



No. 11-088087- -00007-0000

Señor Director
DIRECCION DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Fecha: 2013-05-03 16:26:12 Dep. 2020 DIR.NUEVASCR
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Act. 523 RESPUESTA Folios: 28

Trámite : 011
Evento : 378
Actuación : 444

Referencia : Expediente No. 11088087
Asunto : Solicitud de patente para: **MÉTODO PARA PRODUCIR UN CLAVEL QUE EXHIBE INFLORESCENCIA ALTERADA**
Solicitante : **SUNTORY HOLDINGS LIMITED**
Fecha de solicitud : 14 de julio de 2011

1. Yo, Luz Clemencia DE PÁEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, me refiero al oficio No. **23222** notificado por fijación en lista el 26 de diciembre de 2012.

2. Mediante oficio No. **23222** se puso en conocimiento del solicitante el informe que contiene las observaciones hechas por su Despacho sobre la presente solicitud de patente. Las objeciones presentadas en el oficio se refieren a que las reivindicaciones 1-20 y 22-23 no son patentables, a la falta de claridad del título de la invención y de la reivindicación 21 y a la falta de nivel inventivo de la reivindicación 21 en vista de los documentos D1: WO200501714 y D2: el artículo "Engineering of the rose flavonoid biosyntheti pathway succesfully generated blue-hued flowers accumulating delphinidin".

3. **Respuesta a las objeciones**

De este modo y con el objeto de atender las objeciones arriba mencionadas, se desea presentar las siguientes modificaciones, aclaraciones y argumentos:

"EXCELENCIA LEGAL EN UN MUNDO SIN FRONTERAS"

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3.1 Modificación de una solicitud en trámite

De acuerdo con el artículo 34 de la Decisión 486 de la Comisión de la Comunidad Andina, donde se establece que la modificación de una solicitud es posible en cualquier momento del trámite mientras no implique una ampliación de la solicitud inicial, me permito presentar un nuevo pliego reivindicatorio conformado por una única reivindicación; además nos permitimos presentar un nuevo título.

El título se ha modificado de la siguiente manera:

“Método para producir un clavel que exhibe inflorescencia alterada.”

Se han introducido las siguientes modificaciones al pliego reivindicatorio:

- Se eliminaron las reivindicaciones 1-20 y 22-23.
- Se modificó la reivindicación 21 para incorporar las secuencias de nucleótidos del material genético y ahora corresponde a la reivindicación 1 del presente pliego reivindicatorio.

Por lo anterior, las objeciones formuladas por el Examinador de excepción a la patentabilidad y de claridad se ven superadas. En consecuencia, se solicita atentamente tener en cuenta este nuevo pliego reivindicatorio para el nuevo análisis de patentabilidad.

3.2 Argumentos a favor del nivel inventivo

De acuerdo con el artículo 18, de la Decisión 486 de la Comunidad Andina, el cual se refiere al nivel inventivo de la invención, se tiene lo siguiente:

Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

Consecuentemente, una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo, para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

La presente solicitud sí tiene nivel inventivo

El Examinador objeto la invención de la reivindicación 21 basado en los documentos D1 (WO 2005/017147) y D2 (Plant Cell Physiol. (2007) 48 (11) :1589-1600) argumentando que una persona versada en la materia podría de manera obvia derivar un método para modificar claveles basado en D1, ya que rosas y claveles muestran las mismas rutas metabólicas para el color.

Sin embargo, respetablemente el solicitante manifiesta su desacuerdo con el punto de vista del Examinador por las razones que se explicaran a continuación y solicita que el nivel inventivo de esta solicitud sea reconocido.

Para determinar una invención como obvia, tanto las sugerencias de la invención reivindicada como la expectativa de éxito deben encontrarse en el arte previo, no en lo divulgado en la presente solicitud. Adicionalmente, para determinar lo obvio se requiere que en el arte previo haya sugerencias de todas las limitaciones en una reivindicación. La Oficina de patentes también debe establecer que una persona versada en la materia debió tener una expectativa de éxito razonable para ejercer con la invención reivindicada.

Teniendo en cuenta lo anterior, la reivindicación 1 modificada menciona cuatro diferentes materiales de expresión de genes:

(i) una enzima flavonoide 3',5' hidroxilasa (F3'5'H) no nativa, codificada por la secuencia de nucleótidos de SEQ ID NO:1;

(ii) una enzima dihidroflavonol 4- reductasa DFR no nativa, codificada por la secuencia de nucleótidos de SEQ ID NO: 3;

(iii) secuencias de nucleótidos de sentido y antisentido (*ds carnDFR*) que corresponden al gen DFR del clavel e incorporando un fragmento o fragmentos de la secuencia de nucleótidos de SEQ ID No: 9, regulando por disminución la expresión del gen *DFR* nativo del clavel; y

(iv) o una S-adenosilmetionina: antocianina 3'5' metiltransferasa (ThMT) no nativa codificada por la secuencia de nucleótidos de SEQ ID NO: 11 o una flavona sintasa (ThFNS) codificada por la secuencia de nucleótidos de SEQ ID NO: 13.

Todo el material genético es definido por sus **secuencias específicas**.

Es importante notar que en el documento D1 no se describe para nada la introducción de ThMT o ThFNS.

Ni en el documento D1 o D2, se enseña sobre un clavel genéticamente modificado para exhibir una inflorescencia alterada, o sobre el clavel o su progenie que contiene al menos un material genético expresable que expresa los cuatro diferente materiales de expresión de genes, mencionados previamente y caracterizados por sus secuencias específicas. Si no se enseñan todos los elementos de la reivindicación no habría una expectativa de éxito razonable para hacer el producto de la solicitud predecible.

Además, como se estableció previamente, los documentos D1 y D2 no se relacionan a una planta de clavel, sino a una planta de rosas. Teniendo en cuenta que una rosa es distinta a las especies de clavel y que el cambio de color en claveles requiere de una combinación estructural y/o secuencial delicada de los cuatro materiales de expresión de genes que son definidos por secuencias específicas que se mencionaron arriba, una persona versada en la materia no tendría una expectativa de éxito razonable en introducir/expresar una F3'5'H no nativa, una DFR no nativa que regula por disminución la expresión del gen DFR nativo del clavel e introducir/expresar o una S-adenosilmetionina: antocianina 3'5' metiltransferasa (ThMT) no nativa o una flavona sintasa (ThFNS) no nativa (todos definidos en la reivindicación 1 modificada) a un clavel para lograr el cambio de color (más azul). En otras palabras en un organismo vivo complicado, como lo es la planta del clavel es totalmente inesperado esperar que la

pluralidad del material de expresión de genes pueda funcionar correctamente sin ningún efecto adverso. Por estas razones se presenta que la invención de la nueva reivindicación 1 tiene nivel inventivo sobre D1 y D2.

Los inventores de esta solicitud primero encontraron que si $F3'5'H\uparrow + DFR\uparrow + DFR\downarrow$ nativa + (ThMT $\uparrow$ y ThFNS $\uparrow$), flavona y malvidin son producidas y si $F3'5'H\uparrow + DFR\uparrow +$ nativa $DFR\downarrow + (ThFNS\uparrow)$, flavona y delfinidina son producidas. Teniendo en cuenta que $\uparrow$ significa una regulación por aumento y $\downarrow$ significa una regulación por disminución.

Es importante notar que aunque sea esperado que el efecto del copigmento de flavona y malvidin haga la flor de color más azul, la proporción de las cantidades de flavona y malvidin es crítico para realmente poder llevar a cabo dicho efecto del copigmento en plantas de claveles. Incluso si se introduce el gen de la flavona sintetasa (FNS), el gen flavonoide 3',5' hidroxilasa ($F3'5'H$) y el gen de la antocianina metiltransferasa (MT), una persona versada en la materia no podría anticipar las cantidades y la proporción de los productos en las plantas de claveles. La proporción óptima de la cantidad de flavona y malvidin depende del grado en que los genes son expresados en el clavel. Los inventores encontraron que la combinación específica de los genes FNS, $F3'5'H$, ThMT y ThFNS en la célula hospedera resultaban en la proporción necesaria de flavona y malvidin para hacer la flor del clavel de una apariencia de color más azul en el ambiente.

De forma similar los inventores de esta solicitud encontraron que si $F3'5'H\uparrow + DFR\uparrow + DFR\downarrow$ nativa + ThMT $\uparrow$, malvidin es producida. Antocianina se acumula generalmente en vacuolas. En las plantas de claveles la síntesis de antocianina por medio de delfinidina 3-glucosida se lleva a cabo en el citoplasma, y luego es transferida a vacuolas en donde se les adhiere un sacárido a las antocianinas en la posición 5 y luego el sacárido es malonilado. Anexamos a este memorial el artículo de The Plant Cell, Vol. 22: 3374-3389, Octubre 2010 (Tabla 2, pág. 3382) en donde se muestra que en la glicosilación en la posición 5, delfinidina 3-glucosido es el sustrato óptimo. Sin embargo, si

ThMT es introducido en la planta del clavel, las antocianinas son metiladas en el citoplasma, pero para una persona versada en la materia es totalmente inclaro si dichas antocianinas metiladas son transferidas correctamente a vacuolas. De esta manera, no hay una expectativa razonable de éxito que hiciera el producto reivindicado predecible.

4.4 Conclusiones

Mediante el nuevo pliego reivindicatorio se superaron las objeciones relativas a claridad. Con respecto al nivel inventivo, consideramos que el Examinador está excluyendo el hecho de que el inventor en el momento de solucionar el problema técnico de la solicitud tuvo que emplear habilidad inventiva, pues la presente invención no resulta de la mera extrapolación de las enseñanzas del arte previo, como se afirma. Se está desconociendo el mérito investigativo empleado y que está aportando una ventaja al arte previo.

De ésta forma se demostró la presencia de los requisitos de patentabilidad de toda nueva creación en la presente solicitud, para así ser merecedora de su concesión de patente.

5. Puesto que se ha dado respuesta a todas las objeciones formuladas por el Examinador, se solicita atentamente proceder al otorgamiento de la solicitud de patente. En este sentido, respetuosamente recordamos al Examinador que la patente no podrá ser negada con base en objeciones diferentes a las expuestas en la acción oficial No. 23222, toda vez que al no haber sido trasladadas a mi representada, dicha decisión violaría tanto el tenor literal del artículo 45 de la Decisión 486 como el derecho constitucional al debido proceso. En este evento, y con base en el inciso 2 del artículo 45, respetuosamente solicitamos al Examinador expedir una nueva acción oficial que permita a mi representada contra argumentar las mismas o modificar la solicitud con el fin de cumplir los requisitos de patentabilidad.

6. Anexos

- Formulario de modificaciones en solicitud pendiente
- Nuevo pliego reivindicatorio conformado por 1 reivindicación
- Artículo de The Plant Cell, Vol. 22: 3374-3389, Octubre 2010

Señor Director,


LUZ CLEMENCIA DE PÁEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

 COLO-17511-0841-003-4
FBJ\FBJ\ID-241050\WPC17511
No. M-2013-241050\FBJ

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DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES

1. Identificación del Trámite

☐ Error material

☒ Modificación a una Solicitud en trámite

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural

☐

Persona Jurídica

☒

SUNTORY HOLDINGS LIMITED

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa Micro ☐ Pequeña ☐ Mediana ☐ Otra ☐

Documento de identificación: ☐ C.C. ☐ C.E. ☐ NIT ☐ Otro Número _____

Nacionalidad/País de constitución	Dirección y domicilio del titular	Ciudad
Japón	1-40, Dojimahama 2-chome, Kita-ku, Osaka-shi, Osaka 530-8203, Japón	Osaka
Dirección electrónica		No. Fax
clientes@cavelier.com		2 11 86 50
		Número telefónico
		3 47 36 11

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

Apellidos y Nombre
De Páez Luz Clemencia

Documento de identificación
C.C. 35.456.344

Tarjeta profesional
T.P.A. 23.555

Dirección y domicilio		Ciudad
Carrera 4ª No. 72 –35		Bogotá
Dirección electrónica	No. Fax	Número telefónico
cavelier@cavelier.com	2 11 86 50	3 47 36 11

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general

4. Datos del trámite

Corrección:

Expediente No 11.088.087

Aparece

Debe ser

Modificación:**Modificación al capítulo reivindicatorio.**

Presento nuevo capítulo reivindicatorio compuesto por 1 reivindicación y solicito que el examen de patentabilidad sea realizado sobre estas nuevas reivindicaciones presentadas.

Respetuosamente manifiesto que a pesar de eliminar materia del pliego reivindicatorio originalmente presentado, mi representada no renuncia a la protección que pueda obtener sobre dicha materia y se reserva el derecho de presentar una solicitud divisional que la contenga durante el trámite de la presente solicitud, beneficiándose de la fecha de prioridad inicialmente reivindicada.

**Modificación al capítulo descriptivo.****Modificación al resumen.****Modificación a los dibujos.****Modificación al Título.**

Solicito respetuosamente a su despacho tener en cuenta que el nuevo título de esta solicitud de patente ha quedado como sigue: **"MÉTODO PARA PRODUCIR UN CLAVEL QUE EXHIBE INFLORESCENCIA ALTERADA."**

5. Anexos

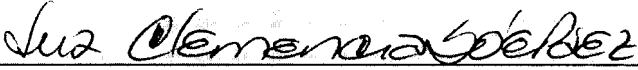
Poder, si fuere el caso.



Documento que contiene las modificaciones o correcciones.



Copia de la solicitud y sus anexos en formato magnético.

6. Firma		
Nombre y apellidos	Firma	
LUZ CLEMENCIA DE PAEZ		
C.C. 35.456.344 DE Usaquén	Tarjeta Profesional 23.555	

FBJVCBWPC17511\ID-241385

REIVINDICACIONES MODIFICADAS

1. Un método para producir un clavel que exhibe inflorescencia alterada, comprendiendo el citado método los pasos de:

introducir en células regenerables de una planta de clavel por lo menos un material genético expresable que expresa

una enzima flavonoide 3',5' hidroxilasa (F3'5'H) no nativa, codificada por la secuencia de nucleótidos de SEQ ID NO:1, o la secuencia de nucleótidos capaz de hibridarse a una forma complementaria de la secuencia de nucleótidos de SEQ ID NO:1 bajo condiciones de astringencia alta;

una enzima dihidroflavonol 4- reductasa DFR no nativa, codificada por la secuencia de nucleótidos de SEQ ID NO:3 o una secuencia de nucleótidos capaz de hibridarse a una forma complementaria de la secuencia de nucleótidos de SEQ ID NO:3 bajo condiciones de astringencia alta;

secuencias de nucleótidos de sentido y antisentido (*ds carnDFR*) que corresponden al gen DFR del clavel y que incorporan un fragmento o fragmentos de la secuencia de nucleótidos de SEQ ID No:9 o una secuencia de nucleótidos capaz de hibridarse a la forma complementaria de la secuencia de nucleótidos de SEQ ID NO: 9 bajo condiciones de astringencia alta, que regula por disminución la expresión del gen *DFR* nativo del clavel; y entre

una S-adenosilmetionina: antocianina 3'5' metiltransferasa (ThMT) no nativa codificada por la secuencia de nucleótidos de SEQ ID NO: 11; o

flavona sintasa (ThFNS) codificada por la secuencia de nucleótidos de SEQ ID NO: 13, y

regenerar a partir de ésta una planta de clavel.

A Novel Glucosylation Reaction on Anthocyanins Catalyzed by Acyl-Glucose–Dependent Glucosyltransferase in the Petals of Carnation and Delphinium

Yuki Matsuba,^{a,1} Nobuhiro Sasaki,^{a,1} Masayuki Tera,^a Masachika Okamura,^b Yutaka Abe,^{a,2} Emi Okamoto,^a Haruka Nakamura,^a Hisakage Funabashi,^a Makoto Takatsu,^a Mikako Saito,^a Hideaki Matsuoka,^a Kazuo Nagasawa,^a and Yoshihiro Ozeki^{a,3}

^a Department of Biotechnology and Life Science, Faculty of Engineering, Tokyo University of Agriculture and Technology, Tokyo 184-8588, Japan

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Glucosylation of anthocyanin in carnations (*Dianthus caryophyllus*) and delphiniums (*Delphinium grandiflorum*) involves novel sugar donors, aromatic acyl-glucoses, in a reaction catalyzed by the enzymes acyl-glucose–dependent anthocyanin 5 (7)-O-glucosyltransferase (AA5GT and AA7GT). The AA5GT enzyme was purified from carnation petals, and cDNAs encoding carnation Dc AA5GT and the delphinium homolog Dg AA7GT were isolated. Recombinant Dc AA5GT and Dg AA7GT proteins showed AA5GT and AA7GT activities in vitro. Although expression of Dc AA5GT in developing carnation petals was highest at early stages, AA5GT activity and anthocyanin accumulation continued to increase during later stages. Neither Dc AA5GT expression nor AA5GT activity was observed in the petals of mutant carnations; these petals accumulated anthocyanin lacking the glucosyl moiety at the 5 position. Transient expression of Dc AA5GT in petal cells of mutant carnations is expected to result in the transfer of a glucose moiety to the 5 position of anthocyanin. The amino acid sequences of Dc AA5GT and Dg AA7GT showed high similarity to glycoside hydrolase family 1 proteins, which typically act as β -glycosidases. A phylogenetic analysis of the amino acid sequences suggested that other plant species are likely to have similar acyl-glucose–dependent glucosyltransferases.

INTRODUCTION

Anthocyanins are one of the colored secondary metabolites responsible for the colorful variations in flowers. Only six types of anthocyanin aglycone are produced in the plant kingdom; the rich variety of flower colors is principally the result of differences in the modification of these compounds with glycosyl and acyl moieties to generate thousands of molecular variants (Tanaka et al., 2008). One of the simplest anthocyanins is 3-mono-O-glucopyranosyl anthocyanidin (anthocyanidin 3-glucoside), which is synthesized using UDP-glucose as a donor molecule and anthocyanidin aglycone as an acceptor by UDP-glucose–dependent anthocyanin 3-O-glucosyltransferase (UA3GT). UA3GT activity has been detected in several plant species, and the gene encoding the enzyme was first identified as the *Bz1* allele in maize (*Zea mays*; Furtek et al., 1988). This identification was rapidly followed by isolation of homologous genes and cDNAs

from many other species. In some plant species, other sugar moieties, such as galactose or arabinose, are conjugated at the 3 position of anthocyanidins (Yonekura-Sakakibara et al., 2009). The other typical glycosylation site is the 5 position of anthocyanidins to give 3,5-di-O-glucopyranosyl anthocyanins. UDP-glucose–dependent 5-O-glucosyltransferase was first identified in *Perilla frutescens*, and the gene encoding the enzyme was determined by differential display (Yamazaki et al., 1999). Subsequently, the enzyme was also shown to be present in *Petunia hybrida* (Yamazaki et al., 2002), *Iris hollandica* (Imayama et al., 2004), and *Gentiana scabra* (Nakatsuka et al., 2008). One exception is *Rosa hybrida* in which the same enzyme sequentially catalyzes glycosylation at the 5 position and the 3 position of the anthocyanidin (Ogata et al., 2005). In some species, a glucosyl moiety is attached to the 3' position of the B ring of anthocyanidin 3-glucoside by a UDP-glucose:anthocyanin 3'-O-glucosyltransferase; the activity of this enzyme, and the gene that encodes the enzyme, were first identified in *G. scabra* (Fukuchi-Mizutani et al., 2003).

Although the glycosylation of anthocyanidins can involve cytosolic enzymes using UDP-sugars as donors (Osmani et al., 2009), not all of the potential glycosylation reactions have been elucidated (Davies, 2009; Yonekura-Sakakibara et al., 2009). Carnations (*Dianthus caryophyllus*) are one of the most important horticultural species grown for their flowers. The major anthocyanin in the typical pink petals of carnations is 3,5-di-O-glucopyranosyl pelargonidin 6''-O-4,6'''-O-1-cyclic malate (Pg3,5cyclicmalylDg) (Nakayama et al., 2000). The enzyme UA3GT is active in carnation petals during the biosynthesis of this anthocyanin; a candidate

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Yoshihiro Ozeki (ozeki@cc.tuat.ac.jp).

 Some figures in this article are displayed in color online but in black and white in the print edition.

 Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.110.077487

cDNA encoding the enzyme has been isolated, but the 5-O-glucosylation reaction has not yet been identified in the crude extract prepared from carnation petals (Ogata et al., 2004). The 7-O-glucosylation reaction for anthocyanins in petals of delphinium (*Delphinium grandiflorum*), cineraria (*Senecio hybrida*), Himalayan blue poppy (*Meconopsis grandis*), and anemone (*Anemone coronaria*) flowers remains similarly uncertain (Davies, 2009; Yonekura-Sakakibara et al., 2009). There are two possible reasons for this failure: first, extremely low activity or instability of UA5GT or UA7GT in the crude extract; second, the glucosyltransferase at the 5 or 7 position does not use UDP-glucose as a donor.

Here, we show that in carnation petals, an acyl-glucose, 1-O- β -D-vanillyl-glucose (VG), acts as the donor molecule in the glucosyl transfer reaction at the 5 position of anthocyanin. VG also acts as the sugar donor in the 7-O-glucosylation reaction in delphinium petals. We also show that this reaction is catalyzed by the acyl-glucose-dependent anthocyanin 5-O-glucosyltransferase (AA5GT) in carnation and by AA7GT in delphinium (Figure 1).

RESULTS

Isolation and Identification of VG from the Petals of Carnation

We screened protein-free extracts of carnation petals prepared using 50% ethanol followed by fractionation on an octa decyl silyl

(ODS) open column and eluted with 5% methanol to identify sugar donor candidates. Contamination by anthocyanins in fractions over 5% methanol precluded their use for purification of the candidates. The presence of candidate donors in the 5% methanol eluate fraction was tested using a crude protein extract prepared from carnation petals and with cyanidin 3-glucopyranoside (Cy3G) as the acceptor. After this reaction, an additional peak was observed in an HPLC chromatogram. The retention time of the peak, its UV-visible spectrum, and mass spectrum corresponded to those expected of cyanidin 3,5-diglucopyranoside (Cy3,5dG). On the basis of this *in vitro* identification, we purified a donor candidate molecule from the 5% methanol eluate fraction by three HPLC purification steps. The purified donor substrate was analyzed by high-resolution electrospray ionization-mass spectrometry, which gave a mass-to-charge ratio (m/z) of 352.95257 (positive ion mode) corresponding to $C_{14}H_{18}NaO_9$. The structure was analyzed using 1H - and ^{13}C -NMR spectroscopy. This analysis indicated that the compound was comprised of one vanillyl moiety and one glucosyl moiety with a bound ester. The anomeric proton 1H -NMR coupling constant of the glucosyl moiety ($J = 7.8$) suggested that the compound might have β -conjugation. Since there was still a possibility of α - and not β -conjugation, we synthesized the α - and β -conjugated molecules (see Supplemental Figure 1 online). The configuration of the anomeric center was confirmed by the coupling constant (α , $J = 3.7$ Hz; β , $J = 8.0$ Hz). This showed that the molecular structure of the donor substrate prepared and

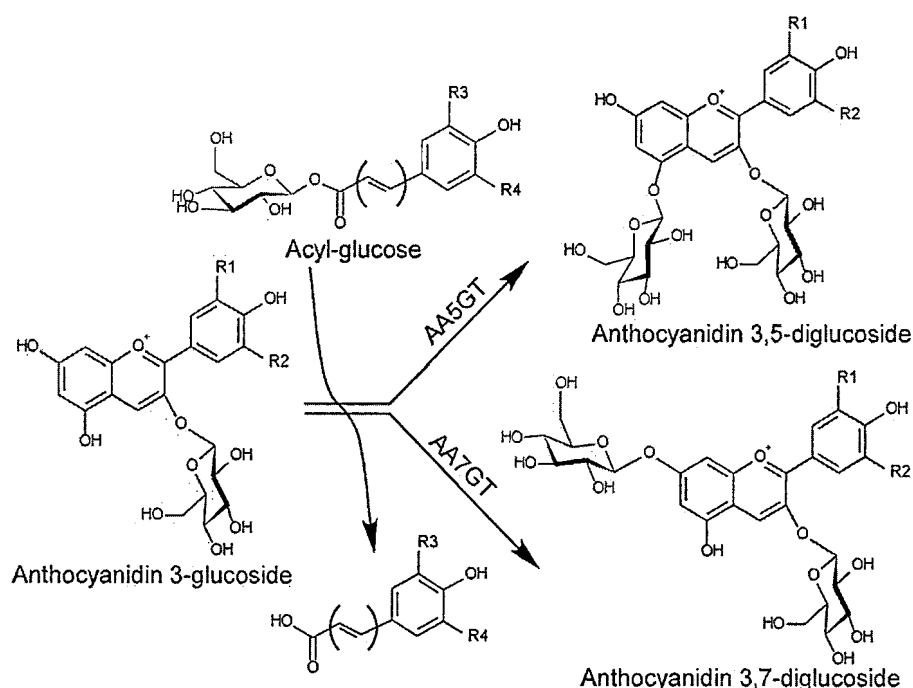


Figure 1. Glucosyl Transfer Reaction Catalyzed by AA5GT and AA7GT.

R1 = H, R2 = H, pelargonidin; R1 = OH, R2 = H, cyanidin; R1 = OH, R2 = OH, delphinidin. Representative acyl-glucose molecules, hydroxycinnamoyl-glucoses (C6-C3-glucoses, HCAGs) (e.g., feruloyl-glucose; R3 = OMe, R4 = H). Hydroxybenzoyl-glucoses (C6-C1-glucoses, lacking two carbons of HCAG shown in parentheses) (e.g., vanillyl-glucose [R3 = OMe, R4 = H] and *p*-hydroxybenzoyl-glucose [R3 = H, R4 = H]).

[See online article for color version of this figure.]

purified from carnation petals was VG. Using chemically synthesized VG and Cy3G as substrates, we found glucosyltransferase activity at the 5 position of anthocyanin in the crude extract prepared from carnation petals (Figure 2A); we designated the enzyme responsible for this activity as AA5GT. We were also able to detect glucosyltransferase activity in crude protein extracts prepared from delphinium petals, again using Cy3G and VG as the substrates (Figure 2B). Since the major anthocyanin in delphiniums, cyanodelphin, is glucosylated at both the 3 and 7 positions of the aglycone (Kondo et al., 1991), the product peak in the chromatogram after the reaction was

expected to be the Cy3,7dG molecule; a subsequent experiment confirmed this.

Purification of AA5GT Protein from Carnation Petals and Isolation of cDNAs for AA5GT and AA7GT from Carnation and Delphinium

Using chemically synthesized VG as the donor molecule, the AA5GT protein was purified from a crude protein extract obtained from 400 g of carnation petals using seven purification steps (see Supplemental Table 1 online). The purified protein

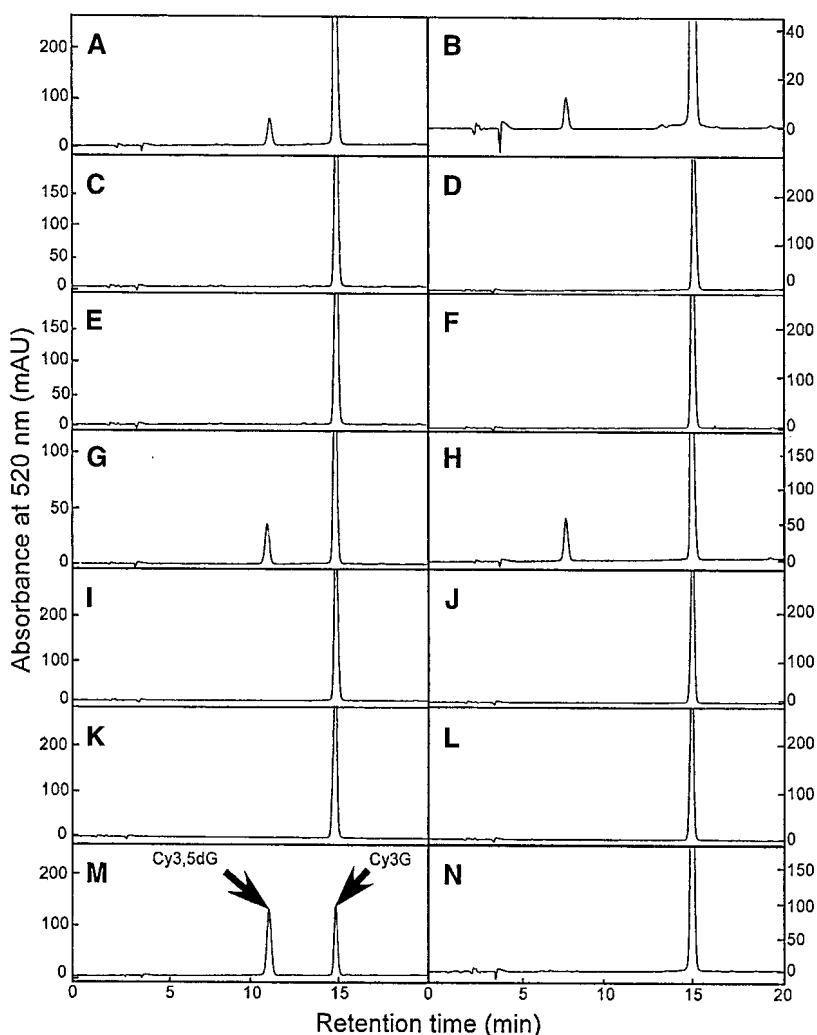


Figure 2. Detection of Dc AA5GT and Dg AA7GT Activity Using Cy3G as an Acceptor.

(A), (C), and (E) Crude protein extracts from carnation petals using VG (A) and UDPG (C) as a donor or lacking any donor (E).

(B), (D), and (F) Crude protein extract from delphinium petals using VG (B) and UDPG (D) as a donor or lacking any donor (F).

(G), (I), and (K) Crude extracts from *E. coli* harboring an expression vector containing Dc AA5GT cDNA using VG (G) and UDPG (I) or lacking any donor (K).

(H), (J), and (L) Crude extracts from *E. coli* harboring an expression vector containing Dg AA7GT cDNA using VG (H) and UDPG (J) as a donor or lacking any donor (L).

(M) Authentic standards of Cy3G and Cy3,5dG. The enzymatic reaction product of each peak fraction separated by HPLC was analyzed by mass spectrometry and confirmed as m/z 611 (positive ion mode).

(N) Crude extracts from negative control *E. coli* harboring *lacZ* using VG as a donor.

mAU, milli absorbance units.

appeared as a single band with a molecular mass of 55 kD on a silver-stained SDS-PAGE (see Supplemental Figure 2 online).

The amino acid sequences of the peptide fragments obtained by lysyl-endopeptidase digestion of the purified AA5GT enzyme were GTQPHVTLLH(S)D, FTPXETELLTG(S), and GLEYNNLV-NAXL; the N terminus sequence was SEFDRLDFPKH and lacked the first Met. Using degenerate primers based on these peptide sequences, a cDNA fragment was obtained with the first-strand cDNA prepared from carnation petals as the template. A full-length cDNA was obtained using 5'- and 3'-rapid amplification of cDNA ends (RACE) and was designated Dc AA5GT cDNA. This cDNA contained an open reading frame of 1506 bp encoding 502 amino acids. The deduced amino acid sequence contained all four peptide fragments described above and contained 30 additional amino acids at the N terminus deleted in the mature protein (see Supplemental Figure 3 online). The molecular mass of the deduced amino acid sequence of Dc AA5GT following removal of the additional sequence was predicted as 54 kD, a close approximation to the 55 kD of the native AA5GT on the SDS-PAGE. Analysis of the additional sequence using WoLF PSORT predicted that it might be a signal sequence for a transit peptide to the vacuole, described in detail later.

The degenerate primers used to isolate Dc AA5GT cDNA were also used to isolate a cDNA candidate encoding acyl-glucose-dependent anthocyanin 7-O-glucosyltransferase (Dg AA7GT) from the first-strand cDNA prepared from delphinium petals. To confirm that the Dc AA5GT and Dg AA7GT cDNAs encoded the AAGT protein, the cDNAs were introduced into an expression vector in which the putative transit peptide was deleted but in which the first Met had been added; the recombinant protein was expressed in *Escherichia coli*. Dc AA5GT and Dg AA7GT activities were detected using Cy3G and VG as the substrates in crude extracts of *E. coli* harboring the expression vector containing Dc AA5GT or Dg AA7GT cDNA (Figures 2G and 2H). As an authentic Cy3,7dG molecule is currently unavailable, the enzymatic product obtained from the Dg AA7GT reaction was isolated and analyzed using electrospray ionization-mass spectrometry and NMR spectrum to confirm that it had the expected characteristics of Cy3,7dG (see Supplemental Figure 4 online).

The deduced amino acid sequences of Dc AA5GT and Dg AA7GT (see Supplemental Figure 3 online) showed high similarities to those of β -glycosidase of *Arabidopsis* (Xu et al., 2004), rice (*Oryza sativa*; Opassiri et al., 2006), and grape (*Vitis vinifera*), indicating that they are GH1 proteins. GH1 proteins are believed to have roles in fundamental processes, such as chemical defense against herbivory (Rask et al., 2000; Morant et al., 2008), lignification (Dharmawardhana et al., 1995), hydrolysis of cell wall-derived oligosaccharides (Leah et al., 1995), and regulation of phytohormones (Falk and Rask, 1995). A phylogenetic tree analysis based on the amino acid sequences of β -glycosidases of various plant species showed that they formed independent clades according to their predicted activities: myrosinases, mannosidases, enzymes concerned with defense responses in monocots and dicots, and lignification, among others (Figure 3; see Supplemental Figure 5 and Supplemental Table 2 online). The alignment analysis revealed that Dc AA5GT and Dg AA7GT amino acid sequences are close to the β -glycosidases BGLU1 to BGLU11 of *Arabidopsis thaliana*.

Although the expression profiles of these glycosidases have been described, their functions are still uncertain (Xu et al., 2004).

Subcellular Localization of Dc AA5GT and Dg AA7GT and Expression of Dc AA5GT in Carnation Petals

The increased knowledge of genome DNA sequences produced by the various genome projects has enabled the identification of all GH1 proteins of *Arabidopsis* (Xu et al., 2004) and rice (Opassiri et al., 2006). Additionally, sequencing data predict that most of these proteins have the transit peptide sequences at the N-terminal region that are necessary for localization in the endoplasmic reticulum (ER), microbody (peroxisome), mitochondrial matrix, plasma membrane, and for sequestration into the vacuoles. As mentioned above, WoLF PSORT predicted that the N-terminal amino acid sequences of Dc AA5GT and Dg AA7GT contain the putative transit peptide necessary for localization in the vacuole. To confirm the subcellular localization of Dc AA5GT and Dg AA7GT, green fluorescent protein (GFP) fusion constructs containing either the full-length or the predicted transit peptide sequences of Dc AA5GT and Dg AA7GT driven by cauliflower mosaic virus (CaMV) 35S promoter were introduced into onion epidermal cells by microprojectile bombardment. We observed a close correspondence in the distribution pattern for Dc AA5GT-transit-peptide:GFP (Figure 4A), Dg AA7GT-transit-peptide:GFP (Figure 4B), and full-length-Dg AA7GT:GFP (Figure 4D) with that of a Gly-rich protein of *Arabidopsis* (GRP5) that is normally located in the vacuoles (Figure 4G) (Mangeon et al., 2009). The full-length-Dc AA5GT:GFP did not show strict localization of fluorescent signals to the vacuole but also had some signals in the cytosol (Figure 4C). It is possible that the distribution of transiently expressed full-length-Dc AA5GT:GFP in onion cells might differ from that of the native Dc AA5GT following removal of the transit peptide in the cells of carnation petals. Nevertheless, our results suggest that the vacuole is a strong candidate location as the final destination of Dc AA5GT and Dg AA7GT.

We examined Dc AA5GT expression profiles, AA5GT activity, and anthocyanin accumulation during petal development to gain further insights into the physiological interrelationships of these factors (Figure 5). Although peak Dc AA5GT expression was observed at stage 2 of petal development, AA5GT activity continued to increase to stage 4. The amount of total anthocyanin was measured by spectrophotometrical analyses of the 80% methanol extract containing 0.1% trifluoroacetic acid and was found to increase at stage 4. HPLC analysis of anthocyanin molecules at each stage of petal development showed that Pg3,5cyclicmalyldG, but not pelargonidin 3-O-malylglucopyranoside (Pg3malyIG), was detectable. As discussed below, if the AA5GT protein is stable, then it is possible that the active protein might accumulate even after stage 3 when transcripts for Dc AA5GT decreased. A sufficient amount of Dc AA5GT protein to transfer glucose at the 5 position might accumulate in cells after stage 2. Expression of Dc AA5GT increased at an earlier stage of petal development than for other genes involved in anthocyanin synthesis, such as *chalcone synthase* (CHS), *dihydroflavonol reductase* (DFR), *anthocyanidin synthase* (ANS), or *UA3GT* (Figure 5C). Dc AA5GT transcripts and AA5GT activity were not

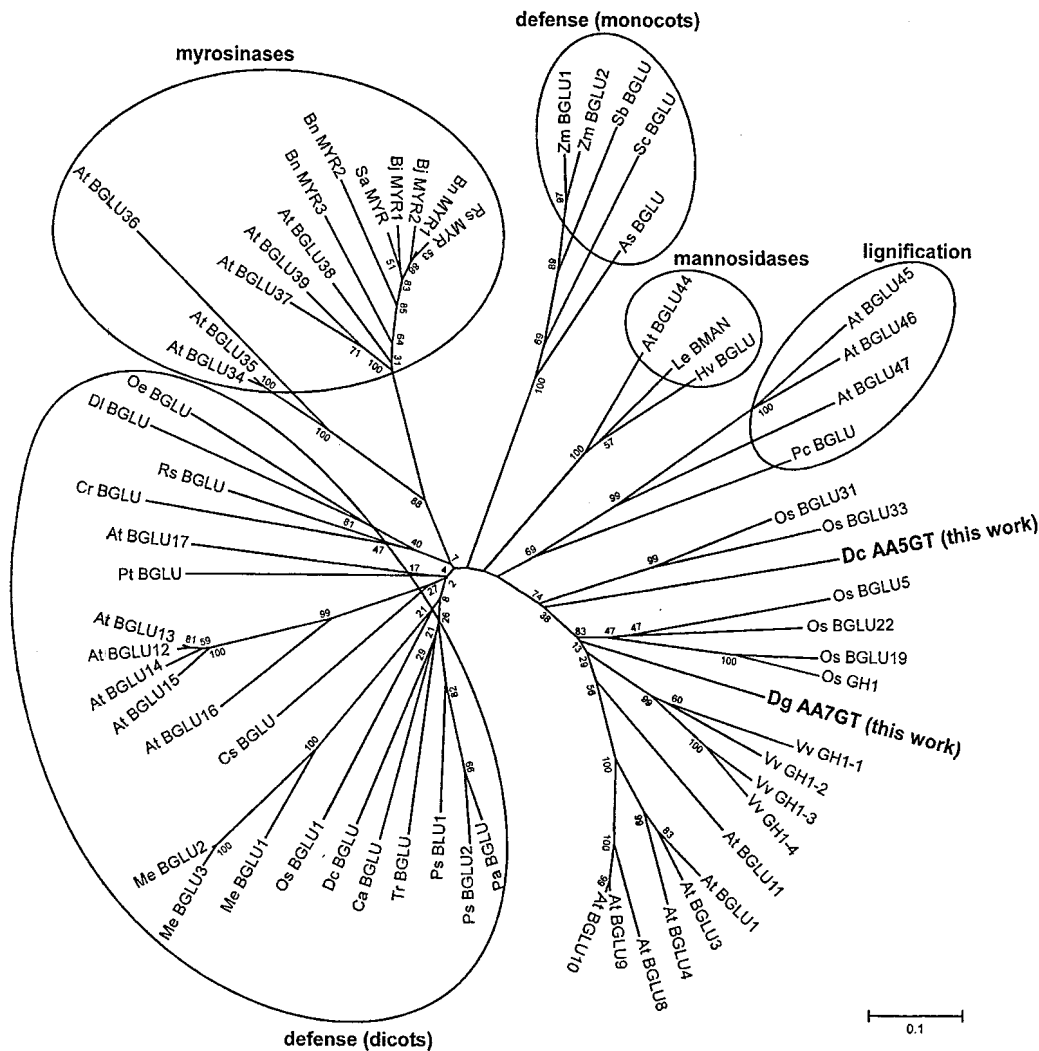


Figure 3. A Molecular Phylogenetic Tree of Glycoside Hydrolase Family 1 Members Based on Amino Acid Sequences.

Putative roles have been ascribed to several genes of *Arabidopsis* (Xu et al., 2004), rice (Opassiri et al., 2006), and grape. Other genes have known biochemical functions. Bar = 0.1 amino acid substitutions/site.

detectable in leaves or stems, nor could we detect expression of *DFR*, *ANS*, or *UA3GT*. Expression of *CHS*, which might be expected because flavonoids accumulate in these tissues, was also difficult to detect (Figure 5C). *Dc AA5GT* transcripts and *AA5GT* activity were not detected in carnation varieties that lack the glucosyl moiety at the 5 position on major anthocyanins (Figure 6A). These results suggest that *Dc AA5GT* might be involved in anthocyanin synthesis in carnation petals.

Anthocyanins glucosylated at the 5 position fluoresce on thin layer chromatographs, whereas those not glucosylated do not fluoresce (Markham, 1982). As is shown in the right panel of Figure 6F, petals that accumulated cyanidin with glucosylation of both the 3 and 5 positions showed bright fluorescence with emission at >580 nm, but those that accumulated cyanidin glucosylated only at the 3 position did not fluoresce when illuminated with excitation light at 520 to 550 nm (Figure 6F, left panel). Thus, fluorescence might be a good marker for the synthesis and accumulation of anthocyanins glucosylated at the

5 position in vivo. We performed a microprojectile bombardment experiment with *Dc AA5GT* cDNA driven by CaMV 35S promoter construct (35S-*Dc AA5GT*) and GFP driven by CaMV 35S promoter (35S-*GFP*); the latter acted as a control for successful introduction and expression of the genes. Petals of a mutant carnation that synthesizes and accumulates cyanidin 3-O-malylglucopyranoside (Cy3malylG) showed bright fluorescent spots after bombardment (Figure 6B). Cells showing fluorescence associated with anthocyanin glucosylation also showed GFP fluorescence (Figure 6C), suggesting that these cells received both 35S-*Dc AA5GT* and 35S-*GFP* and expressed both proteins. By contrast, introduction of a construct with deletion of the sequence for the putative transit peptide to vacuoles did not produce glucosylation-associated fluorescence (Figure 6D). These results strongly suggest that *Dc AA5GT* is expressed and processed in the transformed cells and that it may be involved in the transfer of a glucose moiety to the 5 position of anthocyanin, thereby producing fluorescence upon appropriate

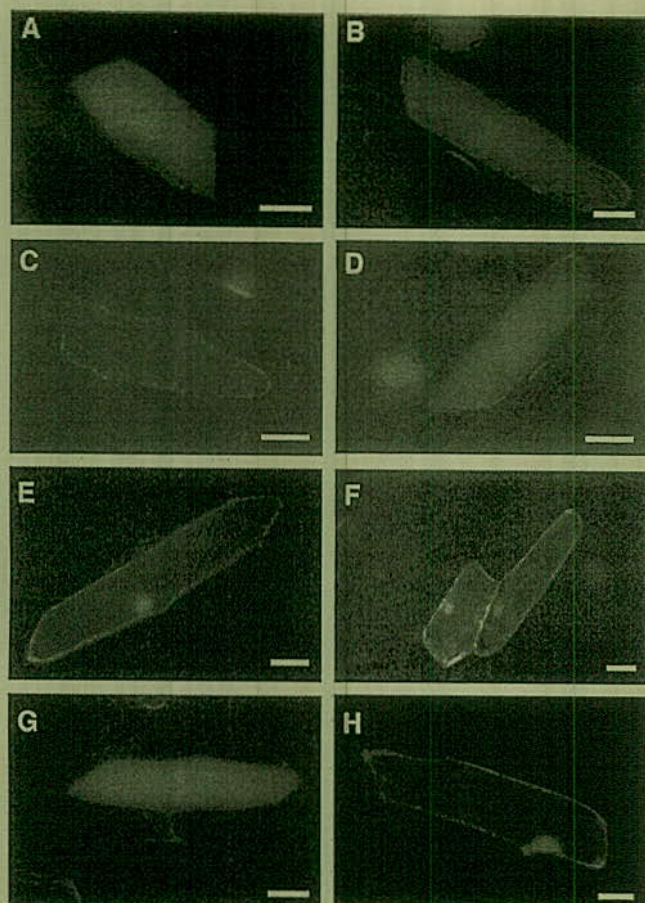


Figure 4. Subcellular Distributions of the GFP Fusion Proteins Expressed in Onion Epidermal Cells.

(A) Dc AA5GT-transit-peptide:GFP.
(B) Dg AA7GT-transit-peptide:GFP.
(C) Full-length-Dc AA5GT:GFP.
(D) Full-length-Dg AA7GT:GFP.
(E) Deleted-transit-peptide-Dc AA5GT:GFP.
(F) Deleted-transit-peptide-Dg AA7GT:GFP.
(G) At GRP5 translationally fused with GFP as a vacuolar protein control.
(H) GFP driven by CaMV 35S promoter.
Bars = 2.5 μ m.

illumination. By contrast to onion epidermal cells in which GFP fluorescence was restricted to the transformed cells, in carnation petals, GFP fluorescence was observed in cells neighboring the transformed cells (Figures 6C and 6E). This effect might be caused by transmission of GFP through the plasmodesmata from the transformed cells to their neighbors. Such transmission has been observed in other bombardment experiments on flower petals (Larsen et al., 2003; Nakatsuka et al., 2005; Shoji et al., 2010).

To investigate the movement of the Dc AA5GT:GFP fusion protein from the transformed cells to their neighbors, a full-length-Dc AA5GT:GFP construct was introduced into epidermal cells of carnation petals. Bright fluorescent spots similar to those in Figure 6B were observed (see Supplemental Figure 6A online). Thus, the Dc AA5GT:GFP fusion protein shows AA5GT activity in transformed carnation cells. However, GFP fluorescence levels in

cells transformed with other constructs, including 35S-At GRP5:GFP, were too low for detection (see Supplemental Figures 6C to 6I online). These results suggest that some unknown mechanism prevents or reduces the generation of fluorescence of GFP fused to AA5/7GT or GRP5 in carnation cells but not onion cells.

Kinetic Properties and Substrate Preferences of Acceptor and Donor Molecules of Dc AA5GT and Dg AA7GT

We measured the linearity of the reaction time course, the amount of enzyme, optimum pH, and temperature of the reaction for purified native Dc AA5GT, recombinant Dc AA5GT, and Dg

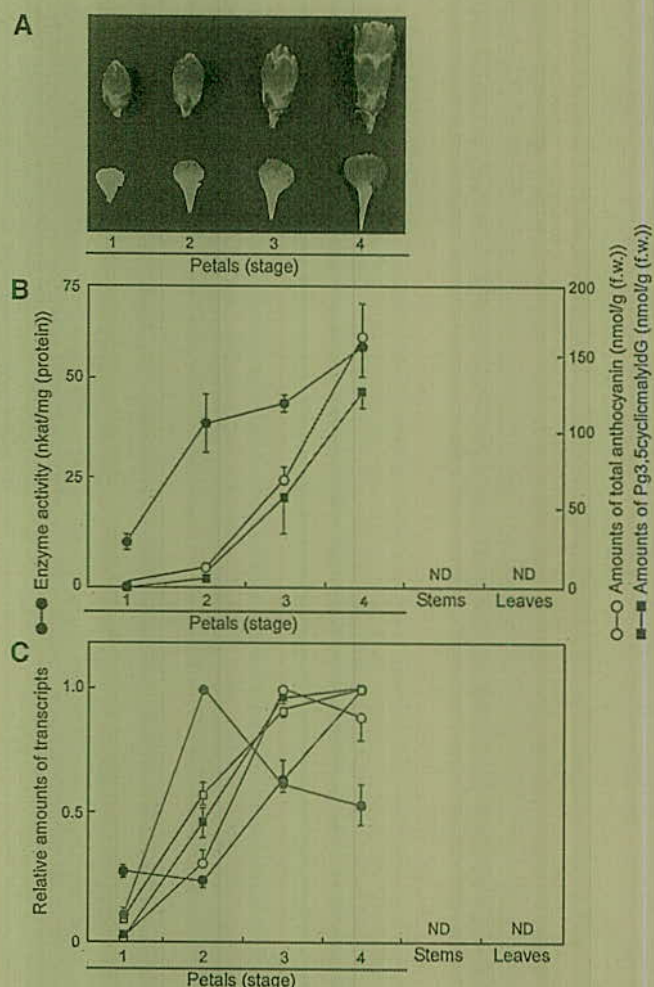


Figure 5. Expression of Dc AA5GT and AA5GT Activity in Crude Protein Extracts and Anthocyanin Levels in Carnation Petals, Stems, and Leaves.

(A) Four different petal development stages are illustrated.
(B) AA5GT activities (closed circles) and levels of total anthocyanin accumulating (open circles) and of Pg3,5cyclicmalydG (closed squares) at different petal stages and in stems and leaves. f.w., fresh weight; ND, not detectable.
(C) Expression levels of Dc AA5GT (red closed circles) and other anthocyanin biosynthetic genes, *CHS* (black closed circles), *DFR* (black open circles), *ANS* (black closed squares), and *UA3GT* (black open squares). Error bars indicate $\pm$ SD.

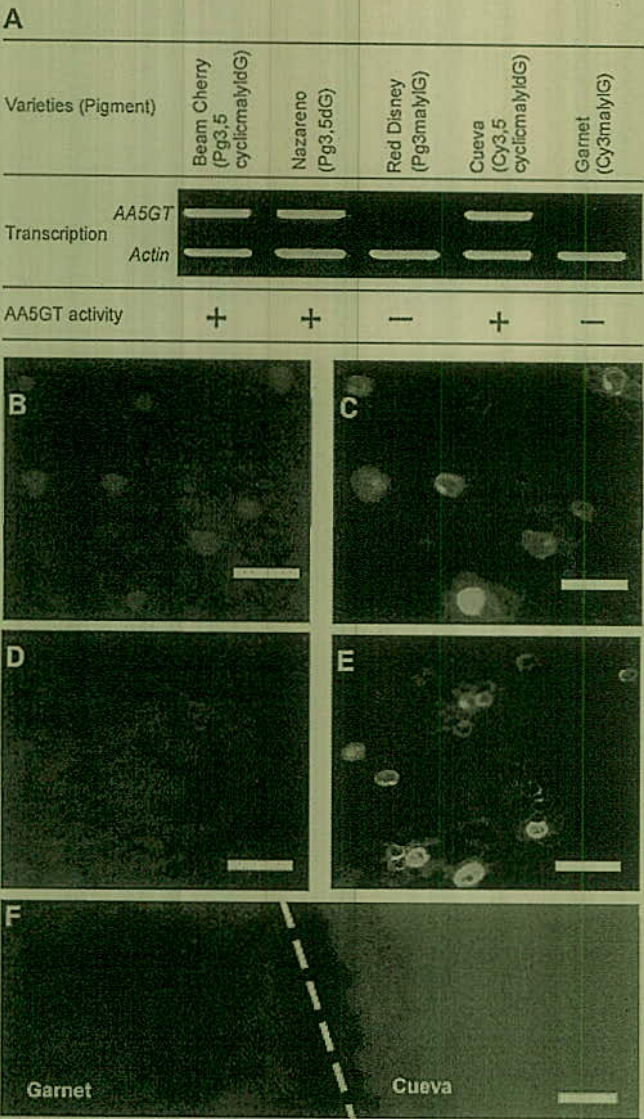


Figure 6. Expression of Dc AA5GT and AA5GT Activity in Different Carnation Varieties and Transient Expression of Dc AA5GT in Petals of the Variety Garnet in Which Anthocyanin Lacks the Glucosyl Moiety at the 5 Position.

(A) Relationship between anthocyanin structures and Dc AA5GT transcription and AA5GT activity in different carnation varieties. Pg3,5cyclicmalydG, 3,5-di-O-glucopyranosyl pelargonidin 6''-O-4,6'''-O-1-cyclic malate; Pg3,5dG, pelargonidin 3,5-di-O-glucopyranoside; Pg3malyG, pelargonidin 3-O-malyglucopyranoside; Cy3,5cyclicmalydG, 3,5-di-O-(glucopyranosyl) cyanidin 6''-O-4,6'''-O-1-cyclic malate; Cy3malyG, cyanidin 3-O-malyglucopyranoside.

(B) to (E) Fluorescence microscopy analysis of petals after transient expression of various constructs in petals of the carnation variety Garnet accumulating Cy3malyG.

(B) Full-length AA5GT.

(D) Mutant AA5GT lacking the putative transit peptide.

(C) and **(E)** GFP cobombardment controls corresponding to **(B)** and **(D)**, respectively.

(F) Fluorescence micrographs of petals of Garnet (left) or Cueva variety (right).

Bars = 10 μ m.

AA7GT (see Supplemental Figure 7 online). Native Dc AA5GT, recombinant Dc AA5GT, and Dg AA7GT proteins continued to catalyze the glucosyltransferase reaction for 48 h when incubated at 30°C (see Supplemental Figure 8 online). Although both the enzymatic reaction product (Cy3,5dG) and the substrate (Cy3G) were decomposed after 6 h of incubation, the AA5GT reaction in the crude protein extract prepared from carnation petals showed linearity until 6 h. These results indicated that the enzymes were stable and active even in the crude extract. The linearity of the enzyme reaction in a 50- μ L reaction mixture was measured using up to 200 ng of purified protein during the course of a 20-min reaction at 30°C (see Supplemental Figure 9 online). Optimum enzyme reactions were found to occur at 45°C and pH 4.5 to 5.0 for native Dc AA5GT, at 35°C and pH 6.0 for recombinant Dc AA5GT, and at 40°C and pH 6.0 for recombinant Dg AA7GT (see Supplemental Figures 10 and 11 online). The slight difference in pH optima of native and recombinant Dc AA5GT might be due to in vivo variation in posttranslational modification of the proteins or to use of a His-tag-fused protein to generate the recombinant AA5GT.

In light of these profiles, assessment of enzyme kinetic parameters was performed using the following conditions: for native Dc AA5GT, 15-min reaction time, pH 5.0, and 30°C; and for recombinant Dc AA5GT and Dg AA7GT, 15-min reaction time, pH 6.0, and 30°C. Native Dc AA5GT (k_{cat} for Cy3G = 0.06 s⁻¹, k_{cat} for VG = 0.01 s⁻¹, K_m for Cy3G = 13.0 μ M, and K_m for VG = 46.5 μ M; see Supplemental Table 3 online) showed similar kinetic parameters to recombinant Dc AA5GT (k_{cat} for Cy3G = 0.07 s⁻¹, k_{cat} for VG = 0.01 s⁻¹, K_m for Cy3G = 6.5 μ M, and K_m for VG = 51.9 μ M; see Supplemental Table 3 online) but differed from Dg AA7GT (k_{cat} for Cy3G = 0.17 s⁻¹, k_{cat} for VG = 0.10 s⁻¹, K_m for Cy3G = 22.9 μ M, and K_m for VG = 260.8 μ M; see Supplemental Table 3 online). The AAGTs could use various acyl-glucose molecules, including benzoyl-glucose derivatives (C6-C1-glucoses) and 1-O- β -hydroxycinnamoyl-glucoses (HCAGs, C6-C3-glucoses) but not flavonoid glucosides (C6-C3-C6-glucoses), as donor substrates (Table 1). By contrast, the AAGTs showed a strict acceptor preference for anthocyanidin 3-glucoside, 3-galactoside, and 3-malyglucoside but showed no preferences for other flavonoids or phenolic compounds (Table 2). The activity of Dc AA5GT was notably higher with HCAG as the donor than with VG. The k_{cat}/K_m value (s⁻¹ M⁻¹) for 1-O- β -d-feruloyl-glucose (FG) (1.9×10^3 ; see Supplemental Table 3 online) was 8.3-fold higher than that for VG (2.3×10^2 ; see Supplemental Table 3 online) in the case of recombinant Dc AA5GT. These analyses indicate that, in vitro, Dc AA5GT has a preference for FG over VG as a donor. Initially, we identified the donor molecule, VG, in an extract obtained using 50% methanol followed by an ODS open column eluted by 5% methanol. This procedure was designed to avoid contamination by anthocyanin interfering with the activity of enzymes prepared from carnation petals. As HCAGs were eluted in the over 5% methanol fraction, they were difficult to identify as active donor molecules for the enzyme reaction in this fraction. The preference of Dc AA5GT for FG over VG as a donor suggests the possibility that HCAGs, including FG rather than VG, might play more important roles as donors in vivo. The levels of six acyl-glucoses in carnation petals were measured: VG was found to show a higher level of

Table 1. Substrate Preferences and Relative Activities of AAGTs for Various Donor Molecules

Substrates	nkat/mg Protein (Relative Activities [%])		
	Native Dc AA5GT	Recombinant Dc AA5GT	Recombinant Dg AA7GT
Phenol glucosides			
1-O-β-D-Vanillyl-glucose	0.34 ± 0.04 (100)	0.76 ± 0.09 (100)	1.02 ± 0.08 (100)
1-O-α-D-Vanillyl-glucose	ND	ND	ND
1-O-β-D-Isovanillyl-glucose	0.11 ± 0.03 (31)	0.17 ± 0.05 (22)	0.27 ± 0.06 (26)
1-O-β-D-Galloyl-glucose	ND	ND	ND
1-O-β-D- <i>p</i> -hydroxybenzoyl-glucose	0.08 ± 0.01 (22)	0.07 ± 0.01 (9)	2.2 ± 0.1 (146)
1-O-β-D-Sinapoyl-glucose	2.8 ± 0.2 (827)	12.6 ± 0.2 (1663)	ND
1-O-β-D-Feruloyl-glucose	4.4 ± 0.1 (1284)	14 ± 1 (1834)	0.04 ± 0.01 (4)
1-O-β-D-Caffeoyl-glucose	1.0 ± 0.1 (295)	1.90 ± 0.06 (250)	0.02 ± 0.01 (2)
1-O-β-D-4-Coumaroyl-glucose	1.5 ± 0.3 (442)	3.69 ± 0.16 (485)	0.08 ± 0.02 (8)
1-O-β-D-Vanillyl-galactose	ND	ND	ND
<i>p</i> -Nitrophenyl-glucoside	ND	ND	ND
Arbutin	ND	ND	ND
Salicin	ND	ND	ND
Populin	ND	ND	ND
Eleutheroside B	ND	ND	ND
Phloridzin	ND	ND	ND
Glucovanillin	ND	ND	ND
Coumarin glucoside			
Esculin	ND	ND	ND
Flavonoid glucosides			
Kaempferol 3-glucoside	ND	ND	ND
Kaempferol 7-neohesperidoside	ND	ND	ND
Apigenin 7-glucoside	ND	ND	ND
Naringenin 7-glucoside	ND	ND	ND
Daidzin	ND	ND	ND
Indole glucoside			
Indican	ND	ND	ND

Donor preferences of AAGT proteins using Cy3G as the acceptor. Relative activity is shown in comparison to 1-O-β-D-vanillyl-glucose, which is arbitrarily set as 100%. All values are the mean ± SD at least three independent determinations. ND, not detectable.

accumulation compared with HCAGs (see Supplemental Table 4 online). However, although the levels of accumulation of HCAGs were lower than of VG, it is still possible that Dc AA5GT could use HCAGs effectively as the *in vivo* donor. In delphiniums, Dg AA7GT showed a preference for 1-O-β-D-*p*-hydroxybenzoyl-glucose (pHBG) as the donor substrate over other acyl-glucoses (Table 1; see Supplemental Table 3 online) and pHBG accumulated in the petals (see Supplemental Table 4 online). Our results suggest that Dg AA7GT might use pHBG as the donor substrate *in vivo*.

As these enzymes belong to the GH1 group of proteins, their glycosidase activities for phenol glucosides, coumarin glucosides, flavonoid glucoside, and indole glucoside were assessed (see Supplemental Table 5 online). AAGTs showed glucosidase activities only for the acyl-glucoses, Cy3,5dG, and Cy3,7dG; however, their efficiency was lower than for glucosyltransferase activities. The efficiency of glucosidase activity of recombinant Dc AA5GT for Cy3,5dG ($k_{cat}/K_m = 4.0 \times 10^2$; see Supplemental Table 6 online) was only 4% of that of its 5-*O*-glucosyltransferase activity for Cy3G ($k_{cat}/K_m = 1.0 \times 10^4$; see Supplemental Table 3 online). In addition, the efficiency of glucosidase activity of Dc AA5GT for VG or FG (k_{cat}/K_m for VG = 1.3×10^2 and k_{cat}/K_m for FG = 1.2×10^2 ; see Supplemental Table 6 online) were 60 and

6%, respectively, of its glucosyltransferase activities for Cy3G (k_{cat}/K_m for VG = 2.3×10^2 and k_{cat}/K_m for FG = 1.9×10^3 ; see Supplemental Table 3 online). Next, we determined the relative extent to which VG and FG were hydrolyzed but not used in the transfer reaction on AA5GT activity. When both Cy3G and VG were present in the reaction mixture, the activity to produce Cy3,5dG and vanillic acid (VA) was almost equal (0.76 and 0.73 nkat/mg protein, respectively; see Supplemental Table 7 online). In the reaction mixture with FG, the activity to produce Cy3,5dG and ferulic acid (FA) was 10.9 and 14.1 nkat/mg protein, respectively (see Supplemental Table 7 online), showing that 77% of glucose molecule released from FG was transferred to Cy3G. In the case of recombinant AA7GT, the efficiency of glucosidase activity for Cy3,7dG ($k_{cat}/K_m = 1.9 \times 10^2$; see Supplemental Table 6 online) was only 3% of that of 7-*O*-glucosyltransferase activity for Cy3G ($k_{cat}/K_m = 7.3 \times 10^3$; see Supplemental Table 3 online). The efficiency of glucosidase activity of Dg AA7GT for VG or pHBG (k_{cat}/K_m for VG = 2.7×10^1 and k_{cat}/K_m for pHBG = 5.3×10^1 ; see Supplemental Table 6 online) was 7 and 9%, respectively, of that of glucose transfer activity from VG or pHBG to Cy3G (k_{cat}/K_m for VG = 3.9×10^2 and k_{cat}/K_m for FG = 5.8×10^2 ; see Supplemental Table 3 online). When Cy3G and either VG or pHBG were present in the reaction mixture, the activity

Table 2. Substrate Preferences and Relative Activities of AAGTs for Various Acceptor Molecules

Substrates	nkat/mg Protein (Relative Activities [%])		
	Native Dc AA5GT	Recombinant Dc AA5GT	Recombinant Dg AA7GT
Flavonoids			
Kaempferol	ND	ND	ND
Kaempferol 3-glucoside	ND	ND	ND
Apigenin	ND	ND	ND
Apigenin 7-glucoside	ND	ND	ND
Naringenin	ND	ND	ND
Naringenin 7-glucoside	ND	ND	ND
Cyanidin	ND	ND	ND
Cyanidin 3-glucoside	0.34 ± 0.04 (100)	0.76 ± 0.09 (100)	1.02 ± 0.08 (100)
Cyanidin 3-galactoside	0.43 ± 0.06 (126)	0.90 ± 0.01 (119)	0.60 ± 0.02 (59)
Cyanidin 3-malylglucoside	0.4 ± 0.1 (128)	1.0 ± 0.2 (131)	0.44 ± 0.06 (43)
Cyanidin 3,5-diglucoside	ND	ND	ND
Cyanidin 3,7-diglucoside	ND	ND	ND
Pelargonidin	ND	ND	ND
Pelargonidin 3-glucoside	0.45 ± 0.07 (131)	1.4 ± 0.3 (181)	1.00 ± 0.03 (98)
Pelargonidin 3-malylglucoside	0.35 ± 0.03 (101)	0.82 ± 0.08 (108)	0.24 ± 0.03 (24)
Delphinidin	ND	ND	ND
Delphinidin 3-glucoside	0.52 ± 0.03 (152)	0.89 ± 0.06 (117)	1.73 ± 0.24 (170)
(+) Catechin	ND	ND	ND
(-) Epicatechin	ND	ND	ND
Naphthols			
1-Naphtol	ND	ND	ND
2-Naphtol	ND	ND	ND
NAA	ND	ND	ND
IAA	ND	ND	ND
Coumarins			
7-Hydroxy-4-methylcoumarin	ND	ND	ND
7-Hydroxy-6-methoxycoumarin	ND	ND	ND
Phenols			
Salicylic acid	ND	ND	ND
Phloroglucin	ND	ND	ND

Acceptor preferences of AAGT proteins using 1-O-β-D-vanillyl-glucose as the donor. Relative activity is shown in comparison to Cy3G, which is arbitrarily set as 100%. IAA, indole-3-acetic acid; NAA, naphthalene-1-acetic acid; ND, not detectable. All values are the mean ± SD at least three independent determinations.

producing Cy3,7dG and VA (1.02 and 1.05 nkat/mg protein; see Supplemental Table 7 online) and Cy3,7dG and *p*-hydroxybenzoic acid (pHBA) (2.2 and 2.8 nkat/mg protein; see Supplemental Table 7 online) showed that 97 and 79% of glucose molecules released from VG and pHBG, respectively, were transferred to Cy3G. The reverse reaction, transferring glucose from Cy3,5dG or Cy3,7dG to VA, FA, pHBA, and UDP was under the level of detection (see Supplemental Table 7 online).

DISCUSSION

Here, we demonstrated a new reaction mechanism for the transfer of a glucose moiety to anthocyanidin 3-glucoside at the 5 or 7 position. This reaction mechanism uses acyl-glucoses as the donors and is mediated by enzymes belonging to the GH1 group of proteins (Figure 3). The carnation variety Garnet had undetectable levels of Dc AA5GT expression or AA5GT activity in its petals but showed modification of anthocyanin at the 5 position in vivo in a microprojectile bombardment experiment

(Figure 6). This result strongly suggested that Dc AA5GT might be responsible for the modification of anthocyanin. In delphinium, Dg AA7GT cDNA was isolated and recombinant Dg AA7GT showed transfer activity for glucose at the 7 position of anthocyanidin 3-glucoside.

Most GH1 proteins have putative signal peptides for localization in ER, microbody, plasma membrane, or for sequestration into the vacuoles (Xu et al., 2004; Opasiri et al., 2006). The Dc AA5GT protein might be transported across membranes since the putative transit peptide is required for the intact enzyme to function in vivo (Figure 6). Although the putative transit peptide of Dc AA5GT and of Dg AA7GT caused accumulation of GFP in vacuoles of onion epidermal cells, the Dc AA5GT:GFP fusion protein was not completely transported into vacuoles (Figure 4C). Nevertheless, the loss of the transit peptide in transcribed Dc AA5GT protein in transformed carnation cells might be responsible for the loss of in vivo AA5GT activity (Figure 6D). These results suggest that after translation in the cytosol, Dc AA5GT and Dg AA7GT proteins might be transported across membranes and function in noncytosolic compartments after

processing of the transit peptide to the mature protein, as shown in the N-terminal amino acid sequence of purified AA5GT protein (see Supplemental Figure 3 online). The first compartment may be ER and the end point may be the vacuoles. To date, we have not been able to definitively identify in which cellular compartment (ER, prevacuolar compartment, or vacuole) the enzyme functions (i.e., the locations where acceptor and donor molecules coexist after transportation across membranes). We detected sites of accumulation of donor molecules in the cells, principally in the vacuoles (see Supplemental Table 4 online); however, we were unable to determine whether these sites were those at which the enzymatic reaction occurred. One possibility is that the AAGT protein might be transported into the vacuole via the vesicle trafficking route and that both anthocyanidin 3-glucoside and acyl-glucoses might be directly transported into the vacuole. Vacuolar compartmentalization of HCAGs has been shown to occur in sweet clover (*Melilotus alba*; Oba et al., 1981). We therefore anticipate that, in carnation petal cells, VG and HCAGs will similarly accumulate at high levels in vacuoles in association with anthocyanins. Consequently, the glucosyl transfer reaction might occur in the vacuole; in which case, we could measure the amount of accumulated anthocyanins and acyl-glucoses produced by the kinetic reaction. As Dc AA5GT has a broad preference for acyl-glucoses, it is possible that Dc AA5GT could use both FG, its preferred donor, and VG, the most abundant donor, in the vacuole of carnation petals. Another possibility is that HCAG is exhausted by Dc AA5GT activity, and as a result, the amount of accumulated HCAG is lower than that of VG. Even if Dg AA7GT prefers pHBG to VG as the donor and the amount of accumulated pHBG is higher than other acyl-glucoses in delphinium, we think it unlikely that pHBG is the main donor molecule for the in vivo Dg AA7GT reaction, as it remains possible that the enzyme preferentially exhausts other potential donors, causing pHBG to accumulate.

Recent studies of an *Arabidopsis* mutant have led to the proposal of an alternative route for transportation of anthocyanins in which the compounds are trafficked through the ER-to-vacuole protein-sorting route (Grotewold, 2004; Poustka et al., 2007; Grotewold and Davies, 2008). If both anthocyanidin 3-glucoside and acyl-glucoses are transported into ER containing AAGT proteins, then it is possible that glucosylation occurs in the ER during this trafficking process to the final destination in the vacuole. It remains unclear whether all three elements, AAGT, donor, and acceptor, are transported into and are present contemporaneously in intermediate cellular compartments where glucosylation might occur or are transported independently along different trafficking pathways to meet and interact in the vacuole. We will seek to gain further insights into the in vivo kinetics of the glucosylation reaction and the transportation of anthocyanidin 3-glucoside and acyl-glucoses in cells in future experiments.

GH1 enzymes are known to have the properties of β -glucosidases (Marana, 2006). The x-ray crystal structures of GH1 enzymes have been solved and the enzyme catalytic sites identified (Barrett et al., 1995; Burmeister et al., 1997; Sanz-Aparicio et al., 1998; Czjzek et al., 2000; Verdoucq et al., 2004). Although the amino acid sequences of Dc AA5GT and Dg AA7GT showed only 45% identity (Figure 3; see Supplemental Figure 3 online), the classic primary structures of GH1 enzymes, namely,

the highly conserved peptide motifs TFNEP and I/VTENG, were conserved as TF/INEA/P and I/VHENG in Dc AA5GT/Dg AA7GT (see Supplemental Figure 3 online). The hydrolysis of a β -glycosidic bond requires the participation of an acid/base catalyst and a nucleophile, which in β -glucosidases are the two glutamic acids in the motifs described above (Sanz-Aparicio et al., 1998). The glutamic acids are conserved at positions 197/388 and 198/403 in the Dc AA5GT and Dg AA7GT amino acid sequence, respectively. Although GH1 proteins have been studied for their glycosidase activity, there have also been attempts to convert the glycosidase activity to a glycosyltransferase activity (Perugino et al., 2004; Hancock et al., 2006; Wang and Huang, 2009). The generation of mutant enzyme proteins belonging to glycoside hydrolase families with glycosyltransferase but not glycosidase activity was achieved by mutation of the catalytic nucleophile residue to a non-nucleophilic one. Interestingly, although both Dc AA5GT and Dg AA7GT enzymes have the conserved nucleophilic glutamic acids, the enzymes also show the ability to transfer a glucose moiety from an acyl-glucose to anthocyanidin 3-glucoside. These new enzymatic properties are assumed to have arisen in the course of evolution of their host species. The specificity of the enzymes for the 5 and 7 positions of anthocyanidin 3-glucoside in Dc AA5GT and Dg AA7GT, respectively, is also of interest; Dc AA5GT cannot transfer a glucose moiety to the 7 position nor can Dg AA7GT do so to the 5 position. This difference in position specificity may be due to differences in the primary amino acid sequences of the two enzymes (e.g., the 210 to 230 amino acid region of the Dg AA7GT amino acid sequence is deleted in Dc AA5GT). It is possible that a comparative x-ray crystallographic analysis of Dc AA5GT and Dg AA7GT will provide information relevant to the differences between the enzymes in the binding site for anthocyanidin 3-glucoside and the catalytic site residue for glucose transfer.

Our results here show that the GH1 proteins Dc AA5GT and Dg AA7GT from two taxonomically distant species, carnations (Caryophyllaceae) and delphiniums (Ranunculaceae), respectively, share a novel glucosyltransferase activity for transferring glucose to the 5 and 7 positions of anthocyanins. This suggests that the glucosylation mechanism using acyl-glucoses as the donor is not species specific but may be a universal mechanism across the plant kingdom. Our identification here of a new glucosylation mechanism acting on anthocyanins suggests that in addition to cytosolic UDP-glucose-dependent glycosylation enzymes other glycosylation enzymes belonging to GH1 in the noncytosolic compartment might contribute to the diversity of secondary metabolites in plants.

METHODS

Plant Materials and Chemicals

The carnation (*Dianthus caryophyllus*) and delphinium (*Delphinium grandiflorum*) varieties used in the study were obtained from horticultural growers and Central Laboratories for Frontier Technology, Kirin Holdings. Petals at different developmental stages, stems, and leaves were harvested; the tissues were immediately frozen in liquid nitrogen and stored at -80°C until required. Flavonoids, including Cy3G, Cy3,5dG, pelargonidin 3-O-glucopyranoside, and pelargonidin 3,5-di-O-glucopyranoside, delphinidin 3-O-glucopyranoside, delphinidin 3,5-di-O-glucopyranoside,

and naphthols, coumarins, phenols, FA, pHBA, UDP, UDP-glucose (UDPG), and UDP-galactose were purchased from Funakoshi Co. or Wako Pure Chemical. HcAGs (sinapoyl-, caffeoyl-, 4-coumaroyl-, and FG) were prepared by enzymatic synthesis as described previously (Matsuba et al., 2008). pHBG was also synthesized using the enzymatic synthesis system, and the ^1H -NMR analysis (400 MHz, CD_3OD) gave the following results: δ 7.93 (2H, dd, H-2, 6), 6.82 (2H, dd, H-3, 5), 5.65 (1H, d, H-Glc1, $J = 8.02$), 3.33 to 3.82 (5H, m, H-Glc). Pelargonidin and cyanidin 3-O-malylglucopyranosides (Pg3malylG and Cy3malylG, respectively) were isolated and purified from carnation petals using flash chromatography and HPLC equipped with an ODS column. Eleutheroside B, daidzin, populin, salicin, phloridzin, glucovanillin, indican, kaempferol 7-neohesperidoside, and arbutin were the kind gift of Yukihiro Goda (National Institute of Health Sciences).

Purification and Identification of VG from the Petals of Carnation

Petals (20 g) from carnation Red Vital plants were placed in 800 mL of 50% ethanol for 72 h at 25°C with shaking. The ethanol solution was then allowed to evaporate and the extracts were redissolved in aqueous 0.1% acetic acid. The solution was loaded on an ODS column (110 mL) equilibrated with aqueous 0.1% acetic acid, and the column was washed with 5 $\times$ volume of aqueous 0.1% acetic acid. The compounds were eluted from the column with 300 mL of 5% methanol in water, which was then allowed to evaporate. The extracts were redissolved in 3 mL of water and centrifuged at 20,400g for 2 min. The extracts were purified using HPLC equipped with a Develosil RPAQUEOUS-AR-5 (i.d. 10 $\times$ 250 mm; Nomura Chemical) by linear gradient elution (5 mL/min) from 7% solvent B (90% methanol) to 20% in solvent A (0.1% acetic acid in water) for 40 min. The active fractions were combined, the solvent allowed to evaporate, and the extracts redissolved in water. They were then further purified using HPLC equipped with an XTerraPrepMS C₁₈ column (i.d. 10 $\times$ 250 mm; Waters) by linear gradient elution (5 mL/min) from 6% solvent B to 10% in solvent A for 40 min. The active fractions were combined, the solvent allowed to evaporate, and the extracts redissolved in water. They were then loaded on a Cosmosil HILIC column (i.d. 10 $\times$ 250 mm; Nacalai Tesque) and separated by linear gradient elution (5 mL/min) from 95% acetonitrile to 90% in water for 40 min. A purified donor compound prepared from carnation petals was analyzed by high-resolution mass spectrometry and NMR. ^1H NMR (400 MHz, CD_3OD), δ 7.62 (1H, dd, H-6), 7.58 (1H, d, H-2), 6.82 (1H, d, H-5), 5.67 (1H, d, H-Glc1, $J = 7.80$), 3.88 (3H, s, CH_3), 3.40 to 3.95 (5H, m, H-Glc), ^{13}C NMR (400 MHz, CD_3OD), δ 56.4 (CH_3), 62.3, 71.1, 74.1, 78.1, 78.8, 96.0 (C-Glc), 113.8 (C-2), 116.3 (C-5), 120.9 (C-1), 125.9 (C-6), 149.1 (C-3), and 154.7 (C-4). The correlation between the glucose 1' proton signal and the VA observed in the HMBC spectrum showed that the VA moiety was linked through its carbonyl carbon, and the ^1H -NMR coupling constant ($J = 7.8$) showed that it had a β configuration.

Chemical Synthesis of 1-O- β - and α -D-Vanillyl-Glucose and Related Compounds

The synthetic route for 1-O- β - and α -D-vanillyl-glucose is summarized in Supplemental Figure 1 online. The detailed steps of the synthesis are as follows.

- (1) A mixture of 4-hydroxy-3-methoxybenzoic acid (500 mg, 3 mmol) (Sigma-Aldrich), potassium carbonate (2100 mg, 15 mmol) (Wako Pure Chemical), and benzyl bromide (Sigma-Aldrich) (1526 mg, 9 mmol) in dimethylformamide (30 mL) was stirred at room temperature for 12 h. Water was added to the reaction mixture, and the organic layer was extracted with ethyl acetate. The organic layer was concentrated under reduced pressure, and the residue was purified by column chromatography (silica gel [Kanto Chemical]

and an 8:1 mixture of hexane:ethyl acetate) to produce the 4-benzyloxy-3-methoxybenzoic acid benzyl ester. The ^1H -NMR analysis (300 MHz, CDCl_3) gave the following result: δ 7.65 (1H, dd, H-6), 7.59 (1H, d, H-2), 7.41-7.26 (9H, m, H-benzylic CH_2), 6.88 (1H, d, H-5), 5.33 (2H, s, H-benzylic CH_2), 5.21 (1H, s, H-benzylic CH_2), 3.92 (3H, s, CH_3).

- (2) A mixture of 4-benzyloxy-3-methoxybenzoic acid benzyl ester (1045 mg, 3 mmol) and lithium hydroxide (250 mg, 6 mmol) (Wako) in a 3:1:1 mixture of tetrahydrofuran (Kanto Chemical), water, and methanol (15 mL) was stirred at 60°C for 17 h. The pH of the reaction mixture was adjusted to make it acidic by addition of HCl and then the mixture was diluted with water. The organic layer was extracted with ethyl acetate and concentrated under reduced pressure. The residue was purified by column chromatography (silica gel and a 6:1 mixture of hexane:ethyl acetate) to produce 4-benzyloxy-3-methoxybenzoic acid. The ^1H -NMR analysis (300 MHz, CDCl_3) gave the following result: δ 7.68 (1H, dd, H-6), 7.60 (1H, d, H-2), 7.45-7.25 (5H, m, H-benzylic CH_2), 6.90 (1H, d, H-5), 5.25 (2H, s, H-benzylic CH_2), 3.92 (3H, s, CH_3).
- (3) A mixture of 4-benzyloxy-3-methoxybenzoic acid (603 mg, 2.3 mmol), *N,N*-dimethyl-4-aminopyridine (287 mg, 2.3 mmol) (Tokyo Kasei Kogyo), and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (2700 mg, 14 mmol) (Kokusaikagaku Co.) in dimethylformamide (10 mL) (Thermo Fisher Scientific) was added to 2,3,4,6-tetra-O-benzyl- β -D-glucopyranose (1081 mg, 2 mmol) (Tokyo Chemical Industry) and stirred at 60°C for 4 h under nitrogen. Water was added to the reaction mixture, which was then extracted with ethyl acetate. The extract was concentrated under reduced pressure, and the residue was purified by column chromatography (silica gel and a 5:1 mixture of hexane-ethyl acetate) to give a 2,3,4,6-tetra-O-benzyl-D-glucosylated product as a diastereomer mixture at C1' (i.e., β - and α -anomers). The MS analysis showed $[\text{M}+\text{Na}]^+$ at m/z 803.14, and the ^1H -NMR analysis (300 MHz, CDCl_3) gave the following result: δ 7.60 (1H, dd, H-6), 7.59 (1H, d, H-2), 7.45-7.26 (60H, m, H-benzylic CH_2), 6.90 (1H, d, H-5), 6.55 (1H, d, H-Glc1 α , $J = 3.21$), 5.84 (1H, d, H-Glc1 β , $J = 7.65$), 5.25 (10H, s, H-benzylic CH_2), 4.96-3.56 (94H, m, CH_3 , H-Glc).
- (4) The diastereomer mixture (200 mg, 0.3 mmol) was dissolved in methanol (15 mL) and then tetrahydrofuran (10 mL) and 10% palladium hydroxide on charcoal (40 mg); this mixture was stirred for 20 h at room temperature under an atmosphere of hydrogen. The reaction mixture was filtered through a pad of Celite, and the filtrates were concentrated under reduced pressure. The two diastereomers were separated using an HPLC equipped with a Develosil RPAQUEOUS-AR-5 column (i.d. 10 $\times$ 250 mm; Nomura Chemical) by an isocratic elution (5 mL/min) in 9% methanol containing 0.9% formic acid for 40 min and monitored at 270 nm. The MS analysis of α -anomer showed $[\text{M}+\text{Na}]^+$ at m/z 353.08304, and the ^1H -NMR analysis (400 MHz, CD_3OD) gave the following result: δ 7.64 (1H, dd, H-6), 7.60 (1H, d, H-2), 6.86 (1H, d, H-5), 6.30 (1H, d, H-Glc1, $J = 3.66$), 3.92 (3H, s, CH_3), 3.40-3.88 (5H, m, H-Glc), and the ^{13}C -NMR analysis (100 MHz, CD_3OD) gave the following result: δ 56.5 (CH_3), 62.3, 71.2, 72.5, 75.6, 76.2, 93.9 (C-Glc), 113.8 (C-2), 116.0 (C-5), 122.1 (C-1), 125.6 (C-6), 148.8 (C-3), 153.4 (C-4), 166.8 (C-7). The MS analysis of β -anomer showed $[\text{M}+\text{Na}]^+$ at 353.08213, and the ^1H -NMR analysis (500 MHz, CD_3OD) gave the following result: δ 7.64 (1H, dd, H-6), 7.61 (1H, d, H-2), 6.86 (1H, d, H-5), 5.68 (1H, d, H-Glc1, $J = 7.79$), 3.91 (3H, s, CH_3), 3.42-3.94 (5H, m, H-Glc), and the ^{13}C -NMR analysis (125 MHz, CD_3OD) gave the following result: δ 56.4 (CH_3), 62.3, 71.1, 74.1, 78.1, 78.9, 96.1 (C-Glc), 113.8 (C-2), 116.0 (C-5), 121.8 (C-1), 125.7 (C-6), 148.8 (C-3), 153.4 (C-4), 166.7 (C-7).

1-O- β -D-isovanillyl- and galloyl-glucoses were chemically synthesized in a similar manner using 3-hydroxy-4-methoxybenzoic acid (Sigma-Aldrich) or 3,4,5-trihydroxybenzoic acid (Wako), respectively, as the starting material instead of 4-hydroxy-3-methoxybenzoic acid. 1-O- β -D-vanillyl-galactose was synthesized using 2,3,4,6-tetra-O-benzyl- β -D-galactopyranose (Sigma-Aldrich) instead of 2,3,4,6-tetra-O-benzyl- β -D-glucopyranose at step 3 above.

The MS analysis of 1-O- β -D-isovanillyl-glucose showed $[M+Na]^+$ at m/z 352.99, and the 1H -NMR analysis (300 MHz, CD_3OD) gave the following result: δ 7.63 (1H, dd, H-6), 7.60 (1H, d, H-2), 6.98 (1H, d, H-5), 5.67 (1H, d, H-Glc1, $J = 8.10$), 3.91 (3H, s, CH_3), 3.42-3.88 (5H, m, H-Glc). The MS analysis of 1-O- β -D-galloyl-glucose showed $[M+Na]^+$ at m/z 354.97, and the 1H -NMR analysis (300 MHz, CD_3OD) gave the following result: δ 7.38 (2H, d, H-2,6), 5.85 (1H, d, H-Glc1, $J = 7.50$), 3.42-3.94 (5H, m, H-Glc). The MS analysis of 1-O- β -D-vanillyl-galactose showed $[M+Na]^+$ at m/z 353.02, and the 1H -NMR analysis (300 MHz, CD_3OD) gave the following result: δ 7.62 (1H, dd, H-6), 7.58 (1H, d, H-2), 6.79 (1H, d, H-5), 5.63 (1H, d, H-Glc1, $J = 8.06$), 3.88 (3H, s, CH_3), 3.34-3.92 (5H, m, H-Glc).

Protein Extraction from the Petals of Carnation and Delphinium

Petals (0.5 g) from carnation Beam Cherry and delphinium Triton Light Blue plants were ground in liquid nitrogen in a mortar and pestle and then thawed in 3 mL of extraction buffer (0.1 M potassium phosphate buffer, pH 7.2) on ice for 30 min. After centrifugation, a 250- μ L aliquot of the supernatant was added to 750 μ L of aqueous saturated ammonium sulfate. The mixture was incubated on ice for 30 min and collected by centrifugation. The precipitate was dissolved in 50 μ L of extraction buffer followed by desalting and exchanging the buffer to 0.1 M citrate buffer, pH 5.6, using a MicroSpin G-25 column (GE Healthcare). The amount of protein in the preparation was measured using a Coomassie Plus Protein Assay Kit (Thermo Fisher Scientific) with BSA as the standard.

Detection of AAGT Activity and Product Identification

AA5GT and AA7GT activities were assessed in a 50 μ L reaction mixture of 0.1 M citrate buffer, pH 5.0 and 6.0, containing 30 to 50 μ g of the crude extracted protein from the petals, recombinant Dc AA5GT, or recombinant Dg AA7GT; the mixture also contained 10 nmol Cy3G as an acceptor and 50 nmol acyl-glucose as a donor. The reaction mixture was incubated at 30°C for 15 to 180 min, and the reaction was terminated by the addition of 2.5 μ L of 20% phosphoric acid. The mixture was centrifuged at 20,000g for 5 min, and the supernatant was subjected to HPLC analysis using an ODS column Develosil-SR-5 (i.d. 4.6 $\times$ 250 mm; Nomura Chemical) with detection at 520 nm. The substrates and products were separated by linear gradient elution (1 mL/min) from 20 to 55% methanol in solvent C (1.5% phosphoric acid in water) for 20 min. For the rapid enzyme assay, the enzyme reaction was performed in 0.1 M citrate buffer, pH 5.6, at 30°C for 30 to 40 min, and products were separated by HPLC using a short Chromolith Performance RP-18e 100-4 column (i.d. 4.6 $\times$ 100 mm; Merck KGaA) with linear gradient elution (3 mL/min) from 18% solvent B to 37% in solvent C for 3 min.

Purification of Dc AA5GT from Carnation Petals

Petals (400 g) from carnation Beam Cherry plants were ground using a blender and suspended in 1500 mL of 10 mM potassium phosphate buffer, pH 7.2, further disrupted using a Polytron (Kinematica), then centrifuged at 15,000g for 30 min. Protein precipitating between 35 and 55% saturated $(NH_4)_2SO_4$ was collected by centrifugation at 15,000g for 30 min. The pellet was dissolved in 1500 mL of 10 mM potassium phosphate buffer, pH 7.2, and dialyzed in the same buffer. The protein solution was applied to a DEAE Sepharose Fast Flow (GE Healthcare) anion exchange column (i.d. 50 $\times$ 120 mm) equilibrated with 10 mM

potassium phosphate buffer, pH 7.2, at a flow rate of 7 mL/min. The column was washed with the same buffer followed by elution with a linear gradient of 0 to 0.8 M NaCl for 250 min in 10 mM potassium phosphate buffer, pH 7.2. The active fraction was combined and dialyzed in 10 mM potassium phosphate buffer, pH 7.2. The protein solution was applied to a TOYOPEARL Butyl-650 M (Tosoh) hydrophobic interaction column (i.d. 50 $\times$ 75 mm) equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing 35% $(NH_4)_2SO_4$, at a flow rate of 7 mL/min followed by washing with the same buffer. The enzyme was eluted with a linear gradient of 35 to 0% $(NH_4)_2SO_4$ for 180 min in 10 mM potassium phosphate buffer, pH 7.2. The active fraction was combined and concentrated using Amicon Ultra-15 (10,000 MWCO; Millipore) and then applied to a Benzamidine Sepharose 4 Fast Flow (high sub) column (i.d. 10 $\times$ 100 mm; GE Healthcare), equilibrated with 10 mM potassium phosphate buffer, pH 7.2, 500 mM NaCl, at a flow rate of 5 mL/min. The column was washed with the same buffer. The enzyme was eluted with a linear gradient of 0 to 100% of 20 mM 4-aminobenzamidine for 10 min in 10 mM potassium phosphate buffer, pH 7.2. The active fraction was combined and concentrated by ultrafiltration and was fractionated by chromatography in a gel filtration column (Superdex 75 10/300 GL; GE Healthcare), equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing 150 mM NaCl, at a flow rate of 0.5 mL/min. The active fraction was combined and concentrated by ultrafiltration and further fractionated using a prepacked Resource ETH hydrophobic interaction column (i.d. 6.4 $\times$ 30 mm; GE Healthcare), equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing 35% $(NH_4)_2SO_4$, at a flow rate of 0.8 mL/min. After washing with the same buffer, the enzyme was eluted with a linear gradient of 35 to 15% $(NH_4)_2SO_4$ for 19 min in 10 mM potassium phosphate buffer, pH 7.2. The active fraction was loaded on a prepacked HiTrap Butyl hydrophobic interaction column (i.d. 7 $\times$ 25 mm; GE Healthcare), equilibrated with 10 mM potassium phosphate buffer, pH 7.2, containing 20% $(NH_4)_2SO_4$, at a flow rate of 1 mL/min. After washing the column with the same buffer, the enzyme protein was eluted with a linear gradient of 20 to 0% $(NH_4)_2SO_4$ for 19 min in 10 mM potassium phosphate buffer, pH 7.2, containing 10% ethylene glycol. The active fraction was combined and concentrated by ultrafiltration. The purified enzyme gave a single protein band on SDS-PAGE by silver staining. The amino acid sequences of the peptide fragments were obtained by lysyl-endopeptidase digestion of purified Dc AA5GT.

Isolation of Dc AA5GT and Dg AA7GT cDNAs and Construction of Expression Vectors

Poly(A)⁺ RNAs from petals of carnation Beam Cherry and delphinium Triton Light Blue plants were prepared as described previously (Sasaki et al., 2005). The cDNA templates for 3'-RACE were synthesized using a 3'-full RACE core kit (Takara Bio). cDNA fragments corresponding to the Dc AA5GT and Dg AA7GT candidates were isolated by degenerate PCR (30 s at 92°C, 30 s at 52°C, and 45 s at 72°C, 35 cycles) performed using the sense primer VGTdgFwd01 and the antisense primer VGTdgRv01 (see Supplemental Table 8 online) and 3'-RACE cDNA as the template. The nucleotide sequence information from the partial cDNA fragments for Dc AA5GT and Dg AA7GT was used to obtain full-length cDNAs with a GeneRacer kit (Invitrogen) according to the manufacturer's protocol. The 5' cDNA ends of Dc AA5GT and Dg AA7GT were isolated by nested PCR. The first PCR was performed using the GeneRacer 5' primer (Invitrogen) and either the DcAA5GTRv1 primer or DgAA7GTRv1 primer (see Supplemental Table 8 online) and the appropriate full-length cDNA as the template. The first PCR products were purified by polyethylene glycol precipitation and used as the template in the second PCR. The second PCR was performed (30 s at 92°C, 30 s at 52°C, and 45 s at 72°C) using a GeneRacer 5' nested primer and a DcAA5GTatg primer or DgAA7GTatg primer (see Supplemental Table 8 online) were both designed to include the putative first

ATG. Each was used with the 3' Site 3' Adaptor primer supplied with the 3'-RACE core kit (Takara Bio) to isolate Dc AA5GT or Dg AA7GT cDNA fragments that contained the putative open reading frame region. Amplified cDNAs were introduced into the pGEM-T Easy vector (Promega), and the sense and antisense strands of the cDNA sequence were determined.

Phylogenetic Analysis

GH1 homologs were obtained by GenBank and BLAST searches. The sequences were aligned using ClustalW (Thompson et al., 1994) and are shown in Supplemental Figure 5 and Supplemental Data Set 1 online. The phylogenetic tree and bootstrap values were obtained using the neighbor-joining algorithm MEGA version 4.1 (Tamura et al., 2007). Bootstrap values were performed with 1000 replications.

Analysis of the Effect of Transient Expression of Dc AA5GT and Dg AA7GT cDNA in Onion Epidermal Cells

Nucleotide sequences corresponding to the putative transit peptide (30 amino acid sequence) of Dc AA5GT and that (38-amino acid sequence) of Dg AA7GT were prepared by PCR. Nucleotide sequences corresponding to the full-length At GRP5 (Mangeon et al., 2009) product were obtained by PCR with the sense primer AtGRP5s and the antisense primer AtGRP5rt (see Supplemental Table 8 online) and using the cDNAs prepared from *Arabidopsis thaliana* seedlings as the template. After the second PCR using these cDNA fragments and primers containing the sequences for the recombination reaction, the fragments were introduced into the pDONR/zeo vector using BP clonase (Invitrogen). These entry vectors for the GATEWAY SYSTEM (Invitrogen) were recombined with GFP chimeric expression destination vector pGWB405 (Nakagawa et al., 2009) for onion epidermal cells. pSK-GFP plasmid harboring a GFP cDNA driven by CaMV 35S promoter in pBluescript SK+ vector was used as the control for onion epidermal cells. Transformation of onion epidermal cells with the plasmids was performed by microprojectile bombardment using the Biolistic PDS-1000/He system (Bio-Rad Laboratories) in accordance with the standard protocol provided by the supplier. Five hundred micrograms of gold particles (diameter 1.0 μ m) coated with 1 μ g of plasmid were used in each bombardment at a helium pressure of 1100 p.s.i. in each shot. The target distance between the stop screen and the onion epidermal segments was set at 6 cm. After bombardment, onion epidermal segments were placed on 0.8% agar gel in Petri dishes and kept in the dark at 22°C for 24 to 48 h. Transformed onion epidermal cells were viewed using a fluorescence microscope.

Heterologous Production of Dc AA5GT and Dg AA7GT in *Escherichia coli* and Purification of Recombinant Proteins

Dc AA5GT and Dg AA7GT cDNAs, in which the nucleotide sequence corresponding to the putative signal peptide was deleted but which included the first Met, were amplified by PCR using pGEM-Dc AA5GT and pGEM-Dg AA7GT cDNAs, respectively, as the template. For Dc AA5GT, the sense primer DcAA5GTs and the antisense primer DcAA5GTrt (see Supplemental Table 8 online) were used. For Dg AA7GT, the sense primer DgAA7GTs and the antisense primer DgAA7GTrt (see Supplemental Table 8 online) were used. The amplified partial cDNAs were introduced into the pTrcHis2 vector using the topoisomerase reaction (pTrcHis2-TOPO cloning kit; Invitrogen) and transformed into *E. coli* BL21(DE3) cells. *E. coli* harboring pTrcHis2-Dc AA5GT or -Dg AA7GT cDNA was inoculated into 500 mL of Luria-Bertani medium containing 50 μ g/mL of ampicillin and cultured overnight at 30°C; 500 μ L of 1 M isopropyl- β -D (-)-thiogalactopyranoside was then added and the culture continued for 24 h at 16°C. The cells were then harvested by centrifugation. Harvested cells were resuspended in 50 mL of 0.1 M potassium phosphate buffer, pH 5.6, and disrupted by sonication (Tomy Seiko). After centrifugation of

the lysate at 20,000g for 2 min, the supernatant was used as a crude protein extract for the AAGT reaction. The Dc AA5GT enzyme was purified from the crude protein extract using four chromatographic steps: DEAE Sepharose Fast Flow (i.d. 25 $\times$ 30 mm), TOYOPEARL Butyl-650 M (i.d. 25 $\times$ 30 mm), HiTrap His (i.d. 7 $\times$ 25 mm; GE Healthcare), and HiTrap Butyl (i.d. 7 $\times$ 25 mm). The Dg AA7GT enzyme was purified from the crude protein using HiTrap His (i.d. 7 $\times$ 25 mm) and HiTrap Butyl (i.d. 7 $\times$ 25 mm) (see Supplemental Figure 7 online). The pTrcHis2-TOPO/lacZ plasmid (Invitrogen) included in the kit was used as the control vector.

Identification of Cy3,7dG as the Product of Recombinant Dg AA7GT

The recombinant Dg AA7GT protein was generated in *E. coli* that harbored an expression vector containing Dg AA7GT cDNA with a deletion of the 5' nucleotide sequences corresponding to the 28 amino acids of the N-terminal hydrophobic region but including the first Met. The Dg AA7GT enzyme was added to an 80-mL reaction mixture at 37°C for 12 h, and the reaction was terminated by addition of 4 mL of 20% phosphoric acid. Denatured proteins in the reaction mixture were precipitated by centrifugation and passed through a filter. The product of the enzyme reaction was purified by two rounds of ODS flash column chromatography. The purified product from the Dg AA7GT enzyme reaction had a molecular mass (MS) [M]⁺ at *m/z* 611 (positive ion mode) corresponding to that expected of Cy3,7dG (C₂₇H₃₁O₁₆: 611). The ¹H-NMR analysis (400 MHz and a 9:1 mixture of CD₃OD:CF₃COOD) gave the following results: δ 3.96–3.33 (10H, m, 3 and 7Glc H), 5.18 (1H, d, 7GlcH1, *J* = 6.47), 5.35 (1H, d, 3GlcH1, *J* = 8.02), 6.84 (1H, s, H8), 7.01 (1H, d, H2'), 7.26 (1H, s, H6), 8.09 (1H, d, H5'), 8.34 (1H, dd, H6'), and 8.98 (1H, s, H4). Peaks were identified as described in a previous report (Hashimoto et al., 2002), and the positions of glucosides were determined from a Nuclear Overhauser Effect difference spectrum (see Supplemental Figure 4 online).

Dc AA5GT Expression, AA5GT Activity, and the Accumulation of Anthocyanin

Total RNAs were obtained as described above from petals of the carnation varieties Beam Cherry, Nazareno, Red Disney, Cueva, and Garnet and the stems and leaves of the carnation variety Beam Cherry. First-strand cDNA was synthesized from 500 ng of total RNA using the oligo(dT)₁₅ primer (Promega) and M-MLV reverse transcriptase (Invitrogen). Specific primers DcAA5GTF and DcAA5GTR (see Supplemental Table 8 online) were designed for Dc AA5GT, and primers DcCHSF and DcCHSR, DcDFRF and DcDFRR, DcANSF and DcANSR, DcU3GTF and DcU3GTR, and DcActinF and DcActinR (see Supplemental Table 8 online) for Dc CHS, Dc DRF, Dc ANS, Dc U3GT, and Actin, respectively. Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio) and the DNA Engine Opticone 2 (Bio-Rad Laboratories). The reaction conditions were 30 s at 95°C and then 35 cycles for each pair of primers, consisting of 5 s at 94°C, 15 s at 58°C, and 10 s at 72°C. The quantification of RT-PCR was performed by Delta Ct. The PCR conditions used to detect Dc AA5GT transcripts in the five varieties of carnation (Figure 6A) were 2 min at 94°C and then 32 cycles for each pair of primers, consisting of 30 s at 94°C, 30 s at 60°C, and 20 s at 72°C. The crude protein extracts used to investigate Dc AA5GT activity were prepared from four petal developmental stages (Figure 5A), from the stems and leaves of the Beam Cherry variety, and also from the petals of the other varieties shown in Figure 6A. AA5GT activity was assessed using the same conditions as described above. Anthocyanins were extracted from the petals, stems, and leaves of Beam Cherry (Figure 5B) and the petals of Nazareno, Red Disney, Cueva, and Garnet (Figure 6A) using 80% methanol containing 0.1% trifluoroacetic acid. The different types of anthocyanin in the extracts (Figure 6A) were identified using an HPLC equipped with a Synergi 4 μ Fusion-RP 80Å (i.d. 4.6 $\times$ 250 mm) by linear gradient elution (1 mL/min) from 5 to 43% acetonitrile in solvent

C for 20 min. Peak identification was based on retention time and cochromatography with the standard. The amounts of total anthocyanin in Beam Cherry (Figure 5B) were measured at an absorbance at 520 nm by spectrophotometrical analyses using Pg3,5dG as the standard. The amounts of 3,5-di-O-(glucopyranosyl) pelargonidin 6''-O-4,6'''-O-1-cyclic malate (Pg3,5cyclicmalylidG) in Beam Cherry were calculated based on the area of HPLC chromatogram at 520 nm using Pg3,5dG as the standard.

Transient Expression of Dc AA5GT cDNA in Carnation Petals in Which Anthocyanin Lacks the Glucosyl Moiety at the 5 Position

Nucleotide sequences corresponding to the full-length Dc AA5GT product were obtained by PCR using the Dc AA5GT cDNA fragment as the template with appropriate primers. Similarly, nucleotide sequences corresponding to the deletion protein (which lacks the N-terminal region corresponding to the transit peptide but includes the first Met) were prepared. cDNA fragments were introduced into pGWB402 Ω (Nakagawa et al., 2009) using the Gateway system (Invitrogen). pSK-GFP plasmid harboring a GFP cDNA driven by CaMV 35S promoter in pBluescript SK+ vector was used as the control for expression of the gene introduced by bombardment into carnation petals. Five hundred micrograms of gold particles (diameter 1.0 μ m) coated with 1 μ g of plasmid were used with the Biolistic PDS-1000/He system (Bio-Rad Laboratories). The conditions used for the bombardment and those of the following incubation were the same as those described for onion epidermal cells in the methods. Transformed carnation epidermal cells were viewed using a fluorescence microscope.

Characterization of the Enzyme Reaction Properties of the AAGT Proteins

Assessment of the enzyme kinetics in the linear range of the reaction time was performed using the following reaction mixtures: 10 μ g of the crude extracts from carnation petals and 24 ng of purified native Dc AA5GT (or purified recombinant Dc AA5GT or Dg AA7GT) in a reaction mixture of 50 μ L of 0.1 M citrate buffer (pH 5.0 for crude extracts for carnation petals and native Dc AA5GT and pH 6.0 for recombinant Dc AA5GT and Dg AA7GT) containing 10 nmol Cy3G and 50 nmol VG, and incubated at 30°C. The linear range between enzyme activity and reaction time (see Supplemental Figure 8 online) or amount of enzyme protein (range of protein amount is shown in the horizontal axis of Supplemental Figure 9 online) was assessed in a 50- μ L reaction mixture consisting of 10 nmol Cy3G and 50 nmol VG in 0.1 M citrate buffer (pH 5.0 for native Dc AA5GT and pH 6.0 for recombinant Dc AA5GT and Dg AA7GT), and incubated at 30°C for 20 min. The optimum reaction temperature for activity of each AAGT was determined by analyses performed in the range 10 to 60°C in 0.1 M citrate buffer, pH 5.0 (305 ng of native Dc AA5GT) or pH 6.0 (242 ng and 246 ng of purified recombinant Dc AA5GT and Dg AA7GT, respectively) containing 10 nmol Cy3G and 50 nmol VG and incubated for 15 min (see Supplemental Figure 10 online). The effect of reaction mixture pH on AAGT activity was determined across the range pH 3.0 to 9.0 using 0.1 M citrate, MES, malate, potassium phosphate, and Tris-HCl buffers containing 305 ng of native Dc AA5GT, 242 and 246 ng of purified recombinant Dc AA5GT and Dg AA7GT, respectively, 10 nmol Cy3G and 50 nmol VG, and incubated at 30°C for 15 min (see Supplemental Figure 11 online).

The K_m values of Cy3G for the AAGT reactions were determined by varying the concentration of Cy3G in the range 10 to 80 μ M at 1 mM acyl-glucose and incubating with 242 to 305 ng purified AAGT protein for 10 min at 30°C in 0.1 M citrate buffer, pH 5.0 (native Dc AA5GT) or pH 6.0 (recombinant Dc AA5GT and Dg AA7GT). The K_m values of acyl-glucose were determined by varying the concentration of acyl-glucose in the range 20 to 80 μ M at 200 μ M Cy3G and incubating with each AAGT protein as above. Preferences for acceptor molecules were assessed for

each substrate by incubating 200 μ M of the substrate with 1 mM VG (AA5GT) or pHBG (AA7GT) in a reaction mixture containing purified AAGT protein. The reaction was terminated by addition of phosphoric acid, and then 15 μ L of the reaction mixture was introduced into an ODS HPLC column (Synergi 4 μ Fusion-RP 80 $\text{\AA}$, i.d. 4.6 $\times$ 250 mm; Phenomenex) to screen for formation of glucosylated acceptor molecules. The substrate and products were separated by linear gradient elution (1 mL/min) from 10 to 80% (for catechin and epicatechin, 10 to 35%) acetonitrile in solvent C for 20 min using HPLC-DAD (LaChrom Elite system; Hitachi Hi-Technologies) monitored at 190 to 600 nm. The possible glucosylated flavonoids, naphthols, coumarins, phenols, and an indole were analyzed by HPLC and compared with the products of a reaction mixture lacking donor substrate. Five hundred micromolar of chemically or enzymatically synthesized donor candidate molecules was incubated with 200 μ M Cy3G as the acceptor in the enzyme reaction mixture, followed by product analysis in the same manner as for the reaction using VG as the donor.

The K_m value of the glucosidase activity of recombinant AA5GT and AA7GT for Cy3,5dG, Cy3,7dG, VG, FG, and pHBG were determined by varying the concentration in the range of 20 to 500 μ M and incubating with each AAGT protein as above.

The other glycosidase activities of recombinant Dc AA5GT and Dg AA7GT for phenol glucosides, a coumarin glucoside, flavonoid glucosides, and an indole glucoside were assessed for each substrate by incubating 1 mM (phenol glucosides, a coumarin glucoside, and an indole glucoside) or 200 μ M (flavonoid glucosides) in a reaction mixture containing purified AAGT protein. The reaction was terminated by addition of phosphoric acid, and then 15 μ L of the reaction mixture was introduced into an ODS HPLC column (Synergi 4 μ Fusion-RP 80 $\text{\AA}$, i.d. 4.6 $\times$ 250 mm) to screen the aglycone molecules and each glycoside. The substrate and products were separated by linear gradient elution (1 mL/min) from 10 to 35% acetonitrile in solvent C for 20 min using HPLC-DAD monitored at 190 to 600 nm. Relative activity is shown in comparison to VG, which is arbitrarily set as 100%.

The reverse glucosyltransferase activities using purified AAGT protein from Cy3,5dG to VA, FA, or UDP and of Cy3,7dG to VA, pHBA, or UDP were determined in a reaction mixture containing 150 ng of each AAGT, 10 nmol of Cy3,5dG or Cy3,7dG, and 50 nmol of VA, FA, pHBA, or UDP and incubated at 30°C for 20 min. The reaction was terminated by addition of phosphoric acid, and then 15 μ L of the reaction mixture for VA, VG, FA, FG, pHBA, or pHBG were introduced into an ODS HPLC column (Synergi 4 μ Fusion-RP 80 $\text{\AA}$ column, i.d. 4.6 $\times$ 250 mm) or of the reaction mixture for UDP or UDPG into a Develosil RPAQUOS RP-5 column (i.d. 4.6 $\times$ 250 mm) to screen the substrate and each glucoside. The substrate and products were separated by linear gradient elution (1 mL/min) from 10 to 45% methanol in solvent C for 20 min (VA, FA, or pHBA) or 100% solvent C elution for 20 min (UDP) using HPLC-DAD monitored at 190 to 600 nm.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL/ DDBJ databases under accession numbers AB507446 (Dc AA5GT) and AB510758 (Dg AA7GT). Accession numbers for sequences used in phylogenetic analysis can be found in Supplemental Table 2 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Schematic Outline of the Procedures for Chemical Synthesis of 1-O- β - and α -D-Vanillyl-glucose.

Supplemental Figure 2. Protein Profiles on an SDS-PAGE Gel at Each Step of Purification of Dc AA5GT from Carnation Petals.

Supplemental Figure 3. Deduced Amino acid Sequences of Dc AA5GT and Dg AA7GT.

Supplemental Figure 4. The ^1H -NMR Spectrum and Nuclear Overhauser Effect Difference Spectrum of Cy3,7dG.

Supplemental Figure 5. Sequence Alignment of GH1 Proteins.

Supplemental Figure 6. Transient Expression of AAGTs:GFP Fusion Constructs in Petals of the Carnation Variety Garnet in Which Anthocyanin Lacks the Glucosyl Moiety at the 5 Position.

Supplemental Figure 7. Protein Profiles on an SDS-PAGE Gel at Each Step of Purification of Recombinant Dc AA5GT and Dg AA7GT.

Supplemental Figure 8. Time Course of Enzyme Reaction of AAGTs.

Supplemental Figure 9. Linearity of the Enzyme Reaction versus the Amount of Enzyme Protein.

Supplemental Figure 10. The Optimum Reaction Temperature for Activity of Each AAGT.

Supplemental Figure 11. The Optimum pH for Activity of Each AAGT.

Supplemental Table 1. Purification of Dc AA5GT from a 400 g Sample of Petals of Carnation Variety Beam Cherry.

Supplemental Table 2. Protein Sequences Used for Construction of the Phylogenetic Tree (Figure 3).

Supplemental Table 3. Kinetic Parameters of the Glucosyltransferase Activities of the AAGTs.

Supplemental Table 4. The Amounts of Six Glucose Esters That Accumulated in Petals of the Carnation Variety Beam Cherry and the Delphinium Variety Triton Light Blue.

Supplemental Table 5. Substrate Preferences and Relative Activities of Glycosidase Activities of Recombinant Dc AA5GT and Dg AA7GT Proteins.

Supplemental Table 6. Kinetic Parameters of the Glucosidase Activities of the AAGT Proteins.

Supplemental Table 7. Comparison of Glucosyltransferase and Glucosidase Activities Using Both Acceptor and Donor or Without Either Substrate.

Supplemental Table 8. Nucleotide Sequences of PCR Primers.

Supplemental Data Set 1. Protein Sequences Used for Construction of the Phylogenetic Tree (Figure 3).

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A Novel Glucosylation Reaction on Anthocyanins Catalyzed by Acyl-Glucose-Dependent Glucosyltransferase in the Petals of Carnation and Delphinium

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