Flavonoid 3',5'-Hydroxylase (F3'5'H) Gene Polymorphisms Co-segregate with Variation in Anthocyanin Composition in the Flower Petals of Lisianthus [Eustoma grandiflorum (Raf.) Shinn.]

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This study investigated genetic polymorphism related to anthocyanin composition variation in lisianthus (Eustoma grandiflorum) flower petals. Three different bands were detected by genomic polymerase chain reaction using specific primers based on the reported flavonoid 3',5'-hydroxylase (F3'5'H) gene sequence. Our genetic study revealed that they were allelic. They were named F3'5'H1-1, F3'5'H1-2, and f3'5'h1-2. F3'5'H1-1 and F3'5'H1-2 differed in the length of the intron. Allele f3'5'h1-2 possessed a retrotransposon insertion in the first exon, but otherwise, its gene sequence was almost identical to that of F3'5'H1-2. This retrotransposon is a Ty1-copia type retrotransposon and was designated as the retrotransposon of Eustoma grandiflorum (rTeg1). The mauve flower line ‘ME’ and red-purple flower line ‘PRP’ were homozygous for F3'5'H1-1 and mostly accumulated cyanidin, with a small amount of delphinidin, in flower petals (the Cy major phenotype). The violet flower line ‘RV’ was homozygous for F3'5'H1-2 and accumulated only delphinidin (the Dp major phenotype). The pink-red flower line ‘AK’ was homozygous for f3'5'h1-2, accumulated pelargonidin, and lacked delphinidin (the Pg major-non Dp phenotype). Segregation analysis of the F2 population from ‘ME’ × ‘RV’ revealed that F3'5'H1-2 was associated with the Dp major phenotype, and the delphinidin major trait was dominant in the Cy major phenotype. All the Cy major phenotype plants in the F2 population possessed homozygous F3'5'H1-1. Genetic analysis of the F2 population from the cross between delphinidin-containing and delphinidin-lacking strains revealed that the homozygous f3'5'h1-2 genotype co-segregated with the Pg major-non Dp phenotype. Expression analysis of the F3'5'H gene demonstrated that an abnormal mRNA was transcribed from the homozygous f3'5'h1-2 line by the insertion of rTeg1. Therefore, these F3'5'H gene polymorphisms can be used as DNA markers for breeding lisianthus based on flower color.

Key Words: DNA marker, flavonoid, flower color, mutation, retrotransposon.

Introduction

Flower color is one of the most important traits for breeding floricultural plants. Plant pigments include porphyrins, carotenoids, flavonoids, betalains, etc. Among these pigments, anthocyanins are the most abundant for flower color (Schwinn and Davies, 2004). The genetic and molecular biological aspects of anthocyanin biosynthesis have been well-studied in several ornamental species such as Petunia spp. (Quattrocchio et al., 1999), Antirrhinum majus (Martin et al., 1991), Ipomoea nil (Morita et al., 2006), and Gentiana triflora (Nakatsuka et al., 2006, 2008; Nishihara et al., 2008). In these species, most of the enzymes involved in the anthocyanin biosynthetic pathway have been isolated and cloned.

Lisianthus [Eustoma grandiflorum (Raf.) Shinn.], also known as the Texas bluebell or prairie gentian, originated from the central and southern United States...
and has been developed as an important ornamental plant through breeding efforts, especially in Japan (Harbaugh, 2006). Although most wild lisianthus plants exhibit a purple flower trait, which is attributed to a delphinidin-type anthocyanin (Dp) pigmentation, cultivars with various flower colors such as white, pale yellow, pale green, pink, red, and magenta have been produced. In addition to Dp, there are other types of anthocyanins, cyanidin-type anthocyanin (Cy) and pelargonidin-type anthocyanin (Pg) that cause various flower colors. As a major anthocyanin, the 3'-hydroxylase (F3'H). F3'5'H plays an important role in the biosynthesis of Dp, which is a major pigment for blue and violet coloration. F3'H plays a role in Cy biosynthesis. The complementary DNA (cDNA) and the genomic DNA sequences that encode F3'5'H have been isolated from petunia (Holton et al., 1993; Kikuchi et al., 1993). This enzyme is a member of the cytochrome P450 family. Evidence for the involvement of F3'5'H in B-ring hydroxylation has been provided by an in vitro assay using a yeast expression system (Holton et al., 1993). Shimada et al. (1999) introduced the cDNA encoding petunia F3'5'H to petunia and tobacco by Agrobacterium-mediated transformation and confirmed in vivo activities and pigment alteration in transgenic plants. F3'H is also a member of the cytochrome P450 family and was first isolated from petunia by Brugliera et al. (1999).

Characterization of the genes involved in anthocyanin biosynthesis, together with biochemical analysis of anthocyanin pigmentation, is important for flower color breeding in lisianthus. Full-length cDNAs of chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, dihydroflavonol 4-reductase, and anthocyanidin synthase were first cloned by Noda et al. (1993; Kikuchi et al., 1993). This enzyme is a member of the cytochrome P450 family and was first isolated from petunia by Brugliera et al. (1999).

Materials and Methods

Plant materials

Seeds of the lisianthus cultivars ‘Mellow Lavender’ and ‘Royal Violet’ were obtained from Takii & Co., Ltd. (Kyoto, Japan). Seeds of the lisianthus cultivars ‘Asuka no Kurenai’ and ‘Papillon Rose Pink’ were obtained from Sakata Seed Corporation (Yokohama, Japan) and Miyoshi & Co., Ltd. (Tokyo, Japan), respectively. Seedlings of the cultivars and their different self- and cross-pollinated progenies were transplanted to a polyethylene house at Kagoshima University Experimental Farm under standard conditions 110 days after sowing as described by Hashimoto et al. (2004) and Uddin et al. (2002).

Young leaves were used for DNA and RNA extraction and petals (not including the throat) were used for RNA extraction at six stages of flower development: 1) bud less than 20-mm long; 2) bud between 20 mm and 30 mm; 3) bud more than 30 mm and tightly closed; 4) bud more than 30 mm and loosely closed; 5) opening flower; and 6) fully opened flower. Extraction was performed and the same materials and stages of flowers were used as described by Takatori et al. (2015). Young leaves and petal samples were immediately frozen in liquid nitrogen and stored at −80°C until DNA and RNA extraction. Early stage and late stage in Figure 4 correspond to stage (2) and (5–6) in Figure 3 respectively.

Cross-pollination and characterization of anthocyanin composition

The mauve flower line ‘ME’ (self-pollinated line derived from the cultivar ‘Mellow Lavender’)(Fig. S1B) and the violet flower line ‘RV’ (self-pollinated line derived from the cultivar ‘Royal Violet’)(Fig. S1D) were crossed to generate an F2 population with 40 plants. The pink-red flower line ‘AK’ (self-pollinated line derived from the cultivar ‘Asuka no Kurenai’) (Fig. S1A) and the red-purple flower line ‘PRP’ (self-pollinated line derived from the cultivar ‘Papillon Rose Pink’) (Fig. S1C) were crossed to generate an F2 population with 19 plants. An F2 population with 67 plants was also generated from an ‘RV’ × ‘AK’ cross.

Samples for anthocyanin analysis were prepared from petals and analysed using high-performance liquid chromatography (HPLC) with procedures described by
Hashimoto et al. (2002), Uddin et al. (2001, 2002), and Shimizu et al. (2011). For the analysis of anthocyanin composition, prepared samples were hydrolysed to obtain the anthocyanin aglycones before analysis by HPLC. Anthocyanin composition was deduced from the anthocyanidin composition obtained from the HPLC profile.

Genotyping of the F3'5'H gene

Genomic DNA was extracted from young lisianthus leaves using the Plant Genomic DNA Extraction Miniprep System (Viogene, New Taipei City, Taiwan) according to the manufacturer’s instructions. Genomic DNA fragments containing the F3'5'H gene were amplified by polymerase chain reaction (PCR). PCR primers for F3'5'H genotyping were designed from lisianthus F3'5'H cDNA sequences (U72654, Nielsen and Podivinsky, 1997; D14589.1, Shimada et al., 1999; AB078957, Noda et al., 2004). The 20 μL reaction mixture consisted of 0.5 units of Ex Taq™ HS, 1× Ex Taq™ Buffer (TAKARA BIO INC., Shiga, Japan), 0.2 mM of each of the four dNTPs, 0.4 μM each of the forward primer (Genotyping-F, 5'-GTA CTT AAA GGT AGG CAG CTG TGG-3') (Fig. S2) and the reverse primer (Genotyping-R, 5'-GGC TTC TCG TCT ATA GGG AGG TAT-3') (Fig. S2), and 1–5 ng of DNA template. The thermal cycling conditions were as follows: 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 4 min, and a final step of 10 min at 72°C. The PCR products were separated by agarose gel electrophoresis.

The binding sites of all the primers used to amplify the lisianthus F3'5'H gene in this study are shown in Figure S2.

Cloning and sequencing

Genomic DNA fragments containing the region between the start codon to stop codon of F3'5'H were amplified by PCR for cloning and sequencing. The 20 μL reaction mixture consisted of 1× PCR buffer for KOD -Plus- (TOYOBO Co., Ltd., Osaka, Japan), 0.2 mM of each of the four dNTPs, 0.4 units of KOD -Plus- DNA polymerase (TOYOBO), 0.3 μM each of the forward primer (Cloning-F, 5'-GGC CGG ATT CTT ACC AAG AT-3') (Fig. S2) and the reverse primer (Cloning-R, 5'-TCA GAA ACA TGG ACG AAC TCC-3') (Fig. S2), and 1–5 ng of DNA template. The thermal cycling conditions for ‘ME’ and ‘RV’ were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 68°C for 3 min, and a final step of 10 min at 68°C. In the PCR of ‘AK’, no amplification was observed when the same forward primer used for ‘ME’ and ‘RV’ amplifications was used. Therefore, PCR for ‘AK’ was performed again using the same method as described above, but using the forward primer (Cloning AK-F, 5'-GCC CGT ACT TAA CGA TAT GCC TGT-3') (Fig. S2) and a thermal cycling extension time of 8 min at 68°C. The PCR products were separated by agarose gel electrophoresis and purified using the Gel-M Extraction System (Viogene) according to the manufacturer’s instructions. Purified fragments were either sequenced directly or cloned into a pCR®-Blunt-II-TOPO® vector (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol prior to sequencing. All sequencing was performed using an ABI PRISM® 3100 Genetic Analyzer (Life Technologies). The F3'5'H sequences reported in this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB383116 (‘ME’), AB383117 (‘RV’), and AB383118 (‘AK’).

Detection and quantification of F3'5'H transcripts

RNA was extracted from 50–100 mg of petals from ‘ME’, ‘RV’, and ‘AK’ using the Plant Total RNA Extraction Miniprep System (Viogene) according to the manufacturer’s instructions. First-strand cDNA was synthesized from DNase I-treated RNA according to the manufacturer’s protocol using the PrimeScript® First-Strand cDNA Synthesis Kit (TAKARA BIO). For detection of F3'5'H transcripts among these strains, PCR was conducted using a 20 μL reaction mixture consisting of 1× Ex Taq™ Buffer, 0.2 mM of each dNTP, 0.5 units of Ex Taq™ HS, 0.4 μM each of the forward primer (Genotyping-F) and the reverse primer (RT-PCR-R, 5'-GAG TGA CCA TAG CAA GAG G-3') (Fig. S2), and approximately 50 ng of first strand cDNA as the template. The thermal cycling conditions were as follows: 94°C for 1 min, followed by 25 cycles of 94°C for 30 s, 67°C for 30 s, 72°C for 2 min, and a final step of 4 min at 72°C. Lisanthius ubiquitin (AB049409) (Oka et al., 2001) was amplified for normalization of PCR using the forward primer (5'-ATC CAG ACC AAG AT-3') and the reverse primer (5'-GGC TTC TCG TCT ATA GGG AGG TAT-3') (Fig. S2). The PCR products were analysed using agarose gel electrophoresis.

For the quantification of F3'5'H gene expression, real-time quantitative polymerase chain reaction (qPCR) was performed using SYBR® Green detection chemistry and run in triplicate on a CFX96 Touch™ qPCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The 10 μL reaction volume contained 1× SYBR® Premix DimerEraser Buffer (TAKARA BIO), 0.3 μM each of the forward primer (qPCR-F, 5'-GCC TCT TGC TGC TAT GGT CAC T-3') (Fig. S2) and the reverse primer (qPCR-R, 5'-TTC TCA ATG ACC CAT AGA ACA CAG A-3') (Fig. S2), and approximately 30 ng cDNA as the template. The thermal cycling conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a melting curve analysis. To normalize qPCR data, the same primer set as above—lisianthus ubiquitin—was used.
Southern hybridization of the retrotransposon inserted in the lisianthus F3’5’H

Genomic DNA was extracted from young lisianthus leaves using a modified CTAB method (Lassner et al., 1989). Genomic DNA (5 μg) was digested with EcoRI, separated by agarose gel electrophoresis, and then blotted onto a Hybond-N+ membrane following the manufacturer’s protocol (GE Healthcare, Chalfont St Giles, UK). The probe for Southern hybridization was prepared by PCR using the forward primer (5’-AGC CTT GGT TCA ACA CCA TC-3’) and the reverse primer (5’-TCG GTA GCA CCA CAA TCA AA-3’), and was subsequently labeled using the Gene Images AlkPhos Direct Labeling and Chemiluminescent Detection System with CDP-Star (GE Healthcare). This primer was designed from the sequence of a retrotransposon inserted in lisianthus F3’5’H. The blot was hybridized overnight with the labeled probe at 65°C. Post-hybridization signal generation was carried out according to the manufacturer’s instructions.

Co-segregation analysis of the F3’5’H genotype and color phenotype in the F2 populations

The F2 populations generated in this study were used for co-segregation analysis of the anthocyanin composition and F3’5’H polymorphism. All PCRs were conducted according to the procedures described above in subsection (3) Genotyping of the F3’5’H gene, except for the F2 population of ‘AK’ × ‘RV’, for which the procedure is described below.

In the F2 population of ‘AK’ × ‘RV’, PCR was conducted using the Ampdirect® Plus PCR system (SHIMADZU Corp., Kyoto, Japan), which facilitates PCR with a crude DNA extract. Genomic DNA was prepared from young lisianthus leaves according to the manufacturer’s instructions and described by Moriya <http://www.an.shimadzu.co.jp/bio/reagents/amp/protocol/p0007.pdf> with some modifications. Twenty milligrams of lisianthus leaves were homogenized in 200 μL of CTAB buffer (20 mM Tris-HCl, 2 M NaCl, 50 mM EDTA, 2% (w/v) hexadecyltrimethylammonium bromide, 0.5% (v/v) 2-mercaptoethanol) and incubated for 10 min at 94°C. After incubation, the sample was centrifuged at 15000 × g for 1 min. The obtained supernatant was diluted 20 times with 1/10 TE and used as the PCR template. The 10 μL reaction mixture consisted of 1 × Ampdirect Buffer, 0.25 units of Ex Taq™ HS, 0.4 μM each of primers, and 1 μL of diluted crude DNA extract. The thermal cycling conditions and primers were as described above in subsection (3) Genotyping of the F3’5’H gene.

Results

Characterization of anthocyanin composition

Anthocyanin-containing samples were extracted from the lisianthus self-pollinated lines used for genetic experiments (Table 1). They were hydrolysed to obtain the aglycones and HPLC analysis was conducted to deduce the anthocyanin composition from the composition of anthocyanidin. The mauve flower line ‘ME’ comprised 69.8% Cy and 27.0% Dp. The red-purple flower line ‘PRP’ comprised 70.3% Cy, 23.9% Pg, and 4.5% Dp. The violet flower line ‘RV’ comprised 100% Dp. The pink-red flower line ‘AK’ comprised 79.3% Pg and 20.7% Cy, but no Dp was detected. In this study, traits that are mainly associated with the accumulation of Cy, such as ‘ME’ and ‘PRP’, were assigned as “the Cy major phenotype”. Similarly, a trait that mainly accumulates Dp like ‘RV’ was assigned as “the Dp-major phenotype”, and a trait that mainly accumulated Pg, but did not accumulate Dp such as ‘AK’ as “the Pg major-non Dp phenotype”.

Genotyping and sequencing of the F3’5’H gene

Figure 1 shows the results of genomic PCR using genotyping primers designed from published cDNA sequences of lisianthus F3’5’H (Nielsen and Podivinsky, 1997; Shimada et al., 1999; Noda et al., 2004). Amplified products of three different lengths were detected among ‘ME’ (approximately 2 kbp), ‘PRP’ (approximately 2 kbp), ‘RV’ (approximately 2.5 kbp), and ‘AK’ (approximately 8 kbp) (Fig. 1). Sequencing of the amplified fragments from the genomic PCR using the cloning primers (Fig. S2) revealed that the F3’5’H gene (from the start codon to stop codon of F3’5’H) comprised 2045 bp in ‘ME’, 2662 bp in ‘RV’, and 8072 bp

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Table 1. Anthocyanin composition of lisianthus lines.

| Line | Flower color | Anthocyanin composition | Major anthocyanin |
|------|--------------|------------------------|-------------------|
|      |              | Pg (%) | Cy (%) | Pn (%) | Dp (%) | Mv and Pt (%) |                  |
| ‘AK’ | Pink-red     | 79.3   | 20.7   | 0.0    | 0.0    | 0.0          | Pg                |
| ‘ME’ | Mauve        | 0.0    | 69.8   | 3.2    | 27.0   | 0.0          | Cy                |
| ‘PRP’| Red-purple   | 23.9   | 70.3   | 1.3    | 4.5    | 0.0          | Cy                |
| ‘RV’ | Violet       | 0.0    | 0.0    | 0.0    | 100.0  | 0.0          | Dp                |

* ‘AK’ = self-pollinated line derived from cultivar ‘Asuka no Kurenai’, ‘ME’ = self-pollinated line derived from cultivar ‘Mellow Lavender’, ‘PRP’ = self-pollinated line derived from cultivar ‘Papillon Rose Pink’, ‘RV’ = self-pollinated line derived from cultivar ‘Royal Violet’.

* Pg = pelargonidin type anthocyanin, Cy = cyanidin type anthocyanin, Pn = peonidin type anthocyanin, Dp = delphinidin type anthocyanin, Mv = malvidin type anthocyanin, Pt = petunidin type anthocyanin.
in ‘AK’. These three types of F3′5′H are allelic as described below. Therefore, we have indicated the ‘ME’ type F3′5′H and ‘RV’ type F3′5′H as F3′5′H 1-1 and F3′5′H 1-2, respectively. We found the retrotransposon (rTeg1, retro transposon of Eustoma grandiflorum 1) inserted in ‘AK’ type F3′5′H and the remaining sequence of ‘AK’ type F3′5′H to be almost identical to that of F3′5′H 1-2 as described below. We have indicated the genotype of rTeg1-inserted F3′5′H as F3′5′h1-2, which is co-segregated to a loss of function mutation, as described below.

Based on a comparison with cDNA sequences reported by Nielsen and Podivinsky (1997), Shimada et al. (1999), and Noda et al. (2004), two exons and one intron were found in the lisianthus F3′5′H gene (Fig. 2A). F3′5′H1-1 and F3′5′H1-2 contained single introns at the same positions, but of different sizes: 512 bp in F3′5′H1-1 and 1129 bp in F3′5′H1-1. Both F3′5′H1-1 and F3′5′H1-2 contained one ORF of 1533 bp encoding a protein of 510 amino acid residues. F3′5′H1-1 and F3′5′H1-2 showed 98% sequence identity with base substitutions at 26 nucleotide positions. These nucleotide differences led to differences in the corresponding amino acid sequences at eight positions: 51, 282, 291, 329, 408, 469, 484, and 509. The coding region of F3′5′H1-1 was almost identical to that of the F3′5′H cDNA, as previously reported by Nielsen and Podivinsky (1997) (UT2654), with nucleotide differences at only two positions. The coding region of F3′5′H1-2 was identical to that of the F3′5′H cDNA sequences, as reported by Shimada et al. (1999) and Noda et al. (2004).

Sequencing and analyses of the inserted retrotransposon in F3′5′h1-2

The F3′5′h1-2 in the Pg major-non Dp phenotype line (‘AK’) included an insertion of 5405 bp in the first exon, and there were 5 bp direct repeats on both sides of this insertion sequence, while the remaining gene sequence was identical to that of F3′5′H1-2. The insertion in F3′5′h1-2 was sequenced and characterized as a novel 5405 bp retrotransposon (rTeg1) (Fig. 2A). This element contained a 4776 bp coding region flanked by two long terminal repeats (LTRs) that were identical in length (216 bp) and sequence. A short, complete, inverted terminal sequence of 5′-TGTT---AACAG-3′ was found at the LTR ends. The rTeg1-flanked direct repeats of 5′-AACAG-3′ are presumed to be the target site duplication (TSD) produced by the insertion of rTeg1 in lisianthus. The primer binding site (PBS) was located downstream of the 5′-LTR end, which is the binding site for a tRNA primer necessary for reverse transcription of the retrotransposon (Kimura et al., 2001). The polypurine tract (PPT), which is necessary to initiate the synthesis of plus-strand DNA (Kimura et al., 2001), was located upstream of the 3′-LTR end. The coding

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**Fig. 1.** Genotyping of the F3′5′H gene in four lisianthus lines: ‘ME’, ‘PRP’, ‘RV’, and ‘AK’. Lane M = 1 kbp DNA size marker and numbers on the left indicate sizes of the DNA marker. F3′5′H1-1, F3′5′H1-2, and F3′5′h1-2 are the three different genomic sequences of F3′5′H.

**Fig. 2.** Genomic structure of the lisianthus F3′5′H gene and Southern blot analysis of rTeg1. (A) Diagram showing the structure of the three different genomic sequences of F3′5′H: F3′5′H1-1, F3′5′H1-2, and F3′5′h1-2. Exons and introns are indicated using boxes and solid lines, respectively. The numbers indicate the sizes (bps) of the fragments. The vertical solid line in exon I shows the position of the rTeg1 insertion. LTR = long terminal repeat; Gag = Group-specific antigen; pol = polypeptide; PR = protease; INT = integrase; RT = reverse transcriptase; PBS = primer binding site; PPT = polypurine tract. The positions of EcoRI sites, and the rTeg1 probe region are indicated. The elements in this figure are not drawn to scale. (B) Southern blot analysis of rTeg1 in ‘ME’, ‘RV’, and ‘AK’. The positions of the molecular weight markers (10 kbp DNA ladder) are indicated on the left.
region of the rTeg1 element can be translated into a protein of 1591 amino acids. This sequence is homologous to the internal coding region of LTR retrotransposons and contains conserved domains belonging to gag (a core protein of virus-like particles) and pol polyproteins. This polyprotein gene pol contains protease, integrase, reverse transcriptase, and RNase H, in that order (Figs. 2 and S3). This order of domains and sequence homology analysis indicated that rTeg1 is a Ty1-copia-like retrotransposon (Bennetzen, 2000; Casacuberta and Santiago, 2003).

**Presence of the rTeg1 retrotransposon in lisianthus**

The presence of rTeg1 was investigated by Southern blot analysis. The profile showed multiple bands in the three lisianthus lines: ‘ME’, ‘RV’, and ‘AK’ (Fig. 2B). This suggests that rTeg1 exists in multiple positions in these lisianthus genomes. The 5.5 kbp fragment was expected to detect only the ‘AK’ samples with homozygous rTeg1 insertion, but we could not distinguish the ‘AK’ specific signal in the corresponding blot site under our experimental conditions.

**Analysis of F3’5’H gene expression**

PCR was performed using cDNA derived from flower petals as a template to examine whether the mRNA expression of the F3’5’H gene was affected by the insertion of the rTeg1 element. Based on the internal sequences of the F3’5’H gene, primers were designed to amplify a 1286 bp fragment. The expected fragment was detected in ‘ME’ and ‘RV’ at the late stages of flower development (Fig. 3A, B). In ‘AK’, the 1286 bp fragment could not be amplified, but amplified fragments of lengths approximately 2.5 kbp and 6.5 kbp (Fig. 3C) were observed. The longer fragment that was presumed to be a 6696 bp fragment included 1286 bp of the F3’5’H gene and 5410 bp of rTeg1 with TSD (Fig. 3D). Partial sequencing of the longer fragment supported this speculation (data not shown). The shorter fragment was subjected to sequence analysis and was revealed to contain a 2482 bp fragment, which was assumed to have resulted from alternative splicing caused by the rTeg1 insertion (Fig. 3D). Thus, only abnormal F3’5’H transcripts were present in ‘AK’.

It is likely that the difference in anthocyanin composition between ‘ME’ and ‘RV’ was affected by the F3’5’H expression level. Although low expression of F3’5’H was observed in the early stages of flower development in ‘RV’, but not in ‘ME’, no major difference in the expression levels of F3’5’H genes was observed between ‘RV’ and ‘ME’ in the later stages (Fig. 3A, B). To confirm this result, we conducted qPCR to compare F3’5’H expression in ‘RV’ and ‘ME’ and it was revealed that there was higher relative gene expression in ‘RV’ than in ‘ME’, but this difference was not significant (Fig. 4).

**Analysis of the flower color segregation ratio and anthocyanin type in the F$_2$ populations**

Cross-pollination between ‘ME’ and ‘RV’ produced an F$_1$ hybrid plant with violet flowers. This indicated that the Dp major phenotype with violet flowers was dominant to the Cy major phenotype with mauve flowers. In the F$_2$ population from ‘ME’ × ‘RV’, the flower color phenotypes segregated into 30 violet-flowered plants and 10 mauve- or pink-flowered plants. Typical flowers in the F$_2$ population are shown in Figure S1 (E–G). The segregation ratio fit well with the 3:1 ratio expected under Mendelian inheritance ($\chi^2 = 0.0$, $\chi^2 < \chi^2_{0.05} = 3.841$). HPLC analysis of petals from 15 of the 30 violet-flowered plants in the F$_2$ population revealed that they all were the Dp major phenotype (Dp: 70.9%–
Cross-pollination between ‘AK’ and ‘PRP’ produced an F₁ hybrid plant with red-purple flowers. This indicated that the red-purple flower phenotype was dominant to the pink-red flower phenotype. In the F₂ population from ‘AK’ × ‘PRP’, the petal anthocyanin phenotype segregated into 15 Cy major phenotypes (Pg: 6.3%–45.3%, Cy: 51.5%–82.4%, Peonidin [Pn]: 0%–1.6%, Dp: 2.1%–17.3%) and four Pg major-non Dp phenotypes (Pg: 89.2%–98.2%, Cy: 1.8%–10.8%) (Table 3). The segregation ratio was consistent with the 3:1 ratio expected under Mendelian inheritance ($\chi^2 = 0.158, \chi^2 < \chi^2_{0.05} = 3.841$). Typical flowers in the F₂ population are shown in Figure S1 (H–J). These results indicate that the Cy major phenotype is dominant to the Pg major-non Dp phenotype. A similar result was observed in the F₂ population from ‘RV’ × ‘AK’, which segregated into 47 violet-flowered plants and 20 pink-flowered plants. Typical flowers in the F₂ population are shown in Figure S1 (K and L). The segregation ratio was consistent with the 3:1 ratio expected under Mendelian inheritance ($\chi^2 = 0.841, \chi^2 < \chi^2_{0.05} = 3.841$). The anthocyanin composition was investigated in 20 violet-flowered plants and seven pink-flowered plants in the ‘RV’ × ‘AK’ F₂ population. All violet-flowered plants were the Dp major phenotype, while all pink-flowered plants were the Pg major-non Dp phenotype (Table S1).

### Table 2. Segregation of the anthocyanin composition in 20 lisianthus F₂ individuals generated from the cross between ‘ME’ and ‘RV’.

| F₂ population | Flower color | Anthocyanin composition | Major anthocyanin |
|---------------|--------------|-------------------------|-------------------|
|               |              | Pg (%) | Cy (%) | Dp (%) | Mv and Pt (%) |            |
| 1             | Mauve        | 0.0    | 89.3   | 10.7   | 0.0         | Cy        |
| 2             | Mauve        | 0.0    | 88.2   | 11.8   | 0.0         | Cy        |
| 3             | Mauve        | 0.0    | 96.7   | 0.5    | 2.8         | Cy        |
| 4             | Pink         | 0.0    | 82.6   | 2.5    | 14.9        | Cy        |
| 5             | Pink         | 17.5   | 81.0   | 1.5    | 0.0         | Cy        |
| 6             | Violet       | 1.2    | 18.6   | 70.9   | 9.3         | Dp        |
| 7             | Violet       | 1.0    | 7.5    | 86.0   | 5.5         | Dp        |
| 8             | Violet       | 1.3    | 8.5    | 79.3   | 10.9        | Dp        |
| 9             | Violet       | 0.0    | 0.0    | 89.6   | 10.4        | Dp        |
| 10            | Violet       | 0.0    | 12.7   | 79.9   | 7.4         | Dp        |
| 11            | Violet       | 0.0    | 1.6    | 93.6   | 4.8         | Dp        |
| 12            | Violet       | 0.0    | 0.0    | 91.0   | 9.0         | Dp        |
| 13            | Violet       | 0.0    | 1.3    | 92.4   | 6.3         | Dp        |
| 14            | Violet       | 0.0    | 15.3   | 75.6   | 9.1         | Dp        |
| 15            | Violet       | 0.0    | 18.4   | 75.1   | 6.5         | Dp        |
| 16            | Violet       | 0.0    | 0.0    | 92.0   | 8.0         | Dp        |
| 17            | Violet       | 1.4    | 3.6    | 87.5   | 7.5         | Dp        |
| 18            | Violet       | 1.0    | 3.2    | 89.7   | 6.1         | Dp        |
| 19            | Violet       | 0.0    | 0.0    | 92.8   | 7.2         | Dp        |
| 20            | Violet       | 0.0    | 12.8   | 80.8   | 6.4         | Dp        |

* ‘ME’ = self-pollinated line derived from cultivar ‘Mellow Lavender’, ‘RV’ = self-pollinated line derived from cultivar ‘Royal Violet’.

* Pg = pelargonidin type anthocyanin, Cy = cyanidin type anthocyanin, Dp = delphinidin type anthocyanin, Mv = malvidin type anthocyanin, Pt = petunidin type anthocyanin.

Co-segregation of the F3’5’H genotype and color phenotype in the F₂ populations

Twenty of the 40 ‘ME’ × ‘RV’ F₂ plants were examined for any relationship between anthocyanin composition and F3’5’H genotype. All plants with the...
homozygous F3’5’H1-1 exhibited the Cy major phenotype, whereas plants with the homozygous and heterozygous F3’5’H1-2 showed the Dp major phenotype (Fig. 5A). These results suggest that the Dp major phenotype and Cy major phenotype co-segregated with F3’5’H1-2 and F3’5’H1-1, respectively, in the F2 population of ‘ME’ × ‘RV’.

‘AK’ × ‘PRP’ F2 plants were also examined by co-segregation analysis. All plants with homozygous f3’5’h1-2 exhibited the Pg major-non Dp phenotype, whereas plants with homozygous and heterozygous F3’5’H1-1 showed the Cy major phenotype (Fig. 5B).

‘RV’ × ‘AK’ F2 plants were examined in co-segregation analysis using the Ampdirect® Plus PCR system for crude DNA extracts. All plants with the pink flower phenotype exhibited only an f3’5’h1-2 band, whereas plants with violet flower phenotypes exhibited the F3’5’H1-2 band (Fig. S4). However, no heterozygous

| F2 population | Flower color  | Anthocyanin composition | Major anthocyanin |
|---------------|---------------|-------------------------|-------------------|
|               |               | Pg (%)      | Cy (%)     | Pn (%)     | Dp (%)     |               |
| 1             | Red-purple    | 32.3        | 63.4       | 1.0        | 3.3        | Cy           |
| 2             | Red-purple    | 13.1        | 80.8       | 0.0        | 6.1        | Cy           |
| 3             | Red-purple    | 13.4        | 82.4       | 0.9        | 3.3        | Cy           |
| 4             | Red-purple    | 36.1        | 60.4       | 0.5        | 3.0        | Cy           |
| 5             | Red-purple    | 8.0         | 75.0       | 0.0        | 17.0       | Cy           |
| 6             | Red-purple    | 28.6        | 67.5       | 0.5        | 3.4        | Cy           |
| 7             | Red-purple    | 25.2        | 71.1       | 0.5        | 3.2        | Cy           |
| 8             | Red-purple    | 40.1        | 57.2       | 0.6        | 2.1        | Cy           |
| 9             | Red-purple    | 37.9        | 58.8       | 0.8        | 2.5        | Cy           |
| 10            | Red-purple    | 27.7        | 68.2       | 0.7        | 3.4        | Cy           |
| 11            | Red-purple    | 32.8        | 61.9       | 1.6        | 3.7        | Cy           |
| 12            | Red-purple    | 45.3        | 51.5       | 0.7        | 2.5        | Cy           |
| 13            | Red-purple    | 37.7        | 59.4       | 0.5        | 2.4        | Cy           |
| 14            | Red-purple    | 6.3         | 76.4       | 0.0        | 17.3       | Cy           |
| 15            | Red-purple    | 24.3        | 70.6       | 0.7        | 4.4        | Cy           |
| 16            | Pink-red      | 89.2        | 10.8       | 0.0        | 0.0        | Pg           |
| 17            | Pink-red      | 94.3        | 5.7        | 0.0        | 0.0        | Pg           |
| 18            | Pink           | 97.2        | 2.8        | 0.0        | 0.0        | Pg           |
| 19            | Pink           | 98.2        | 1.8        | 0.0        | 0.0        | Pg           |

Table 3. Segregation of the anthocyanin composition in 19 lisianthus F2 individuals generated from the cross between ‘AK’ and ‘PRP’.

z ‘AK’ = self-pollinated line derived from cultivar ‘Asuka no Kurenai’, ‘PRP’ = self-pollinated line derived from cultivar ‘Papillon Rose Pink’.
y Pg = pelargonidin type anthocyanin, Cy = cyanidin type anthocyanin, Pn = peonidin type anthocyanin, Dp = delphinidin type anthocyanin.

Fig. 5. Co-segregation of F3’5’H genotype and anthocyanin phenotype in the lisianthus F2 populations generated from crosses between ‘ME’ × ‘RV’ (A) and ‘AK’ × ‘PRP’ (B). The lane numbers in the (A) and (B) represent the same plants as the numbers in Tables 2 and 3, respectively. Lane M = 1 kbp DNA size marker and numbers on the left indicate DNA marker size. (A) Lanes 1–5, ‘ME’ × ‘RV’ plants with Cy major phenotype; lanes 6–20, ‘ME’ × ‘RV’ plants with Dp major phenotype. (B) Lanes 1–15, ‘AK’ × ‘PRP’ plants with the Cy major phenotype; lanes 16–19, ‘AK’ × ‘PRP’ plants with the Pg major and non-Dp phenotype.
plants were observed among the plants with the Dp major phenotype, which exhibited only the F3'5'H1-2 signal. The long product $f3'5'h1-2$ may not have amplified well owing to competition between amplified products in heterozygous plants. These results suggest that the Pg major-non Dp phenotype co-segregated with $f3'5'h1-2$.

Discussion

Our results showed that at least three alleles of the F3'5'H gene—F3'5'H1-1, F3'5'H1-2, and $f3'5'h1-2$ (Figs. 1 and 2)—were present in lisianthus. F3'5'H1-1 was detected in Cy major phenotype plants, F3'5'H1-2 was detected in Dp major phenotype plants, and $f3'5'h1-2$ was present in Pg major-non Dp phenotype plants. The flavonoid B-ring hydroxylation of lisianthus is controlled by multiple alleles named $H^c$, $H^f$, $H^p$, and $H^o$ (Hashimoto et al., 2004). Based on the inheritance of flower phenotypes in this study, the Dp major phenotype line ‘RV’ was presumed to have the genotype $H^cH^f$ with homozygous F3'5'H1-2. The Cy major phenotype lines, ‘PRP’ and ‘ME’, were presumed to have the genotype $H^pH^o$ with homozygous F3'5'H1-1. The Pg major-non Dp phenotype line ‘AK’ was presumed to have the genotype $H^pH^o$ with homozygous $f3'5'h1-2$.

Mauve-flowered plants and pink-flowered plants were observed in the F$_2$ population of ‘ME’ × ‘RV’ (Table 2; Fig. S1). However, there was no distinct difference in anthocyanin composition between mauve-flowered plants and pink-flowered plants (Table 2). Therefore, this difference in flower color may be due to another factor such as a difference in the amount of anthocyanin accumulation, co-pigmentation or anthocyanin modification. Although methylated anthocyanins were not detected in the parental line ‘RV’ (Table 1), small amounts of Pt and Mv were detected in some plants in the F$_2$ population (Table 2). This may be due to the effect of environmental conditions or the genetic background of ‘ME’.

The insertion of a 5405 bp transposable element, rTeg1, was observed in the non-Dp accumulating line ‘AK’ (Fig. 2). Although the amino acid sequence of the protease was poorly conserved, the rTeg1 element appeared to encode almost all the structural and enzymatic proteins necessary for transposition (Figs. 2 and S3). In addition, Southern blot analysis showed that multiple copies of rTeg1 were present in the lisianthus genome (Fig. 2B). Therefore, rTeg1 can be considered a potentially autonomous retrotransposon in lisianthus. Many active retrotransposons have been identified in various plants, such as Tnt1 (Grandbastien et al., 1989) and Tto1 in tobacco (Hirochika, 1993), BARE-I in barley (Suoniemi et al., 1996), TOS-17 in rice (Hirochika et al., 1996), RIRE1 in Oryza sativa (Nomura et al., 1997), Tar1 in Trifolium aestivum (Matsuoka and Tsunewaki, 1997), OARE-1 in oat (Kimura et al., 2001), and LORE1 in legumes (Madsen et al., 2005). Further study is needed to determine whether rTeg1 is an active retrotransposon.

Gene expression analysis showed the presence of two abnormal amplified fragments (one longer and one shorter) in petals of ‘AK’ (Fig. 3C, D). Sequence analysis revealed that the shorter fragment comprised abnormal transcripts of the F3'5'H gene caused by insertion of the rTeg1 element. This transcript of $f3'5'h1-2$ could not be translated into a functional F3'5'H enzyme because of a nonsense mutation caused by a stop codon in the left LTR of rTeg1 (Fig. 3D). The resulting truncated protein of 136 amino acids lacked the protein core of F3'5'H, including parts of the I-helix and heme-binding regions (Nielsen and Podivinsky, 1997). The same stop codon was also detected by partial sequencing of the longer fragment (data not shown). Thus, a detectable amount of normal F3'5'H transcript was not present in the petals of ‘AK’. These results agree with those reported by Nielsen and Podivinsky (1997) and Noda et al. (2004), who did not detect the normal F3'5'H gene transcript in pink-flowered lisianthus lines that lacked Dp, but accumulated Pg. Previous research has shown that inserting the transposable element GsTRIM1 in the F3'5'H gene in the Gentiana scabra ‘Momokorin’ also caused two alternative F3'5'H transcripts, neither of which could produce the functional F3'5'H enzyme because of a nonsense mutation within the GsTRIM1 element (Nakatsuka et al., 2006). Furthermore, genetic study of the F$_2$ populations from ‘RV’ (F3'5'H1-2) × ‘AK’ ($f3'5'h1-2$) and ‘PRP’ (F3'5'H1-1) × ‘AK’ ($f3'5'h1-2$) revealed that homozygous rTeg1 insertion co-segregated with the non-Dp flower phenotype. Nielsen and Podivinsky (1997) reported that only a single copy of the F3'5'H gene is present in the lisianthus genome. Taking the previous and present studies together indicates that non-Dp accumulating type lisianthus cannot produce functional F3'5'H because of an rTeg1 insertion.

Co-segregation of anthocyanin composition and DNA polymorphisms of the F3'5'H gene were also observed in the F$_2$ population from ‘ME’ (F3'5'H1-1) × ‘RV’ (F3'5'H1-2). All F$_2$ plants with homozygous F3'5'H1-1 exhibited the Cy major phenotype. In contrast, homozygous and heterozygous F3'5'H1-2 genotypes exhibited the Dp major phenotype with violet flowers. Variations in the expression levels and patterns of the F3'5'H and F3'H genes are associated with flower color variation in some plants (Johnson et al., 2001; Tanaka et al., 2010; Sato et al., 2011). In the present study, we anticipated marked variations in F3'5'H expression between ‘ME’ and ‘RV’. However, such marked variation in F3'5'H expression was rarely detected between these lines (Figs. 3 and 4). Takatori et al. (2015) investigated the lisianthus F3'H expression among ‘ME’, ‘RV’, and ‘AK’—the same materials as used in this study—and reported no marked expression variation among them. In addition, the F3'H gene is
mainly expressed during the early stages of flower development (Takatori et al., 2015). This expression pattern is consistent with that of FLS (Noda et al., 2004). The lisianthus F3’H enzyme may contribute significantly to the B-ring hydroxylation step of the flavonol biosynthesis, but not that of the anthocyanin. This hypothesis is supported by the result in this study that Cy was not the main pigment in the F3’5’H deficient line ‘AK’. Which genes are responsible for the biosynthesis of the Cy that accumulates in the Cy major phenotype plants? Several studies have shown that the F3’5’H enzyme has F3’H activity (Olsen et al., 2010; He et al., 2013; Wang et al., 2014), and it is possible that lisianthus F3’5’H in Cy major phenotype plants also has F3’H activity. Moreover, it is conceivable that the eight substitutions in F3’5’H gene, coding for amino acid residues observed between ‘ME’ (F3’5’H1-1) and ‘RV’ (F3’5’H1-2), may cause variations in anthocyanin composition. Although these substitutions are not within the important P450 enzyme domain (Nielsen and Podivinsky, 1997), it remains unclear whether this variation affects the flower color phenotype. Alternatively, another gene closely linked to the F3’5’H gene may cause this flower variation in lisianthus. Transformation experiments should be performed to confirm the relationship between the F3’5’H gene and anthocyanin composition. DNA markers for the flower color phenotype targeted at anthocyanin biosynthesis-related genes have been developed for several ornamental plants (Nakajima et al., 2005; Matsubara et al., 2006; Nakatsuka et al., 2012; Nishihara et al., 2015). The only previous study of DNA markers in lisianthus found that a 94 bp deletion of the ANS gene co-segregated with the acyanic flower phenotype (Shimizu et al., 2011). Our study revealed that DNA polymorphisms of the F3’5’H gene are useful DNA markers for flower color breeding in lisianthus. Furthermore, when used with purified DNA samples, these DNA markers were co-dominant and could also be used to distinguish heterozygous plants from the segregating population.

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