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Co-pigmentation and flavonoid glycosyltransferases in blue *Veronica persica* flowers ☆

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ABSTRACT

Glycosylation is one of the key modification steps for plants to produce a broad spectrum of flavonoids with various structures and colors. A survey of flavonoids in the blue flowers of *Veronica persica* Poiret (Lamiales, Scrophulariaceae), which is native of Eurasia and now widespread worldwide, led to the identification of highly glycosylated flavonoids, namely delphinidin 3-*O*-(2-*O*-(6-*O*-*p*-coumaroyl-glucosyl)-6-*O*-*p*-coumaroyl-glucoside)-5-*O*-glucoside (1) and apigenin 7-*O*-(2-*O*-glucuronosyl)-glucuronide (2), as two of its main flavonoids. Interestingly, the latter flavone glucuronide (2) caused a bathochromic shift on the anthocyanin (1) toward a blue hue in a dose-dependent manner, showing an intermolecular co-pigment effect. In order to understand the molecular basis for the biosynthesis of this glucuronide, we isolated a cDNA encoding a UDP-dependent glycosyltransferase (UGT88D8), based on the structural similarity to flavonoid 7-*O*-glucuronosyltransferases (F7GAT) from Lamiales plants. Enzyme assays showed that the recombinant UGT88D8 protein catalyzes the 7-*O*-glucuronosylation of apigenin and its related flavonoids with preference to UDP–glucuronic acid as a sugar donor. Furthermore, we identified and functionally characterized a cDNA encoding another UGT, UGT94F1, as the anthocyanin 3-*O*-glucoside-2″-*O*-glucosyltransferase (A3Glc2″GlcT), according to the structural similarity to sugar–sugar glycosyltransferases classified to the cluster IV of flavonoid UGTs. Preferential expression of *UGT88D8* and *UGT94F1* genes in the petals supports the idea that these UGTs play an important role in the biosynthesis of key flavonoids responsible for the development of the blue color of *V. persica* flowers.

1. Introduction

Blue flowers are rare in nature. Limited examples includes dayflower (*Commelina communis*) and cornflower (*Centaurea cyanus*), both of which produce natural supramolecules known as metalloanthocyanins in their petals (Kondo et al., 1992; Shiono et al., 2005). Those metalloanthocyanins are formed through stoichiometric self-association of metal-pigment complexes, and metal complexation of flavonoid glycosides and intermolecular hydrophobic association confer pure blue coloration on flowers (Goto and Kondo, 1991; Yoshida et al., 2009). Recent studies on the metalloanthocyanins from two *Salvia* species with blue petals showed that the pigment complex consists of six molecules each of diglycosyl anthocyanins and diglycosyl flavones, although the composition of the flavonoids within the complex is different between the two species (Kondo et al., 2001; Mori et al., 2008). In the case of the Himalayan blue poppy (*Meconopsis grandis*) has a diglucosyl flavonol as a component of the metal complex-pigment in the sky-blue flower (Yoshida et al., 2006).

Co-pigmentation, in addition to the metal complex-pigment, is also well known to cause bathochromic shift by an intermolecular association that colored anthocyanin pigments and colorless/transparent co-pigments, such as flavone glycosides stack hydrophobically in an aqueous solution (Goto and Kondo, 1991). Co-pigmentation occurs only in solution in a non-stoichiometrical way, and confers a bluish color on flowers and fruits of plants, and also during fermentation processes of wines (Boulton, 2001). Flavones/flavonols *per se* exhibit pale yellow color; however, their role as co-pigments and UV-absorbents seem to be more significant on flower coloration, serving as key modulators of anthocyanin pigments for pollinator attraction (Asen et al., 1972;

Abbreviations: MeCN, acetonitrile; UDP, uridine diphosphate; GlcA, glucuronic acid; Glc, glucose; $^{1}H\{^{13}C\}$-HMBC, heteronuclear multiple bond correlation.

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Thompson and Meinwald, 1972). For instance, the *C*-glucosyl flavone isovitexin in the petals of the garden iris (*Iris ensata* Thunb.) is known to be a strong co-pigment co-existing with the iris anthocyanin, delphinidin 3-*O*-*p*-coumaroyl-rutinoside-5-*O*-glucoside (Yabuya et al., 1997). Moreover, apigenin 7-*O*-glucuronide and luteolin 7-*O*-glycoside have been reported as a strong co-pigment in snapdragon (*Antirrhium majus*) and wishbone flowers (*Torenia hybrida*), respectively (Asen et al., 1972; Aida et al., 2000). It is noteworthy that co-pigmentation efficiency depends on the structural affinity of modified pigments to co-pigments, as well as their concentrations, and vacuolar pH of the solution (Fukui et al., 2003). The fact that flavonoid composition in flowers with similar color varies among different species highlights the importance of the structural diversity of this class of pigments for developing ornamentals with novel flower colors (Katsumoto et al., 2007).

Plant secondary metabolites, or specialized metabolites, have extraordinary diverse core structures that are often further elaborated through modifications including glycosylation (Harborne and Baxter, 1999). Specific plant lineage has specialized flavonoids with unique glycosylation pattern, that are a consequence of the general differentiation of regio-specificity of the UGT enzyme followed by local differentiation of sugar donor specificity in a lineage-specific manner (Vogt and Jones, 2000; Noguchi et al., 2008, 2009). For example, flavone 7-*O*-glucuronide, a specialized metabolite of Lamiales plants, is produced by a flavonoid 7-*O*-glucuronosyltransferase (F7GAT), which is considered to be locally differentiated from flavonoid 7-*O*-glucosyltransferase (F7GlcT) by acquiring the binding specificity to UDP–glucuronic acid (UDP–GlcA) instead of UDP–glucose (Harborne, 1963; Yoshida et al., 1993; Hirotani et al., 1998; Yamazaki et al., 2003; Noguchi et al., 2009).

Here, we describe identification and functional characterization of two novel UGTs responsible for the blue flower color of *Veronica persica* Poiret (Lamiales, Scrophulariaceae), which is native to Mediterranean area and domesticates throughout temperate zone (Fig. 1A). Most recently, flavonoids that accumulated in the flowers of this plant were also reported (Mori et al., 2009) after this submission was received. Here we show the cloning of the two *V. persica* UGTs, one is involved in the biosynthesis of anthocyanin glycosides, and the other in the biosynthesis of flavone glucuronide co-pigments. Observation of bathochromic shift of the anthocyanin **1** by the addition of flavone glucuronide **2** *in vitro* further underscores the significance of the glycosylation of these major flavonoids for color development of the blue petals of *V. persica*.

2. Results and discussion

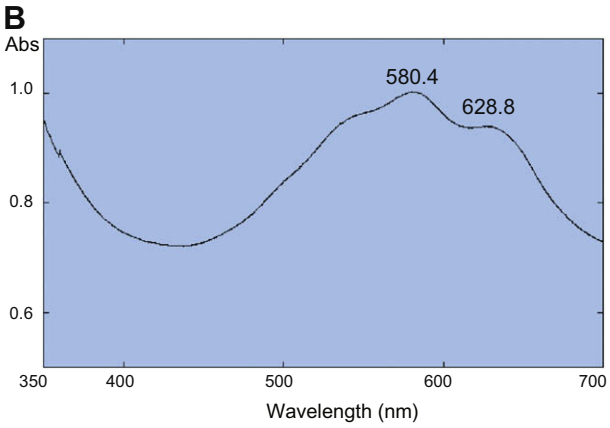
2.1. Measurement of pH and the $L^*a^*b^*$ color of Veronica petals

Vacuolar pH significantly affects the coloration of anthocyanin pigments and varies in each species. As the vacuole is the largest organella in petal cells, the value of pressed petal juice is often measured as the approximate vacuolar pH (Fukada-Tanaka et al., 2000; Verweij et al., 2008). The pH value of the petal juice of *V. persica* was estimated to be 6.2, which is relatively high compared to that of carnation and rose petals (Fukui et al., 2003; Katsumoto et al., 2007). The intact petal showed characteristic spectra with the two λ_{max} peaks at 580.4 and 628.8 nm (Fig. 1B). The petal surface colors in an $L^*a^*b^*$ colorimetric value were L^*; 48.01, a^*; 17.67, b^*; −33.49 and the hue was 297.8° (Fig. 1C).

2.2. Identification of flavonoid glycosides

High-resolution time-of-flight MS (HR-TOF-MS) analysis of isolated compounds **1** and **2** gave molecular ions at *m*/*z* 1081.2876, $[M]^+$ (calcd. 1081.2825, err: +4.7 ppm) and 623.1251, $[M+H]^+$ (calcd. 623.1248, err: +0.5 ppm), respectively. These values correlated to the masses calculated using their molecular formulae: $C_{51}H_{53}O_{26}$ and $C_{27}H_{26}O_{17}$, respectively. The λ_{max} of **1** was 541 nm in 10% MeCN in H_2O with 0.1% TFA.

After acid hydrolysis, the HPLC analysis of **1** indicated the presence of delphinidin (ret. time: 4.0 min, λ_{max}: 538 nm). We were able to establish full assignment data for the ^1H NMR and ^{13}C NMR spectra for **1** and **2** (see Supplementary Tables 1 and 2, respectively), using $^1H\{^{13}C\}$-HSQC, $^1H\{^{13}C\}$-HMBC, TOCSY, DQF-COSY, and ROESY. The ^1H NMR and ^{13}C NMR spectroscopic data of **1** indicated the presence of three glucosyl residues and two *p*-coumaroyl moieties. In the case of compound **1**, the analysis of the $^1H\{^{13}C\}$-HMBC established that the hydroxyl groups at the



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molar ratio	L *	a *	b *	H K	max (nm)	OD
Petal	48.01	17.67	-33.49	297.8	580, 629	
in vitro						
1 : 0	43.52	42.28	-56.75	306.6	615	1
1 : 1	41.94	38.64	-59.36	303.0	614	1.021
1 : 2	40.65	36.79	-60.30	301.4	632	1.031
1 : 3	39.96	34.98	-60.34	300.1	630	1.038
1 : 4	39.46	33.41	-60.02	299.1	639	1.045
1 : 5	39.59	31.65	-59.18	298.1	641	1.035
1 : 6	39.84	29.92	-58.13	297.2	645	1.037

Fig. 1. *Veronica persica* flowers and co-pigment effect. (A) Bluish flowers of *Veronica persica* (B) absorption spectra of intact petals of *V. persica* (C) $L^*a^*b^*$ color value and visible spectral data of co-pigment assay. Co-pigment analyses of compound **1** with **2** were measured in a McIlvaine buffer (pH 6.2). Compound **1** was dissolved in 1 mM concentration, and **2** was added in 1–6 equivalent to **1**. The hue (H) was shifted to blue in molar ratio dependent. The molar ratio is anthocyanin **1**: flavone **2**.