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Changes in the Expression of Anthocyanin Biosynthetic Genes during Apple Development

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Abstract. **Cyanidin 3-galactoside was the primary anthocyanin in red 'Tsugaru' apples [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.]. The concentration of cyanidin 3-galactoside in the skin decreased from 20 to 62 days after full bloom (DAFB), then increased rapidly after 104 DAFB. Small amounts of cyanidin 3-arabinoside and cyanidin 3-glucoside were detected at 122 and 133 DAFB (harvest). The expression of five anthocyanin biosynthetic genes of *chalcone synthase* (*MdCHS*), *flavanone 3-hydroxylase* (*MdF3H*), *dihydroflavonol 4-reductase* (*pDFR*), *anthocyanidin synthase* (*MdANS*), and *UDP glucose-flavonoid 3-O-glucosyltransferase* (*pUFGluT*) was examined in the skin of red and nonred apples. In general, the expression of anthocyanin biosynthetic genes in red apples was strong in juvenile and ripening stages. The expression of *MdCHS, MdF3H, pDFR*, and *MdANS* was observed before ripening stage when anthocyanin was not detected. In contrast, the expression of *pUFGluT* was detected in the development stage only when anthocyanin was detected. However, the expression of all five genes was observed at 20 DAFB in fruit bagged after fertilization, and anthocyanin was not detected. The expression of *MdCHS*, *MdF3H*, *pDFR*, and *MdANS*, excluding *pUFGluT*, was detected at 98 DAFB in fruit bagged after 30 DAFB, and anthocyanin was not detected. These results suggest that *pUFGluT* may be closely related to the anthocyanin expression in apple skin at the ripening stage.**

The color of apple (*Malus sylvestris* var. *domestica*) skin primarily results from the composition of anthocyanin, flavonoid, carotenoid, and chlorophyll (Lancaster, 1992). Two kinds of color exist, one that shows red coloring, and one that does not. However, anthocyanin formation can be observed at the juvenile stage in both types of fruit. The mechanism of nonred cultivars that produce anthocyanin only at the time of cell division is unknown. At the ripening stage, the anthocyanin produced through the flavonoid biosynthesis pathway accumulates in red cultivars, but flavonoid accumulates in nonred cultivars (Ju et al., 1995a). In red cultivars, the degree of pigmentation is a very important factor for marketing. Anthocyanin accumulation in the skin is influenced by environmental factors such as light, temperature, and nitrogen concentration. Of these, light is the strongest factor (Saure, 1990). Little or no anthocyanin is produced in apples in which the skin is not exposed to light. The relationship between anthocyanin formation and the wavelength of light was investigated, and UV radiation was found to induce anthocyanin formation (Proctor, 1974).

Some enzymes are associated with anthocyanin formation. The activity of phenylalanine ammonia-lyase (PAL), the first enzyme in phenylpropanoid pathway, increased in apple skin with UV radiation (Arakawa, 1986). However, since the activity of PAL increased despite the absence of anthocyanin, it has been concluded that PAL is not a regulatory enzyme of anthocyanin formation (Ju et al., 1995b; Wang et al., 2000). Although few studies have analyzed the genes which regulate anthocyanin biosynthesis in fruit skins, the expression of their genes was investigated in grape berries (*Vitis vinifera* L.) (Boss et al., 1996). The anthocyanins of 'Shiraz' grape berries result from the composition of the glucoside of delphinidin, cyanidin, petunidin, malvidin, and peonidin, but malvidin-based anthocyanins are the principal ones. Cyanidin-based anthocyanins have only been reported as anthocyanin in apples (Mazza and Velioglu, 1992). In addition, time of appearance differs with grape berries. Anthocyanin appears at the beginning of fruit development in apples, whereas in grape berries it only appears during the ripening stage.

This study investigated two specific areas to clarify the regulatory mechanism of anthocyanin biosynthesis in apples. One is the changes in expression of five structural genes from the anthocyanin biosynthetic pathway during fruit development. The other area is the relationship between light and the expression of these genes.

Materials and Methods

Plant material. Five randomly selected 12- or 13-year-old 'Tsugaru' (red apples) growing in an open field at Hiroshima Prefectural University or 'Oorin' (nonred apples) apple trees growing in an open field at Akita Fruit Tree Experiment Station, grafted onto Malling 26 (M. 26) rootstocks, were used in this study in 2000

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and 2001. Thirty or more apples (depending on the fruit growth stage) were collected at regular intervals from 10 d after full bloom (DAFB) until harvest (138 DAFB) for 'Tsugaru.' 'Oorin' apples were sampled in the same manner at 20 DAFB and at 165 DAFB (harvest). 'Tsugaru' or 'Oorin' fruit were bagged at the two different development stages (juvenile and ripening) to examine the relationship between light and the expression of anthocyanin pathway genes. Flowers were wrapped in a double paper bag immediately after fertilization, and the expression of genes was examined at 20 DAFB (juvenile stage) ('Tsugaru' and 'Oorin'). Fruit were bagged at 30 DAFB, and the expression of genes was compared at 98 DAFB (ripening stage) (Tsugaru). Bagging the fruit this way blocks nearly all light and generally delays ripening. Oxygen concentrations in bagged fruit have not yet been reported. The skin color of two positions on the longitudinal part of the fruit was measured by a color-difference meter (CR-200; Minolta, Tokyo) immediately after harvest. Hue angle (0º = red-purple, 90º = yellow, 180º = blue-green, 270º = blue) was calculated according to the method reported by McGuire (1992). After the skin was peeled with a knife, it was immediately frozen in liquid nitrogen for the extraction of anthocyanin and RNA. These samples were stored at –80 ºC until analysis.

Anthocyanin and cyanidin analysis. Anthocyanin was extracted from 0.5 g of skin with 15 mL 0.1% (v/v) HCl-methanol for 1 d at 4 ºC in the dark. After filtration, the extract was determined by high performance liquid chromatography (HPLC) [Gulliver series; Japan Spectroscopic (JASCO), Tokyo] with an ODS column of Mightysil RP-18 (Kanto Chemical, Tokyo; 4.6 mm i.d. × 25 cm) by a slight modification of Tada et al. (1996). Solvent A was 150 mM phosphoric acid and solvent B was composed of 150 mM phosphoric acid, 3.3 M acetic acid, and 4.8 M acetonitrile. Starting conditions were 75% A and 25% B, then a linear gradient to 65% A and 35% B for 30 min, held 5 min, then returned to initial conditions within 5 min. A column at 35 ºC with a flow rate of 1 $mL \cdot min^{-1}$ and UV detector at 520 nm were used. Cyanidin was extracted from 1 g of skin according to the method of Terahara et al. (1993). Samples hydrolyzed in 2 M HCl at 80 ºC for 20 min were analyzed by HPLC. Analytical conditions were the same as for anthocyanin measurement.

Confirmation of anthocyanins. Cyanidin 3-galactoside, 3-glucoside, and 3-arabinoside were isolated from the skin sample through an XAD-2000 column, PVP column and preparative ODS-HPLC according to a report by Terahara et al. (2001). These cyanidin 3-monosides were obtained as dark red powders of trifluoroacetic acid salts, and their purity was over 95% by HPLC analysis. Their structures were confirmed on the basis of the combined results of electrospray ionization/time-of-flight mass spectrometry (ESI/TOFMS) spectra (Mariner Biospectrometry Work Station, Applied Biosystems, Foster City, Calif.), acid hydrolysis, and measurements of UV-VIS spectra (JASCO V-550 UV/VIS spectrometer, Tokyo).

The molecular weights and formulas of cyanidin 3-galactoside, 3-glucoside and 3-arabinoside were determined by ESI/TOFMS measurement whose spectra for anthocyanins gave especially clear molecular mass peaks (Knox et al., 2001). To confirm the kind of anthocyanidin and its glycoside, after their acid hydrolysis, according to method of Terahara et al. (1993), the analysis was done on a cellulose TLC with standard anthocyanidins (pelargonidin, cyanidin, and delphinidin) and standard sugars (glucose, galactose, arabinose, and xylose).

Isolation of *MdCHS, MdF3H* and *MdANS* clones. *Chalcone synthase* (*CHS*), *flavanon 3-hydroxylase* (*F3H*), and *anthocyanidin synthase* (*ANS*) were identified in the course of expressed sequence tag analysis from a cDNA library of apple flower buds (M. Wada, unpublished). Total RNA was isolated from flower buds according to the CTAB method as reported by Kotoda et al. (2000). From the total RNA, first- and second-strand cDNAs were synthesized using an Oligo (dT) primer (a cDNA synthesis system plus kit, Amersham Pharmacia, Little Chalfont, U.K.). After ligation with an *Eco*RI/*Bam*HI adaptor, double-strand cDNAs longer than 300-bp were fractionated using electrophoresis on an agarose gel. Then they were ligated into the *Eco*RI site of pBluescript II SK+ vectors (Stratagene, La Jolla, Calif.). Following the transformation of DH5a, plasmid DNAs were extracted for nucleotide sequencing from randomly selected bacterial white colonies. A sequence analysis was then performed using the fluorescence detection method on a Hitachi SQ5500 automated sequencer (Hitachi, Tokyo). BLAST searches were performed on the data from the sequence, which yielded three clones showing strong homology to anthocyanin biosynthetic genes *CHS*, *F3H* and *ANS*. These cDNAs have the complete coding region for *CHS*, *F3H*, and *ANS* which were expressed as *MdCHS, MdF3H*, and *MdANS*, respectively, in this study.

Isolation of *pDFR* and *pUFGluT* fragments. The partial fragments for *dihydroflavonol 4-reductase* (*DFR*) and *UDP glucose: flavonoid 3-O-glucosyltransferase* (*UFGluT*) were amplified by reverse transcription-polymerase chain reaction (RT-PCR) using specific primers based upon the corresponding genes from 'Fuji' (AF117268 for *DFR* and AF117267 for *UFGluT*). Total RNA for RT-PCR was isolated from fruit skin using the CTAB method. From the total RNA of skins, first-strand cDNA was synthesized using a first-strand cDNA synthesis kit (Amersham Pharmacia). The *DFR* probe was amplified with primers 5'-gagtccgaatccgtttgtgtca-3' and 5'-atgtttgtgggggctgtcgatg-3', and the *UFGluT* probe was amplified with primers 5'-tccctttcactagccatgcaag-3' and 5'-gtggaggatgga-gtttttacc-3'. Amplified fragments were cloned into the pCRII vector with a TA cloning system (Invitrogen, San Diego, Calif.). Four clones were respectively sequenced according to the fluorescence detection method using a sequencer (model 377A; Perkin-Elmer Applied Biosystems, Foster City, Calif.) and demonstrated to be identical at the nucleotide level. Two cDNAs acquired from the partial fragments for *DFR* and *UFGluT* were expressed as *pDFR* and *pUFGluT* in this study.

The apple cDNAs of *MdCHS, MdF3H, MdANS, pDFR*, and *pUFGluT* used in this study were deposited in DNA data bank of Japan (DDBJ). Their accession numbers are: *MdCHS* = AB074485, *MdF3H* = AB074486, *MdANS* = AB074487, *pDFR* = AB074488, and *pUFGluT* = AB074489.

Analysis of RNA. Total RNA was isolated from the skin sample using the method reported by Loulakakis et al. (1996). The Northern blot analysis was performed using the method of Kobayashi et al. (2001). In order to reveal the expression of structural genes, 20 µg of total RNA was separated by electrophoresis in a 1.2% agarose gel containing 0.66 M formaldehyde, and then it was transferred to a nylon membrane. The membranes were then hybridized with digoxigenin (DIG) labeled probes using a PCR DIG probe synthesis kit (Boehringer Mannheim, Mannheim, Germany). The DNA clones that were used as probes were isolated using PCR. Hybridization occurred for 16 h at 50 ºC within a hybridization buffer of DIG Easy Hyb (Boehringer Mannheim, Germany). The membranes were washed twice in (2× SSC) (150 mM NaCl and 15 mM trisodium citrate, pH 7.0), 0.1% (w/v) SDS at room temperature for 5 min each, and then washed twice in 0.1× SSC, 0.1% SDS at 68 ºC for 15 min each.

Statistical analysis. In Figs. 1 and 4, data are presented as means ± SE, and student's *t* test was used to determine the significance in Fig. 4 (SAS Institute, Cary, N.C.).

Results and Discussion

Cyanidin 3-galactoside, 3-glucoside, and 3-arabinoside respectively gave the molecular cations at *m/z* 449 [$C_{21}H_{21}O_{11}^+$], 449 [$C_{21}H_{21}O_{11}^+$], and 419 [$C_{20}H_{19}O_{10}^+$] corresponding to their molecular formulas and the fragmentation ion at *m/z* 287 [$C_{15}H_{11}O_6^+$] of cyanidin. Under acid hydrolysis, they gave cyanidin as anthocyanidin and gave the corresponding sugars, galactose, glucose, and arabinose, respectively. Moreover, UV-VIS data of their 0.1% HCl-methanol solutions showed that each sugar was attached to a 3-position of cyanidin because all values of E440/Evis.max were large (>20%) as pointed out by Harborne (1985).

The principal anthocyanin in the skin of 'Tsugaru' apples detected in this study was cyanidin 3-galactoside, followed by cyanidin 3-arabinoside and the lowest was cyanidin 3-glucoside (Fig. 1). Cyanidin 3-galactoside was detected at 10 DAFB, then decreased, and began increasing again after 103 DAFB. Both cyanidin 3-arabinoside and cyanidin 3-glucoside were detected after only 110 DAFB. Also in 'Oorin', cyanidin 3-galactoside and cyanidin 3-arabinoside were detected at 20 DAFB (data not presented). In 'Tsugaru,' cyanidin which was the highest at 10 DAFB decreased gradually until 94 DAFB, and then increased again toward 138 DAFB (Fig. 1). The concentrations of cyanidin did not always correlate with those of cyanidin 3-galactoside which is the primary anthocyanin in 'Tsugaru' apples. At 10 DAFB, when the concentration of cyanidin was the highest, the color of the apple fruit was brick red which happens to be the color of cyanidin. This implies that most cyanidin may fail to be glycosylated because the sugar concentration in the fruit was very low at 10 DAFB. That is, the formation of glycosides may not depend on the concentration of cyanidin, but rather sugar concentration in the fruit because it is high at the ripening stage. In contrast, the concentration of cyanidin was the lowest at 94 DAFB when the anthocyanin was not detected, and the skin color was green. Therefore, cyanidin may not contribute to the fruit color if the concentration is below a certain level. This concentration was less than 262 μM·kg^{-1} fresh weight in our study.

In 'Tsugaru,' there were generally two peaks in the expression of anthocyanin pathway genes. One was from 10 DAFB until 73 DAFB, and the other was during ripening starting at 101 DAFB (Fig. 2). However, the expression of *MdCHS, MdF3H, pDFR*, and *MdANS* was detected even at 94 DAFB when anthocyanin was not observed. The minimum level of detection of cyanidin 3-galactoside by HPLC was 50 nM. In contrast, in the analysis repeated three times, the expression of *pUFGluT* was not detected at 94 DAFB, but was detected on all other days on which the fruit was tested for *pUFGluT*.

CHS catalyzes the first committed step in the pathway to flavonols and anthocyanins (Fig. 3). Ju et al. (1995a) showed that CHS activity in the skin of 'Delicious' and 'Ralls' did not change significantly during the entire fruit development stages. In the skin of bagged 'Tsugaru' fruit, in which anthocyanin was not detected, *MdCHS* expression was reduced but did not disappear (Fig. 4). These results suggest that *MdCHS* may not regulate directly the expression of anthocyanin in 'Tsugaru.'

In 'Oorin' at 165 DAFB (harvest), the expressions of only *MdF3H* and *pDFR* were observed (Fig. 4). F3H and DFR catalyze flavanone to leucoanthocyanidin (Fig. 3). The reddening of apples is caused by anthocyanin. However, nonred apples accumulate flavonols and proanthocyanidins in the skin (Lancaster, 1992). Flavonoid concentration remained relatively high during the entire fruit development of apples (Ju et al., 1995a). Therefore, it is considered that the expression of *MdF3H* and *pDFR* during development indicates the presence of flavonols and proanthocyanidins. In our study with 'Tsugaru,' cyanidin was observed throughout fruit

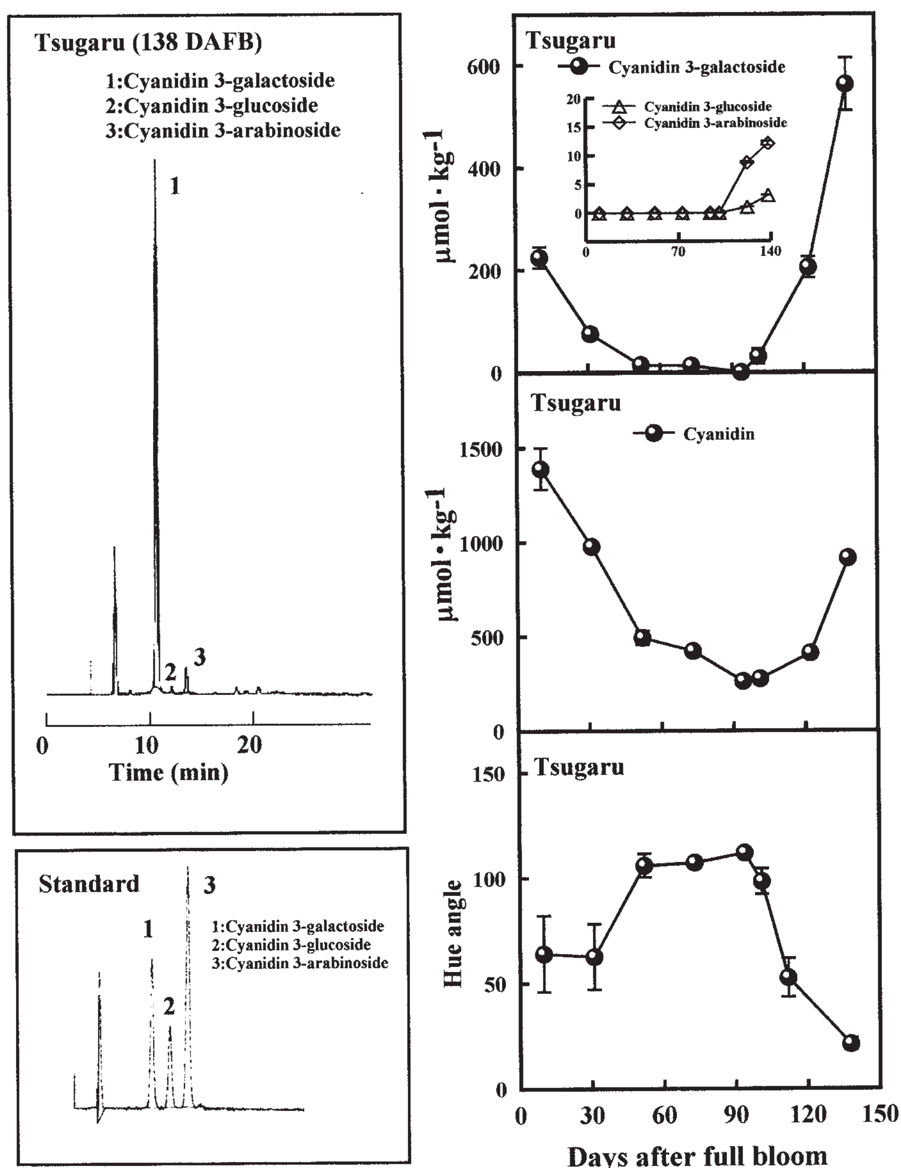

Fig. 1. Chromatogram of analytical HPLC of anthocyanins in 'Tsugaru' apples at 138 DAFB in the year 2000, and changes in cyanidin 3-galactoside, cyanidin 3-arabinoside, cyanidin 3-glucoside, cyanidin and hue angle in the skin of 'Tsugaru'. Data of anthocyanin, cyanidin, and hue angle are means ± SE of five fruit. Hue angle = [(ATAN (b/a)/2π) × 360º, 0º = red-purple, 90º = yellow, 180º = blue-green, 270º = blue]. SE is smaller than the symbol when error bar is not shown.

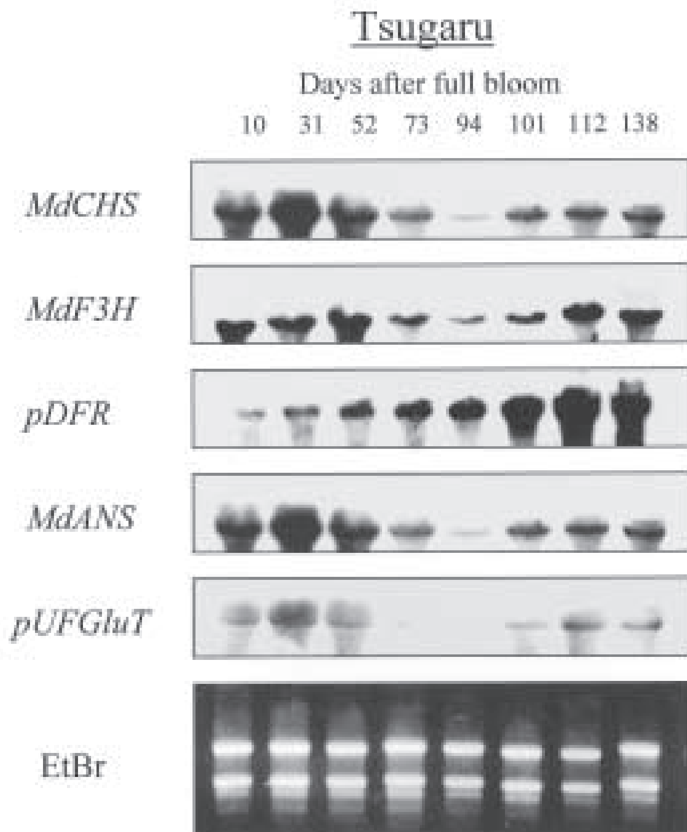

Fig. 2. Northern blot analysis of RNA from 'Tsugaru' skin during fruit development stages in the year 2000. Total RNAs (20 µg) were hybridized with probes of cDNA clones for *MdCHS*, *MdF3H*, *pDFR*, *MdANS*, and *pUFGluT*. The analysis was repeated three times. Bottom panel shows the ethidium bromide-stained gel as a loading control.

development (Fig. 1). The cyanidin was also detected in 'Oorin' at 165 DAFB when anthocyanin was not observable (Fig. 4). Leucoanthocyanidin changes to anthocyanidin through the action of ANS (Fig. 3). The cyanidin concentrations changed similarly as the expression of *MdANS* (Figs. 1 and 2). Proanthocyanidins such as catechin which are formed from leucoanthocyanidin existed in both red and nonred apple skins (Kondo et al., 2001). When measuring the expression of *MdANS* in bagged fruit in which anthocyanin did not appear, it was found to be much lower than the expression in nonbagged fruit with red color (Fig. 4). The expression of *MdCHS* was also similar to that of *MdANS* but *pUFGluT* was not detected (Fig. 4). This shows that *MdCHS*, *MdANS*, and *pUFGluT* in the skin of 'Tsugaru' are determined by the intensity of light. Small amounts of *MdCHS* and *MdANS* still appear in bagged fruit under dark conditions, but these levels are too low for anthocyanin to form. This conclusion is based on *MdCHS* and *MdANS* not being detected in 'Oorin' at 165 DAFB (Fig. 4).

MdANS was also detected in the skin at 94 DAFB in 'Tsugaru' where anthocyanin did not appear (Fig. 2). In contrast, *MdANS* was not detected at 165 DAFB in 'Oorin' (Fig. 4). In 'Tsugaru,' the lowest expression of *MdANS* was 94 DAFB, before coloring began, when the skin color was most green (Fig. 1). In bagged 'Tsugaru' at 98 DAFB and 'Oorin' at 165 DAFB, the cyanidin concentrations were almost the same. However, the skin color was different as that of 'Oorin' was greener than that of 'Tsugaru.' These observations suggest that the yellow color of the skin may be due to the expression of *MdANS*. *ANS* was also detected in the white-skin cultivar of grape berries at harvest (Kobayashi et al., 2001). Therefore, ANS may not regulate directly anthocyanin production.

In our study, a region of the cDNA encoding *UFGluT* was used as a probe. UFGluT catalyzes the 3-*O*-glucosylation of anthocyanidins to form cyanidin 3-glucoside (Gantet et al., 1993). The anthocyanin detected in our study was almost entirely cyanidin 3-galactoside at both the beginning of fruit development stage and the ripening stage. Cyanidin 3-galactoside is formed from cyanidin by UDP-galactose-3-*O*-galactosyltransferase (UFGalT) (Miller et al., 1999). In *Vigna mungo*, the deduced amino acid sequence of *UFGalT* showed 42% identity with that of *UFGluT* (Mato et al., 1998). A previous report (Honda et al., 2002) showed that the deduced amino acid sequence of the *UFGluT* from 'Fuji' possessed sequences in some regions that are similar to the *UFGluT* or *UFGalT* in other plants. That is, *UFGluT* and *UFGalT* cannot be clearly distinguished by their deduced amino acid sequences. Thus, the probe used in our northern analyses may be of *UFGalT*. *pUFGluT* was not detected in cases where anthocyanin did not

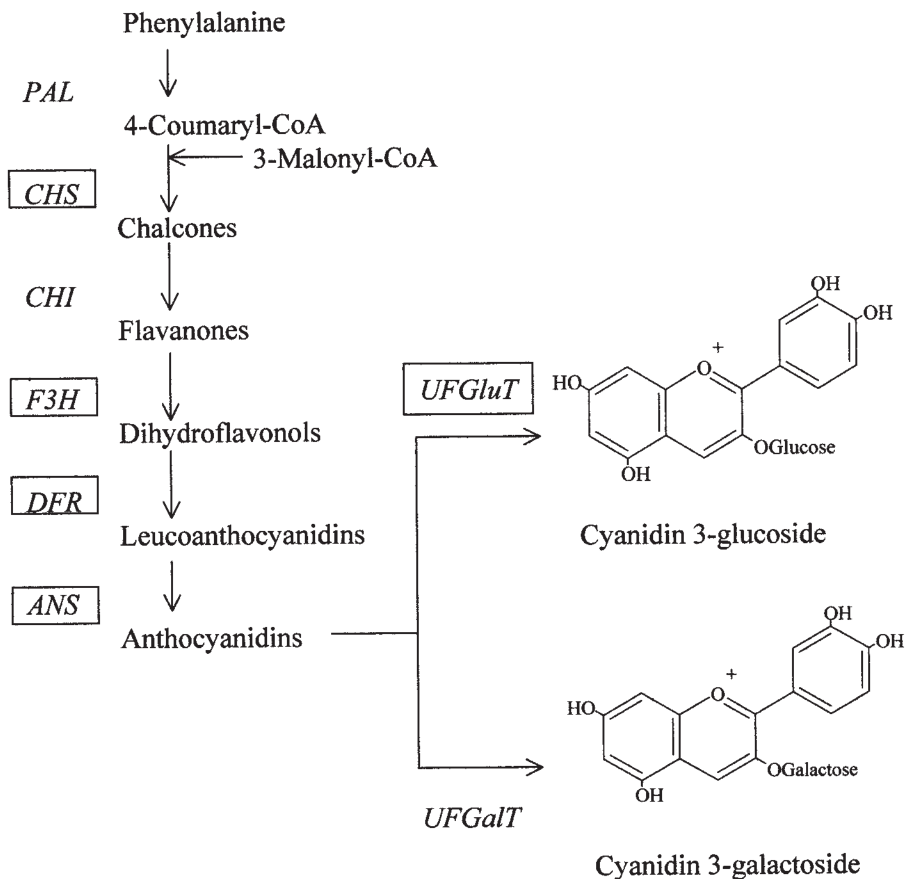

Fig. 3. Scheme of the anthocyanin biosynthetic pathway in apple skin. The genes analyzed by Northern blot in this study are boxed. PAL = phenylalanine ammonia-lyase, CHS = chalcone synthase, CHI = chalcone isomerase, F3H = flavanone 3-hydroxylase, DFR = dihydroflavonol 4-reductase, ANS = anthocyanidin synthase, UFGluT = UDP glucose = flavonoid 3-*O*-glucosyltransferase, UFGalT = UDP galactose = flavonoid 3-*O*-galactosyltransferase.

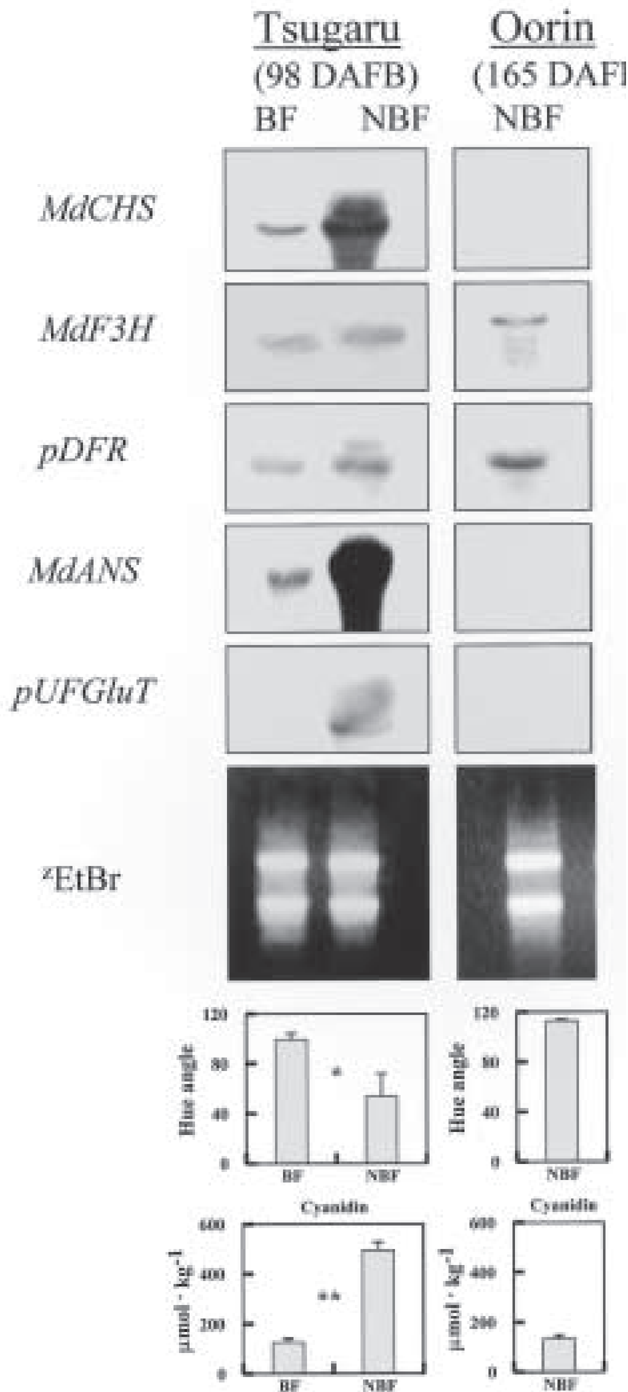

Fig. 4. The expression of anthocyanin biosynthetic genes, hue angle, and cyanidin concentrations in the skins of bagged and nonbagged 'Tsugaru' apples at 98 DAFB in the year 2000, and of 'Oorin' apples at 165 DAFB. The 'Tsugaru' fruit was bagged at 30 DAFB and anthocyanin was not detected in bagged fruit at 98 DAFB. Northern blots are of total RNA (20 μg) probed with cDNA clones for *MdCHS*, *MdF3H, pDFR, MdANS*, and *pUFGluT*. The analysis was repeated three times. Hue angle = [(ATAN (b/a)/2π) × 360º, 0º = red-purple, 90º = yellow, 180º = blue-green, 270º = blue]. BF = bagged fruit. NBF = nonbagged fruit. DAFB = days after full bloom. [*,**]Significant at $P \leq 0.05$ or 0.01, respectively. [z]See Fig. 2.

appear in 'Tsugaru' at 94 DAFB, 'Oorin' at 165 DAFB, nor bagged 'Tsugaru' at 98 DAFB. This result implies that UFGluT may be an enzyme which plays a role in the accumulation of anthocyanin in apple fruit. However, *MdCHS, MdF3H, pDFR, MdANS*, and *pUFGluT* were all detected at 20 DAFB in both bagged fruit without anthocyanin and nonbagged fruit with anthocyanin (Fig. 5). Apple

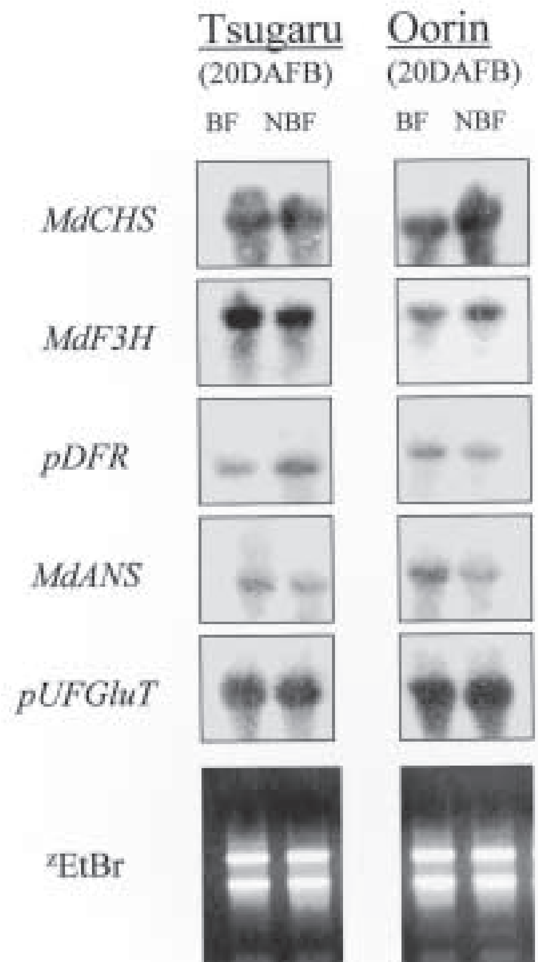

Fig. 5. The expression of anthocyanin biosynthetic genes in the skins of bagged and nonbagged 'Tsugaru' and 'Oorin' apples at 20 DAFB in the year 2001. The fruit was bagged after fertilization and anthocyanin was not detected in bagged fruit at 20 DAFB. Northern blots are of total RNA (20 μg) probed with cDNA clones for *MdCHS*, *MdF3H, pDFR, MdANS*, and *pUFGluT*. The analysis was repeated three times. BF = bagged fruit. NBF = nonbagged fruit. DAFB = days after full bloom. [z]See Fig. 2.

skin at the beginning of the fruit development stage is rich in flavonoids and procyanidins (Ju et al., 1995a; Kondo et al., 2001). UFGluT glucosylates including both flavonols and anthocyanins in petunia (Lancaster, 1992). The results of these reports suggest that the role of UFGluT may differ with the fruit development stage. However, the behavior of *pUFGluT* in our study shows that UFGluT may be closely related to the expression of anthocyanin at the ripening stage.

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