Reverted glutathione S-transferase-like genes that influence flower color intensity of carnation (Dianthus caryophyllus L.) originated from excision of a transposable element

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A glutathione S-transferase-like gene, DeGSTF2, is responsible for carnation (Dianthus caryophyllus L.) flower color intensity. Two defective genes, DeGSTF2mu with a nonsense mutation and DeGSTF2-dTacl containing a transposable element dTacl, have been characterized in detail in this report. dTacl is an active element that produces reverted functional genes by excision of the element. A pale-pink cultivar ‘Daisy’ carries both defective genes, whereas a spontaneous deep-colored mutant ‘Daisy-VPR’ lost the element from DeGSTF2-dTacl. This finding confirmed that dTacl is active and that the resulting reverted gene, DeGSTF2rev1, missing the element is responsible for this color change. Crosses between the pale-colored cultivar ‘06-LA’ and a deep-colored cultivar ‘Spectrum’ produced segregating progeny. Only the deep-colored progeny had DeGSTF2rev2 derived from the ‘Spectrum’ parent, whereas progeny with pale-colored flowers had defective forms from both parents, DeGSTF2mu and DeGSTF2-dTacl. Thus, DeGSTF2rev2 had functional activity and likely originated from excision of dTacl since there was a footprint sequence at the vacated site of the dTacl insertion. Characterizing the DeGSTF2 genes in several cultivars revealed that the two functional genes, DeGSTF2rev1 and DeGSTF2rev2, have been used for some time in carnation breeding with the latter in use for more than half a century.

Key Words: anthocyanin, CACTA, Dianthus, glutathione S-transferase, S locus, transposable element.

Introduction

Carnation, Dianthus caryophyllus L., is one of the most important ornamentals in the world, and many cultivars with different flower colors have been developed. Flower pigments are mainly anthocyanin and chalcone derivatives, and most of the genes involved in pigment biosynthesis in carnation have been identified (Abe et al. 2008,Britsch et al. 1993, Itoh et al. 2002, Mato et al. 2000, Matsuba et al. 2010, Momose et al. 2013, Ogata et al. 2004, Yoshimoto et al. 2000).

A genetic study conducted more than half a century ago concluded that the S locus controls the amount of anthocyanin in carnation petals. A dominant S results in deep flower colors such as red, deep pink, crimson and purple, whereas a recessive s results in pale colors such as salmon, light pink and lavender (Mehlquist and Geissman 1947). These investigators also reported that a mutable svar allele in some varieties causes variegation of deep-color within a pale background color, for instance red stripes on a salmon background.

Flower color intensity is regulated by the amount of pigments that accumulate in petal cells. The anthocyanin content of flower petals results from biosynthesis and transport to vacuoles where the pigment accumulates. Vacuolar sequestration of pigments requires transporter(s) and glutathione S-transferase (GST); however, the glutathionation of anthocyanin by GST is controversial because anthocyanin-glutathione conjugates have not been found (Koes et al. 2005). The GST-like protein rather than a glutathionation activity is thought to be required, but the mechanisms for flavonoid transport into vacuoles are not fully understood.
(Zhao and Dixon 2010). Anthocyanin-related GST genes were found in various plant species, including An9 of Petunia hybrida (Alfenito et al. 1998), TT19 of Arabidopsis thaliana (Kitamura et al. 2004), PfGST1 of Perilla frutescens (Yamazaki et al. 2008), VvGST1 and VvGST4 of Vitis vinifera (Conn et al. 2008) and CkmGST3 of Cyclamen sp. (Kitamura et al. 2012) after the B2Z gene of Zea mays that is necessary for vacuolar anthocyanin accumulation was revealed to encode a GST (Marrs et al. 1995).

The fl3 mutant of carnation, whose flower colors are pale due to a low anthocyanin content, was reported to be complemented by the GST genes of maize (BZ2) or petunia (An9), suggesting that FL3 encodes a GST (Larsen et al. 2003). The report proposed that the mutable fl3-m allele that was thought to contain a transposable element caused spots or sectors of deep color to appear on light-colored backgrounds when the activating locus Rfl3-m, a putative transposase acting on fl3-m, was present. However, neither the gene nor the transposable element causing the mutable phenotype has been identified.

A carnation gene named DcGSTF2 that putatively encodes a protein of 217 amino acids was recently identified and shown to be responsible for flower color intensity (Sasaki et al. 2012). The gene belongs to the Phi class of GST proteins and was classified within a clade including An9 and TT19 by phylogenetic analysis of its amino acid sequence. Furthermore, this report described two defective genes in a pale pink-flowered cultivar ‘Daisy’ and its mutant ‘Daisy-VP’ that has variegated flowers with deep-pink stripes on a pale-pink background. The first gene is DcGSTF2-mu that contains a nucleotide substitution in the third exon resulting in a stop codon at an unexpected position, thereby putatively encoding a truncated protein of 101 amino acids. The second defective gene is DcGSTF2-dTac1 that has a non-autonomous CACTA-type transposable element, dTac1, in the first intron of the gene (GenBank accession no. AB688111). Analysis of DNA from deep-pink regions in variegated ‘Daisy-VP’ found the footprint sequence instead of the dTac1 insertion in DcGSTF2-dTac1 suggesting that excision of the transposable element resulted in deep color stripes through the recovery of gene function.

Here, we confirm that dTac1, present in the DcGSTF2-dTac1, is an active transposable element and that movement of the element resulted in reversion of the gene. We also report two functional genes produced by excision of the element have been used in carnation breeding for many years.

Materials and Methods

Plant materials

The carnation cultivar ‘Daisy’ with stable pale-pink flowers and two flower color mutants that originated from the cultivar, ‘Daisy-VP’ with variegated flowers and ‘Daisy-VPR’ with deep-pink flowers (Fig. 1A–1C), were grown in a greenhouse. The carnation cultivar ‘Spectrum’ was obtained from the National Institute of Agrobiological Sciences Genebank.

A breeding line ‘06LA’ (Fig. 1D) pollinated by ‘Spectrum’ (Fig. 1E) produced eight seeds. The seeds were sown and grown in a greenhouse until flowering. The leaves of the other cultivars were harvested from plants in growers’ greenhouses.

Pigment analysis

Petals of Daisy-VP were divided into pale and deep-colored tissues. Carnation petals were extracted with 10% acetic acid and 10μl aliquots were analyzed using an HP1100 system with a photodiode array detector (Agilent Technologies-Yokokawa Analytical Systems) and an Inertsil ODS-2 column (4.6 mm x 250 mm, GL Sciences) at 40°C with a flow rate of 0.8 ml/min. Absorption spectra were monitored at 220–600 nm. A linear gradient of 10–50% of solvent B (1.5% H2PO4, 40% acetic acid, 50% acetonitrile) in solvent A (1.5% H2PO4) was run over a 40 min period. Anthocyanin, flavonols and cinnamic acid derivatives were identified based on their absorption spectra and quantified based on their absorption at 530 nm, 360 nm and 330 nm, respectively. The mean and standard error of three independent experiments were calculated.

Genomic DNA analysis of GST genes

DNA was isolated from 100 mg leaves using a DNeasy Plant Mini Kit (Qiagen). The position of PCR primers used in this study and the primer sequences are shown in Fig. 2 and Table 1, respectively.

GST genes were amplified by PCR with primers P1 and P3 for fragment 1 and primers P2 and P4 for fragment 2 (Fig. 2). PCR conditions were as follows: an initial denaturation step at 95°C for 3 min was followed by 30 cycles at 95°C for 30 sec, 56°C for 30 sec and extension at 72°C for 5 min with a final 3 min extension at 72°C. The PCR products were gel purified using MagExtractor (Toyoobo) and sequenced directly or after cloning into pCR 4-TOPO using the TOPO TA Cloning Kit (Invitrogen). DNA sequences were determined using an ABI PRISM 310 Genetic
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Analyzer (Applied Biosystems). PCR with primers P2 and P5 was carried out to see if the DcGSTF2-dTac1 allele was present. The insertion of a dTac1 transposable element was confirmed by PCR with primers P3 and P6, as well. The conditions for both PCR were same as those mentioned above but the extension time at 72°C was 30 sec.

Results

Analysis of GST genes in a deep-colored flower mutant

‘Daisy-VP’ (Fig. 1C), a completely deep pink-flowered mutant, was obtained from the variegated ‘Daisy-VP’ (Fig. 1B). Analysis of the petal pigment revealed that the deep-pink and the pale-pink regions of ‘Daisy-VP’ and ‘Daisy-VPR’, respectively, had the same anthocyanin, 3,5-di-O-(β-glucopyranosyl) pelargonidin 6″-O-4,6″-O-1-cyclic malate. Because growth stage or growth conditions affect the amount of the anthocyanin, the regions of a variegated flower were compared in order to reduce such a fluctuation. The amount of anthocyanin was seven times larger in the deep-pink regions (3.63 ± 0.32 μmol/g f.w) than in the pale-pink regions (0.52 ± 0.03 μmol/g f.w.). There were no differences in flavonol composition or content between the two regions. Since the difference in anthocyanin content between the two regions was the only difference, the color intensity was attributed to the anthocyanin content. This result suggested that reversion of DcGSTF2 function which is required for anthocyanin accumulation was involved in the color change.

‘Daisy-VP’ was previously reported to have flawed DcGSTF2 genes, DcGSTF2mu and DcGSTF2-dTac1. Since reversion of the gene was suggested, the GST genes of the mutant ‘Daisy-VPR’ were characterized to prove that the color change resulted from excision of the transposable element, dTac1. As clearly shown in Fig. 3A, dTac1 was not amplified from the GST gene of ‘Daisy-VPR’. Excision was confirmed by the sequences of fragment 2. Fragment 2 of ‘Daisy-VPR’ contained two sequences, DcGSTF2mu and DcGSTF2rev1 (reverted from DcGSTF2-dTac1). The latter gene has a footprint at the site where dTac1 was inserted (arrowhead). Another defective gene, DcGSTF2rev2, has an incomplete third exon due to a nucleotide substitution from CAA to TAA resulting in a stop codon. The positions for primers P1-P6 used in this study are indicated by horizontal arrows. Sequences of the GST genes were analyzed using PCR-amplified fragment 1 (with primers P1 and P3) and fragment 2 (with primers p2 and P4).

Table 1. Primer sequences used in this study

| No. | Sequence (5’ to 3’) | Note                      |
|-----|---------------------|----------------------------|
| P1  | TAATCAAGTAAAGAAAATGAGGT | DcGSTF2 5’ UTR forward   |
| P2  | GTGCAACCAACCAATGAGAA | DcGSTF2 1st intron forward |
| P3  | TCTGCGTTGCTTACGAGTA | DcGSTF2 1st intron reverse |
| P4  | GTCTGCTCAAACTGACGCAG | DcGSTF2 3’ UTR reverse   |
| P5  | TACCGGTGCTTGGCCGACTA | dTac1 upstream reverse    |
| P6  | GAAGTTAGGGGAGAAGCTG | dTac1 downstream forward  |

Fig. 2. Schematic structure of the GST genes. Coding regions (gray boxes) are separated by two introns (lines). DcGSTF2 (top) is the wild type gene. In a defective gene named DcGSTF2-dTac1 a transposable element, dTac1, is inserted into the first intron between the duplicated target sequences, TGG. The element is flanked by terminal inverted repeats (open triangles). In the reverted genes, DcGSTF2rev1 and 2, footprint sequences, TGCAATGG and TGCCGG, respectively are present at the site where dTac1 was inserted (arrowhead). Another defective gene, DcGSTF2mu, has an incomplete third exon due to a nucleotide substitution from CAA to TAA resulting in a stop codon. The positions for primers P1-P6 used in this study are indicated by horizontal arrows. Sequences of the GST genes were analyzed using PCR-amplified fragment 1 (with primers P1 and P3) and fragment 2 (with primers p2 and P4).
transposable elements, \textit{dTac1} was shown to be an active transposable element. The difference between ‘Daisy-VP’ and the mutant ‘Daisy-VPR’ is the existence of \textit{dTac1} and the footprints. Since \textit{dTac1} was present in the intron, the gene without the element must function normally even if the footprint sequence is present. Removal of an active transposable element, \textit{dTac1}, from \textit{DcGSTF2-dTac1} most likely resulted in the mutation by reversion of the \textit{GST} gene.

\textit{Another reverted GST gene by dTac1 excision}

An old red-flowered cultivar ‘Spectrum’ (Fig. 1E, 1F right) is heterozygous for the \textit{S} locus (Mehlquist and Geissman 1947). The DNA sequences of fragments 1 and 2 revealed that ‘Spectrum’ has \textit{DcGSTF2} with a footprint (\textit{DcGSTF2rev2}) at the \textit{dTac1} insertion site in addition to \textit{DcGSTF2-dTac1}. \textit{DcGSTF2rev2} contains a different footprint sequence from \textit{DcGSTF2rev1}, TGGCCGG (Fig. 2). The footprint indicates that the gene was reverted from \textit{DcGSTF2-dTac1} by transposition of \textit{dTac1} before ‘Spectrum’ was established since the cultivar must have a functional \textit{GST} gene. \textit{DcGSTF2rev2} is implied to be functional by the same reason as the reverted gene in ‘Daisy-VPR’.

A cross between carnation breeding line ‘06LA’ with pale-colored flowers (lavender, Fig. 1D, 1F left) and ‘Spectrum’ was expected to produce deep (heterozygous \textit{Ss}) and pale (homozygous \textit{ss}) progeny in a 1:1 ratio. Here, ‘06LA’ was confirmed by the sequence to have only a defective \textit{DcGSTF2mu}. We also confirmed by PCR that \textit{DcGSTF2-dTac1} was present only in the ‘Spectrum’ male parent (Fig. 3B). The cross yielded five progeny with deep (purple or crimson) and three progeny with pale (lavender or pale pink). Thus, \textit{DcGSTF2rev2} was found only in plants with deep-colored flowers. These results demonstrated that \textit{DcGSTF2rev2} is the functional gene for deep color. Furthermore, the results were consistent with the hypothesis that the \textit{S} locus codes for \textit{DcGSTF2} derivatives and that \textit{DcGSTF2-dTac1} and \textit{DcGSTF2mu} are the genes for the recessive \textit{s} and \textit{DcGSTF2rev2} for the dominant \textit{S}. The transposable element \textit{dTac1} must be stable in the ‘Spectrum’ cultivar since no footprints other than \textit{DcGSTF2rev2} were detected in the progeny. This stability simplified the interpretation of the results from this test cross.

\textit{Distribution of GST genes in cultivars}

Carnation cultivars with pale and deep-colored flowers were evaluated for the types of \textit{GST} genes present in their genomes. We chose cultivars with pale-pink flowers as pale-colored materials and cultivars with red and deep-pink flowers as deep-colored materials because pale pink was reported to be brought about by a recessive \textit{s} and red or deep pink by a dominant \textit{S} (Mehlquist and Geissman 1947). The distribution of genes in each of the cultivars is shown in Table 3. The defective \textit{DcGSTF2-dTac1} was detected in all four pale-colored cultivars tested (Fig. 3C). ‘Lucia’, ‘Opal’ and ‘Toja’ have \textit{DcGSTF2mu} as their second gene. In contrast, ‘Fantasia’ has only \textit{DcGSTF2-dTac1} since only a single fragment of the expected size from \textit{DcGSTF2-dTac1} amplified with primers P3 and P6.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Line & \textit{DcGSTF2rev2} & \textit{DcGSTF2mu} & \textit{DcGSTF2-dTac1} \\
\hline
06LA & − & + & − \\
Spectrum & + & − & + \\
Deep\textsuperscript{a} & + & + & − \\
Pale\textsuperscript{b} & − & + & + \\
\hline
\end{tabular}
\caption{GST genes present in the pale-colored parent ‘06LA’ (lavender), deep-colored parent ‘Spectrum’ (red) and their progeny.}
\textsuperscript{a} progeny with deep-colored flowers, purple or crimson.
\textsuperscript{b} progeny with pale-colored flowers, lavender or pale pink. +: present, −: absent.
\end{table}
and defective genes, respectively. These two genes have been used for a long time in carnation breeding as functional genes present in the old ‘Spectrum’ cultivar, these two genes have repeatedly in carnation breeding. The defective genes, DcGSTF2-dTac1, have also been widely utilized. Since both DcGSTF2rev1 and DcGSTF2rev2, the origin of both genes must be DcGSTF2mu. DcGSTF2-dTac1, have also been widely utilized. Since both DcGSTF2rev1 and DcGSTF2rev2 are present in the old ‘Spectrum’ cultivar, these two genes have been used for a long time in carnation breeding as functional and defective genes, respectively.

**Table 3. GST genes present in the cultivars**

| Cultivar | Flower color | DcGSTF2rev1 | DcGSTF2rev2 | DcGSTF2mu | DcGSTF2-dTac1 |
|----------|--------------|-------------|-------------|------------|---------------|
| Lucia    | pale pink    | -           | -           | +          | +             |
| Opal     | pale pink    | -           | -           | +          | +             |
| Toja     | pale pink    | -           | -           | +          | +             |
| Fantasia | pale pink    | -           | -           | -          | +             |
| Tanga    | red          | +           | -           | +          | -             |
| Vital    | deep pink    | -           | +           | -          | -             |

+: present, -: absent

![Image](https://example.com/image.png)

**Fig. 4.** Analysis of fragment 2 of the GST gene in cultivars. Primers P2 and P4 were used for PCR amplifications. Pale-colored cultivars, Lucia (1), Opal (2), Toja (3), Fantasia (4) and dark-colored cultivars, Tanga (5), Vital (6). The arrow indicates the expected fragment size of DcGSTF2-dTac1.

**dTac1** was detected (Fig. 4). The cultivars with deep-colored flowers, ‘Tanga’ (red) and ‘Vital’ (deep pink), have DcGSTF2mu and DcGSTF2rev1 or DcGSTF2rev2. Since footprints are present in DcGSTF2rev1 and DcGSTF2rev2, the origin of both genes must be DcGSTF2-dTac1. Thus, in addition to DcGSTF2 two dominant genes originating from DcGSTF2-dTac1 by excision of dTac1 have been used repeatedly in carnation breeding. The defective genes, DcGSTF2mu and DcGSTF2-dTac1, have also been widely utilized. Since both DcGSTF2rev2 and DcGSTF2-dTac1 are present in the old ‘Spectrum’ cultivar, these two genes have been used for a long time in carnation breeding as functional and defective genes, respectively.

**Discussion**

Two reverted genes, DcGSTF2rev1 and DcGSTF2rev2, were found to contain footprints at the dTac1 insertion site, indicating that these functional genes were derived from DcGSTF2-dTac1 after removal of dTac1. This non-autonomous transposable element belonging to the CACTA family was confirmed to be an active element since the deep-colored ‘Daisy-VPR’ mutant was derived from ‘Daisy-VP’ by excision of the element. Because dTac1 is a non-autonomous element, the movement must have required a functional transposase(s) located in genomic regions other than the GST gene.

Although the genetic relationship between $S$ reported by Mehlquist and Geissman (1947) and FL3 reported by Larsen et al. (2003) is unclear, these two independent reports studied the same phenotype and described a genetic factor for dilute anthocyanin coloration. In the former report, the $s_{var}$ allele was hypothesized to be responsible for variegated flower color. In the latter study, the mutable allele $fl3-m$ was hypothesized to contain a transposable element and the $Rfl3-m$ activating locus was proposed to code for a transposase acting on $fl3-m$. Neither the transposable element nor the transposase for this mutable allele have been identified yet. Because the variegation of ‘Daisy-VP’ flowers is likely due to the movement of dTac1 from DcGSTF2-dTac1, $s_{var}$ and $fl3-m$ could encode DcGSTF2-dTac1. Although ‘Spectrum’ has a dTac1-containing gene, Mehlquist and Geissman (1947) did not propose that the cultivar has $s_{var}$, but rather their study suggested the presence of the recessive $s$. This proposal means that ‘Spectrum’ showed a stable phenotype.

On the other hand, ‘Daisy-VP’ had unstable variegated flowers and resulted in ‘Daisy-VPR’. Thus, the functional transposase gene must be present in ‘Daisy-VP’. An unresolved issue is the reason for the activation of dTac1 which would explain why ‘Daisy’ is basically stable but ‘Daisy-VP’ has variegated flowers. During vegetative propagation, spontaneous activation of dTac1 movement could have occurred. Some epigenetic or other mechanism on the transposase and/or dTac1 could inhibit the mobile activity of the element in ‘Daisy’. ‘Daisy-VP’ is thought to be free from such inhibition at least in the petals. To understand the mechanism for such a difference between ‘Daisy’ and ‘Daisy-VP’, the transposase of dTac1 needs to be identified.

Another question to be tackled is why the defective GST gene results in pale-colored flowers and not white flowers. In the pale-colored flowers caused by defective GST genes, anthocyanin pigment is present although at a low level; thus, pigment accumulation occurs at a reduced efficiency. Three possibilities exist to explain low accumulation of pigment unless the pigment accumulates in the cytosol. First, the defective genes might not lose function completely and could have limited function; second, the DcGSTF2 gene could be dispensable and the third possibility is that an alternative vacuolar transport system in which the GST gene is not
involved could be present. Since the function of the GST gene in anthocyanin transportation has not been fully elucidated, the answer awaits further investigation.

Molecular studies of flower color mutants have identified not only genes for pigmentation but also active transposable elements. Two transposable elements belonging to the hAT (Ac/Ds) family have been identified as active elements in carnation and shown to be involved in variegation or bud mutation (Itoh et al. 2002, Momose et al. 2013). In this study, dTac1, a member of the CACTA (En/Spm) family of non-autonomous transposable elements, is also involved in variegation and bud mutation of flower color intensity. Non-autonomous elements could be advantageous for crop improvement because activation and stabilization of the elements can be achieved by crossing to control the transposase gene at another locus distant from the elements. In plants such as carnations, ornamentals or fruit trees, it is highly probable that active transposable elements in the genome can function as endogenous mutagens to effectively destroy genes during crop improvement.

In this study, a transposable element, dTac1, was shown to be active. Excision of the element from DcGSTF2-dTac1 created two reverted genes, DcGSTF2rev1 and DcGSTF2rev2. These two reverted genes and two previously reported defective genes, DcGSTF2-dTac1 and DcGSTF2mu, are likely to have been widely used in carnation breeding for many years.

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