Plant Biotechnology 29, 223–227 (2012)
DOI: 10.5511/plantbiotechnology.12.0120a

Short Communication

Special Issue

Identification of the *glutathione S-transferase* gene responsible for flower color intensity in carnations

Nobuhiro Sasaki[1], Yuzo Nishizaki[1], Yasuhiro Uchida[1], Eigo Wakamatsu[1], Naoyuki Umemoto[2], Masaki Momose[2], Masachika Okamura[2], Hiroyuki Yoshida[3], Masaatsu Yamaguchi[4], Masayoshi Nakayama[5], Yoshihiro Ozeki[1,*], Yoshio Itoh[1,a]

[1] Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan; [2] Central Laboratories for Frontier Technology, Kirin Holdings Co., Ltd., Sakura, Tochigi 329-1414, Japan; [3] Applied Plant Research Laboratory, Japan Tobacco, Inc., Oyama, Tochigi 323-0808, Japan; [4] Faculty of Enviromental Horticulture, Minami Kyushu University, Miyazaki 885-0035, Japan; [5] Institute of Floricultural Science, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-8519, Japan
* E-mail: ozeky@cc.tuat.ac.jp Tel & Fax: +81-42-388-7239

Received December 22, 2011; accepted January 20, 2012 (Edited by N. Ohtsubo)

Abstract Two cDNAs with homology to *glutathione* S-*transferase* (*GST*) were isolated from the carnation (*Dianthus caryophyllus*); these cDNAs are termed here *DcGSTF1* and *DcGSTF2*. Phylogenetic analysis suggested that both DcGSTF1 and DcGSTF2 belonged to the Phi class of GSTs. *DcGSTF2* showed high levels of transcription at late stages of petal development when anthocyanin biosynthesis is most active. Sequencing of *DcGSTF2* indicated that it consisted of three exons and two introns. A truncated *DcGSTF2* gene, resulting from the insertion of a CACTA-type transposable element, was found in the genome of a mutable flower line bearing deep pink sectors on pale pink petals. A full length *DcGSTF2* gene driven by a continuous expression promoter was introduced into the epidermal cells of carnations with pale pink petals. The transformed cells were deep pink. These results suggest that the *DcGSTF2* gene is responsible for flower color intensity in carnations.

Key words: Anthocyanin, carnation, glutathione *S*-transferase, transposable element, vacuolar transport.

Carnations (*Dianthus caryophyllus*) are one of the most popular cultivated flowers around the world and this interest has led to the breeding of a large number of varieties with differently colored flowers. The pigmentation of the flowers is derived from the anthocyanins in the cells of the petals. The structures of anthocyanin molecules in the carnation have been determined (Nakayama et al. 2000). Our group is investigating the genes responsible for carnation flower color and is striving to identify the enzymes catalyzing the reaction steps in the synthesis of anthocyanin molecules. These investigations identified the genes *phenylalanine ammonia-lyase* (*PAL*) (Yoshimoto et al. 2000) and *dihydroflavonol reductase* (*DFR*), which is disrupted by the insertion of transposable elements (Itoh et al. 2002); we also found anthocyanin modification enzymes, such as two types of glucosyltransferase that utilize UDP-glucose (Ogata et al. 2004) or acyl-glucose (Matsuba et al. 2010) as the glucose-donor, and a malyltransferase (Abe et al. 2008). Mutation of the genes encoding anthocyanin biosynthetic enzymes can lead to changes in the colors of the carnation flowers (Itoh et al. 2002; Nishizaki et al. 2011). The variations in flower color usually result from changes to the anthocyanin molecules. By contrast, flower color intensity is thought to be determined by the amount of anthocyanin present. Analysis of several carnation varieties that have pale pink petals confirmed that the plants had a reduced anthocyanin content in their petals without any alteration in the composition of the anthocyanins (data not shown). The pale color in petal epidermal cells can be restored to a deeper color by the introduction of maize *Bronze2* (*Bz2*) or petunia *Anthocyanin9* (*An9*) genes using a microprojectile bombardment method (Larsen et al. 2003). As *Bz2* and *An9* encode glutathione *S*-transferase (GST)-like proteins, one possible explanation of the pale pink coloration in some carnation varieties is disruption of a *GST-like* gene. Here, we isolated two

Abbreviations: DFR, dihydroflavonol reductase; GFP, green fluorescent protein; GST, glutathione S-transferase; MATE, the multidrug and toxin extrusion protein; PAL, phenylalanine ammonia-lyase; RACE, rapid amplification of cDNA ends.

[a] Deceased.

This article can be found at http://www.jspcmb.jp/
Published online June 15, 2012

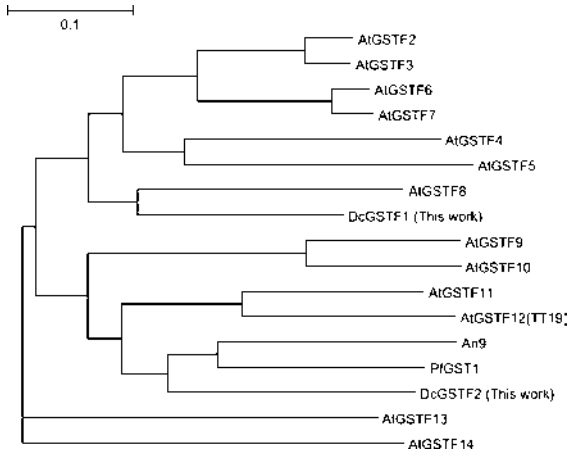

Figure 1. Unrooted phylogenetic tree of Phi class GSTs, including those of *Arabidopsis*, *DcGSTF1* and *DcGSTF2*, and *Perilla frutescens PfGST1*, based on their amino acid sequences. Multiple alignments of the amino acids sequences were determined using the CLUSTALW algorithm, which is available on the GENOMENET server (http://www.genome.jp), and the phylogenetic tree was constructed using GNETYX-TREE (GENETYX corp., Tokyo, Japan). Bar = 0.1 amino acid substitutions/site. The accession numbers for the sequences are: *An9*, Y07721; *DcGSTF1*, AB688110; *DcGSTF2*, AB688111; *PfGST1*, AB362191; *AtGSTF2*, At4g02520; *AtGSTF3*, At2g02930; *AtGSTF4*, At1g02950; *AtGSTF5*, At1g02940; *AtGSTF6*, At1g02930; *AtGSTF7*, At1g02920; *AtGSTF8*, At2g47730; *AtGSTF9*, At1g02930; *AtGSTF10*, At2g30870; *AtGSTF11*, At3g03190; *AtGSTF12*, At5g17220; *AtGSTF13*, At3g62760; *AtGSTF14*, At1g49860.

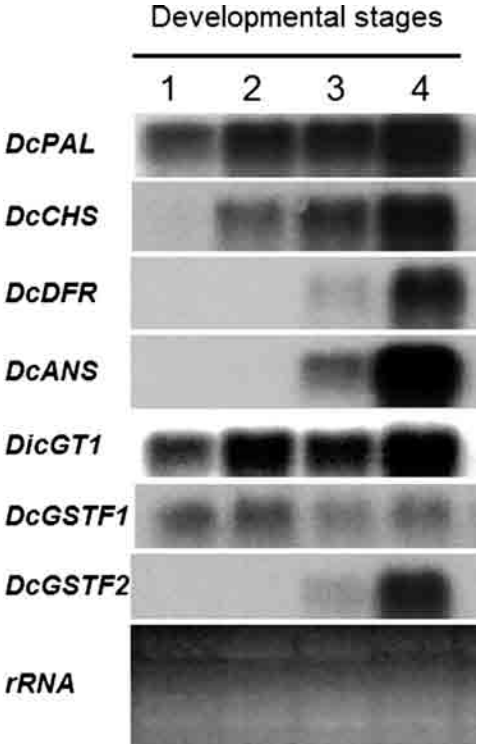

Figure 2. Expression profiles at different stages of carnation petal development. The cDNA fragments used as probes were obtained by PCR using gene-specific primers designed from the DNA sequence information deposited in genome databases. The ^{32}P-labeled probes were hybridized with total RNAs bound to the membrane. Hybridization was performed at 65°C overnight. Membranes were washed twice with a low stringency buffer (2×SSC, 0.5% SDS) at room temperature followed by two washes with a high stringency buffer (0.1×SSC, 0.1% SDS) at 65°C. *DicGT1* was used as the anthocyanin 3-glucosyltransferase gene (Ogata et al. 2004). The membranes were exposed to X-ray films. The DDBJ accession numbers of the genes are: *DcPAL*, AB041361; *DcCHS*, AF267173; *DcDFR*, AB071787; *DcANS*, U82432; *DicGT1*, AB191245.

cDNAs with homology to *GST*; these cDNAs were obtained by PCR using first strand cDNA prepared from carnation petals as the template and degenerate primers based on conserved amino acid sequences in the GST protein. The 5′- and 3′-regions of both cDNAs were obtained by 5′- and 3′-rapid amplification of cDNA ends (RACE) using a GENE Racer kit (Invitrogen, Carlsbad, CA, USA). Full-length cDNAs were obtained by PCR using primers designed for the 5′-untranslated region (UTR) (DcGSTF1, 5′-ATA ACC TCT CAA TTT TCT CTC TAA -3′; DcGSTF2, 5′-TAA TCA AGT AAG AAA AAA TGG GAG T-3′) and 3′-UTR (DcGSTF1, 5′-TCA CTG ACT CAC TGT ATA ACA CGA -3′; DcGSTF2, 5′-TGT GCC CTG TAT GGC TGT ATG TTC T-3′). A homology search of the deduced amino acid sequences of the amplified fragments revealed that these GST homologs belonged to the Phi class of GST proteins (Dixon and Edwards 2010). Therefore, the two GSTs were designated DcGSTF1 and DcGSTF2 in accordance with the consensus nomenclature (Edwards et al. 2000). *DcGSTF1* cDNA contained an ORF of 624 bp encoding 208 amino acids; the *DcGSTF2* cDNA contained an ORF of 651 bp encoding 217 amino acids. The amino acid sequences of DcGSTF1 showed 45% identity to Dc2GSTF2. A phylogenetic tree analysis based on the amino acid sequences revealed that DcGSTF2 fell into a clade that includes An9 and AtGSTF12 (TT19). This implies that DcGSTF2 might be involved in transportation of anthocyanin into the vacuole in carnations, in a similar manner to An9 and TT19 in the accumulation of anthocyanin/flavonoids in the petunia and *Arabidopsis*, respectively (Alfenito et al. 1998; Kitamura et al. 2004).

The expression profiles of *DcGST* genes at different stages of flower development were analyzed by northern hybridization. We found that *DcGSTF2* mRNA accumulated at later developmental stages, a pattern similar to that reported for other 'late' genes involved in anthocyanin biosynthesis, such as *DFR*, *ANS* and an anthocyanin 3-glucosyltransferase named *DicGT1* (Ogata et al. 2004) (Figure 2). By contrast, *DcGSTF1* was expressed at all stages of flower development. These results suggest that DcGSTF2 might be involved in the transportation of anthocyanin molecules from the cytosol into the vacuole.

To determine whether DcGSTF2 is involved in transportation of anthocyanin molecules into the vacuole *in vivo*, we performed a complementation experiment

using the pale pink flower variety '03L-3' (Figure 3A). *DcGSTF2* and *green fluorescent protein* (*GFP*), driven by a cauliflower mosaic virus 35S (CaMV35S) promoter, were co-introduced into epidermal cells of 03L-3 petals by a microprojectile bombardment method. After 48 h incubation at 27°C, the cells were analyzed by fluorescence microscopy and green fluorescence was observed in transformed cells; the cells appeared deep pink when observed by bright field microscopy (Figure 3B). When only the *GFP* construct was introduced into the epidermal cells, no deep pink spots were detected although fluorescence arising from the GFP was observed (Figure 3C). These results confirm that DcGSTF2 is actively involved in anthocyanin accumulation in the vacuole.

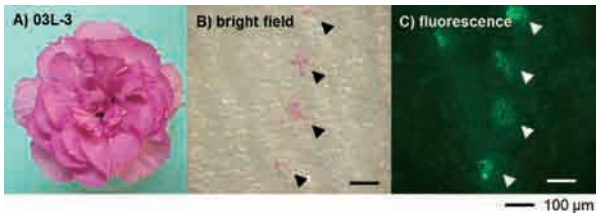

Figure 3. Complementation analysis of the *DcGSTF2* gene in the '03L-3' variety, which has pale pink petals. (A), Flower from an '03L-3' plant showing its typical pale pink pigmentation. (B), Bright field and (C) fluorescence images of epidermal cells co-expressing *DcGSTF2*, driven by the CaMV35S promoter, and GFP.

Genomic DNAs were extracted from the leaves of 'Scania' (which has deep red flowers) and 03L-3 plants (which have pale pink flowers) using the cetyl trimethyl ammonium bromide (CTAB)-CsCl ultracentrifugation method. Using these DNAs as templates, the *DcGSTF2* genomic region was amplified by PCR with the primers 5′-TAA TCA AGT AAG AAA AAT GGG AGT -3′ and 5′-TGT GCC CTG TAT GGC TGT ATG TTC T-3′. Both varieties yielded a DNA fragment of ca. 1.9 kbp; these fragments were cloned into pBluescript SK^+ vector and their DNA sequences were determined. Comparison of these DNA sequences with that of *DcGSTF2* cDNA showed that the latter consisted of three exons and two introns, similar to other GST members of the Phi class (Dixon and Edwards 2010). The *DcGSTF2* genomic region, termed here *DcGSTF2mu*, from the '03L-3' variety contained ten nucleotide substitutions in the third exon compared to that of 'Scania'. One of these substitutions in the third exon was associated with the alteration of a glutamine codon to a stop codon (Figure 4C, asterisk), which might cause a loss of function and lead to the formation of pale flower colors. The phenotypic change could result from the transport of lower amounts of anthocyanins by either DcGSTF1 (in

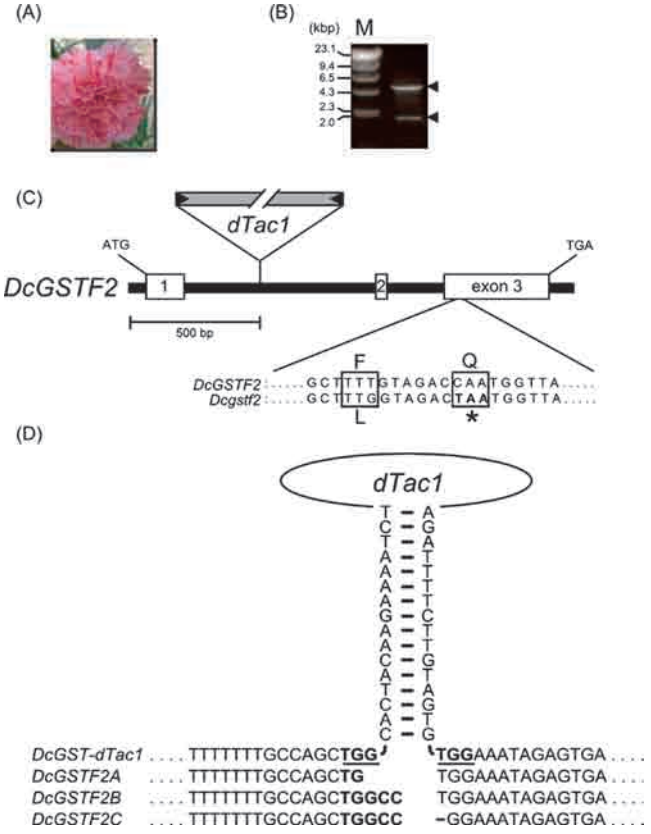

Figure 4. *DcGSTF2* gene structures and the insertion site of *dTac1*. (A), Flower from the variegated variety 'Daisy-VP'. (B), Genomic PCR using 'Daisy-VP' DNA as the template and primers designed to amplify *DcGSTF2*. (C), Diagram of the structure of *DcGSTF2* and its variant. (D) The DNA sequences of the *dTac1* insertion region and footprints.

place of DcGSTF2) or other trafficking mechanisms, such as the route mediated by the multidrug and toxin extrusion protein (MATE) (Gomez et al. 2009), or via the endoplasmic reticulum-mediated system (Poustka et al. 2007).

The carnation variety 'Daisy-VP' has variegated pale pink and deep pink flowers (Figure 4A). It has been reported that the variegation is caused by the insertion of a transposable element into the genes involved in the anthocyanin synthetic pathway (Iida et al. 2004; Itoh et al. 2002). We speculated that the variegated phenotype of 'Daisy-VP' might be due to insertion of a transposable element into the *DcGSTF2* gene. We amplified the *DcGSTF2* genomic region by PCR using the gene specific primers described above, and also extracted genomic DNA from the deep pink parts of the flowers of 'Daisy-VP' for use as a template. Two major bands, ca. 4.5 kbp and 1.9 kbp, were detected on the agarose gel (Figure 4B). Sequencing of the 4.5 kbp fragment amplified from 'Daisy-VP' showed a 3,640 bp insertion at the first intron. The inserted sequence contained 16 bp terminal inverted repeats starting with 5′-CACTA-3′; additionally, the inserted element generated a 3 bp target site duplication (TGG) adjoining the insertion site. These characteristic sequences indicated that the insertion was a CACTA-type DNA transposon. We termed this transposable element *dTac1*; the insert sequence contained an incomplete ORF suggesting it was a non-autonomous element. Four other DNA fragments were also obtained from shorter amplicons. Three of these contained inaccurate footprints at the *dTac1* detachment site (Figure 4C). The fourth corresponded to the *DcGSTF2mu* genomic region. These results suggest that 'Daisy-VP' is heterozygous and harbors genomic *DcGSTF2* truncated by *dTac1* and *DcGSTF2mu*.

Several trafficking mechanisms, such as MATE-mediated, GST-ABC transporter mediated and endoplasmic reticulum-mediated, have recently been proposed to be involved in the transport of secondary metabolites (Ozeki et al. 2011). In *Arabidopsis*, carnation, petunia, and maize, the GST protein mediated mechanism seems to be the main one for trafficking anthocyanins. *Bz2* of maize is grouped in the Tau class of GSTs. By contrast, *Arabidopsis TT19*, carnation *DcGSTF2*, *Perilla frutescens PfGST* (identified as encoding the protein involved in anthocyanin transportation; Yamazaki et al. 2008), and petunia *An9* are in the Phi class. The difference in classification of *Bz2* and these other genes suggests that the GST-mediated anthocyanin transport mechanism of monocotyledons evolved separately from that of dicotyledons.

Acknowledgments

This research was supported by Grants from the Research and Development Program for New Bio-industry Initiatives to Y. O.

References

Abe Y, Tera M, Sasaki N, Okamura M, Umemoto N, Momose M, Kawahara N, Kamakura H, Goda Y, Nagasawa K, Ozeki Y (2008) Detection of 1-*O*-malylglucose: pelargonidin 3-*O*-glucose-6″-*O*-malyltransferase activity in carnation (*Dianthus caryophyllus*). *Biochem Biophys Res Commun* 373: 473–477

Alfenito MR, Souer E, Goodman CD, Buell R, Mol J, Koes R, Walbot V (1998) Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione *S*-transferases. *Plant Cell* 10: 1135–1149

Dixon DP, Edwards R (2010) Glutathione transferases. *Arabidopsis Book* 8: e0131

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci* 5: 193–198

Gomez C, Terrier N, Torregrosa L, Vialet S, Fournier-Level A, Verriès C, Souquet J-M, Mazauric JP, Klein M, Cheynier V, Ageorges A (2009) Grapevine MATE-type proteins act as vacuolar H^+-dependent acylated anthocyanin transporters. *Plant Physiol* 150: 402–415

Iida S, Morita Y, Choi JD, Park KI, Hoshino A (2004) Genetics and epigenetics in flower pigmentation associated with transposable elements in morning glories. *Adv Biophys* 38: 141–159

Itoh Y, Higeta D, Suzuki A, Yoshida H, Ozeki Y (2002) Excision of transposable elements from the *chalcone isomerase* and *dihydroflavonol 4-reductase* genes may contribute to the variegation of the yellow-flowered carnation (*Dianthus caryophyllus*). *Plant Cell Physiol* 43: 578–585

Kitamura S, Shikazono N, Tanaka A (2004) *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. *Plant J* 37: 104–114

Larsen ES, Alfenito MR, Briggs WR, Walbot V (2003) A carnation anthocyanin mutant is complemented by the glutathione *S*-transferases encoded by maize *Bz2* and petunia *An9*. *Plant Cell Rep* 21: 900–904

Matsuba Y, Sasaki N, Tera M, Okamura M, Abe Y, Okamoto E, Nakamura H, Funabashi H, Takatsu M, Saito M, Matsuoka H, Nagasawa K, Ozeki Y (2010) A novel glucosylation reaction on anthocyanins catalyzed by acyl-glucose-dependent glucosyltransferase in the petals of carnation and delphinium. *Plant Cell* 22: 3374–3389

Nakayama M, Koshioka M, Yoshida H, Kan Y, Fukui Y, Koike A, Yamaguchi M (2000) Cyclic malyl anthocyanins in *Dianthus caryophyllus*. *Phytochemistry* 55: 937–939

Nishizaki Y, Matsuba Y, Okamoto E, Okamura M, Ozeki Y, Sasaki N (2011) Structure of the acyl-glucose-dependent anthocyanin 5-*O*-glucosyltransferase gene in carnations and its disruption by transposable elements in some varieties. *Mol Genet Genomics* 286: 383–394

Ogata J, Itoh Y, Ishida M, Yoshida H, Ozeki Y (2004) Cloning and heterologous expression of cDNAs encoding flavonoid glucosyltransferases from *Dianthus caryophyllus*. *Plant Biotechnol* 21: 367–375

Ozeki Y, Matsuba Y, Abe Y, Umemoto N, Sasaki N (2011) Pigment biosynthesis I. Anthocyanins. In: Ashihara H, Crozier A, Komamine A (eds) *Plant Metabolism and Biotechnology.* Wiley, West Sussex, pp 321–342

Poustka F, Irani NG, Feller A, Lu Y, Pourcel L, Frame K, Grotewold E (2007) A trafficking pathway for anthocyanins overlaps with the endoplasmic reticulum-to-vacuole protein-sorting route in Arabidopsis and contributes to the formation of vacuolar

inclusions. *Plant Physiol* 145: 1323–1335
Yamazaki M, Shibata M, Nishiyama Y, Springob K, Kitayama M, Shimada N, Aoki T, Ayabe S, Saito K (2008) Differential gene expression profiles of red and green forms of *Perilla frutescens* leading to comprehensive identification of anthocyanin biosynthetic genes. *FEBS J* 275: 3494–3502
Yoshimoto Y, Higeta D, Itoh Y, Yoshida H, Hasebe M (2000) Isolation and characterization of a cDNA for phenylalanine ammonia-lyase (PAL) from *Dianthus caryophyllus* (carnation). *Plant Biotechnol* 17: 325–329