Suppression subtractive hybridization identifies genes induced in response to UV-B irradiation in apple skin: isolation of a putative UDP-glucose 4-epimerase

Yusuke Ban¹, Chikako Honda², Hideo Bessho³, Xiao-Ming Pang²* and Takaya Moriguchi¹,2,†

¹ Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki, 305-8572 Japan
² National Institute of Fruit Tree Science, Tsukuba, Ibaraki, 305-8605 Japan
³ National Institute of Fruit Tree Science, Morioka, Iwate, 020-0123 Japan

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Abstract

Suppression subtractive hybridization (SSH) successfully identified 11 cDNAs in apple skin with highly induced expression as a result of ultraviolet (UV)-B irradiation. Apart from three putative flavonoid biosynthetic genes, chalcone synthase (CHS; A5C), flavanone-3-hydroxylase (F3H; B5F), and flavonol synthase (FLS; D1F), five clones (A1H, A10E, B11G, D5F, and D11H) were induced by low temperature (17 °C) as well, which is also known to induce anthocyanin accumulation in apple skin. Moreover, four clones (A1H, A10E, B11G, and D11H), showing higher expression levels in the skin, accumulated higher anthocyanin concentrations than their counterparts. Of the four clones, only A10E, a putative UDP-glucose 4-epimerase (UGE), was deemed to play an important role in anthocyanin accumulation in apple skin based on the facts that: (i) its transcription level was higher in the deep red cultivar, ‘Jonathan’, than in the pale red cultivar, ‘Tsugaru’; and (ii) it could reversibly catalyse UDP-glucose to UDP-galactose, and the latter molecule is a major sugar donor for cyanidin-glycoside in apple. Therefore, the full-length cDNA of A10E was isolated by rapid amplification of cDNA ends (RACE) and designated as *MdUGE1*. Further analysis demonstrated that UGE enzymatic activity was positively correlated with anthocyanin accumulation in apple skin. Thus, *MdUGE1* isolated by SSH could play an important role in anthocyanin biosynthesis in apple skin in concert with other flavonoid biosynthetic genes.

Key words: Anthocyanin, apple (*Malus* × *domestica*) skin, gene expression, suppression subtractive hybridization, UDP-glucose 4-epimerase (UGE), UV-B.

Introduction

Ultraviolet (UV)-B (280–320 nm) can cause damage to biomolecules, such as DNA, RNA, proteins, and lipids (Jansen et al., 1998; Gerhardt et al., 1999; Frohnmeyer and Staiger, 2003). In DNA, cyclobutane–pyrimidine dimers and pyrimidine–pyrimidine dimers are formed during irradiation of plants with UV-B (Britt, 1999). UV-B irradiation also evokes photomorphogenic responses, such as hypocotyl growth inhibition and induction of UV-B-protecting pigmentation, which are considered to be different from DNA damage signalling pathways (Kucera et al., 2003). It has been reported that *Arabidopsis thaliana* displays several phenotypic responses after treatment with UV-B, including inhibition of hypocotyl growth, cotyledon expansion, phototropic curvature, biosynthesis of anthocyanins and flavonols, and stomatal opening (Kim et al., 1998; Boccalandro et al., 2001; Eisinger et al., 2003; Suesslin and Frohnmeyer, 2003; Shinkle et al., 2004). Among these, anthocyanin accumulation is an important plant response for protection from UV-B irradiation.

Anthocyanins are plant secondary products best known as the characteristic red, blue, and purple pigments of plant tissues (Winkel-Shirley, 2001). These pigments increase in response to UV-B irradiation and absorb UV-B (Winkel-Shirley, 2002). Indeed, the mutation of *A. thaliana chalcone synthase* (CHS) and *chalcone isomerase* (CHI), which
encode flavonoid biosynthetic enzymes, resulted in UV-hypersensitive phenotypes (Li et al., 1993), providing direct evidence for the physiological role of anthocyanins in the protection against UV-B damage and subsequent cell death by DNA dimerization and breakage.

Apart from its biological importance noted above, anthocyanin accumulation is an important determinant in consumer preference and the marketability of fruits, including apple. There is ample evidence to suggest that both the activities of anthocyanin biosynthetic enzymes and the expression of their genes are highly induced by UV-B irradiation. Dong et al. (1995) have shown that the enzymatic activities of phenylalanine ammonia lyase and CHI increased 10–20-fold by UV-B irradiation. In addition, Ubi et al. (2006) demonstrated that the mRNA levels of CHS, flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3-O-glucosyltransferase (UGF) were induced by UV-B irradiation. More recently, Takos et al. (2006) reported that the light-induced MYB gene could regulate anthocyanin biosynthetic genes in apple. However, other UV-B-responsive genes involved in anthocyanin biosynthesis are still unknown in apple. Thus, the identification of genes whose expression is induced by UV-B irradiation is a useful strategy for elucidating the mechanisms underlying the physiological process leading to pigmentation.

Microarrays have been widely used for the isolation of differentially expressed genes in model plants, such as A. thaliana and maize (Brosche et al., 2002; Casati and Walbot, 2004; Ulm et al., 2004). In apple, although about 180 000 expressed sequence tag (EST) sequences have been deposited in public databases (Newcomb et al., 2006; Park et al., 2006), no commercial microarray for apple has become available so far. Therefore, differential screening and suppression subtractive hybridization (SSH) are alternative strategies that have been widely used to identify differentially expressed genes (Liang and Pardee, 1992; Diatcheko et al., 1996). In this study, SSH was used to identify genes that are up-regulated by UV-B irradiation. The functions of the identified genes were annotated by a homology search. Moreover, to investigate the possible involvement of these genes in anthocyanin biosynthesis in apple skin, the expression patterns were analysed in two apple cultivars that differed in their skin colour: ‘Tsugaru’ (a pale-red cultivar) and ‘Jonathan’ (a deep-red cultivar) with or without UV-B irradiation combined with temperature treatments (17 °C or 27 °C). Among the isolated genes, A10E, a putative UDP-glucose 4-epimerase (UGE), was further characterized as a candidate gene that contributed to red coloration in apple. Finally, the usefulness of SSH to identify genes induced by UV-B and possible physiological roles of UGE are discussed.

Materials and methods

Plant materials

Apple (Malus domestica) fruits of ‘Tsugaru’ (a pale-red cultivar), ‘Jonathan’ (a deep-red cultivar), and ‘Orin’ (a non-red cultivar) were obtained from the orchard of the National Institute of Fruit Tree Science at Morioka, Japan in 2004, 2005, and 2006. In this area, the commercial harvest times of ‘Tsugaru’, ‘Jonathan’, and ‘Orin’ are in mid-September, mid-October, and late October, respectively. ‘Tsugaru’, ‘Jonathan’, and ‘Orin’ fruits were harvested about 1 week later than the commercial harvest times, which correspond to about 120 d after full bloom (DAFB), 150 DAFB, and 165 DAFB, respectively. ‘Tsugaru’ fruits were harvested at 93 DAFB and 122 DAFB in 2004, and at 16, 37, 60, 79, 102, and 116 DAFB in 2005; ‘Jonathan’ fruits were harvested at 106 and 154 DAFB in 2004; and ‘Orin’ fruits were harvested at 166 DAFB in 2006. 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 RNA isolation and protein extraction.

For UV-B and temperature treatment, ‘Tsugaru’ fruits were bagged on the tree about 1 month before commercial harvest (122 DAFB in 2004 and 116 DAFB in 2005). All bagged fruits were harvested at the commercial harvest time for both years and kept in the incubator at 17 °C for 3 h in darkness according to Ubi et al. (2006). After 3 h of pre-conditioning, the entire skin, including 1 mm of the cortical tissue, from seven fruits (in 2004) and 10 fruits (in 2005) was immediately collected after removing the bags, and these were regarded as control samples (untreated).

For UV-B irradiation, 21 fruits (in 2004) and 63 fruits (in 2005) were exposed to UV-B irradiation (emission peak at 320 nm) after removing the bags. Eighteen fruits (in 2005) were kept at 27 °C for 48 h, and the remaining 45 fruits (in 2005) were kept at 17 °C for 12 (nine fruits), 48 (27 fruits), and 94 h (nine fruits). In 2004, all 21 fruits were kept at 17 °C for 1, 6, and 24 h (seven fruits for each treatment).

For UV-B null treatment (without UV-B), 21 fruits (in 2004) and 63 fruits (in 2005) after removing the bags were kept under a UV-B filter (Mitsubishi Chemical MKV Company, Tokyo, Japan), which can block UV-B irradiation. In 2005, 18 fruits were kept at 27 °C for 48 h, and the remaining 45 fruits were kept at 17 °C for 12 (nine fruits), 48 (27 fruits), and 94 h (nine fruits). In 2004, all 21 fruits were kept at 17 °C for 1, 6, and 24 h (seven fruits for each treatment).

In all treatments, after the desired exposure time, skin samples including 1 mm of the cortical tissue were collected from the upper half of each fruit sample. Collected skin samples were stored as noted above for RNA isolation, protein extraction, and anthocyanin measurement.

Suppression subtractive hybridization (SSH)

Total RNA from all samples was isolated using a modified hot borate method (Wan and Wilkins, 1994). Poly(A)⁺ RNA was purified using Oligotex®-dT30 <Super> (TaKaRa, Shiga, Japan). SSH was carried out between UV-B-treated skin (‘tester’) and UV-B-untreated skin (‘driver’) using the PCR-Select Subtractive Hybridization kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions except for the modification of the first- and second-round PCR conditions. To prevent the amplification of undesired sequences, PCR cycles were optimized (first PCR, 25 cycles; second PCR, eight cycles) according to von Stein et al. (1997).

The cDNA library obtained from SSH was subjected to evaluation of its subtraction efficiency. PCR analysis of the subtraction efficiency basically followed the manufacturer’s instructions. The apple ubiquitin (Ub) gene was employed as a
Identification of UV-B-responsive genes in apple skin

**Northern blot analysis**

Total RNA (5 μg) was electrophoresed on a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham Biosciences) by capillary transfer. Insert DNAs of the identified cDNA clones were labelled with digoxigenin-dUTP (Roche Diagnostics) using primers 1 and 2R (Clontech) and in PCRs. Membranes were hybridized and washed as described above.

**Measurement of total anthocyanin concentration**

Total anthocyanin was extracted according to the method of Dong et al. (1995). A 1 g aliquot of peel discs randomly chosen from each sample was placed in 5 ml of hydrochloric acid/methanol (1:99, v/v) at 4 °C for at least 6 h. Absorbance of each extract (100 μl) was measured at 530, 620, and 650 nm with a spectrophotometer (UV-1600, Shimadzu, Kyoto, Japan). The concentration of anthocyanin was calculated according to the methods of Siegelman and Hendriks (1958).

**Isolation of full-length A10E cDNA**

A10E isolated by SSH contained a 3′-untranslated region (UTR); therefore, 5′-rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNA. A 5 μg aliquot of total RNA from 'Tsugaru' apple skin was used to prepare RACE-Ready cDNAs with a SMART™ RACE cDNA Amplification Kit (Clontech) following the user manual. The 5′-RACE was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) with a 5′ gene-specific primer that was designed based on the sequence of A10E. The obtained cDNA ends were ligated into pCR 2.1 (Invitrogen) and sequenced as described above. The resultant sequences were aligned with A10E to obtain full-length cDNA. The full-length gene was designated as MdUGE1 and was deposited in the DDBJ database under the accession number AB272752.

A similarity search of the MdUGE1 sequence was carried out using the BLASTN program against apple EST collections (270 490 ESTs, November 30, 2006) retrieved from GenBank dbEST. In order to find the contigs formed by apple ESTs showing similarity to MdUGE1, computational analysis was performed using gap4 (Staden Package, http://staden.sourceforge.net/).

**UDP-glucose 4-epimerase assay**

Enzyme extraction from apple skin was carried out essentially as described by Dong et al. (1995). A 5 g aliquot of skins from ripened ‘Jonathan’, ‘Orin’, and ‘Tsugaru’ and from UV-B-treated and -untreated ‘Tsugaru’ was ground using a mortar and pestle in liquid nitrogen until a fine powder was obtained. Polyclar AT was then added at a rate of 40% of the gram fresh weight and mixed. A 15 ml aliquot of 50 mM TRIS-HCl (pH 8.5) containing 20 mM 2-mercaptoethanol and 1 mM phenylmethylsulphonyl fluoride (PMSF) was added to the samples, and the mixture was gently stirred until completely thawed. After the homogenate was centrifuged at 12 000 g for 20 min at 4 °C, the supernatant was filtered with Miracloth (Calbiochem, San Diego, CA, USA). To remove polysaccharides, ammonium sulphate was added to the filtered solution to give 30% saturation, and the mixture was centrifuged at 20 000 g for 10 min at 4 °C. Protein was precipitated from the supernatant by adding ammonium sulphate to 75% final saturation and pelleted by centrifuging at 20 000 g for 10 min at 4 °C. The protein pellet was resuspended in 250 μl of 10 mM TRIS-HCl (pH 8.5) containing 20 mM 2-mercaptoethanol and 1 mM PMSF. Before the enzyme assay, the extracts were de-salinated by using Microcon YM-3 (Millipore, Bedford, MA, USA).

Measurement of UGE activity was carried out according to the methods described by Dörmann and Benning (1998). A 5 μl aliquot of protein solution was incubated with 5 μl of 22 μM UDP-[U-14C]glucose (11.1 GBq mmol⁻¹, American Radiolabeled Chemical, Inc., St Louis, MO, USA) for 10 min at room temperature. The reaction was stopped, and UDP-glucose, UDP-galactose, and a known amount of UDP-[U-14C]glucose was hydrolysed with

**DNA sequencing and sequence data analysis**

Sequencing was performed by using the BigDye® Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems) and analysed with the Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems). All the inserted sequences were checked for homology using the BLASTX program against the GenBank/EMBL/DDBJ non-redundant sequence database. The sequence (B11G) sharing no homology hit using the BLASTX program was further searched against the data set for plant (all Chlorophyta and Streptophyta) EST sequences of the GeneBank dbEST.

**Screening using reverse northern**

A total of 384 white clones were randomly picked up and cultured for differential screening. Inserts of 384 cDNA clones were amplified by PCR (30 cycles of 95 °C for 10 s and 68 °C for 3 min) using primers 1 and 2R (Clontech). To prepare membranes for reverse northern, the PCR products were denatured with 0.6 M NaOH and spotted onto a 10×12 cm nylon filter, Hybond N⁺ (Amersham Biosciences, Piscataway, NJ, USA). The spotted DNAs were fixed on a nylon filter by UV cross-linking. The cDNA probes from UV-B-treated and -untreated skins were synthesized using a Super Script First-strand Synthesis System for RT-PCR (Invitrogen) in the presence of digoxigenin-dUTP (Roche Diagnostics, Mannheim, Germany). A 7.5 μl aliquot of total RNA from each skin was mixed with 1 μl of an oligo(dT) primer and 2 μl of DIO DNA Labeling Mix (Roche Diagnostics). The mixture (10 μl) was incubated at 65 °C for 5 min and chilled on ice for 1 min. Then, 2 μl of a 10× RT buffer, 4 μl of 25 mM MgCl₂, 2 μl of 0.1 M dithiothreitol (DTT), and 1 μl of RNase OUT™ were added and incubated at 42 °C for 2 min. Subsequently, 1 μl of Superscript II was added to the reaction mixture and incubated at 42 °C for 50 min and 72 °C for 15 min. A 1 μl aliquot of RNase H was added and incubated at 37 °C for 20 min. Pre-hybridization (1 h) and hybridization (overnight) were carried out in a high SDS hybridization buffer containing 50% formamide, 5× SSC, 2% blocking solution (Roche Diagnostics), 0.1% lauroylsarcosine, and 7% SDS at 42 °C. After hybridization, the membranes were washed with 2× SSC (75 mM NaCl and 7.5 mM tri-sodium citrate, pH 7.0), 0.1% SDS at 68 °C for 15 min, followed by washing twice with 0.1× SSC, 0.1% SDS at 68 °C for 15 min. The detection was performed according to the manufacturer’s instructions using a DIG-CSPD system (Roche Diagnostics), and the membranes were exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

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1 μl of 0.5 M HCl at 95 °C for 5 min. Then, 1 μl of 0.5 M NaOH and 0.5 μl of carrier monosaccharides (0.5 mg ml⁻¹ glucose and 0.5 mg ml⁻¹ galactose) were added, and the samples were spotted onto silica TLC plates (Silica K60 F260, Merck, Darmstadt, Germany). The plates were developed with 90% (v/v) acetone for 3 h, with filter paper attached to the top of the plates for better separation, and exposed to an imaging plate (BAS-IP MS 2025, Fuji Film) for 24 h. Radioactive images were obtained with a scanner (BAS 1800 II, Fuji Film), and the spots containing galactose were quantified using an Image Gauge (Fuji Film). Protein was measured (BAS 1800 II, Fuji Film), and the spots containing galactose were separated, and exposed to an imaging plate (BAS-IP MS 2025, Fuji Film) for 24 h. Radioactive images were obtained with a scanner (BAS 1800 II, Fuji Film), and the spots containing galactose were quantified using an Image Gauge (Fuji Film). 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-galactose formed per min per mg protein) from the three independent samples.

**Results**

**Anthocyanin accumulation in apple skin with UV-B irradiation**

To determine the samples (apple skin) for the construction of the subtracted cDNA library, the anthocyanin concentrations of the skin samples were measured (Fig. 1). Under 1, 6, 12, and 24 h UV-B irradiation, there were no significant differences in the anthocyanin concentrations between UV-B-treated and UV-B-untreated skins, but the anthocyanin concentration was significantly induced by 48 h and 94 h UV-B irradiation. We assumed that known UV-B-responsive genes such as **CHS**, **F3H**, and **UFGT** could be selected predominantly by SSH in the sample from 48 h UV-B irradiation, possibly due to the large differences in anthocyanin concentrations between the UV-B-treated and -untreated skins. Therefore, in this study, skins from 48 h UV-B irradiation were used for SSH to increase the opportunity for identifying genes other than the flavonoid biosynthetic genes.

**Identification of differentially expressed genes**

Using SSH, a subtracted cDNA library was constructed, and its subtraction efficiency was evaluated (Fig. 2). For the unsubtracted cDNA, the apple Ub gene was amplified after 18 cycles; however, for the subtracted cDNA, the Ub gene product was only detected after 38 cycles. The reduction of apple Ub gene abundance in the subtracted cDNA ensures that SSH was successfully achieved. Therefore, this result for Ub indicated that the quality of the subtracted cDNA could be sufficient to identify the genes induced by UV-B.

In order to select the UV-B-induced genes, reverse northern analysis was applied to screen the subtracted cDNA library using the cDNAs from UV-B-treated or -untreated skin as probes. In total, 384 cDNA clones were used for reverse northern analysis. Screening was performed in two replicates for each probe. As a result, 30 candidate cDNA clones that were expressed at higher levels in UV-B-treated skin than in the UV-B-untreated counterpart were obtained. Sequence analysis showed that the 30 selected cDNA clones corresponded to 17 independent genes. Further confirmation using northern blot analysis demonstrated that six clones among 30 were not induced by UV-B irradiation. The remaining 24 clones were successfully confirmed to be induced by UV-B irradiation at 17 °C, as shown in Fig. 3. Among these clones, 11 clones corresponded to the same cDNA A5C, and four others corresponded to another cDNA D1F. As a result, 11 representative clones that showed high hybridization signals in the UV-B-treated skin were finally selected (Table 1).

**Expression of the selected clones in apple skins with UV-B and temperature treatment**

The expression of the selected UV-B-responsive genes (Table 1) was analysed in the skin with or without UV-B irradiation combined with temperature treatment (Fig. 3). The expression levels of all genes were quite low in the untreated samples. The expression of all genes was preferably induced by UV-B irradiation regardless of the temperature conditions. The expression of three putative flavonoid biosynthetic genes, namely A5C (**CHS**), B5F (**F3H**), and D1F (**flavonol synthase, FLS**), was suppressed by high temperature treatment in the skins with and without UV-B irradiation. A1H, A10E, B11G, D5F, and D11H showed the same expression patterns as these flavonoid biosynthetic genes. On the other hand, the expression of B7H, D1C, and D10H was not suppressed by high temperature; on the contrary, it was induced.
Effect of UV-B and temperature treatments for 48 h on apple fruit anthocyanin accumulation

The anthocyanin concentrations in the apple skins used for northern blot analysis (Fig. 3) are shown in Fig. 4. Untreated apple skin showed a low anthocyanin concentration. Anthocyanin accumulation was enhanced by the UV-B and/or temperature treatments. UV-B, in particular, significantly stimulated anthocyanin synthesis regardless of the temperature conditions, but the synergetic effects of UV-B and low temperature (17 °C) were greatest. However, there were no significant differences in anthocyanin accumulation without UV-B irradiation after treatment at 17 °C or 27 °C.

Expression of the selected clones during apple fruit ripening

Since five clones, A1H, A10E, B11G, D5F, and D11H, showed the same expression patterns as those of flavonoid biosynthetic genes, the expression of these clones during apple fruit ripening was examined. The expression patterns of these clones are shown in Fig. 3. The expression levels were determined using semi-quantitative RT-PCR. The PCR products were visualized by gel electrophoresis and quantified using a scanning densitometer. The expression levels of these clones were highest in the control samples, except for clone D11H, which showed a lower expression level than the other clones.

Table 1. Identified apple cDNAs induced by UV-B irradiation

<table>
<thead>
<tr>
<th>Reference clone (bp) cDNA size</th>
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<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1H 506</td>
<td>Putative α7 proteasome subunit (Nicotiana tabacum; Q93X34)</td>
<td>c^{-54}</td>
<td>AB273181</td>
</tr>
<tr>
<td>A5C 628</td>
<td>Chalcone synthase (Malus domestica; Q4TZJ6)</td>
<td>c^{-114}</td>
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<tr>
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<td>B11G 141</td>
<td>Mdas9006L19.g1 (similar to early light-induced protein) (Malus domestica; DR996688)</td>
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<td>Non-specific lipid transfer protein (Fragaria ananassa; Q4PLT5)</td>
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<td>AB273187</td>
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<tr>
<td>D1F 353</td>
<td>Flavonol synthase (Malus domestica; Q52T39)</td>
<td>2e^{-56}</td>
<td>AB273188</td>
</tr>
<tr>
<td>D5F 790</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (Capsicum annuum; Q8VWP2)</td>
<td>c^{-127}</td>
<td>AB273189</td>
</tr>
<tr>
<td>D10H 488</td>
<td>Ubiquitinating enzyme (Arabidopsis thaliana; Q4TZ05)</td>
<td>8e^{-68}</td>
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<td>D11H 397</td>
<td>Putative glucosyltransferase (Oryza sativa; Q9AUV3)</td>
<td>1e^{-47}</td>
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Fig. 2. Confirmation of SSH efficiency using the apple ubiquitin gene (Ub). PCR was performed on the subtracted (lanes 1–5) and unsubtracted (lanes 6–10) cDNA with the Ub primers. The numbers of PCR cycles are indicated above the panel. Lane M indicates the 100 bp ladder marker.

Fig. 3. Expression levels of the selected clones in ‘Tsugaru’ apple skins with UV-B and temperature treatments. The treatment conditions are indicated above the panel: with UV-B (+) and without UV-B (–); 17 °C or 27 °C. The untreated control is indicated as U. In the lower right panel, the total RNA on the gel is stained with ethidium bromide to confirm equivalent loading. The putatively identified gene names are shown in parentheses. PA7, α7 proteasome subunit; CHS, chalcone synthase; UGE, UDP-glucose 4-epimerase; F3H, flavanone-3-hydroxylase; Pftf, FtsH-like protein Pftf precursor; ELIP, early light-induced protein; LTP, non-specific lipid transfer protein; FLS, flavonol synthase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; E2, ubiquitinating enzyme; GT, glucosyltransferase.

Identification of UV-B-responsive genes in apple skin

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<tr>
<td>D1F 353</td>
<td>Flavonol synthase (Malus domestica; Q52T39)</td>
<td>2e^{-56}</td>
<td>AB273188</td>
</tr>
<tr>
<td>D5F 790</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase (Capsicum annuum; Q8VWP2)</td>
<td>c^{-127}</td>
<td>AB273189</td>
</tr>
<tr>
<td>D10H 488</td>
<td>Ubiquitinating enzyme (Arabidopsis thaliana; Q4TZ05)</td>
<td>8e^{-68}</td>
<td>AB273190</td>
</tr>
<tr>
<td>D11H 397</td>
<td>Putative glucosyltransferase (Oryza sativa; Q9AUV3)</td>
<td>1e^{-47}</td>
<td>AB273191</td>
</tr>
</tbody>
</table>
apple fruit ripening was analysed to obtain insights into their possible involvement in the apple red pigmentation. For this purpose, two apple cultivars that differed in their skin colour were used: ‘Tsugaru’ (a pale-red cultivar) and ‘Jonathan’ (a deep-red cultivar) (Fig. 5). The anthocyanin concentrations of these two cultivars during the ripening process had already been measured by Honda et al. (2002) and Ubi et al. (2006). According to their reports, there was no detectable accumulation of anthocyanin in ‘Tsugaru’ and ‘Jonathan’ at 93 DAFB and 106 DAFB, respectively, but anthocyanin accumulations were noticeably induced in ‘Tsugaru’ and ‘Jonathan’ at 122 DAFB and 154 DAFB, respectively. In addition, the final anthocyanin concentration of ‘Jonathan’ was much higher than that of ‘Tsugaru’. In this study, the expression levels of A1H, A10E, B11G, and D11H increased in both cultivars with fruit ripening. The expression level of D5F increased with fruit ripening in ‘Jonathan’ but not in ‘Tsugaru’, i.e., no consistent expression patterns were observed, as in the four other clones. Of the four clones, only A10E showed a higher expression level in ‘Jonathan’ than in ‘Tsugaru’, suggesting that the expression pattern of A10E was positively correlated with the anthocyanin concentration.

Molecular cloning, expression analysis, and enzymatic activity of clone A10E

Since the expression pattern of A10E was in accordance with the anthocyanin accumulation in apple skin, an attempt was made to obtain a full-length cDNA using the RACE method. The obtained A10E sequence contained a 3’-UTR; therefore, only 5’-RACE PCR was carried out. The resultant sequence of a putative UGE (MdUGE1) contained an open reading frame encoding 350 amino acid residues with the first in-frame ATG at nucleotide position 90 and a stop codon at position 1142 (data not shown). The alignment of the deduced amino acid sequence with UGE proteins from other species showed that MdUGE1 possessed an NAD+-binding domain as well as catalytic amino acid residues serine, tyrosine, and lysine in the activation site (Fig. 6), suggesting that MdUGE1 possibly encodes a functional UGE enzyme.

In order to determine how many other UGE genes are expressed in apple, a similarity search of the MdUGE1 sequence against apple EST collections was carried out. The result of the search showed that the MdUGE1 sequence had matches with 15 apple ESTs with an E-value of <e^-100 and identity of <95%, suggesting identical or very similar sequences (Table 2), while no ESTs estimated as isoforms derived from different loci (E-value of >e^-100) were detected. Moreover, these ESTs grouped into a single contig. These results indicated that 15 apple ESTs are likely to be derived from MdUGE1.

The expression levels of MdUGE1 were examined using the RNA isolated from ‘Tsugaru’ flower bud, mature fruit flesh, and apple skin at various ripening stages (Fig. 7). MdUGE1 was highly expressed in fruit skin at 16, 37, 60,

![Fig. 4](image-url) Levels of anthocyanin accumulation in ‘Tsugaru’ apple skin under UV-B and temperature treatments. The treatment conditions are indicated below each column: with UV-B (+) and without UV-B (−); 17°C or 27°C. The untreated sample is indicated as U. The values are the averages of five replications. The error bars indicate the SE. Different letters in the panel represent a significant difference at P <0.01 using a Tukey test for LSD.

![Fig. 5](image-url) Expression levels of the selected clones during ripening in apple skin of ‘Tsugaru’ (a pale-red cultivar) and ‘Jonathan’ (a deep-red cultivar). The sampling date is indicated as the days after full bloom (DAFB). In the lower panel, total RNA on the gel was stained with ethidium bromide to confirm equivalent loading. The names in parentheses indicate the putatively identified gene names. PAT, 27 proteasome subunit; UGE, UDP-glucose 4-epimerase; ELIP, early light-induced protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GT, glucosyltransferase.
and 116 DAFB; however, its expression level was quite low in flower bud, mature fruit flesh, and fruit skin at 79 DAFB and 102 DAFB.

When the UGE activity was compared in skin with or without UV-B irradiation, the activity was significantly higher in the UV-B-treated skin than in the UV-B-untreated skin (Fig. 8). Furthermore, the activities of UGE in ‘Jonathan’ (a deep-red cultivar), ‘Tsugaru’ (a pale-red cultivar), and ‘Orin’ (a non-red cultivar) skins were positively correlated with the anthocyanin concentration, i.e. they were higher in ‘Jonathan’, moderate in ‘Tsugaru’, and lower in ‘Orin’ (Fig. 8).

**Discussion**

SSH was successfully applied to isolate genes induced by UV-B irradiation in apple skin. Eleven cDNAs were isolated from UV-B-irradiated apple skin and characterized on the basis of their sequence similarities to other plants (Table 1). Three of them, i.e. A5C (CHS), B5F (F3H), and D1F (FLS), were putative flavonoid biosynthetic genes. It is generally known that flavonoid biosynthetic genes are UV-B-responsive genes in various plants (Christie et al., 1994; Leyva et al., 1995; Dixon and Paiva, 1995; Shvarts et al., 1997; Sanchez-Ballesta et al., 2000; Hasegawa et al., 2001; Winkel-Shirley, 2002). In apple, the expression of both CHS and F3H was also enhanced by UV-B irradiation (Ubi et al., 2006). Therefore, successful isolation of putative CHS and F3H indicated that the
subtracted cDNA library obtained herein was worthy of further analysis. In contrast, there have been no reports regarding FLS expression during apple skin coloration, because it has been believed that FLS does not directly contribute to anthocyanin accumulation. In this study, it was shown for the first time that FLS mRNA also accumulated in response to UV-B and low temperature treatment (Fig. 3). These results indicated that UV-B and low temperature treatment might comprehensively activate the genes in the flavonoid pathway, such as FLS, more than the genes specific to anthocyanin biosynthesis in apple, as reported in other plants (Dixon and Paiva, 1995; Winkel-Shirley, 2002).

Apart from three putative flavonoid biosynthetic genes (A5C, B5F, and D1F), the expression of five other clones (A1H, A10E, B11G, D5F, and D11H) was also induced by both UV-B and low temperature (Figs 3, 4). In addition to the response to UV-B and temperature treatments, the expression levels of four genes, A1H, A10E, B11G, and D11H, were high in the red-coloured skin, as were those of flavonoid biosynthetic genes (Honda et al., 2002). However, the expression of A1H, B11G, and D11H was not completely correlated with the anthocyanin concentrations when two cultivars that differed in their skin colour traits were compared, and only A10E showed a higher expression level in the deep-red apple cultivar ('Jonathan') than in the pale-red cultivar ('Tsugaru') (Fig. 5).

Based on the results of a homology search, A1H was identified to be a putative \( \alpha \)7 proteasome subunit, a part of the 20S proteasome, which is a component of the 26S proteasome (Smalle and Vierstra, 2004). In eukaryotes, the 26S proteasome is essential to degrade ubiquitin-conjugated proteins. In addition, putative ubiquitinating enzyme (E2; D10H) expression was also induced by UV-B irradiation in this study (Fig. 3). Therefore, both A1H and D10H might play an important role in the turnover of proteins that are injured by UV-B and/or low-temperature treatment. B11G showed a high similarity to early light-inducible protein (ELIP), and it has been proposed that ELIP could act in the chloroplast either as a chlorophyll pigment carrier or as a sink for excess excitation energy (Montané and Kloppstech, 2000; Adamska et al., 2001). In apple fruits, chloroplasts were degraded as the fruits ripened, with a gradual loss of chlorophyll (Merzlyak et al., 1999), but apple ELIP was actually expressed strongly in the red-coloured skin (Fig. 3). Therefore, apple ELIP might have different functions from those previously reported (Montané and Kloppstech, 2000; Adamska et al., 2001). D11H is a homologous gene of glucosyltransferase (GT), which encodes an enzyme transferring the nucleotide sugar to small molecular weight and lipophilic acceptors. Although UFGT has been isolated from apple skin (Honda et al., 2002), the similarity between UFGT and D11H was low, indicating the involvement of D11H in a different physiological process from anthocyanin accumulation. Thus, the functions of these three clones are still ambiguous in apple, and further studies are necessary to reveal their precise functions. Nevertheless, these isolated clones will be useful tools for analysing UV-B impact in apple skin.

Generally, the expression levels of the flavonoid biosynthetic genes were high in the redder apple cultivars (Honda et al., 2002), although other factors including a transcription factor gene could also be involved in anthocyanin accumulation apart from the flavonoid biosynthetic genes. The expression of a putative UGE gene was higher in the red skin portion than in the non-red portion, indicating its important role in anthocyanin biosynthesis of apple skin in concert with other flavonoid biosynthetic genes. Therefore, the full-length cDNA of A10E (MdUGE1) was isolated by RACE PCR. The deduced amino acid sequence of MdUGE1 showed high similarities with UGEs from other species (Fig. 6). The motif for the NAD\(^+\)-binding domain, GXXGXXG, and characteristic sequences (serine, tyrosine, and lysine) that are deemed to be located in the human UGE catalytic site (Thoden et al., 2001) were also conserved in MdUGE1.

UGE is the enzyme that catalyses the reversibleimerization of UDP-galactose and UDP-glucose. Since UDP-galactose is synthesized via two pathways, namely directly from UDP-glucose by UGE or by galactokinase and galactose-1-phosphate uridylyl transferase in the presence of \( \alpha \)-galactose, UGE is one of the essential enzymes for the de novo biosynthesis of UDP-galactose. So far, it has been shown that the UGE4-deficient mutant of A. thaliana (ROOT HAIR DEFICIENT 1: RHD1) showed an approximately 20% decrease in cell wall-bound galactose.

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**Fig. 8.** UDP-glucose 4-epimerase activity in apple skins from UV-B-treated and -untreated ‘Tsgar’ and those from ripened ‘Jonathan’, ‘Orin’, and ‘Tsgar’. UV-B treatments were performed at 17 °C. The values are the averages of three replications. The error bars indicate the SE. The asterisks in the right panel show a significant difference at \( P < 0.05 \) using a Tukey test for LSD.
(Seifert et al., 2002). In potato tubers, overexpression of UGE increased the cell wall galactose content (Oomen et al., 2004). These reports suggest that UGE plays an important role in the biosynthesis of cell wall polysaccharides (e.g. xylglucan and rhamnogalacturonan). An EST search showed that MdUGE1 was mainly found in the active tissues, such as young fruitlets, young roots, and root tips (Table 2). Since cell growth occurs exponentially in root tips and during the early developmental stages of fruits (Pratt, 1988), UDP-galactose, a product of UGE, might be used for cell wall construction in these tissues. In this study, MdUGE1 was highly expressed in the young fruitlets (Fig. 7), which supported the above scenario. MdUGE1 was also highly expressed in mature fruits (Fig. 7). UDP-galactose is a sugar donor for cyanidin, and the resultant cyanidin 3-galactoside is a major anthocyanin pigment, which comprised >80% of the total cyanidin 3-glycosides in the red-coloured apple skin (Lancaster, 1992). In fact, UGE activity was higher in the apple skins with anthocyanin accumulation (Fig. 8), indicating the possible contribution of this enzyme to red coloration. Moreover, anthocyanin accumulation in apple skin is also observed at the fruitlet stage (Lancaster, 1992). Taking these results into consideration, in fruits, UDP-galactose biosynthesized by MdUGE1 may be utilized in the biosynthesis of both cell wall polysaccharides and anthocyanin at the early fruit developmental stage, and may be exclusively used for anthocyanin biosynthesis at the later stages.

Collectively, it can be concluded that: (i) SSH could efficiently isolate the genes responding to UV-B irradiation; (ii) the expression of some of the selected genes, namely A1H, A10E, B11G, D5F, and D11H, was also enhanced by low temperature; and (iii) among the selected genes, only the expression pattern of A10E was positively correlated with the anthocyanin concentration. The full-length of A10E (MdUGE1) could contribute to anthocyanin biosynthesis by supplying the UDP-galactose in apple skin.

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**References**


Montané M-H, Kloppstech K. 2000. The family of light-harvesting-related proteins (LHCs, ELIPs, HLIPs): was the harvesting of light their primary function? Gene 258, 1–8.


