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Original article

Anthocyanin biosynthetic genes are coordinately expressed during red coloration in apple skin

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Abstract

Five genes of anthocyanin biosynthetic enzymes, chalcone synthase (CHS; EC 2.3.1.74), flavanone 3-hydroxylase (F3H; EC 1.14.11.9), dihydroflavonol 4-reductase (DFR; EC 1.1.1.219), anthocyanidin synthase (ANS; EC 1.14.11.X), and UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGluT; EC 2.4.1.X), were isolated, and their expression was investigated to elucidate the molecular mechanism for red coloration in apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] skin. In 'Orin,' a yellow apple cultivar, no significant levels of anthocyanin were detectable, whereas in 'Jonathan' and 'Fuji,' both red apple cultivars, anthocyanin concentrations increased during fruit development. At the ripe stage, the level of anthocyanin concentration was about three times higher in 'Jonathan' than in 'Fuji.' The accumulation of transcripts for the five genes was induced at the later developmental stages in all three cultivars. The levels for the expression of the five genes basically corresponded to the anthocyanin concentrations; that is, the induction of the genes in 'Orin' was less pronounced, and that in 'Fuji' and 'Jonathan' was notable, with much higher expression levels in 'Jonathan' than in 'Fuji'. These results indicate that the five genes are coordinately expressed during fruit development and that their levels of expression are positively related to the degree of anthocyanin concentration. This is the first report that characterizes the relationship between the expression of anthocyanin biosynthetic genes and apple fruit coloration.

Keywords: Anthocyanin; Apple skin; Flavonoid biosynthetic genes; Fruit development; Gene expression; Red coloration

1. Introduction

The red coloration of apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] skin is derived from chemical components called anthocyanins that belong to a class of flavonoids. The accumulation of anthocyanins is influenced by environmental stimuli, such as light, temperature, and nutrition [13,19], as well as by genetic factors. The regulatory mechanisms of anthocyanin biosynthesis attract interest because red skin coloration is an important factor in market acceptance for many apple cultivars. There are distinguished varieties of red-skinned apples: (a) red cultivars, such as 'Jonathan' and 'Starking Delicious,' which bear well-colored fruits; (b) red cultivars, such as 'Fuji' and 'Jonagold,' which are pale in color under certain conditions; and (c) non-red cultivars that accumulate some anthocyanins after shade-pretreatment by bagging, such as 'Orin,' 'Golden Delicious,' and 'Mutsu.'

During the past decade, cloning of structural genes encoding enzymes in the anthocyanin biosynthetic pathway and the identification of genes encoding transcription factors that regulate the expression of structural genes have

Abbreviations: ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; CTAB, cetyltrimethylammonium bromide; DAFB, days after full bloom; DFR, dihydroflavonol 4-reductase; EDTA, ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid; EST, expressed sequence tag; F3H, flavanone 3-hydroxylase; HPLC, high performance liquid chromatography; PAL, phenylalanine ammonia-lyase; RT–PCR, reverse transcription–polymerase chain reaction; UFGalT, UDP galactose: flavonoid 3-*O*-galactosyltransferase; UFGluT, UDP glucose: flavonoid 3-*O*-glucosyltransferase

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contributed to our understanding of the molecular basis of anthocyanin biosynthesis in higher plants, such as maize, snapdragon, and petunia [6]. In fruit trees, the anthocyanin biosynthetic genes from grapes (*Vitis vinifera* L.) have been investigated carefully [20], and UDP glucose:flavonoid 3-*O*-glucosyltranseferase (UFGluT) was found to be a key enzyme controlling the color red in grape skin [1]. More recently, Kobayashi et al. [11] found that there were no differences in either coding or promoter sequences of the UFGluT gene between white-skinned grape cultivars ('Italia' and 'Muscat of Alexandria') and their red-skinned sport cultivars ('Ruby Okuyama' and 'Flame Muscat'). They concluded that a mutation of a regulatory gene controlling UFGluT gene expression caused the change from white to red in the sports.

In apples, on the other hand, there have been only a few molecular studies on the anthocyanin biosynthesis pathway. Dong et al. [3] investigated the changes in the expression of the anthocyanin biosynthetic genes during apple flower development and showed that the genes were controlled by both development and light. Cheng et al. [2] isolated co-dominant randomly amplified polymorphic DNA (RAPD) markers linked to apple skin color, which showed that the trait of red skin color was primarily controlled by one or more dominant genes at a single locus in apple. To date, however, little is known about the roles of these genes on the anthocyanin accumulation during red coloration in apple skin.

There are two peaks of anthocyanin biosynthesis in apple fruits, one in young fruitlets and the other in ripening fruits. We focused on the later stage, which is economically important for market value, and studied the relationship between anthocyanin accumulation and the expression of anthocyanin biosynthetic genes at this stage. In this study, we describe the isolation of the five genes of anthocyanin biosynthetic enzymes, chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UFGluT (Fig. 1), from 'Jonathan.' We also indicate that the five genes are coordinately expressed during apple fruit development and that the levels of expression are positively correlated with the degree of anthocyanin concentrations in ripening fruits of 'Orin,' 'Fuji,' and 'Jonathan.'

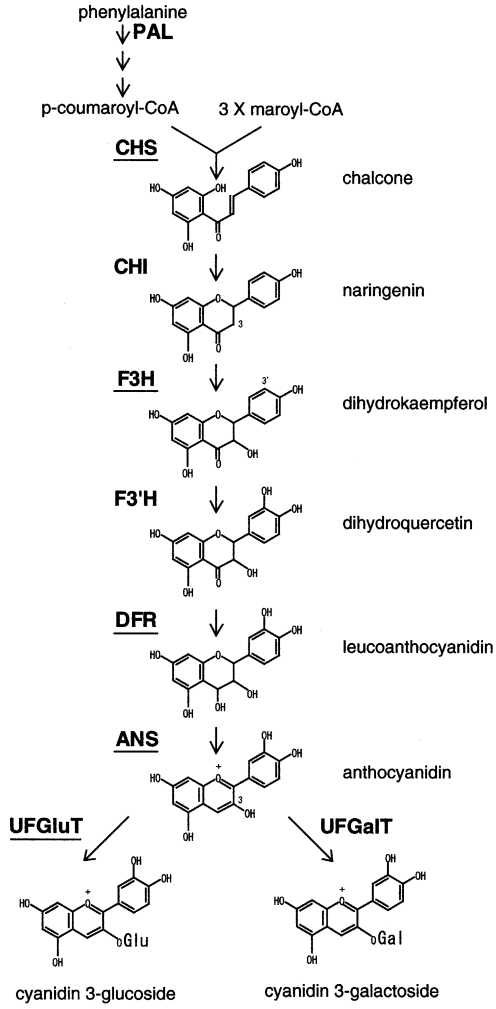

Fig. 1. The putative anthocyanin biosynthetic pathway in apple skin. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGluT, UDP glucose:flavonoid 3-*O*-glucosyltransferase; UFGalT, UDP galactose:flavonoid 3-*O*-galactosyltransferase; glu, glucose; gal, galactose. The enzymes whose genes were used for Southern and northern blot analyses are underlined.

2. Results

2.1. Gene cloning and sequence analysis

The cDNAs containing the complete coding region (*MdCHS*, *MdF3H*, and *MdANS*) or the cDNA fragments (*pDFR* and *pUFGluT*) for the anthocyanin biosynthetic genes were isolated from 'Jonathan' in two ways. First, in the course of expressed sequence tag (EST) analysis from a cDNA library of 'Jonathan' floral buds, we identified *MdCHS*, *MdF3H*, and *MdANS*. The *CHS* partial sequence in *Malus* sp. (DDBJ accession no. X68977) was previously reported, and our *MdCHS* from 'Jonathan' was the first isolated gene with the complete coding region. The coding region of *MdCHS* was 1176 bp long, with a 391-deduced amino acid sequence and a predicted molecular mass of 42.6 kDa. *MdCHS* was highly homologous with the *CHS*s from other plants. The deduced amino acid sequence of MdCHS showed 89.7% identity with that of *Citrus sinensis* CHS (AB009350), 89.7% identity with that of *Lycopersicon esculentum* CHS (X55195), 89.2% identity with that of *V. vinifera* CHS (X75969), 87.7% identity with that of *Antirrhinum majus* CHS (X03710), and 83.9% identity with that of *Zea mays* CHS (X60204) (data not shown). In addition to *MdCHS*, cDNA clones for *MdF3H* and *MdANS* containing the complete coding regions were also isolated from this library. Both of the corresponding genes have already been isolated from 'Fuji' (AF117270 and

AF117269, respectively). The coding sequence of 'Jonathan' *MdF3H* (1098 bp long) showed 94.5% identity with that of 'Fuji' *F3H* at the nucleotide level, and the deduced amino acid sequence of MdF3H showed 96.7% identity with that of 'Fuji' F3H at the amino acid level (data not shown). The coding sequence of 'Jonathan' *MdANS* (1074 bp long) was substituted by only two nucleotides and one amino acid residue compared with that of 'Fuji' *ANS* (data not shown).

Secondly, the partial fragments for *DFR* and *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*). A single band of the respective expected size (1.0 kbp for *DFR* and 1.2 kbp for *UFGluT*) was amplified from cDNA synthesized from the total RNA of 'Jonathan' fruit skin, then sub-cloned into a pCRII vector, and finally sequenced to confirm the homology. The partial fragments for *pDFR* (943 bp long) and *pUFGluT* (1175 bp long) of 'Jonathan' showed 99.6% and 98.8% identities to those of 'Fuji' at the nucleotide level, respectively.

These clones of *MdCHS*, *MdF3H*, *MdANS*, *pDFR*, and *pUFGluT* have been deposited in DDBJ under the accession numbers AB074485, AB074486, AB074487, AB074488, and AB074489, respectively.

2.2. Southern blot analysis

To estimate the copy number of these structural genes in the apple genome, the genomic DNA from 'Jonathan' leaves was digested with *Eco*RI, *Bam*HI, *Dra*I, *Kpn*I, *Hin*dIII, or *Xba*I, and Southern blot analysis was performed under high stringency using the five genes listed above as the probes (Fig. 2). Hybridization with *MdCHS* as the probe gave one to three major fragments in the *Dra*I, *Hin*dIII, and *Xba*I digestions. A number of weak bands were observed in all digestions, indicating that *CHS* was encoded by a multigene family in the apple genome. Two major fragments and another weak fragment hybridized with the *MdF3H* probe were detected in the *Dra*I and *Hin*dIII digestions, and two major fragments were found in the *Xba*I digestion. *pDFR* showed two weak fragments in the *Bam*HI digestion, two major fragments in the *Dra*I digestion, and three major fragments in the *Eco*RI digestion. *MdANS* showed one major band in the *Dra*I digestion, two weak bands in the *Kpn*I digestion, and three major bands in the *Xba*I digestion. *pUFGluT* showed one major band in the *Bam*HI digestion, three major bands in the *Dra*I digestion, and two bands in the *Eco*RI digestion. Collectively, these results suggested that a few copies for each *F3H*, *DFR*, *ANS*, and *UFGluT* might be present in the apple genome.

2.3. Total anthocyanin accumulation

To investigate anthocyanin accumulation, we chose three apple cultivars, 'Orin,' 'Fuji,' and 'Jonathan,' which differed in fruit color genotypes according to a report by Cheng et al. [2]. Apple skin was collected three times during fruit development, and total anthocyanin concentrations were spectrophotometrically measured at 530 nm (Fig. 3). Since the ripe stages differed among the three cultivars, the last harvest dates were dependent on them. The harvest dates were described as days after full bloom (DAFB). In 'Orin,' a significant accumulation of anthocyanin was not detected in any stage (data not shown). In 'Fuji,' no anthocyanin accumulation occurred at 106 DAFB, but it was notably induced at 142 DAFB, resulting in a 4.2-fold increase from 142 to 176 DAFB. In 'Jonathan,' no significant accumulation of anthocyanin was detectable at 106 DAFB; thereafter, a rapid accumulation from 142 to 154 DAFB occurred, with a 2.2-fold increase during the last 12 d.

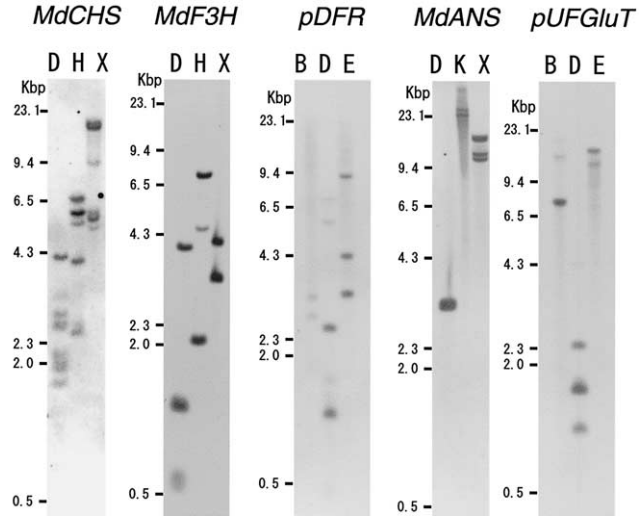

Fig. 2. Southern blot analysis of the five anthocyanin biosynthetic genes in the 'Jonathan' apple. Each lane contained 10 μg of genomic DNA from 'Jonathan' leaves digested with each restriction enzyme. B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I. Positions of the molecular mass markers are shown at left.

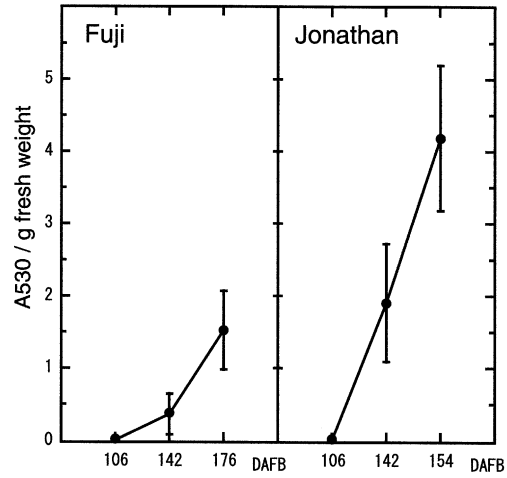

Fig. 3. Change of total anthocyanin concentrations during fruit maturation. Fruits of 'Fuji' and 'Jonathan' were harvested on the indicated day. Three to six skin disks were used for each measurement. Vertical lines indicate standard deviation. DAFB, days after full bloom.

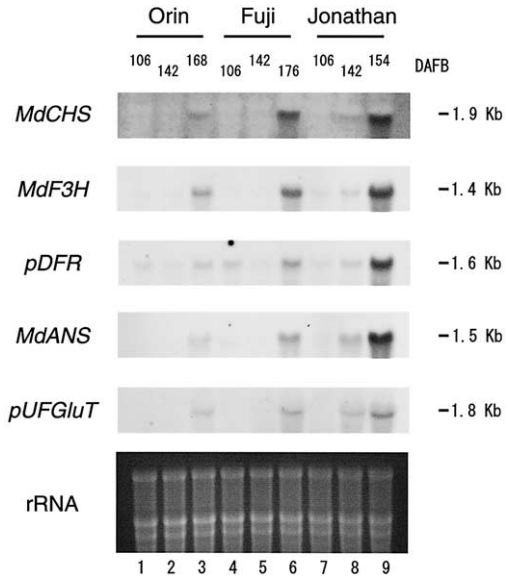

Fig. 4. Northern blot analysis of the five anthocyanin biosynthetic genes during the ripening of 'Orin,' 'Fuji,' and 'Jonathan' fruits. Apple fruits were harvested on the indicated day. Each lane contained 10 μg of total RNA from apple skin. In the lower panel, the total RNA on the gel was stained with ethidium bromide to confirm equivalent loading. DAFB, days after full bloom. Lanes 1–3, 'Orin;' lanes 4–6, 'Fuji;' lanes 7–9, 'Jonathan.'

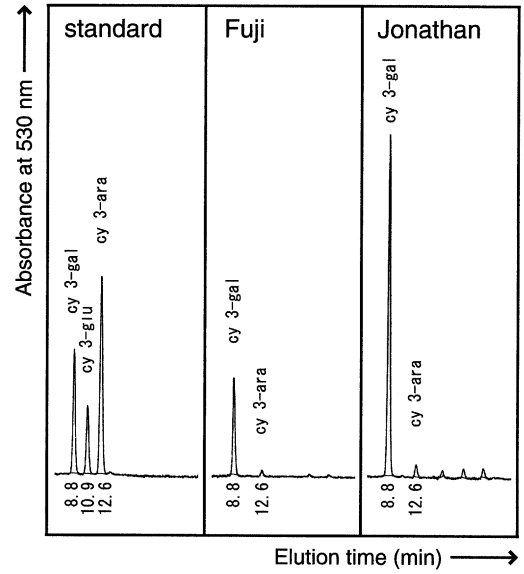

Fig. 5. HPLC analysis of anthocyanins in 'Fuji' and 'Jonathan.' The identity and the molar ratio were established by chromatography of authentic standards. The concentration of each standard is 8.3 ppm. cy 3-gal, cyanidin 3-galactoside; cy 3-glu, cyanidin 3-glucoside; cy 3-ara, cyanidin 3-arabinoside.

2.4. Expression patterns of the five anthocyanin biosynthetic genes during fruit development

Northern blot analysis was done using the same probes as in Southern blot analysis. Each of the five probes generated one band corresponding to the expected size in the hybridization (Fig. 4). In 'Orin,' the expression of the five anthocyanin biosynthetic genes was hardly detectable at both 106 and 142 DAFB (lanes 1 and 2); a very low expression of *MdCHS*, *MdF3H*, *pDFR*, *MdANS*, and *pUFGluT* was then observed at the ripe stage (168 DAFB; lane 3). In 'Fuji,' the accumulation of the transcripts corresponding to the five genes was hardly detectable at 106 and 142 DAFB (lanes 4 and 5), and all five genes were induced at the ripe stage (176 DAFB; lane 6). In 'Jonathan,' little expression of the five biosynthetic genes was detected at 106 DAFB (lane 7), and a slight expression was observed for *MdF3H*, *pDFR*, *MdANS*, and *pUFGluT* at 142 DAFB (lane 8). At the ripe stage (154 DAFB), all five genes were induced at high levels (lane 9). These results indicated that the five genes are coordinately expressed during fruit development and that the levels of expression are positively related to the degree of anthocyanin concentration. However, considering that the anthocyanin accumulation had partially started at 142 DAFB (Fig. 3), the beginning for the significant induction of the five genes was delayed.

2.5. HPLC analysis of anthocyanin in apple skin

Previous works using paper chromatography revealed that the major pigment in the red skin of apple cultivars was cyanidin 3-galactoside and that the minor pigments identified were 3-glucoside and 3-arabinoside [21,23]. Recently, Lister et al. [14] showed by reverse-phase high performance liquid chromatography (HPLC) that the apple peel of 'Splendour' contained cyanidin 3-galactoside. Gómez-Cordovés et al. [5] also showed by reverse-phase HPLC that cyanidin 3-galactoside was the major anthocyanin in the 'Starking Delicious' apple. However, there is no available information by HPLC on 'Orin,' 'Fuji,' or 'Jonathan.' Thus, the molecular components of the anthocyanin pigments in the three cultivars, 'Orin,' 'Fuji,' and 'Jonathan,' were analyzed by reverse-phase HPLC. A representative result of the chromatograms is shown in Fig. 5. None of the 3-glycosides was detected by HPLC, not even in the ripe fruit of 'Orin' at 168 DAFB (data not shown). In 'Fuji' at 176 DAFB, 3-glucoside was not detected; instead, 3-galactoside and 3-arabinoside were identified with a molar ratio of 95 to 5. Furthermore, 3-glucoside was also not detected, and 3-galactoside and 3-arabinoside were identified with a molar ratio of 96 to 4 in 'Jonathan' at 154 DAFB.

3. Discussion

We demonstrated that anthocyanin accumulation was positively correlated with the expression of at least the five anthocyanin biosynthetic genes (*MdCHS*, *MdF3H*, *pDFR*, *MdANS*, and *pUFGluT*) in apple skin (Figs. 3, 4) and also suggested that the levels for the expression of the five genes basically corresponded to the degree of anthocyanin concentration; that is, the induction of the genes in a yellow cultivar, 'Orin,' was less pronounced, and that in the red

cultivars, 'Fuji' and 'Jonathan,' was notable, with much higher expression levels in 'Jonathan' than in 'Fuji.' At present, the reason for the time lag between the notable induction of the gene expression and the anthocyanin accumulation is still unclear. We assume that slight expression of the genes may be sufficient to produce some color, which might act as a trigger for anthocyanin accumulation. A little expression of the biosynthetic genes did not induce anthocyanin accumulation in ripening fruits of 'Orin' at 168 DAFB, but gave rise to its accumulation in immature fruits of 'Jonathan' at 142 DAFB, suggesting that there are other factors controlling red coloration during fruit ripening in addition to the regulation of gene expression.

Some enzymes involved in the anthocyanin biosynthetic pathway in apple skin were studied during fruit development. Lister et al. [16] demonstrated that the activities of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), chalcone isomerase (CHI; EC 5.5.1.6), and UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT; EC 2.4.1.X) in the 'Splendour' apple were correlated with anthocyanin accumulation during fruit ripening. Ju et al. [7,8] showed that PAL activity was not correlated with anthocyanin accumulation in the 'Delicious' apple and that CHS activity was not correlated with anthocyanin accumulation in the 'Delicious' and 'Ralls' apples exposed to light, whereas UFGalT activity was positively correlated with its production in both apple varieties. Moreover, they found that the rapid accumulation of anthocyanin was correlated with an increase in DFR activity in the 'Delicious' apple [9]. In this paper, we could not find a key gene whose expression was induced accompanied by anthocyanin accumulation in the present apple cultivars, such as *UFGluT* in grape [1,11]. These results indicate that one single gene was not responsible for the anthocyanin accumulation but at least five genes were involved in the red coloration of apple skin.

The main anthocyanin identified in apple skin is cyanidin 3-galactoside, while the cyanidin 3-glucoside level is very low (Fig. 5). This suggests that the key enzyme controlling red coloration is not UFGluT but UFGalT in apple skin. Recently, the genes for UFGalT have been isolated from two plants, *Vigna mungo* [17] and *Petunia hybrida* [18]. Recombinant UFGalT from *P. hybrida* transferred the galactosyl moiety only to flavonoids, but not to anthocyanidins [18], whereas recombinant UFGluT from *V. vinifera* transferred the glucosyl moiety primarily to anthocyanidins [4]. The deduced amino acid sequence of the apple UFGluT from 'Fuji' possesses sequences in some regions that are similar to the UFGluTs and also to the UFGalTs from other plants (Fig. 6). The putative UDP-glucose binding domain [10] in the apple UFGluT region from 358 to 381 was conserved among the UFGluTs and UFGalTs from other plants. The amino acid sequences of the UFGluTs from *V. vinifera*, *Z. mays*, and *Hordeum vulgare* showed 52.6%, 37.5%, and 35.9% identity, respectively, to the UFGluT from 'Fuji' (Table 1). The amino acid sequences of the UFGalTs from *P. hybrida* and *V. mungo* showed 48.2% and 44.1% identity, respectively, to the UFGluT from 'Fuji' (Table 1). Since it is not possible to discriminate the substrate specificities (glucosyl moiety or galactosyl moiety, and anthocyanidins or flavonoids) based on their deduced amino acid sequences, we cannot predict whether or not the apple UFGluT functions as UFGalT transferring the galactosyl moiety to cyanidin. Lister et al. [15] reported that a crude extract of flavonoid glycosyltransferase from apple skin specially transferred the glycosyl moiety from sugar nucleotide donors to the 3-position of the flavonoid nucleus and that UDP-galactose showed the highest activity compared with other sugar donors. Further characterization of substrate and sugar specificity of the apple UFGluT will be necessary to investigate its role in fruit coloration.

4. Methods

4.1. Plant materials

All apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] samples were obtained from the orchard of the National Institute of Fruit Tree Science at Morioka, Japan. Whole 'Jonathan' flower buds were collected in the middle of the growing season (from June to September) for the construction of a cDNA library. Young 'Jonathan' leaves were collected for genomic DNA isolation. Fruits from the 'Orin,' 'Fuji,' and 'Jonathan' varieties were periodically harvested at a later developmental stage, and the entire skin was removed with 1 mm of the cortical tissue for anthocyanin measurement and RNA isolation. Samples were frozen in liquid nitrogen and stored at –80 °C until analyzed.

4.2. Isolation of MdCHS, MdF3H, and MdANS clones from 'Jonathan'

Total RNA was extracted from 'Jonathan' whole flower buds in the middle of the growing season (June to September) according to a cetyltrimethylammonium bromide (CTAB)-based method by Kotoda et al. [12]. First- and second-strand cDNAs were synthesized from total RNA by using an oligo(dT) primer (a cDNA Synthesis System Plus kit, Amersham, Little Chalfont, UK). Following ligation with an *Eco*RI/*Bam*HI adaptor, double-stranded cDNAs that were more than 300 bp long were fractionated by electrophoresis on an agarose gel and ligated into the *Eco*RI site of pBluescript II SK$^+$ vectors (Stratagene, La Jolla, CA, USA). After transformation with DH5α, bacterial white colonies were randomly selected, and the plasmid DNAs were extracted for nucleotide sequencing. Sequence analysis was conducted by the fluorescence detection method on a Hitachi SQ5500 automated sequencer (Hitachi, Tokyo, Japan). BLAST searches were done against the sequence data, and three clones, *MdCHS*, *MdF3H*, and *MdANS*, showed high homology to the anthocyanin biosynthetic genes, *CHS*, *F3H*, and *ANS*, respectively, from other plants.

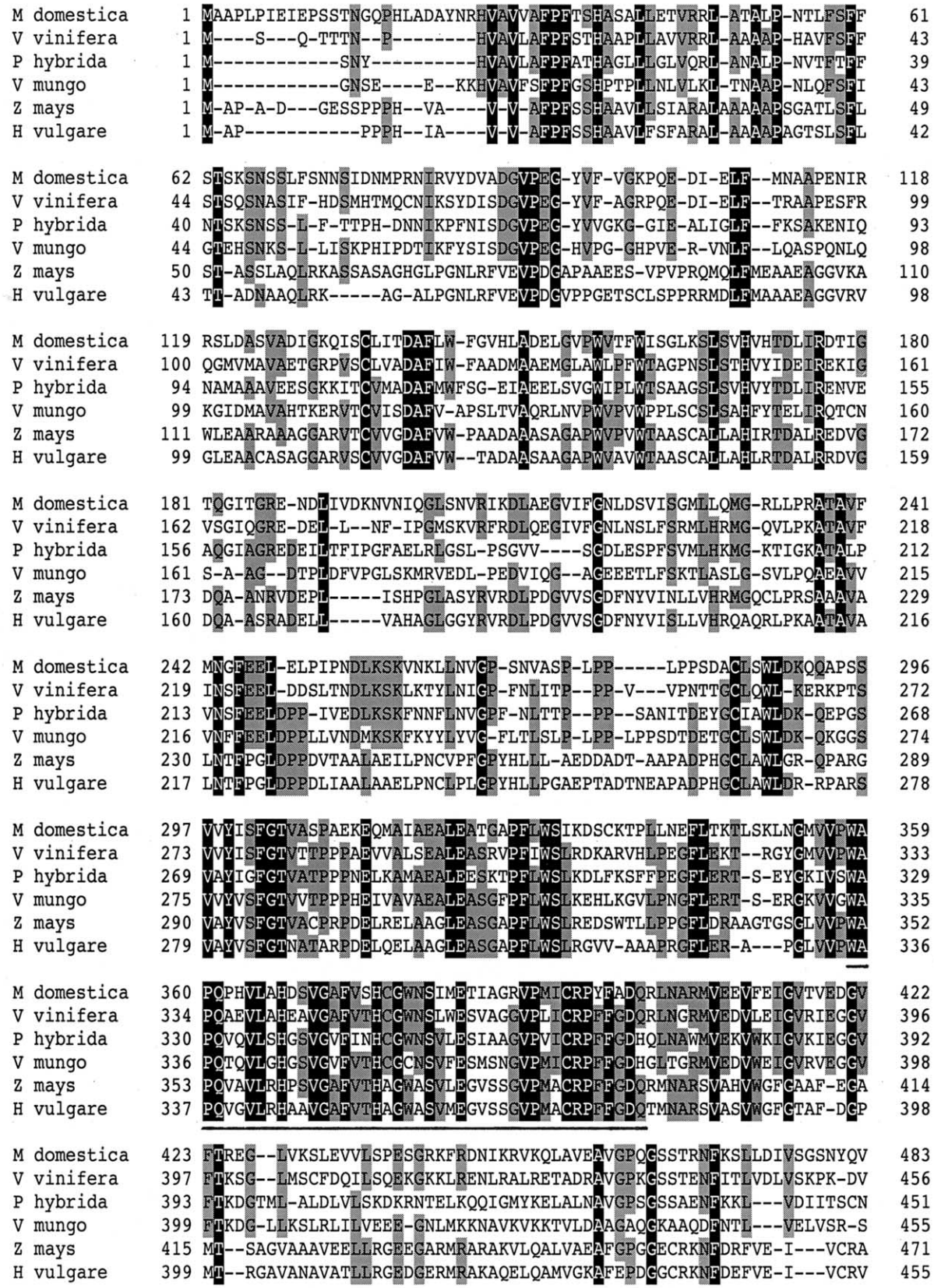

Fig. 6. Alignment of the deduced amino acid sequence of UDP glucose:flavonoid 3-*O*-glucosyltransferase (UFGluT) from *Malus domestica* (AF117267) with UFGluT from *Vitis vinifera* (AF000372), UDP galactose:flavonoid 3-*O*-galactosyltransferase (UFGalT) from *Vigna mungo* (AB009370), UFGalT from *Petunia hybrida* (AF165148), UFGluT from *Zea mays* (X13502), and UFGluT from *Hordeum vulgare* (X15694). Identical amino acids are indicated by black shading; the similarities are shown by gray shading. The putative UDP-glucose binding domain is underlined.

Table 1
Comparison of the deduced amino acid sequence of flavonoid glycosyltransferases from higher plants. Accession numbers are given in Fig. 6

Organism	*M. domestica*	*V. vinifera*	*P. hybrida*	*V. mungo*	*Z. mays*	*H. vulgare*
M. domestica	100.0					
V. vinifera	52.6	100.0				
P. hybrida	48.2	52.6	100.0			
V. mungo	44.1	47.7	48.9	100.0		
Z. mays	37.5	40.3	37.4	39.1	100.0	
H. vulgare	35.9	40.3	35.6	37.8	73.6	100.0

4.3. Isolation of pDFR and pUFGluT fragments from 'Jonathan'

Total RNA for RT–PCR was isolated from 'Jonathan' fruit skin according to the CTAB method. First-strand cDNA was synthesized from total RNA of 'Jonathan' skin using a first-strand cDNA synthesis kit (Amersham Pharmacia). *pDFR* was amplified by using the primers 5'-gagtccgaatccgtttgtgtca-3' and 5'-atgtttgtgggggctgtcgatg-3', and *pUFGluT* was amplified by the primers 5'-tccctttcactagccatgcaag-3' and 5'- gtggaggatggagtttttacc -3' based on the sequences of the genes corresponding to those of 'Fuji' (AF117268 for *DFR* and AF117267 for *UFGluT*). PCR for isolating *pDFR* was performed on the first-strand cDNA under the following reaction condition: three cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C; then, 27 cycles of 30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C. PCR for isolating *pUFGluT* was performed under the following reaction condition: three cycles of 30 s at 94 °C, 30 s at 45 °C, and 90 s at 72 °C; then, 27 cycles of 30 s at 94 °C, 30 s at 50 °C, and 90 s at 72 °C. Amplified fragments were cloned into the pCRII vector using a TA cloning system (Invitrogen, San Diego, CA, USA) and sequenced by the fluorescence detection method using a model 377A sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

4.4. Genomic DNA extraction and Southern blot hybridization

Genomic DNA was isolated from mature leaves of a 'Jonathan' tree according to a CTAB-based method modified by Yamamoto and Mukai (unpublished). Approximately 1 g of apple leaves was ground to a fine powder in liquid nitrogen and mixed with 10 ml of an isolation buffer (10% polyethylene glycol 6,000, 0.35 M sorbitol, 0.1 M Tris–HCl (pH 8.0), and 0.5% 2-mercaptoethanol). The mixture was centrifuged at $7150 \times g$ at 4 °C for 15 min, and the pellet was suspended with 5 ml of a lysis buffer (0.35 M sorbitol, 0.1 M Tris–HCl (pH 8.0), and 0.5% 2-mercaptoethanol). After the addition of 0.5 ml of 10% lauroylsarcosine, the mixture was kept at room temperature for 10 min, mixed with an equal volume of 2× CTAB (2% CTAB, 0.1 M Tris–HCl (pH 9.5), 20 mM ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid (EDTA), 1.4 M NaCl, and 1% 2-mercaptoethanol), and incubated at 65 °C for 20 min. The mixture was then extracted with an equal volume of chloroform/isoamylalcohol (24:1, v/v), and nucleic acids in the aqueous phase were precipitated with an equal volume of isopropanol and pelleted by centrifugation at $7150 \times g$ at 4 °C for 10 min. Collected nucleic acids were dissolved in 0.75 ml of a TE solution (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA) containing 2 μg ml^{-1} RNase A and kept at 4 °C overnight. DNA was extracted once with an equal volume of phenol and once with an equal volume of chloroform/isoamylalcohol (24:1, v/v), and precipitated with an equal volume of isopropanol. After being collected by centrifugation at $7150 \times g$ at 4 °C for 10 min, DNA was washed once with ethanol/water (7:3, v/v), dried, and dissolved in approximately 0.2 ml of the TE solution. The quantity of the extracted genomic DNA was determined by electrophoresis on a 0.7% agarose gel. After being digested with *Bam*HI, *Dra*I, *Eco*RI, *Hin*dIII, *Kpn*I, or *Xba*I, the genomic DNA (10 μg) was electrophoresed on 0.8% agarose gels and blotted on nylon membranes (Hybond-N, Amersham Pharmacia). Plasmid cDNA fragments of *MdCHS*, *MdF3H*, *pDFR*, *MdANS*, and *pUFGluT* from 'Jonathan' were used to generate the probes using a DIG DNA labeling kit (Roche Diagnostics, Mannheim, Germany). Prehybridization (2 h) and hybridization (overnight) were done in a hybridization buffer containing 5× SSC, 1% blocking reagent (Roche Diagnostics), 0.1% lauroylsarcosine, and 0.02% SDS at 68 °C. After hybridization, the membranes were washed twice at 68 °C for 15 min with 2× SSC and 0.1% SDS and then twice at 68 °C for 15 min with 0.1× SSC and 0.1% SDS. The detection was performed according to the protocol recommended by the supplier using a DIG-CSPD system (Roche Diagnostics), and the membranes were exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

4.5. Total RNA extraction and northern blot hybridization

Total RNA of 'Orin,' 'Fuji,' and 'Jonathan' was isolated from the fruit skin. Approximately 200 μg of total RNA were extracted from 10 g for each sample. Total RNA (10 μg) was electrophoresed on 1.1% agarose gels containing 5.0% (v/v) formaldehyde and then blotted on nylon membrane (Hybond-N). The loading and transferring equivalent of RNA was confirmed by staining the gels with ethidium bromide and the nylon membrane with methylene blue. Hybridization was done in a hybridization buffer containing 50% formamide, 5× SSC, 2% blocking, 0.1% lauroylsarcosine, and 0.02% SDS at 68 °C using the same

probes as in Southern blot analysis. Washing and detection were performed by the same procedure as used in Southern blot hybridization.

4.6. Measurement of total anthocyanin concentration

Three to six peel disks were randomly chosen for each sample. Apple peel disks were placed in 25 volumes of hydrochloric acid/methanol (1:99, v/v) at 4 °C at least for 6 h. Absorbance of each extract was measured at 530 nm with a spectrophotometer (UV-1600, Shimadzu, Kyoto, Japan).

4.7. HPLC analysis

HPLC was carried out with a Gulliver system (JASCO Corporation, Tokyo, Japan) according to a method by Tada et al. [22]. Anthocyanins of apple skin were extracted in 30 volumes of hydrochloric acid/methanol (0.1:99.9, v/v) and analyzed on a reversed-phase column (4.6 mm × 250 mm, Mightsyl RP-18 GP Aqua, Kanto Chemicals, Tokyo, Japan). The solvent system used was a linear gradient of 25% to 35% solvent B (phosphoric acid/acetic acid/acetonitrile/water, 1.5:20:25:53.5, v/v/v/v) in solvent A (phosphoric acid/water, 1.5:98.5, v/v) over a period of 30 min. Samples were eluted at 40 °C and a flow rate of 1 ml min^{-1}. The HPLC elutes were monitored by absorbance at 530 nm, and the peaks were identified by comparing their retention time with authentic standards.

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