Characterization of the Recombinant UDP:flavonoid 3-O-galactosyltransferase from Mangifera indica ‘Irwin’ (MiUFGalT3) involved in Skin Coloring

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Red mangos (Mangifera indica L.) accumulate appreciable amounts of cyanidin-based anthocyanins in the skin, and previous studies showed that these anthocyanins contain galactose as a sugar moiety. To date, two UDP:flavonoid 3-O-glycosyltransferase (UFGT)-like genes named MiUFGT1 and MiUFGT3 (MiUFGalT3) have been isolated from mango ‘Irwin’ peel as anthocyanin-related UFGT genes, but the function of the proteins of the genes have not yet been elucidated. In this study, we characterized recombinant MiUFGT1 and MiUFGalT3 expressed in Escherichia coli. In the presence of quercetin as an acceptor, rMiUFGT1 showed marginal glucosylation activity, while rMiUFGalT3 exhibited significant galactosylation activity 20-fold higher than its glucosylation activity. Specificity analysis using purified MiUFGalT3 found that rMiUFGalT3 almost equally accepts anthocyanidins and flavonols. The anthocyanins extracted from the ‘Irwin’ skins were cyanidin 3-O-galactoside and 7-O-methylcyanidin 3-O-galactoside by instrumental analyses, which is consistent with previous results obtained for other red mango cultivars. The results suggest that MiUFGalT3 is responsible for the red coloration of ‘Irwin’ mango fruit skins.

Key Words: anthocyanin, light-induced coloration, phylogeny, sugar moiety.

Introduction

Anthocyanins, the largest subclass of plant flavonoid, are water-soluble pigments with colors ranging from red to blue and purple. They are the main pigments in flowers and fruits of many plant species and are synthesized from phenylalanine through the flavonoid biosynthetic pathway, which is one of the most well understood pathways among plant secondary metabolisms (Grotewold, 2006; Tanaka et al., 2008). In the final step of this pathway, the anthocyanidin aglycon is glycosylated by a glycosyltransferase, increasing its stability and water solubility, and decreasing its reactivity, while changing its spectral characteristics, and allowing it to be transported into the vacuole (Fukuchi-Mizutani et al., 2003; Prior and Wu, 2006; Vogt and Jones, 2000). Thus, the expression and activity of glycosyltransferases are important factors for anthocyanin accumulation in plant organs. Glycosyltransferases associated with anthocyanin biosynthesis are referred to as UDP:flavonoid glycosyltransferases (UFGTs), and several studies on UFGTs were conducted related to the coloration of flowers or fruits in a number of plant species such as grape (Ford et al., 1998), kiwifruit (Liu et al., 2018; Montefiori et al., 2011), and petunia (Yamazaki et al., 2002). In grapes, UFGT gene expression is crucial and appears to be the key regulatory factor for anthocyanin accumulation in the berry skin of red cultivars (Boss et al., 1996a, b; Kobayashi et al., 2001, 2002). Similarly, correlated expression of UFGT genes with anthocyanin accumulation was reported in several plant species such as kiwifruit (Liu et al., 2018; Montefiori et al., 2011), petunia (Yamazaki et al., 2002).
The color of a mature mango (Mangifera indica L.) fruit varies depending on the cultivar, ranging from green to yellow, orange, or red. As in other crops, the red coloration of mango fruit skin is due to anthocyanin accumulation. Recently, several molecular studies of anthocyanin biosynthesis in mangoes have been published (Bajpai et al., 2018; Hoang et al., 2015; Kanzaki et al., 2019; Karanjalker et al., 2018a, b). In our previous study, we isolated two UFGT genes, MiUFGT1 and MiUFGT3, from mango fruit skin and showed that the expression of both genes was upregulated by light exposure, which induced anthocyanin accumulation in the fruit skin (Kanzaki et al., 2019). This suggests that these two genes play important regulatory roles in anthocyanin accumulation in mangoes. The amino acid sequences of these genes exhibited high similarity to other plant UFGTs and contained the conserved plant secondary product glycosyltransferase (PSPG) box characteristic of the plant glycosyltransferase family. Although the presence of a glutamine and a histidine residue at the C-terminus end of the PSPG box in MiUFGT1 and MiUFGT3, respectively, implied that MiUFGT1 and MiUFGT3 use glucose and galactose as sugar donors, respectively, the actual function and roles in the biosynthetic pathway was not investigated. As the major anthocyanins in mango skin are 7-O-methylcyanidin 3-O-β-D-galactopyranoside and cyanidin 3-O-galactoside in some cultivars (Berardiní et al., 2005a, b; Lopez-Cobo et al., 2017), the expression and activity of UDP-galactose: flavonoid 3-O-galactosyltransferases (UGalTs) are considered essential factors for anthocyanin accumulation in mango fruit.

In this study, we investigated the functional and biochemical characterization of the recombinant protein of the two mango UFGTs, MiUFGT1 and MiUFGT3 to elucidate the role of these enzymes in anthocyanin biosynthesis in mango fruit skin. The rMiUFGT1 protein showed marginal glucosylation and no galactosylation activity, while rMiUFGT3 showed significant galactosylation activity. Thus, MiUFGT3 is a UGalT (MiUFGalT3) and may play a crucial role in anthocyanin accumulation in red mango fruit.

**Materials and Methods**

**Expression of MiUFGT1 and MiUFGalT3 in Escherichia coli**

The coding sequences of MiUFGT1 (Accession No.; LC474860) and MiUFGalT3 (Accession No.; LC474861) were separately PCR amplified, and subcloned into a pColdI expression vector (Takara Bio Inc., Shiga, Japan) fused to a glutathione S-transferase (GST) tag using an In-Fusion HD Cloning Kit (Takara Bio) to generate His- and GST-tagged fusion proteins. The primer pairs of 5'-TTCCAGGGCCCGAGATGCTG AAGCAAAAAGAAACCC-3' and 5'-ACCGAGCTCCTCA TATGCTAGGACGAGATAATCTGAACAAG-3' and of 5'-TTCCAGGGCCCGGGGAGTGCTCTAAACACT GCCAC-3' and 5'-ACCGAGCTCCTATGCTCAATCA ACCCACCCTGGCCTTG-3' were used to amplify the respective genes. The GST tag sequence was artificially synthesized by a manufacturer (Fasmac Co., Ltd., Atsugi, Japan), and amplified by PCR for application in the In-Fusion reaction. After sequence confirmation, the resulting plasmids pColdI-MiUFGT1 or MiUFGalT3 were used to transform *E. coli* Chaperone Competent Cells pGro7/BL21 cells (TaKaRa Bio). The transformants were grown in 40 mL of LB medium containing 50 mg·L⁻¹ ampicillin and 20 mg·L⁻¹ chloramphenicol for 4–5 h with shaking at 37°C at 200 rpm. When the OD600 value reached 0.4–0.6, the culture was exposed to 15°C for 30 min, and then isopropyl β-D-thiogalactopyranoside (IPTG) and L-arabinose were added to the culture for protein induction at final concentrations of 0.1 mM and 0.5 mg·mL⁻¹, respectively. The culture was further incubated at 15°C for 24 h with shaking at 200 rpm.

**Western blot analysis**

Expression of the recombinant MiUFGT1 and MiUFGalT3 in *E. coli* was examined by Western blot analysis. The cell-free extracts (20 μg) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and the separated proteins transferred to a PVDF membrane (Immobilon-P; MilliporeSigma, MA, USA). The image was processed with ImageJ (National Institutes of Health).

**Purification of the recombinant MiUFGalT3**

The *E. coli* cells expressing the recombinant MiUFGalT3 were harvested by centrifugation, resuspended in 4 mL lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X100, and 1 mM DTT, and disrupted by sonication on ice. Cell debris was removed by centrifugation, and the resulting soluble fraction was applied onto a column containing His60 Ni Superflow Resin (Clontech Laboratories, Inc., CA, USA). After eluting the bound protein according to the manufacturer’s instructions, the enzyme was further
purified by Mono Q 5/50 GL column chromatography (GE Healthcare Life Sciences). The elution was performed with a linear gradient of 0–1 M NaCl in 10 mM Tris-HCl buffer (pH 8.0). The purified preparation was dialyzed against HEPES-KOH buffer (pH 7.5) and stored at −30°C in 50% glycerol until use. Protein concentration was determined using a Qubit™ protein assay kit (Thermo Fisher Scientific, MA, USA). The purity of the protein was confirmed by SDS-PAGE with Coomassie Brilliant Blue R-250 staining.

**Enzyme assay**

Quercetin, kaempferol, and quercetin 3-O-glucoside (Q3Glc) were purchased from Sigma (St. Louis, MO, USA), while cyanidin 3-O-galactoside (Cy3Gal) and UDP-glucose (UDP-Glc) were from Fluka (St. Gallen, Switzerland). Petunidin was obtained from Tokiwa Phytochemical Co., Ltd. (Sakura, Japan), and peonidin and quercetin 3-O-galactoside (Q3Gal) were obtained from Extrasynthese (Lyon, France). 7-Methoxyquercetin was purchased from Cayman Chemical (Ann Arbor, MI, USA), and UDP-galactose (UDP-Gal) from Merck (Darmstadt, Germany).

A standard reaction mixture (total volume of 25 μL) contained 100 mM HEPES-KOH (pH 7.5), 75 mM KCl, 100 μM MnCl₂, 1 mM DTT, 10% glycerol, 100 μM of acceptors, 600 μM UDP-sugar donors, and 0.35 μg of purified rMiUFGalT3. The reactions were initiated by mixing the substrate solution with the enzyme solution, both of which were separately pre-incubated for 3 min at 25°C. The mixtures were incubated for short time periods (~13 s) at 25°C to minimize the degradation of anthocyanidins. The reactions were terminated by adding 50 μL of 100% MeOH containing 1% HCl (for anthocyanidins) or 100% MeOH (for flavonols). The mixtures were vortexed and centrifuged at 13,000 rpm for 5 min to precipitate the proteins.

To compare the activity between MiUFGT1 and MiUFGalT3, 100 μM quercetin was incubated with 10 mg of the E. coli cell-free extracts expressing the enzymes in the presence of either UDP-Gal or UDP-Glc. The assay was conducted for 10 min or 60 min. Identities of quercetin 3-O-glycoside were verified by co-elution with the standard samples and the absorption spectra of the peaks obtained in the HPLC analysis (described below).

The optimum pH of the enzyme activity was determined using 20 μM of cyanidin and 600 μM UDP-Gal, as the substrates. Sodium citrate (pH 4–6), MES-KOH (pH 5–7), HEPES-KOH (pH 7–8), and glycine-NaOH (pH 8.5–9.5) buffers were used at 100 mM. The effect of temperature (35–60°C) on enzymatic activity was examined using various flavonols and anthocyanidins at a concentration of 25 μM in the presence of 600 μM UDP-Gal. In this assay, the relative activities for flavonols and anthocyanidins were calculated and expressed as quercetin 3-O-galactoside (Q3Gal) and cyanidin 3-O-galactoside (Cy3Gal) equivalents, respectively.

**HPLC analysis of reaction products**

The reaction products (20 μL) were analyzed by reverse-phase HPLC using a Prominance LC20A instrument (Shimadzu Corporation, Kyoto, Japan) equipped with a C18 YMC-ODS A analytical column (4.6 × 150 mm inner diameter, particle size 5 μm; YMC Co., Ltd., Kyoto, Japan) protected by a guard column as previously reported (Ikegami et al., 2009). The elution was carried out at 37°C at a flow rate of 1 mL·min⁻¹ using a solvent system of A [2% (v/v) acetic acid] and B (100% MeOH) in which solvent B was increased from 5% to 40% in the first 5 min and then to 70% in the next 15 min. The elution was monitored by a photodiode array detector SPD-M20A (Shimadzu), and Cy3Gal was quantified at a wavelength of 525 nm. When flavonols were used as acceptors, the reaction mixtures were analyzed similarly, except that solvent B was increased from 30% to 100% in the first 13 min and the elution was monitored at 360 nm.

**Extraction of anthocyanins from ‘Irwin’ mango fruit skins and component analysis**

The mature fruit skins of ‘Irwin’ mangoes were lyophilized after being frozen in liquid nitrogen. The colored pericarp tissue (approximately 0.1 g) was then scraped off using razor blades and anthocyanins extracted with 200 μL of aqueous acetone [80% (v/v) containing 1% ascorbic acid]. After the organic phase was evaporated in vacuo, the aqueous phase was applied onto a 200 mg C18 YMC Dispo SPE cartridge (YMC). After washing the cartridge with 1.5 mL of 0.01% HCl and ethyl acetate to remove non-anthocyanin substances, anthocyanins were eluted in 0.5 mL of acidified MeOH (0.01% hydrochloric acid) and manually collected as a red fraction. The obtained fraction was then subjected to HPLC with C18 Inertsil ODS-2 analytical column (5 μm, 250 × 6.0 mm inner diameter; GL Sciences, Tokyo, Japan) for separation, and the major peak fraction collected manually. The elution condi-
tions were as described above for anthocyanin analysis except that solvent B was increased from 10% to 40% in 50 min. To the collected fraction, 1/5 volume of 11.7 M HCl was added and the mixture incubated at 90°C for 50 min to hydrolyze the sugar linkage. The acid-hydrolyzed products were then subjected to high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) to detect the liberated sugars. HPAEC-PAD was carried out using a Dionex ICS-3000 (Thermo Fisher Scientific) system equipped with a CarboPac PA1 column (2 × 250 mm). The elution was performed at 30°C under constant flow (0.25 mL·min⁻¹) of 13.8 mM NaOH and 5 mM sodium acetate for 20 min. The column was washed with 200 mM NaOH for 10 min before the next run. Known concentrations of glucose (Glc) and galactose (Gal) were used as the standards.

The hydrolyzed samples were also analyzed by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) to detect aglycon. LC-ESI-MS analysis was carried out using an LCMS-8030 system (Shimadzu) equipped with an InertSustain C18 HP column (2.1 × 100 mm; GL Sciences). The elution was performed at 30°C under a flow rate of 0.2 mL·min⁻¹ with a linear gradient of 5–60% acetonitrile in 0.1% formic acid. The spectra were obtained in the positive ion mode to scan m/z 200–500.

Results
Heterologous expression of MiUFGT1 and MiUFGalT3 in E. coli
To examine the catalytic activities of MiUFGT1 and MiUFGalT3, we expressed as His- and GST-tagged fusion proteins using the pColdI vector in E. coli cells carrying the chaperon plasmid pGro7. Western blot analysis of the cell-free extracts obtained from the recombinant cells found that both proteins are expressed as soluble forms (calculated, 79 and 80 kDa, respectively) (Fig. 1A). The expression level of rMiUFGalT3 was estimated to be 4.6 times higher than rMiUFGT1, as judged by the peak area in the HPLC chromatograms and the band intensities in the Western blot analysis.

A model structure of MiUFGT1 was constructed using the grape VvGT1 as a template (PDB ID: 2C1Z; 51% identical in amino acid sequence) in the SWISS-MODEL server (Offen et al., 2006; Waterhouse et al., 2018). When the two structures were compared, two amino acid residues involved in the recognition of UDP
in VvGT1 were replaced with other amino acids in MiUFGT1 (Supplementary Fig. 1). The two residues were identical in VvGT1 and MiUFGalT3, both of which demonstrated marked glycosyltransferase activity. Accordingly, we constructed single and double replacement mutants of MiUFGT1 (I142T and/or N278T) and expressed them in *E. coli* cells using the same method as for the wild-type protein. However, neither glucosyltransferase nor galactosyltransferase activity was increased in these mutants as compared with the wild-type enzyme-expressing cells (data not shown). Based on these findings, we decided to focus on MiUFGalT3 in the following experiments.

**Purification and characterization of rMiUFGalT3**

rMiUFGalT3 was purified to homogeneity by affinity chromatography followed by anion-exchange chromatography (Fig. 1B). When the purified enzyme was incubated with UDP-Gal and cyanidin and the resulting reaction mixture was analyzed by HPLC, a new peak corresponding to Cy3Gal appeared in the chromatogram as shown in Figure 3. The optimum pH of cyanidin galactosyltransferase activity was found to be pH 7.5 (HEPES-KOH buffer), while the optimal temperature of quercetin galactosyltransferase activity was determined to be 55°C (Supplementary Fig. 2). Note that quercetin was used for the thermostability assay due to the heat lability of cyanidin.

Acceptor specificity of rMiUFGalT3 was determined using six anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin) and four flavonols (kaempferol, 7-O-methylquercetin, myricetin, and quercetin) (Tables 1 and 2). The activities are expressed as cyanidin 3-O-galactoside and quercetin 3-O-galactoside equivalents by measuring the absorbance of 520 nm and 360 nm, respectively. Previous reports indicated that the absorption coefficients of these compounds differed by 1.1- to 1.25-fold (Jordheim et al., 2007). The specificity analysis suggested that rMiUFGalT3 is relatively tolerable against 3', 4', and 5' modification, but does not accept an anthocyanidin modified with a methoxy group at either the 3' or 5' positions (malvidin). A similar specificity pattern was also observed when flavonols were used as acceptors. The specific activity of the enzyme for cyanidin was 1.2

![Fig. 3. HPLC analysis of reaction products catalyzed by rMiUFGalT3.](image)

### Table 1. The relative activity of rMiUFGalT3 towards various anthocyanidins.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Relative activity (%)</th>
<th>Substrate</th>
<th>Structure</th>
<th>Relative activity (%)</th>
</tr>
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<td>Pelargonidin</td>
<td><img src="image" alt="Pelargonidin" /></td>
<td>54</td>
</tr>
<tr>
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<td>67</td>
<td>Delphinidin</td>
<td><img src="image" alt="Delphinidin" /></td>
<td>99</td>
</tr>
<tr>
<td>Petunidin</td>
<td><img src="image" alt="Petunidin" /></td>
<td>69</td>
<td>Malvidin</td>
<td><img src="image" alt="Malvidin" /></td>
<td>nd&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>x</sup> The acceptor was added at the final concentration of 10 μM.

<sup>y</sup> Note that activity was calculated as cyanidin-3-O-galactoside (Cy3Gal) equivalents.

<sup>x</sup> nd, not detected.
times higher than quercetin at the acceptor concentration of 25 μM. Notably, rMiUFGalT3 accepted 7-O-methylquercetin equally to quercetin, which strongly suggests the ability of this enzyme to transfer Gal to 7-O-methylcyanidin (described below).

While using quercetin as an acceptor, we found that quercetin inactivated rMiUFGalT3 through an unknown mechanism; therefore, the kinetic parameters were determined using cyanidin as an acceptor. Sigmoidal curves were obtained for both UDP-Gal and cyanidin, and the plots fitted to the Hill equation (Fig. 4). The $k_{cat}$ and $K_{0.5}$ values for UDP-Gal were calculated to be 1.8 s$^{-1}$ and 240 μM, while those for cyanidin were 1.8 s$^{-1}$ and 6.9 μM. Hill coefficients were 2 and 6, respectively.

### Table 2. The relative activity of rMiUFGalT3 towards various flavonols.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Structure</th>
<th>Relative activity (%) $^\text{y}$</th>
</tr>
</thead>
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<tr>
<td>Myricetin</td>
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<td>83</td>
</tr>
</tbody>
</table>

$^z$ The acceptor was added at the final concentration of 10 μM.

$^y$ Note that activity was calculated as quercetin-3-O-galactoside (Q3Gal) equivalents.

### Analysis of anthocyanins in 'Irwin' fruit skin

Finally, we examined the composition of anthocyanins in the ‘Irwin’ mango skins. Anthocyanins were extracted from the skins and separated from flavonols. As a result, two peaks appeared in the HPLC chromatogram (Fig. 5A). The small peak that eluted earlier likely corresponded to Cy3Gal because the retention time, as well as the absorption spectra obtained in the photodiode array, were the same as those obtained for the authentic compound (Fig. 5B). Note that Cy3Glc was eluted slightly faster than Cy3Gal (data not shown). To investigate the chemical compound corresponding to the larger peak, the latter peak fraction detected in the HPLC chromatogram was manually collected and subjected to acid-hydrolysis (Fig. 6). Sugar content analysis using HPAEC-PAD showed that the hydrolysate contained Gal and Glc at a molar ratio.
of 98.8 to 1.2. LC-ESI-MS analysis with scanning from m/z 200 to 500 found that the hydrolysate contained a compound with a molecular mass of 301.00 Da, which is consistent with that of an anthocyanidin substituted with one methoxy- and three hydroxy groups at A- and/or B-rings (calculated: 301.07 Da). Peonidin is the only known anthocyanidin species with such modifications other than 7-O-methylcyanidin 3-O-galactoside; however, the retention time of the isolated anthocyanin from the mango (10.4 min) differed from that of peonidin O-galactoside, which was synthesized by incubating peonidin with UDP-Gal in the presence of MiUFGalT3 (9.7 min) (Fig. 5C). The latter peak was therefore attributable to 7-O-methylcyanidin 3-O-galactoside, which has been previously isolated as the major anthocyanin species contained in red ‘Tommy Atkins’ mango peels (Berardini et al., 2005b). Taken together, the results suggest that the anthocyanins of ‘Irwin’ fruit skins are mainly comprised of Cy3Gal and 7-O-methylated Cy3Gal. The tolerance of rMiUFGalT3 for accepting 7-O-methyl substitution at A-ring (e.g. 7-O-methylquercetin) was noted above.

**Discussion**

The anthocyanins extracted from the red ‘Irwin’ mango skins were found to be cyanidin 3-O-galactoside and 7-O-methylcyanidin 3-O-galactoside, which agrees with previous results obtained for the ‘Tommy Atkins’ red mango (Berardini et al., 2005b) and some other red cultivars (Berardini et al., 2005a, b; Lopez-Cobo et al., 2017). This indicates that the primary sugar moiety of mango anthocyanins is mainly galactose, at least among red cultivars. Therefore, MiUFGalT3, which has galactosyltransferase activity, may be an important enzyme in red mango cultivars for anthocyanin accumulation.

Glycosyltransferases (GTs) involved in plant secondary metabolism including flavonoid biosynthesis, were assigned to family 1 (Bowles et al., 2006; Vogt and Jones, 2000). These family 1 GTs recognize UDP-sugars by a motif located close to the C-terminus, which is one of the few regions with significant sequence similarity among members. The so-called PSPG motif is comprised of 44-amino acids residues (Gachon et al., 2005). Comparison of the amino acid sequences of the region and subsequent site-directed mutagenesis studies indicated that donor specificity may in part be determined by the last amino acid residue in the PSPG motif. For example, Gln is specified for UDP-Glc while His is used for UDP-Gal (Kubo et al., 2004). However, the extent to which the residue contributes to donor specificity is controversial (Offen et al., 2006; Ono et al., 2010). The corresponding residues of MiUFGT1 and MiUFGT3 (MiUFGalT3) are Gln and His, respectively (Kanzaki et al., 2019). rMiUFGT1 expressed in E. coli as a GST fusion showed weak glucosyltransferase activity, suggesting that MiUFGT1 may have mutations at the sites associated with its activity. In this study, we examined two amino acid substitutions in MiUFGT1 (I142T and/or N278T), but the exact sites related to the activity were not elucidated. Further investigation will be needed to clarify the factors affecting MiUFGT1’s activity and exact role in mango fruit skin. On the other hand, rMiUFGalT3 showed significant galactosyltransferase activity 20 times higher than its glucosyltransferase activity. Although we have no clear explanation as to the mechanistic basis underlying the sigmoidal substrate saturation curve obtained for the enzyme, the kinetic parameters were comparable with those obtained for grape flavonoid glucosyltransferase (Kcat = 30 μM and 700 μM for quercetin and UDP-Glc, respectively; kcatal = 0.1 s−1; Offen et al., 2006).

Plants often accumulate flavonols and anthocyanidins as a form of glycoside in the flowers, seeds, and fruits. VvGT1, which is assumed to be important for grape coloration, glucosylates cyanidin with 50-fold higher efficiency than quercetin, and its galactosyltransferase activity is less than 8% of its glucosyltransferase activity (Ford et al., 1998; Offen, et al., 2006). On the other hand, VvGT5 and VvGT6, which share 91% amino acid sequence identity and are located in tandem on a chromosome different from that containing VvGT1, use UDP-glucuronic acid and UDP-Gal/Glc, as sugar donors, respectively, and use flavonols as acceptor substrates (Ono et al., 2010). In the present study, the functional and biochemical nature of the two recombinant UFGTs were elucidated, but it is possible that other plant GTs such as MiUFGT4, which was isolated in our previous study (Kanzaki et al., 2019), contribute in part to the glycosylation of the diverse phenolic compounds in the mango peel (Berardini et al., 2005a). Further investigation is required to understand the divergence and division in the roles of glycosyltransferase family mem-

![Fig. 6. HPAEC-PAD analysis of acid-hydrolysates of the peak fraction collected from HPLC eluent (RT = 10.4 min, see Fig. 5). The chromatograms between 10 min and 13 min of retention time are enlarged and shown in inset. Gal and Glc were used as standards. The molar ratio of Gal/Glc in the acid-hydrolysates was calculated to be 98.8/1.2.](image-url)
bers in mangoes. UFGTs that have a His residue at the end of PSPG box and specifically use UDP-Gal as a donor were identified from different origins including *Vigna mungo* (Ishikura and Mato, 1993), *Petunia hybrida* (Miller et al., 1999), *Aralia cordata* (Kubo et al., 2004), *Diospyros kaki* (Ikegami et al., 2009), *Daucus carota* (Xu et al., 2016), *Actinidia chieniens* (Liu et al., 2018), etc. The phylogenetic tree constructed using these plant UFGTs (Supplementary Fig. 3) revealed the occurrence of a small cluster comprised only of galactosyltransferases; however, MiUFGalT3 and VmUFGaIT were classified into different clusters together with LcUFGT and GmUFGT, respectively, which use UDP-Glc as a donor. The above mentioned VvGT5 and VvGT6 form a distant cluster with the same ancestral node as several glucosyltransferases and galactosyltransferase (VmUFGaIT). Considering UFGTs from the same plant origins are scattered in the tree and exhibit different specificities, their phylogeny may represent the divergent evolutionary processes of flavonol and anthocyanin biosynthesis in plants. The evolution of UFGTs may thus concur in each plant not only with special and temporal separation of flavonoid biosynthesis, but also with the biosynthesis of donor substrates, UDP-glycosides.

In conclusion, MiUFGalT3, which was referred to as MiUFGT3 in our previous study (Kanzaki et al., 2019), was found to have relatively high galactosyltransferase activity. Considering the expression profile reported previously (Kanzaki et al., 2019) and its functional preference for UDP-galactose as a sugar donor, MiUFGalT3 rather than MiUFGT1 plays a crucial role in the light-induced red coloration of the mango fruit skin. Our findings may provide an important clue to elucidate the mechanism of anthocyanin accumulation in mango fruit skin. Further investigation is required to clarify the evolutionary process and key factors determining sugar donor preferences of plant UFGTs.

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