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T. Nakajima · K. Matsubara · H. Kodama
H. Kokubun · H. Watanabe · T. Ando

Insertion and excision of a transposable element governs the red floral phenotype in commercial petunias

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Abstract Commercial cultivars of *Petunia hybrida* with red flowers (red petunias) accumulate cyanidin 3-glucoside as a main floral anthocyanin pigment. The conversion of anthocyanidin 3-glucosides to anthocyanidin 3-rutinosides is catalyzed by a UDP-rhamnose: anthocyanidin 3-glucoside-rhamnosyltransferase (RT). In red petunias, the *RT* gene is known to be disrupted by the insertion of a transposable element (*dTph3*). We have cloned the *dTph3*-like element (called *dTph3-C*) from a red petunia. Unlike *dTph3*, *dTph3-C* harbored a perfect terminal inverted repeat. In addition, an excision product (so-called "footprint") of *dTph3*/*dTph3-C* was found in another red petunia. The *RT* transcripts harboring this footprint could not produce the RT enzyme because a stop codon was created in the footprint sequence. The genotypes of the 42 commercial petunias which exhibit different anthocyanin pigmentation were determined by multiplex PCR. In this technique, the amplified products from normal, *dTph3*/*dTph3-C*-inserted, and footprint-retaining *RT* genes can be separated from one another. Our results indicate that the red-floral phenotype of commercial petunias is governed by insertion and excision events of a transposable element in the coding region of the *RT* gene.

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T. Nakajima · K. Matsubara · H. Kokubun
Graduate School of Science and Technology,
Chiba University, 1-33 Yayoi-cho, Inage-Ku,
Chiba 263-8522, Japan

H. Kodama · T. Ando (✉)
Faculty of Horticulture, Chiba University, 648 Matsudo,
Chiba 271-8510, Japan
E-mail: andot@faculty.chiba-u.jp
Tel.: +81-47-3088808
Fax: +81-47-3088809

H. Watanabe
Center of Environment, Health, and Field Science,
Chiba University, 6-2-1 Kashiwanoha, Kashiwa,
Chiba 277-0882, Japan

Introduction

Cultivars of *Petunia hybrida* (petunias) have been bred since the early 1830s (Paxton 1836) and are now available as a popular bedding plant. Modern petunia flowers are diverse in color, with anthocyanin pigments accounting for most of the floral colors except for white and yellow (Wiering and De Vlaming 1984; Winkel-Shirley 2001).

Two dihydroflavonols, dihydroquercetin and dihydromyricetin, are the precursors of anthocyanin pigments in petunias. Dihydromyricetin is synthesized via hydroxylation of dihydroquercetin (or dihydrokaempferol, another dihydroflavanol). These two dihydroflavonols are subjected to sequential modification involving the same enzymes (Fig. 1). Dihydroquercetin and dihydromyrcetin are converted by reduction and glycosylation into cyanidin 3-glucoside and delphinidin 3-glucoside (designated delphinidin), respectively. Then a rhamnosyl group is attached to these anthocyanidin 3-glucoside molecules by a UDP-rhamnose: anthocyanidin 3-glucoside-rhamnosyltransferase (RT). Three additional reactions, namely, acylation, glucosylation, and methylation, lead to the formation of peonidin 3-(acyl)-rutinoside-5-glucoside (designated peonidin) and malvidin 3-(acyl)-rutinoside-5-glucoside (designated malvidin; Fig. 1). The main anthocyanin pigment found in red- or salmon-colored petunias (red petunias) is cyanidin 3-glucoside (designated cyanidin) (Ando et al. 2000), suggesting that RT activity is deficient in these petunias (Wiering and De Vlaming 1973, 1984). Deficiencies in RT activity should cause the accumulation of delphinidin in the synthetic pathway from dihydromyricetin to malvidin (Fig. 1) and because the amount of delphinidin is associated with an undesirable flower shape (crumpled corolla-limb), petunias with high levels of delphinidin are not commercially available (Ando et al. 2004). In contrast, active rhamnosylation should occur in petunias accumulating peonidin or malvidin in the flower. Consequently, commercial petunias are classified into three

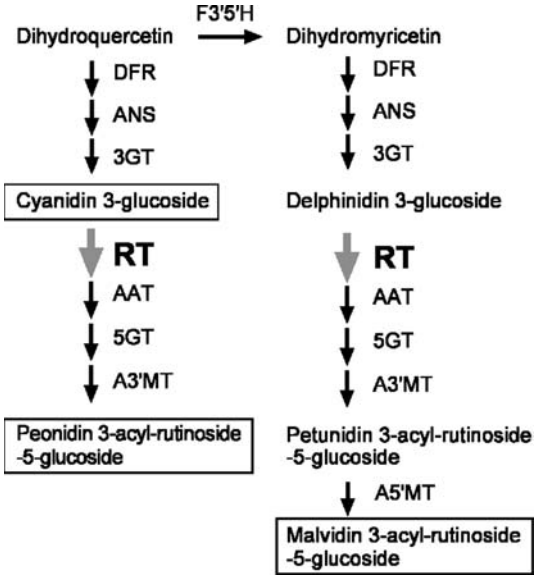

Fig. 1 Biosynthetic pathways for the main anthocyanin pigments in petunias. The major floral anthocyanidins found in commercial petunias are *boxed*. Enzyme names are abbreviated as follows: *F3′5′H* flavonoid 3′,5′-hydoxylase, *DFR* dihydroflavonol 4-reductase, *ANS* anthocyanidin-synthase, *3GT* UDP-glucose, flavonoid 3-*O*-glucosyltransferase, *RT* UDP-rhamnose, anthocyanidin 3-glucoside-rhamnosyltransferase, *AAT* aromatic-acyltransferase, *5GT* UDP-glucose, anthocyanin 5-*O*-glucosyltransferase, *A3′MT* anthocyanin 3′-methyltransferase, *A5′MT* anthocyanin 5′-methyltransferase

groups—those accumulating cyanidin, peonidin (pink to rose color) and malvidin (burgundy and blue color), respectively.

The activity of RT is governed by a dominant allele at the *Rt* locus (Maizonnier et al. 1984; Wiering and De Vlaming 1984), and the *RT* gene at this locus has been isolated (Brugliera et al. 1994; Kroon et al. 1994). In petunia lines defective for RT activity, insertions of two independent transposable elements block expression of the *RT* gene (Kroon et al. 1994). A transposable element (*dTph1*) can be inserted into the promoter region of the *RT* gene, thereby blocking transcription. Another element (*dTph3*) is a 442-bp non-autonomous element which is inserted into the protein-coding region of the *RT* gene. The investigation reported here aimed to find new mutations of the *RT* gene in commercial red petunias and to develop a molecular marker for determining the genotype of the *Rt* locus.

Materials and methods

Plant materials and classification by anthocyanin pigments

Seeds of commercial petunias (*Petunia* × *hybrida* Vilm., Solanaceae) were obtained from Bodger Seeds (El Monte, Calif.; Celebrity series), Goldsmith Seeds (Gilroy, Calif.; Ultra series), the PanAmerican Seed Co. (West Chicago, Ill.; Madness series), and Sakata Seed Co. [Yokohama, Japan; Baccara (=Merlin) and Falcon series]. Plants were grown in a greenhouse following standard practices for garden petunias. The methodology for classification of petunias according to their main floral anthocyanidin pigments (Table 1) has been previously described (Ando et al. 2004).

Table 1 Commercial petunias used and classification by their main anthocyanidin

Series	Cultivar name	Abbreviation	Main anthocyanidin
Baccara	Baccara Blue	BL	Malvidin
	Baccara Plum	PU	Malvidin
	Baccara Pink	PK	Peonidin
	Baccara Rose	RS	Peonidin
	Baccara Cherry Rose	CHRS	Peonidin
	Baccara Red	RD	Cyanidin
	Baccara Salmon	SN	Cyanidin
	Baccara Light Salmon	LS	Cyanidin
Celebrity	Celebrity Burgundy	BY	Malvidin
	Celebrity Neon	NE	Malvidin
	Celebrity Blue	BL	Malvidin
	Celebrity Sky Blue	SKBL	Malvidin
	Celebrity Lilac	LL	Malvidin
	Celebrity Hot Pink	HTPK	Peonidin
	Celebrity Red	RD	Cyanidin
	Celebrity Salmon	SN	Cyanidin
	Celebrity Scarlet	ST	Cyanidin
Falcon	Falcon Burgundy	BY	Malvidin
	Falcon Blue	BL	Malvidin
	Falcon Mid Blue	MIBL	Malvidin
	Falcon Lilac	LL	Malvidin
	Falcon Blush Pink	BHPK	Peonidin
	Falcon Rose	RS	Peonidin
	Falcon Deep Rose	DPRS	Peonidin
	Falcon Red	RD	Cyanidin
	Falcon Salmon	SN	Cyanidin
	Falcon Pastel Salmon	PASN	Cyanidin
	Falcon Coral	CO	Cyanidin
Madness	Burgundy Madness	BY	Malvidin
	Midnight Madness	MN	Malvidin
	Plum Madness	PU	Malvidin
	Pink Madness	PK	Peonidin
	Rose Madness	RS	Peonidin
	Red Madness	RD	Cyanidin
	Simply Madness	SI	Cyanidin
	Coral Madness	CO	Cyanidin
Ultra	Ultra Burgundy	BY	Malvidin
	Ultra Blue	BL	Malvidin
	Ultra Plum	PU	Malvidin
	Ultra Pink	PK	Peonidin
	Ultra Rose	RS	Peonidin
	Ultra Red	RD	Cyanidin

PCR analysis of genomic DNA

Total DNA was prepared from petunia leaves according to the method of Walbot and Warren (1988). Plants homozygous for the *Rt* and *rt* alleles, cvs. Celebrity Blue and Celebrity Scarlet, respectively, were used for these initial DNA extractions. The genomic region corresponding to the *RT* cDNA from nucleotides 565 to 1,711

(GenBank accession number X71059) was amplified using the RT-2nd-Uni primer 5′-CTCAGACCTCTG-TTACCTCAGTCAG-3′ and the RT-RV primer 5′-AGAAACTGAACTCTCATCC-3′.

When the footprint sequence was identified, the genomic region corresponding to the *RT* cDNA from nucleotides 1,015 to 1,711 was amplified from total DNA prepared from a red petunia, Ultra Red. PCR was performed with the RT-For2 primer 5′-GGTTTCT-GGAAAGAGTGAAAGACA-3′ and the RT-RV primer. The resultant fragments were cloned into a plasmid vector, pGEM-T Easy vector (Promega, Madison, Wis.) and then sequenced. All sequencing was performed by asymmetric PCR employing a DNA sequencer (Applied Biosystems, Foster City, Calif.).

Multiplex PCR

The 50-μl reaction mixtures consisted of approximately 100 ng total DNA, 1× ExTaq buffer, 0.2 m *M* each deoxynucleotide triphosphate, 1 m *M* each primer and 2.5 U ExTaq (TaKaRa, Japan). Amplification was performed as follows: an initial denaturation for 5 min at 94°C, followed by 30 cycles of 94°C for 45 s, 61°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. This multiplex PCR used the RT-For2 primer, the Foot-RV primer (5′-ATGTTCTGGTAAAGA-CCATCTTCAT-3′) and the Foot-FW primer (5′-GCTGGTGACTCACCAGTGG-3′). The PCR products were separated by agarose gel electrophoresis.

Northern blot analysis

Flowers and flower buds were harvested at developmental stages defined as follows: stage 1, closed bud (less than 10 mm in length); stage 2, enlarged closed bud with slight pigmentation (about 20 mm in length); stage 3, clearly pigmented bud (more than 30 mm in length); stage 4, bud just before opening; stage 5, fully opened flower. Total RNA was isolated using an RNeasy Plant Mini kit (Qiagen, Valencia, Calif.) according to the manufacturer's protocol. Preparation of the blot was as previously described (Kodama et al. 1991). Blots were hybridized with a [^{32}P]-labelled genomic DNA fragment of the *RT* gene. The same blot was rehybridized with the [^{32}P]-labelled *dTph3-C* fragment (accession no. AB191689), and the hybridized probes were then visualized with an FLA-2000 imaging system (Fuji Photo Film).

Results and discussion

Characterization of the *dTph3* sequence in the *RT* gene

Forty-two commercial cultivars of five representative series were selected (Table 1), and expression of the *RT* gene was studied in each of these plants. Initially the *RT* genomic sequence corresponding to the *RT* cDNA from nucleotides 565 to 1,711 (Kroon et al. 1994) was amplified. The amplified sequence from a petunia accumulating malvidin in the flower, Celebrity Blue, was identical to the previously reported *RT* gene (Kroon et al. 1994). In contrast, a longer fragment was amplified from a cultivar accumulating cyanidin, Celebrity Scarlet. This fragment harbored a 438-bp insertion that was strongly homologous in sequence (97.7%) with the transposable element *dTph3* (Kroon et al. 1994). To distinguish it from *dTph3*, we termed this insertion sequence *dTph3-C*. Both *dTph3* and *dTph3-C* are non-autonomous transposable elements since they are too small to encode full-length transposons. The insertion position of *dTph3-C* in the *RT* gene and the 8-bp target site (CTGGTGAG) were identical to those of *dTph3* (Kroon et al. 1994). Kroon et al. (1994) suggested that the *dTph3* copy presently at the *Rt* locus can no longer transpose as a result of structural defects in the terminal inverted region. Unlike *dTph3*, *dTph3-C* had perfect terminal inverted repeats (Fig. 2), implying that the mobility of *dTph3-C* has not been lost. The inverted repeat sequences of the *dTph3-C* were identical to the left inverted sequence of the *dTph3* but not to the right one. In this respect, *dTph3* appears to be a more divergent form than *dTph3-C*. In the remainder of this paper, we have not discriminated between *dTph3-C* and *dTph3* since these two sequences share extremely high similarities. Unless otherwise stated, *dTph3* and *dTph3-C* are collectively referred to as *dTph3s*.

Expression of the *RT* gene

We next investigated expression of the *RT* gene by Northern blot analysis. The amount of *RT* transcripts

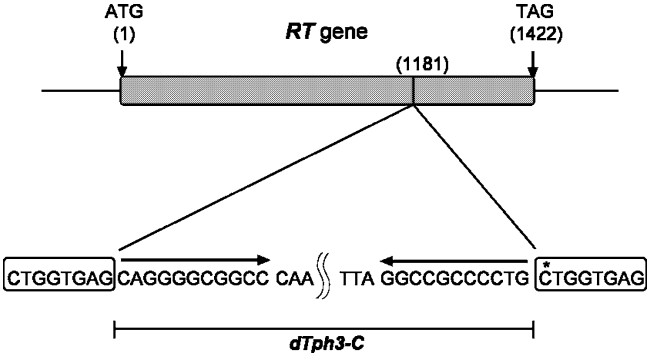

Fig. 2 Structure of *dTph3-C*. The *shaded box* represents the exon of the *RT* gene. The position of the *dTph3-C* insertion is indicated. The target site and its duplication are *boxed*. A nucleotide marked with an *asterisk* in the duplicated target site indicates the missing base after excision of *dTph3s* (see also Fig. 4). The terminal inverted repeats are shown by *arrows*. Nucleotides corresponding to the normal *RT* gene are *numbered* (beginning with the initiation codon in position 1) and their position numbers are shown in *parentheses*. The central part of the *dTph3-C* element has been omitted from the figure

increased markedly in the pigmented buds just before flowering at stage 4 (see Materials and methods). The flower buds at stage 4 were harvested from plants of the nine cultivars of the Celebrity series. Transcripts approximately 1.6 kb long were expressed in cultivars accumulating malvidin or peonidin in the flower. This 1.6-kb transcript was not found in cultivars accumulating cyanidin; instead two different transcripts, approximately 1.3 kb and 2 kb in length, respectively, were detected (Fig. 3). The 1.3-kb transcript had previously been cloned, and its sequence terminated within the *dTph3* sequence (Kroon et al. 1994). Brugliera et al. (1994) also detected longer *RT* transcripts in their study, and they assumed that these transcripts were generated by read-through transcription of the *dTph3* sequence and terminated close to, or at, the original site of the *RT* gene. To demonstrate that these two alternative RNA sequences were generated by incorrect termination of transcription around *dTph3s*, the same blot was hybridized with the *dTph3-C* probe. The *dTph3-C* probe successfully hybridized to both the 1.3- and 2-kb transcripts (Fig. 3), indicating that these two *RT* transcripts were generated by *dTph3s*-mediated aberrant processing and contained *dTph3s* sequences.

Detection of the footprint sequence

Accumulation of malvidin and peonidin was associated with the presence of 1.6-kb *RT* transcripts (Fig. 3). In cultivars accumulating cyanidin, this 1.6-kb transcript was deficient, and only aberrant transcripts with the transposable element sequence were detected. This transcript profile was well conserved in 41 out of the 42 cultivars tested in total (data not shown). One cultivar, Ultra Red (RD in Fig. 4a), was the exception. This cultivar accumulated cyanidin in the flower, but it also produced both the 1.6- and 1.3-kb transcripts as seen, for example, in a cultivar accumulating peonidin, Ultra Rose (RS in Fig. 4a). These 1.3-kb transcripts contained

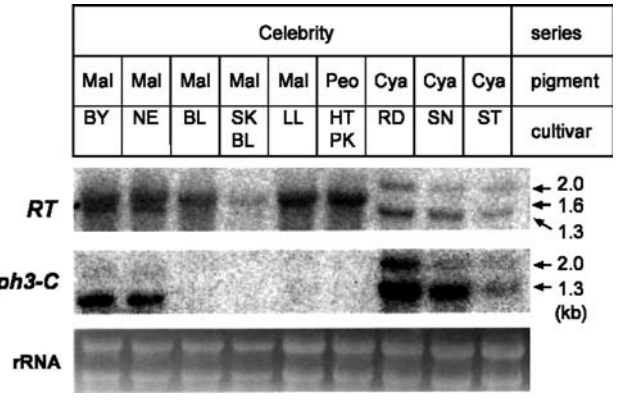

Fig. 3 Expression of the *RT* gene. Total RNA was purified from flower buds of cultivars of the Celebrity series. *RT* transcripts were detected by Northern blot analysis with the total RNA (10 μg per lane). The same blot was reprobed with a *dTph3-C* sequence. The equivalence of RNA loading among lanes was demonstrated by ethidium bromide staining of rRNA bands. Abbreviations for each cultivar name are listed in Table 1. The main pigments in each cultivar are indicated *above* each lane. Malvidin, peonidin, and cyanidin are abbreviated as *Mal*, *Peo*, and *Cya*, respectively

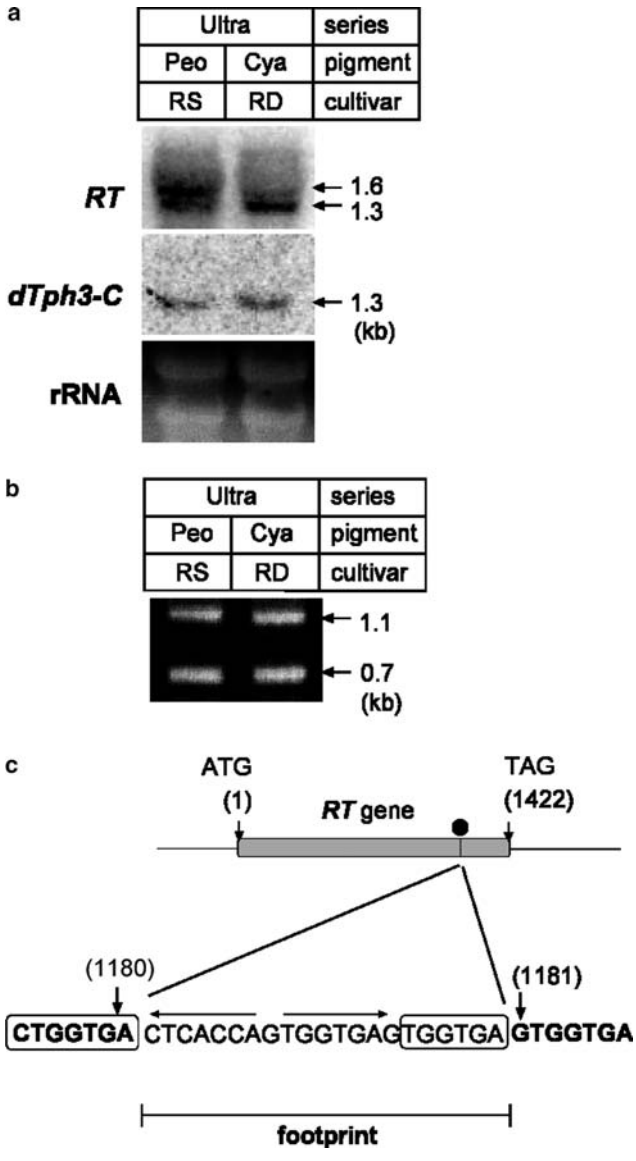

Fig. 4 Identification of the excision product of *dTph3s*. **a** Expression of the *RT* gene in the petunias Ultra Rose (*RS*) and Ultra Red (*RD*). Total RNA prepared from flower buds was subjected to Northern blot analysis. Following hybridization with the *RT* probe, the same blot was rehybridized with a *dTph3-C* probe. Pigments are abbreviated as indicated in the legend for Fig. 3. **b** PCR amplification of the genomic region of the *RT* gene. The genomic region corresponding to bases 1,015–1,711 of the *RT* cDNA (see GenBank accession number X71059) was amplified using the RT-For2 and RT-RV primers. **c** The footprint sequence. The 0.7-kbp fragment amplified from Ultra Red was cloned and its sequence determined. The *shaded box* shows the exon of the *RT* gene. The insertion position of *dTph3s* is marked with a *closed circle*. *Numbers in parentheses* correspond to nucleotide positions in the normal *RT* gene, as described in the legend of Fig. 2. The direction of the 6-bp repeated sequences (TGGTGA) is indicated by an *arrow*. The target site and its duplicated sequence are *boxed*. The *bold letters* indicate the sequences of the *RT* gene

part of the *dTph3s* sequence (Fig. 4a), but the *dTph3-C* probe gave no signal in the position corresponding to the 1.6-kb fragment. If the 1.6-kb transcript found in Ultra Red encodes the normal RT protein, the cyanidin pigment would be rhamnosylated and methylated further. Amplification of the 3′ genomic region of the *RT* gene produced 1.1- and 0.7-kbp fragments in these two cultivars (Fig. 4b). The *Rt* locus of Ultra Rose was heterozygous and consisted of two different alleles, namely, a dominant normal allele and a mutant allele with *dTph3s*. The 1.1-kbp fragment corresponded to the mutant allele containing *dTph3s*, whereas the 0.7-kbp fragment was derived from the normal allele. To confirm this, we cloned the 0.7-kbp fragment amplified from Ultra Red and determined its sequence. We found a short additional sequence in this 0.7-kb fragment (Fig. 4c). When a transposable element is excised, a few extra base pairs, the so-called "footprint", are left behind (Feschotte et al. 2002; Kidwell and Lisch 1997). The footprint following the excision of *dTph3s* consisted of the duplicated target site separated by inverted repetitions of part of the target site (Fig. 4c). A nucleotide (C) in the 3′ downstream duplicated target site was missing following the excision of *dTph3s* (Fig. 4c, see also Fig. 2). The characteristics of this footprint were very similar to *dTph1*-induced footprints (van Houwelingen et al. 1999). Translation of the *RT* transcript from this mutant allele would be terminated in the footprint region by a newly created stop codon. In Ultra Red, *dTph3s* was excised by unidentified autonomous *dTph3* elements *in trans*, resulting in the generation of a new stable recessive mutant allele for the *RT* gene. Thus, the *rt* locus of Ultra Red was heterozygous and consisted of two independent mutant alleles, namely the *dTph3s*-inserted and footprint-retaining alleles.

Kroon et al. (1994) indicated that in some petunias, transcription of the *RT* gene was inhibited by the insertion of *dTph1* into the promoter region of the *RT* gene. However, among the 42 commercial cultivars tested here, none was found to harbor *dTph1*, and all phenotypes could be explained by the *RT* mRNA and the *dTph3s*-induced derivatives found in all tested plants (data not shown).

Determination of genotypes in commercial petunias

Our results indicate that petunias accumulating cyanidin in the flower have a *dTph3s*-insertion allele and/or the corresponding excision allele. To investigate whether or not the floral color phenotype can be explained by the presence of these mutant alleles of the *RT* gene, we determined the genotype of each cultivar by PCR. The 0.67-kbp 3′ genomic region corresponding to the *RT* cDNA from nucleotides 1,015 to 1,683 (Kroon et al. 1994) was amplified with the RT-For2/Foot-RV primers. When *dTph3s* is inserted in the gene, the longer fragment (1.1 kbp in length) can be amplified using these primers (Fig. 5a). The mutant allele with the footprint can be amplified as a unique 0.48-kbp fragment with a footprint-specific primer (Foot-FW) and the Foot-RV primer. Multiplex PCR with these primers can discriminate *dTph3s*-related mutant alleles from the normal dominant *Rt* allele (Fig. 5b).

Thus, the genotypes of the 42 different cultivars shown in Table 1 were compared with their floral color phenotypes. Electrophoretograms of two representative

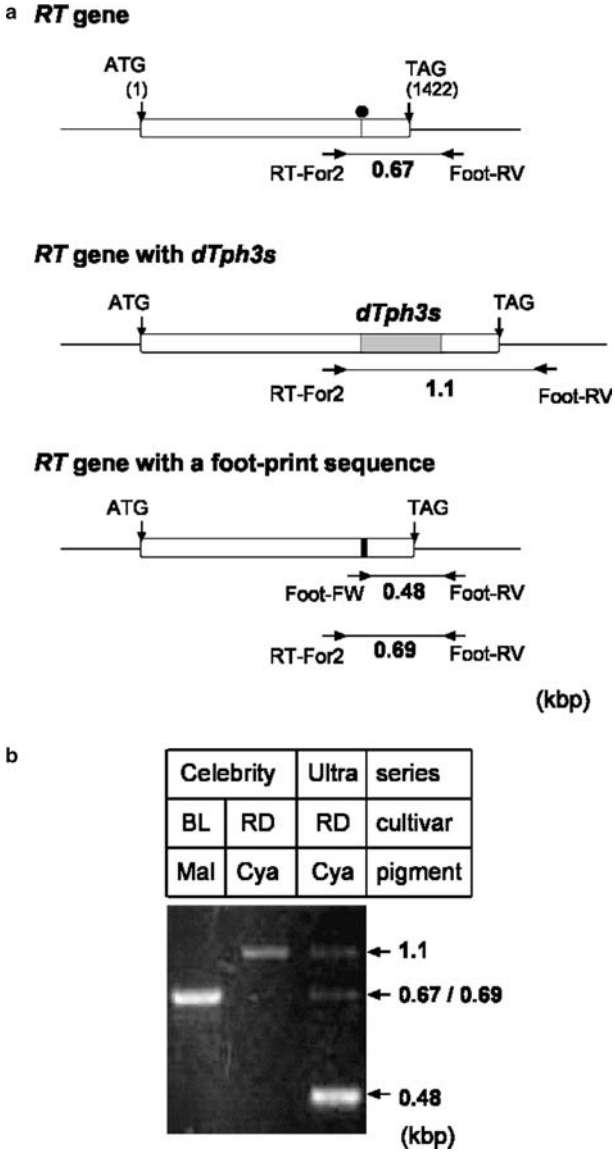

Fig. 5 Multiplex PCR of the *RT* gene. **a** Schematic drawing of the amplified region. *White boxes* represent the exon of the *RT* gene. The amplified regions and their lengths are shown with their corresponding primers. The insertion position of *dTph3s* is marked with a *closed circle*. The *dTph3s* element and its excision product are shown by a *shaded box* and a *black box*, respectively. **b** Detection of normal, *dTph3s*-inserted and footprint-retaining *RT* genes. Total DNA from the petunias Celebrity Blue (*BL*), Celebrity Red (*RD*), and Ultra Red (*RD*), was subjected to multiplex PCR analysis. The main pigments are *abbreviated* as indicated in the legend for Fig. 3 and are shown *above* each lane

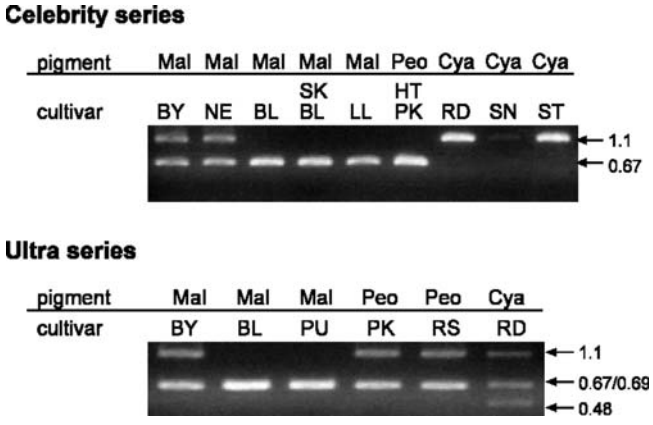

Fig. 6 *RT* genotypes in commercial petunias. Multiplex PCR was carried out as shown in Fig. 5, analyzing total DNA from 42 commercial petunias (Table 1). The main anthocyanin pigment of each cultivar is abbreviated as shown in the legend for Fig. 3. Abbreviations for cultivars are listed in Table 1. Amplified fragments of the cultivars of two representative series, Celebrity and Ultra, are shown

series, Celebrity and Ultra, are shown in Fig. 6. A 0.67-kbp fragment was always detected in petunias accumulating peonidin or malvidin, and some of these were heterozygous since both the normal allele and the *dTph3s*-inserted allele were detected. The 0.67-kbp fragment was not amplified in plants accumulating cyanidin. The mutant allele with the footprint sequence was detected only in Ultra Red plants. The main anthocyanin pigments of other cultivars from Baccara, Falcon, and Madness series were highly consistent with the phenotype predicted from the genotypes as determined by the multiplex PCR (data not shown).

Our results confirmed that red floral color in petunias can be accounted for by the accumulation of cyanidin, which indicates that this phenotype is governed by the transposon-inserted allele and its excision allele in the *RT* gene. In the breeding of petunias, these transposon-related mutations have been utilized for the expression of commercially important red floral color. The transposition capability of *dTph3s* in the *RT* gene would be retained since the excision event was evident in the cultivar, Ultra Red. This result suggests that the mutant allele with the *dTph3s* insertion is unstable, contrary to the opinion of Kroon et al. (1994). Alternatively, the newly found mutant allele with the footprint sequence would be stable and, consequently, of more value in the breeding of red petunias than would the *dTph3s*-inserted mutant allele. In terms of the natural variation in the *RT* gene, the multiplex PCR revealed that the genotypes of potential parental species of garden petunias such as *Petunia integrifolia* (Hook.) Schinz et Thell., *Petunia inflata* R. E. Fr., and *Petunia axillaris* (Lam.) Britton, Sterns et Poggenb. (Sink, 1984) were homozygous for the dominant *Rt* allele (data not shown). Thus, the transposition of *dTph3s* may occur in the breeding process of commercial petunias. Other natural species of petunias are now collectively under investigation, as will be reported elsewhere. Taken together, our multiplex PCR technique as applied to the *RT* gene provides a rapid and reliable method for genotype determination and would enable more convenient breeding of the next generation of petunias.

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