Identification of the glucosyltransferase gene that supplies the \( p \)-hydroxybenzoyl-glucose for 7-polyacylation of anthocyanin in delphinium

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Abstract

In delphiniums (\textit{Delphinium grandiflorum}), blue flowers are produced by the presence of 7-polyacylated anthocyanins. The polyacyl moiety is composed of glucose and \( p \)-hydroxybenzoic acid (pHBA). The 7-polyacylation of anthocyanin has been shown to be catalysed by two different enzymes, a glucosyltransferase and an acyltransferase; both enzymes utilize \( p \)-hydroxybenzoyl-glucose (pHBG) as a bi-functional (Zwitter) donor. To date, however, the enzyme that synthesizes pHBG and the gene that encodes it have not been elucidated. Here, five delphinium cultivars were investigated and found to show reduced or undetectable 7-polyacylation activity; these cultivars synthesized delphinidin 3-O-rutinoside (Dp3R) to produce mauve sepals. One cultivar showed a deficiency for the acyl-glucose-dependent anthocyanin 7-O-glucosyltransferase (AA7GT) necessary for mediating the first step of 7-polyacylation. The other four cultivars showed both AA7GT activity and \( Dg \text{AA7GT} \) expression; nevertheless, pHBG accumulation was significantly reduced compared with wild-type cultivars, whereas \( p \)-glucosyl-oxybenzoic acid (pGBA) was accumulated. Three candidate cDNAs encoding a UDP-glucose-dependent pHBA glucosyltransferase (\( Dg \text{pHBAGT} \)) were identified. A phylogenetic analysis of \( Dg \text{pHBAGT} \) amino acid sequences showed a close relationship with UGTs that act in acyl-glucose synthesis in other plant species. Recombinant \( Dg \text{pHBAGT} \) protein synthesized pHBG and had a high preference for pHBA \textit{in vitro}. Mutant cultivars accumulating pGBA had very low expression of \( Dg \text{pHBAGT} \), whereas expression during the development of sepals and tissues in a wild cultivar showed a close correlation to the level of accumulation of pHBG. These results support the conclusion that \( Dg \text{pHBAGT} \) is responsible for \textit{in vivo} synthesis of pHBG in delphiniums.

Key words: Acyl-glucose-dependent anthocyanin 7-O-glucosyltransferase, delphinium, \( p \)-glucosyl-oxybenzoic acid, \( p \)-hydroxybenzoylic acid glucosyltransferase, \( p \)-hydroxybenzoyl-glucose, 7-polyacylated anthocyanin.

Introduction

Plants produce a wide range of secondary metabolites that serve important ecological functions (\textit{Dixon}, 2001). Among these secondary metabolites, anthocyanins are the major pigments of plants and which contribute to the reproduction...
process through recruitment of pollinators and animal seed dispersal agents (Jürgens et al., 2000). Anthocyanins have six major types of basic structure (anthocyanidins) that behave as chromophores and provide different colours for plant organs. The structure of an anthocyanidin molecule varies with the number of hydroxyl residues at the B ring of the aglycone; for example, pelargonidin has a single residue at the 4’ position, cyanidin has two residues, at the 3’ and the 4’ positions, and delphinidin has three residues, at the 3’, 4’, and 5’ positions. The differences in the number and positions of the residues determine the colour of the chromophore: pelargonidin is orange-red, cyanidin is crimson, and delphinidin is red-purple (Tanaka et al., 2008). Although plants have a limited number of basic anthocyanidin structures, the further modification of these structures by the addition of sugars and organic acids gives rise to many thousands of anthocyanin molecular species, yielding wide variations in colour (Andersen and Jordheim, 2006). In several plant species with violet-blue flowers, such as delphinium (Delphinium grandiflorum), Canterbury bells (Campanula medium), cineraria (Senecio cruentus), Chinese bell flower (Platycedron grandiflorum), and anemone (Anemone coronaria), modification of delphinidin with sugars at the 7 position plays an important role in the generation of the flower colour (Davis, 2009; Yoshida et al., 2009).

Plants with blue flowers are widely appreciated and enjoyed; however, only a limited number of species are able to produce blue pigmentation. For this reason, a considerable effort has been expended by horticulturists to ‘blue’ the flowers of commonly grown plants. The recent progress in plant molecular biology has led to the successful breeding of transgenic carnations (Tanaka et al., 1998; Mol et al., 1999) and roses (Katsumoto et al., 2007), and such varieties have found a ready commercial market. This progress has also enabled the production of chrysanthemums with blue flowers (Brugliera et al., 2013; Noda et al., 2013). These transgenic carnations, roses, and chrysanthemums synthesize delphinidin following the introduction of the flavonoid 3’,5’-hydroxylase gene, which is not normally present. In species in which endogenous dihydroflavonol 4-reductase (DFR) has a low efficiency for catalysing dihydroquercetin and/or dihydrokaempferol, then it is necessary to suppress expression of the endogenous DFR gene during generation of transgenic plants. It is also essential to introduce an exogenous DFR gene from another species that has high efficiency for catalysis of dihydromyricetin (Tanaka et al., 1998; Katsumoto et al., 2007). However, the flower colour in these transgenic plants is not a vivid blue but rather a purplish-blue. The same blueish hue is developed in the flowers of other plant species, although they employ different mechanisms to achieve this colouration. Typically, these mechanisms involve metal ions, variations in vacuolar pH, and intermolecular and intramolecular co-pigmentation (Yoshida et al., 2009). In species such as Asiatic dayflowers (Commelina communis), cornflowers (Centaurea cyanus), and hydrangeas (Hydrangea macrophylla), which can have blue petals, and in tulips (Tulipa gesneriana cv. Murasakizuisho), in which the bottom of the perianth can be blue, metal ions such as Mg$, Fe$^+$, and Al$^{3+}$, and flavones, flavonols, or 5-O-acylquinic acids play important roles. In vitro analyses showed that they can associate with anthocyanins to produce a range of shades of blue by varying their relative concentrations (Takeda et al., 1985; Goto and Kondo 1991; Shiono et al., 2005, 2008; Shoji et al., 2007). In Asiatic dayflowers and cornflowers, the structures of metal complexes have been determined by X-ray crystal structure analysis (Kondo et al., 1992; Shiono et al., 2005). In contrast to these species, blue morning glory (Ipomoea tricolor cv. Heavenly Blue) accumulates tricaffeoylated anthocyanin (heavenly blue anthocyanin) in its petals (Kondo et al., 1987); although the anthocyanin component does not change before or after anthesis, the colour of the petals changes from red to blue apparently in association with an increase in vacuolar pH to approximately pH 7.7 (Yoshida et al., 1995).

Intermolecular co-pigmentation, which does not require metal ions but is a hydrophobic association between anthocyanin and flavone, flavonol, or tannin, can also produce blue flower coloration (Yoshida et al., 2009). For example, in the case of blue Veronica persica, apigenin 7-O-((2-O-glucuronosyl)-glucuronic) is the main flavonoid co-existing with delphinidin 3-O-(2-O-(6-O-p-coumaroyl)-glucosyl)-6-O-p-coumaroyl-glucoside)-5-O-glucoside. Apigenin causes a bathochromic shift in absorbance on the anthocyanin (Ono et al., 2010). Polyacylation of delphinidin (i.e. modification with two or more linked glucosyl and aromatic residues) likewise causes a bathochromic shift to produce a blue colour by intramolecular co-pigmentation. In some species with blue flowers, such as delphinium, Canterbury bells, cineraria, and Chinese bell flower, the 7 position of delphinidin is polyacylated (Honda and Saito, 2002; Saito et al., 2007; Yoshida et al., 2009). Blue coloration in delphinium sepal is derived from the 7-polyacylated anthocyanins violdelphin (delphinidin 3-O-rutinoside-7-O-(4-O-(6-O-(p-hydroxybenzoyl)-glucosyl)-oxybenzoyl)-glucoside)) and cyanodelphin (delphinidin 3-O-rutinoside-7-O-(3-O-(6-O-(4-O-(6-O-(p-hydroxybenzoyl)-glucosyl)-oxybenzoyl)-glucosyl)-oxybenzoyl)-glucosyl)-6-O-(4-O-(6-O-(p-hydroxybenzoyl)-glucosyl)-oxybenzoyl)-glucoside) (Fig. 1) (Hashimoto et al., 2002). The polyacyl moieties in delphinium are composed of glucose and p-hydroxybenzoic acid (pHBA) molecules in which each pHBA is linked to glucose moieties through an ester bond. Recently, 7-polyacylation in delphinium was shown to be catalysed by vacuolar acyl-glucose-dependent glycosyltransferases and acyltransferases that utilize p-hydroxybenzoyl-glucose (pHBG) as the glucosyl and acyl donor, respectively. Thus, pHBG has been termed a ‘Zwitter’ donor in recognition of the fact that it can act in a bi-functional manner (Fig. 1) (Nishizaki et al., 2013).

In vivo, aromatic organic acids are often conjugated with a glucose via an ester bond to form an acyl-glucose or through an O-glucoside bond to form a glucoside (Molgaard and Ravin, 1988; Herrmann, 1989). Glucosides are believed to be vacuolar storage molecules until activation or recruitment to the cytosol after a glucosidase reaction that occurs in the vacuole (Yazaki et al., 1995; Dixon, 2001; Dick et al., 2012). Recent research led to the proposal that acyl-glucoses can additionally function
**Materials and methods**

**Plant materials and reagents**

The *D. grandiflorum* cultivars Triton Light Blue (TLB), Blue Magic (BM), White Candle (WC), and Aurora Mauve Blue (AMV) were purchased from a flower market. Summer Wine (SW), Magic Fountain Cherry Blossom (MFCB), Patricia Johnson (PJ), and Cassis Pink (CP) were a gift from the Ehime Research Institute of Agriculture, Forestry and Fisheries. The flowers of delphiniums consist of large sepals and much smaller central petals. In some delphinium cultivars, the colours of the sepals and petals differ; for example, the cultivars AMV, BM, and TLB have colourless petals, whereas SW and PJ have darkish petals (Fig. 2). Since attractive coloration of the sepals is more valuable in marketing terms than the colour of the small petals, this study concentrated on pigmentation of sepals. Sepals were collected from WC cultivar plants at different floral developmental stages, defined as S1–S4 (see Fig. 7A); leaves and stems were also collected from these plants. Only stage 3 or 4 sepals were collected from the other cultivars. The collected tissues were immediately frozen in liquid nitrogen and stored at –80 °C until use.

**Protein extraction from sepals of *D. grandiflorum***

For the measurement of the activities of acyl-glucose-dependent anthocyanin 7-O-glucosyltransferase (AA7GT) and...
UDP-glucose-dependent pHBA glucosyltransferase (pHBAGT), 1 g of frozen tissue was ground into powder in liquid nitrogen using a mortar and pestle and then placed into 5 ml of extraction buffer [0.1 M potassium phosphate (pH 7.5) and 14 mM 2-mercaptoethanol]. Cell debris was removed by centrifugation at 15 000 g for 5 min. Proteins were precipitated using 80% ammonium sulphate and suspended with the extraction buffer, followed by desalting; the suspension was placed in a G-25 spin column (GE Healthcare, Piscataway, NJ, USA) and the buffer was replaced with 0.1 M citrate buffer (pH 5.5) for AA7GT, or 0.1 M TRIS-HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol for pHBGAT. The standard AA7GT enzymatic reaction was performed in a reaction mixture consisting of 3 nmol cyanidin 3-O-glucoside (Cy3G), 45 nmol pHBR, 2.4 μmol citrate buffer (pH 5.5), and 5–25 μg of crude protein in a total volume of 30 μl; the mixture was incubated at 30 °C for 1 h. The standard pHBGAT enzymatic reaction was performed in a reaction mixture consisting of 25 nmol pHBA, 125 nmol UDP-glucose, 2.4 μmol TRIS-HCl buffer (pH 7.5) containing 14 mM 2-mercaptoethanol, and 8–40 μg of crude protein in a total volume of 50 μl; the mixture was incubated at 30 °C for 10 min. All reactions were stopped by the addition of 20% phosphoric acid to a final concentration of 1%. Protein concentrations were quantified using a Coomassie brilliant blue (CBB) protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) with bovine serum albumin as the standard.

Isolation and identification of p-glucosyl-oxybenzoic acid extracted from sepals of Aurora Mauve Blue

p-Glucosyl-oxybenzoic acid (pGBA) was extracted from 200 g of frozen tissue from AMV plants using 80% methanol containing 0.1% trifluroacetic acid (TFA) at 4 °C. The extract was evaporated and pGBA was separated by flash liquid chromatography (YFLC-AI-580, Yamazen Corp., Osaka, Japan) and reverse-phase columns (Hi-Flash columns, ODS-S: 50 μm, i.d. 20 × 65 mm, followed by a column with i.d. 26 × 100 mm, Yamazen) using a linear gradient elution (20 ml min⁻¹) of 0–30% methanol in 0.1% aqueous TFA for 20 min. Purified pGBA (20 μg) was analysed by electrospray ionization-mass spectrometry (ESI-MS, AccuTOF MS, JMS-T100LC, JEOL Ltd, Tokyo, Japan) and nuclear magnetic resonance (¹H NMR, ¹³C NMR, ¹H(¹³C) HMQC, ¹H(¹³C) HMBC, TOCSY, H-H COSY, and NOESY) in CD₃SOCD₃ on a JNM-ECA-500 spectrometer (JEOL). For ¹H NMR (500 MHz) and ¹³C NMR (125 MHz), chemical shifts were referenced to the residual solvent (CD₃SOCD₃) signals at δH 2.50 and δC 39.5.

Measurement of pHBG and pGBA accumulation

Total pHBG and pGBA were extracted from 1 g of frozen S4 stage sepals using 100 ml of 80% methanol containing 0.1% TFA at 4 °C overnight; each extract was then evaporated and redissolved in 5 ml of 100% methanol. pHBG and pGBA were analysed by HPLC at an absorbance of 260 nm and their relative quantities were estimated from peak areas using synthetic pHBG and purified pGBA as standards. Each assay was repeated at least three times.

Cloning of DgpHBAGT cDNA from D. grandiflorum and quantitative RT-PCR expression analysis of DgpHBAGT

The methods used to isolate total RNA and to synthesize first-strand cDNAs from the sepals of each delphinium cultivar were described previously (Nishizaki et al., 2013). Candidate pHBGAT cDNA fragments were obtained using the degenerate primers UGTdgFwd (5'-AAAYAACNTTTYATCTCGGGTT-3') and UGTdgRev (5'-CATNGTNSWRTTCCANCCRCARTG-3'), which were designed to correspond to the amino acid sequence of NNPFIPW (sense) and MTSNWGCN (antisense), respectively. The template cDNA was synthesized from total RNA obtained from sepal of D. grandiflorum. The 5' and 3' cDNA ends of DgpHBAGT, DgpUGT2, and DgpUGT3 were amplified with a SMARTer RACE cDNA Amplification Kit (Clontech Laboratories, Inc., Mountain View, CA, USA).

Quantitative real-time PCR (RT-PCR) was performed using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio, Ohtsu, Japan), the DNA Engine Opticon 2 (Bio-Rad Laboratories, Hercules, California, USA), and cDNAs from S3 sepals as the template. The following primer sets were used: DgAA7GT, DgAA7GTQFwd (5'-GGCGTCTTGTGGCAATCTA-3') and DgAA7GTQRev (5'-GGAAACCTGGCTCCTCTTTGG-3'); Dgp HBAGT, DgpHBAGTQFwd (5'-TGGGCTGGGAAC TCTTCTAT-3') and DgpHBAGTQRev (5'-CCTTCTCCACCTTC AAGGCCG-3'); DgpUGT2, DgpUGT2QFwd (5'-CCCCGGG

![Fig. 2. The flower phenotypes of seven delphinium cultivars and the major anthocyanins that are accumulated in each cultivar. AMV, Aurora Mauve Blue; SW, Summer Wine; MFCB, Magic Fountain Cherry Blossom; PJ, Patricia Johnson; CP, Cassis Pink; TLB, Triton Light Blue; BM, Blue Magic. mAU, milli absorbance units.](https://academic.oup.com/jxb/article-abstract/65/9/2495/523874/2498-Nishizaki-et-al)
GTATTGCTG(TGTGCTG)C-3'); and DgUGT3QRev (5′-CTGCGGCGGACCAGTGTCGAGG-3′) and DgUGT3QFwd (5′-CTGCGGCGGACCAGTGTCGAGG-3′). These primer sets were designed using GENETIX Ver.11 Primer3 (Genetyx Co., Tokyo, Japan). The reaction conditions for the degenerate PCR and quantitative RT-PCRs were the same as described previously (Nishizaki et al., 2013).

Phylogenetic analysis

The amino acid sequences of the UGT gene products were obtained from GenBank and BLAST searches and used for the phylogenetic analysis. The sequences were aligned using ClustalW (Thompson et al., 1994). The phylogenetic tree and bootstrap values were obtained using the Neighbor–Joining algorithm MEGA version 5.05 (Tamura et al., 2007). Bootstrap values were performed with 1000 replications.

Heterologous expression and purification of DgpHBAGT

Full-length cDNAs of DgpHBAGT, DgUGT2, and DgUGT3 were amplified by PCR using the following primer sets: DgpHBAGT Fwd (5′-CATGGGAGTGACAAACTTCATCAC-3′) and DgpHBAGTTrtRev (5′-AGATTTGATGTCATCTCTCCAGATGTG-3′); DgUGT2 Fwd (5′-ATGGCAACACACAGGAAGACC-3′) and DgUGT2TrtRev (5′-CAATTCTTCTTGCTTCTCTTCTTCTATCA-3′); and DgUGT3 Fwd (5′-ATGGGCACTCACTCGTCTATGAG-3′) and DgUGT3TrtRev (5′-ATTTACTCACTCACTCGTCTATGAG-3′). The amplicons were analysed using the pTrcHis2 TOPO Expression Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The enzymatic properties of DgpHBAGT were characterized using a glutathione S-transferase (GST) fusion protein produced by introducing the full-length DgpHBAGT cDNA coding sequence into the pDEST15 plasmid (Invitrogen). Escherichia coli strain KRX (Promega Biosciences, LLC, San Luis, CA, USA) was used as the host for expression of recombinant DgpHBAGT, DgUGT2, DgUGT3, and GST–DgpHBAGT. Escherichia coli recombinant cells were inoculated into 200 ml of Luria–Bertani medium containing 50 μg ml−1 ampicillin and grown at 30 °C until an A600 of 0.5 was reached. After the addition of 200 μl of 1 M isopropyl-β-D-thiogalactopyranoside (pTrcHis2) or 20% (w/v) rhamnose (pDEST15), the E. coli cells were cultured at 16 °C for 24 h. The cells were harvested by centrifugation, and resuspended in 10 ml of 0.1 M potassium phosphate buffer (pH 7.5), 14 mM 2-mercaptoethanol, and disrupted by sonication (UD-201, Tomy Seiko, Tokyo, Japan). After centrifugation of the lysate at 15000 g for 5 min, the supernatant containing recombinant DgpHBAGT, DgUGT2, or DgUGT3 was applied to a G-25 spin column (GE Healthcare) to change the buffer to 0.1 M potassium phosphate buffer (pH 7.5) containing 14 mM 2-mercaptoethanol. These solutions were then used as the crude protein extracts. Enzyme assays were carried out using the same conditions as described above for the crude extract prepared from delphinium sepal. The GST–DgpHBAGT supernatant was further purified using a pre-packed GSTrap HP column (GE Healthcare) and analysed by SDS–PAGE (Supplementary Fig. S3 available at JXB online).

Analysis of the enzymatic properties of GST–DgpHBAGT

The conditions for the reactions to analyse enzymatic properties of GST–DgpHBAGT were as follows: a total volume of 50 μl of TRIS–HCl buffer (pH 7.5) containing 5 μg of GST–DgpHBAGT protein; 2.5 mM UDP-glucose as the glucose donor; 1 mM pHBA; and 20 mM MgCl2. The reactions were allowed to run for 10 min at 30 °C. The pH and Mg2+ concentrations were varied in different experiments. To determine the optimal pH of the reaction, the reaction buffers MES (pH 5.0–6.0), MOPS (pH 6.5 and 7.0), and TRIS–HCl (pH 7.5–8.5) were used. Optimal Mg2+ concentration was determined using the reaction mixture described above except for varying the Mg2+ concentration between 0 and 50 mM. The reactions were stopped by the addition of 20% phosphoric acid solution to a final concentration of 1%. The Km values of pHBA, p-coumaric acid, and ferulic acid for GST–DgpHBAGT were determined by varying the concentration of pHBA, p-coumaric acid, or ferulic acid in the range 0.0625–4 mM at 5 mM UDP-glucose.

HPLC conditions

HPLC was used to separate the pHBG or pGBA extracted from the sepal or the AA7GT and pHBAGT reaction products in the crude protein extract reaction mixtures from delphinium sepal. The separations were carried out for 20 min using an HPLC–photodiode array detector system (LaChrome Elite, Hitachi High-Technologies Corp., Tokyo, Japan) equipped with an ODS column (i.d. 4.6 × 250 mm, Wakopak Handy ODS, Wakо Pure Chemical Industries, Ltd, Shiga, Japan), and a linear gradient elution (1 ml min−1) of 5–26% acetonitrile in 1.5% aqueous phosphoric acid. The reaction products in crude protein extracts containing DgpHBAGT, DgUGT2, or DgUGT3 recombinant proteins were analysed using the same HPLC conditions. The enzymatic properties of GST–DgpHBAGT were analysed using a short ODS column (i.d. 4.6 × 50 mm, COSMOSIL 5C8–MS-II; Nacalai Tesque, Kyoto, Japan) and the short method [linear gradient elution (1.5 ml min−1) of 10–20% (pHBA) or 25–80% (p-coumaric acid and ferulic acid) methanol in 1.5% aqueous phosphoric acid for 5 min] as for the HPLC experiments.

Results

Identification of delphinium cultivars that accumulate delphinidin 3-O-rutinoside and detection of AA7GT activity in crude extracts from sepal

In some delphinium cultivars, Dp3R has been identified as the major anthocyanin (Hashimoto et al., 2002). A survey of various delphinium cultivars using HPLC showed that the cultivars AMV, SW, MFCB, PJ, and CP accumulated Dp3R (Fig. 2). The TLB and BM cultivars, which have blueish flowers, synthesize and accumulate cyanodelphin and violdelphin, respectively (Fig. 2). Dp3R is the substrate for the modification reaction at the 7 position of anthocyanin by AA7GT to produce Dp3R7G (Fig. 1) (Matsuba et al., 2010; Nishizaki et al., 2013). This AA7GT reaction is generally thought not to occur in the sepal of cultivars that accumulate Dp3R, such as AMV, SW, MFCB, PJ, and CP. Crude protein extracts prepared from these cultivars were incubated with pHBG and Cy3G at 30 °C for 60 min. After termination of the reactions, the reaction mixtures were analysed by HPLC. Cy3,7dG was present on all HPLC chromatographs with the exception of that of CP sepal (Supplementary Fig. S1A at JXB online). The quantitative RT-PCR analyses showed that DgAA7GT expression could not be detected in CP but was present in the other cultivars (Supplementary Fig. S1B). These results indicate that the failure to modify anthocyanin at the 7 position in CP plants was caused by the loss of function of AA7GT.

Isolation of p-glucosyl-oxybenzoic acid in delphinium cultivars accumulating anthocyanin that has not been 7-polyacylated

The analyses described above showed that the delphinium cultivars AMV, SW, MFCB, PJ, and CP accumulated...
anthocyanins that had not been polyacylated at the 7 position (Dp3R); these cultivars, with the exception of CP, had both AA7GT activity and DgAA7GT expression (Supplementary Fig. S1 at JXB online). The level of pHBG in sepals from each cultivar was quantified using HPLC. As reported previously, pHBG was detected in the cultivars TLB, BM, and WC, which synthesize and accumulate cyanodelphin, violdelphin, and Dp3R7G, respectively, (Nishizaki et al., 2013). In contrast, pHBG was present at a very low level in the cultivars AMV, SW, MFCB, and PJ (black bars in Fig. 3). Interestingly, HPLC analysis showed that sepals from AMV, SW, MFCB, and PJ plants contained an unknown hydroxybenzoyl derivative with a similar absorption spectrum (peak at 250 nm) to pHBG (peak at 260 nm) but with a different retention time (Fig. 4). This unidentified compound was below the level of detection in sepals of TLB and CP plants. To identify this compound, it was first extracted and purified from AMB sepals. Mass spectrometric analysis showed that the molecular ion of the compound was observed at m/z=300.4, corresponding to the molecular mass of pHBA bound to glucose. The compound was also analysed by 1H NMR and 13C NMR spectroscopy using 1H{13C} HMQC, 1H{13C} HMBC, H-H COSY, and NOESY. The 1H NMR spectrum showed the presence of one glucose moiety and one benzoyl moiety. The anomic proton of the glucose moiety was assigned at δ 4.98 (d, J=6.87 Hz). An 1H{13C} HMBC signal between the carboxyl carbon of the benzoyl moiety and the anomic proton of the glucose moiety and a NOESY signal between the anomic proton of the glucose moiety and H-3 and H-5 of the benzoyl moiety was observed; thus, the compound is pGBA (Supplementary Fig. S2 at JXB online). The amount of pGBA in S4 stage sepals of each cultivar was quantified (grey bars in Fig. 3). This analysis indicated that accumulation of pGBA or pHBA was in an inverse relationship in the different cultivars.

Detection of pHBAGT activity and isolation of cDNAs

Acyl-glucose is expected to be synthesized by UGT with an acyl moiety as an acceptor (Leznicki and Bandurski, 1988; Mock and Strack, 1993). The crude protein extracts were prepared from AMV, SW, MFCB, PJ, and CP plants, and each extract was incubated with UDP-glucose and pHBA at 30 ºC for 10 min. After termination of each reaction, the production of pHBG was analysed on an HPLC chromatogram: CP showed clear production of pHBG, but the levels were lower in AMV, SW, MFCB, and PJ plants (asterisks in Fig. 5). Thus, pHBAGT activity occurred in the CP cultivar, but was absent or repressed in the AMV, SW, MFCB, and PJ cultivars.

In order to isolate a cDNA encoding pHBAGT, three candidate cDNAs (DgUGT1, DgUGT2, and DgUGT3) were amplified by PCR using degenerate primers based on the conserved amino acid sequences of Gomphrena globosa sinapic acid glucosyltransferase (GgSGT), Arabidopsis thaliana hydroxybenzoic acid glucosyltransferase (AtUGT84A1), and Fragaria xananassa cinnamic acid glucosyltransferase...
Figure 5. Detection of pHBAGT activities in crude extracts prepared from sepals of delphinium cultivars. The triangles and asterisks on the chromatograph indicate peak pHBA and pHBG, respectively. AMV, Aurora Mauve Blue; SW, Summer Wine; MFCB, Magic Fountain Cherry Blossom; PJ, Patricia Johnson; CP, Cassis Pink. mAU, milli absorbance units.

(FaGT2) (Lim et al., 2002; Landmann et al., 2007; Matsuba et al., 2008). A phylogenetic analysis showed that the deduced amino acid sequences of DgUGT1 and DgUGT3 were close to those of UGTs with activity in acyl-glucose synthesis (highlighted by blue font in Fig. 6). DgUGT2 belonged to a different clade that included flavonoid glucosyltransferases.

Analysis of UGT gene expression

To identify the cDNA encoding the pHBAGT enzyme, a quantitative RT-PCR analysis of expression of the DgUGT1, 2, and 3 genes was performed in combination with an investigation of pHBAGT activities and pHBG levels in S1–S4 stage sepals and in leaves and stems of the WC cultivar (Fig. 7). In this cultivar, the highest level of pHBG accumulation was detected in S4 stage sepals; no accumulation was detected in leaves or stems (Fig. 7B). pHBAGT activity was very low in leaves and stems; the highest levels of activity were seen in S3 and S4 stage sepals (Fig. 7C). DgUGT2 expression was detected in sepals, but higher levels of expression were observed in leaves and stems. Expression of DgUGT1 and DgUGT3 was relatively high in sepals; the highest level of DgUGT1 expression occurred at the S3 stage, whereas that of DgUGT3 occurred at the S4 stage (Fig. 7D).

Expression of DgUGT genes in stage S3 sepals of all cultivars was analysed by quantitative RT-PCR. DgUGT1 expression was present at low levels in the AMV, SW, MFCB, and PJ cultivars; however, high levels of expression were found in the TLB, BM, WC, and CP cultivars (Fig. 7E). This analysis showed that the amount of synthesized and accumulated pHBG was closely correlated with the DgUGT1 expression level (compare the black and white bars in Figs 3 and 7E). The patterns of expression of DgUGT2 and DgUGT3 were uncorrelated with pHBG accumulation in S3 stage sepals (Fig. 7E). In contrast, the amount of accumulated pGBA in S4 stage sepals of the AMV, SW, MFCB, and PJ cultivars showed an inverse relationship with the level of DgUGT1 expression (grey bars in Fig. 3).

Glucosylation of pHBA is catalysed by DgpHBAGT

The three candidate cDNAs (DgUGT1, DgUGT2, and DgUGT3) were introduced into E. coli expression vectors in order to determine whether the recombinant proteins had the ability to attach a glucose moiety to pHBA in a reaction mixture containing UDP-glucose as the glucose donor. The reactions were performed using crude protein extracts and, after termination, the reaction mixtures were subjected to an HPLC analysis. pHBG was detected on the HPLC chromatogram of the reaction mixture containing DgUGT1, but not in those containing DgUGT2 or DgUGT3 (Fig. 8). Hence, DgUGT1 is designated as DgpHBAGT.

To characterize the enzymatic properties of DgpHBAGT, a GST fusion protein was generated in E. coli cells. After purification of the GST–DgpHBAGT protein, optimal activity was found to occur at pH 7.5 (Supplementary Fig. S3A, B at JXB online). Many plant glucosyltransferases have been shown to be activated in the presence of a divalent cation, with a preference for magnesium (Taguchi et al., 2000; Jackson et al., 2001; Hefner et al., 2002; Landmann et al., 2007). For this reason, the effects of varying the Mg$^{2+}$ concentration were tested; the highest pHBAGT activity occurred at 20 mM MgCl$_2$ at pH 7.5 (Supplementary Fig. S3C at JXB online). This concentration was therefore used in the subsequent assays of kinetic parameters. The kinetics of GST–DgpHBAGT were examined in reactions with 5 mM UDP-glucose and different concentrations of pHBA, p-coumaric acid, or ferulic acid. GST–DgpHBAGT displayed typical Michaelis–Menten behaviour, and the resulting plots for pHBA, p-coumaric acid, or ferulic acid were used to determine its kinetic parameters (see Table 1 for details). The catalytic efficiency values ($K_{cat}/K_m$) for the three substrates revealed that GST–DgpHBAGT showed a preference for pHBA over p-coumaric acid and ferulic acid.

Discussion

The blue coloration of delphinium flowers is produced by 7-polyacylated anthocyanins such as violodelphin and cyanodelphin. Previously, it was reported that 7-polyacylation is catalysed by a glucosyltransferase and an acyltransferase; additionally, pHBG was shown to act as a bi-functional (Zwitter) glucosyl and acyl donor (Fig. 1) (Nishizaki et al., 2013). In this modification process, the synthetic pathway for the starter acceptor molecule Dp3R7G, which is synthesized from Dp3R by AA7GT, has been elucidated (Matsuba et al., 2010); however, the enzyme(s) required for synthesis of the
Fig. 6. A molecular phylogenetic tree of UGT members based on amino acid sequences. UGTs which have acyl-glucose synthesis activity are indicated in blue font. The accession numbers of the nucleotide sequences for the proteins used in the alignment are as follows: AcGaT (AB103471), ATUGT72E2 (NM_126067), ATUGT73G6 (NM_129234), ATUGT74B1 (BT001160), ATUGT74F2 (BT010327), ATUGT75C1 (AK226538), ATUGT78D1 (NM_102790), ATUGT78D2 (NM_121711), ATUGT78D3 (NM_121709), ATUGT79B1 (BT033073), ATUGT84A1 (BT015796), ATUGT84B1 (NM_127900), AsUGT74H6 (EU496509), AsUGT74C6 (EU496520), BnUGT84A9a (AF287143), BpUGAT (AB190262), Cm1,2RhaT (AY048882), Cs1,6RhaT (DQ119035), DgUGT1 [DgpHBAGT] (AB889521), DgUGT2 (AB889522), DgUGT3 (AB889523), FaGT2 (AY663785), GgSGT (AB362221), Gt5GT7 anthocyanin 5 GT, GtA3GT anthocyanin 3 GT, GtA3GT anthocyanin 3' GT, AcGaT anthocyanin 3 galactosyltransferase, VfF3GT flavonoid 3 GT, AtUGT78D1 flavonol 3 rhamnosyltransferase, AtUGT78D2 flavonoid 3 GT, AtUGT78D3 flavonol 3 arabinosyltransferase, AtUGT73C6 flavonol 7 GT, DgUGT2 (This work), GlA3'GT anthocyanin 3' GT, SbUGT baicailein 7 GT, AtUGT72E2 hydroxycinnamyl 4 GT, BpUGAT anthocyanin 3-glucoside: 2" glucuronosyltransferase, Cm1,2RhaT flavonoid 7-glucoside: 2" rhamnosyltransferase, AtUGT79B1 anthocyanin 3-glucoside: 2" xylosyltransferase, In3GT anthocyanin 3-glucoside: 2" GT, Cs1,6RhaT flavonoid 7-glucoside: 6" rhamnosyltransferase, Ph3GRhaT anthocyanin 3-glucoside: 6" rhamnosyltransferase.

Ac, Aralia cordata; At, Arabidopsis thaliana; As, Avena strigosa; Bn, Brassica napus; Bp, Bellis perennis; Cm, Citrus maxima; Cs, Citrus sinensis; Dg, Delphinium grandiflorum; Fa, Fragaria-ananassa; Gg, Gomphrena globosa; Gt, Gentiana triflora; Ih, Iris×hollandica; In, Ipomoea nil; Nt, Nicotiana tabacum; Pf, Petunia×hybrida; Sb, Scutellaria baicalensis; Vv, Vitis vinifera. Bar=0.1 amino acid substitutions/site.
donor molecule pHBG have not yet been elucidated. The analyses here have clarified one aspect of this process by confirming that the *DgpHBAGT* gene encodes the enzyme that synthesizes pHBG.

A number of delphinium cultivars, such as AMV, SW, MFCB, PJ, and CP, have mauve flowers and accumulate Dp3R as the major anthocyanin (Fig. 2). This study has shown that the CP cultivar was deficient in either AA7GT activity or expression of *AA7GT* (Supplementary Fig. S1 at JXB online). Although other cultivars had both AA7GT activity and *AA7GT* expression, they nevertheless accumulated Dp3R (Fig. 2). Quantification of pHBG in S4 stage sepals showed that...
the AMV, SW, MFCB, and PJ cultivars had low levels; in contrast, they had relatively high levels of pGBA. Both pHBA and pGBA are thought to be synthesized by glucosylation of pHBA by UGTs. pHBA, a C₆-C₁ unit, may be synthesized from C₆-C₃ substances such as p-coumaric acid. The formation of benzoic acid requires the removal of two carbon units from a phenylpropanoid residue; the catabolic steps of this reaction have been well studied, especially with regard to the biosynthesis of plant volatile organic compounds (Dudareva et al., 2013). In the case of pHBA, investigations using hairy roots of Daucus carota suggest that p-coumaric acid is a precursor (Sircar and Mitra, 2008; Sircar et al., 2011). Accumulation of pGBA implied that the pHBA synthetic pathway was active in the AMV, SW, MFCB, and PJ cultivars, and that these cultivars were either deficient in or suppressed to a considerable extent the glucosylation activity needed to synthesize pHBG in vivo.

One of the UGTs isolated by degenerate PCR, DgpHBAGT, was active in vitro in the synthesis of pHBG, other than DgUGT2 and DgUGT3 (Fig. 8). The expression profile of DgpHBAGT was positively correlated with both pHBG activity and accumulation of pHBG in sepals at stages S1–S4, and in leaves and stems of WC cultivar plants; notably, expression was low in the AMV, SW, MFCB, and PJ cultivars (Fig. 7E). However, recombinant GST–DgpHBAGT was able to transfer a glucose moiety to p-coumaric acid and ferulic acid in addition to pHBA in vitro (Table 1). Many of the UGTs that attach plant secondary metabolites to a glycosyl moiety have been reported to show broad acceptor preference in vitro but strict glycosyl donor preference (Fraissinet-Tachet et al., 1998; Lee and Raskin, 1999; Taguchi et al., 2000; Landmann et al., 2007; Ozeki et al., 2011). For example, a recombinant GgSGT can attach a glucose moiety to four hydroxycinnamic acids as acceptors to generate sinapoyl-, feruloyl-, caffeoyl-, and p-coumaroyl-glucoses (Matsuba et al., 2008). Likewise, recombinant GST–DgpHBAGT has broad acceptor preference for pHBA, p-coumaric acid, and ferulic acid, although its catalytic efficiency for glucosyl transfer activity is higher for transfer to pHBA than to p-coumaric acid or ferulic acid (Table 1). This property supports the interpretation that the main catalytic role of DgpHBAGT in vivo is synthesis of pHBG, but that the protein also has the lesser role of synthesizing p-coumaroyl-glucose or feruloyl-glucose. This hypothesis is consistent with the observation that acyl-glucose accumulation, except for pHBG, was absent or occurred at a low level in delphinium sepals (Nishizaki et al., 2013).

Although crude extracts from the AMV, SW, MFCB, and PJ cultivars showed low activity for synthesizing pGBA using pHBA and UDP-glucose (triangles in Fig. 5), these cultivars showed relatively high accumulation of pGBA and pHBG compared with the TLB, BM, WC, and CP cultivars (Fig. 3, grey bars). In the latter, pGBA was either below the detection level or present at a very low level (Fig. 3). In cells of the wild-type cultivars TLB, BM, WC, and CP, pHBA is synthesized in the cytosol and then efficiently glucosylated by DgpHBAGT and transferred into the vacuole. However, in the mutant cultivars AMV, SW, MFCB, and PJ, the DgpHBAGT gene shows reduced expression, and excess pHBA may accumulate in the cytosol. This suggests that, in these mutant cultivars, UGT(s) other than DgpHBAGT are involved in the catalysis step of other secondary metabolic pathways and, through having broad acceptor preferences, convert the accumulated pHBA in the cytosol to pGBA, which is then transported into the vacuole. This substitute activity by other UGT(s) may, however, be too inefficient in vitro to allow conversion of pHBA to pGBA using UDP-glucose (Fig. 5), but may be sufficient Publishers use this service to check for duplicate content.
Analyses of acceptor preferences were performed with UDP-glucose as the donor. The reaction products were calculated from the area of the HPLC chromatograms recorded at 260 nm (p-hydroxybenzoic acid) or 330 nm (p-coumaric acid and ferulic acid).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nkat mg$^{-1}$)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxybenzoic acid</td>
<td>0.851 ± 0.066</td>
<td>13.00 ± 0.681</td>
<td>1.042 ± 0.054</td>
<td>1.231 ± 0.034</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>1.142 ± 0.106</td>
<td>6.841 ± 0.405</td>
<td>0.548 ± 0.032</td>
<td>0.484 ± 0.015</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>1.126 ± 0.037</td>
<td>3.286 ± 0.048</td>
<td>0.263 ± 0.004</td>
<td>0.234 ± 0.006</td>
</tr>
</tbody>
</table>

All values are means ± SD of least three independent determinations.

to catalyse conversion of most of the pHBA in vivo to pGBA. In the presence of DgpHBAGT, most of the pHBA is efficiently catalysed to pHBG; the possible occurrence of substitute enzyme activity could not be detected in in vitro reactions in the presence of such strong pHBAGT activity.

In conclusion, it is suggested that DgpHBAGT is the gene encoding pHBAGT. This suggestion is supported by the expression profile of DgpHBAGT, which shows a close phenotypic correlation with that of pHBAGT enzyme activity, the comparative levels of accumulation of pHBG in wild and mutant cultivar plants, the close relationship between the DgpHBAGT gene expression profile and level of pHBAGT activity and pHBG accumulation, and the biochemical data on enzyme activities of the recombinant proteins. Thus, DgpHBAGT is responsible for pHBG synthesis through encoding the enzyme that supplies a Zwitter donor in delphinium.

**Supplementary data**

Supplementary data are available at JXB online.  
**Figure S1.** AA7GT activities and expression profile of DgAA7GT in wild-type and mutant cultivars.  
**Figure S2.** Characterization of the structure of p-glucosyl-oxynbenzoic acid (pGBA).  
**Figure S3.** Characterization of the enzymatic properties of GST–DgpHBAGT.

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**References**


