in aqueous media. Their structure and thus their colour vary with the pH. At pH 1 they are highly coloured as flavylium cations. With increasing pH the colour gradually fades as colorless pseudobases are formed. In mild alkali blue anhydrobases are formed. The reaction is reversible unless strong alkali is used, which produces irreversible changes. The structural changes occur in anthocyanins with changes of pH as shown in Figure 3 (Gross, 1987). Anthocyanins in fruit and vegetables show their highest colour intensity in the flavylium ion form (Harborne and Williams, 1995). It has been demonstrated that anthocyanin stability is influenced by substituents in their structures, sugars and acyl groups (Giusti and Wrolstad, 2003).

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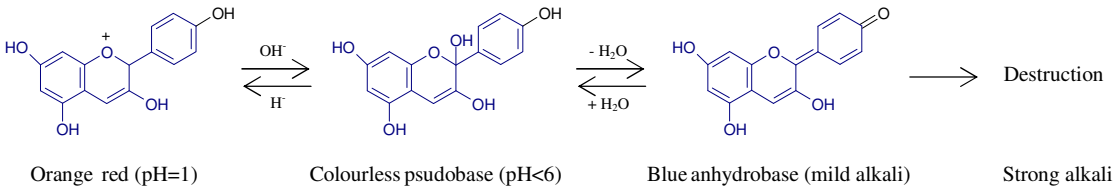


Figure 3 Structural changes of anthocyanins with pH.

3.5 Plant growth regulators

Many researchers have reported effects of plant growth regulators on anthocyanin accumulation in fruits. Anthocyanin synthesis can be enhanced by exogenous ethylene or the ethylene-releasing compound, ethephon (2-chloroethyl phosphonic acid) in apple (Gómez-Cordovés *et al.*, 1996) and rabbit eye blueberry (Ban *et al.*, 2007). Ethephon treatment increased UFGalT activity and also promoted anthocyanin formation in both 'Delicious' and 'Ralls' apples (Ju *et al.*, 1995a). In grapes, ethylene enhanced anthocyanin accumulation and expression of anthocyanin genes (El-Kereamy *et al.*, 2003). Conversely, application of aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor, reduced anthocyanin accumulation and delayed colour development and ripening in apple (Wang and Dilley, 2001; Whale *et al.*, 2008). Treatment with 1-MCP inhibited phenylalanine ammonia lyase (PAL) activity, and lowered increases in anthocyanin and phenolic contents in strawberry (Jiang *et al.*, 2001). Treatment with methyl jasmonate enhanced anthocyanin content while treatment with ethylene or 1-MCP alone, or ethylene + 1-MCP had no effect on anthocyanin accumulation in 'Fuji' apple (Rudell and Mattheis, 2008).

3.6 Storage conditions

Anthocyanin can be changed in many fruits which depend on storage temperature and atmosphere conditions. UV-B and low temperature were important factors for anthocyanin accumulation in apple by inducing the expression of the

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anthocyanin biosynthetic genes, especially CHS, ANS and UFGluT genes (Ubi *et al.*, 2006). Low temperature induced an accumulation of total anthocyanin content during storage in strawberry (Cordenunsi *et al.*, 2005) and grape (Romero *et al.*, 2008). Holcroft *et al.* (1998) reported that the arils of pomegranates stored in air were deeper red than the initial controls and carbon dioxide-enriched atmospheres at 10^{o}C.

4. Analysis of gene expression

Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. In all organisms, gene expression can be divided into two main phases; transcription and translation. Transcription is the production of copying DNA into RNA and translation is the production of a protein or polypeptide to the specifications of a mRNA template. Several methods have been developed to study the expression of a specific RNA and a specific protein (Dale and von Schantz, 2003).

4.1 Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) represents a highly sensitive and powerful tool for analyzing RNA (Freeman *et al.*, 1999). This is a specific method useful for the detection of rare transcripts or for the analysis of samples available in limiting amounts of RNA (Erlich, 1989). It is possible to detect a specific mRNA in a single cell. This has obvious advantages, in being to detect low abundance mRNA, or to analyze gene expression in cells that are difficult to obtain in large numbers (Dale and von Schantz, 2003). Furthermore, this method can be conducted on a large number of samples and/or many different genes in the same experiments (Freeman *et al.*, 1999). Quantification of mRNA can be determined by semi-quantitative or quantitative methods (Ferre, 1992). Semi-quantitative RT-PCR method involves determination of the levels of the target mRNA based on the use an internal control, which is a housekeeping gene such as actin, glyceraldehyde 3-phosphate dehydrogenase (GADPH), and elongation factor (ELF). It is typically a constitutive gene that is transcribed at a relatively constant level. The housekeeping gene's products are

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typically needed for maintenance of the cell. Housekeeping genes are used as internal standards in quantitative polymerase chain reaction since it is generally assumed that their expression is unaffected by experimental conditions. Housekeeping mRNA levels can be adjusted in each sample and used to standardize samples for containing the same amount of the housekeeping genes. Based on the assumption that the level of housekeeping genes is constant, the relative levels of target mRNA in each sample can be determined (Giambernardi and Klebe, 2000).

4.2 Real-time PCR

In order to robustly detect and quantify expression from small amounts of RNA, amplification of the gene transcript is necessary. The PCR is a traditional method for amplifying DNA; for mRNA-based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase. A traditional method uses agarose gel electrophoresis for detection of PCR amplification at the final phase or end-point of the PCR reaction (Erlich, 1989). Development of PCR technologies based on reverse transcription permit measurement of DNA amplification during PCR in real time. The amplified product of real-time PCR is measured at each PCR cycle throughout the PCR reaction. Compared with classical PCR, the main advantages of real-time PCR are high sensitivity, high specificity, good reproducibility and rapidity to provide reliable data. Typically, the time of a whole real-time PCR run ranges from 20 min to 2 h (Bustin, 2000; Gachon *et al.*, 2004). Real-time PCR is therefore a very convenient method for studies with a limited amount of starting material, or for assessing the expression of a high number of genes from minute quantities of RNA. Basically, real-time quantitative PCR may be used for quantifying DNA or RNA abundance, leading to two majors types of applications: foreign DNA (e.g. transgenes or contaminating micro-organisms) detection and quantification, and gene expression studies (Gachon *et al.*, 2004). Real-time qPCR has become a routine and robust approach for measuring the expression of genes of interest, validating microarray experiments, and monitoring biomakers. The future of qPCR remains bright as the technology becomes more rapid, cost-effective, easier to use, and capable of higher throughput (VanGuilder *et al.*, 2008).

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Real-time qPCR allows quantification of PCR products in 'real time' during each PCR cycle, yielding a quantitative measurement of PCR products accumulated during the course of the reaction. Real-time reactions are carried out in a thermocycler that permits measurement of a fluorescent detector molecule, which decreases post-processing steps and minimizes experimental error. This is most commonly achieved through the use of fluorescence-based technologies, including probe sequences that fluoresce upon hydrolysis (TaqMan; Applied Biosystems, Foster City, CA, USA) or hybridization (LightCycler; Roche, Indianapolis, IN, USA), fluorescent hairpins or intercalating dyes (SYBR Green). SYBR Green is an example of an intercalating dye that fluoresces upon binding to the double-stranded DNA, much like the workhorse dye, ethidium bromide. Following primer-mediated replication of the target sequence during PCR, multiple molecules of SYBR Green bind to the product and emit a strong fluorescent signal that is easily detected. Intercalating dyes are inexpensive and simple to use compared to sequence probes and, because they are not sequence-specific and can be used for any reaction. However, because they do not discriminate between gene sequences, they cannot be used for multiplexed analyses (Zipper *et al.*, 2004; VanGuilder *et al.*, 2008).
A disadvantage of SYBR Green is equally incorporated into every amplicon, and should unspecific sequences be amplified, the signal measured would correspond to both non-specific and specific products, thereby compromising the accurate quantification of the latter (Gachon *et al.*, 2004).

The most commonly used method to analyze from real-time qPCR analysis is relative quantification. It relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. The $2^{-\Delta\Delta C_T}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. This method upon on two assumptions. The first is that the reaction is occurring with 100% efficiency. In other words, the amount of product doubles with each cycle of PCR. The relative expression of a gene of interest in relation to another gene, mostly to an appropriate reference gene, can be calculated on the basis of 'delta crossing point' (ΔC_P) or 'delta delta threshold cycle' ($\Delta\Delta C_T$) values (Livak and Schmittgen, 2001). A 'C_T' is defined as the number of

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cycles necessary to reach this threshold of fluorescence. A calibration curve is drawn from parallel runs using known initial amounts of the specific target. Recently, various mathematical models have been established to calculate the relative expression ratio, based on the comparison of the distinct cycle differences.
The relative expression can be determined by various methods including efficiency corrected calculation (Pfaffl, 2001).

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MATERIALS AND METHODS

Fruit of Malay apple (*Syzygium malaccense* (L.) Merr. & L.M. Perry) cultivars locally called 'Mamieo' and 'Saraek'which both cultivars exhibit red and white skin, were obtained from the sames orchards in the Nonthaburi province of central Thailand. Flowers were tagged at full bloom. Fruits were bagged at stage 2. Samples of 30 fruit were collected randomly from several trees, throughout the growing season. Developmental stages were divided into 8 stages: stage 1, 21 days 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 (Figure 4A-B).

1. Study of fruit growth and quality of Malay apples during fruit growth and ripening

1.1 Fruit growth

Fruit growth was determined in each fruit development stage. Fresh weight was measured in individual fruit using a digital balance (AND, Tokyo, Japan). Fruit width and fruit length were measured using a digital vernier caliper (Insize, IL, USA).

1.2 Fruit quality

Fruit firmness was measured using a hand-held fruit firmness tester (Effegi, Alfonsine, Italy), equipped with a cylindrical plunger 0.5 cm in diameter. The plunger was inserted to a depth of 0.5 cm into the fruit skin at 2 positions on opposite sides of each fruit. The force was recorded in Newtons (multiply by 9.807).

Soluble solids content (SSC) of the flesh juice was measured using a hand-held refractometer (Atago, Tokyo, Japan) and calibrated with distilled water, while titratable acidity (TA) of the flesh juice was determined from a 5 mL aliquot by

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titration with 0.1 N NaOH with 1% phenolphthalein as an indicator and results are given as grams of citric acid per 100 mL. The SSC/TA ratio was also calculated for each sample.

1.3 Fruit skin colour

Fruit skin colour was measured using a Minolta CR-300 chromameter (Minolta, Osaka, Japan) as L^*, a^*, b^* values (CIE L a b) and converted to hue angle (colour wheel, with red-purple at an angle of 0^o, yellow at 90^o, bluish-green at 180^o). The colour reading was taken thrice at the top, middle, and bottom region of each fruit and then averaged to estimate a value for each fruit. After fruit skin colour measurement, the fruit skin was peeled off carefully with a knife. Samples of fruit skin were frozen immediately in liquid nitrogen and stored at -80^oC until use for next experiments.

2. Study of total anthocyanins and individual anthocyanins in the fruit skin of Malay apples during fruit growth and ripening

2.1 Total anthocyanins

Total anthocyanins were extracted from the Malay apple fruit skin according to the method described by Piccaglia *et al.* (2002). One gram of each sample was homogenized with 20 mL of methanol:HCl (99:1 v/v) using a Polytron PT 2100 (Kinematica AG, Luzern, Switzerland) with 1.2 mm diameter head at speed 12 for 1 min, and then shaken for 6 h at 4^oC in darkness. The aqueous phase was removed and the pellets were re-extracted four times with 20 mL of methanol:HCl within 24 h and then adjusted to a final volume of 100 mL with methanol:HCl. The combined aqueous extracts were centrifuged at 8,000 x *g* for 10 min at 4^oC to remove the pellet. The absorbance of the supernatant was measured at 530 nm, using a Genesys 10UV spectrophotometer (Thermo Spectronic, Rochester, USA). Total anthocyanin content was expressed as cyadinin equivalents (molar extinction = 34,300). All measurements

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were made in triplicate. The samples were kept in an amber bottle at -80°C until individual anthocyanins were analyzed.

$$\text{Total anthocyanin (mg/kg)} = \frac{A \times MW \times DF \times 1000}{\varepsilon L \times Wt}$$

A = Absorbance
ε = Cyanidin-3-*O*-glucoside molar extinction (34,300)
MW = Anthocyanin molecular weight (287)
DF = Dilution factor
Wt = Sample weight (mg)
L = Cell path length (usually 1cm)

2.2 Individual anthocyanins

Individual anthocyanin compounds were analyzed by HPLC and LC-MS, using a modification of the method described by Stevenson *et al.* (2006). Samples were filtered through syringe filter, 0.45 µm nylon membrane (Chromtech, Minnesota, USA) before injections. Twenty microlitres of individual samples were analyzed using 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, California, USA). The second was a Synergi 250 mm x 4.6 mm column, filled with Polar-RP 4 µm diameter particles, 8 nm pore size (Phenomenex, California, USA). Two mobile phases were used: (A) acetonitrile + formic acid (99:1) and (B) acetonitrile/water/formic acid (5:92:3). Flow rate was 1 mL/min at a column temperature of 45°C. A gradient consisting of mobile phase A was 0% at zero time and ramped linearly to 20% at 20 min, 30% at 26 min, 50% at 28.5 min, 95% between 32 and 35 min and back to 0% between 36 and 42 min. The individual anthocyanin compounds were analysed by using the retention times and comparison with standard solutions of known concentration, at 520 nm.

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

3. Study of enzyme activities in the fruit skin of Malay apples during fruit growth and ripening

Enzymes were extracted from the Malay apple fruit skin according to Lister *et al.* (1996a) with slight modification. Three grams of frozen fruit skin at each stage (Figure 4A-B) were extracted with 15 mL 50 mM Na_2HPO_4/KH_2PO_4 buffer (pH 7.0) containing 50 mM Na ascorbate; 18 mM 2-mercaptoethanol; 2 g polyvinylpyrrolidone (PVP-40) and 0.1% Triton X-100. The extraction sample was homogenized using a Polytron PT 2100 (Kinematica AG, Luzern, Switzerland) with 1.2 mm diameter head at speed 12 for 1 min. The sample was incubated on ice for 10 min, then centrifuged at 20,000 x *g* for 20 min to remove particulate matter. Ammonium sulphate was added to the supernatant at 35% saturation, followed by centrifugation at 20,000 x *g* for 20 min to remove the PVP, then more ammonium sulfate was added to reach a final saturation of 80%. This fraction was centrifuged at 20,000 x *g* for 20 min. Afterwards, the pellet was re-suspended in 3 mL of extraction buffer without PVP and Triton X-100, and dialyzed with dialysis buffer (extraction buffer without PVP and Triton X-100) overnight to give a partially purified extract that was used for PAL and UFGT enzyme activity. All enzyme extractions steps were carried out at 4°C. The enzyme extract was frozen in liquid nitrogen and stored at -80°C until enzyme activity analysis.

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3.1 Phenylalanine ammonia lyase (PAL)

PAL enzyme activity was measured using the method of Lister *et al.* (1996b) with slight modification. The reaction mixture comprised 200 µL of enzyme extract and 800 µL of 60 mM borate buffer (pH 8.8). The reaction was initiated by the addition 200 µL of L-phenylalanine solution (10 mg/L) and incubated at 30°C in a water bath for 1 h. The reaction was stopped by adding 125 µL of 40% (w/v) trifluoroacetic acid and then centrifuged at 12,000 x *g* for 5 min at 4°C to pellet the denatured protein. PAL enzyme activity was measured by measuring the absorbance at 290 nm using a Genesys 10UV spectrophotometer (Thermo Spectronic, Rochester, USA). Triplicate assays were performed for each enzyme extract. One unit of PAL was defined as the yield of 1 micromoles of *trans*-cinnamic acid per mg of protein.

3.2 UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT)

UFGT enzyme activity was measured using the method of Lister *et al.* (1996a) with slight modification. The reaction mixture comprised 200 µL of enzyme extract, 200 µL 50 mM bicine buffer (pH 8.5), 30 µL 1 mM quercetin, and 20 µL 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 the addition 150 µL of 20% (w/v) trichloroacetic acid in methanol and then centrifuged at 12,000 x *g* for 5 min at 4°C and the supernatant was stored at -80°C until quantification by HPLC. Samples were filtered through syringe filter, 0.45 µm nylon membrane (Chromtech, Minnesota, USA) before injections. Analysis of quercetin-3-*O*-glucose was performed using a 50 mm NH_2 column, Inersil®, 5 µm particle diameter, with a 4.0 x 10 mm HPLC guard column, Inersil®, oDS-3, 5 µm particle diameter. Flow rate was 1 mL/min at a column temperature of 30°C. The injection volume was 20 µL. The mobile phase was acidified water (0.66 M formic acid) (A) and acidified methanol (0.66 M formic acid) (B) in a linear gradient, where A:B ratio changed from 50:50 to 15:85 in 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*-glucose).

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One unit of UFGT was defined as the yield of 1 mol of quercetin-3-*O*-glucoside per second.

3.3 Protein content

Protein content in the enzyme extracts was measured using the method described by Bradford (1976), using bovine serum albumin (BSA) as a standard (0, 20, 40, 60, 80, and 100 mg/mL). Specific activity of the enzyme was expressed as units per mg protein.

4. Cloning and characterization of anthocyanin biosynthesis genes of Malay apples during fruit growth and ripening

This study consisted of an investigation into gene expression of the enzymes involved in anthocyanin biosynthesic pathway. The gene expression of these enzymes was carried out using semi-quantitative RT-PCR and real-time PCR technique.

4.1 Isolation and cloning of anthocyanin biosynthesis genes of Malay apples

4.1.1 Total RNA extraction

Approximately 5 g of frozen samples were ground in a mixing mill MM 301 (Retsch, Germany) under liquid N_2, kept in the RNAse-free Falcon tubes and then stored at -80^{o}C until use. Total RNA was isolated from 2 g of fruit skin tissue as described by López-Gómez and Gómez-Lim (1992). Ground tissue was added to 15 mL of lysis buffer containing 2% SDS, 1% β-mercaptoethanol, 50 mM EDTA, 150 mM Tris base with pH adjusted to 7.5 with 1 M boric acid and 0.5 g polyvinyl polypyrolidone (PVPP) in the RNAse-free Falcon tubes at room temperature, and the contents were vortexed until well mixed. The mixture was homogenized using a Polytron PT2100 (Kinematica AG, Switzerland) with 1.2 mm diameter head at speed 12 for 1 min. Then 1.5 mL of 5 M potassium acetate and 4 mL of chilled absolute ethanol were added to the mixture. The mixture was shaken vigorously for 1 min, then 15 mL of chloroform:

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isoamyl alcohol (24:1 v/v) was added, shaken vigorously for 5 min and then centrifuged at 7,000 x *g* for 20 min at 4°C. The top aqueous phase was transferred to a new RNAse-free Falcon tubes, and 10 mL of phenol: chloroform: isoamyl alcohol (25:24:1 v/v) added. The mixture was shaken vigorously for 5 min and centrifuged at 7,000 x *g* for 20 min at 4°C. The top aqueous phase was again transferred to a new RNAse-free Falcon tube, and 15 mL of chloroform: isoamyl alcohol added. The mixture was shaken and centrifuged at the same speed. The top aqueous phase was transferred to a RNAse-free Oakridge tube and the volume recorded. RNA was then precipitated with 8 M LiCl to obtain a 3 M final concentration at 4°C overnight. After centrifugation at 12,000 x *g* for 20 min at 4°C, the supernatant was carefully poured off and the pellet was washed with 3 mL of 3 M LiCl . The RNA pellet was re-suspended in 350 µL DEPC-water and transferred to an autoclaved microtube and then 35 µL of 3 M potassium acetate and 962.5 µL of chilled absolute alcohol were added. The RNA was precipitated at -80ºC for 1 h. The mixture was centrifuged in a microcentrifuge at 12,000 x *g* for 30 min at 4°C, washed once with 500 µL of chilled 70% ethanol, and re-suspended in 50 µL of chilled DEPC-water. After that RNA was quantified by measuring the absorbance at 260 nm. One absorbance unit at 260 nm corresponded to approximately 40 µg/mL (Sambrook and Russel, 2001). The RNA concentration of each sample was estimated in µg/mL by the following equation: RNA concentration = A260 x dilution factor x 40 µg/mL. Quality of the purified total RNA was determined by calculating the A260/A280 ratio. The ratio of between 1.8-2.0 indicated good quality of the RNA and qualitatively checked using gel electrophoresis on a 1% (w/v) agarose gel.

4.1.2 cDNA synthesis (Reverse Transcription Reaction)

Five micrograms of total RNA was treated with deoxyribonucleaseI (Dnase I, RNase free, Fermentas, Burlington, Canada) to remove contaminating genomic DNA. The first strand cDNA was synthesized from 4 µg of total RNA following the protocol of Omniscript RT kit (Qiagen, Hilden, Germany) as a template for anthocyanin biosynthesis genes. Fifty-fold diluted cDNA was used for semi-quantitative RT-PCR and quantitative real-time RT-PCR. The synthesized

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cDNA was used as a template to amplify the targeted genes by PCR. The first strand cDNA was further used as a template in PCR reaction or stored at -20°C until use.

4.1.3 PCR amplification of Malay apple cDNA fragments

Malay apple anthocyanin biosynthesis genes were isolated from fruit at stage 8 (Figure 4A-B), using forward and reverse degenerate primers designs which were based on conserved regions of similars genes in the National Center for Biotechnology Information (NCBI) database. The sequences of the primers are described in Table 3. The reaction mixture (50 µL) consisted of 10x PCR buffer (5 µL), 50 mM $MgCl_2$ (2 µL), 10 mM dNTPs (1 µL), 10 µM of each primer (5 µL), cDNA (1 µL), Platinum Taq (0.2 µL) and water (30.8 µL) (Invitrogen, California, USA). The amplification reactions were initially held at 94°C for 5 min with 1 cycle and then subjected to 35 cycles at the following condition: 94°C for 30 s and then 54°C for 30 s (*SmPAL*, *SmCHI*) or 56°C for 30 s (*SmDFR, SmCHS*, *SmF3H*, *SmLDOX*, *SmUFGT*), and 72°C for 1 min with a final period at 72°C for 10 min, 1 cycle.

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Table 3 Degenerate primers of genes encoding enzymes in the anthocyanin biosynthetic pathway.

Gene name	Sequence	Annealing temperature (ºC)	Expected Size (bp)
SmPAL	F-GYDATYTTYGCWGARGTBATG	54	381
(GU233756)	R-AGATTNGAHGGYAABCCRTTGTTG		
SmCHS	F-CAGCCCAAGTCCAAAATCAC	56	550
(GU233757)	R-CAGCCCAARTCCAARATCAC		
SmCHI	F-TMGTYWCVGGYCCBTTTGAG	54	484
(GU233758)	R-CYTYGMTBCWGGVGAAAC		
SmF3H	F-TKGCYTAYAAYSAMTTYAGC	56	794
(GU233759)	R-GTTYTGGAAWGTNGCWATBG		
SmDFR	F-YTCWTGGCT SGTCATGAGRC	56	574
(GU233760)	R-SCAGWDATGAGGCTYGGHG		
SmLDOX	F-ARAARGAGAAGTATGCHAAYGASC	56	580
(GU233761)	R-CCAYGARATYCTMACCTTYTCC		
SmUFGT	F-TSTTCWCNTTCTTCARCAC	56	947
(GU233762)	R-GAGYYCCADCCRCAATGYGWYAC		

4.1.4 PCR products cloning and DNA sequencing

After visualizing the PCR product by gel electrophoresis on 1% agarose, the amplified PCR fragments from each gene were purified using a gel extraction (QIAprep® Gel Extraction, Qiagen, Hilden, Germany) and cloned into pGEM-T Vector (pGEM®-T Easy Vector System, Promega, California, USA) as described in the supplier's instructions.

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1. Ligations using the pGEM®-T Easy vectors

The ligation reactions were set up using 5 µL of 2x Rapid Ligation Buffer, 1 µL of pGEM-T Vector (50 ng), 3 µL of PCR product and 1 µL of T4 DNA Ligase to a final volume of 10 µL. The reactions were mixed gently by pipetting and then incubated overnight at 4°C.

2. Transformations using the pGEM®-T Easy vector ligation reactions

Transformation of the vector with inserted DNA was conducted using *Escherichia coli* DH5α cells. Two LB/ampicillin/X-Gal plates for each ligation reaction were prepared, and equilibrated at room temperature prior to plating. The 100 µL frozen DH5-α tube(s) were removed from -80°C storage and placed in an ice bath until just thawed (about 10 min). The ligation reaction (10 µL) was carefully put into each competent cell. The tubes were gently flicked to mix and then placed on ice for 30 min. The cells were heat-shocked for 45 s in a water bath at exactly 42°C without shaking and immediately returned to ice for 2 min. Then 1 mL SOC medium was added to the tubes and incubated for 1.5 h at 37°C with shaking (~150 rpm). Each transformation culture (100 mL) was plated onto duplicate LB/ampicillin/X-Gal plates and incubated overnight (16-24 h) at 37°C. White colonies were selected for the next step.

3. Purification of plasmid DNA using QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany)

Single white colonies containing the inserted genes were cultured in 5 mL LB medium overnight. Cells were centrifuged at 7,000 x *g* for 10 min and only the pellet cells collected. The pellet cells were re-suspended in 250 µL of re-suspension buffer and transferred to a 1.5 mL microtube. Then, 250 µL of lysis buffer was added and the tube gently inverted to mix. Three hundred and fifty µL of neutralization buffer were added and the tube inverted immediately and then

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centrifuged at 12,000 x *g* for 10 min. The supernatant was transferred to the spin column by pipetting. The tube was then centrifuged for 1 min and the flow-through discarded. The spin column was washed by adding 700 μL of washing buffer and centrifuged for 1 min, the flow-through discarded, and centrifuged again to remove the residual washing buffer. DNA was eluted by adding 50 μL of elution buffer, incubated 1 min in the room temperature and centrifuged for 1 min. DNA was kept at -20^{o}C until used for cutting with the *Eco*RI restriction enzyme (New England Biolabs, Inc., USA) and the insert size was checked by gel electrophoresis before sequencing.

4. DNA sequencing and analysis

The sequence analysis of the clone was conducted by automatic sequencing using the ABI PRISM® 377 DNA sequencer (Applied Biosystems, California, USA). The sequences of amplified all fragments were compared with the genes in the Genbank database using the BLAST program from NCBI. The DNA and protein sequences were analyzed by the Vector NTI 10.0 program (Invitrogen, USA) and submitted to GenBank (NCBI). A new pair of specific primers for the gene were encoded for all genes (Table 4) and used for semi-quantitative RT-PCR and quantitative real time RT-PCR. Specific primers were tested for specificity using plasmid amplification and the product analyzed on a 1.2% (w/v) agarose gel stained with ethidium bromide. All primers were designed with Vector NTI 10.0 (Invitrogen, USA). The size of qPCR products ranged from 100 to 200 bp.

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Table 4 Specific primers of genes encoding enzymes in the anthocyanin biosynthetic pathway.

Gene name	Sequence	Expected size (bp)
SmPAL (GU233756)	F-CACAAACTGAAGCATCATCCTGGC R-GCTTCTGAAGTGGGTCCATCTCGT	121
SmCHS (GU233757)	F-GACCCCGTACCTGAAATCG R-TTGGAGGTTGATGGTGAGC	126
SmCHI (GU233758)	F-GGCTCAATACGCAGAGAAGG R-GGCGATTGCGTGAATAGG	159
SmF3H (GU233759)	F-GCAATGGGATTGGAGAAGG R-GCAAGAGGGTGATGGTTCC	148
SmDFR (GU233760)	F-GCTCGCCAAGAAGATGACC R-AACGAGACTTGGGATGATGC	121
SmLDOX (GU233761)	F-TTCTTCCACCTCGTTTACCC R-GAGTGCCGATAGGATTTTGG	135
SmUFGT (GU233762)	F-AACACTGCCGATTCCATAGC R-ATGTCCTCCTGTGGCTTCC	125

4.2 Expression analysis of anthocyanin biosynthesis genes of Malay apples during fruit growth and ripening

4.2.1 Analysis of gene expression by semi-quantitative RT-PCR

Semi-quantitative RT-PCR was conducted for studying the pattern of gene expression. Fifty-fold diluted cDNA was used. To find the suitable cycle of thermocycler for normalization of *Syzygium* cDNA, the *SmACT* primers were used as an internal control to normalize all sample loadings. Transcripts were amplified under the following conditions: 94^{o}C for 5 min with 1 cycle, 54^{o}C for 30 s with 28 cycles (*SmCHI*) or 30 cycles (*SmPAL*), 56^{o}C for 30 s with 28 cycles (*SmDFR, SmUFGT*), 60^{o}C for 30 s with 26 cycles (*SmACT*) or 60^{o}C for 30 s with 28 cycles (*SmLDOX*) or

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30 cycles (*SmCHS*, *SmF3H*), and 72°C for 30 s with a final period at 72°C for 10 min, 1 cycle. The reaction components were used for semi-quantitative RT-PCR as shown in Table 5.

Table 5 The components of reactions for semi-quantitative RT-PCR.

Components	Volume (μL)
10x PCR buffer	2.5
25 mM $MgCl_2$	2.5
10 μM dNTPs	0.5
10 μM forward primer	1.0
10 μM reverse primer	1.0
Taq polymerase enzyme	0.25
cDNA template	2.0
Distilled water	15.25
Total volume	25.0

The PCR product (5μL) was analyzed using gel electrophoresis on a 1.2% (w/v) agarose at 100 volts for 30 min. The gel was stained in 2.5 μg/ml ethidium bromide (EtBr) solution for 15 min. The RNA bands were visualized under UV transilluminator and photographed by SYNGENE BIO IMAGINE Gel Documentation (Syngene, England).

4.2.2 Analysis of gene expression by quantitative real-time RT-PCR

Data were confirmed by quantitative real-time RT-PCR. Fifty-fold diluted cDNA was used. qPCR was performed using an ABI Prism® 7000 real-time PCR machine (Applied Biosystems, California, USA) using SYBR green (QuantiTect™ SYBR® Green PCR kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. All reactions were performed in triplicate using 3 μL of diluted template (50x), 1 μL of each forward and reverse primer (2.5μM), and 12.5 μL of 2x Master mix to a final volume of 25 μL. Reactions were initiated by

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15 min at 95°C 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 melting curve were included in every run. The melting peak, dissociation curve and sequencing were analyzed to confirm the expected product rather than primer dimer. The data were normalized to actin transcript levels (*SmACT*, GU233755) to minimize variation in cDNA template levels. The primers of *SmACT* primers were designed with Vector NTI 10.0 (Invitrogen, CA, USA) as described above (Forward 5'-TGCCATTCAGGCTGTCCTTTCC -3' and Reverse 5'-CCCAGCCAGGTCAAGACGAAGA -3'). Direction of the PCR product was measured by monitoring the increase in fluorescence caused by the binding of SYBR® Green dye to double-stranded DNA. A fluorescence threshold was set manually to ΔRn on the log fluorescence scale to determine the fractional cycle number (C_T value) at which the fluorescence passed the detection threshold.

The expression levels were shown as a ratio relative to the fruit development stage 3. The ratio of the calibrator was set to the nominal value of 1. The relative expression was analyzed as transcript abundance ratio of target gene to reference gene by using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Formular of relative gene expression is $2^{-\Delta\Delta C_T}$.

$$\Delta\Delta C_T = (C_{T\ Target} - C_{T\ Reference})_{Time\ x} - (C_{T\ Target} - C_{T\ Reference})_{Time\ 0}$$

Where: C_T is the number of PCR cycle

Time x is any time point

Time 0 represents the 1x expression of the target gene normalized to *SmACT*

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5. Statistical analysis

Using a SAS package, experimental data were analyzed statistically by ANOVA and significance of the differences between means was estimated by Duncan's new multiple range test (DMRT) at $p \leq 0.05$. Experimental data were the average of three replications $\pm$ standard error (SE). All experiments were repeated at least twice.

6. The experimental time and places

The experiments were carried out during December 2006-October 2009 and the research was conducted at the places as described below:

1. Malay apple orchards in Nonthaburi Province.

2. Postharvest Technology Center, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom.

3. Center of Excellence on Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom.

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RESULTS

1. Study of fruit growth and quality of Malay apples during fruit growth and ripening

All Malay apples were identified to 8 stages of development, determined by classes of fruit size. The fruit of all stages of development are shown with the distal part down. The appendix seen at the distal end in young fruit is not the peduncle but the remaining stamen. The fruit of Malay apple (in contrast to Java apple) has no clear peduncle (Figure 4A-B).

1.1 Fruit growth

1.1.1 Fruit growth of Malay apple cv. Mamieo

Fruit sizes (width, length and fresh weight) of Malay apple cv. Mamieo in both red and white fruits were not significantly different and increased gradually during the early fruit growth from stage 1 to stage 2. After that fruit sizes increased rapidly from stage 2 to stage 6 and then increased gradually again during ripening from stage 6 to stage 8. The fruit size of red fruit was significantly smaller than that of white fruit in the same stage of fruit growth and ripening. The fruit growth pattern showed single sigmoidal curve in both red and white fruits (Figure 5A-C, Appendix Table 1 and 2).

1.1.2 Fruit growth of Malay apple cv. Saraek

Fruit sizes (width, length and fresh weight) of Malay apple cv. Saraek in both red and white fruits were not significantly different and increased gradually during the early fruit growth from stage 1 to stage 3. After that fruit sizes increased rapidly from stage 3 to stage 6 and then increased gradually again during ripening from stage 6 to stage 8. At fully ripe fruit, the fruit size of red fruit was

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significantly larger than that of white fruit. The fruit growth pattern showed single sigmoidal curve in both red and white fruits (Figure 6A-C, Appendix Table 3 and 4).

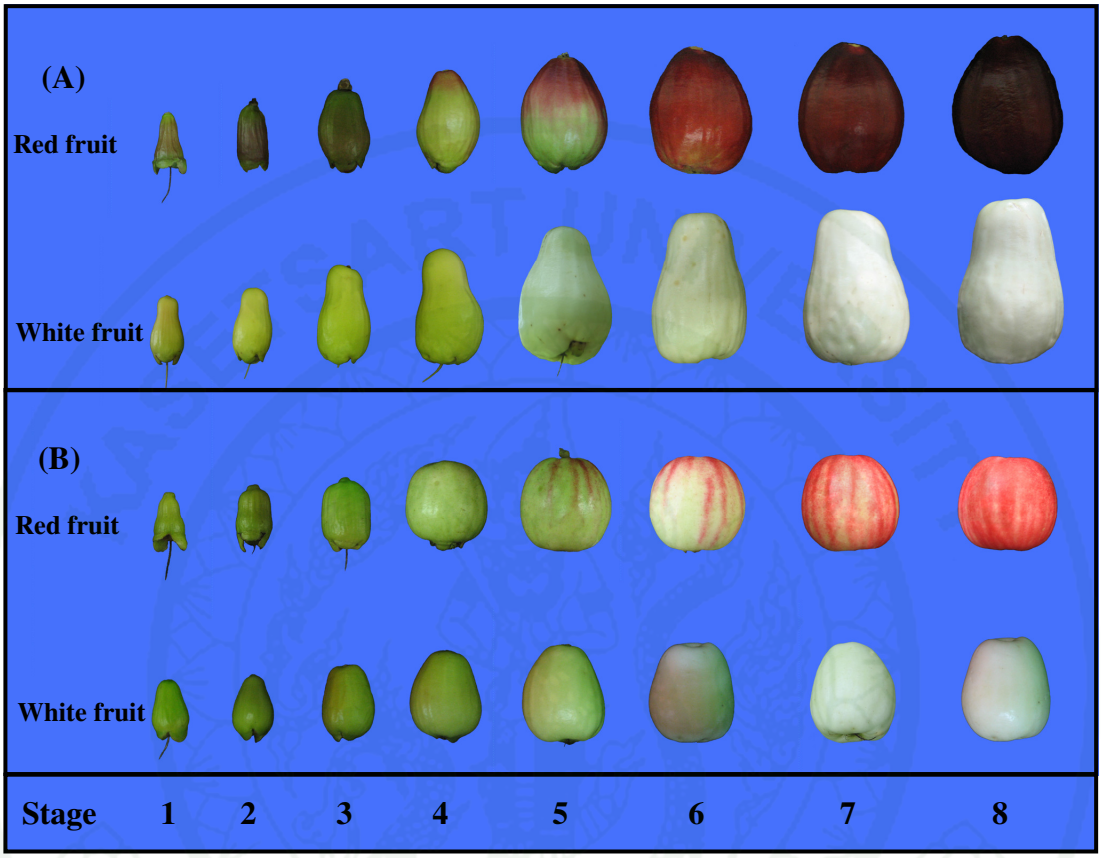


Figure 4 Stages of fruit development in the red and white Malay apple cvs. Mamieo (A) and Saraek (B).

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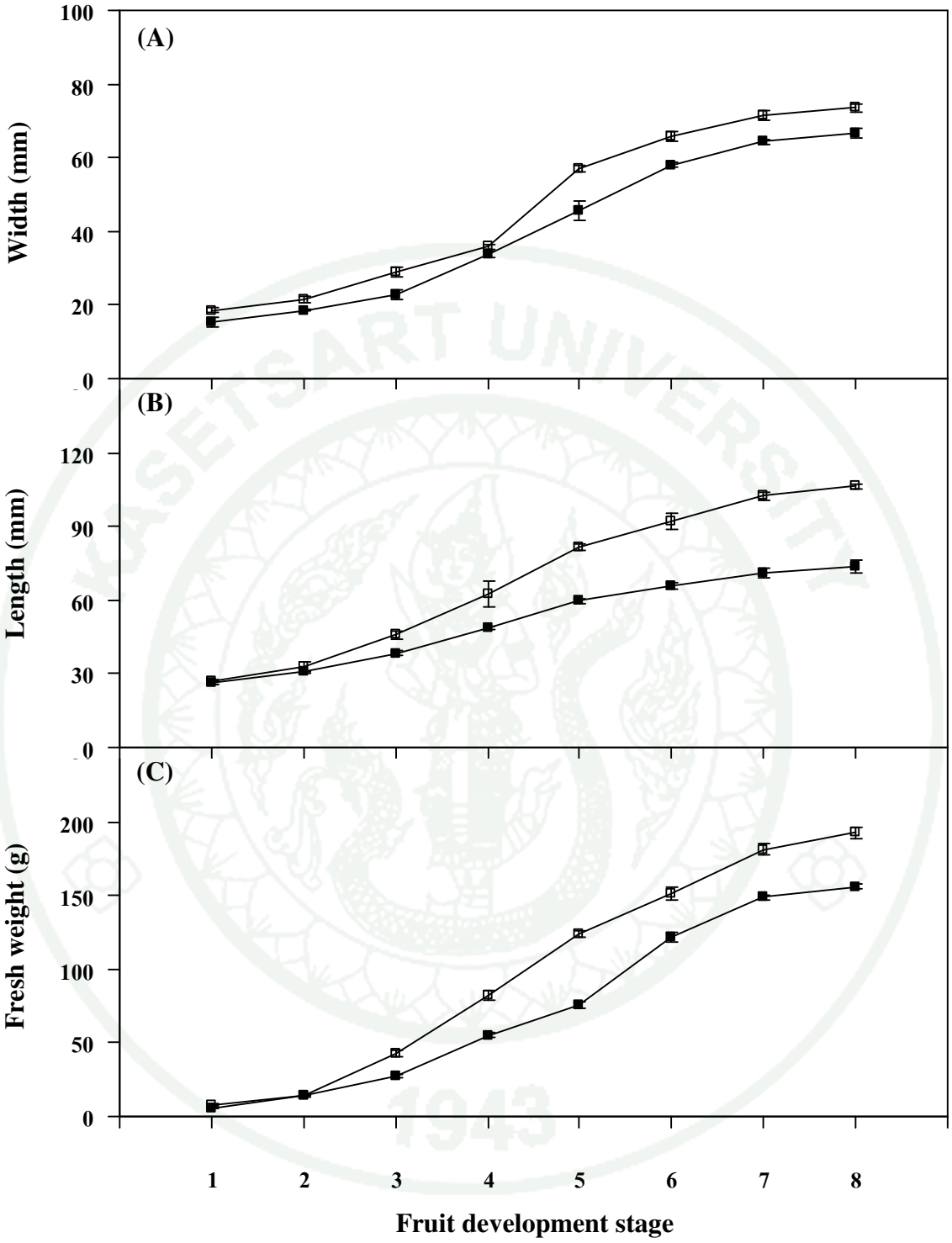


Figure 5 Changes in fruit size (width, length and fresh weight) of red (■) and white (□) Malay apple cv. Mamieo during fruit growth and ripening. Data are means ± SE of three replications.

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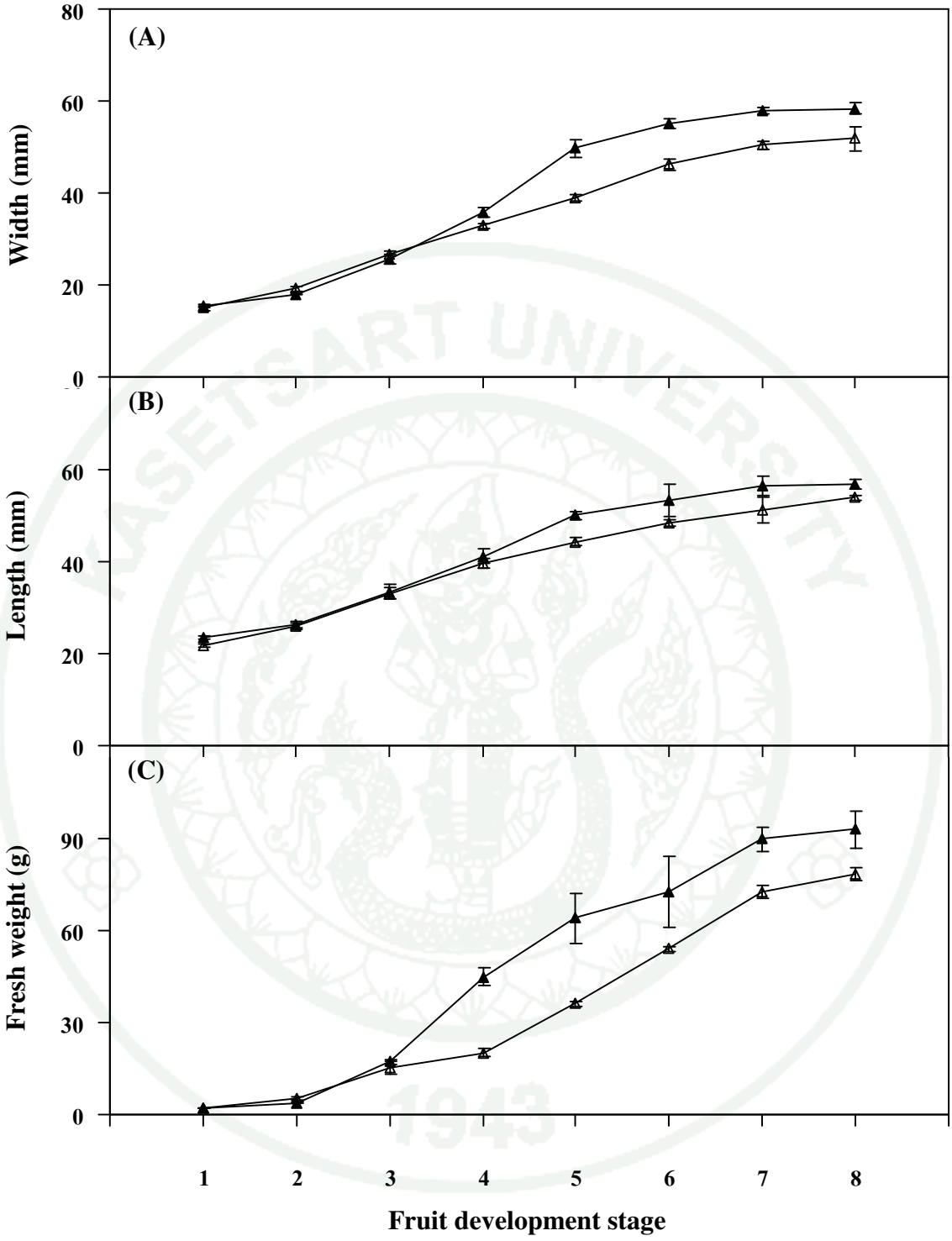


Figure 6 Changes in fruit size (width, length and fresh weight) of red (▲) and white (Δ) Malay apple cv. Saraek during fruit growth and ripening. Data are means ± SE of three replications.

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1.2 Fruit skin colour

1.2.1 Fruit skin colour of Malay apple cv. Mamieo

Red fruit at stage 4 of development had a red colour at the proximal part of the fruit. The red colour subsequently covered the remainder of the fruit. The colour became darker over time, and fruit were dark purple by stage 8 of development. Fruit of the white had a light green colour all over the whole fruit from stage 1 to stage 4. The green changed to white or creamy colour during ripening from stage 5 to stage 8 (Figure 4A-B). There was significant difference in parameters L*, a* and b* values and hue values between red and white fruits at all stages. The L* value of red fruit did not change from stage 1 to stage 5, and then decreased sharply during ripening from stage 6 to stage 8 (Figure 7A-D, Appendix Table 5). The a* value did not change from stage 1 to stage 4, and then increased rapidly with colour development until stage 7 and decreased slightly at stage 8 (Figure 7B). The hue values decreased significantly and correlated closely with red colouration during fruit growth and ripening (Figure 7D). In white fruit, the L* value increased slightly, while the a* and hue values did not change during fruit growth and ripening (Figure 7A-B,D , Appendix Table 6). The b* value increased slightly from stage 1 to stage 5, and then decreased rapidly during ripening from stage 6 to stage 8 (Figure 7C, Appendix Table 6).

1.2.2 Fruit skin colour of Malay apple cv. Saraek

Red fruit at stage 5 had a red streak colour at the proximal part of the fruit and red developed forward at stage 8. Fruit of the white had light green colour all over the whole fruit from stage 1 to stage 5 and changed to white or creamy colour during ripening from stage 5 to stage 8 (Figure 4C and 4D). The L* value of red fruit remained stable during the early stage from stage 1 to stage 3, and then decreased sharply during ripening from stage 6 to stage 8 (Figure 8A, Appendix Table 7). The a* value did not change during the early stage from stage 1 to stage 3, and then increased rapidly with colour development until stage 8 (Figure 8B). The hue values

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decreased significantly and correlated closely with red colouration during fruit growth and ripening from stage 4 to stage 8 (Figure 8D). In white fruit, the L*, a* and hue values did not change from stage 1 to stage 5 and increased slightly during ripening from stage 6 to stage 8 while the hue values did not change during fruit growth and ripening (Figure 8A-B,D, Appendix Table 8). The b* value decreased slightly from stage 1 to stage 5, and then decreased rapidly during ripening from stage 6 to stage 8 (Figure 8C, Appendix Table 8).

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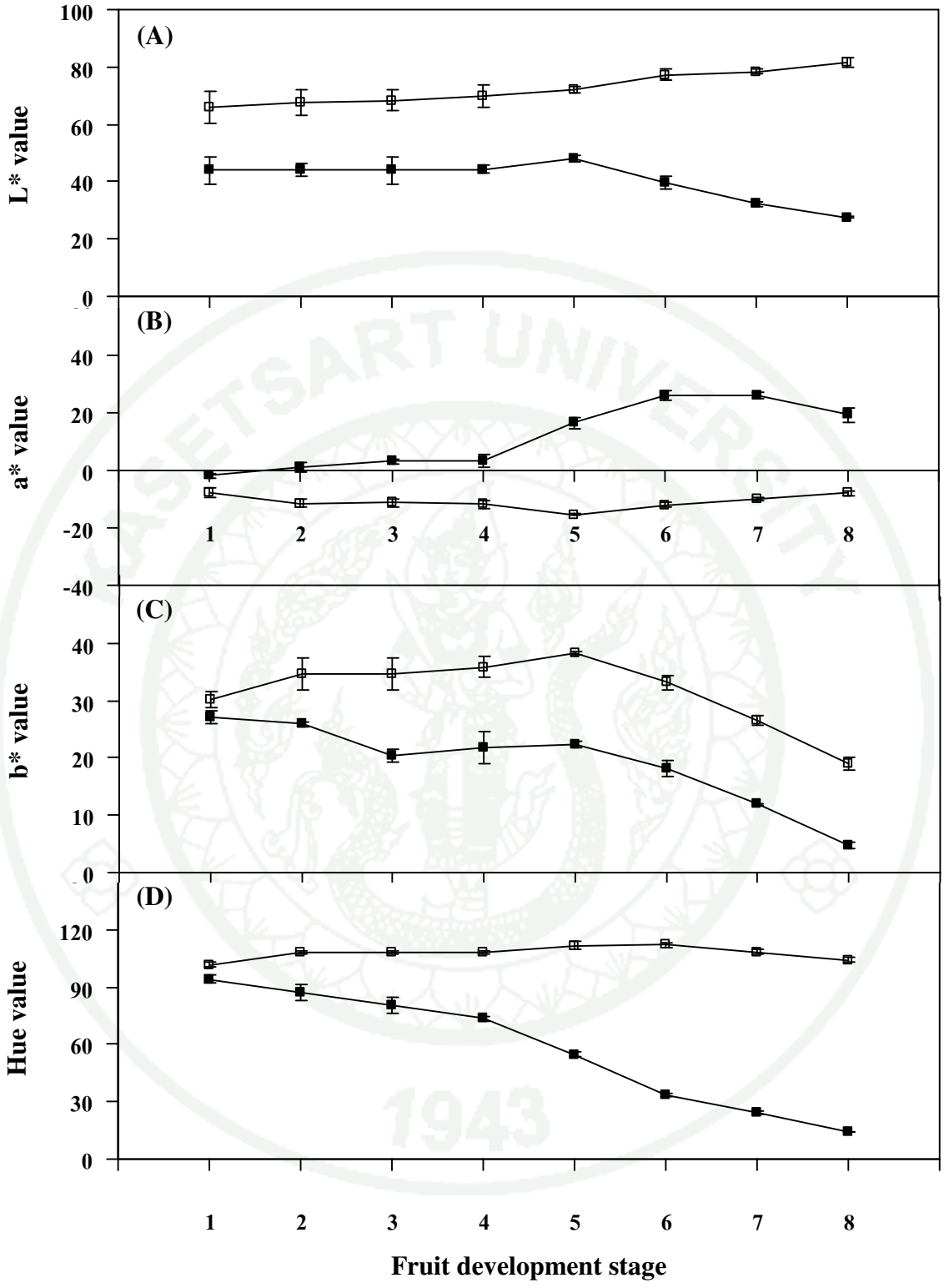


Figure 7 Changes in fruit skin colour (L*, a*, b* and hue value) of red (■) and white (□) Malay apple cv. Mamieo during fruit growth and ripening. Data are means ± SE of three replications.

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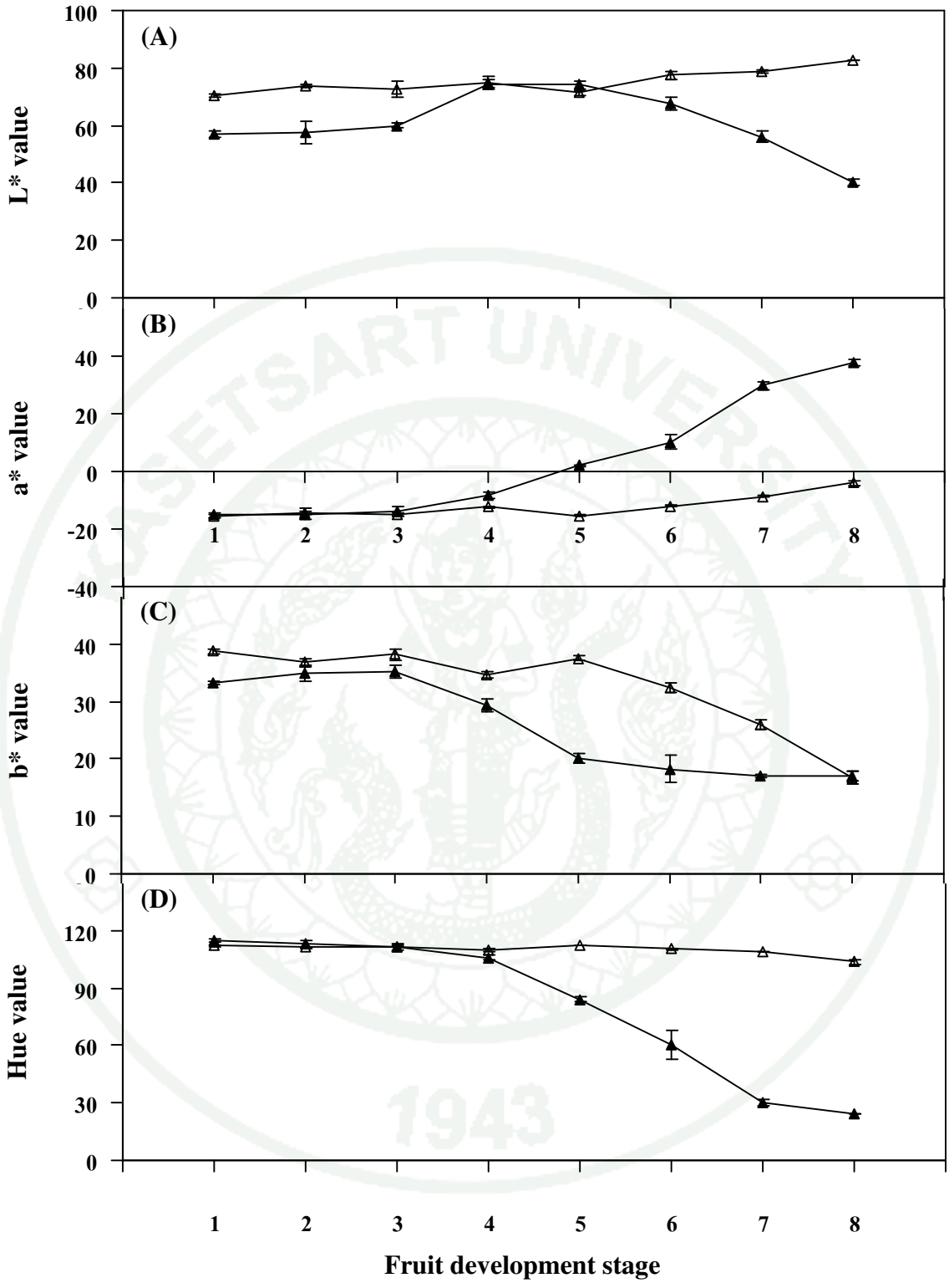


Figure 8 Changes in fruit skin colour (L*, a*, b* and hue value) of red (▲) and white (Δ) Malay apple cv. Saraek during fruit growth and ripening. Data are means ± SE of three replications.

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1.3 Fruit quality

1.3.1 Fruit quality of Malay apple cv. Mamieo

Fruit firmness of Malay apple cv. Mamieo in both red and white fruits was significantly different and decreased sharply during ripening from stage 6 to stage 8. Fruit firmness of red fruit was significantly less than that of white fruit in the same stage of fruit ripening. SSC and SSC/TA ratio increased rapidly, while TA decreased rapidly during ripening from stage 6 to stage 8 (Figure 9A-D, Appendix Table 9 and 10).

1.3.2 Fruit quality of Malay apple cv. Saraek

Fruit firmness of Malay apple cv. Saraek in both red and white fruits was significantly different and decreased sharply during ripening from stage 6 to stage 8. SSC and SSC/TA ratio increased rapidly, while TA decreased rapidly during ripening from stage 6 to 8 (Figure 10A-D, Appendix Table 11 and 12).

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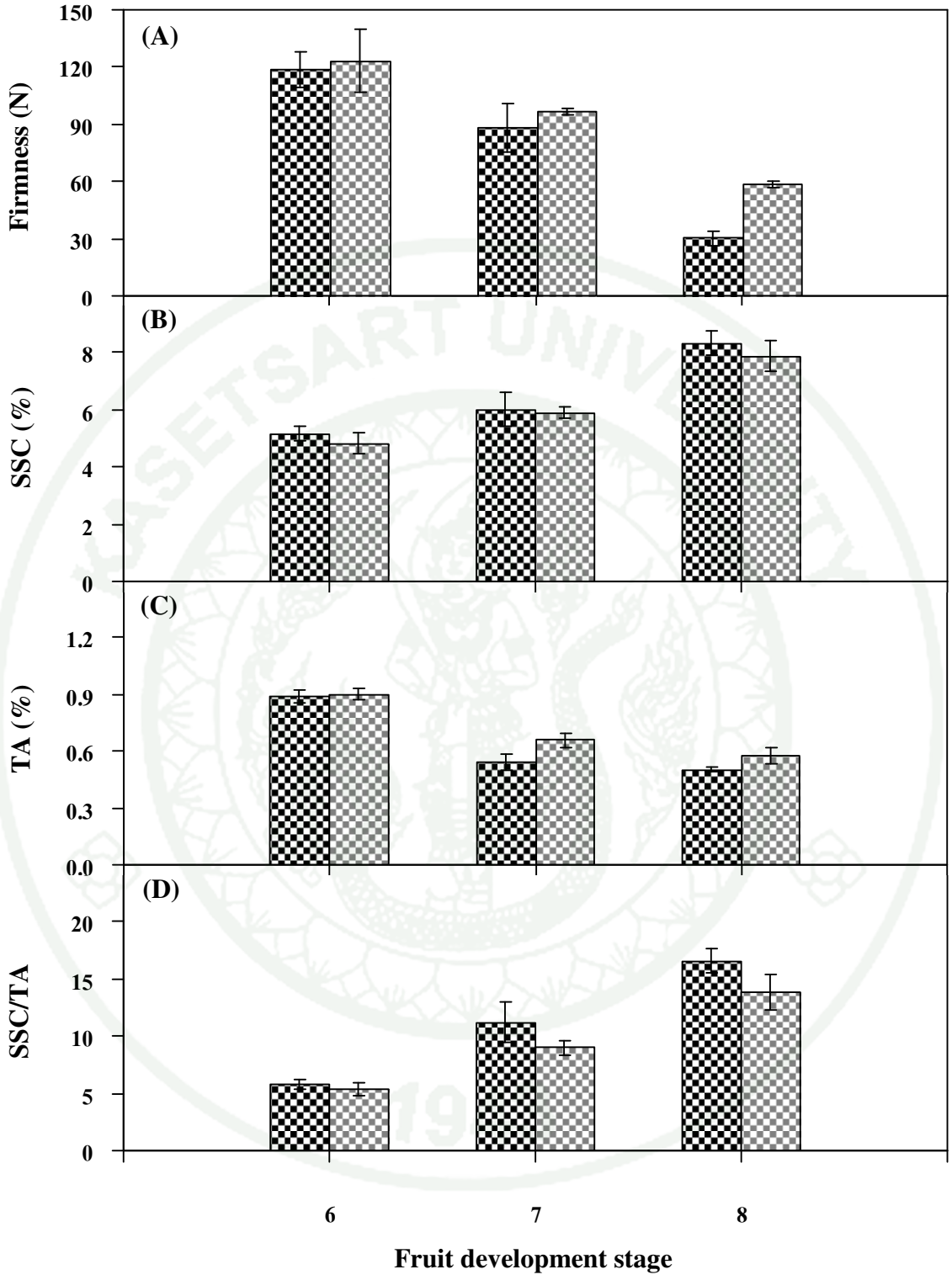


Figure 9 Changes in fruit quality (firmness, SSC, TA and SCC/TA) of red (■) and white (□) Malay apple cv. Mamieo during fruit ripening. Data are means ± SE of three replications.

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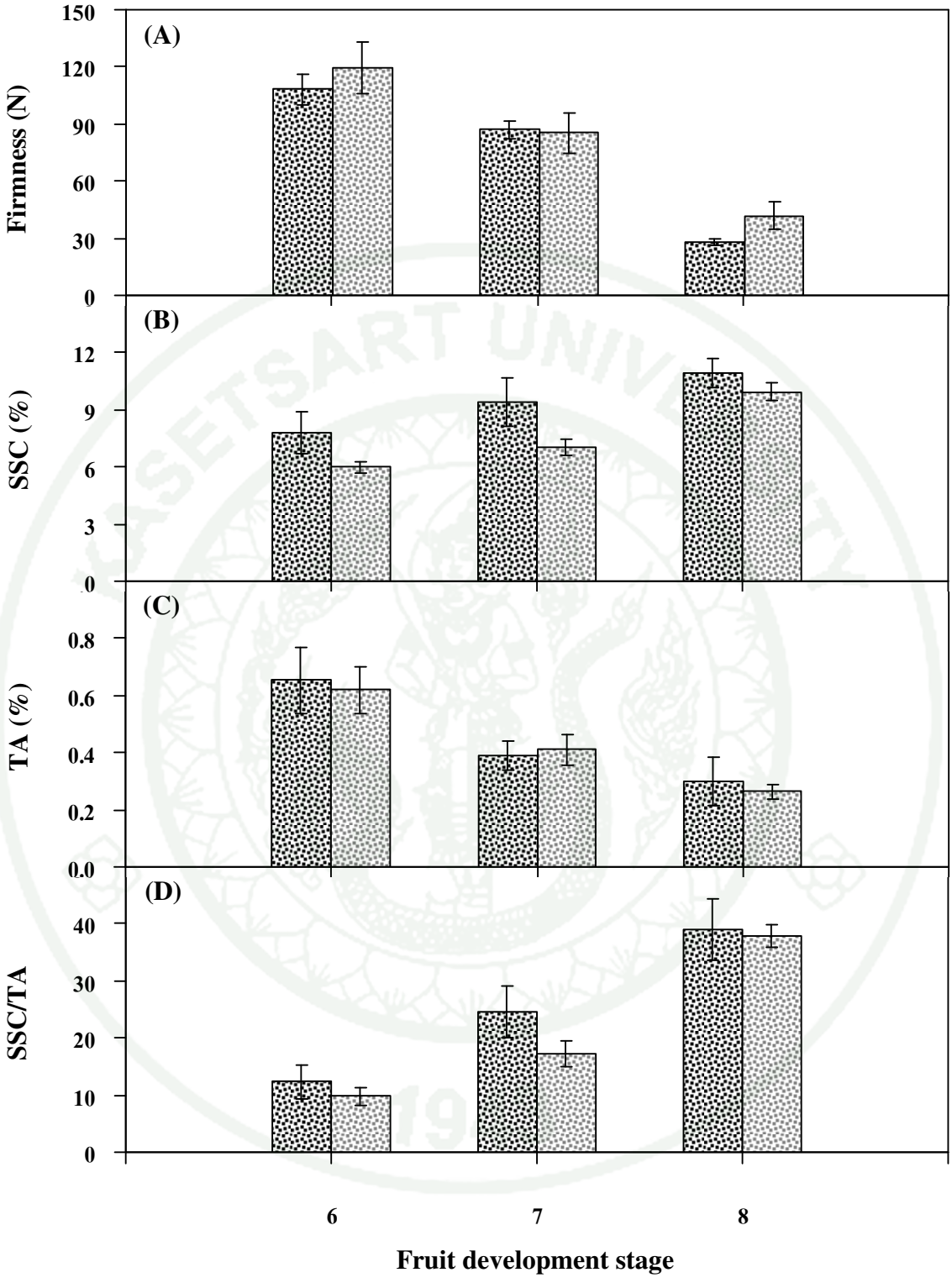


Figure 10 Changes in fruit quality (firmness, SSC, TA and SCC/TA) of red (▣) and white (□) Malay apple cv. Saraek during fruit ripening. Data are means ± SE of three replications.

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2. Study of anthocyanins in the fruit skin of Malay apples during fruit growth and ripening

2.1 Anthocyanins in the fruit skin of Malay apple cv. Mamieo

Total anthocyanin contents were significantly different in all fruit developmental stages. Total anthocyanin content in the red skin fruit increased gradually from stage 1 to stage 5, and then increased more rapidly during stage 6 to stage 8. A 26-fold increase was found between stage 1 and stage 8 whereas hue values decreased sharply (Figure 11A, Appendix Table 13). The anthocyanin content was closely associated with red colour development of the fruit. Hue value and total anthocyanin content were closely associated with red colour development of fruit skin (Figure 4A). At stage 1 the skin of the white fruit contained anthocyanin levels that were similar to those in the red fruit. However, in contrast to the red fruit a decrease in anthocyanin levels was found at later stages of development (Figure 11B, Appendix Table 14).

Anthocyanins in the skin of both red and white fruit at the fully ripe stage (stage 8) were identified by LC-MS and compared with data in the literature. The anthocyanins in the red fruit were mainly cyanidin-3,5-*O*-diglucoside with *m/z* 611 $[M]^+$ ion (peak 1), peonidin-3,5-*O*-diglucoside with *m/z* 625 $[M]^+$ ion (peak 2), cyanidin-3-*O*-glucoside with *m/z* 449 $[M]^+$ ion (peak 3), pelargonidin-3-*O*-glucoside with *m/z* 433 $[M]^+$ ion (peak 4) and peonidin-3-*O*-glucoside with *m/z* 463 $[M]^+$ ion (peak 5) (Figure 13A, Table 6, Appendix Figure 1). By far the largest peak of the LC data was cyanidin-3-*O*-glucoside. This was further confirmed the identity of this compound by comparison with an authentic standard of cyanidin-3-*O*-glucoside. The data of the red fruit were in contrast with those of the white, where LC-MS analysis revealed no detectable anthocyanins in the fruit skin (Figure 13B). This seems in contrast with the data of Figure 11B, which are based on spectrophotometric analysis at 530 nm. These data indicated low levels of material absorbing at this wavelength. In the absence of anthocyanins in the LC-MS data of Figure 11B seem to show compounds other than anthocyanins that show absorption at 530 nm.

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The level of cyanidin-3-*O*-glucoside was quantified by HPLC. It gradually increased during fruit growth and ripening on the tree, reaching its maximum at the last stage (stage 8) studied and the pattern of changes was similar to total anthocyanin content (Figure 11A and 15A, Appendix Table 13). In contrast, anthocyanin peak was not detected in white skin fruit (Appendix Table 14).

2.2 Anthocyanins in the fruit skin of Malay apple cv. Saraek

Total anthocyanin content was significantly different in all fruit development stages. Total anthocyanin content in the fruit skin of the red increased gradually from stage 1 to stage 6, and then increased more rapidly during stage 7 to stage 8. A 9-fold increase was found between stage 1 and stage 8 whereas hue values decreased sharply (Figure 12A, Appendix Table 15). The anthocyanin content was closely associated with red colour development of the fruit. Hue value and total anthocyanin content were closely associated with red colour development of fruit skin (Figure 4B). At stage 1 the skin of the white fruit contained anthocyanin levels that were similar to those in the red fruit. However, in contrast to the red fruit a decrease in anthocyanin levels was found at later stages of development (Figure 12B, Appendix Table 16).

Anthocyanins in the skin of both red and white fruit at the fully ripe stage (stage 8) were identified by LC-MS and compared with data in the literature. The anthocyanins in the red fruit were mainly cyanidin-3,5-*O*-diglucoside with *m/z* 611 $[M]^+$ ion (peak 1), cyanidin-3-*O*-glucoside with *m/z* 449 $[M]^+$ ion (peak 2), pelargonidin-3-*O*-glucoside with *m/z* 433 $[M]^+$ ion (peak 3) and peonidin-3-*O*-glucoside with *m/z* 463 $[M]^+$ ion (peak 4) (Figure 14A, Table 7, Appendix Figure 2). By far the largest peak of the LC data is cyanidin-3-*O*-glucoside. This was further confirmed the identity of this compound by comparison with an authentic standard of cyanidin-3-*O*-glucoside. The data of the red fruit are in contrast with those of the white fruit, where LC-MS analysis revealed no detectable anthocyanins in the fruit skin (Figure 14B).

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The level of cyanidin-3-*O*-glucoside was quantified by HPLC. It gradually increased during fruit growth and ripening, reaching its maximum at the last stage studied and the pattern of changes was similar to total anthocyanin content (Figure 12A and 15B, Appendix Table 15). In contrast, anthocyanin peak was not detected in white skin fruit (Appendix Table 16).

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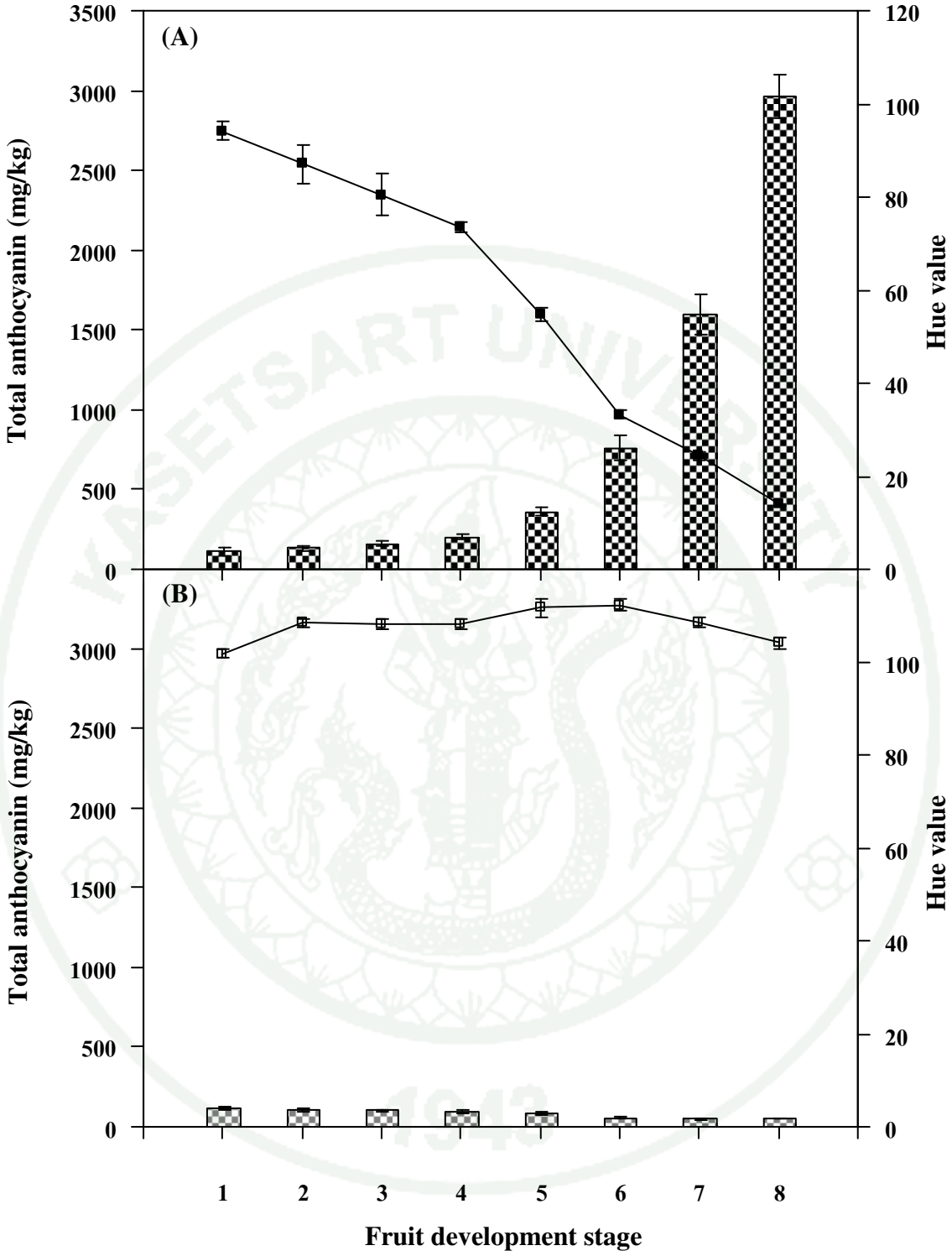


Figure 11 Changes in total anthocyanin content and hue value in the fruit skin of red (▩, ■) and white (▨, □) Malay apple cv. Mamieo during fruit growth and ripening. Data are means ± SE of three replications.

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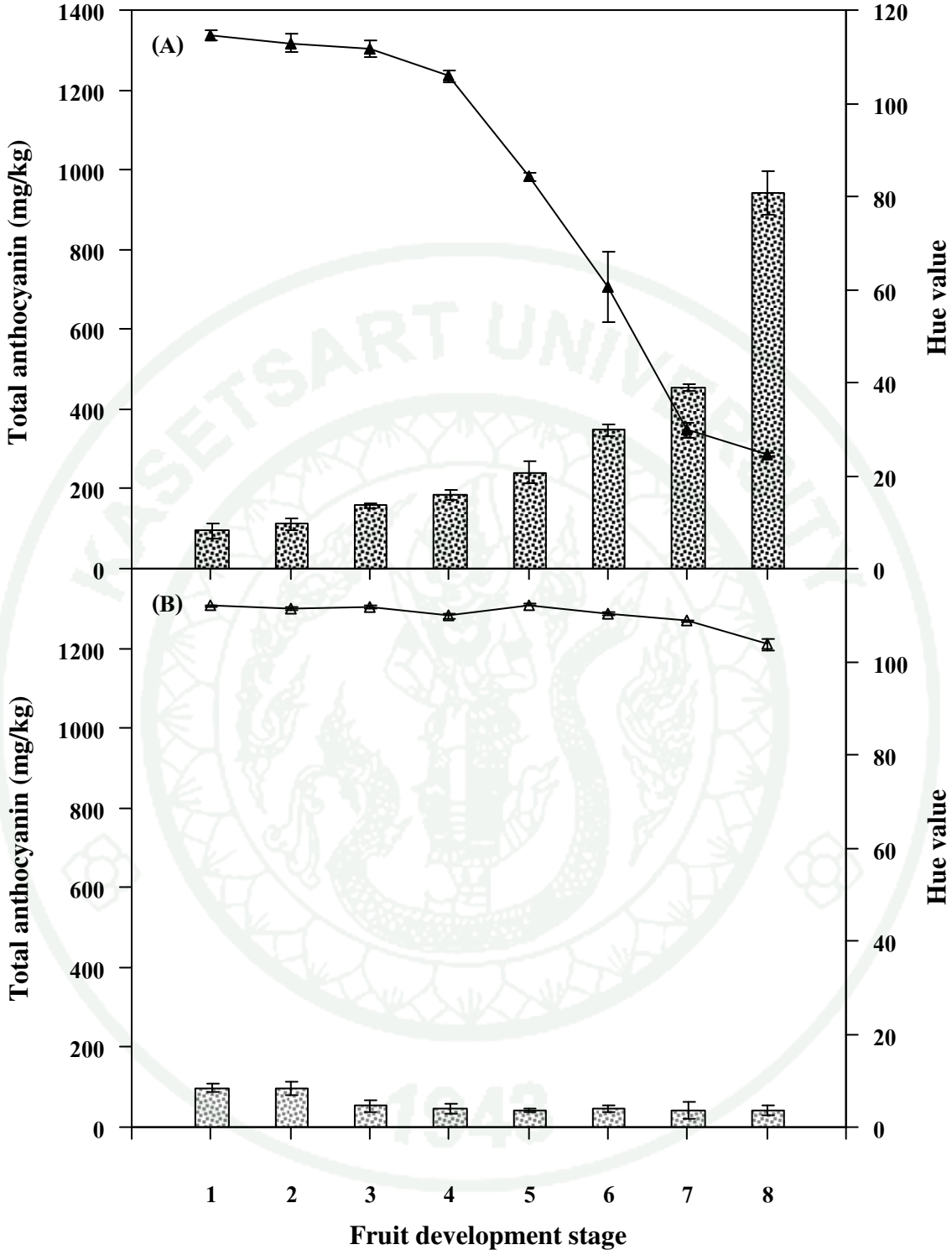


Figure 12 Changes in total anthocyanin content and hue value in the fruit skin of red (▣, ▲) and white (□, Δ) Malay apple cv. Saraek during fruit growth and ripening. Data are means ± SE of three replications.

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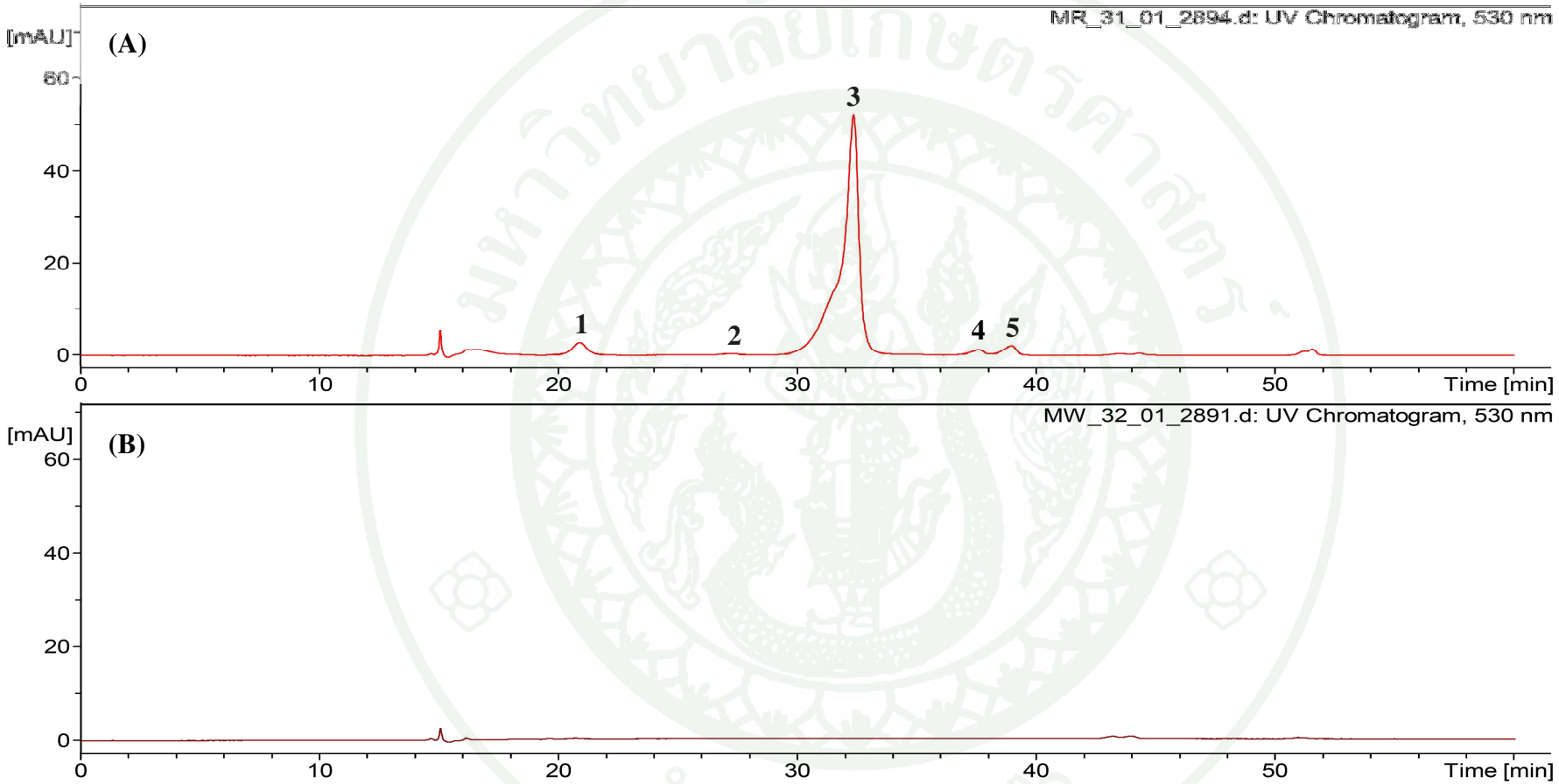


Figure 13 Anthocyanin profiles of the fruit skin in red (A) and white (B) Malay apple cv. Mamieo at the fully ripe fruit stage (developmental stage 8), using LC-MS. Peaks were (1) cyanidin-3,5-*O*-diglucoside, (2) peonidin-3,5-*O*-diglucoside, (3) cyanidin-3-*O*-glucoside, (4) pelargonidin-3-*O*-glucoside, and (5) peonidin-3-*O*-glucoside.

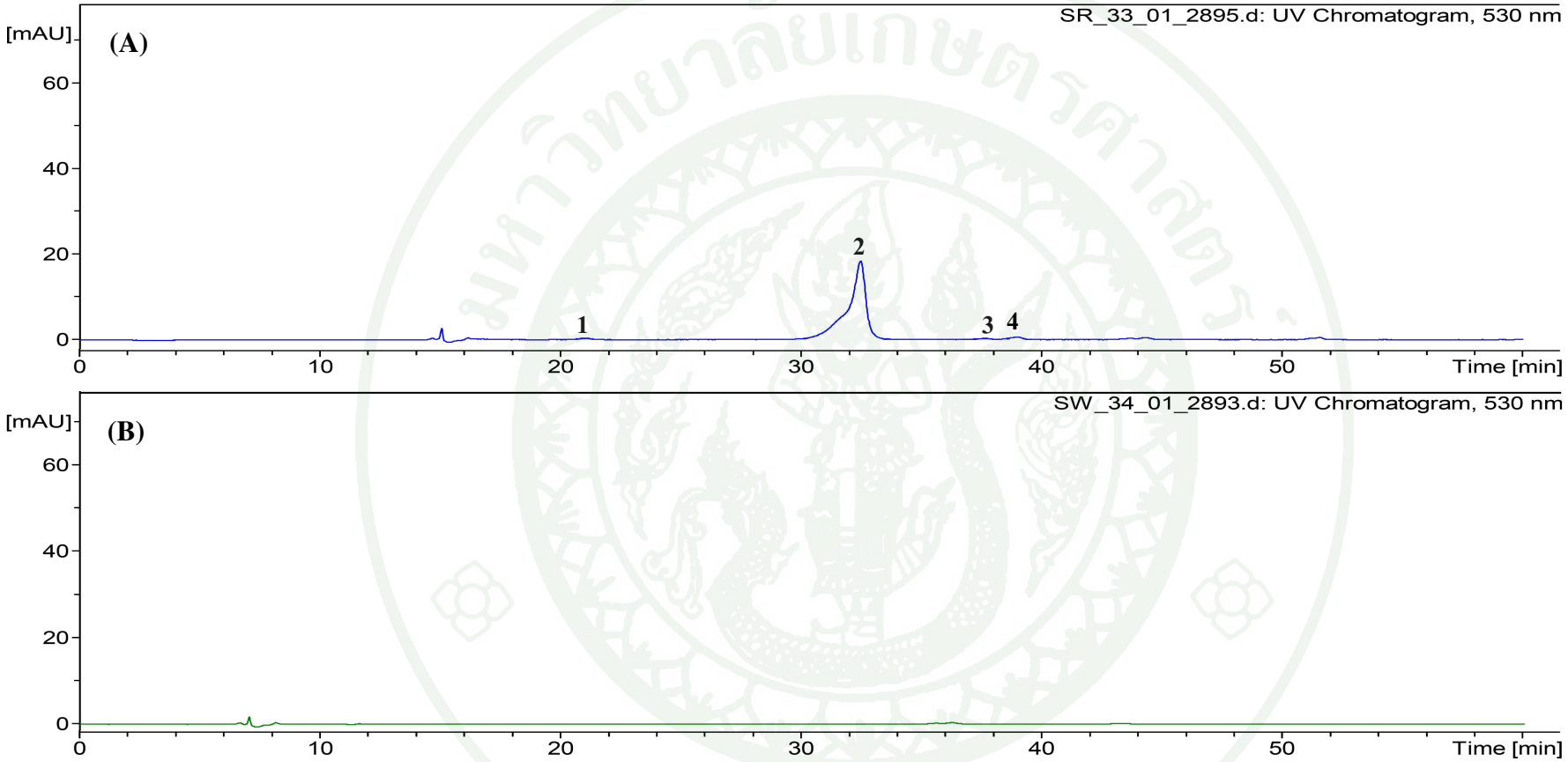


Figure 14 Anthocyanin profiles of the fruit skin in red (A) and white (B) Malay apple cv. Saraek at the fully ripe fruit stage (developmental stage 8), using LC-MS. Peaks were (1) cyanidin-3,5-*O*-diglucoside, (2) cyanidin-3-*O*-glucoside, (3) pelargonidin-3-*O*-glucoside, and (4) peonidin-3-*O*-glucoside.

Table 6 Chromatographic and spectroscopic characteristics of the anthocyanins found in the red skin of Malay apple cv. Mamieo, using LC-MS.

Peak no.	Retention time (min)	M+ (m/z)	M+ aglycone (m/z)	Anthocyanin
1	21.2	611	449,287	cyanidin-3,5-*O*-diglucoside
2	27.4	625	463,301	peonidin-3,5-*O*-diglucoside
3	31.2	449	287	cyanidin-3-*O*-glucoside
4	37.8	433	271	pelargonidin-3-*O*-glucoside
5	39.1	463	301	peonidin-3-*O*-glucoside

Table 7 Chromatographic and spectroscopic characteristics of the anthocyanins found in the red skin of Malay apple cv. Saraek, using LC-MS.

Peak no.	Retention time (min)	M+ (m/z)	M+ aglycone (m/z)	Anthocyanin
1	21.2	611	449,287	cyanidin-3,5-*O*-diglucoside
2	31.8	449	287	cyanidin-3-*O*-glucoside
3	37.8	433	271	pelargonidin-3-*O*-glucoside
4	39.2	463	301	peonidin-3-*O*-glucoside

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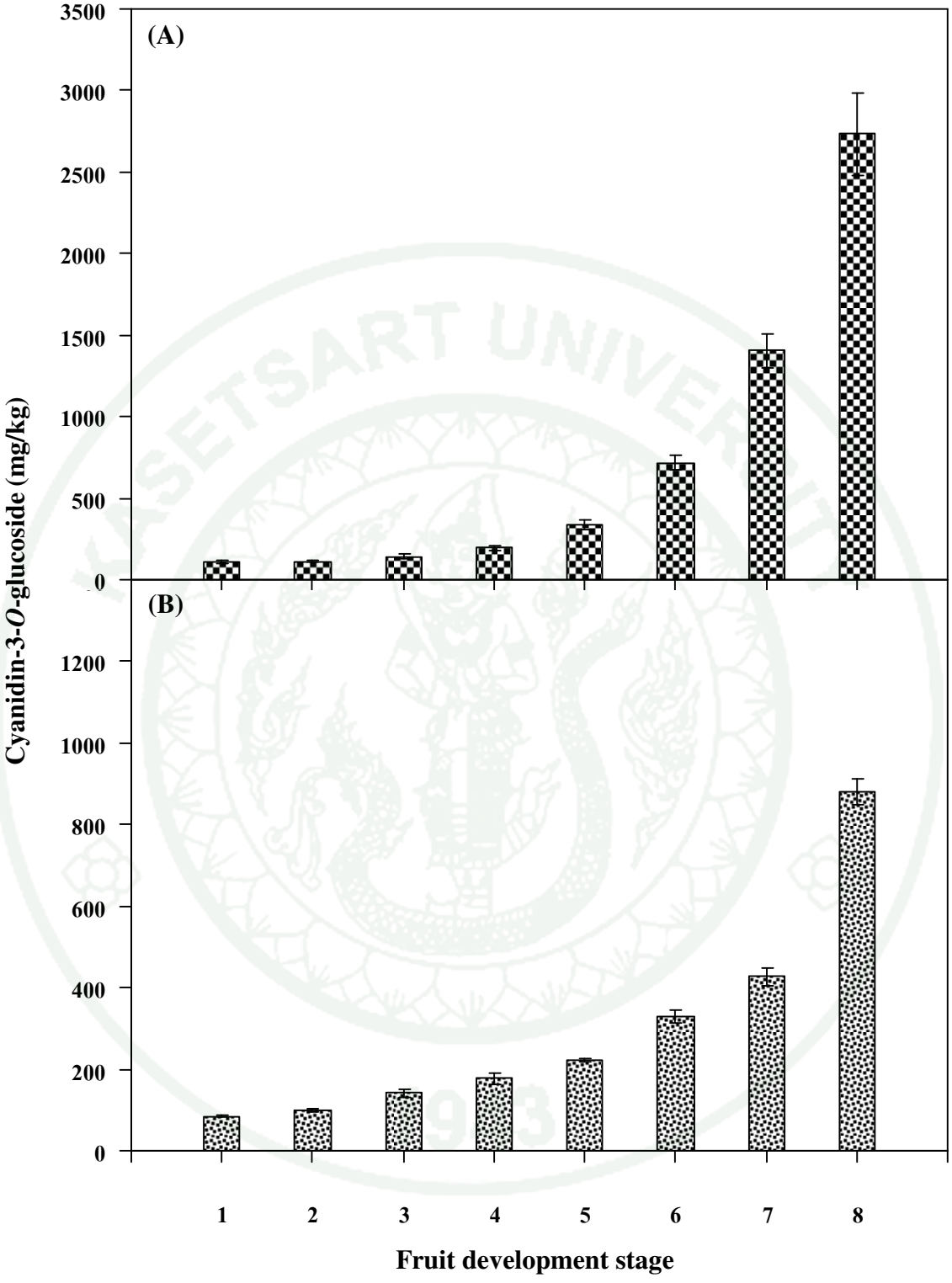


Figure 15 Changes in cyanidin-3-*O*-glucoside content of the fruit skin in red Malay apple cvs. Mamieo (▪) and Saraek (▨) during fruit growth and ripening. Data are means ± SE of three replications.

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3. Study of enzyme activities in the fruit skin of Malay apples during fruit growth and ripening

3.1 Enzyme activities in the fruit skin of Malay apple cv. Mamieo

3.1.1 PAL activity

The PAL activity in the red and white skin fruit remained stable during early fruit growth from stage 1 to stage 2. After that the PAL activity in the red skin fruit increased continuously from stage 3 to stage 8. At stage 3 the PAL activity in the white skin fruit was higher than in the red fruit. It slightly increased from stage 3 to stage 5, and then decreased rapidly during ripening (stage 6 to stage 8). However, the white PAL activity was higher at stage 5 than at stage 3, then was lower at stage 6 than stage 5, and again lower at stage 7 and 8 compared with stage 6 (Figure 16A, Appendix Table 17 and 18).

3.1.2 UFGT activity

The UFGT activity in the skin of red fruit gradually increased until stage 7, and then had increased rapidly by stage 8 (Figure 16B, Appendix Table 17). It correlated with total anthocyanin contents and L*, a* and b* values. However, the skin of white fruit showed UFGT activities that were below the detection level, at all identified stages of fruit growth and ripening on the tree (Figure 16B, Appendix Table 18).

3.2 Enzyme activities in the fruit skin of Malay apple cv. Saraek

3.2.1 PAL activity

The PAL activity in the red skin fruit increased continuously during fruit growth and ripening from stage 1 to stage 7 and then increased rapidly

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from stage 7 to stage 8. However, the PAL activity in the white skin fruit increased slightly during fruit growth and ripening (Figure 17A, Appendix Table 19 and 20).

3.2.2 UFGT activity

The UFGT activity in the skin of red fruit remained stable during the early stage from stage 1 to stage 2 and gradually increased until stage 8 (Figure 17b, Appendix Table 19). It correlated with total anthocyanin contents and L*, a* and b* values. However, the white skin fruit showed UFGT activities below the detection level, at all identified stages of fruit growth and ripening (Figure 17B, Appendix Table 20).

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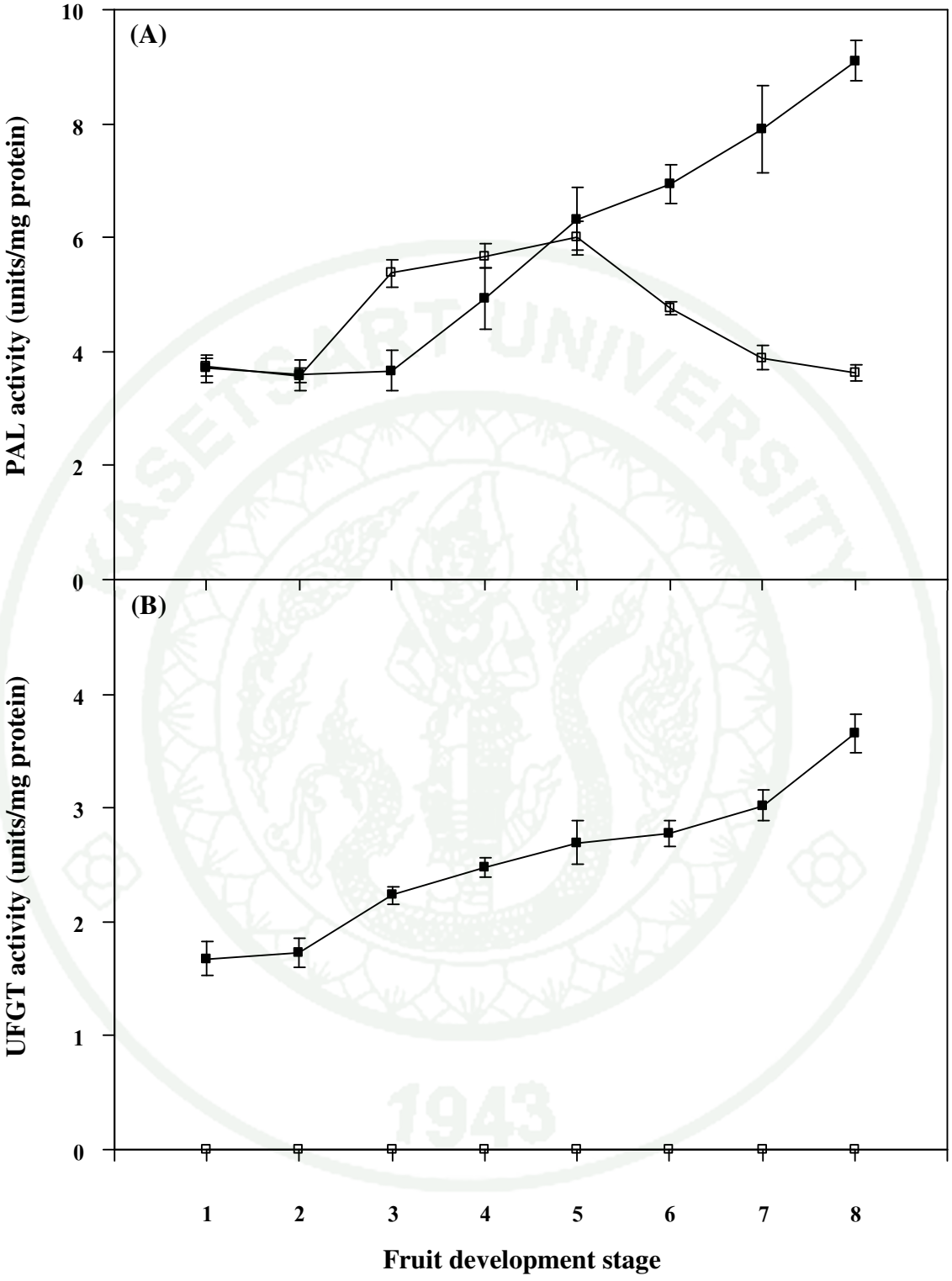


Figure 16 Changes in phenylalanine ammonia lyase (PAL) and UDP- glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) activities in the fruit skin of red (■) and white (□) Malay apple cv. Mamieo during fruit growth and ripening. Data are means ± SE of three replications.

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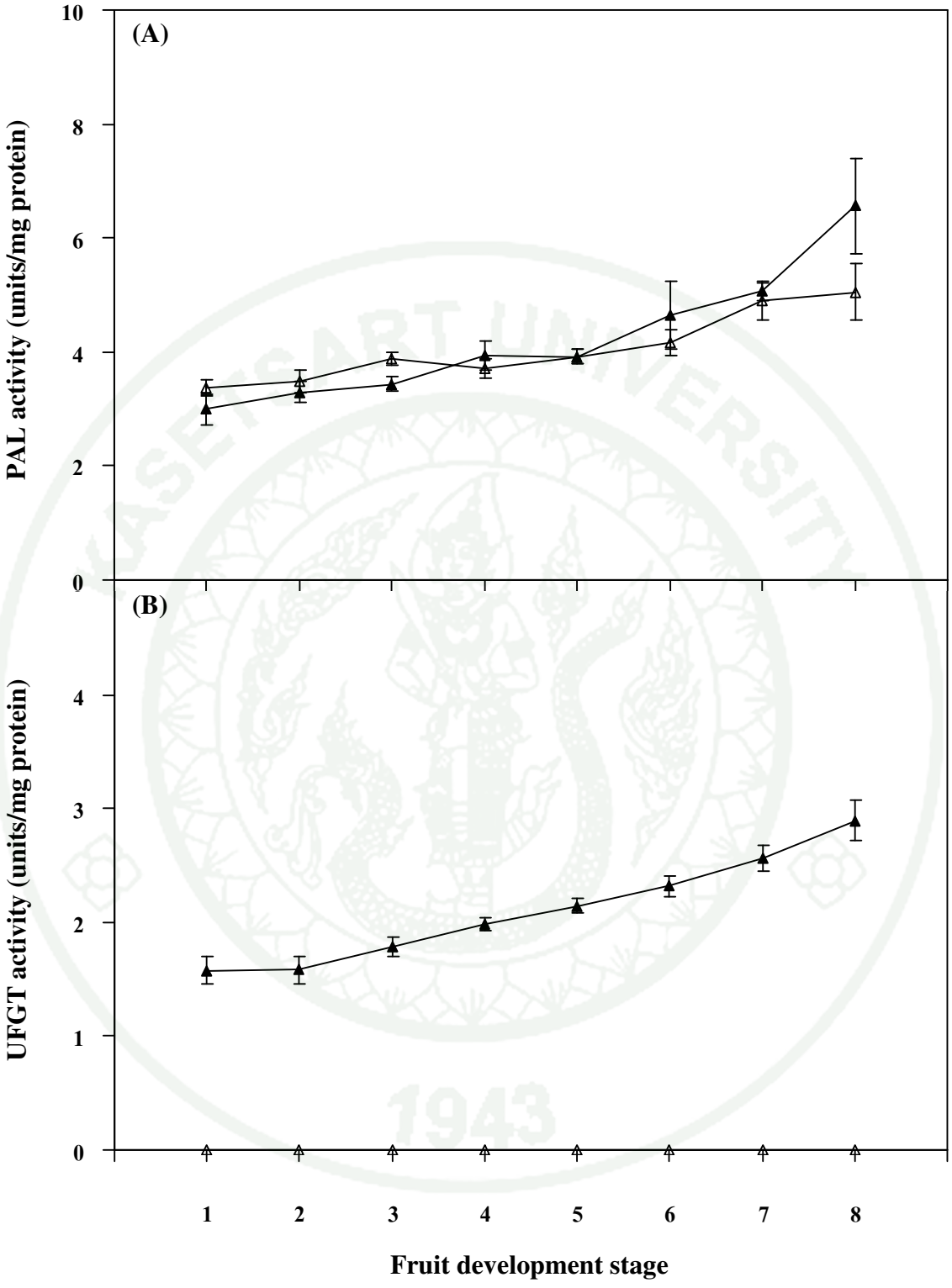


Figure 17 Changes in phenylalanine ammonia lyase (PAL) and UDP- glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) activities in the fruit skin of red (▲) and white (Δ) Malay apple cv. Saraek during fruit growth and ripening. Data are means ± SE of three replications.

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4. Cloning and characterization of anthocyanin biosynthesis genes of Malay apples during fruit growth and ripening

4.1 Isolation and cloning of anthocyanin biosynthesis genes of Malay apple fruit

In the skin of red fruit cv. Mamieo at the fully ripe stage (stage 8), all Malay apple anthocyanin biosynthesis genes were isolated and characterized using forward and reverse degenerate primers (Table 3). The isolated partial coding sequences of all genes were translated to deduced amino acid using Vector NTI 10.0 program (Invitrogen, USA) and then both sequences of nucleotide and amino acid were aligned using ClustalW program (http://www.ebi.ac.uk/clustalw). The isolated partial coding sequences showed considerable homology to other plant anthocyanin biosynthetic genes (accession no. GU233756- GU233762).

1) Phenylalanine ammonia lyase (PAL)

The partial-length cDNA was termed *SmPAL* (accession no. GU233756) and had a 381 bp transcript encoding a predicted protein of 126 amino acids (Figure 18 and 19). After alignment of the partially deduced amino acid sequence with other plants, *SmPAL* gene of Malay apple shared 96-97% homology with *Citrus clementina* x *Citrus reticulata*, *Vitis vinifera*, *Litchi chinensis*, *Rubus idaeus* and *Pyrus communis*, respectively (Table 8). Highly conserved of *SmPAL* gene of Malay apple skin was shown in Figure 20.

2) Chalcone synthase (CHS)

The partial-length cDNA was termed *SmCHS* (accession no. GU233757) and had a 550 bp transcript encoding a predicted protein of 182 amino acids (Figure 21 and 22). After alignment of the partially deduced amino acid sequence with other plants, *SmCHS* gene of Malay apple shared 69-70% homology with *Citrus sinensis*, *Vitis vinifera*, *Malus domestica*, *Pyrus communis* and *Fragaria ananassa*,

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respectively (Table 8). Highly conserved of *SmCHS* gene of Malay apple skin was shown in Figure 23.

3) Chalcone isomerase (CHI)

The partial-length cDNA was termed *SmCHI* (accession no. GU233758) and had a 484 bp transcript encoding a predicted protein of 161 amino acids (Figure 24 and 25). After alignment of the partially deduced amino acid sequence with other plants, *SmCHI* gene of Malay apple shared 70-74% homology with *Malus* sp.**,** *Citrus sinensis***,** *Vitis vinifera***,** *Fragaria ananassa* and *Pyrus communis*, respectively (Table 8). Highly conserved of *SmCHI* gene of Malay apple skin was shown in Figure 26.

4) Flavanone 3-hydroxylase (F3H)

The partial-length cDNA was termed *SmF3H* (accession no. GU233759) and had a 794 bp transcript encoding a predicted protein of 264 amino acids (Figure 27 and 28). After alignment of the partially deduced amino acid sequence with other plants, *SmF3H* gene of Malay apple shared 87-91% homology with *Garcinia mangostana*, *Malus domestica*, *Citrus sinensis*, *Fragaria ananassa*, *Vitis vinifera*, respectively (Table 8). Highly conserved of *SmF3H* gene of Malay apple skin was shown in Figure 29.

5) Dihydroflavonol 4-reductase (DFR)

The partial-length cDNA was termed *SmDFR* (accession no. GU233760) and had a 574 bp transcript encoding a predicted protein of 191 amino acids (Figure 30 and 31). After alignment of the partially deduced amino acid sequence with other plants, *SmDFR* gene of Malay apple shared 75-82% homology with *Fragaria ananassa*, *Garcinia mangostana*, *Malus domestica*, *Vitis vinifera*, *Citrus sinensis*, respectively (Table 8). Highly conserved of *SmDFR* gene of Malay apple skin was shown in Figure 32.

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6) Leucoanthocyanidin dioxygenase (LDOX)

The partial-length cDNA was termed *SmLDOX* (accession no. GU233761) and had a 580 bp transcript encoding a predicted protein of 192 amino acids (Figure 33 and 34). After alignment of the partially deduced amino acid sequence with other plants, *SmLDOX* gene of Malay apple shared 88-92% homology with *Vitis vinifera*, *Malus domestica*, *Citrus sinensis*, *Garcinia mangostana*, *Fragaria ananassa*, respectively (Table 8). Highly conserved of *SmLDOX* gene of Malay apple skin was shown in Figure 35.

7) UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT)

The partial-length cDNA was termed *SmUFGT* (accession no. GU233762) and had a 947 bp transcript encoding a predicted protein of 351 amino acids (Figure 36 and 37). After alignment of the partially deduced amino acid sequence with other plants, *SmUFGT* gene of Malay apple shared 51-62% homology with *Malus domestica*, *Citrus sinensis*, *Fragaria ananassa*, *Garcinia mangostana*, *Vitis vinifera*, respectively (Table 8). *SmUFGT*, the gene involved in the last step of the synthesis of glucose-based anthocyanins biosynthetic pathway, showed somewhat lower identity (51-62%), at the amino acid level, with genes in other species. Highly conserved of *SmUFGT* gene of Malay apple skin was shown in Figure 38.

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```
  1 CTGACCACTT GACTCACAAA CTGAAGCATC ATCCTGGCCA AATAGAAGCT GCAGCTATAA
 61 TGGAGCACAT TTTGGATGGT AGTTCTTATG TCAAGGCGGC CAAGAAGTTG CACGAGATGG
121 ACCCACTTCA GAAGCCGAAG CAAGATCGGT ATGCGCTGAG GACTTCGCCT CAGTGGTTAG
181 GACCTCAGGT GGAAGTGATC CGTGCATCCA CCAAGTCAAT TGAGCGAGAG ATCAATTCAG
241 TTAATGACAA CCCTTTGATC GATGTGTCGA GGAACAAGGC CTTACATGGC GGTAATTTCC
301 AGGGCACCCC TATTGGTGTC TCAATGGACA ATACCCGGTT GGCTATAGCT TCTATTGGAA
361 AACTCATGTT TGCTCAATTC A
```

Figure 18 The partial nucleotide sequences of *SmPAL* cDNA fragment (accession no. GU233756).

```
  1 DHLTHKLKHH PGQIEAAAIM EHILDGSSYV KAAKKLHEMD PLQKPKQDRY ALRTSPQWLG
 61 PQVEVIRAST KSIEREINSV NDNPLIDVSR NKALHGGNFQ GTPIGVSMDN TRLAIASIGK
121 LMFAQF
```

Figure 19 The partial deduced amino acid sequences of *SmPAL* cDNA fragment.

```
Citrus          AIFAEVMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYVKAAKKLHEIDPLQKPK 352
Grape           AIFAEVMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYVKEAKKLHEMDPLQKPK 341
Lychee          AIFAEVMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYIKAAKKLHEMDLLQKPK 355
Raspberry       AIFAEVMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYVKAAEKLHEQDPLQKPK 232
Pear            AIFAEVMHGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSSYVKAAKKLHEQDPLQKPK 351
Malay apple     --------------DHLTHKLKHHPGQIEAAAIMEHILDGSSYVKAAKKLHEMDPLQKPK 46
                             ******************************:* *:**** * *****

Citrus          QDRYALRTSPQWLGPQIEVIRFATKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGV 412
Grape           QDRYALRTSPQWLGPHIEVIRASTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGV 401
Lychee          QDRYALRTSPQWLGPQIEVIRFSTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGV 415
Raspberry       QDRYALRTSPQWLGPQIEVIRFSTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGV 292
Pear            QDRYALRTSPQWLGPQIEVIRYSTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGV 411
Malay apple     QDRYALRTSPQWLGPQVEVIRASTKSIEREINSVNDNPLIDVSRNKALHGGNFQGTPIGV 106
                ***************:::**** :***********************************

Citrus          SMDNTRLAIAAIGKLMFAQFSELVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCS 472
Grape           SMDNTRLAIAAIGKLMFAQFSELVNDFYNNGLPSNLSGSRNPSLDYGFKGAEIAMASYCS 461
Lychee          SMDNTRLAIASIGKLLFAQFSEPVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCS 475
Raspberry       SMDNTRLAIASIGKLMFAQFSELVNDFYNNGLPSNLSGGRDPSLDYGFKGAEIAMASYCS 352
Pear            SMDNTRLAIASIGKLMFAQFSELANDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCS 471
Malay apple     SMDNTRLAIASIGKLMFAQF---------------------------------------- 126
                **********:****:****
```

Figure 20 Alignment of partial deduced amino acid sequences of *SmPAL* cDNA fragment with other plants, citrus (accession no. CAB42793), grape (accession no. ABM67591), lychee (accession no. ACR15762), raspberry (accession no. AAG22550) and pear (accession no. ABB70117). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed.

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```
  1 CAGCCCAAGT CCAAGATCAC CCACCTGGTG TTCTGCACCT ACAGCAGCGT CGACATGCCG
 61 GGCTGCGACT ACCAGCTCAC CAAGCTCTTG GGCCTCCGCC CAACCGTCAA GCGGTACATG
121 ATGTACCAGG TGGGTTGCTA CGGTGGCGGC ATGGTCGTCC GCCTCGCCAA GGACCTCGCC
181 GAGAACAACA AGGGGGCTCG CGTCCTCGTC GTGTGCGCCG AGATCACCAT GGCCGCCTTC
241 CACGGGCCCA ACGAGGCCTT CATGGAGACC CTCCTGGGCC ACGCCCTGTT CGGCGACGGC
301 GCCTCCGCGC TCATCGTGGG CGCAGACCCC GTACCTGAAA TCGAGAGGCC CCTGTACGAG
361 ATTGTCTCCG CGTCCCAGAC CCTCATCCCC AACAGCGAGG GCGCCATCAG CGGACAGGTC
421 CGCCAGATCG GGCTCACCAT CAACCTCCAA AAGGACGTTC CGGCCTTTAT CTCTAAGAAC
481 ATTGAGAAGT GCATGACGGA CGCATTCCAC CCTCTGGGCA TCACGGACTG GAATTCACTC
541 TTCTGGATAA
```

Figure 21 The partial nucleotide sequences of *SmCHS* cDNA fragment (accession no. GU233757).

```
  1 PKSKITHLVF CTYSSVDMPG CDYQLTKLLG LRPTVKRYMM YQVGCYGGGM VVRLAKDLAE
 61 NNKGARVLVV CAEITMAAFH GPNEAFMETL LGHALFGDGA SALIVGADPV PEIERPLYEI
121 VSASQTLIPN SEGAISGQVR QIGLTINLQK DVPAFISKNI EKCMTDAFHP LGITDWNSLF
181 WI
```

Figure 22 The partial deduced amino acid sequences of *SmCHS* cDNA fragment.

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```
Citrus          DKSMIKKRYMYLTEEILKENPNMCAYMAPSLDARQDIVVVEVPKLGKEAATKAIKEWGQP 120
Grape           DKSMIKKRYMHLTEEILKENPNVCEYMAASLDARQDMVVVEVPKLGKEAAAKAIKEWGQP 120
Apple           DKSMIKKRYMYLTEEILKENPSVCEYMAPSIDARQDMVVVEVPKLGKEAAIKAIKEWGQP 120
Pear            DKSMIKKRYMYLTEEILKENPSVCEYMAPSIDARQDMVVVEVPKLGKEAATKAIKEWGQP 120
Strawberry      DKSMIKKRYMYLTEEILKENPSMCEYMAPSLDARQDMVVVEIPKLGKEAAVKAIKEWGQP 120
Malay apple     ------------------------------------------------------------P 1
                                                                             *

Citrus          KSKITHLIFCTTSGVDMPGADYQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAKDLAEN 180
Grape           KSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRFMMYQQGCFAGGTVLRLAKDLAEN 180
Apple           KSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN 180
Pear            KSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN 180
Strawberry      KSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMYQQGCFAGGTVLRLAKDLAEN 180
Malay apple     KSKITHLVFCTYSSVDMPGCDYQLTKLLGLRPTVKRYMMYQVGCYGGGMVVRLAKDLAEN 61
                *******:*** *.*****.************:*** **** **:.** *:*********

Citrus          NKGARVLVVCSEITAVTFRGPADTHLDSLVGQALFGDGAAAVIVGADPDTSVERPLYQLV 240
Grape           NKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAVIVGSDPIPGVEKPMFELV 240
Apple           NKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAVIIGADPVPEVEKPLFELV 240
Pear            NKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAVIIGADPVPEVEKPLFELV 240
Strawberry      NRGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDGAAAIIVGSDPLPEVDRPLFELV 240
Malay apple     NKGARVLVVCAEITMAAFHGPNEAFMETLLGHALFGDGASALIVGADPVPEIERPLYEIV 121
                *:********:*** .:*:** ::.:::*:*:*******:*:*:*:** . :::*::::*

Citrus          STSQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLSEAFAPLGISDWNSIFW 300
Grape           SAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLNEAFQPLGIKDWNSIFW 300
Apple           SAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLNEAFKPIGISDWNSLFW 300
Pear            STAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLNEAFKPIGISDWNSLFW 300
Strawberry      SAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGLISKNIEKSLNEAFKPLNITDWNSLFW 300
Malay apple     SASQTLIPNSEGAISGQVRQIGLTINLQKDVPAFISKNIEKCMTDAFHPLGITDWNSLFW 181
                *::**::*:*:***.*::*::***::* ****.:*******.:.:** *:.*.****:**

Citrus          IAHPGGPAILDQVESKLGLKEEKLKATRQVLSEYGNMPSACVLFILDEMRKKSAEEAKAT 360
Grape           IAHPGGPAILDQVEEKLALKPEKLRSTRHVLSEYGNMSSACVLFILDEMRRKSAEEGLKT 360
Apple           IAHPGGPAILDQVEAKLALKPEKLEATRQVLSDYGNMSSACVLFILDEVRRKSAEKGLKT 360
Pear            IAHPGGPAILDQVEAKLALKPEKLEATRQVLSDYGNMSSACVLFILDEVRRKSAEKGLKT 360
Strawberry      IAHPGGPAILDQVEAKLALKPEKLEATRHILSEYGNMSSACVLFILDEVRRRSAANGHKT 360
Malay apple     I------------------------------------------------------------ 182
                *
```

Figure 23 Alignment of partial deduced amino acid sequences of *SmCHS* cDNA fragment with other plants, citrus (accession no. ACQ41890), grape (accession no. BAB84111), apple (accession no. BAB92996), pear (accession no. AAX16494) and strawberry (accession no. BAE17122). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed. The dot (.) means that the semi-conserved substitutions are observed.

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```
  1 TGCGGGGGCG CGAGGTTTGG AGATCCAGGG GAAGTTCATC AAGTTCACGG CGATCGGCGT
 61 CTACCTGGAG GACGCGGCGC TGCCGTCCCT CGCTGCCAAG TGGGGCGGCA AGAGCGCGGA
121 GGAGCTGGCC GACTGCGTCG AGTTCTTCAG GGACATCGTC ACGGGTCCGT TCGAGAAGTT
181 CTCTCGGGTG ACAATGATAT TGCCATTGAC GGGGGCTCAA TACGCAGAGA AGGTCACTGA
241 GAACTGCGTC AAATACTGGC AATCTGTTGG TACTTACACT GATGCCGAGG CTGCTGCAGT
301 TGAGAAATTC TGCAAGACTT TCAAGGACAA AACCTTCCCG CCCGGTTCCT CGATCCTATT
361 CACGCAATCG CCAAATGGGT CATTGACGAT TGCCTTCTCT GAAGACGGAT CCGTACCCGA
421 AGCCTCGAAC ATGGTGATCG AGAACAGACA ACTCACTGAG GCAGTATTGG AGTCGATCAT
481 TGGA
```

Figure 24 The partial nucleotide sequences of *SmCHI* cDNA fragment (accession no. GU233758).

```
  1 AGARGLEIQG KFIKFTAIGV YLEDAALPSL AAKWGGKSAE ELADCVEFFR DIVTGPFEKF
 61 SRVTMILPLT GAQYAEKVTE NCVKYWQSVG TYTDAEAAAV EKFCKTFKDK TFPPGSSILF
121 TQSPNGSLTI AFSEDGSVPE ASNMVIENRQ LTEAVLESII G
```

Figure 25 The partial deduced amino acid sequences of *SmCHI* cDNA fragment.

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```
Apple           ------------------------------------------------------------
Citrus          MNPSPSVTELQVENVTFTPSVQPPGSTKSHFLGGAGERGLEIEGKFVKFTAIGVYLEDDA 60
Grape           MSQVPSVTAVQVENVLFPPSVKPPGSTNDLFLGGAGVRGLEIQGKFVKFTAIGVYLENSA 60
Strawberry      --MAQSVTGIQIGGMSFPPSVKPPGSGNTFFLGGAGVRGMEIQGNFVKFTAIGVYLEDKA 58
Pear            MAPPPSLAGLQVEATAFPPSVKPPGSSNTLFLGGAGVRGLEIQGNFVKFTAIGVYLEDNA 60
Malay apple     ----------------------------------AGARGLEIQGKFIKFTAIGVYLEDAA 26

Apple           --VDALKWKGKTAEELSESVEFFRDIVTGPFEKFIQVTMILPLTGQQYSEKVSENCVFFW 58
Citrus          VPLLAGKWKGKTAEELTESVEFFRDVVTGPFEKFMKVTMILPLTGAQYSEKVAENCIAIW 120
Grape           VPTLAVKWKGKTVEELADSVDFFRDVVTGPFEKFTKVTTILPLTGRQYSDKVSENCVAFW 120
Strawberry      VPALAVKWKGKTAEELTESVEFFREIVTGPFEKFTQVTMILPLTGQQYSEKVSENCVAIW 118
Pear            VPLLAVKWKGKTAEELSESVEFFRDIVTGPFEKFIQVTTILPLTGQQYSEKVSENCVAFW 120
Malay apple     LPSLAAKWGGKSAEELADCVEFFRDIVTGPFEKFSRVTMILPLTGAQYAEKVTENCVKYW 86
                   * ** **:.***::.*:***::******** :** ****** **::**:***:  *

Apple           KSVGIYTDLEGKAIEQFIDVFKDQNFPPGASILFTQSPKGSLTISFSKDASMPEATNAVI 118
Citrus          KFFGIYTDAEAKAIEKFTEVFKDEIFPPGSSILFTQSP-GSLTISFSKDGSIPKDGVAVI 179
Grape           KSVGIYTDAEAKAIEKFNEVLKDETFPPGNSILFTHSPLGALTMSFSKDGSLPEVGNAVI 180
Strawberry      KKFGIYTDAEAKAIEKFIEVFKDQTFPPGASILFTQSPDGSLTIGFSKDGCIPEVGNAVI 178
Pear            KSVGIYTDAEGKAIEKFIEVFKDQNFPPGASILFTQSPKGSLTICFSKDASVPEAANAVI 180
Malay apple     QSVGTYTDAEAAAVEKFCKTFKDKTFPPGSSILFTQSPNGSLTIAFSEDGSVPEASNMVI 146
                : .* *** *. *:*:* ..:**: **** *****:** *:**: **:*..:*:    **

Apple           ENKLLSETVLESIVGKHGVSPATKQSLAARLSQLL---------NG----CK------- 157
Citrus          ESNLLSEAVLESMIGKNGVSPAAKKSLAERLSALLN-----VTSDKMK----------- 222
Grape           ENKLLTEAVLESIIGKHGVSPEAKKSLAARLSELFC-----KEAGDEKIEAEKVAPVAC 234
Strawberry      ENKLLSESVLESIIGKPGVSPEARKSVATRLSELLKESDHCVAGNGKVDECTKEAEVKA 237
Pear            ENKLLSEAVLESILGKHGVSPAAKRSLAARLSELL---------NG----CK------- 219
Malay apple     ENRQLTEAVLESIIG---------------------------------------------- 161
                *.. *:*:****::*
```

Figure 26 Alignment of partial deduced amino acid sequences of *SmCHI* cDNA fragment with other plants, apple (accession no. CAA48774), citrus (accession no. BAA36552), grape (accession no. CAA53577), strawberry (accession no. BAE17121) and pear (accession no. ABQ08639). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed. The dot (.) means that the semi-conserved substitutions are observed.

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```
  1 TGCCTACAAC GAATTTAGCA ACGAGATCCC CGTGATATCC CTGGCGGGGA TCGACGAAGT
 61 CGGCGGCCGG AGGGAGGAGA TTTGCAGGAA GATCGTGGAG GCGTGCGAGG ACTGGGGGGT
121 CTTCCAGGTG GTCGACCACG GGGTAGACGC GGGGCTCATC GCCGACATGA CCCGGCTCGC
181 GCGTGAGTTC TTCGCGCTGC CCCCGGAGGA GAAGCTCCGG TTCGACATGT CCGGCGGGAA
241 GAAAGGGGGG TTCATCGTCT CGAGCCATCT CCAAGGTGAA GCCGTCCAGG ACTGGCGCGA
301 GATAGTGACC TACTTCTCAT ACCCGGTCCG AACCCGAGAC TACTCCCGAT GGCCCGACAA
361 GCCGGAGGGA TGGAAGTCGG CGACGCAGCA GTACAGCGAG AAGCTCATGG GCCTCGCTTG
421 CAAGCTCCTG GAGGTCCTCT CGGAGGCAAT GGGATTGGAG AAGGAGGCGC TGACCAAGGC
481 GTGTGTGGAC ATGGACCAGA AGGTGGTGGT GAACTACTAC CCCAAATGCC CGCAGCCCGA
541 CCTCACGCTC GGGCTCAAGC GCCACACCGA CCCGGGAACC ATCACCCTCT TGCTCCAGGA
601 CCAGGTGGGT GGCCTCCAGG CCACCAGAGA CGGTGGCAAG AACTGGATCA CCGTCCAGCC
661 CGTGGAAGGG GCTTTCGTGG TCAACCTAGG TGATCACGGT CATTTCCTGA GCAACGGGAG
721 GTTCAAGAAC GCGGACCACC AGGCGGTGGT GAACTCCAAC TACAGCCGCC TCTCCATAGC
781 TACATTCCAG AACA
```

Figure 27 The partial nucleotide sequences of *SmF3H* cDNA fragment (accession no. GU233759).

```
  1 AYNEFSNEIP VISLAGIDEV GGRREEICRK IVEACEDWGV FQVVDHGVDA GLIADMTRLA
 61 REFFALPPEE KLRFDMSGGK KGGFIVSSHL QGEAVQDWRE IVTYFSYPVR TRDYSRWPDK
121 PEGWKSATQQ YSEKLMGLAC KLLEVLSEAM GLEKEALTKA CVDMDQKVVV NYYPKCPQPD
181 LTLGLKRHTD PGTITLLLQD QVGGLQATRD GGKNWITVQP VEGAFVVNLG DHGHFLSNGR
241 FKNADHQAVV NSNYSRLSIA TFQN
```

Figure 28 The partial deduced amino acid sequences of *SmF3H* cDNA fragment.

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```
Mangosteen      MAPTPTTLTALEGETILRASFVRDEDERPKVAYNQFSNDVPVISLEGIDEGGQKRAEICK 60
Apple           MAPPATTLTSIAHEKTLQQKFVRDEDERPKVAYNEFSNEIPIISLAGIDEVEGRRAEICK 60
Citrus          MAP--STLTALAGEKTLNPSFVRFQDERPKVAYNEFSNEIPVISLAGIDDVGGKRAEICK 58
Strawberry      MAPTPTTLTAIAGEKTLQQSFVRDEDERPKVAYNQFSNDIPIISLSGIEEVEGRRAEICK 60
Grape           MAP--TTLTALAGEKTLQSSFVRDEDERPKVAYNDFSNEIPVISLEGIDEVGGRRDEICR 58
Malay apple     ------------------------------AYNEFSNEIPVISLAGIDEVGGRREEICR 29
                                              ***:***::*:*** **::   :* ***:

Mangosteen      KIVEACEEWGIFQVVDHGVDTKLVSEMTRLARAFFALPPEEKLRFDMSGGKKGGFIVSSH 120
Apple           KIVEACEDWGIFQIVDHGVDAELISEMTGLAKEFFDLPSEEKLRFDMSGGKKGGFIVSSH 120
Citrus          KIVEACEDWGIFQVVDHGVDAKLISDMTRLATEFFALPPEEKLKFDMSGGKKGGFIVSSH 118
Strawberry      KIVEACEDWGVFQIVDHGIDPKLISEMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSH 120
Grape           KIVEACEDWGIFQVVNHGVDSNLISEMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSH 118
Malay apple     KIVEACEDWGVFQVVDHGVDAGLIADMTRLAREFFALPPEEKLRFDMSGGKKGGFIVSSH 89
                *******:**:**:*:**:*. *:::** **  ** **.****:****************

Mangosteen      LQGEAVQDWREIVTYFSYPTRTRDYSRWPDKPDGWVDVTKDYGDQLMGLACKLLEVLSEA 180
Apple           LQGEAVQDWREIVTYFLYPIRHRDYSRWPDKPEAWREVTKKYSDELMGLACKLLGVLSEA 180
Citrus          LQGEVVKDWREIVTYFSFPKQSRDYSRWPDKPEGWMEVTKEYSDKLMGVACKLLEVLSEA 178
Strawberry      LQGEAVQDWREIVTYFSYPVRHRDYSRWPDKPEGWRDVTTQYSDELMGLACKLLEVLSEA 180
Grape           LQGEAVQDWREIVTYFSYPLRTRDYSRWPDKPEGWRSVTQEYSEKLMGLACKLLEVLSEA 178
Malay apple     LQGEAVQDWREIVTYFSYPVRTRDYSRWPDKPEGWKSATQQYSEKLMGLACKLLEVLSEA 149
                ****.*:********* :* : **********:.* ..* .*.::***:***** *****

Mangosteen      MGLEKEALTKACVDMDQKIVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATR 240
Apple           MGLDTEALTKACVDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATR 240
Citrus          MGLEKEALTKACVDMDQKIVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATK 238
Strawberry      MGLEKEALTKACVDMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATR 240
Grape           MDLDKDALTNACVDMDQKVVVNFYPQCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATR 238
Malay apple     MGLEKEALTKACVDMDQKVVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATR 209
                *.*:.:***:********:***:**:*********************************:

Mangosteen      DNGETWITVQPIEGAFVVNLGDHGHFLSNGRFRNADHQAVVNSNCSRLSIATFQNPAPDA 300
Apple           DDGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATFQNPAQDA 300
Citrus          DNGKTWITVQPIEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNSSRLSIATFQNPAPEA 298
Strawberry      DGGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNHSRLSIATFQNPAQEA 300
Grape           DGGKTWITVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNHSRLSIATFQNPAPEA 298
Malay apple     DGGKNWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNYSRLSIATFQN----- 264
                *.*:.******:*************:******:********** **********
```

Figure 29 Alignment of partial deduced amino acid sequences of *SmF3H* cDNA fragment with other plants, mangosteen (accession no. ACM62745), apple (accession no. BAB92997), citrus (accession no. BAA36553), grape (accession no. ABM67589) and strawberry (accession no. AAU04792). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed. The dot (.) means that the semi-conserved substitutions are observed.

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```
  1 CTCTTGGCTG GTCATGAGGC TGCTCGAGCG CGGCTACACC GTCCGTGCCA CCGTCCGCGA
 61 CCCCGGTAAC ATGAAGAAGG TGAAGCACCT GCTGGACCTG CCCCAGGCCA AGACGCACTT
121 GACGCTGTGG AAGGCCGACC TCGCCGACGC GGGGAGCTTC GACGAGCCCA TCCATGGCTG
181 CACCGGCGTG TTCCATGTGG CCACGCCCAT GGATTTCGAG TCCAAGGACC CCGAGAATGA
241 GGTGATAAAA CCGACGGTGG AGGGAGTTCT GAGCATCATG AGAGCATGCG CCAAGGCGAA
301 GACCGTCCGG CGGCTCGTGT TCACCTCCTC GGCCGGGACC CTCGACGTCC AGGAACACCG
361 GAAGCCCGTC TACTACGACG ACGACTGGAG CGACATGGAC TTCGTGCTCG CCAAGAAGAT
421 GACCGGATGG ATGTACTTTG TATCGAAGAC GTTGGCAGAG AAAGCAGCTT GGAAATTTGC
481 GGAAGAGAAC AACATCGACC TGATCAGCAT CATCCCAAGT CTCGTTGTGG GCCCCTTCAT
541 CATGCCTTCA ATGCCACCAA GCCTCATAAC TGCA
```

Figure 30 The partial nucleotide sequences of *SmDFR* cDNA fragment (accession no. GU233760).

```
  1 SWLVMRLLER GYTVRATVRD PGNMKKVKHL LDLPQAKTHL TLWKADLADA GSFDEPIHGC
 61 TGVFHVATPM DFESKDPENE VIKPTVEGVL SIMRACAKAK TVRRLVFTSS AGTLDVQEHR
121 KPVYYDDDWS DMDFVLAKKM TGWMYFVSKT LAEKAAWKFA EENNIDLISI IPSLVVGPFI
181 MPSMPPSLIT A
```

Figure 31 The partial deduced amino acid sequences of *SmDFR* cDNA fragment.

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```
Strawberry      MGLGAESGSVCVTGASGFVGSWLVMRLLEHGYTVRATVRDPANLKKVRHLLELPQAATRL 60
Mangosteen      --MGSQNEIVCVTGASGFIGSWLVMRLLERGYTVRATVRDPDNAKKVQHLLELPKAKTHL 58
Apple           --MGSESESVCVTGASGFIGSWLVMRLLEHGYTVRATVRDPTNQKKVKHLLDLPKAETHL 58
Grape           --MGSQSETVCVTGASGFIGSWLVMRLLERRLTVRATVRDPTNVKKVKHLLDLPKAETHL 58
Citrus          --MGSIAETVCVTGASGFIGSWLIMRLLERGYAVRATVRDPDNKKKVKHLLELPKASTHL 58
Malay apple     --------------------SWLVMRLLERGYTVRATVRDPGNMKKVKHLLDLPQAKTHL 40
                                    ***:*****:  :******** * ***:***:**:* *:*

Strawberry      TLWKADLDVEGSFDEAIKGCTGVFHVATPMDFESEDPENEVIKPTINGMLDIMKACLKAK 120
Mangosteen      TLWKAELGIEGSFDEAIQGCSGVFHVATPMDFESKDPENEVIKPTIDGMIDILKSCAKAK 118
Apple           TLWKADLADEGSFDEAIQGCSGVFHVATPMDFESKDPENEVIKPTINGLLDILKACQKAK 118
Grape           TLWKADLADEGSFDEAIKGCTGVFHVATPMDFESKDPENEVIKPTIEGMLGIMKSCAAAK 118
Citrus          TLWKADLAEEGNFDEAIRGCTGVFHLATPMDFESKDPENEVIRPTINGMVSIMRACKNAK 118
Malay apple     TLWKADLADAGSFDEPIHGCTGVFHVATPMDFESKDPENEVIKPTVEGVLSIMRACAKAK 100
                *****:*   *.***.*:**:****:********:*******:**::*:::.*:::*  **

Strawberry      TVRRVVFTSSAGAVAIEEHRKEVYSENNWSDVVFCRKVKMTGWMYFVSKTLAEQAAWKFA 180
Mangosteen      -VRRIVFTASAGALDVEEHRRPVYDENCWSDLEFINSVKMTGWMYFVSKTKAERAAWKFA 177
Apple           TVRKLVFTSSAGTVNVEEHQKPVYDESNWSDVEFCRSVKMTGWMYFVSKTLAEQAAWKYA 178
Grape           TVRRLVFTSSAGTVNIQEHQLPVYDESCWSDMEFCRAKKMTAWMYFVSKTLAEQAAWKYA 178
Citrus          TVRRLVFTSSAGTLDVEEHRKPVYDETSWSDLDFVRSVKMTGWMYFVSKTLAEQAAWKFA 178
Malay apple     TVRRLVFTSSAGTLDVQEHRKPVYYDDDWSDMDFVLAKKMTGWMYFVSKTLAEKAAWKFA 160
                 **::***:***:: ::**:  ** :  ***: *    ***.******** **:****:*

Strawberry      KENNIDFITIIPTLVIGPFLAPSMPPSLISGLSPLTGNEAHYGIIKQCQYVHLDDLCQSH 240
Mangosteen      KENNLDFISIIPSLVVGPFIMQSMPPSLISALALITGNEGHYTILKQGHYVHLDDLVESH 237
Apple           KENNIDFITIIPTLVIGPFLMPSMPPSLITGLSPILRNESHYGIIKQGQYVHLDDLCLSH 238
Grape           KENNIDFITIIPTLVVGPFIMSSMPPSLITALSPITGNEAHYSIIRQGQFVHLDDLCNAH 238
Citrus          EENNIDFISIIPSLVVGPFLTSSMPPSLITALSPITRNEAHYPIIKQGQFVHLDDLCSAH 238
Malay apple     EENNIDLISIIPSLVVGPFIMPSMPPSLITA----------------------------- 191
                :***:*:*:***:**:***:  *******:.

Strawberry      IFLYEHPKAEGRYICSSHDATIHDIAKLLNEKYPKYNVPKKFKGIEENLTNIHFSSKKLK 300
Mangosteen      IYLYENPKAEGRYICSNYDVNIFELANMLNKKYPEYNIPTTFKGIEENLPSVIFSSKKLL 297
Apple           IYLYEHPKAEGRYICSSHDATIHELVKMLREKYPEYNIPTKFKGIDDNLEPVHFSSKKLR 298
Grape           IYLFENPKAEGRYICSSHDCIILDLAKMLREKYPEYNIPTEFKGVDENLKSVCFSSKKLT 298
Citrus          IFLFEHPNAKGRYICSSHPATILELAKFLREKYPEFNVPTEFEDVDENMKNMLFSSKKLT 298
Malay apple     ------------------------------------------------------------

Strawberry      EMGFEFKHSLEDMFTGAVDACREKGLLPLPQ-EEETEKR--------RAG 341
Mangosteen      DHGFEFKYTLDDMFQGAVETCRKKGLIPLSHFNNDAK------------- 334
Apple           EIGFEFKYSLEDMFVGAVDACRAKGLIPIPIPAEKTEAAEESNLVDVKVG 348
Grape           DLGFEFKYSLEDMFTGAVDTCRAKGLLRPSHEKPVDGKT----------- 337
Citrus          DLGFKFKYSLDDMFTGAVDTCRAKGLLPLLCENHVSEVSI---------- 338
Malay apple     ------------------------------------------------
```

Figure 32 Alignment of partial deduced amino acid sequences of *SmDFR* cDNA fragment with other plants, strawberry (accession no. AAU12363), mangosteen (accession no. ACM62744), apple (accession no. AAD26204), grape (accession no. AAS00611) and citrus (accession no. CAA53578). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed. The dot (.) means that the semi-conserved substitutions are observed.

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```
  1 AGAAGGAGAA GTATGCCAAT GACCAGGGCT CCGGGATGAT CCAGGGCTAC GGGAGCAAGC

 61 TTGCCAACAA CGCCAGTGGG CGGCTCGAGT GGGAAGACTA CTTCTTCCAC CTCGTTTACC
121 CTGAGGACAA GCGCGACTTG TCCATCTGGC CCAAGACCCC AAGCGACTAC ACAGTTGCGA
181 CGAGCGAGTA TGCCAAGCTG CTGAGACAGC TCGCGACCAA AATCCTATCG GCACTCTCAA
241 TTGGTCTAGG ACTTGAAGTA GGGAGGTTGG AGAAGGAAGT CGGCGGGCTA GAGGAAATGC
301 TGCTTCAGAT GAAGATCAAC TACTACCCGA AATGCCCCCA ACCAGAGCTA GCCCTAGGAG
361 TTGAGGCTCA CACGGATGTG AGTGCTCTGA CTTTTATCCT CCACAACATG GTCCCCGGAC
421 TTCAGCTCTT CTATGAGGGC AAGTGGGTCA CGGCCAAATG TGTCCCCAAC TCCATCGTCA
481 TGCACATTGG GGACACAATT GAGATCCTGA GCAATGGCAA GTACAAGAGC ATCCTCCACC
541 GCGGGCTGGT CAACAAGGAA AAGGTGAGAA TCTCATGGAA
```

Figure 33 The partial nucleotide sequences of *SmLDOX* cDNA fragment (accession no. GU233761).

```
  1 KEKYANDQGS GMIQGYGSKL ANNASGRLEW EDYFFHLVYP EDKRDLSIWP KTPSDYTVAT
 61 SEYAKLLRQL ATKILSALSI GLGLEVGRLE KEVGGLEEML LQMKINYYPK CPQPELALGV
121 EAHTDVSALT FILHNMVPGL QLFYEGKWVT AKCVPNSIVM HIGDTIEILS NGKYKSILHR
181 GLVNKEKVRI SW
```

Figure 34 The partial deduced amino acid sequences of *SmLDOX* cDNA fragment.

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```
Grape           SEDEVVRERCREELKKAAMEWGVMHLVNHGISDDLINRVKVAGETFFNLPMEEKEKYAND 118
Apple           SDNEKVRAKCREKLKKATVDWGVMHLVNHGISDELMDKVRKAGKAFFDLPIEQKEKYAND 120
Citrus          SEDRVEREKCREELKKAAMDWGVMHLVNHGISDDLTERVKRAGQAFFDQPVEEKEKYANE 118
Mangosteen      SEDKEVRDKCWDELMKAAKEWGVMHLVNHGISNELTEKVKIAGEAFFQLPIEEKEKYAND 117
Strawberry      SNDIKVREKCREDLKEAAVEWGVMHLINHGISDELMERVKKAGKAFFDLPIEQKEKYAND 120
Malay apple     ------------------------------------------------------KEKYAND 7
                                                                       ******:

Grape           QASGKIAGYGSKLANNASGQLEWEDYFFHLIFPEDKRDMTIWPKTPSDYVPATCEYSVKL 178
Apple           QASGKIQGYGSKLANNASGQLEWEDYFFHCVYPEDKRDLSIWPQTPADYIEATAEYAKQL 180
Citrus          QASGKIQGYGSKLANNASGQLEWEDYFFHLIYPEDKRDMSIWPKTPSDYTEATSEYARQL 178
Mangosteen      QGSGMIQGYGSKLANNASGRLEWEDYFFHLVFPEEKRDLSIWPKTPSDYIEVTSEYARQL 177
Strawberry      QASGKIQGYGSKLANNASGQLEWEDYFFHCVYPEDKRDLSIWPQTPSDYIVATSEYAKEL 180
Malay apple     QGSGMIQGYGSKLANNASGRLEWEDYFFHLVYPEDKRDLSIWPKTPSDYTVATSEYAKLL 67
                *.** * ************:********* ::**:***::***:**:**  .*.**:  *

Grape           RSLATKILSVLSLGLGLEEGRLEKEVGGMEELLLQKKINYYPKCPQPELALGVEAHTDVS 238
Apple           RELATKVLKVLSLGLGLDEGRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVS 240
Citrus          RSLATKILAVLSLGLGLEEGRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDVS 238
Mangosteen      RALATKVLSALSLCLGLEEGRLEKEVGGIEELALQMKINYYPKCPQPELALGVEAHTDVS 237
Strawberry      RGLATKILSILSLGLGLEEGRLEKEVGGLEELLLQMKINYYPKCPQPELALGVEAHTDIS 240
Malay apple     RQLATKILSALSIGLGLEVGRLEKEVGGLEEMLLQMKINYYPKCPQPELALGVEAHTDVS 127
                * ****:*  **: ***: *********:**: ** **********************:*

Grape           ALTFILHNMVPGLQLFYEGKWVTAKCVPNSIIMHIGDTIEILSNGKYKSILHRGLVNKEK 298
Apple           ALTFILHNMVPGLQLFYEGKWVTAKCVPNSIVMHIGDTLEILSNGKYKSILHRGMVNKEK 300
Citrus          ALTFILHNMVPGLQLFYKDKWVTAKCVPNSIILHIGDTIEILSNGEYKSILHRGLVNKEK 298
Mangosteen      ALTFILHNMVPGLQLFYEGEWVTAKCVPNSIIMHIGDTLEILSNGKLKSILHRGVVNKEK 297
Strawberry      ALTFILHNMVPGLQLFYGGKWVTAKCVPNSIVMHIGDTLEILSNGKYKSILHRGLVNKEK 300
Malay apple     ALTFILHNMVPGLQLFYEGKWVTAKCVPNSIVMHIGDTIEILSNGKYKSILHRGLVNKEK 187
                ***************** .:***********::*****:******: *******:*****

Grape           VRISWAVFCEPPKEKIILKPLPETVSETEPPLFPPRTFSQHIQHKLFRKTQEALLSK--- 355
Apple           VRISWAVFCEPPKEKIILKPLPETVSEDEPAMFPPRTFAEHIQHKLFRKSQEALLPK--- 357
Citrus          VRISWAVFCEPPKDKIILKPLPETVSEQKPAMFPPRTFQQHIEHKLFRRTQDALLSDEE- 357
Mangosteen      VRISWAVFCEPPKDKIILKPLPELVSETEPPMFPPRTFSQHIQHKLFRKNQDDVGPN--- 354
Strawberry      VRISWAVFCEPPKEKIILKPLPETVSEEEPAMFPPRTFFEHIQHKLFRQSQEALVSTKES 360
Malay apple     VRISW---------------------------------------------------- 192
                *****
```

Figure 35 Alignment of partial deduced amino acid sequences of *SmLDOX* cDNA fragment with other plants, grape (accession no. ABM67590), apple (accession no. BAB92998), citrus (accession no. AAT02642), mangosteen (accession no. ACM62747) and strawberry (accession no. AAU12369). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed. The dot (.) means that the semi-conserved substitutions are observed.

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```
  1 TTGTTCTTCT TTTTCAACAC TGCCGATTCC ATAGCCTCCA TTTTTTCGGC TTCCAATGAC
 61 TTGCCCAACG TGAGAGCCTA CGACGTGGGA GACGGAGTGC TGGACGGTTA TGTGCGGGTG
121 GGGAAGCCAC AGGAGGACAT CGAGTTGTTC CTGAGGGCGG CCCCAGCCAA CTTCAGGAAG
181 GGGATGGAGG AGGCGGTGGC CAAGACAGGG AGGAGGGTGA GCTGCTTGGT CACCGATGCC
241 TTCTTCTGGT TCTGCGCGGA TATGGCAGCG GAGATGGAGT TGCCGTGGGT GGCCTTCTGG
301 ACTGCTGGCC CTGCCCCTCT CTCCGCCCAT CTCTACACCG AGCATCTCAG GCAAACTCTC
361 GGCGTCTGCC GAGGAATTGA AGGGCGAGAA GATGAGACCC TGCACTTCAT CCCAGGAATG
421 TCGAAAGTAC GCATCAGGGA CTTGCCAGAA GGAGTCGTGT TCGGGAACTT GGACTCACTC
481 TTCTCCCGCA TGCTTTGCGA CATGGGGAGA GCCTTGCCGA GAGCAGCTGC GGTGTTCTTG
541 AACAGCTTCG AGGAGTTGGA CCCAACCATA ACCGCTGATC TCAAGTCCAA GCTCAACAAC
601 TTCCTCAACG TGGGTCCCTT CAACCTCATA GCCACTCCAC CTCGTGCTTC CGACGAAAGC
661 AGGTGCATGT CGTGGCTGGA CGGACAAGGA AGGGCCTCCG TTGCCTACAT TAGCTTTGAG
721 TCAGTGACGG TGCCTCCTCG GAAGGAGATT GTGGAACTAG CAGACGCCTT GGAAGCGAGC
781 CAAGTACCCT TCATATGGTC TCTCAAGGAC CATTCAAGAG AGAATCTGCC AGAAGGGTTT
841 CTGAAGAGGA CCGAGACAAG AGGGATGATG GTGGCATGGG CACCACAGGA GGAGATACTG
901 AGGCACGATG CAGTGGGCGC ATTCGTGACA CATTGCGGAT GGGGCTC
```

Figure 36 The partial nucleotide sequences of *SmUFGT* cDNA fragment (accession no. GU233762).

```
  1 LFFFFNTADS IASIFSASND LPNVRAYDVG DGVLDGYVRV GKPQEDIELF LRAAPANFRK
 61 GMEEAVAKTG RRVSCLVTDA FFWFCADMAA EMELPWVAFW TAGPAPLSAH LYTEHLRQTL
121 GVCRGIEGRE DETLHFIPGM SKVRIRDLPE GVVFGNLDSL FSRMLCDMGR ALPRAAAVFL
181 NSFEELDPTI TADLKSKLNN FLNVGPFNLI ATPPRASDES RCMSWLDGQG RASVAYISFE
241 SVTVPPRKEI VELADALEAS QVPFIWSLKD HSRENLPEGF LKRTETRGMM VAWAPQEEIL
301 RHDAVGAFVT HCGWG
```

Figure 37 The partial deduced amino acid sequences of *SmUFGT* cDNA fragment.

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```
Apple           MAAPLPIEIEPSSTNGQPHLADAYNRHVAVVAFPFTSHASALLETVRRLATALPNTLFSF 60
Citrus          MAQT----------------QSQPRPHIAVLNFPFSTHASSVPSIIKRLAVSSPTAMFTF 44
Strawberry      MAS--------NQAGG----------HVAVLAFPFSTHAAPLLNIVCRLAAAAPSTLFSF 42
Mangosteen      MTKP----------------TTDDHPHVAVLAFPFGTHAAPLLSITHHLAALSPSTHFSF 44
Grape           MSQT----------------TTN--PHVAVLAFPFSTHAAPLLAVVRRLAAAAPHAVFSF 42
Malay apple     ------------------------------------------------------LFFF 4
                                                                       * *

Apple           FSTSKSNSSLFSNNSIDNMPRNIRVYDVADGVPEGYVFVGKPQEDIELFMNAAPENIRRS 120
Citrus          FSTPQSNKALFSTGQQRHLPSNVKPYDVSDGVPEGHVFSGKRQEDIELFMNAADANFRKA 104
Strawberry      FNTKQSNSSILASDTSVLRYTNVCVCEVADGVPEGYVFVGKPQEDIELFMKAAPDNFRKC 102
Mangosteen      FGTPSSNSFILSSN--TNLPPNVKPYDVWDGTPDGYAYTGDVQEEMGLFISAAHESFRKG 102
Grape           FSTSQSNASIFHDS-MHTMQCNIKSYDISDGVPEGYVFAGRPQEDIELFMRAAPESFRQG 101
Malay apple     FNTADSIASIFSAS--NDLP-NVRAYDVGDGVLDGYVRVGKPQEDIELFLRAAPANFRKG 61
                *.* .*   ::  .     *:   :: **. :*:.  *  **:: **: **  .:*:

Apple           LDASVADIGKQISCLITDAFLWFGVHLADELG--VPWVTFWISGLKSLSVHVHTDLIRDT 178
Citrus          VEAAVAETGRPLTCLVTDAFIWFAAEMARDWNN-VPWIPCSPAGPNSLSAHLYTDIIRDK 163
Strawberry      LEASVAESGREVSCLVTDSFFWFGAHMADDMGG-VPWVPFWTAGPASLSAHVHTDLIRNT 161
Mangosteen      VDRAVEESGRRVSCLMSDAFFWFGKEMAEEIGGGVMWVPFWTAGPHALSSHLYTDFIRES 162
Grape           MVMAVAETGRPVSCLVADAFIWFAADMAAEMGV--AWLPFWTAGPNSLSTHVYTDEIREK 159
Malay apple     MEEAVAKTGRRVSCLVTDAFFWFCADMAAEMEL--PWVAFWTAGPAPLSAHLYTEHLRQT 119
                :  :* . *: ::**::*:*:**  .:* :      *:.   :*  .** *::*: :*:.

Apple           IGTQG-ITGRENDLIVDKNVNIQGLSNVRIKDLAEGVIFGNLDSVISGMLLQMGRLLPRA 237
Citrus          IGTQ--SQNQDQQ----LIHFIPGMNKIRVADLPEGVVSGDLDSVFSVMVHQMGRQLPKA 217
Strawberry      TSGDC-HDEKE------TITVIAGMSKVRPQDLPEGIIFGNLESLFSRMLHQMGLMLPLA 214
Mangosteen      FAGD--VTQREDE----LLSSIPGMSRVRVCDLPEGVVFGRLDSLFSQMLHKMGQALPKA 216
Grape           IGVSG-IQGREDE----LLNFIPGMSKVRFRDLQEGIVFGNLNSLFSRMLHRMGQVLPKA 214
Malay apple     LGVCRGIEGREDE----TLHFIPGMSKVRIRDLPEGVVFGNLDSLFSRMLCDMGRALPRA 175
                 .      ::        * *:..:*  ** **:: * *:*::* *:  **  ** *

Apple           TAVFMNGFEELELPIPNDLKSKV-NKLLNVGPSNVASPLPPLPPS----------DACLS 286
Citrus          AAVFINSFEELDPELTNHLKTKFNKKFLSVGPFKLLLASDQQPSSA---TDLDDKYGCLA 274
Strawberry      TAVFINSFEELDPVITNDLKSKF-KRFLNVGPLDLLEPTASAATTTPQTAEAVAGDGCLS 273
Mangosteen      DAVFINSFEELDPTFTNDLKSKL-KCCLNIGPFNLISPPAQVP----------DTYGCIP 265
Grape           TAVFINSFEELDDSLTNDLKSKL-KTYLNIGPFNLITPPPVVP----------NTTGCLQ 263
Malay apple     AAVFLNSFEELDPTITADLKSKL-NNFLNVGPFNLIATPPRAS----------DESRCMS 224
                 ***:*.****:  :. .**:*. :  *.:** .:  .    .            *:

Apple           WLDKQQ-APSSVVYISFGTVASPAEKEQMAIAE-----ALEATGAPFLWSIKDSCKTPLL 340
Citrus          WLDKQKKKPASVAYVGFGTVATPSPNEIAAIAEDQPGPSLEASKVPFIWSLRHRSQANLP 334
Strawberry      WLDKQK-AAS-VVYVSFGSVTRPSPEELMALAE-----ALEASRVPFLWSLRDNLKNPQL 326
Mangosteen      WLDKQQ--LASVAYVSFGSATIPLPHELVALAE-----ALEDRKVPFIWSLKDNAKVHLP 318
Grape           WLKERK--PTSVVYISFGTVTTPPPAELVALAE-----ALEASRVPFIWSLRDKASVHLP 316
Malay apple     WLDGQG--RASVAYISFESVTVPPRKEIVELAD-----ALEASQVPFIWSLKDHSRENLP 277
                **. :    : *.*:.* :.: *   *   :*:     :**   .**:**::.
```

Figure 38 Alignment of partial deduced amino acid sequences of *SmUFGT* cDNA fragment with other plants, apple (accession no. AAD26203), citrus (accession no. AAS00612), strawberry (accession no. AAS89832), mangosteen (accession no. ACM62748) and grape (accession no. BAB41024). The asterisk (*) means the amino acid residues conserved in that column are identical in all sequences in the alignment. The colon (:) means that the conserved substitutions have been observed. The dot (.) means that the semi-conserved substitutions are observed.

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```
Apple           NEFLTKTLSKLNGMVVPWAPQPHVLAHDSVGAFVSHCGWNSIMETIAGRVPMICRPYFAD 400
Citrus          NGFLER--TRSDGIVVDWATQVNVLAHEAVGVFVTHCGWGSILESIAAGVPMIGRPFFGD 392
Strawberry      DEFLSK--GKLNGMVVPWAPQPTGPGAWFSWSFVTHCGWNSVLESVAGGVPLICRPFFGD 384
Mangosteen      DGFLET--TKFQGIVIPWAPQAKVLGHKAVGVFITHCGWNSLLETIVGGVPVICRPFYGD 376
Grape           EGFLEK--TRGYGMVVPWAPQAEVLAHEAVGAFVTHCGWNSLWESVAGGVPLICRPFFGD 374
Malay apple     EGFLKR--TETRGMMVAWAPQEEILRHDAVGAFVTHCGWG-------------------- 315
                : **     .  *::: **.*          *::****.
```

Figure 38 (Continued).

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Table 8 Alignment of deduced amino acid sequences of anthocyanin biosynthesis genes from *Syzygium malaccense* with other plants.

Gene name	Species	Identities (%)	Accession no.
SmPAL	*Pyrus communis*	97	ABB70117
	Citrus clementina x *Citrus reticulata*	96	CAB42793
	Litchi chinensis	96	ACR15762
	Rubus idaeus	96	AAG22550
	Vitis vinifera	96	ABM67591
SmCHS	*Fragaria ananassa*	70	BAE17122
	Malus domestica	70	BAB92996
	Pyrus communis	70	AAX16494
	Citrus sinesis	69	ACQ41890
	Vitis vinifera	69	BAB84111
SmCHI	*Pyrus communis*	74	ABQ08639
	Citrus sinesis	72	BAA36552
	Fragaria ananassa	72	BAE17121
	Vitis vinifera	72	CAA53577
	Malus sp.	70	CAA48774
SmF3H	*Vitis vinifera*	91	ABM67589
	Fragaria ananassa	90	AAU04792
	Citrus sinesis	89	BAA36553
	Malus domestica	88	BAB92997
	Garcinia mangostana	87	ACM62745
SmDFR	*Citrus sinensis*	82	CAA53578
	Vitis vinifera	81	AAS00611
	Malus domestica	79	AAD26204
	Garcinia mangostana	77	ACM62744
	Fragaria ananassa	73	AAU12363
SmLDOX	*Fragaria ananassa*	92	AAU12369
	Citrus sinensis	89	AAT02642
	Garcinia mangostana	89	ACM62747
	Malus domestica	89	BAB92998
	Vitis vinifera	88	ABM67590

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Table 8 (Continued).

Gene name	Species	Identities (%)	Accession no.
SmUFGT	*Vitis vinifera*	62	BAB41024
	Garcinia mangostana	56	ACM62748
	Citrus sinensis	52	AAS00612
	Fragaria ananassa	52	AAS89832
	Malus domestica	51	AAD26203

4.2 Expression analysis of anthocyanin biosynthesis genes of Malay apple during fruit growth and ripening

During the fruit developmental stages 3 to 8 the expression of the seven isolated genes, all encoding enzymes of the anthocyanin biosynthetic pathway, was determined by semi-quantitative RT-PCR. Transcription levels of all seven genes were confirmed by quantitative RT-PCR.

4.2.1 Gene expression in the fruit skin of Malay apple fruit cv. Mamieo

Transcript levels of all seven genes were detectable in the skin of both red and white Malay apple fruit cv. Mamieo, with the exception of *SmUFGT* which was below the detection limit in the white fruit (Figure 39 and 40A-N, Appendix Table 21 and 22). In the red fruit a high transcript abundance of *SmPAL* was found during ripening (Figure 40A). In the white fruit the transcript abundance of a *SmPAL* increased slightly during the early growth, whereas low transcript abundance was found during ripening (Figure 40H). The transcript abundance of *SmCHS*, *SmCHI*, *SmF3H*, *SmDFR* and *SmLDOX* was similar in red and white fruits (Figure 40B-F and 40I-M). In red *SmUFGT* a more than 160-fold gradual increase was found from stage 3 to stage 7. The transcript abundance of *SmUFGT* decreased between stage 7 and stage 8 (Figure 40G).

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4.2.2 Gene expression in the fruit skin of Malay apple fruit cv. Saraek

Transcript levels of all seven genes were detectable in the skin of Malay apple fruit cv. Saraek in both red and white fruit, with the exception of *SmUFGT* which was below the detection limit in the white fruit(Figure 41 and 42A-N, Appendix Table 23 and 24). The transcription levels of Malay apple fruit cv. Saraek was similar in the Malay apple fruit cv. Mamieo. In the red fruit the transcript abundance of a *SmPAL* increased slightly during early fruit growth and high transcript abundance was found during ripening (Figure 42A). In the white fruit the transcript abundance of a *SmPAL* remained stable during fruit growth and ripening (Figure 42H). The transcript abundance of *SmCHS*, *SmCHI*, *SmF3H*, *SmDFR* and *SmLDOX* was similar in red and white fruits (Figure 42B-F and 42I-M). In red fruit, *SmUFGT* gradual increase was found from stage 3 to stage 7 and then increased rapidly at stage 8, with a more than 80 fold (Figure 42G).

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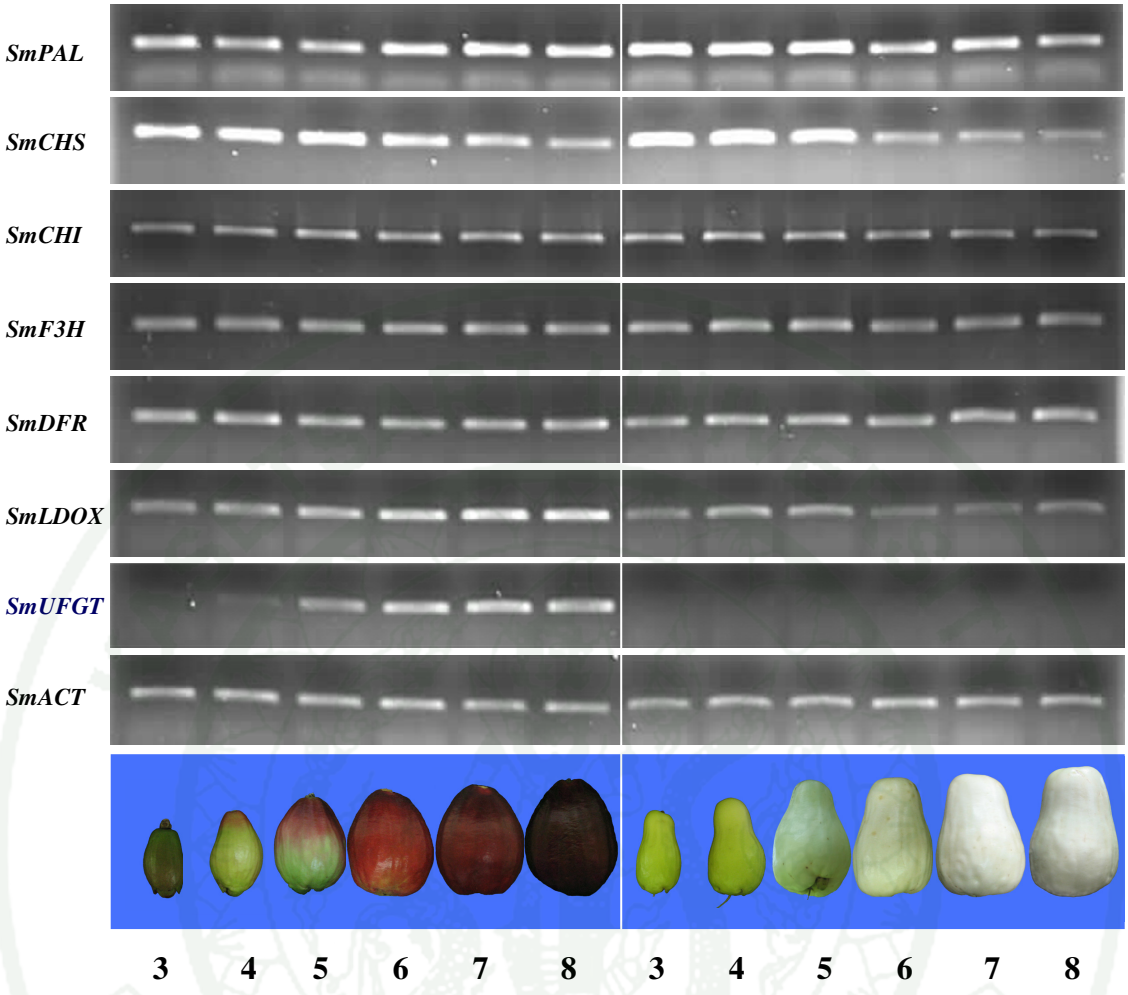


Figure 39 Semi-quantitative RT-PCR of anthocyanin biosynthesis genes in the fruit skin of red and white Malay apple cv. Mamieo during fruit growth and ripening.

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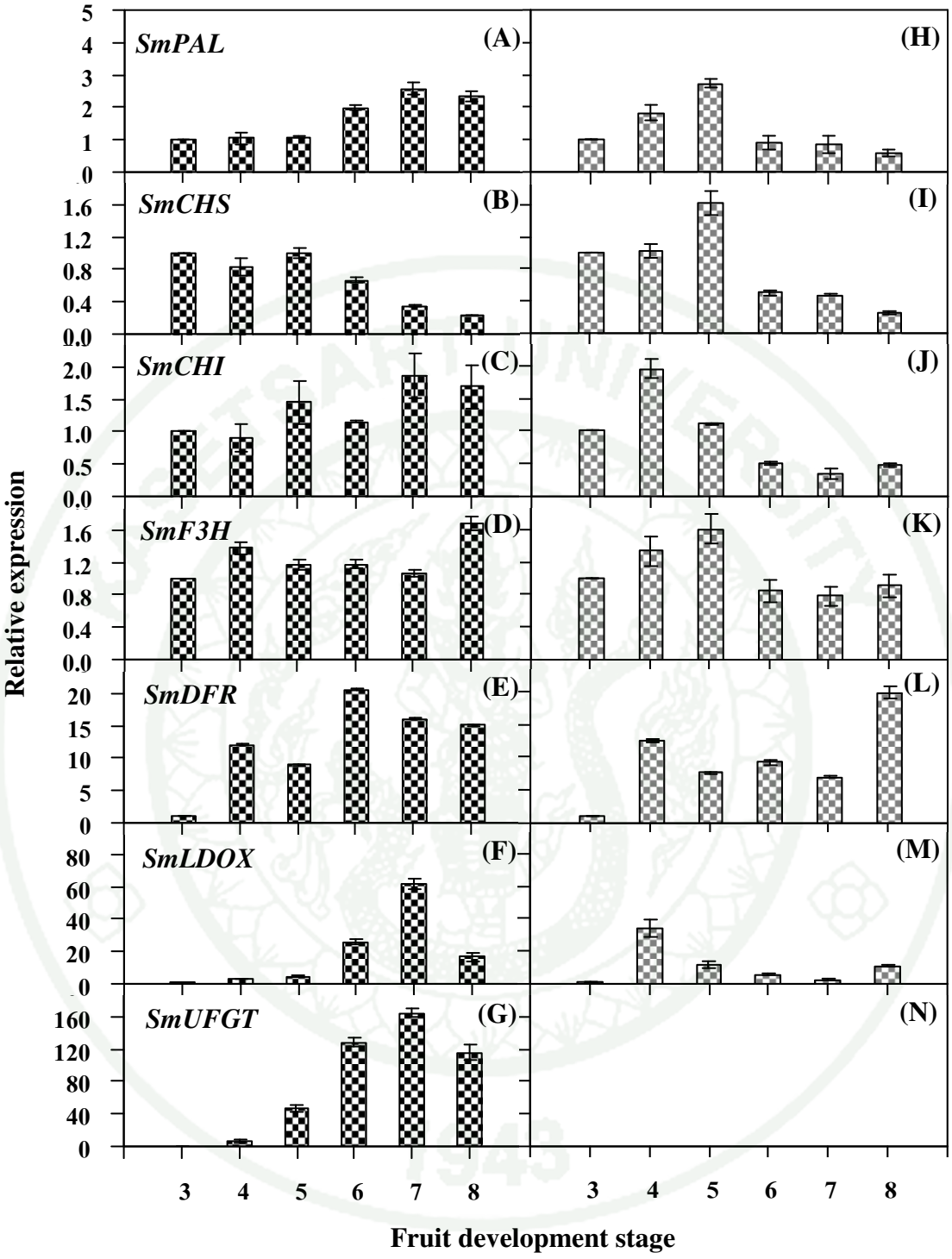


Figure 40 Relative expression of anthocyanin biosynthesis genes in the fruit skin of red (■) and white (□) Malay apple cv. Mamieo during fruit growth and ripening. The column height indicates the relative mRNA abundance, relative to stage 3 which was set at 1.0. All real time-PCR reactions were normalized using the C_T value of an isolated *SmACT* gene. Data are means ± SE of three replications.

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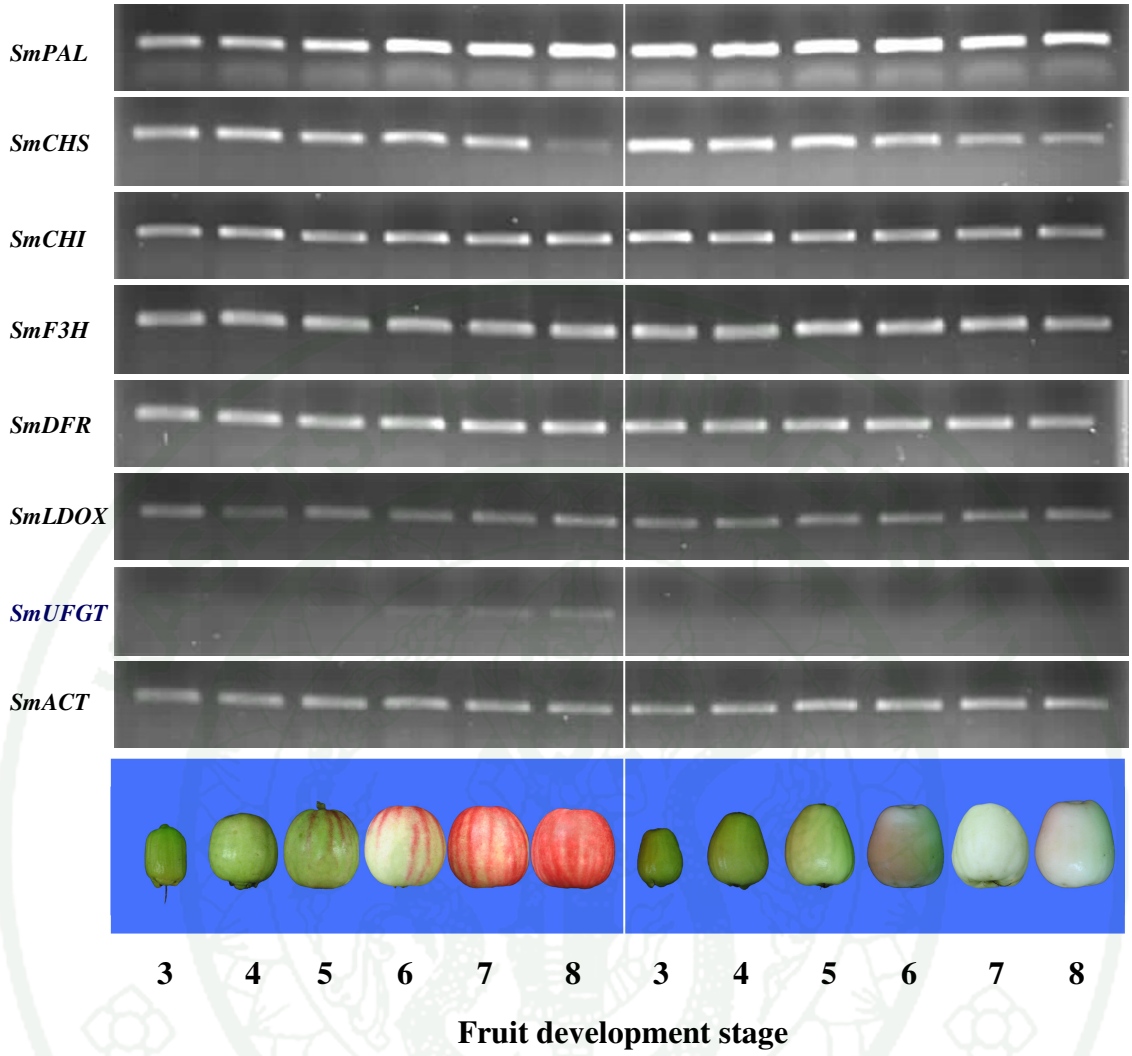


Figure 41 Semi-quantitative RT-PCR of anthocyanin biosynthesis genes in the fruit skin of red and white Malay apple cv. Saraek during fruit growth and ripening.

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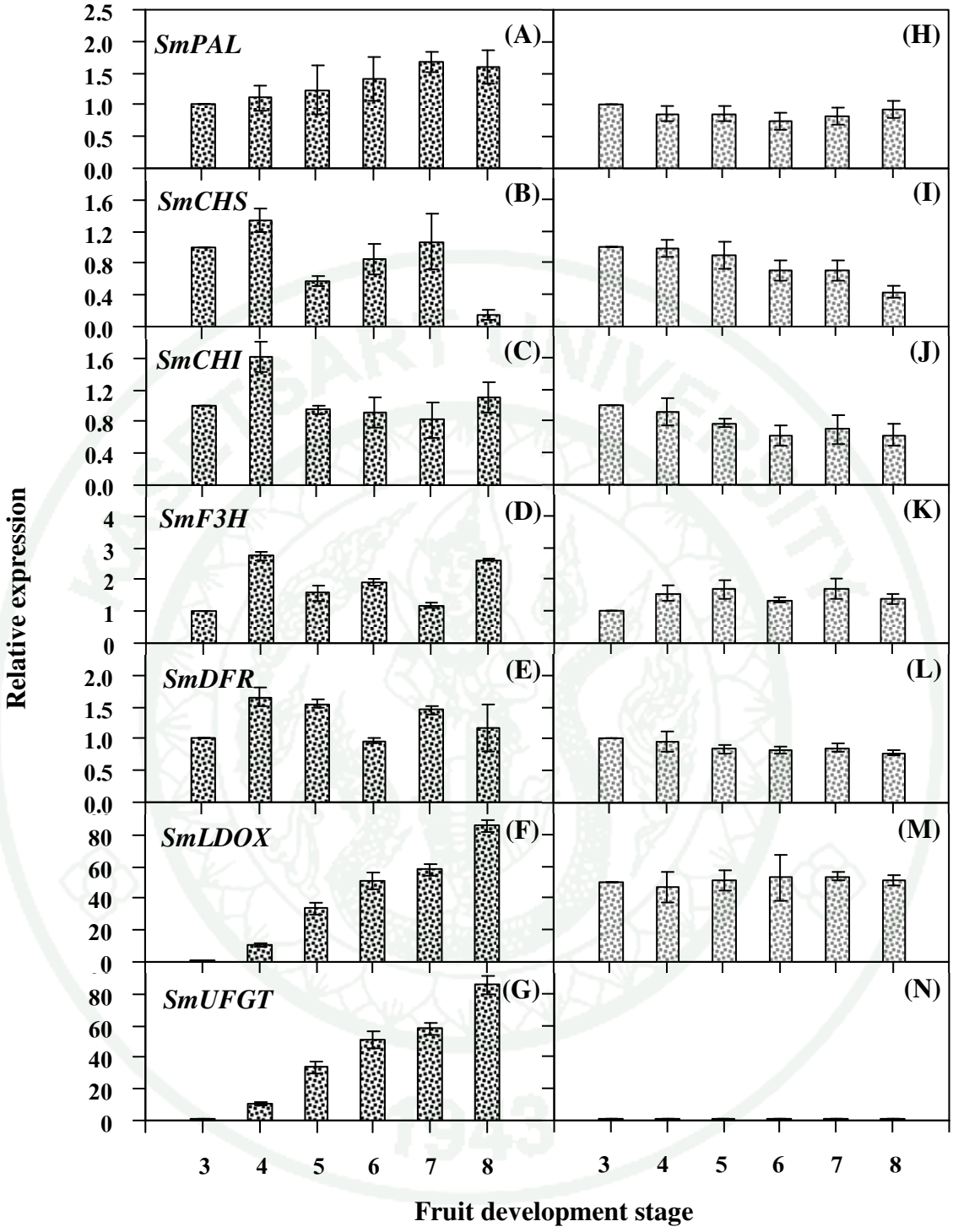


Figure 42 Relative expression of anthocyanin biosynthesis genes in the fruit skin of red (▨) and white (▨) Malay apple cv. Saraek during fruit growth and ripening. The column height indicates the relative mRNA abundance, relative to stage 3 which was set at 1.0. All real time-PCR reactions were normalized using the C_T value of an isolated *SmACT* gene. Data are means ± SE of three replications.

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DISCUSSION

1. Study of fruit growth and quality of Malay apples during fruit growth and ripening

The fruit growth pattern showed single sigmoidal curve in all Malay apple cultivars, with a growing period of 63 days reaching a maximum at stage 8 (Figure 5A-C and 6A-C) similar to many fruits such as loquat (Cuevas *et al*., 2003), and guava (Hernández *et al*., 2007).

Red Malay apple cvs. Mamieo and Saraek are tropical fruit that develops red skin colour during fruit growth and ripening on the tree similar to 'Hass' avocado (Cox *et al*., 2004), pear (Steyn *et al*., 2004) and 'Red Delicious' apple (Ben-Yehudah *et al*., 2005). Red colouration in the fruit skin of red Malay apple cvs. Mamieo and Saraek develops rapidly to dark purple and red streak, respectively which correlated closely with hue values during ripening. In contrast, fruit of white Malay apple cvs. Mamieo and Saraek showed light green colour all over the whole fruit and changed to white or creamy colour during ripening (Figure 4A-B, 7D and 8D). This indicated that development of red colouration of fruit skin is clearly different between red and white skin fruit. Their characteristic is of interest for the fruit model to study about anthocyanin biosynthesis, because the fruit skin exhibits red colour in red fruit and white colour in white fruit.

Fruits of all Malay apple cultivars were harvested during ripening at stage 6 to stage 8. Fruit quality is generally dependent on the fruit development stage. Firmness and TA decreased sharply, while SSC and SSC/TA ratio increased rapidly, during ripening from stage 6 to stage 8 (Figure 9A-D and 10A-D). These results suggest that at stage 8 of fruit development stage reaches a physiological maturity of Malay apple.

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2. Study of anthocyanins in the fruit skin of Malay apples during fruit growth and ripening

As fruit of red Malay apple cvs. Mamieo and Saraek, total anthocyanin content increased strongly and correlated closely with decrease in colour hue during the ripening until reaching a maximum value at the fully ripe stage (Figure 11A and 12B). A similar increase of anthocyanin levels during fruit ripening has also been reported in many other fruits such as strawberry (Gil *et al*., 1997), lychee (Rivera-López *et al*., 1999), apple (Awad and de Jager, 2002), blueberries (Kalt *et al*., 2003), muscadine grape (Lee and Talcott, 2004), grape (Sedade *et al*., 2008) and mangosteen (Palapol *et al*., 2009b). This suggested that anthocyanin accumulation of red Malay apple correlated well with red colour development. The fruit of white Malay apple cvs. Mamieo and Saraek at stage 1 contained anthocyanin levels that were similar to those in the red fruit. In contrast to the red fruit, a decrease in anthocyanin levels was found at later stages of development (Figure 11A-B and 12A-B). Kalt *et al*. (2003) have reported that anthocyanin contents almost undetectable in green unripe blueberries.

LC-MS Analyses of individual anthocyanins in the fruit skin of Malay apple at the fully ripe stage. Chromatographic and spectroscopic characteristics obtained for the anthocyanin peaks are presented in Table 6 and 7. Five compounds of glucose-based anthocyanins in the fruit skin of red Malay apple cvs. Mamieo and Saraek were detected namely cyanidin-3,5-*O*-diglucoside, peonidin-3,5-*O*-diglucoside, cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside and peonidin-3-*O*-glucoside, except peonidin-3,5-*O*-diglucoside which was not found in the fruit skin of Malay apple cv. Saraek. A anthocyanin was not detected in the fruit skin of white Malay apple (Figure 13A and 14B). The identity of these compounds was illustrated by comparison of their chromatographic and spectroscopic characteristics with data in library and confirmed by mass analysis (Giusti *et al*., 1999). After the identification of individual anthocyanins using LC-MS, the content of major anthocyanin was also quantified by HPLC. The major anthocyanin found in the fruit skin of red Malay apple cv. Mamieo and Saraek was cyanidin-3-*O*-glucoside and this was confirmed by authentic standard of cyanidin-3-*O*-glucoside. Other anthocyanins in red fruit were present at too low

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concentration compared to cyanidin-3-*O*-glucoside. Its increase accounted for most of the increase in the total anthocyanin content, during the late stages of fruit growth and during ripening. Red colour development of the fruit skin closely correlated with a strong increase in cyanidin-3-*O*-glucoside content during fruit growth and ripening (Figure 15A-B). To our knowledge, this is the first time that cyanidin-3-*O*-glucoside has been mainly found in the red Malay apple fruit. The content of cyanidin-3-*O*-glucoside showed a similar trend and accounted for almost of the total anthocyanins in red fruit during fruit growth and ripening. This information on individual anthocyanins will be of help in illustrating the anthocyanin biosynthetic pathway in the fruit skin of Malay apple. Cox *et al.* (2004) reported a similar increase in cyanidin-3-*O*-glucoside levels during ripening in avocado cv. Hass, which was correlated red colouration of the skin. The major anthocyanins in 'Wonderful' pomegranate juice also included cyanidin-3-*O*-glucoside (Holcroft *et al*., 1998). In 'Pink Lady' apple, the concentration of cyanidin-3-*O*-galactoside increased during maturation and ripening coinciding with an increase in red blush (Whale and Singh, 2007).

3. Study of enzyme activities in the fruit skin of Malay apples during fruit growth and ripening

PAL and UFGT are important enzymes in anthocyanin biosynthesis in several plant tissues (Lancaster, 1992). Changes in PAL and UFGT activities during fruit growth and ripening of red and white Malay apple cv. Mamieo and Saraek were compared with changes in total anthocyanin contents. PAL activity in the fruit skin of red Malay apple increased continuously during fruit growth and ripening. PAL activity showed a positive correlation with total anthocyanin content. Similarly PAL activity was also found in white skin fruit. This indicated that PAL may not be a key enzyme regulating anthocyanin synthesis in white fruit of Malay apple, which is similar to apple (Saure, 1990; Ju *et al*., 1995b). The increase in PAL activity in red skin fruit during fruit growth and ripening involved in an increase of anthocyanin biosynthesis while PAL activity showed in white skin fruit may be involed in biosynthesis of other phenolic compounds. PAL is the first enzyme in the biosynthetic

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pathway of a wide range of phenylpropanoid compounds such as phenols, anthocyanins, flavonoids and lignins, which are abundant in apple skin (Lancaster *et al*., 1994). PAL is the key enzyme for the phenylpropanoid pathway and not specific to anthocyanin biosynthesis. Anthocyanins are probably the major phenolic compounds being synthesized in red skin of many fruits during ripening and total anthocyanin contents correlated well with PAL activity. According to Ju *et al*. (1995b), PAL catalyze a reaction to produce precurcors of anthocyanin synthesis, under conditions of sufficient precursors, changes in anthocyanin accumulation can occur independently of changes in PAL activity. Changes in anthocyanin accumulation in apple fruit can exist independently of changes in PAL activity; the increase of anthocyanin during additional enzymes between leucocyanidin and cyanidin glycosides. In ripened 'Jonathan' apples, an anthocyanin accumulation decreased, even though PAL activity was relatively high. Therefore, maximum PAL activity is not the regulating factor for anthocyanin accumulation in ripe apple (Wang *et al*., 2000).

UFGT activity was also positively correlated with anthocyanin accumulation during fruit growth and ripening of the red fruit. Similar to Malay apple, in ripening strawberry fruit, the changes in UFGT activity paralleled those in PAL activity (Given *et al.* 1988). These data might suggest a parallel upregulation of the activities of several enzymes involved in anthocyanin biosynthesis. In this study furthermore suggest that the absence of UFGT activity does not block PAL activity. At stage 3 of fruit development in Malay apple cvs. Mamieo (Figure 16) and Saraek (Figure 17), when the white fruit lacked UFGT activity, the white fruit PAL activity was even higher than that in the red fruit. PAL activity, although required for anthocyanin biosynthesis, is also required for the synthesis of several other compounds. Although a correlation is often found between PAL activity and anthocyanin synthesis in fruit skin, exceptions have also been reported (Wang *et al.*, 2000). These data also suggest independence of the regulation of PAL and UFGT activities.

Ju *et al*. (1995a) reported that the importance of UDP-galactose:flavonoid 3-*O*-glucosyltransferase (UFGalT) in regulating anthocyanin biosynthesis in 'Delicious'

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and ‘Ralls’ apples probably depends on the availability of its precursor namely cyanidin. In strawberry fruit, UFGT activity has been shown to parallel with the PAL activity (Given *et al*., 1988). This suggests that anthocyanin biosynthesis is closely associated with developmental stages of the fruit. In strawberry (*Fragaria* x *ananassa*), the flavonoid 3-*O*-glucosyltransferase activity is not essential for redirection from flavonol to anthocyanin formation during fruit ripening. 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 was concomitant with the rise in anthocyanin biosynthesis (Mori *et al*., 2005). This indicated that UFGT is required for anthocyanin biosynthesis in red colouration of many fruits. Therefore, a key step for regulation of anthocyanin biosynthesis in Malay apple is likely to be at the final step of the pathway controlled by UFGT rather than a step controlled by PAL.

4. Cloning and characterization of anthocyanin biosynthesis genes of Malay apples during fruit growth and ripening

In this study the regulatory mechanism and gene expression involved in anthocyanin biosynthesis of Malay apple cvs. Mamieo and Saraek were clarified in comparison with red (red skin) and white (white skin) fruit.

4.1 Characterization of the red Malay apple fruit

The increasing red and purple colour during fruit ripening Malay apple was closely related with an increase, in the skin, of the total anthocyanin content, the levels of five glucose-based anthocyanins, the activity of two enzymes involved in anthocyanin synthesis, and the expression of seven isolated genes that encode enzymes in the biosynthesis of glucose-based anthocyanins. We isolated partial coding sequences of seven genes involved in the anthocyanin biosynthetic pathway. The translated sequences showed high homology to genes encoding enzymes in the anthocyanin pathway in fruits of other plants especially grape, strawberry and pear. The amino acid sequences of *SmPAL*, *SmCHS*, and *SmCHI* were close to those of pear

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(*Pyrus communis*), the amino acid sequences of *SmF3H*, *SmDFR*, and *SmUFGT* were close to those of grape (*Vitis vinifera*), and the amino acid sequences of *SmLDOX* were close to those of strawberry (*Fragaria ananassa*) (Table 8).

All transcripts isolated showed increased expression or a transient increase in expression during fruit growth and ripening. In both the red Malay apple cvs. Mamieo and Saraek, the transcript abundance of *SmPAL* was expressed during fruit growth and ripening, which correlated with PAL activity (Figure 40A and 42B). Therefore, expression of *SmPAL* gives further support to the suggestion that PAL is not a key gene regulating anthocyanin biosynthesis in Malay apple fruit similar to apple (Saure, 1990; Ju *et al*., 1995b). In strawberry, transcript abundance of PAL and CHS did not change markedly during fruit development (Li *et al*., 2001). It has been reported that enzymatic activity of PAL has a peak during fruit ripening, which correlated with the rapid accumulation of anthocyanin in strawberry (Given *et al*., 1988; Cheng and Breen, 1991). During the early stages of fruit development, transcript abundance of CHI and DFR genes was high although there was no anthocyanin accumulation (Li *et al*., 2001).

While the transcript levels of a *SmUFGT* increased markedly with red colouration during fruit growth and ripening. In the red Malay apple cv. Mamieo, the largest increase (more than 160 fold) was found in the transcript of the isolated *SmUFGT* , from development stage 3 to stage 7 and declined at the final stage (stage 8) while the largest increase (more than 80 fold), from development stage 3 to stage 8 in the red Malay apple cv. Saraek (Figure 40G and 42G). This increase in *SmUFGT* expression was correlated with an increase in fruit skin colour, with total anthocyanin content, cyanidin-3-*O*-glucoside content, and UFGT enzyme activity. The expression pattern of the isolated *SmUFGT* was similar to that of an *UFGT* in strawberry fruit (Almeida *et al.* 2007). Expression of *UFGT* catalayzes anthocyanidin glycosylation at a final step of the anthocyanin biosynthetic pathway, was detected in grape fruit skin and contained high anthocyanin levels. The role of UFGT in regulating anthocyanin accumulation in apple skin has been previously reported (Boss *et al*., 1996; Honda *et al*., 2002; Kondo *et al*., 2002). While some studies reported a specific correlation

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between anthocyanin biosynthesis and UFGT expression (Honda *et al*., 2002; 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). In this present study, anthocyanins in the fruit skin of red Malay apple are glucosylated and the UFGT gene encodes a glucosyl-transferase, suggested that the same glucosyl-transferase enzyme utilizes anthocyanins as a substrate. Based on anthocyanin identification, these results correlated with cyanidin pigments in Malay apple fruit. In bilberry (*Vaccinium myrtillus*), it has been demonstrated a coordinated expression of flavonoid biosynthetic genes in relation to accumulation of anthocyanins, proanthocyanidins, and flavonols in developing fruit (Jaakola *et al*., 2002).

4.2 Characterization of the white Malay apple fruit

Thus far, several mutants of the anthocyanin pathway have been partially or fully characterized. At least three categories of mutants can be distinguished: a) those lacking the expression of one or more structural genes encoding enzymes of anthocyanin biosynthesis, b) those lacking expression of transcription factors, and c) those lacking the expression of other genes, including those encoding proteins involved in the vacuolar sequestration of anthocyanins. Examples of the first category are mutations found in species such as tomato (shoots), *Arabidopsis* (seeds) and *Petunia* and *Antirrhinum* (flowers). These mutants include *CHS* (Sommer and Saedler, 1986; O'Neill *et al.*, 1990; Kubo *et al.*, 2007), *CHI* (Holton and Cornish, 1995; Kubo *et al.*, 2007), *F3H* (Martin *et al.*, 1991), *DFR* (Martin *et al.* 1985; Wang *et al.* 1993; Goldsbrough *et al.*, 1994) and *LDOX/ANS* (Martin *et al.*, 1991). Examples of the second category are genes encoding factors in the MYB (Walker *et al.*, 2007) and bHLH (Sweeney *et al.*, 2006) families, and some regulatory factors containing WD40 repeat sequences (Carey *et al.*, 2004). An example of the third category is a gene encoding a glutathione S-transferase involved in vacuolar sequestration of anthocyanins (Larsen *et al.*, 2003). In this study, it is identified a white fruit in the

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first category. The white fruit of Malay apple cvs Mamieo and Saraek lacked UFGT activity, and showed expression of a range of genes encoding enzymes in the anthocyanin biosynthetic pathway, except *SmUFGT*.

The fruit skin of both white Malay apple cvs. Mamieo and Saraek had no detectable levels of anthocyanin (based on LC-MS data), no detectable UFGT activity, and no detectable expression of the isolated *SmUFGT*, while several other genes encoding enzymes in the biosynthetic pathways showed considerable expression. The data thus indicate that the white fruit is correlated with the absence of the last step in anthocyanin synthesis in Malay apple, which involves 3-*O*-glucosylation. It should be noted that the present data do not exclude that the lesion is present in a regulatory gene rather than in the *SmUFGT* gene itself. As noted in the introduction, Walker *et al.* (2007) found that white grapes showed no UFGT activity. They found that the white grapes had a detectable, albeit very low level of *UFGT* transcript. The sequence of the transcript in the red and white grapes was the same, showing that the mutation was not in the *UFGT* gene. It was established that a mutation in a MYB transcription factor was the cause of the low anthocyanin level. In contrast, we now found no detectable *UFGT* transcript in the white fruit. This might indicate that the white fruit is either in the gene itself or due to a transcription factor mutation. Point mutations in At5G17050 were the cause of low anthocyanin levels in the seed coat of three mutant *Arabidopsis* lines (Kubo *et al.*, 2007). At5G17050 encodes an anthocyanidin 3-*O*-glucosyltransferase (UDP-glucose:flavonoid 3-*O*-glucosyl transferase). Anthocyanidins such as cyanidin and pelargonidin as well as flavonols such as kaempferol and quercetin are accepted substrates of this enzyme (TAIR, http://arabidopsis.org/servlets). The protein encoded by this gene thus seems to show a function that is very similar to the presently isolated *SmUFGT*. A T-DNA insertion mutation in At5G17050 also had low amounts of anthocyanin (Tohge *et al.*, 2005). Since the anthocyanin levels in the *Arabidopsis* mutants were not zero, the data indicated that the gene was very important for total anthocyanin production but was complemented by one or more other genes. Indeed, *Arabidopsis* contains at least two genes (At5G17030 and At5G54060) with a function very similar to that of At5G17050. It is not known how many *UFGT* genes are

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expressed in Malay apple fruit skin, but if more than one gene is present they were apparently unable to produce a detectable amount of *UFGT* transcript.

The present data also show an interesting contrast with those of Yuan *et al.* (2009), who investigated the difference between gene expression in four cultivars each of green and red cabbage. The green cultivars contained negligible amounts of anthocyanins. Compared with the red cultivars, the green ones showed (much) lower expression of *PAL*, *CHS*, *F3H*, *DFR* and *LDOX*/*ANS*. This difference in a series of anthocyanin biosynthetic pathway genes indicated a role of transcription factors. Indeed, differences were observed in the expression of at least two such genes (both encoding *MYB* factors) which seemed to regulate the concomitant constitutive expression of nearly all anthocyanin biosynthesis pathway genes in red cabbage. Mano *et al.* (2007) compared the purple, anthocyanin-rich flesh of several sweet potato cultivars with cultivars that have low levels of flesh anthocyanins. A *MYB* transcript was specific for red flesh, and transformation experiments indicated that expression of this *MYB* gene was sufficient for the coordinated induction of all structural anthocyanin biosynthesis genes. Similarly, Schwinn *et al.* (2006) suggested that variation in MYB activity is a key determinant in the variation of anthocyanin levels.

Additionally, compared to an apple cultivar with white fruit flesh, a cultivar with red fruit flesh showed high transcript abundance of several genes encoding anthocyanin pathway enzymes (*CHS*, *CHI*, *F3H*, *DFR*, *LDOX*/*ANS* and *UFGT*). The coordinated expression of these genes was regulated by the combination of a MYB and a bHLH transcription factor, which are known to bind to each other for effective transcriptional control (Espley *et al.*, 2007). Work with *Zea mays* also showed that a MYB and a bHLH transcription factor were necessary and sufficient for the induction of all the genes in the anthocyanin biosynthetic pathway (Quattrochio *et al.* 2002).

These data suggest the coordinated activation, by transcription factors such as MYB and bHLH, of all genes encoding enzymes involved in anthocyanin

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biosynthesis. This is in contrast to what this study found, as in the white Malay apple all genes were expressed with the exception only of *UFGT*. This data might therefore suggest the hypothesis that the Malay apple mutant is not a MYB or bHLH transcription factor mutant. However, other data show that combinations of transcription factors can also affect parts of the anthocyanin pathway. For example, Quattroccio *et al.* (2002) reported that an2 (a MYB transcription factor) and jaf 12 (a bHLH transcription factor) activated *DFR* in *Petunia* flowers, but did not activate *CHS* and *F3H*. Moreover, it was found that both in flowers of *Antirrhinum majus* and *Petunia hydrida* (a combination of) transcription factors did not act on the genes involved in the early steps in the anthocyanin biosynthetic pathway, but did act on genes involved in later steps. In this study data are different from these findings. So the question is whether some transcription factors have been shown to be specific to only one or two of the genes encoding enzymes in the biosynthetic pathway. Such a transcription factor has apparently not been found, but a WD40 transcriptional regulator seems rather specific. In various plants, *transparent testa glabrous1* (*TTG1*), a WD40 protein (Walker *et al.* 1999), is known to regulate genes such as *DFR* and *ANS*. To determine whether At5G17050, a *F3GT* in *Arabidopsis*, was regulated by *TTG1*, the expression of *UFGT* was examined in a *ttg1* background. The expression of *DFR* was reduced, but that of *UFGT* was not (Kubo *et al.* 2007). The literature data, therefore, cannot be used to suggest that the white fruit is not in a transcription factor or in a transcriptional regulator. However, if the white fruit is not in the production of such a protein, it can be in the *UFGT* gene. Indeed, as noted earlier, mutations in single genes that encode anthocyanin biosynthesis enzymes, including a mutation in the At5G17050 *UFGT* in *Arabidopsis* (Kubo *et al.* 2007), has previously been shown to result in a drastic reduction of anthocyanin levels. The mutation in 4 alleles of *UFGT* in *Arabidopsis* has been found to be due to a) in one allele a change from G to A in nucleotide 810, which resulted in a change of codon 270 from tryptophan to a stop codon, b) in the three other alleles a change of C to T in nucleotide 958, resulting in the change of codon 320 from glutamine to a stop codon (Kubo *et al.* 2007).

In this study data furthermore suggest that the absence of UFGT activity does not block PAL activity. At stage 3 of fruit development, when the white fruit

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lacked UFGT activity, the white fruit PAL activity was even higher than that in the red fruit. PAL activity, although required for anthocyanin biosynthesis, is also required for the synthesis of several other compounds.

In conclusion, the fruit growth pattern of Malay apple cvs. Mamieo and Saraek in both red and white fruits is single sigmoidal curve. Cyanidin-3-*O*-glucoside is the major anthocyanin in the red/purple skin of the fruit in red Malay apple cv. Mamieo and Saraek. The cyanidin-3-*O*-glucoside content markedly increased during fruit growth and ripening. This increase was correlated with colour development, with the activity of UFGT (the enzyme that apparently catalyses the last step of anthocyanin synthesis) and the expression of an isolated *SmUFGT*. The white fruit, showing a white fruit skin, lacked detectable anthocyanins, had no detectable UFGT activity, and no detectable expression of the isolated *SmUFGT*. It did show PAL activity and showed the normal increase transcript abundance of six other genes isolated, all encoding enzymes in the anthocyanin biosynthetic pathway (*CHS*, *CHI*, *F3H*, *DFR* and *LDOX*). The white fruit might be in the gene itself or in a transcription factor. The data suggested that the white fruit involves inhibition of *UFGT* expression at the final step of anthocyanin biosynthetic pathway. The proposed anthocyanin synthesis of Malay apple can be illustrated in Figure 43.

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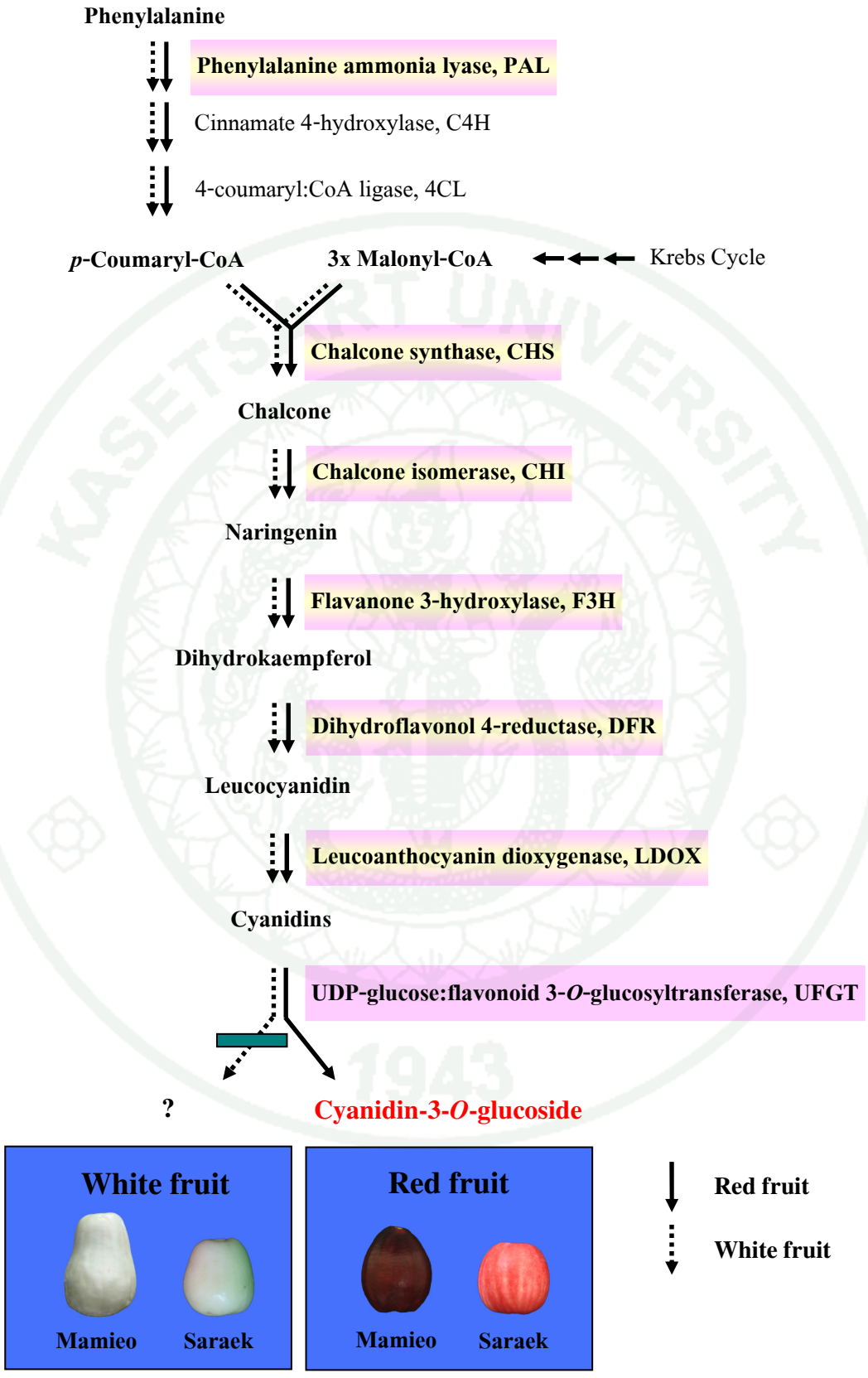


Figure 43 The proposed anthocyanin synthesis of Malay apple fruit.

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CONCLUSIONS

Study on anthocyanin biosynthesis and gene expression of Malay apples during fruit growth and ripening can be concluded as following:

1. The fruit growth pattern of both red and white Malay apple fruit cvs. Mamieo and Saraek exhibits a single sigmoidal curve.

2. The skin of Malay apple cvs. Mamieo and Saraek can develop from green colour to red colour in both red fruits and white colour in both white fruits during fruit growth and ripening.

3. Total anthocyanins content in the skin of red Malay apple fruit cv. Mamieo was higher than red Malay apple fruit cv. Saraek.

4. The skin of red Malay apple fruit cv. Mamieo contains five glucose-based anthocyanins (cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, cyanidin-3,5-*O*-diglucoside and peonidin-3,5-*O*-diglucoside).

5. The skin of red Malay apple fruit cv. Saraek contains four glucose-based anthocyanins (cyanidin-3-*O*-glucoside, pelargonidin-3-*O*-glucoside, peonidin-3-*O*-glucoside and cyanidin-3,5-*O*-diglucoside).

6. Cyanidin-3-*O*-glucoside is the major anthocyanin in the skin of red Malay apple fruit cvs. Mamieo and Saraek. The cyanidin-3-*O*-glucoside content markedly increases during fruit growth and ripening. The accumulation cyanidin-3- *O*-glucoside during fruit growth and ripening is correlated with colour development, activities of phenylalanine ammonia lyase and UDP-glucose:flavonoid 3-*O*-glucosyltransferase.

7. Anthocyanin was not detected in white Malay apple fruits cvs. Mamieo and Saraek.

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8. Transcript levels of all seven genes (*SmPAL*, *SmCHS*, *SmCHI*, *SmF3H*, *SmDFR, SmLDOX* and *SmUFGT*) involved in anthocyanin biosynthesis were detectable in the skin of red and white Malay apple fruit cvs. Mamieo and Saraek, with the exception of *SmUFGT* which was below the detection limit in the white fruit.

9. *SmUFGT* is a key biosynthetic gene in Malay apple pigmentation.

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APPENDIX

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Appendix Table 1 Changes in fruit size (width, length and fresh weight) of red Malay apple cv. Mamieo during fruit growth and ripening.

Fruit development stage	Width[1] (mm)	Length[1] (mm)	Fresh weight[1] (g)
1	15.23h	26.33h	5.97h
2	18.58g	30.68g	14.44g
3	22.90f	38.43f	27.48f
4	33.65e	48.70e	54.96e
5	45.75d	59.58d	76.02d
6	58.10c	65.83c	131.60c
7	64.30b	71.00b	148.74b
8	66.68a	73.55a	155.79a
F-test	**	**	**
CV (%)	3.28	2.58	2.34

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 2 Changes in fruit size (width, length and fresh weight) of white Malay apple cv. Mamieo during fruit growth and ripening.

Fruit development stage	Width[1] (mm)	Length[1] (mm)	Fresh weight[1] (g)
1	18.54h	26.69h	8.22h
2	21.44g	32.59g	14.51g
3	28.86f	45.84f	43.07f
4	35.85e	62.57e	82.49e
5	56.94d	81.52d	123.71d
6	65.83c	91.92c	170.98c
7	71.35b	102.35b	181.40b
8	73.49a	106.32a	192.46a
F-test	**	**	**
CV (%)	2.23	3.77	3.02

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 3 Changes in fruit size (width, length and fresh weight) of red Malay apple cv. Saraek during fruit growth and ripening.

Fruit development stage	Width[1] (mm)	Length[1] (mm)	Fresh weight[1] (g)
1	15.54g	23.43g	2.17f
2	18.06f	26.38f	3.85f
3	25.64e	33.46e	17.12e
4	35.75d	41.20d	44.92d
5	49.70c	50.06c	64.03c
6	55.18b	53.35b	82.61b
7	57.76a	56.58a	89.77a
8	58.37a	56.77a	93.02a
F-test	**	**	**
CV (%)	2.82	4.09	11.99

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 4 Changes in fruit size (width, length and fresh weight) of white Malay apple cv. Saraek during fruit growth and ripening.

Fruit development stage	Width[1] (mm)	Length[1] (mm)	Fresh weight[1] (g)
1	15.09h	21.76h	2.22h
2	19.16g	26.08g	5.15g
3	26.83f	33.09f	15.24f
4	32.87e	39.75e	25.26e
5	38.94d	44.38d	36.08d
6	46.18c	48.50c	64.09c
7	50.37b	51.19b	72.53b
8	51.81a	53.96a	78.41a
F-test	**	**	**
CV (%)	3.33	3.20	4.09

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 5 Changes in fruit skin colour (L*, a*, b* and hue value) of red Malay apple cv. Mamieo during fruit growth and ripening.

Fruit development stage	L* value[1]	a* value[1]	b* value[1]	Hue value[1]
1	44.01a	-1.92e	27.03a	94.23a
2	44.31a	1.03d	25.93a	87.13b
3	43.90a	3.07d	20.46b	80.45c
4	44.40a	3.28d	21.80b	73.39d
5	48.13a	16.56c	22.39b	54.88e
6	39.57b	26.34a	18.12c	33.29f
7	32.20c	25.89a	11.98d	24.40g
8	27.53d	19.18b	4.86e	14.00h
F-test	**	**	**	**
CV (%)	5.77	12.49	5.60	3.60

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 6 Changes in fruit skin colour (L*, a*, b* and hue value) of white Malay apple cv. Mamieo during fruit growth and ripening.

Fruit development stage	L* value[1]	a* value[1]	b* value[1]	Hue value[1]
1	65.92c	-7.71a	30.14c	101.78d
2	67.55bc	-11.48bc	34.57b	108.33b
3	68.29bc	-11.27bc	34.64b	108.01b
4	69.82bc	-11.80c	35.84ab	108.20b
5	72.16b	-15.37d	38.15a	111.66a
6	77.31a	-11.95c	33.17b	112.19a
7	78.49a	-9.77b	26.50d	108.41b
8	81.55a	-7.87a	19.01e	103.99c
F-test	**	**	**	**
CV (%)	3.82	8.95	4.75	1.00

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 7 Changes in fruit skin colour (L*, a*, b* and hue value) of red Malay apple cv. Saraek during fruit growth and ripening.

Fruit development stage	L* value[1]	a* value[1]	b* value[1]	Hue value[1]
1	56.86cd	-15.21f	33.12b	114.66a
2	57.69cd	-14.77f	34.18ab	112.94a
3	59.86c	-14.03f	35.17a	111.72a
4	74.19a	-8.37e	29.40c	105.85b
5	74.12a	2.06d	20.17d	84.16c
6	67.59b	10.26c	18.28e	60.65d
7	56.13d	29.84b	17.08e	29.81e
8	40.10e	37.82a	17.012e	24.35f
F-test	**	**	**	**
CV (%)	2.83	28.02	3.93	3.18

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 8 Changes in fruit skin colour (L*, a*, b* and hue value) of white Malay apple cv. Saraek during fruit growth and ripening.

Fruit development stage	L* value[1]	a* value[1]	b* value[1]	Hue value[1]
1	70.61e	-15.60e	38.74a	111.91a
2	73.64cd	-14.47d	36.86c	111.41a
3	72.47de	-15.24e	38.19ab	111.74a
4	75.12c	-12.47c	34.68d	109.75b
5	71.27e	-15.29e	37.52bc	112.17a
6	77.40b	-12.08c	32.48e	110.36b
7	78.84b	-8.81b	26.05f	108.66c
8	82.70a	-4.12a	16.77g	103.65d
F-test	**	**	**	**
CV (%)	1.54	3.21	2.04	0.45

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 9 Changes in fruit quality (firmness, SSC, TA and SSC:TA) of red Malay apple cv. Mamieo during fruit ripening.

Fruit development stage	Firmness[1] (N)	SSC[1] (%)	TA[1] (%)	SSC:TA[1]
6	118.67a	5.15c	0.89a	5.79c
7	88.14b	6.00b	0.54b	11.20b
8	30.16c	8.33a	0.50b	16.58a
F-test	**	**	**	**
CV (%)	5.77	12.49	5.60	3.60

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 10 Changes in fruit quality (firmness, SSC, TA and SSC:TA) of white Malay apple cv. Mamieo during fruit ripening.

Fruit development stage	Firmness[1] (N)	SSC[1] (%)	TA[1] (%)	SSC:TA[1]
6	122.96a	4.83c	0.90a	5.37c
7	96.85b	5.90b	0.66b	9.00b
8	58.11c	7.88a	0.57c	13.80a
F-test	**	**	**	**
CV (%)	10.43	6.44	5.23	10.76

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 11 Changes in fruit quality (firmness, SSC, TA and SSC:TA) of red Malay apple cv. Saraek during fruit ripening.

Fruit development stage	Firmness[1] (N)	SSC[1] (%)	TA[1] (%)	SSC:TA[1]
6	108.13a	7.80b	0.65a	12.29c
7	86.95b	9.43ab	0.39b	24.50b
8	28.20c	10.95a	0.30b	38.97a
F-test	**	**	**	**
CV (%)	7.14	11.32	19.62	27.82

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 12 Changes in fruit quality (firmness, SSC, TA and SSC:TA) of white Malay apple cv. Saraek during fruit ripening.

Fruit development stage	Firmness[1] (N)	SSC[1] (%)	TA[1] (%)	SSC:TA[1]
6	119.65a	5.98c	0.62a	9.81c
7	85.32b	7.00b	0.41b	17.26b
8	41.68c	9.93a	0.26c	37.76a
F-test	**	**	**	**
CV (%)	13.07	5.16	13.54	9.23

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 13 Changes in total anthocyanin content and cyanidin-3-*O*-glucoside content of red Malay apple skin cv. Mamieo during fruit growth and ripening.

Fruit development stage	Total anthocyanin content[1] (mg/kg)	Cyanidin-3-*O*-glucoside content[1] (mg/kg)
1	113.86f	108.25e
2	132.26ef	112.31e
3	161.88ef	141.73e
4	204.81e	195.65e
5	360.95d	336.93d
6	760.68c	714.38c
7	1596.13b	1405.24b
8	2965.74a	2735.29a
F-test	**	**
CV (%)	6.28	9.22

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 14 Changes in total anthocyanin content and cyanidin-3-*O*-glucoside content of white Malay apple skin cv. Mamieo during fruit growth and ripening.

Fruit development stage	Total anthocyanin content[1] (mg/kg)	Cyanidin-3-*O*-glucoside content[1] (mg/kg)
1	112.50a	0
2	104.49ab	0
3	101.45bc	0
4	94.02cd	0
5	85.46d	0
6	55.25e	0
7	49.44e	0
8	53.59e	0
F-test	**	na
CV (%)	7.27	na

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

na = no analysis

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Appendix Table 15 Changes in total anthocyanin content and cyanidin-3-*O*-glucoside content of red Malay apple skin cv. Saraek during fruit growth and ripening.

Fruit development stage	Total anthocyanin content[1] (mg/kg)	Cyanidin-3-*O*-glucoside content[1] (mg/kg)
1	94.76g	84.40g
2	111.58g	98.15g
3	158.81f	142.28f
4	186.34e	176.74e
5	241.06d	223.71d
6	347.10c	329.24c
7	453.11b	426.63b
8	941.20a	881.32a
F-test	**	**
CV (%)	5.24	3.56

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 16 Changes in total anthocyanin content and cyanidin-3-*O*-glucoside content of white Malay apple skin cv. Saraek during fruit growth and ripening.

Fruit development stage	Total anthocyanin content[1] (mg/kg)	Cyanidin-3-*O*-glucoside content[1] (mg/kg)
1	97.40a	0
2	96.14a	0
3	53.69b	0
4	47.21b	0
5	41.89b	0
6	44.52b	0
7	42.75b	0
8	41.82b	0
F-test	**	na
CV (%)	15.75	na

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 17 Changes in phenylalanine ammonia lyase (PAL) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) activities of red Malay apple skin cv. Mamieo during fruit growth and ripening.

Fruit development stage	PAL activity[1] (unit/mg protein)	UFGT activity[1] (unit/mg protein)
1	3.70e	1.67e
2	3.59e	1.73e
3	3.66e	2.24d
4	4.93d	2.48cd
5	6.33c	2.69bc
6	6.94bc	2.78bc
7	7.91b	3.02b
8	9.10a	3.66a
F-test	**	**
CV (%)	11.52	9.01

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 18 Changes in phenylalanine ammonia lyase (PAL) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) activities of mutant Malay apple skin cv. Mamieo during fruit growth and ripening.

Fruit development stage	PAL activity[1] (unit/mg protein)	UFGT activity[1] (unit/mg protein)
1	3.73d	0
2	3.57d	0
3	5.37b	0
4	5.68ab	0
5	6.00a	0
6	4.76c	0
7	3.89d	0
8	3.63d	0
F-test	**	na
CV (%)	5.62	na

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 19 Changes in phenylalanine ammonia lyase (PAL) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) activities of red Malay apple skin cv. Saraek during fruit growth and ripening.

Fruit development stage	PAL activity[1] (unit/mg protein)	UFGT activity[1] (unit/mg protein)
1	2.99d	1.57f
2	3.29d	1.58f
3	3.44d	1.79ef
4	3.94cd	1.98ed
5	3.92cd	2.14cd
6	4.64bc	2.32bc
7	5.07b	2.57b
8	6.56a	2.89a
F-test	**	**
CV (%)	13.55	8.65

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 20 Changes in phenylalanine ammonia lyase (PAL) and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) activities of white Malay apple skin cv. Saraek during fruit growth and ripening.

Fruit development stage	PAL activity[1] (unit/mg protein)	UFGT activity[1] (unit/mg protein)
1	3.37c	0
2	3.47bc	0
3	3.88bc	0
4	3.71bc	0
5	3.92bc	0
6	4.16b	0
7	4.89a	0
8	5.06a	0
F-test	**	na
CV (%)	8.98	na

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

na = no analysis

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Appendix Table 21 Relative expression of anthocyanin biosynthesis genes in the skin of red Malay apple cv. Mamieo during fruit growth and ripening.

Fruit development stage	Genes[1]						
	SmPAL	*SmCHS*	*SmCHI*	*SmF3H*	*SmDFR*	*SmLDOX*	*SmUFGT*
3	1.00c	1.00a	1.00b	1.00d	1.00d	1.00d	1.00d
4	1.05c	0.83b	0.90b	1.38b	12.02c	3.26d	6.45d
5	1.09c	0.99b	1.46ab	1.17c	8.93c	4.77d	46.76c
6	1.98b	0.67c	1.15ab	1.18c	20.60a	26.06b	128.41b
7	2.56a	0.35d	1.86a	1.06d	16.01b	61.88a	164.23a
8	2.35a	0.23e	1.69a	1.68a	15.07b	16.78c	115.26b
F-test	**	**	**	**	**	**	**
CV (%)	15.87	15.63	12.84	15.15	12.17	17.93	11.68

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

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Appendix Table 22 Relative expression of anthocyanin biosynthesis genes in the skin of white Malay apple cv. Mamieo during fruit growth and ripening.

Fruit development stage	Genes[1]						
	SmPAL	*SmCHS*	*SmCHI*	*SmF3H*	*SmDFR*	*SmLDOX*	*SmUFGT*
3	1.00c	1.00b	1.00b	1.00c	1.00e	1.00c	0
4	1.81b	1.02b	1.95a	1.32b	12.50b	34.15a	0
5	2.73a	1.62a	1.11b	1.60a	7.59d	11.55b	0
6	0.89c	0.51c	0.52c	0.84d	9.21c	5.80bc	0
7	0.85c	0.48c	0.34c	0.79d	7.03d	2.49c	0
8	0.59d	0.25d	0.48c	0.91c	19.74a	11.16b	0
F-test	**	**	**	**	**	**	na
CV (%)	13.70	18.73	11.09	19.83	8.21	18.66	na

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

** = significantly different at $P \leq 0.01$

na = no analysis

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Appendix Table 23 Relative expression of anthocyanin biosynthesis genes in the skin of red Malay apple cv. Saraek during fruit growth and ripening.

Fruit development stage	Genes[1]						
	SmPAL	*SmCHS*	*SmCHI*	*SmF3H*	*SmDFR*	*SmLDOX*	*SmUFGT*
3	1.00	1.00ab	1.00ab	1.00d	1.00bc	1.00bc	1.00d
4	1.11	1.34a	1.61a	1.56b	1.66a	0.48d	10.55cd
5	1.23	0.57bc	0.95ab	1.68a	1.55ab	0.58cd	33.56bc
6	1.41	0.85bc	0.91ab	1.34b	0.97c	0.43d	51.31b
7	1.68	1.07ab	0.82b	1.69a	1.46ab	1.09b	58.07b
8	1.60	0.15c	1.11ab	1.38b	1.17ab	1.74a	85.80a
F-test	ns	**	*	**	**	**	**
CV (%)	13.21	17.60	17.69	11.28	12.60	17.68	17.43

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

* = significantly different at $P \leq 0.05$

** = significantly different at $P \leq 0.01$

ns = non-significantly different

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Appendix Table 24 Relative expression of anthocyanin biosynthesis genes in the skin of white Malay apple cv. Saraek during fruit growth and ripening.

Fruit development stage	Genes[1]						
	SmPAL	*SmCHS*	*SmCHI*	*SmF3H*	*SmDFR*	*SmLDOX*	*SmUFGT*
3	1.00a	1.00a	1.00a	1.00a	1.00	1.00	0
4	0.86bc	0.98a	0.91a	0.78a	0.96	0.94	0
5	0.86bc	0.89b	0.78ab	0.84a	0.84	1.02	0
6	0.74d	0.71c	0.61b	0.67b	0.83	1.06	0
7	0.82cd	0.71c	0.69b	0.85a	0.86	1.07	0
8	0.93ab	0.44c	0.62b	0.69b	0.78	1.02	0
F-test	**	*	*	*	ns	ns	na
CV (%)	5.76	17.17	13.47	10.00	15.02	15.83	na

[1] Mean values followed by different letters in the same column are significantly different at $P \leq 0.05$ using DMRT

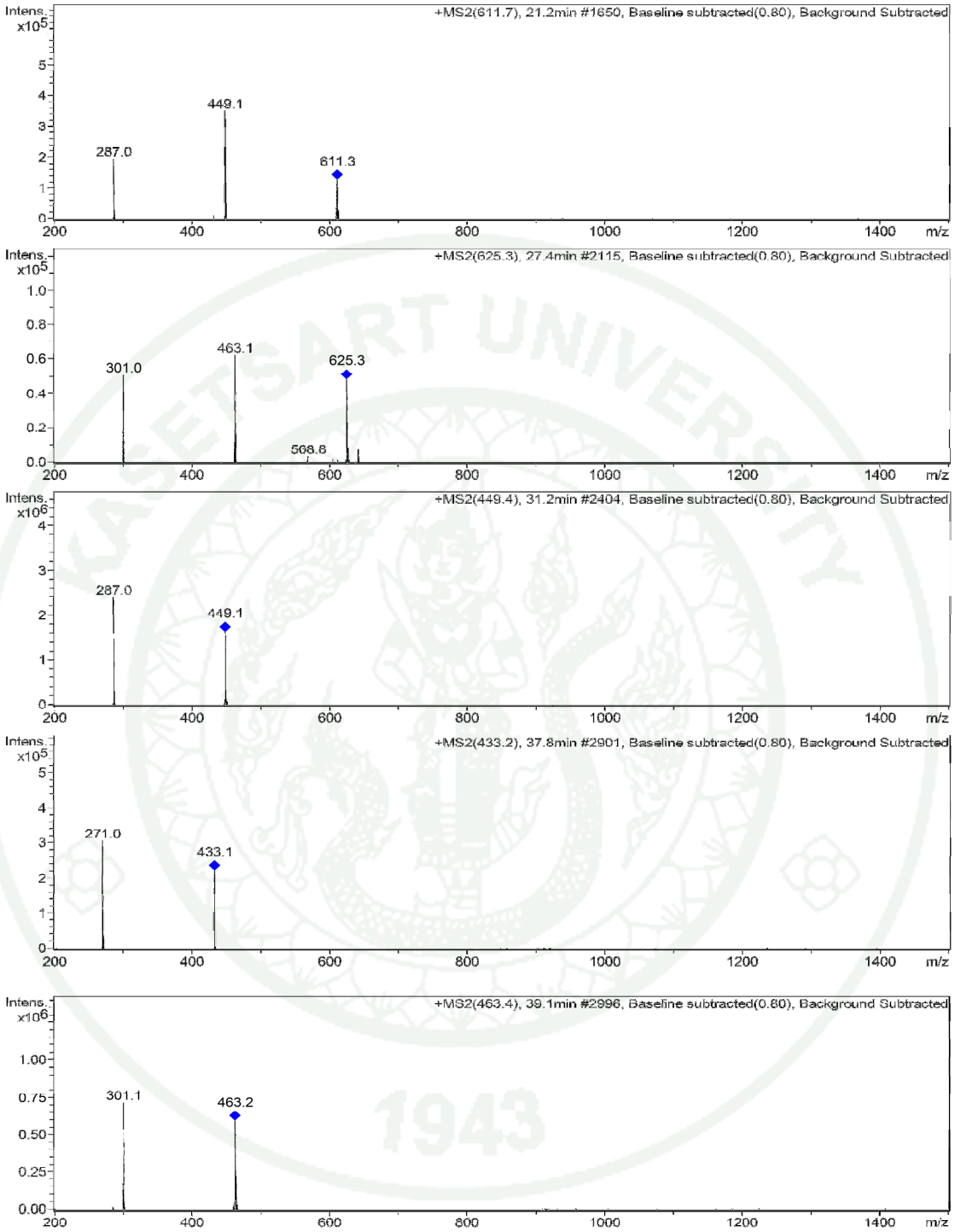
* = significantly different at $P \leq 0.05$

** = significantly different at $P \leq 0.01$

ns = non-significantly different

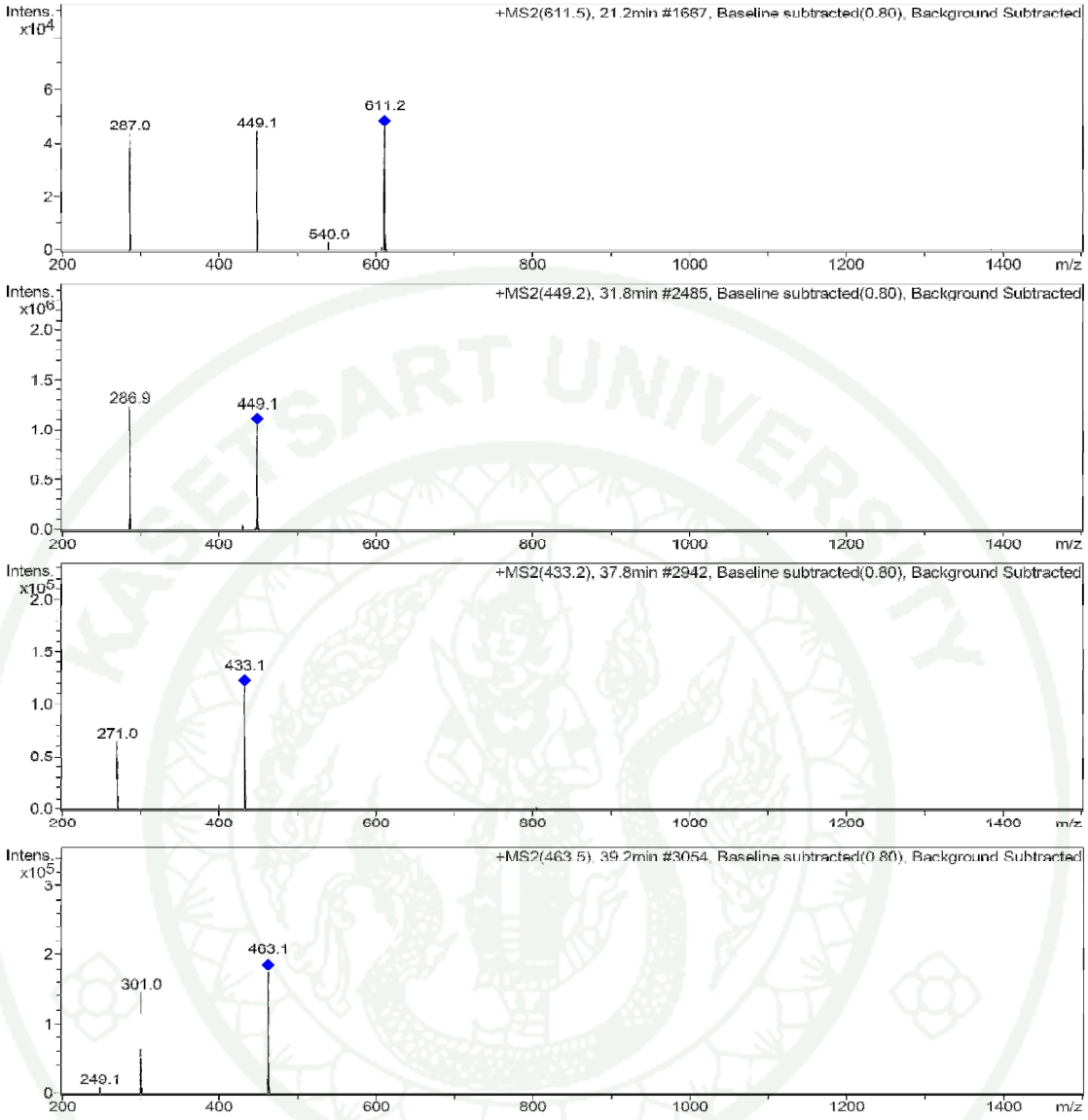
na = no analysis

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Appendix Figure 1 LC-MS of anthocyanin in the skin of red Malay apple cv. Mamieo at the fully ripe stage (development stage 8).

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Appendix Figure 2 LC-MS of anthocyanin in the skin of red Malay apple cv. Saraek at the fully ripe stage (development stage 8).

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