

UDP-sugar biosynthetic pathway: contribution to cyanidin 3-galactoside biosynthesis in apple skin

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Abstract UDP-galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT) is responsible for cyanidin 3-galactoside (cy3-gal) synthesis from cyanidin (cy) and UDP-galactose (UDP-gal) which are, respectively, catalyzed by anthocyanidin synthase (ANS) and UDP-glucose 4-epimerase (UGE). To clarify the contribution of UDP-galactose pathway to cy3-gal accumulation in apple skin, we analyzed the contents of UDP-gal and UDP-glucose (UDP-glu), cy, and, cy3-gal contents along with UGE activity. We confirmed that transcript levels for apple *ANS* and *UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT)* coincided with anthocyanin accumulation in three apple cultivars differing in their skin colors. During fruit development, changes in level of cy coincided with that of cy3-gal, whereas UDP-gal and UGE activity showed no similar trend with cy3-gal. Significant correlation was not observed between the changes in UGE activity and UDP-sugar contents. The effect of temperature

and UV-B radiation (different environmental conditions) on the accumulation of UDP-sugars, cy and cy3-gal, and UGE activity were also investigated in a pale-red cultivar. High temperature tended to depress the accumulation of both UDP-sugars and cy concomitant with the decrease in cy3-gal content irrespective of UV-B radiation. Although there was no high inhibition of both cy and UDP-sugars at low-temperature without UV-B, cy3-gal accumulation was highly depressed. UGE activity was highest at low temperature with UV-B, but not much different under other conditions. Most of the parameters under different environmental conditions were significantly correlated with each other. Based on these results, contribution of UDP-sugar biosynthetic pathway to anthocyanin biosynthesis under different environmental conditions as well as during fruit development is discussed.

Keywords *Anthocyanidin synthase (ANS)* · Anthocyanin · Apple (*Malus × domestica*) skin · Cyanidin · Cyanidin 3-galactoside · UDP-galactose · UDP-glucose · *UDP-glucose 4-epimerase (UGE)* · *UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT)*

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Abbreviations

Cy	Cyanidin
Cy3-gal	Cyanidin 3-galactoside
DAFB	Days after full bloom
UDP-gal	UDP-galactose
UDP-glu	UDP-glucose
UV	Ultraviolet

Introduction

The red coloration of apple (*Malus × domestica*) skin is derived from anthocyanins. Since anthocyanin accumulation

is an important determinant in consumer preference and the marketability of apple, considerable efforts have been invested to clarify the mechanism underlying the red coloration. It has been proposed that the development of red coloration is influenced by genetic factors and various environmental factors. As genetic factors, the structural genes in the anthocyanin biosynthetic pathway have been isolated from apple and their expression during fruit development analyzed. The expression of the structural genes, including apple *chalcone synthase* (*MdCHS*), *flavanone 3-hydroxylase* (*MdF3H*), *dihydroflavonol 4-reductase* (*pMdDFR*), *anthocyanidin synthase* (*MdANS*), and *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*pMdUFGT*) are positively induced with increase in anthocyanin accumulation in the skin of apple fruit (Honda et al. 2002). Recently, *MYB* genes isolated from apple skin (*MdMYB1* and *MdMYBA*) responsible for anthocyanin accumulation (Tacos et al. 2006; Ban et al. 2007a), were also reported. The expression of *MYB* genes positively correlated with the degree of anthocyanin accumulation. On the other hand, environmental factors have also been intensively investigated, and both low-temperature and ultraviolet (UV)-B radiation (280–320 nm) are important factors that stimulate the production of anthocyanins (Saure 1990; Lancaster 1992; Ubi 2004). Several lines of biochemical evidence suggest that the activities of anthocyanin biosynthetic enzymes and the expression of their genes are highly induced by both low-temperature and UV-B radiation (+UV-B). Dong et al. (1995) showed that the enzymatic activities of phenylalanine ammonia lyase (PAL) and chalcone isomerase (CHI) were increased 10- to 20-fold by +UV-B. The mRNA levels of *CHS*, *F3H*, *DFR*, *ANS*, and *UFGT* were up-regulated by both low-temperature and +UV-B (Ubi et al. 2006). In addition, the expression of *MdMYBA* was also induced by low-temperature and +UV-B (Ban et al. 2007a). Most of the genes involved in anthocyanin biosynthesis appear to participate in the development of red coloration in apples under the regulation of temperature and UV-B. Thus, the identification of genes whose expression is affected by temperature and UV-B is a useful strategy for elucidating the mechanisms underlying the physiological process leading to pigmentation.

In our previous study, we performed suppression subtractive hybridization with the apple skin irradiated with UV-B to isolate other genes participating in anthocyanin biosynthesis, apart from those located in the flavonoid biosynthetic pathway and *MYB*. As a result, we identified an apple *UDP-glucose 4-epimerase* (*MdUGE1*), which is not only a UV-B-, but also a low-temperature-responsive gene in apple skin (Ban et al. 2007b). Functional analysis of *MdUGE1*, including expression analysis using different tissues/cultivars at different developmental stages of apple fruit ripening and measurement of UGE enzymatic activity,

revealed a positive correlation with anthocyanin level in apple skin. Since UGE can reversely catalyze the conversion of UDP-glucose (UDP-glu) into UDP-galactose (UDP-gal), and the latter molecule is a major sugar donor for cyanidin-glycoside in apple pigments, we hypothesized that *MdUGE1* could contribute to anthocyanin biosynthesis by supplying the UDP-gal in apple skin because cyanidin 3-galactoside (cy3-gal) accounts for >80% of the total cyanidin 3-glycosides in red skin (Lancaster 1992).

The results obtained from the previous study encouraged us to focus on the involvement of UDP-sugars in anthocyanin biosynthesis. In addition, Yonekura-Sakakibara et al. (2008) showed that UDP-rhamnose produced by UDP-rhamnose synthase (RHM1) is used for flavonol rhamnosylation, and RHM1 plays a major role in supplying UDP-rhamnose for flavonol modification in *Arabidopsis*. This result indicated that supplying UDP-rhamnose can affect flavonoid biosynthesis in *Arabidopsis*. In the apple skin, a predominant anthocyanin, cy3-gal, is synthesized from cyanidin (cy) and UDP-gal by UDP-galactose:flavonoid 3-O-galactosyltransferase (UFGaT) activity. However, intensive studies have, thus far, been focused on anthocyanin biosynthetic pathway with little or no available information on UDP-sugars, which are one of the substrates for UFGaT in anthocyanin biosynthesis in the apple skin. In this study, to clarify the relationships between UDP-sugars, cy, and cy3-gal in apple skin, we measured the contents of UDP-gal, UDP-glu, cy, and cy3-gal and investigated the expression analysis of *MdANS* and *MdUFGT* in three apple cultivars differing in their skin colors, non-red, pale red, and red. Fluctuations of these compounds and UGE activity were monitored in the developing fruit of these apple cultivars. Furthermore, the effects of temperature and UV-B on the accumulation of UDP-gal, UDP-glu, cy, and cy3-gal were analyzed in the pale-red apple cultivar ‘Tsugaru’ along with UGE activity. To the best of our knowledge, this is the first report relating cy3-gal accumulation to the supply of UDP-sugars in apple skin.

Materials and methods

Plant materials

All apple (*Malus × domestica* Borkh.) fruits were obtained from the orchard of the National Institute of Fruit Tree Science at Morioka, Japan in 2005 and 2007. ‘Orin’ fruits were harvested at 35, 64, 98, 126, and 170 days after full bloom (DAFB) in 2007; ‘Tsugaru’ fruits were harvested at 37, 60, 79, 102, and 116 DAFB in 2005; and ‘Jonathan’ fruits were harvested at 37, 79, 102, 116, and 144 DAFB in 2005. It should be noted that no marked differences in the average climatic conditions were observed between 2005 and 2007

(data not shown). The entire skin, including 1 mm of the cortical tissue, was collected, immediately frozen in liquid nitrogen, and stored at -80°C until needed for the experiments.

Temperature and UV-B treatments were performed as described by Ubi et al. (2006). Here we designated different temperature treatments and \pm UV-B radiation as “under different environmental conditions”. To avoid the induction of anthocyanin biosynthesis by sunlight, ‘Tsugaru’ fruits were bagged on the trees about 1 month before commercial harvest in 2005. All bagged fruits were harvested at the commercial harvest stage and kept in the incubator at 17°C for 3 h in darkness. After pre-conditioning, the treatments were performed in the incubator after removing the bags. The treatments were (1) 17°C with UV-B radiation ($17^{\circ}\text{C} + \text{UV-B}$), (2) 17°C without UV-B radiation ($17^{\circ}\text{C} - \text{UV-B}$), (3) 27°C with UV-B radiation ($17^{\circ}\text{C} + \text{UV-B}$), and (4) 27°C without UV-B radiation ($17^{\circ}\text{C} - \text{UV-B}$). We designated 17° and 27°C as low- and high-temperature treatments, respectively. These treatments were maintained for 94 h; then the skin samples (including 1 mm of the cortical tissue) were collected from half of each fruit sample that had been most directly exposed to the light source (i.e. the upper half). Collected skin samples were immediately frozen in liquid nitrogen and then stored at -80°C until needed for experiments.

RNA gel blot analysis

Total RNA was isolated from apple fruit skin samples according to the method of Wan and Wilkins (1994). Total RNA (5 μg) was electrophoresed on a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham Biosciences, Piscataway, NJ, USA) by capillary transfer. The full-length cDNAs of *MdANS* (accession number AB074487) and *MdUFGT* (AB461385) were labeled with digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany) using M13 forward and reverse primers in the PCR reaction. Our *MdUFGT* shared 98% homology with other apple UFGTs, which has been shown to be involved in anthocyanin accumulation (Li et al. 2002; Takos et al. 2006); thus, it could not be a *flavonoid 3-O-glucosyltransferase* but a *UDP-galactose: flavonoid 3-O-galactosyltransferase*, which needs to be further elucidated in future studies. Prehybridization, hybridization, washing, and detection were carried out according to Ban et al. (2007a).

Measurement of UDP-gal and UDP-glu concentration

Extraction and detection of UDP-sugars from apple skin was carried out essentially as described by Ryll and Wagner (1991). Five grams of apple skin was ground with

mortar and pestle in liquid nitrogen until a fine powder was obtained. One milliliter of 0.5 M perchloric acid was added to the sample, and the mixture was stored on ice for 2 min. The insoluble compounds were subsequently precipitated by centrifugation at 2,000g for 3 min at 0°C . The supernatant was neutralized to pH 6.5 by adding cooled 2.5 M potassium hydroxide in 1.5 M dipotassium hydrogenphosphate, and stored on ice for 2 min. The potassium perchlorate precipitate was finally removed by centrifugation at 2,000 g for 1 min at 0°C . The supernatant was filtered with Millex[®]-HA filter (0.45 μm , Millipore, Bedford, MA, USA). The extracted samples were analyzed by HPLC. The HPLC system consisted of an HPLC pump (L-7100, Hitachi, Tokyo, Japan), a UV-VIS detector (L-7420, Hitachi), an integrator (D-7500, Hitachi), a column oven (L-7300, Hitachi) and a Supelcosil LC-18T column (4.6 mm i.d. \times 150 mm, Supelco, Bad Homburg, Germany). Solvent A composed of 100 mM potassium dihydrogenphosphate, 100 mM dipotassium hydrogenphosphate, and 8 mM tetrabutylammonium hydrogensulphate. The pH of solvent A was adjusted to 5.3 by adding phosphoric acid. Solvent B was composed of 70% solvent A plus 30% methanol. The gradient was 100% solvent A for 2.5 min, 0–40% solvent B for 14 min, 40–100% solvent B for 1 min, 100% solvent B for 6 min, and 100–0% solvent B for 1 min. The flow-rate was 1.5 ml min^{-1} . Temperature was set to 30°C . The HPLC elutes were monitored by absorbance at 254 nm, and the peaks were identified by comparing their retention time with authentic standards.

Measurement of anthocyanin composition and concentration

Anthocyanins were extracted from apple skin in 5–10 volumes of hydrochloric acid/methanol (1:99, v/v) at 4°C at least for 12 h. After filtration, the extract was determined by HPLC with a Gulliver system (Jasco Corporation, Tokyo, Japan) and an ODS column (4.6 mm i.d. \times 250 mm, Mightsyl RP-18, Kanto Chemicals, Tokyo, Japan) according to the method of Tada et al. (1996). The solvent system used was a linear gradient of 25–35% solvent B (150 mM phosphoric acid, 3.3 M acetic acid and 4.8 M acetonitrile) in solvent A (150 mM phosphoric acid) over a period of 30 min. Samples were eluted at 35°C and a flow-rate of 1 ml min^{-1} . The HPLC elutes were monitored by absorbance at 520 nm, and the peaks were identified by comparing their retention time with authentic standards.

Estimation of cyanidin concentration

Due to the absence of a direct method of measuring the quite unstable cyanidin in apple, we estimated the cyanidin content by measuring the acid-hydrolyzed

samples. The data obtained from acid-hydrolyzed samples derived from both, anthocyanin and proanthocyanidin. In this study, we focused on the strength of metabolic-flow on cyanidin biosynthetic pathway and therefore, considered that the data obtained from acid-hydrolyzed sample can be used for this study. One gram of each sample was hydrolyzed in 2 M HCl at 100°C for 30 min, and then analyzed by HPLC (Terahara et al. 1993). Analytical conditions were the same as for anthocyanin measurement.

Measurement of UGE activity

Protein extraction and measurement of UGE activity were carried out according to Ban et al. (2007b) with slight modifications. Two grams of skin samples from ‘Orin’, ‘Tsugaru’, and ‘Jonathan’ during fruit development was used for protein extraction. Since protein could not be extracted from young fruitlets using the method reported previously (Ban et al. 2007b), polyvinylpyrrolidone (average molecular weight 40,000; PVP-40) was added at a rate of 10% (w/v, gram fresh weight) instead of polyclar AT. Protein was measured according to Bradford (1976). UGE activity was estimated by the galactose synthesized, which was calculated by comparison with the known amount of D-[U-¹⁴C] glucose and expressed as an average (pmol UDP-gal formed per min per mg protein) from the three independent samples.

Statistical analysis

All the data presented are mean values of three replicates, except for the data presented in Fig. 1b. Statistical analysis was performed using the Turkey’s LSD test. Correlation analysis was performed to show the relationships among UDP-gal, UDP-glu, cy, and, cy3-gal contents, as well as UGE activity based on the corresponding data pairs.

Results

Anthocyanin composition, concentration, and gene expression in mature fruit skin samples at commercial harvest

Analysis of anthocyanin composition and concentration in the skin samples of the ripened fruits collected at commercial harvest was carried out by HPLC in ‘Orin’ (non-red cultivar), ‘Tsugaru’ (pale-red cultivar) and ‘Jonathan’ (deep-red cultivar) (Fig. 1a). Three peaks corresponding to cy3-gal, cyanidin 3-arabinoside, and cyanidin 3-glucoside were obtained from HPLC analy-

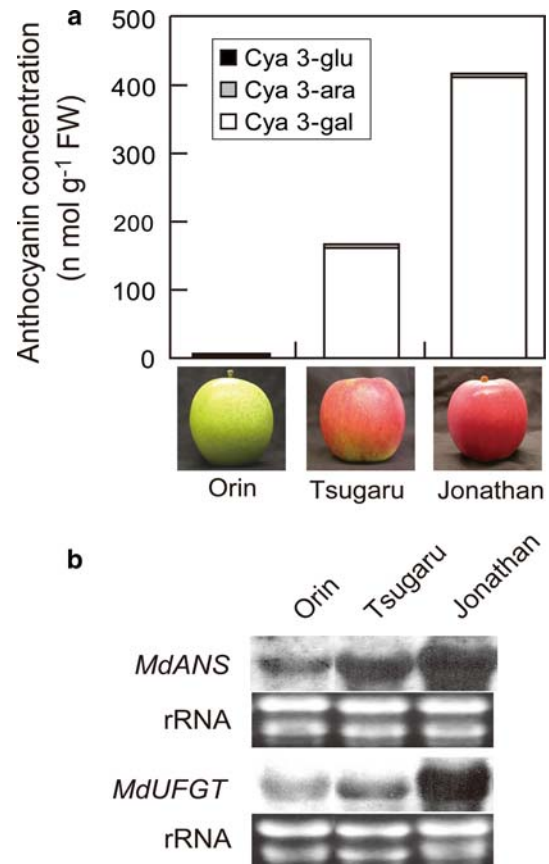


Fig. 1 **a** Anthocyanin composition in ripened fruit skin of ‘Orin’ (non-red cultivar), ‘Tsugaru’ (pale-red cultivar), and ‘Jonathan’ (deep-red cultivar). The values are the averages of three replications. **b** Expression analysis of *MdANS* and *MdUFGT* in the same materials. Ethidium bromide staining showing rRNA loading

sis. The total amount of anthocyanin was 0.7 nmol g⁻¹ fresh weight (FW) in ‘Orin’, 160.9 nmol g⁻¹ FW in ‘Tsugaru’ and 411.4 nmol g⁻¹ FW in ‘Jonathan’. In all cultivars, cy3-gal was detected and was the predominant compound, accounting for more than 96% of the total anthocyanins, while cyanidin 3-glucoside and cyanidin 3-arabinoside were not detected in ‘Orin’.

The apple skins used for HPLC analysis were also used for expression analysis of *MdANS* and *MdUFGT*, which encode the enzymes catalyzing the final steps for the biosynthesis of cy and cy3-gal, respectively. *MdANS* was highly expressed in both ‘Tsugaru’ and ‘Jonathan’, but much less in non-red skin cultivar, ‘Orin’ (Fig. 1b). The expression level of *MdUFGT* showed similar trend to that of *MdANS*. Generally, cultivars that accumulated high levels of cy3-gal showed relatively higher expression levels of *MdANS* and *MdUFGT* (Fig. 1a, b), indicating positive correlations between the transcript levels and anthocyanin accumulation.

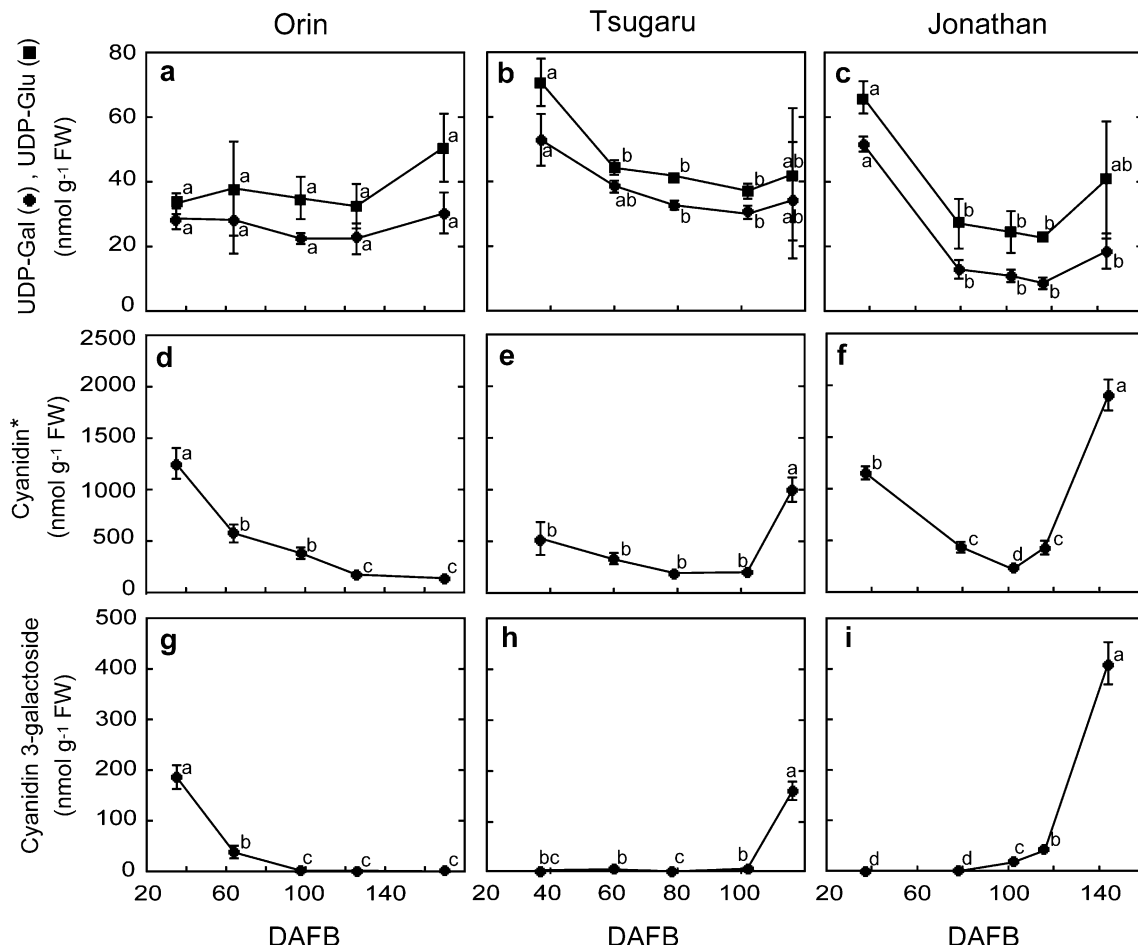


Fig. 2 Concentration of UDP-galactose and UDP-glucose (a–c), cyanidin (d–f) and cyanidin 3-galactoside (g–i) in fruit skins at different developmental stages of ‘Jonathan’, ‘Tsugaru’, and ‘Orin’. The values

are means ± SE (*n* = 3). Dots labeled with different letters are significantly different at *P* < 0.05. *Based on estimates from acid-hydrolyzed samples

Changes in UDP-gal, UDP-glu, cy, and cy3-gal content in fruit skins during fruit development

The content of UDP-gal, UDP-glu, cy, and cy3-gal in the fruit skins of the three apple cultivars, ‘Orin’, ‘Tsugaru’, and ‘Jonathan’ were determined during fruit development (Fig. 2). The content of UDP-gal and UDP-glu fluctuated similarly within each cultivar (Fig. 2a–c). In ‘Tsugaru’ and ‘Jonathan’, UDP-gal and UDP-glu were highest at early fruit developmental stage (37 DAFB in both ‘Jonathan’ and ‘Tsugaru’), decreased slightly during the middle developmental stage (60–102 DAFB in ‘Tsugaru’ and 79–116 DAFB in ‘Jonathan’), and then slightly increased at ripened stage (116 DAFB in ‘Tsugaru’ and 144 DAFB in ‘Jonathan’), albeit not always significant (Fig. 2b, c). Significant differences were prominent between the early and middle phases of fruit development. In ‘Orin’, the content of UDP-gal and UDP-glu were nearly constant until 126 DAFB, and

slightly increased at 170 DAFB (Fig. 2a). Significant differences were not detected between the developmental phases. In these three cultivars, the content of UDP-glu was always higher than that of UDP-gal (Fig. 2a–c). The changes in cy contents showed similar patterns to that of cy3-gal in ‘Orin’ and ‘Tsugaru’ (Fig. 2d, e, g, h). In ‘Orin’, cy and cy3-gal were highest at 35 DAFB and then significantly decreased from about 60 DAFB. In ‘Tsugaru’, cy and cy3-gal remained nearly constant and then significantly increased at 116 DAFB. In ‘Jonathan’, the content of cy did not correlate with that of cy3-gal at early developmental stage (Fig. 2f, i), but rather showed concomitant increase at the ripened stage. The content of cy was detected at 37 DAFB, then significantly decreased, and started to increase sharply after 102 DAFB reaching the highest level at 144 DAFB. On the other hand, cy3-gal was not detected until 102 DAFB, when it significantly increased reaching the highest level at 144 DAFB.

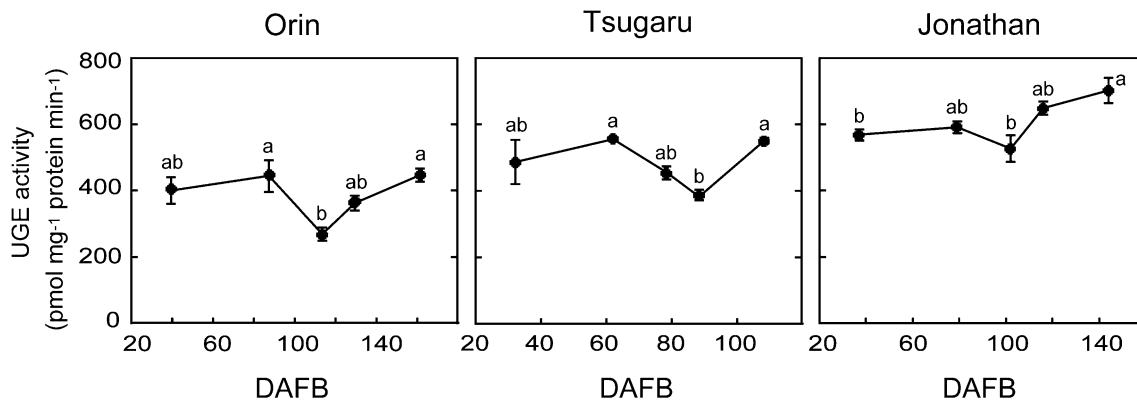


Fig. 3 UGE activities in fruit skins at different ripening stages of ‘Jonathan’, ‘Tsugaru’, and ‘Orin’. The values are means \pm SE ($n = 3$). Dots labeled with different letters are significantly different at $P < 0.05$

Changes in UGE activity in fruit skins during fruit development

The levels of UGE activity fluctuated similarly in ‘Orin’, ‘Tsugaru’, and ‘Jonathan’ (Fig. 3). In these three cultivars, the level of UGE activity decreased during the middle developmental stage, and then increased at ripened stage. The levels at ripened stage were higher than that at early fruit developmental stage, albeit not always significant. Overall, UGE activity was high in ‘Jonathan’, medium in ‘Tsugaru’, and low in ‘Orin’, concomitant with anthocyanin contents in these apple cultivars, especially at the last harvest stage. It is noteworthy that the UGE activity measured in this study was about 5-times higher than that in the previous report (Ban et al. 2007b), which might be possibly ascribed to the addition of PVP-40 as a phenolic-absorbing substance instead of polyclar AT for protein extraction. In apple fruits, Gyulakhmedov et al. (2006) also showed the reduction of ATP-dependent phosphofructokinase activity in a buffer containing polyclar AT, compared to PVP-40.

Relationships among UDP-gal, UDP-glu, cy, and cy3-gal contents and UGE activity in fruit skins during fruit development

The result of correlation analyses among UDP-gal, UDP-glu, cy, and cy3-gal contents and UGE activity in fruit skins during fruit development is shown in Table 1. Highly significant correlation values were observed only between UDP-gal and UDP-glu and between cy and cy3-gal. Relatively high positive correlation was observed between UGE activity and either cy or cy3-gal content, albeit not statistically different. Interestingly, negative correlation values, albeit quite low, were obtained between cy3-gal and UDP-sugars and between UGE activity and UDP-gal.

Table 1 Correlation values among UDP-gal, UDP-glu, cy and cy3-gal content and UGE activity during fruit development

Correlation value	UDP-Gal	UDP-Glu	Cy ^a	Cy3-Gal	UGE
UDP-Gal	–				
UDP-Glu	0.926**	–			
Cy	0.117	0.197	–		
Cy3-Gal	–0.179	–0.084	0.870**	–	
UGE	–0.142	0.019	0.476	0.449	–

^a Based on estimates from acid-hydrolyzed samples

** $P \leq 0.01$

Effects of temperature and UV-B treatments on UDP-gal, UDP-glu, cy, and cy3-gal contents in fruit skins

The contents of UDP-gal, UDP-glu, cy, and cy3-gal in the fruit skins of ‘Tsugaru’ under different environmental conditions are shown in Fig. 4. The contents of both UDP-sugars in the skin under low-temperature were higher than that in the skin under high temperature, regardless of UV-B. However, a significant decrease in both UDP sugars was observed when the temperature was increased to 27°C without UV-B (Fig. 4a, b). The content of cy was enhanced under +UV-B at low temperature, but not significantly at high temperature (Fig. 4c), although +UV-B induced cy accumulation. Accumulation of cy3-gal was greatly enhanced by a combination of low-temperature and +UV-B (Fig. 4d). However, content of cy3-gal was repressed by the high-temperature treatment, regardless of UV-B. In addition, cy3-gal accumulation was not stimulated under –UV-B, despite low-temperature condition.

Effects of temperature and UV-B treatments on UGE activity in fruit skins

UGE activity in the fruit skins of ‘Tsugaru’ under different environmental conditions is shown in Fig. 5. UGE activity

Fig. 4 Concentration of UDP-galactose (a), UDP-glucose (b), cyanidin (c), and cyanidin 3-galactoside (d) in ‘Tsugaru’ fruit skins under different environmental conditions. The values are means ± SE (*n* = 3). The treatment conditions are indicated in the bottom of panels: +UV-B (+) or - UV-B (-), and 17 or 27°C. Bars labeled with different letters are significantly different at *P* < 0.05. *Based on estimates from acid-hydrolyzed samples

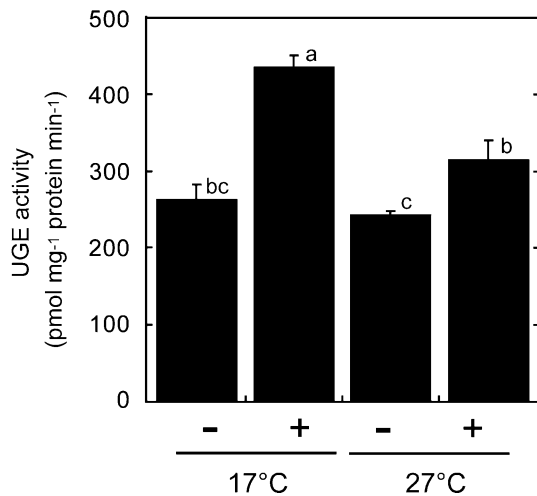
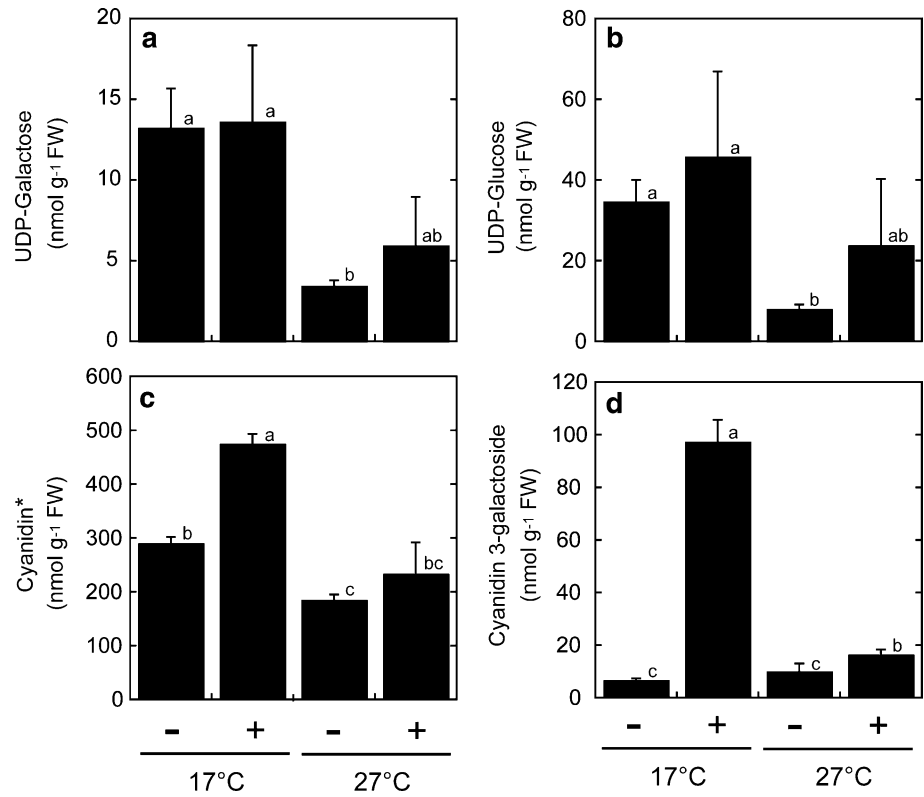


Fig. 5 UGE activities in ‘Tsugaru’ fruit skins under different environmental conditions. The treatment conditions in the bottom of panel were shown in Fig. 4. The values are means ± SE (*n* = 3). Bars labeled with different letters are significantly different at *P* < 0.05

was greatly enhanced by a combination of low-temperature and +UV-B. However, UGE activity was repressed by the high-temperature treatment: repression was relatively more pronounced in -UV-B treatment. Moreover, UGE activity was not induced under -UV-B even at low-temperature with levels nearly similar to those under -UV-B at high temperature.

Relationships among UDP-gal, UDP-glu, cy, and cy3-gal contents and UGE activity under different environmental conditions

The result of correlation analyses among UDP-gal, UDP-glu, cy, and cy3-gal contents and UGE activity in fruit skins under different environmental conditions is shown in Table 2. Most of the parameters analyzed were highly significantly correlated with each other with the correlation value between UGE activity and cy3-gal content being the highest (*r* = 0.956). UGE also showed high correlation with cy (*r* = 0.906). Significant correlations were observed between UDP-gal and both UGE or cy3-gal.

Discussion

In apple skin, the predominant anthocyanin (cy3-gal) is synthesized from cy and UDP-gal by UDP-sugar-specific UFGaT activity. UFGaT have already been thought to be the most important enzyme in the development of red pigmentation for apple (Li et al. 2002). One substrate, cy, is synthesized from cinnamic acid derivatives via flavonoid biosynthetic pathway. The final step for cyanidin biosynthesis is conversion of leucocyanidin into cyanidin by ANS activity. The other substrate, UDP-gal, can be synthesized via two pathways, namely, directly from UDP-glu by UGE or by galactokinase and galactose-1-phosphate uridy

Table 2 Correlation values among UDP-gal, UDP-glu, cy and cy3-gal content and UGE activity under different environmental conditions

Correlation value	UDP-Gal	UDP-Glu	Cy ^a	Cy3-Gal	UGE
UDP-Gal	–				
UDP-Glu	0.945**	–			
Cy	0.823**	0.921**	–		
Cy3-Gal	0.546*	0.720**	0.925**	–	
UGE	0.550*	0.773**	0.906**	0.956**	–

^a Based on estimates from acid-hydrolyzed samples

* $P \leq 0.05$; ** $P \leq 0.01$

transferase in the presence of galactose. However, the metabolome analysis showed that galactose could not be detected in apple skins (Rudell et al. 2008). Therefore, UGE may be responsible for the final step of UDP-gal biosynthesis in apple skin. In this study, we investigated the contribution of UDP-gal pathways to anthocyanin (cy3-gal) biosynthesis in apple skin by focusing on the content of UDP-sugars, cy, and cy3-gal, along with the UGE activity and the expression of genes corresponding to the final enzymatic steps of the biosynthetic pathway, i.e., *ANS* and *UFGT* in apple skin.

As a first step, we analyzed the anthocyanin content in three apple cultivars differing in their skin colors and performed expression analysis of *MdANS* and *MdUFGT* in mature fruit skins of these cultivars, because several studies suggested that positive correlations existed among the expression levels of anthocyanin biosynthetic genes including *ANS* and *UFGT* and anthocyanin contents (e.g. Honda et al. 2002; Ubi et al. 2006). When the transcript levels were transformed to the relative values based on the rRNA intensities in RNA gel blot analysis, the levels of *MdANS* and *MdUFGT* were positively correlated with the cy3-gal contents (data not shown). From these results, we confirmed that cy biosynthetic pathway could be involved in anthocyanin biosynthesis in apple skin.

When cy, UDP-gal, and cy3-gal contents were measured in ‘Orin’ (non-red cultivar), ‘Tsugaru’ (pale-red cultivar), and ‘Jonathan’ (deep-red cultivar) during apple fruit development, the content of UDP-gal did not significantly correlate with that of cy3-gal (Fig. 2). On the other hand, the content of cy was highly correlated with that of cy3-gal (Fig. 2), except for ‘Jonathan’ at early developmental stages (from 37 to 79 DAFB). In addition, cy3-gal was scarcely detected in the ‘Orin’ skin at 170 DAFB despite sufficient amount of UDP-gal. This trend was also supported by statistical correlation analysis, in which significant positive correlation values were obtained only between UDP-gal and UDP-glu and between cy and cy3-gal (Table 1). This may suggest that the cy biosynthetic pathway, rather than the UDP-gal biosynthetic pathway could

influence anthocyanin biosynthesis during fruit development in the orchard, which is comparable to under low-temperature and UV-B radiation.

It has been well known that temperature and UV-B can affect the cy3-gal content via the induction of flavonoid biosynthetic genes such as *ANS* and a *MYB* (Tako et al. 2006; Ban et al. 2007a), but there is paucity of information on the effects of temperature and UV-B on UDP-gal and UDP-glu contents. Therefore, we investigated the effects of temperature and UV-B on UDP-sugars, cy, and cy3-gal contents in the skins of a pale-red apple cultivar, ‘Tsugaru’ (Fig. 4). High temperature (27°C) seemed to give a greater impact for the inhibition of both UDP-gal and UDP-glu accumulation than UV-B. Since we used detached fruits, alterations in sugar contents in fruits, including UDP-sugars were not derived from new translocation events from other organs but from metabolism within the fruits themselves. Thus, reduction of UDP-gal and UDP-glu contents by high-temperature treatment might imply that the sources for UDP-glu, such as glucose 1-phosphate are also affected by temperature. It has been reported that the activities of sucrose phosphate synthase and sucrose synthase in fruits are affected by high and low-temperatures (Lingle et al. 1987; Holland et al. 2005), which suggest that enzymatic activities responsible for sugar-metabolism in apple skin may be negatively affected by high temperature. In our present study, both temperature and UV-B significantly affected the cy level (Fig. 4c) and the synergistic effect of low-temperature (17°C) and +UV-B markedly enhanced cy3-gal accumulation (Fig. 4d), concomitant with high-UGE activity (Fig. 5). It is worth noting that under low-temperature (17°C) and –UV-B, there was no high inhibition of UDP-sugars and cy accumulation, but the accumulation of cy3-gal was largely inhibited. Therefore, under low-temperature conditions, other UV-B responsive factor(s), other than those affecting the UDP-gal and cy biosynthetic pathways, might be involved in anthocyanin biosynthesis. Since the expression level of *MdUFGT* was the most induced by UV-B radiation in various apple cultivars (Ubi et al. 2006), *UFGT* appears to be one of the UV-B responsive factors. However, the possibility that other UV-B responsive factors may be involved in this phenomenon cannot be ruled out. The candidate UV-B responsive factor is the protein related to the sequestration of anthocyanins, such as a glutathione S-transferase (GST)-like protein and an ATP-binding cassette (ABC) transporter (Marrs et al. 1995; Alfenito et al. 1998; Goodman et al. 2004; Kitamura et al. 2004). The gene coding GST-like protein, *TT19*, is also induced by UV-B in *Arabidopsis* (Brown et al. 2005). A further investigation of these genes is necessary to clarify this issue.

In this study, the level of UGE activity was not correlated with UDP-gal concentration during fruit development,

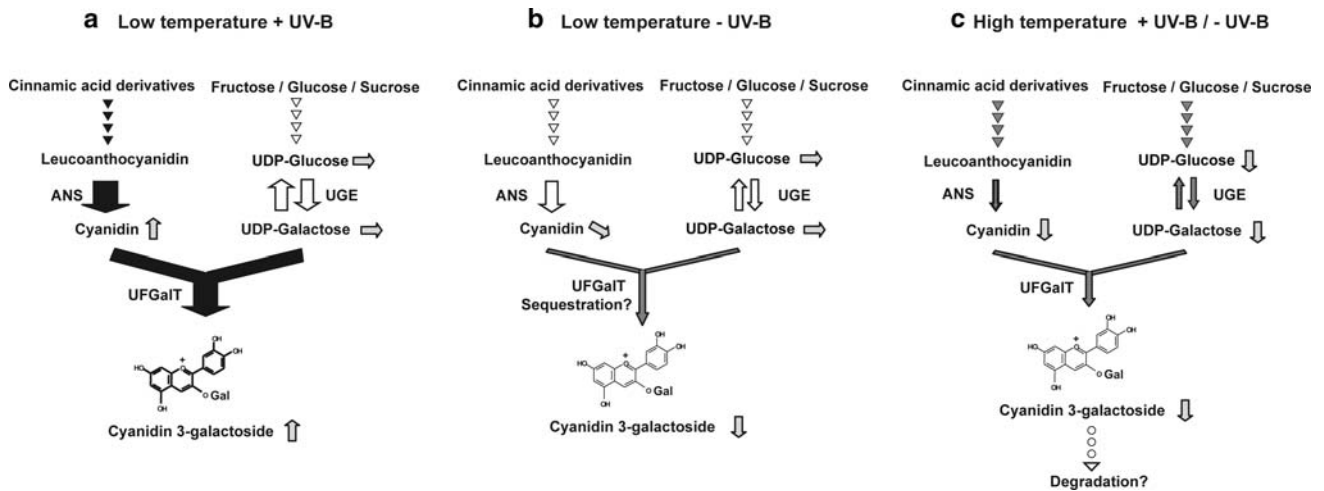


Fig. 6 A hypothetical scheme for the relationship among UDP-sugars, cyanidin, and cyanidin 3-galactoside at different temperature and UV-B conditions in apple skins. The arrows closed in black and gray indicate effective pathway for increment and decrement of anthocyanin accumulation, respectively. The width of arrows (except arrows beside metabolites) indicates the flow-rate of each metabolite. The arrows

shown beside the metabolite name indicate the trend of its content. Arrows pointing upward (a) indicate increase in metabolite; arrows pointing rightward (b) indicate no change in metabolite; arrows pointing diagonally downward (b, c) indicate a slight decrease in metabolite; while the arrows pointing downward (b, c) indicate a decrease in metabolite

although a moderate but significant correlation was observed under inductive environmental conditions (Tables 1, 2). It should be noted that the conversion of UDP-glu into UDP-gal by UGE is a reversible reaction (Fig. 6). We supposed that the positive relationship between UGE activity and UDP-gal content toward fruit ripening, invariably suggest a negative relationship between UGE activity and UDP-glu content. However, no such positive or negative correlation of UGE activity with the respective UDP-gal and UDP-glu was revealed at least in the fruit skins of ‘Orin’, ‘Tsugaru’, and ‘Jonathan’ during development (Figs. 2, 3) and in the fruit skins of ‘Tsugaru’ under different temperature and UV-B conditions (Figs. 4, 5). Similar result was also reported in *Arabidopsis thaliana* (Dörmann and Benning 1998). Transgenic *Arabidopsis* plants expressing the functional *UGE* in sense or anti-sense orientation resulted in a range of plant lines with different UGE expression and activities. However, no alterations were observed in the content of UDP-gal and UDP-glu, albeit a maximal reduction in UGE activity of 10% compared to the wild type (Dörmann and Benning 1998). Several reports suggest that UDP-gal synthesized by UGE is utilized for the biosynthesis of cell wall polysaccharides (e.g., xyloglucan and rhamnogalacturonan) (Seifert et al. 2002; Oomen et al. 2004). UDP-gal and UDP-glu are also utilized as a sugar donor for anthocyanin and sucrose biosynthesis in apple skin, respectively. Thus, we considered that the lack of correlation between UGE activity and UDP-sugars could be ascribed to rapid conversion of UDP-sugars into polysaccharides, cy3-gal and/or sucrose. In addition, UDP-gal and UDP-glu contents fluctuated with relative

constant ratio throughout fruit developmental stages in this study (Fig. 2a–c; Tables 1, 2). In *Cryptococcus neoformans*, UGE1 and UGE2 regulate the UDP-glu/UDP-gal equilibrium (Moyrand et al. 2008). Taking these facts into consideration, MdUGE1 may play a role to maintain the homeostatic levels of UDP-gal and UDP-glu in apple skin and not to regulate the direction between UDP-gal and UDP-glu, which remains to be clarified.

Comparatively, clear differences in relationships among the UDP sugars and cy-3 gal are discernible during fruit development and under different environmental conditions (Tables 1, 2); significant positive correlation was observed only between UDP-gal and UDP-glu and between cy and cy3-gal during fruit development (corresponding to low-temperature and +UV-B that provides suitable condition for anthocyanin biosynthesis), while all of the parameters were significantly correlated with each other under different environmental conditions. These results suggested that cy pathway could be the rate-limiting step for anthocyanin biosynthesis under suitable conditions. Conversely, all parameters could play an important role for anthocyanin accumulation in varying degrees under unsuitable conditions such as under high temperature and/or –UV-B. Based on our results, we illustrated the biosynthetic flows for anthocyanin (cy3-gal) accumulation under different environmental conditions as well as during fruit development (Fig. 6). Under low-temperature (17°C) and +UV-B, whose condition is comparable to during fruit development in the orchard, all flavonoid biosynthetic enzymes including ANS and UFGaIT may be activated, leading to increase in cy content and in transferring ability of UDP-gal. Similarly,

UGE activity is also induced (Ban et al. 2007b), but induction of UGE does not always lead to the increase in UDP-gal contents. Thus, determinant pathway for cy3-gal biosynthesis appears not to be the UDP-gal biosynthetic pathway but rather the cy biosynthetic pathway (Fig. 6a). However, under low-temperature and –UV-B, less accumulation of cy3-gal was observed despite no depression of UDP sugars and less depression of cy synthesis. Therefore, UV-B responsive factor(s), other than UDP-gal and cy biosynthetic pathways, may be involved in anthocyanin biosynthesis (Fig. 6b). On the other hand, under high temperature (27°C), less increase in cy and UDP-gal contents and a decrease in transferring ability of UDP-gal were apparent with or without UV-B. Thus, both less UDP-gal and cy contents could be one of the rate-limiting factors for UFGalT activity under high temperature, thereby depressing anthocyanin biosynthesis (Fig. 6c). Furthermore, degradation by high temperature was proposed in grape (Mori et al. 2007), which may also contribute to the less cy3-gal accumulation under high temperature. The hypothetical scheme shown in Fig. 6 can be applied at least to pale-red cultivars, such as ‘Tuguru’, in which temperature and UV-B can significantly impact anthocyanin reddening. We also suppose that this scheme could be applied to deep-red cultivars, such as ‘Jonathan’. However, since the red coloration can be kept to a certain level regardless of temperature and UV-B in deep-red cultivars (Ubi et al. 2006), these effects may not be so pronounced as compared to the pale-red cultivars. Thus, given the constant level of UDP-gal accumulation at low temperature irrespective of UV-B treatment and its decline at high temperature, temperature control may be more important for UDP-sugar accumulation. For practical purposes, however, both UV-B and low-temperature are necessary for anthocyanin accumulation.

References

- Alfenito MR, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferase. *Plant Cell* 10:1135–1149
- Ban Y, Honda C, Hatsuyama Y, Igarashi M, Bessho H, Moriguchi T (2007a) Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. *Plant Cell Physiol* 48:958–970
- Ban Y, Honda C, Bessho H, Pang XM, Moriguchi T (2007b) Suppression subtractive hybridization identifies genes induced in response to UV-B radiation in apple skin: isolation of a putative UDP-glucose 4-epimerase. *J Exp Bot* 58:25–34
- Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brown AB, Cloix C, Jiang GH, Kaiserli E, Herzyk P, Kliebenstein DJ, Jenkins GI (2005) A UV-B-specific signaling component orchestrates plant UV protection. *Proc Natl Acad Sci USA* 102:18225–18230
- Dong Y, Mitra D, Kootstra A (1995) Postharvest stimulation of skin color in Royal Gala apple. *J Am Soc Hortic Sci* 120:95–100
- Dörmann P, Benning C (1998) The role of UDP-glu epimerase in carbohydrate metabolism of *Arabidopsis*. *Plant J* 13:641–652
- Goodman CD, Casati P, Walbot V (2004) A multidrug resistance-associated protein involved in anthocyanin transport in *Zea mays*. *Plant Cell* 16:1812–1826
- Gyulakhmedov SG, Omarov YA, Mamedov ZM, Kuliev AA (2006) Isolation and study of active ATP-dependent phosphofructokinase from apple fruits *Pyrus domestica* Borkh. *Appl Biochem Microbiol* 42:534–538
- Holland N, Menezes HC, Lafuente MT (2005) Carbohydrate metabolism as related to high-temperature conditioning and peel disorders occurring during storage of citrus fruit. *J Sci Food Agri* 53:8790–8796
- Honda C, Kotoda N, Wada M, Kondo S, Kobayashi S, Soejima J, Zhang Z, Tsuda T, Moriguchi T (2002) Anthocyanin biosynthetic genes are coordinately expressed during red coloration in apple skin. *Plant Physiol Biochem* 40:955–962
- Kitamura S, Shikazono N, Tanaka A (2004) *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *Plant J* 37:104–114
- Lancaster JE (1992) Regulation of skin color in apples. *Crit Rev Plant Sci* 10:487–502
- Li Z, Sugaya S, Gemma H, Iwahori S (2002) Flavonoid biosynthesis and accumulation and related enzyme activities in the skin of ‘Fuji’ and ‘Oorin’ apples during their development. *J Jpn Soc Hortic Sci* 71:317–321
- Lingle SE, Lester GE, Dunlap JR (1987) Effect of postharvest heat treatment and storage on sugar metabolism in polyethylene-wrapped muskmelon fruit. *HortScience* 22:917–919
- Marrs KA, Alfenito MR, Lloyd AM, Walbot V (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature* 375:397–400
- Mori K, Goto-Yamamoto N, Kitayama M, Hashizume K (2007) Loss of anthocyanins in red-wine grape under high temperature. *J Exp Bot* 58:1935–1945
- Moyrand F, Lafontaine I, Fontaine T, Janbon G (2008) *UGE1* and *UGE2* regulate the UDP-glucose/UDP-galactose equilibrium in *Cryptococcus neoformans*. *Eukaryot Cell* 7:2069–2077
- Oomen RJFJ, Dao-Thi B, Tzitzikas EN, Bakx EJ, Schols HA, Visser RGF, Vincken J-P (2004) Overexpression of two different potato UDP-Glc 4-epimerases can increase the galactose content of potato tuber cell walls. *Plant Sci* 166:1097–1104
- Rudell DR, Mattheis JP, Curry EA (2008) Prestorage ultraviolet-white light irradiation alters apple peel metabolome. *J Agri Food Chem* 56:1138–1147
- Ryll T, Wagner R (1991) Improved ion-pair high-performance liquid chromatographic method for the quantification of a wide variety of nucleotides and sugar-nucleotides in animal cells. *J Chromatogr* 570:77–88
- Saure MC (1990) External control of anthocyanin formation in apple. *Sci Hortic* 42:181–218
- Seifert GJ, Barber C, Wells B, Dolan L, Roberts K (2002) Galactose biosynthesis in *Arabidopsis*: genetic evidence for substrate channeling from UDP-D-galactose into cell wall polymers. *Curr Biol* 12:1840–1845
- Tada H, Terahara N, Motoyama E, Shimomura K, Ishimaru K (1996) Anthocyanins in *Lobelia chinensis* hairy roots. *Plant Tiss Cult Lett* 13:85–86
- Takos AM, Jaffé FW, Jacob SR, Bogs J, Robinson SP, Walker AR (2006) Light induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. *Plant Physiol* 142:1216–1232
- Terahara N, Sakanashi T, Tsukui A (1993) Anthocyanins from the berries of haskaap, *Lonicera caerulea* L. *J Home Econ Jan* 44:197–201

- Ubi BE (2004) External stimulation of anthocyanin biosynthesis in apple fruit. *J Food Agric Environ* 2:65–70
- Ubi BE, Honda C, Bessho H, Kondo S, Wada M, Kobayashi S, Moriguchi T (2006) Expression analysis of anthocyanin biosynthetic genes in apple skin: Effect of UV-B and temperature. *Plant Sci* 170:571–578
- Wan C, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal Biochem* 223:7–12
- Yonekura-Sakakibara K, Tohge T, Matsuda F, Nakabayashi R, Takayama H, Niida R, Watanabe-Takahashi A, Inoue E, Saito K (2008) Comprehensive flavonol profiling and transcriptome coexpression analysis leading to decoding gene-metabolite correlations in *Arabidopsis*. *Plant Cell* 20:2160–2176