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Short Communication

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*)

Yoshio Itoh [1, 3], Daisuke Higeta [1], Akane Suzuki [1], Hiroyuki Yoshida [2] and Yoshihiro Ozeki [1]

[1] *Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, Koganei, Tokyo, 184-8588 Japan*
[2] *Japan Tobacco Inc. Applied Plant Research Center, 1900 Idei, Oyama, Tochigi, 323-0808 Japan*

In the 'Rhapsody' cultivar of the carnation, which bears white flowers variegated with red flecks and sectors, a transposable element, *dTdic1*, belonging to the *Ac/Ds* superfamily, was found within the dihydroflavonol 4-reductase (*DFR*) gene. The red flecks and sectors of 'Rhapsody' may be attributable to a reversion to DFR activity after the excision of *dTdic1*. The yellow color of the carnation petals is attributed to the synthesis and accumulation of chalcone 2′-glucoside. In several of the carnation cultivars that bear yellow flowers variegated with white flecks and sectors, both the chalcone isomerase (*CHI*) and *DFR* genes are disrupted by *dTdic1*.

Key words: Carnation — Chalcone isomerase — *Dianthus caryophyllus* — Dihydroflavonol 4-reductase — *dTdic1* — Transposable element.

Abbreviations: ANS, anthocyanidin synthase; AT, anthocyanin acyltransferase; C4H, cinnamate-4-hydroxylase; CHGT, UDP-glucose: chalcone 2′-glucosyltransferase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNL, flavone synthase; PAL, phenylalanine ammonia-lyase; UFGT, UDP-glucose:flavonoid glucosyltransferase.

The nucleotide sequence reported in this paper for *Dianthus caryophyllus* DFR pseudogene mRNA encoding dihydroflavonol 4-reductase and carrying the *dTdic1* transposable element, has been submitted to DDBJ/EMBL/GenBank under the accession number AB071787.

The cyanic color of most flowers comes from anthocyanins, whereas betacyanins produce this color in the flowers of Centrosparmae, excluding *Dianthus* (Brouillard and Dangles 1993). All the structural genes encoding the enzymes involved in anthocyanin synthesis have been isolated (Fig. 1) (Heller and Forkmann 1993, Holton and Cornish 1995). Some regulatory genes controlling the expression of the structural gene(s) responsible for flower color have been identified (Goodrich et al. 1992, Weiss et al. 1993, de Vetten et al. 1997, Quattrocchio et al. 1998, Spelt et al. 2000). When the expression of some of the structural gene(s) involved in the biosynthetic pathway of the C_6–C_3–C_6 structure of anthocyanin aglycone, or the genes regulating this pathway, are repressed by mutation, the anthocyanin biosynthetic pathway is blocked and acyanic flowers are produced. For example, the white flowers of the carnation *Dianthus caryophyllus* cv. 'Kaly', in which flavanone glycosides accumulate, are caused by a deficiency in the transcripts of the flavanone 3-hydroxylase (F3H) gene (Mato et al. 2000).

The unusual mosaic appearance of flower petal colors has long been attractive not only to breeders, but also to geneticists and other scientists. The unstable mutations present in variegated flowers were described by Mendel (1866–1873). The unstable expression of anthocyanin is particularly easily recognized in flowers. This instability results in cyanic spots, sectors, and flecks on the usually acyanic or pale-colored backgrounds. Some of these variegations are produced by viral infections. Others are caused by periclinal or mericlinal chimeras. The most interesting instance of unstable anthocyanin expression is caused by the mobility of transposable elements. In cells in variegated areas, a transposable element is excised from a gene encoding an enzyme involved in a pigment biosynthetic pathway or from a gene involved in the transcriptional regulation of these enzymes. Several such structural genes and their regulatory genes have been identified by transposon tagging (Lister et al. 1993, Inagaki et al. 1994, de Vetten et al. 1997, Quattrocchio et al. 1998, van Houwelingen et al. 1998, Iida et al. 1999).

Yellow acyanic flower colors are produced from carotenoids in sunflower, from aurones in cosmos and snapdragon, and from betaxanthins in Centrosparmae, excluding *Dianthus* (Ootani and Hagiwara 1969, Toyama et al. 1992, Stafford 1994, Bartley and Scolnik 1995, Nakayama et al. 2000). In the carnation, chalcones impart the yellow and orange colors to flowers. We have previously isolated cDNAs for phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), F3H, dihydroflavonol 4-reductase (DFR), and anthocyanidin synthase (ANS) from red carnation petals, and have elucidated the

[3] Corresponding author: E-mail, yoss@cc.tuat.ac.jp; Fax, +81-42-388-7239.

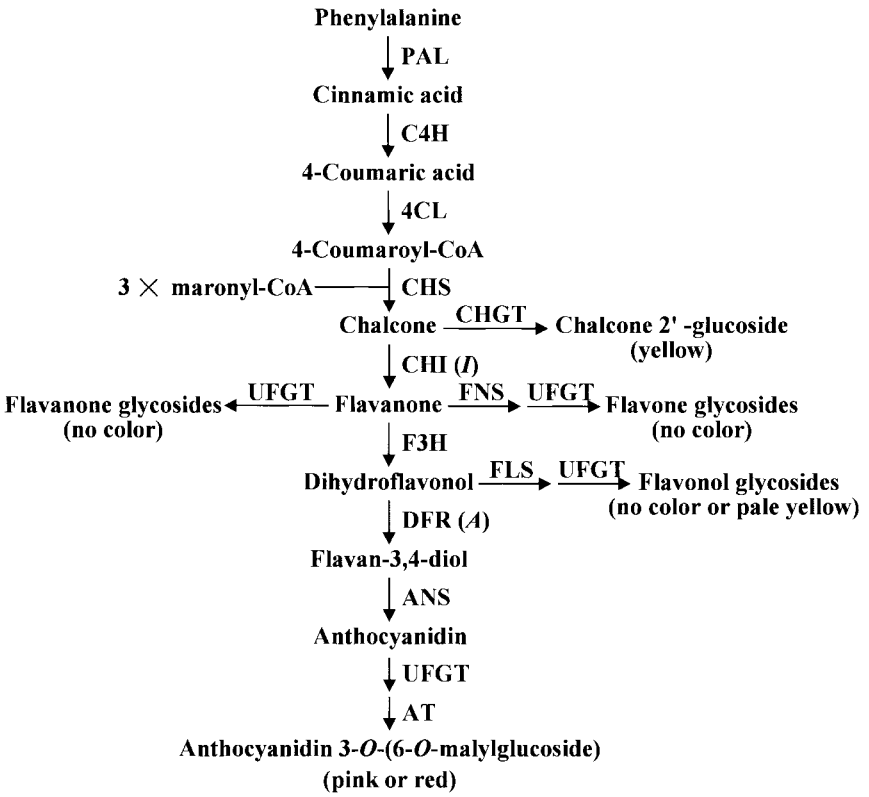

Fig. 1 A simplified pathway for anthocyanin and flavonoid biosynthesis in carnation. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-glucose:flavonoid glucosyltransferase; AT, anthocyanin acyltransferase; CHGT, UDP-glucose: chalcone 2′-glucosyltransferase; FNL, flavone synthase; FLS, flavonol synthase.

relationship between the expression of these genes and anthocyanin synthesis in cyanic and white acyanic flowers (Mato et al. 2000, Yoshimoto et al. 2000, Mato et al. 2001).

The carnation is one of the most important ornamental plants in the world. Since Mehlquist and Geissman (Mehlquist 1940, Mehlquist and Geissman 1947) reported the first genetic studies of carnation flower color, many studies have followed. Forkmann and Dangelmayr (1980) demonstrated that the *I* gene regulates CHI enzyme activity (see Fig. 1). They showed that the *ii* genotype results in yellow flowers containing chalcone 2′-glucoside as the major pigment, but that flavonols and anthocyanins are synthesized by genotypes containing the wild-type alleles at this locus, which then produce flowers that are white or red, respectively (Forkmann and Dangelmayr 1980). Loss of the *A* gene product, which acts downstream from the *I* gene product (Mehlquist and Geissman 1947), results in the loss of DFR activity (Stich et al. 1992).

In a number of mutants involving flower color, mutable loci have been observed in some inbred lines with variegated flower phenotypes (e.g. Fig. 2B). Recently, Larsen (1996) genetically analyzed the mutable loci *fl1-m*, *fl2-m*, *fl3-m*, and *fl4-m* in the carnation in detail. She proposed that the *fl1-m*, *fl2-m*, and *fl3-m* loci correspond to the *CHI* gene, the *DFR* gene, and the glutathione S-transferase (*GST*) gene, respec-

Fig. 2 Carnation phenotypes used in this study. (A) ‘Symphony Rose’; (B) ‘Rhapsody’; (C) ‘7109–01’; (D) ‘J-66’; (E) ‘J-99’.

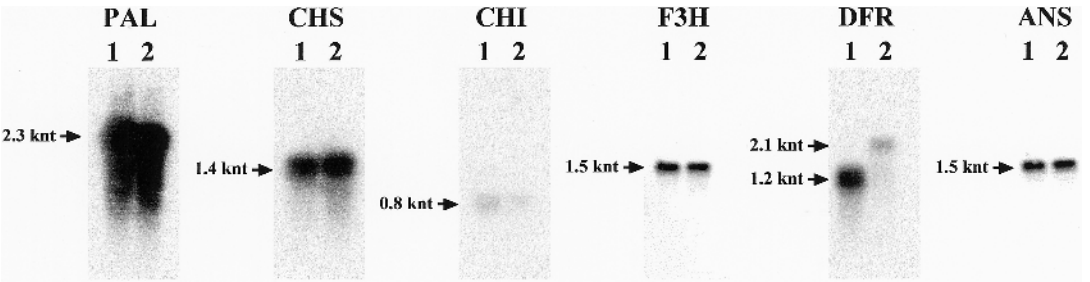

Fig. 3 Northern analysis of the expressed transcripts of the *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, and *ANS* genes. Poly(A)$^+$ RNAs were prepared from the petals at stage 2 of 'Symphony Rose' (lane 1; see Fig. 2A) and 'Rhapsody' (lane 2; see Fig. 2B). Northern blots were hybridized with carnation PAL, CHS, CHI, F3H, DFR, and ANS cDNAs as probes.

tively (Larsen 1996). Only at the completion of our own independent cloning studies did we find the *fl1-m* nucleotide sequence published in the DDBJ/EMBL/GenBank in 2001 (Larsen and Briggs, accession number AF250367).

Our research has focused on the relationship between variegated flower colors and the expression of enzymes involved in the anthocyanin synthetic pathway, using cDNA probes. Here, we report that the transposable element *dTdic1* disrupts the *DFR* gene of the 'Rhapsody' cultivar, which bears white flowers variegated with red flecks and sectors (Fig. 2B). *dTdic1* belongs to the *Ac/Ds* superfamily first reported by Larsen and Briggs in 2001 (accession number AF250367). Independently of their research, we report here the isolation of CHI cDNA and the *CHI* gene carrying *dTdic1* from mutable carnations bearing yellow flowers variegated with white flecks and sectors (Fig. 2C).

We found transcripts of the *DFR* gene from the petals of the 'Rhapsody' cultivar, here designated *gDicDFR1*, that were approximately 2,100 nucleotides (nt) (Fig. 3). A nearly full-length 1,215-bp cDNA from the carnation *gDicDFR1*gene expressed in the petals has been reported (Min et al. accession number Z67983; Mato et al. 2000, Mato et al. 2001). Therefore, the *gDicDFR1* transcripts from 'Rhapsody' should be approximately 900 nt longer than the corresponding transcripts from 'Symphony Rose'. Isolating *gDicDFR1* cDNA from the petals of 'Rhapsody' by RACE–PCR revealed that a transposable element, *dTdic1*, was inserted 37 bp downstream from the adenine residue of the initiating methionine (Fig. 4A). *dTdic1* was reported by Larsen and Briggs (accession number AF250367) as an *Ac/Ds*-like transposable element of 876 bp, with 7-bp terminal inverted repeats, that was inserted into the first exon of the *CHI* gene with 8-bp direct repeats as the target duplication. The *dTdic1* sequence we isolated from the 'Rhapsody' *gDicDFR1* gene was identical to that inserted into the *CHI* gene and reported by Larsen and Briggs, but in the reverse orientation. The target duplication (CTAGACG) in the 'Rhapsody' *gDicDFR1* gene was 7 bp rather than 8 bp long, with an extra 2-bp sequence (CC) at one end (Fig. 4A). The remaining sequence of the *gDicDFR1–dTdic1* cDNA was almost identical to that of the wild-type *gDicDFR1* gene reported previously (Mato et al. 2000, Mato et al. 2001). These results indicate that the *gDicDFR1* gene in which the first exon is interrupted by *dTdic1* is transcribed and the introns spliced correctly, but the *dTdic1* sequence is retained in the mature poly(A)$^+$ RNA. In some cases, not only *Ds* elements (which are defective *Ac* elements) but also defective *En/Spm* elements can act as fairly good introns and are spliced from RNA (Wessler et al. 1987, Kim et al. 1987, Wessler 1989, Menssen et al. 1990, Grioux et al. 1994). However, our results indicate that *dTdic1* in the *gDicDFR1* gene does not act as an intron and is not spliced from the pre-RNA.

We identified two *DFR* genes in the carnation cultivars used in this study. However, three copies of *DFR* genes were observed in other lines not investigated here (data not shown). We found that *dTdic1* was inserted into the first exon of the *gDicDFR1* gene. Two footprint sequences caused by somatic excision of the *dTdic1* element were found in the sequence from vegetatively propagated 'Rhapsody' (Fig. 4A). Sequence alterations at the empty donor site after *dTdic1* excision may produce a frame shift or create a stop codon, as shown by 'footprint 1' in Fig. 4A. If the excision did not disturb the coding frame or give rise to a stop codon, as shown by 'footprint 2' in Fig. 4A, the *gDicDFR1* gene would produce almost the same transcripts as the wild-type *gDicDFR1* gene. Although we have no genetic evidence, it is possible that the *gDicDFR1* gene from which the somatic excision of *dTdic1* occurred expresses the DFR enzyme involved in the anthocyanin synthetic pathway in the petals of 'Rhapsody', resulting in variegated flower color (Fig. 2B).

Among several breeding lines producing yellow flowers, we found mutable lines with a variegated flower color phenotype that displays yellow petals with white flecks and sectors. The white flecks or sectors are hard to distinguish on the yellow background of the petals. The genomic sequence of the *CHI* gene, designated *gDicCHI1* here, of cultivar '7019-01' (Fig. 2C) was isolated by inverse PCR (IPCR). Analysis of *gDicCHI1* revealed that *dTdic1* was inserted 20 bp downstream from the adenine residue of the initiating methionine

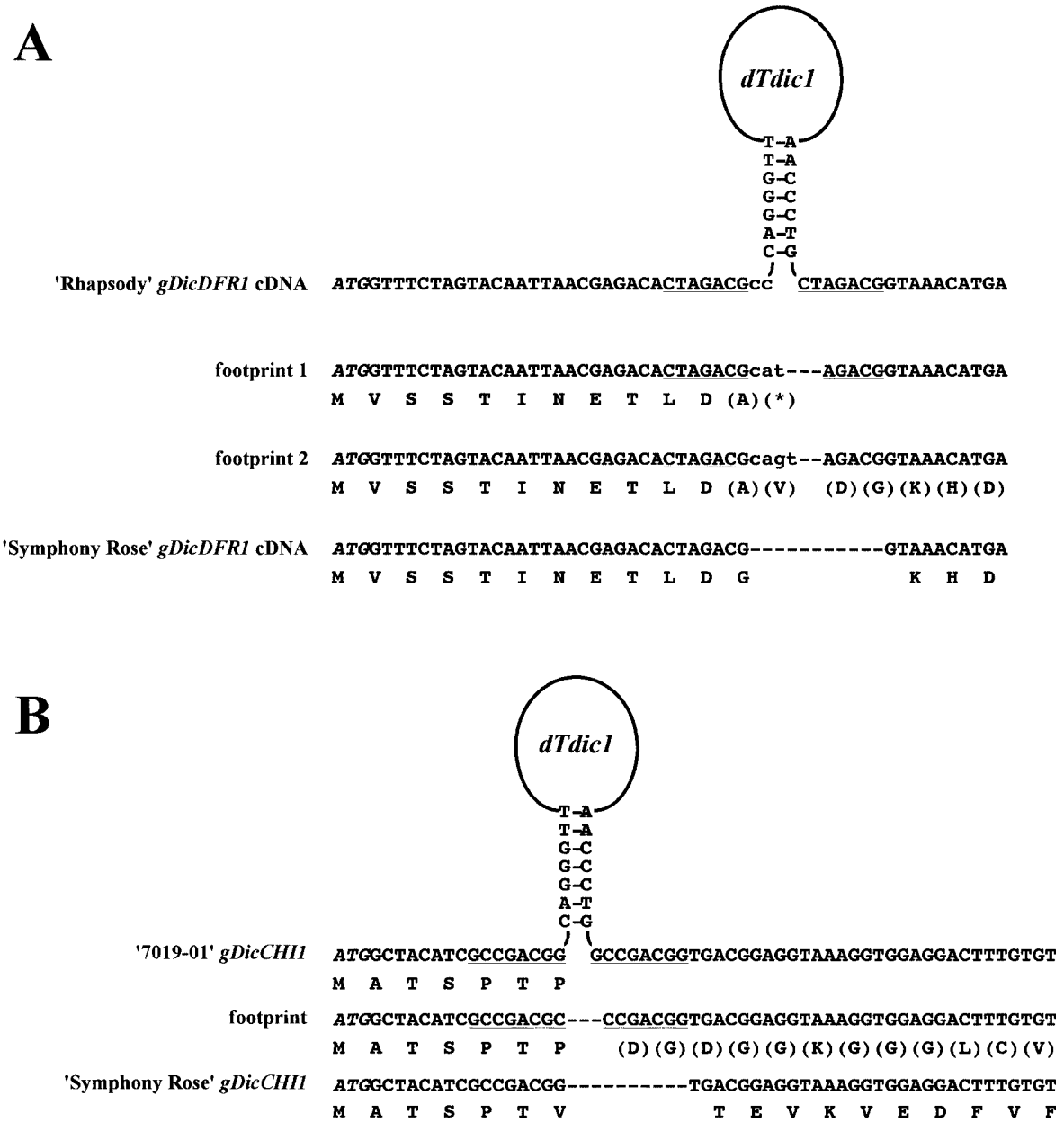

Fig. 4 Structures of the cDNAs of *gDicDFR1* in 'Rhapsody' (A) and the *gDicCHI1* genes in '7019-01' (B) compared with those of 'Symphony Rose'. '*ATG*' in italics corresponds to the initiating methionine. Amino acid sequences in parentheses indicate where sequences differ from the original. (A) cDNAs from the petals of 'Rhapsody' isolated in this study and from 'Symphony Rose' (Mato et al. 2000, Mato et al. 2001). *dTdic1* has 7-bp terminal inverted repeats and the 7 bp underlined (CTAGACG) show the target duplication sequence. Footprint sequences 1 and 2 were obtained by RACE–PCR from the same poly(A)$^+$ RNA used to isolate the 'Rhapsody' *DFR* cDNA carrying *dTdic1*. Nucleotides shown in small capitals different from the *DFR* cDNA sequence isolated from 'Symphony Rose'. (B) "Footprint" sequence was obtained from genomic DNA prepared from either '7019-01' or 'J-66'.

(Fig. 4B). The insertion point and the orientation of *dTdic1* and the sequence of the 8-bp target duplication were identical to that reported by Larsen and Briggs in 2001 (accession number AF250367), and the remaining nucleotide sequence of the *gDicCHI1* gene was almost identical to the Larsen–Briggs sequence.

The 'J-66' cultivar is one of the parent plants of '7019-01'. We isolated from 'J-66' a DNA fragment of the *gDicCHI1* gene harboring *dTdic1*, with an identical nucleotide sequence to the '7019-01' sequence amplified by IPCR. Furthermore, in the same IPCR reactions, DNA fragments were amplified that were shorter than those of the *gDicCHI1* gene harboring

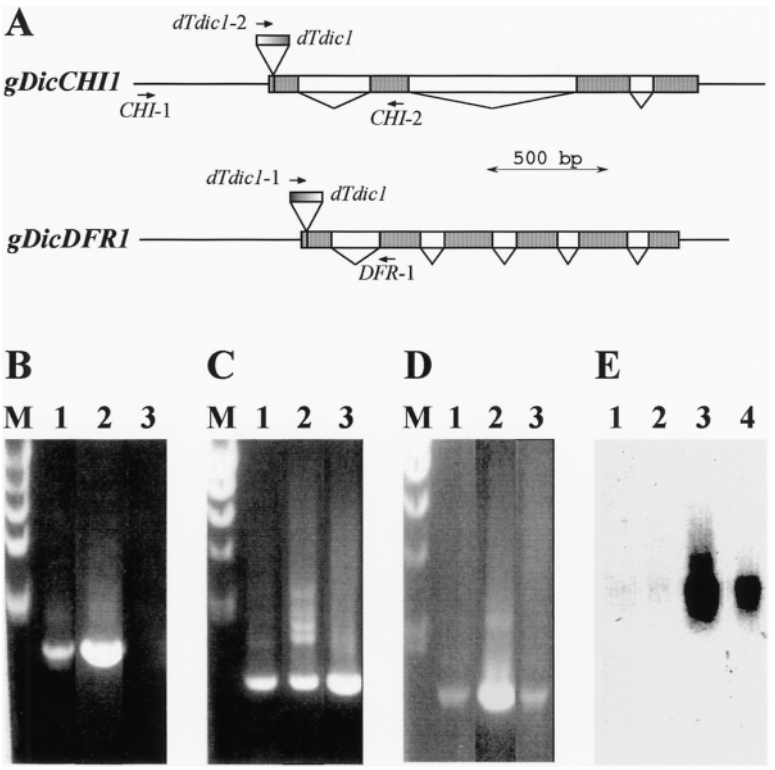

Fig. 5 Survey of the *gDicCHI1* and *gDicDFR1* genes carrying *dTdic1*. (A) Positions of primers used to detect the *gDicCHI1* and *gDicDFR1* genes carrying *dTdic1*. Hatched and white boxes show open reading frames and introns, respectively. The length of *dTdic1* is not shown to scale. (B) PCR analysis with *dTdic1*-2 and *CHI*-2 primers to detect the *gDicCHI1* gene harboring *dTdic1*. (C) PCR with *CHI*-1 and *CHI*-2 primers to detect the *gDicCHI1* gene without *dTdic1*. (D) PCR with *dTdic1*-1 and *DFR*-1 primers to detect the *gDicDFR1* gene carrying *dTdic1*. *dTdic1* was inserted into the *gDicDFR1* gene in the opposite orientation relative to the *CHI* gene, so that *dTdic1*-1 and *dTdic1*-2 primers were used to detect the *gDicDFR1-dTdic1* and *gDicCHI1-dTdic1* genes, respectively. The sources of the genomic DNA used as PCR templates were as follows: lane 1, 'J-66' (Fig. 2D); lane 2, '7019-01' (Fig. 2C); lane 3, 'J-99' (Fig. 2E). (E) Northern hybridization of poly(A)$^+$ RNA prepared from petals of carnation lines, as described above, and 'Rhapsody' (lane 4), with the *gDicCHI* cDNA fragment used as probe. 'J-99' (Fig. 2E), which has the genotype *I/i* and *A/a*^{m} (Yoshida, unpublished data), was used here as a negative control for the PCR reaction in B and a positive control for northern hybridization in E. 'J-99' does not contain the *gDicCHI1-dTdic1* gene, but does contain the *gDicDFR-dTdic1* gene.

dTdic1 when genomic templates prepared from either '7019-01' or 'J-66' were used. We found identical footprint sequences in '7019-01' and 'J-66', indicating that somatic excision events had occurred to generate the same footprint sequences in '7019-01' as in 'J-66' (Fig. 4B). The somatic excision of *dTdic1* from the *gDicCHI1* gene in '7019-01' and 'J-66' was also confirmed in DNA fragments amplified by PCR using primers specific for the *gDicCHI1* gene (*CHI*-1 and *CHI*-2, Fig. 5A) and genomic DNA prepared from either '7019-01' or 'J-66' (Fig. 5C, lane 1 and 2).

The *gDicDFR1*gene carrying *dTdic1* in 'Rhapsody' was transcribed and the introns spliced. However, the *dTdic1* sequence was not spliced out, and the poly-A tail sequence was added (Fig. 3, DFR, lane 2). The transcripts from the *gDicCHI1* gene carrying *dTdic1* in yellow carnations of the 'J-66' and '7019-01' lines were expected to be approximately 1.7 kbp long (0.8 kbp of *gDicCHI1* exons and the 0.9-kbp *dTdic1* element). These transcripts were, however, below the level of detection (Fig. 5E, lanes 1 and 2, respectively). One explanation for the lack of transcripts for *gDicCHI1-dTdic1* or their very low levels is that *dTdic1* was inserted in the reverse orientation relative to the *gDicDFR1-dTdic1* gene. In the *wx-m8* and *bz-13* mutants of maize, the same *dSpm* element was inserted into the *Wx* and *Bz* genes, respectively, but in the opposite orientation in *wx-m8* and *bz-13*. The *Wx* transcripts were below the level of detection in *wx-m8* containing the *dSpm* insertion, whereas the insertion of *dSpm* into *bz-13* in the reverse orientation did not interfere with the accumulation of the *Bz* gene product (Fedoroff 1989).

The carnation has two copies of the *CHI* gene in the lines used here, whereas in other lines not investigated in this paper, four copies of *CHI* were observed (data not shown). We have no direct genetic evidence that the *gDicCHI1* gene is indeed responsible for the anthocyanin synthetic pathway in carnation petals. However, we examined the *gDicCHI1* and *gDicDFR1* genes carrying the *dTdic1* transposable element in several

breeding lines of carnation, using PCR with specific primers and nucleotide sequencing of the amplified fragments. All 16 breeding lines of yellow-flowered carnations analyzed here, including 'J-66 and '7019-01', contain nucleotide sequences in the proximal regions of the *dTdic1* insertion sites in both the *gDicCHI1* and *gDicDFR1* genes that are identical to those of '7019-01' and 'Rhapsody', respectively (data not shown). We have no genetic evidence that both the *gDicCHI1* and *gDicDFR1* genes are responsible for the anthocyanin synthetic pathway in carnation petals. However, assuming that both gene products are involved in the anthocyanin synthetic pathway in petals, it is possible that the insertion of *dTdic1* into the *gDicCHI1* gene might abolish the production of CHI enzymes in the petals, which may cause the accumulation of chalcone. Conversely, the revertant *gDicCHI1* gene from which *dTdic1* was excised would produce an active enzyme, resulting in the generation of red flecks and sectors on yellow petals. The yellow acyanic flowers of the carnation, however, have a second copy of *dTdic1* inserted into the *gDicDFR1* gene, which may block the anthocyanin synthetic pathway at the dihydroflavonol step, producing white acyanic flecks and sectors on yellow petals, which are not easily distinguishable on the yellow background (Fig. 2C, D). To confirm this possibility, further genetic experiments on these cultivars are required to clarify whether the *gDicCHI1* and *gDicDFR1* genes are responsible for the anthocyanin synthetic pathway in carnation petals.

A deficiency of CHI activity in carnation petals cannot cause the production of a stable yellow color by chalcone, because chalcone can convert spontaneously to flavanone without CHI activity (Moustafa and Wong 1967). The yellow color of carnation petals is attributed to the synthesis and accumulation of chalcone 2′-glucoside. The hydroxyl residue at the 2′ position of chalcone must be coupled with glucose by UDP-glucose:chalcone 2′-glucosyltransferase (CHGT, Fig. 1) to arrest the spontaneous circularization of chalcone that converts it to flavanone, and to transport and store chalcone in vacuoles. It is thought that CHGT activity may play an important role in the production of the stable yellow color in flowers. Isolation of CHGT cDNA from yellow-flowered carnations is currently in progress.

The carnation (*Dianthus caryophyllus*) cultivars 'Symphony Rose' bearing red flowers (Fig. 2A) and 'Rhapsody' (Supra Stripe™) (Fig. 2B) were used for cloning the *gDicCHI1* and *gDicDFR1* cDNAs, respectively. The inbred carnation lines, '7019-01', bearing yellow flowers variegated with white flecks and sectors (Fig. 2C), and 'J-66' (Fig. 2D) were used to isolate the *gDicCHI1-dTdic1* gene. J-66 is one parent of '7019-01'; the other parent is 'J-028' which bears yellow flowers (not shown). The petals of the flowers of '7019-01' appeared completely yellow at first glance, but thorough examination revealed infrequent and narrow flecks or sectors of whitish or cream color on the petals. It was very difficult to identify the infrequent and narrow white or cream flecks or sectors on yellow backgrounds, especially in photographs. A breeding line of the pink-flowered carnation 'J-99' (Fig. 2E), which has the genotype *I/i* and A/a^m (Yoshida, unpublished data), was used here as a negative control for the PCR reaction in Fig. 5B and a positive control for Northern hybridization in Fig. 5E. 'J-99' does not contain the *gDicCHI1-dTdic1* gene, but does contain the *gDicDFR-dTdic1* gene.

Total RNA was prepared from red petals of closed flower buds at stage 2 (Fig. 5A in Yoshimoto et al. 2000) of the carnation cultivars 'Rhapsody' and 'Symphony Rose' using a modified guanidinium thiocyanate–CsCl ultracentrifugation method (Chirgwin et al. 1979). Poly(A)$^+$ RNA was prepared from total RNA using Oligotex-dT_{30} <Super> (TaKaRa Biochemicals, Japan) as described in the manual from the supplier. Full-length cDNAs from 'Rhapsody' were prepared using the GeneRacer™ Kit (Invitrogen, The Netherlands), and used as the templates in the amplification of *gDicDFR1* cDNA fragments by LA–PCR (TaKaRa) with the *gDicDFR1*-specific primer (5′-CAAGACTCGACAGCTGCTGTGTACATGTC-3′) and the GeneRacer™ 5′ Primer. This was followed by amplification with the primer 5′-AGTTTCTTCGACGAGAATTG-TACTTGC-3′ and the GeneRacer™ 5′ Nested Primer, to isolate the 5′ region of the *gDicDFR1* cDNA. The LA–PCR mixture (10 μl) was prepared according to the supplier's recommendations using 1 μl of the cDNA mixture and 4 pmol of each primer. The mixture was incubated for 1 min at 94°C, and then 0.5 U TaKaRa LA–*Taq* DNA polymerase was added. The PCR was performed for 35 amplification cycles (30 s at 92°C; 45 s at 53°C; 1 min at 72°C). The probe for Northern hybridization was prepared from the cloned fragment of the 3′ region of the *gDicDFR1* cDNA.

To clone the *gDicCHI1* cDNA fragment, the first cDNA strand was synthesized from poly(A)$^+$ RNA prepared from petals of 'Symphony Rose' using the First-Strand cDNA Synthesis Kit (TaKaRa), and used as a template for PCR. The degenerate primers used to amplify the *gDicCHI1*cDNA fragment by LA–PCR were 5′-CARBNMHYVVWTCCTYGSYG-GYGCHGG-3′ and 5′-AMHVNYTYGTTCWCKAWHAC-3′, corresponding to the consensus nucleotide sequences of other *CHI* cDNAs previously reported. The reaction mixture and the LA–PCR conditions were as described above. The amplified fragment was cloned and used to a probe for *gDicCHI1* sequences in Northern hybridization analyses.

Genomic DNAs were prepared from the leaves of carnation cultivars by the cetyl-trimethyl-ammonium bromide (CTAB) method previously described (Ozeki et al. 1993). LA–PCR was performed with genomic DNA as the template to isolate the exons and introns of the *gDicCHI1I* gene using as primer sequences the 5′ and 3′ ends of the nucleotide sequence of the *gDicCHI1* cDNA: 5′-GTGAATTAGTTTAAGAGTTTA-CGAACTTCTCAACACGTTA-3′ and 5′-TGTTTAAAATAAT-GTTACAAGTGGAG-3′, respectively. The sequences of the 5′ upstream and 3′ downstream regions of the *gDicCHI1* gene were isolated by inverse PCR (IPCR). Genomic DNA (100 ng) was digested with *Eco*RI, *Hin*dIII, *Xba*I, or *Bam*HI, followed

by phenol extraction and ethanol precipitation. The digested DNA was circularized by self-ligation with the TaKaRa Ligation Kit version II, then ethanol precipitated. Using circularized genomic DNA (50 ng) as template, nested LA-PCR was performed under the same condition as described above with the primer pairs 5′-ATATCCCGGAAAAATTCATGAGATTCAACCAAAGCAGTTACACT-3′ and 5′-AAGGCCGTGGATAAGTTCATCGAGGTCTTCAAGGACGAGAAT-3′, followed by 5′-AGGAGCTAACTCAAGGAGACCACTT-3′ and 5′-TCAGTCGCCTTCTGG-TTCACTAACG-3′.

All PCR products were separated by agarose gel electrophoresis and subcloned into the Bluescript SK+ plasmid to determine the nucleotide sequences.

Poly(A)$^+$ RNA prepared from the petals of inbred carnation lines at stage 2, as described above, were separated by 1.5% formaldehyde–denaturing agarose gel electrophoresis, blotted, and fixed on nylon membrane filters. RNAs were then hybridized with various ^{32}P-labeled cDNAs of carnation (PAL, Yoshimoto et al. 2000; CHS, F3H, and ANS, Mato et al. 2000, Mato et al. 2001) at 65°C. The filters were washed twice at room temperature with 2× SSC, 0.5% SDS solution for 15 min, then twice at room temperature with 0.1× SSC, 0.1% SDS for 10 min, and finally twice at 65°C with 0.1× SSC, 0.1% SDS for 30 min. The filters were then exposed to X-ray film at –80°C.

To detect *dTdic1* in the *gDicCHI1* and *gDicDFR1* genes, genomic DNA was prepared from the leaves of inbred carnation lines, as described above. This DNA was used as the template for LA–PCR with the following primer pairs: 5′-CAGGGGTTTTAAATATCGGTATCG-3′ and 5′-AGGAGCTAACTCAAGGAGACCACTT-3′ (termed *dTdic1*-2 and *CHI*-2, respectively, in Fig. 5A); 5′- GTGAATTAGTTTAAGAGTTTACGAACTTCTCAACACGTTA-3′ and 5′-AGGAGCTAACTCAAGGAGACCACTT-3′ (*CHI*-1 and *CHI*-2, respectively); and 5′-CCGTCGGCCAGGGTTCAAAATCTCGGCCGAGTTGACTCGT-3′ and 5′-AGCAAGCAGCGTGGAAGTACGCAGC-3′ (*dTdic1*-1 and *DFR*-1, respectively).

The transposable element isolated here was first reported by Larsen and Briggs (accession number AF250367, designated '*Dianthus caryophyllus chalcone isomerase 1* (*Fl1*) gene, *Fl1-m* allele, interrupted by *dTdic1* transposon, complete sequence'). The name '*dTdc1*' has been changed to '*dTdic1*' in this paper on the basis of a future paper to be published by Larsen and Briggs, to avoid confusion with the 'defective *Tdc1*' element that was isolated by us from carrot (*Daucus carota*) (Ozeki et al. 1997).

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