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Cloning and structural analysis of the anthocyanin pigmentation locus *Rt* of *Petunia hybrida*: characterization of insertion sequences in two mutant alleles

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Summary

Anthocyanin biosynthesis in flowers of *Petunia hybrida* is controlled by the regulatory genes *an1*, *an2* and *an11*. Seven classes of cDNA clones homologous to transcripts that are down-regulated in *an1*⁻, *an2*⁻ and *an11*⁻ mutants were isolated via differential cDNA cloning. Genetic mapping, antisense RNA experiments and analyses of mutant alleles demonstrated that one class of clones originated from the *Rt* locus. The *rt* gene has no introns and encodes a protein with homology to mammalian glucuronosyl transferases and flavonoid 3-*O*-glucosyltransferase (UF3GT) encoded by the *bz1* gene from *Zea mays*. As the *Rt* locus controls the rhamnosylation of reddish anthocyanin-3-*O*-glucosides which is the first in a series of modifications that finally yield magenta or blue/purple coloured anthocyanins, this suggests that *rt* encodes an anthocyanin rhamnosyl transferase.

Molecular analysis of two mutant *rt* alleles showed that their expression is blocked by different DNA insertion elements. Mutability of the *rt-vu15* allele results from the presence of a 284 bp transposable element (*dTph1*) in the *rt* promoter region, causing a block in transcription. The protein coding region of the *rt-r27* allele contains a 442 bp insertion (*dTph3*) resulting in premature polyadenylation of *rt* transcripts. Although *dTph3* cannot transpose, it has sequence characteristics of transposable elements, suggesting that it is a defective member of a new family of transposable elements.

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Introduction

The action of genes controlling the biosynthesis of anthocyanin pigments can be detected visually. This has facilitated the isolation of numerous mutants, in which pigmentation is blocked or spatially altered. Perhaps the largest collection of loci that control anthocyanin biosynthesis is available in *Petunia hybrida*. About 30 genes that control pigmentation of specific floral organs have been identified by mutations (see De Vlaming *et al.*, 1984; Wiering and De Vlaming, 1984 for listings), and they can be divided in two different classes. One class of loci controls the activity of a single enzyme from the anthocyanin biosynthetic pathway and they appear to contain the structural gene encoding that enzyme. This includes the *Po* locus controlling chalcone isomerase (CHI) activity (Van Tunen and Mol, 1987), the *An3* locus controlling flavanone 3-hydroxylase (F3H) activity (Britsch *et al.*, 1991), the *An6* locus controlling dihydroflavonol 4-reductase (DFR) activity (Beld *et al.*, 1989) and the loci *Mt1*, *Mt2*, *Mf1* and *Mf2* controlling activity of four different anthocyanin methyltransferase (iso)enzymes (Jonsson *et al.*, 1984a) (cf. Figure 1). Complementation experiments in which genes isolated from wild-type petunia lines were introduced into mutants proved that the *Po* and *An6* locus contain the structural genes coding for the enzymes CHI and DFR respectively (Huits *et al.*, unpublished data; Van Tunen *et al.*, 1991).

A second class of loci controls the activity of multiple enzymes of the pathway, indicating that they encode a regulatory factor. This includes the *An1*, *An2* and *An11* loci controlling activity of DFR, UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UF3GT), UDP-glucose: flavonoid 5-*O*-glucosyltransferase (UF5GT) and four anthocyanin methyl transferases (Beld *et al.*, 1989; Gerats *et al.*, 1984, 1985a; Jonsson *et al.*, 1984b). In *an1*⁻, *an2*⁻ and *an11*⁻ mutants steady-state *dfr* mRNA levels (Beld *et al.*, 1989) and *dfr* transcription rates (Quattrocchio *et al.*, 1993) are reduced, indicating that these regulatory genes encode or control a transcription factor. Interestingly these regulatory mutants accumulate dihydroflavonols (Gerats *et al.*, 1982) and express *chs*, *chi* and *f3h* genes (Beld *et al.*, 1989; Quattrocchio *et al.*, 1993), implying that in *P. hybrida* the first and the second part of the anthocyanin biosynthetic pathway are under a different transcriptional control. In flowers of *Antirrhinum majus* the

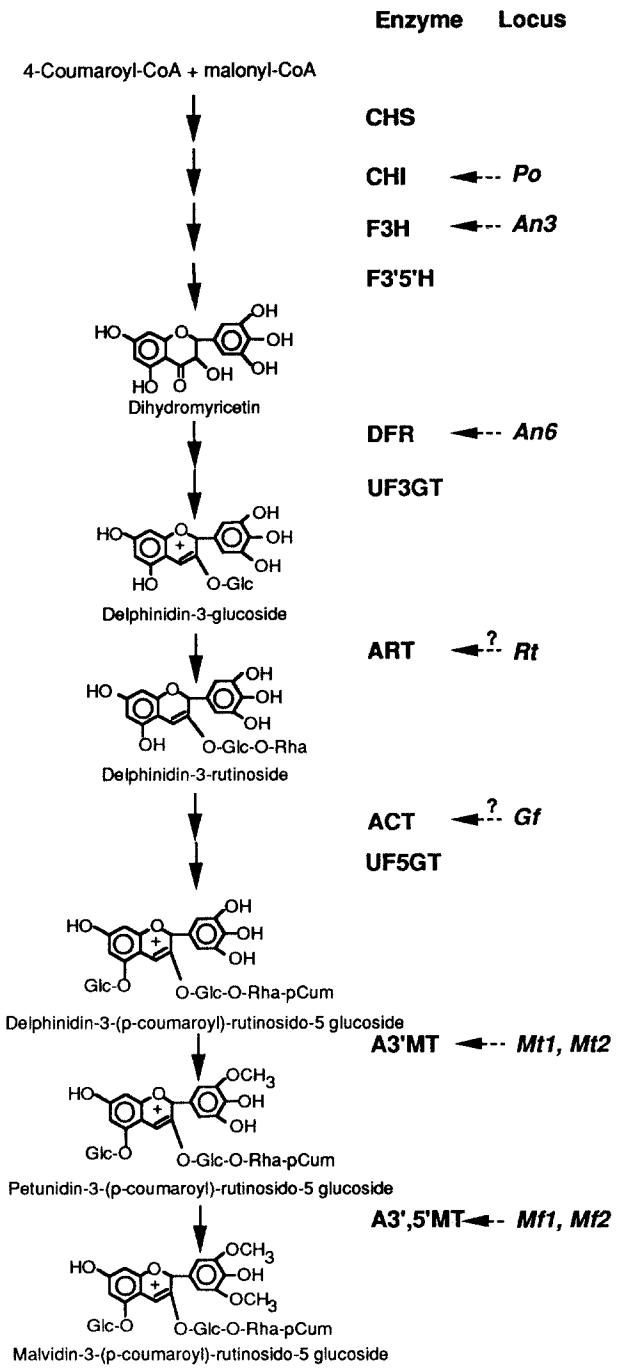

Figure 1. Simplified scheme of the anthocyanin biosynthetic pathway in flowers of *P. hybrida*.
Structures of intermediates of the pathway are given only as far as they are relevant to this paper. Names of enzymes are given in bold capital letters: CHS = chalcone synthase; CHI = chalcone flavanone isomerase; F3H = flavanone 3-hydroxylase; F3'5'H = dihydroflavonol 3',5'-hydroxylase; DFR = dihydroflavonol 4-reductase; UF3GT = UDPG flavonoid: 3-*O*-glucosyltransferase; UF5GT = UDPG flavonoid: 5-*O*-glucosyltransferase; A3'MT = SAM anthocyanin 3'-methyltransferase; A3'5'MT = SAM anthocyanin 3',5'-methyltransferase. Names of genetic loci controlling the activity of these enzymes are given in *italics*. The genetic control of ART (anthocyanin rhamnosyltransferase) and ACT (anthocyanin acyltransferase) has not been demonstrated directly, but is inferred from accumulated products in mutant flowers. Glc = glucose; Rha = rhamnose; pCum = *p*-coumaric acid.

first and second part of the anthocyanin pathway are also under a differential control, but the regulatory division occurs here before *f3h* (Martin *et al.*, 1991). In maize kernels however regulatory genes of the *R* and *C1* family from *Zea mays* appear to control the whole pathway as a single unit (see Dooner *et al.*, 1991 for a review).

To study the molecular basis of this differential gene regulation it is necessary to clone both the regulatory *An* genes and a number of their target genes. Transposon-tagged alleles have been described for the regulatory genes *an1*, *an2* and *an11* as well as for some of their target genes, which can aid in their isolation (Gerats *et al.*, 1989). One difficulty with this approach is that transposable elements in *P. hybrida* are poorly characterized at the molecular level. Therefore we decided first to clone the target genes via a differential cDNA screening and to characterize mutant alleles. In a second step the transposable elements thus isolated can be used to clone the regulatory genes.

Here we report the isolation of seven classes of cDNA clones homologous to transcripts that are down-regulated in $an1^-$, $an2^-$ and $an11^-$ mutants. Antisense RNA experiments, RFLP mapping and molecular analysis show that one of these originated from the *Rt* locus. The *Rt* locus controls rhamnosylation of anthocyanins and encodes a protein with homology to several sugar transferases suggesting that it is an anthocyanin-rhamnosyl transferase. A non-autonomous transposable element (*dTph1*) and a transposon-like insertion sequence (*dTph3*) were isolated from two mutant *rt* alleles.

Results

Isolation of a set of an1 *regulated genes*

In *Petunia hybrida* lines with a mutation at the *An1* locus the activity of multiple enzymes from the anthocyanin biosynthetic pathway is strongly reduced (Gerats *et al.*, 1984, 1985a; Jonsson *et al.*, 1984b), as well as the steady-state level of *dfr* mRNA (Beld *et al.*, 1989). Based on these findings we assumed that the *an1* gene regulates the mRNA levels for several anthocyanin biosynthetic genes and that it should be possible to clone the cDNAs for any of these genes using a differential cDNA screening strategy. Therefore a cDNA library was constructed based on mRNA from corolla limb tissue of the line V26 ($An1^+$). Duplicate filters were taken and hybridized with first strand cDNA from an $An1^+$(V26) and an $an1^-$ line (W162). Two hundred and seventy plaques out of 30 000 showing substantially stronger hybridization to the $An1^+$ cDNA probe than to the $an1^-$ cDNA probe were identified. Thirty five plaques, which did not hybridize to previously cloned pigmentation genes (*chs*, *chi* and *dfr*) were purified to homogeneity. Pairwise cross-

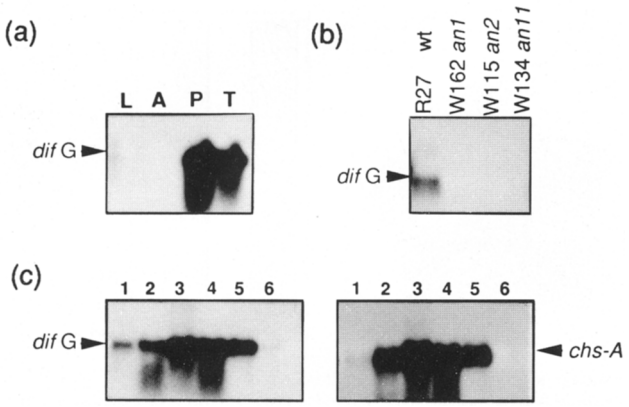

Figure 2. Organ specificity, temporal regulation and genetic control of *difG* mRNA levels.
(a) RNA gel blot analysis of RNA extracted from different organs of the line V26. L = leaf; A = anther; P = petal limb; T = petal tube (pool of all developmental stages). (b) *difG* transcript levels in petals from different regulatory pigmentation mutants. (c) Accumulation of *difG* mRNA and *chs* mRNA in petals during during flower development. The numbers above each lane correspond with the developmental stage of the buds (Van Tunen *et al.*, 1988).

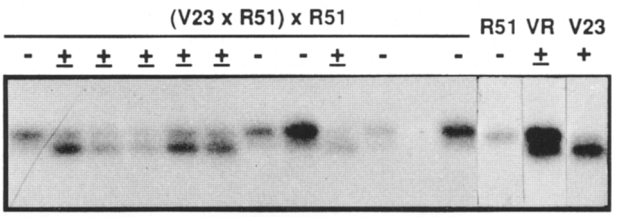

Figure 3. Linkage of *difG* cDNA and the *Rt* locus.
DNA gel blot of *Hin*dIII digested DNA from the lines V23 and R51, an F_1 plant (VR) and backcross progeny (V23×R51)×V23, probed with *difG*. The allelic status at the *Rt* locus is indicated above each lane (– = *rtrt*; ± = *Rtrt* and + = *RtRt*). Note that both V23 and R51 contain in addition to the strongly hybridizing *difG* fragment a second weakly hybridizing fragment of intermediate size. This weakly hybridizing band does not segregate, because it is not polymorphic. Backcross progeny plants in which the hybridization signal of the lowest (R51) band is at least equal to the upper (V23) band were scored heterozygous for the V23 and R51 *difG* allele. In plants homozygous for the V23 allele of *difG* strong hybridization is only seen to the uppermost (V23) band and at most a faint hybridization to the somewhat smaller non-polymorphic fragment.

hybridizations demonstrated that these 35 clones represented seven distinct classes of genes. To verify if any of these clones is involved in anthocyanin biosynthesis, the largest cDNA clone from each class, further indicated as *difA, difC, difE, difF, difG, difH* and *difI*, was characterized with regard to spatial and temporal expression. As an example we show in Figure 2 the organ-specific and developmental expression of *difG*. The results show that the *difG* gene is expressed in the limb and tube of the petal, but not in leaves or anthers (Figure 2a). The accumulation of *difG* during development of the petal limb is similar to that of *chs* (Figure 2c). In addition, *difG* expression appears to be controlled by the regulatory pigmentation genes *an1*, *an2* and *an11* as the *difG* mRNA level was down-regulated in petal limbs of the corresponding mutants (Figure 2b). The expression of the six other classes (*difA*, *difC*, *difE*, *difF*, *difH* and *difI*) displayed a spatial, temporal and genetic control similar to *difG* (data not shown). This indicates that they may represent as yet unidentified clones from anthocyanin biosynthetic genes.

About 25 loci controlling anthocyanin biosynthesis in the flower of *P. hybrida* have been identified via mutations (De Vlaming *et al.*, 1984; Wiering and De Vlaming, 1984). To test if the different classes of cDNA clones could have originated from one of these loci, we localized the position of the gene corresponding to each cDNA class using restriction fragment length polymorphisms (RFLPs). The F_1 hybrid of the two *P. hybrida* lines V23 and R51 (VR hybrid) used in these analyses is heterozygous for at least one marker on each of the seven chromosomes which allows chromosomal localizations in one single backcross with either parent (Wallroth *et al.*, 1986). Among a total of 75 plants from the two backcross progenies [V23×R51] × V23 (35 plants) and [V23×R51] × R51 (40 plants) we detected no cross-overs between the *Rt* locus on chromosome 6 and the RFLP generated by *difG* (Figure 3). Since two more genetically defined pigmentation genes, *an1* and *an2*, map very close to this region of chromosome VI (De Vlaming *et al.*, 1984), it is possible that *difG* originated from the *An1*, the *An2* or the *Rt* locus. The availability of mutable alleles for *an1*, *an2* as well as *rt* led us to investigate *difG* in more detail.

difG *cDNAs originate from the* Rt *locus*

One way to identify the function of a cryptic gene is to inactivate its expression *in planta* by antisense RNA (Van der Krol *et al.*, 1988). To verify if *difG* encodes a protein that is involved in anthocyanin biosynthesis, an antisense *difG* RNA expressing construct under the control of the CaMV 35S promoter was introduced into *P. hybrida* line V26 by *Agrobacterium tumefaciens* mediated transformation. Figure 4 shows the flowers of some of these transformants. Eight transformants had flowers that were indistinguishable from normal V26 flowers (Figure 4; flower 1), in two other transformants alteration of the colour shade was observed in some sectors of the flower (flowers 2 and 3). Similar pigmentation patterns were observed previously in transgenic plants containing antisense or sense *chs* and *dfr* gene constructs (Napoli *et al.*, 1990; Van der Krol *et al.*, 1988, 1990). Analysis of the accumulated flavonoid-aglycones by thin layer chromatography (TLC) showed that corolla limbs of the flowers exhibiting a phenotypic change contained a mixture of delphinidin and petunidin whereas wild-type V26 corolla limbs (V26 is *mf1*$^-$, *mf2*$^-$) only contained petunidin

Figure 4. Flower pigmentation patterns of transgenic *P. hybrida* V26 plants containing an antisense *difG* cDNA construct.
(a) Flowers of transgenic *P. hybrida* V26 plants harbouring an antisense *difG* construct display phenotypes ranging from wild-type flowers (flower 1) to flowers with altered pigmentation in sectors varying in size (flowers 2 and 3).
(b) Thin layer chromatography analysis shows that flowers exhibiting an antisense phenotype contain a mixture of petunidin (P) and delphinidin (D) (lane 1). In wild-type flowers only petunidin is detected (lane 2).

(Figure 4b). This indicates that the antisense *difG* construct blocks one of the reactions in between delphinidin and petunidin, which may be either the rhamnosylation, 5-*O*-glucosylation, acylation or methylation (cf. Figure 1). The antisense *difG* phenotype differs from that of *an1*$^-$ and *an2*$^-$ mutants, which completely lack anthocyanins in the flower limb due to interference with an earlier step in the pathway (De Vlaming *et al.*, 1984; Wiering and De Vlaming, 1973). Therefore it is unlikely that *difG* originated from the *An1* or *An2* locus. The antisense phenotype is similar to that of *rt*$^-$, *gf*$^-$ and *mt1*$^-$, *mt2*$^-$ mutants. As the *Gf* and *Mt1* and *Mt2* loci map on other chromosomes than *difG*, it can be excluded that *difG* orginated from these loci. However, both the antisense phenotype and the chromosomal position are consistant with the hypothesis that *difG* originated from the *Rt* locus.

To further test whether *difG* could have originated from the *Rt* locus, we made use of an *rt*-mutable allele originally described by Cornu (1977). This allele was maintained in our laboratory in the line Vu15 and is referred to as *rt-vu15.* We tested whether reversion of the rt-mutable phenotype correlated with the excision of a transposable element from the *difG* gene. Among 180 progeny plants, originating from a single seed capsule, obtained by selfing of an *rt*-mutable parent, we found six plants with evenly coloured reddish/purple flowers indicating that excision of an element at the *Rt* locus had occurred in sporogenic cells of the parent. Figure 5 shows a DNA gel blot analysis of DNA from two plants with an *rt*-mutable and two with an Rt revertant phenotype. At moderate stringency the *difG* probe detects three bands of 5.1 kb, 3.1 kb and 1.8 kb, respectively. High stringency washing of the blot results in melting of the hybrids with the 5.1 kb band in both the mutant and the revertants indicating that this fragment contains a gene that is related, but not identical, to *difG*. The identity of

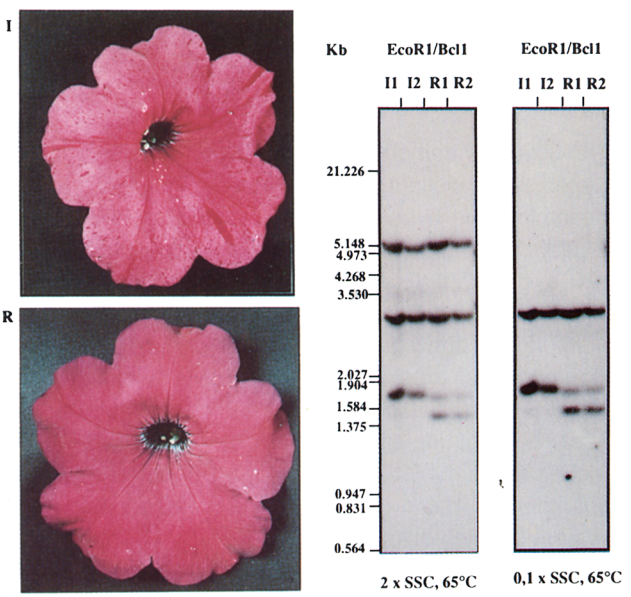

Figure 5. DNA gel blot analysis of *rt*-mutable and revertant plants.
Phenotypes of the flowers from the line Vu15 carrying an unstable *rt* allele (I) and a revertant thereof (R) are shown on the left. On the right a DNA gel blot of two plants with an *rt* mutable (I1 and I2) or a revertant *rt* allele (R1 and R2) probed with *difG*. The blot was initially washed at moderate stringency (2×SSC), exposed and subsequently washed at high stringency (0.1×SSC) and exposed again. The size of marker fragments are indicated in the margin.

this latter gene remains unknown at present. The gene from which the *difG* cDNA originated is cut at an internal *Bcl*I site, resulting in two hybridizing fragments of 1.8 kb and 3.1 kb (cf. Figure 7). Analysis of the two revertant plants reveals a novel band of 1.5 kb, which is smaller than the equivalent band of 1.8 kb present in the *rt*-mutable plants. The same size difference between the original mutable and the revertant allele was found with several other restriction digests and double digests of

the same DNAs (data not shown). Figure 5 shows that the signals of the original (mutable) 1.8 kb band and the novel (revertant) 1.5 kb band in the revertant plants (R1 and R2) are of approximately half of the intensity of the signal for the equivalent 1.8 kb band in the *rt*-mutable plants (I1 and I2). This can be accounted for by heterozygosity for the Rt^+ revertant allele with the mutable *rt* allele on the other chromosome. Indeed, progeny obtained by selfing of revertant plants displayed a 3:1 segregation of the Rt-revertant versus the rt-mutable phenotype (not shown).

Taken together these data provide direct evidence that *difG* originated from the *Rt* locus and that mutability of the *rt-vu15* allele is caused by excision of a mobile element of about 300 bp.

The Rt *locus encodes a protein with homology to mammalian glucuronosyl-transferases and to flavonoid 3-O-glucosyl transferase encoded by the* Bz1 *-locus of* Zea mays

To analyse the structure of the *Rt* locus in more detail we screened genomic and cDNA libraries of the wild-type line V26 with *difG* under high stringency conditions to obtain a full-size cDNA and genomic clones. From a genomic V26 library we obtained four positive clones of which one contained the whole gene colinear with the *rt* cDNA and a 4.7 kb *Hin*dIII/*Sal*I fragment was subcloned into pBluescript and used for further analyses.

Structural analysis of the *rt* gene was performed by sequencing both strands of part of the genomic subclone and of a full length cDNA clone. Comparison of the genomic and cDNA sequences revealed that the V26 *rt* gene contains no introns (Figure 6a). Using primer extension analysis we mapped the CAP site of the mRNA at a G residue 48 bp upstream of the translation start codon (data not shown). As the cDNA clone 5' end started at +15 and the region between nucleotide +1 and +15 does not contain a 3' splice site, we can exclude the presence of an intron and conclude that the upstream region represents the *rt* promoter. The presence of a putative TATA box, TATATAAAT, at position −26 to −18 further supports this conclusion. The original *difG* cDNA and the full size cDNA clone terminated at two distinct positions with a stretch of A residues, indicating that at least two polyadenylation sites are present in the *rt* mRNA.

The cDNA sequence contains an open reading frame of 1422 bp which specifies a protein of 473 amino acids. To find out whether the *rt* gene encodes an enzyme or a regulatory protein, we screened the Genbank data library with the amino acid sequence of the predicted RT polypeptide. Strikingly, the protein with the highest homology to RT was found to be UF3GT: (E.C. 2.4.1.91) encoded by the *bz1* gene from *Z. mays*. Figure 6b shows the region from amino acid 275 to 478 of the RT protein sequence in which the degree of homology was the highest. In addition, this region of RT shares homology with other sugar transferases; in particular the bilirubin UDP-glucuronosyl transferases from human, rat and mouse (Figure 6b). Three distinct sugar transferase are active in the anthocyanin pathway: UF3GT, ART and UF5GT (see Figure 1). We infer that *Rt* encodes ART, as an rt^- mutation blocks rhamnosylation of anthocyanins (Wiering and De Vlaming, 1973), but does not affect UF3GT nor UF5GT enzyme activity (Gerats *et al.*, 1983; Jonsson *et al.*, 1984b) (see Discussion for further details).

Molecular characterization of two mutant rt *alleles*

Even though mutable alleles have been described for a large number of genes (Doodeman *et al.*, 1984a, 1984b; Gerats *et al.*, 1989), until now only one insertion element (*dTph1*) with sequence features of a transposable element has been identified in *P. hybrida* (Gerats *et al.*, 1990). This led us to characterize the mutable *rt* allele in the line Vu15 (Cornu, 1977, cf. Figure 5) in more detail. Using various combinations of primers (see Figure 7b) we amplified different regions of the *rt* gene from plants harbouring the wild-type, the *rt* mutable and the revertant allele and compared the size of the products. In this way the transposon insertion was mapped in the promoter region of the gene in between primers 1 and 3 (data not shown). Amplification products from the plants carrying the *rt* mutable allele were cloned in plasmid vectors and sequenced. Comparison with the wild-type sequence (V26) shows that a 284 bp insertion is present in the TATA box of the *rt-vu15* allele (Figure 7c). This insertion has typical characteristics of a transposable element, such as 12 bp terminal inverted repeats flanked by an 8 bp target site duplication. In addition the element is highly homologous to *dTph1* (three mismatches out of 284 bp), a small insertion sequence isolated from the *dfrC* locus (Gerats *et al.*, 1990).

RNA gel blot analysis demonstrated that *rt*-mutable flowers contain severely reduced *rt* mRNA levels compared with the Rt^+ revertants (Figure 7a), whereas the mRNA levels of two other anthocyanin biosynthetic genes, *chs* and *f3h* were similar (data not shown). At low intensity however, a slightly longer *rt* mRNA is detected in the *rt*-mutable plants. It may be that this RNA originated from an alternative, less efficient, transcription start site within the *dTph1* element. Alternatively it may have derived from the second gene with homology to the *rt* probe.

A large number of *P. hybrida* lines are available which contain a stably mutated *rt* allele. Because it was not clear whether they all harbour the same or different rt^- alleles we screened several *P. hybrida* lines by RNA gel blot analysis. Figure 7a shows that in all Rt^+ lines, a tran-

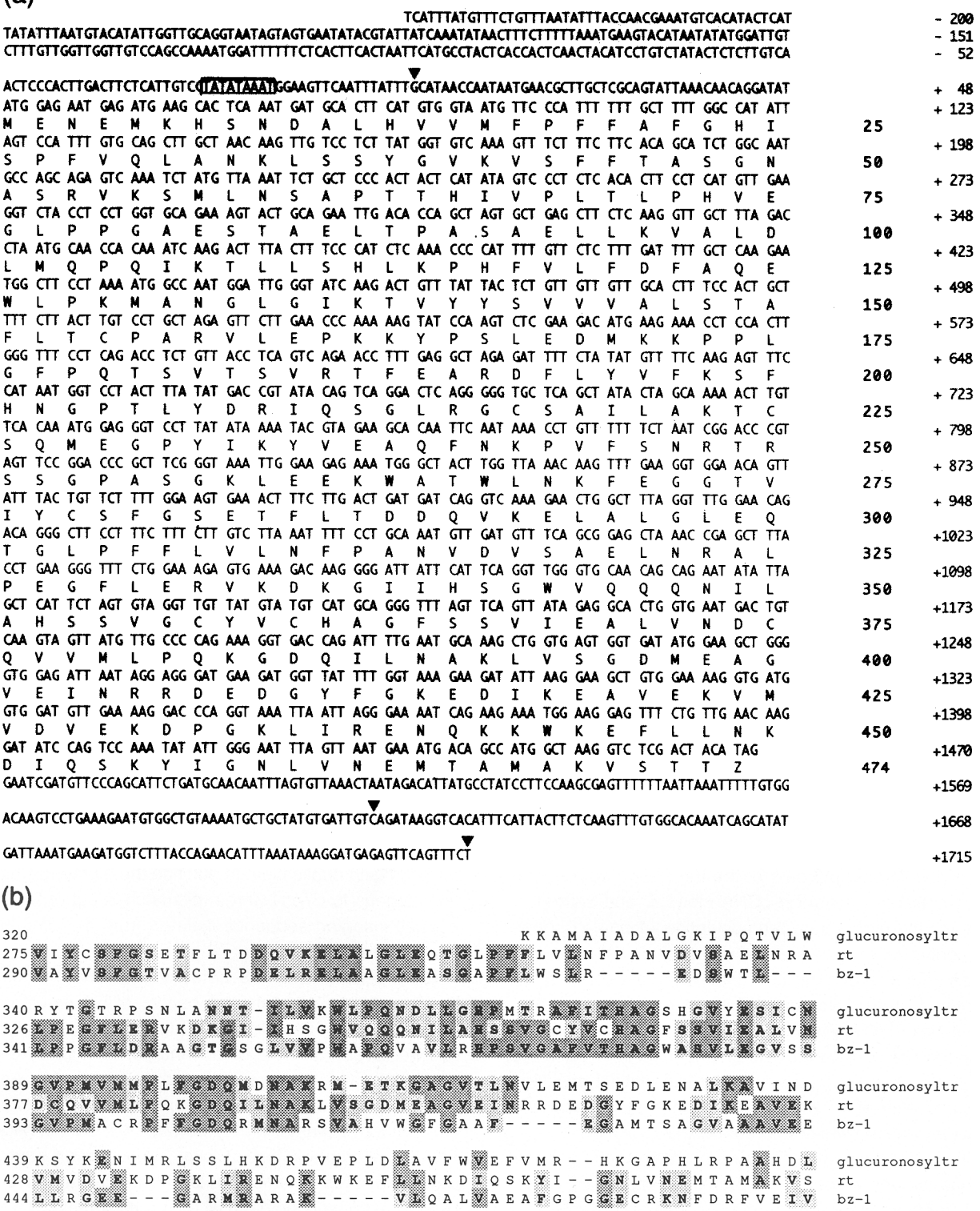

Figure 6. Nucleotide and deduced amino acid sequence of the gene encoded by the *Rt* locus of *P. hybrida* line V26.
(a) Nucleotide position +1 represents the CAP site as determined by primer extension analysis and is marked by an arrowhead. The putative TATA sequence is boxed. The arrowheads in the 3' untranslated region mark the sites where poly(A) tails were present in two cDNA clones. The amino acid sequence corresponding to the open reading frame is presented beneath the nucleotide sequence. Numbers in bold refer to amino acid residues.
(b) Sequence alignment of part of the predicted V26 RT protein, a human bilurubin UDP-glucuronosyltransferase (Sato *et al.*, 1990) and the protein encoded by the *Bz1* locus (Furtek *et al.*, 1988; Ralston *et al.*, 1988) from *Z. mays*. Sequence identity is indicated by bold face symbols and dark shading; sequence similarity is indicated by bold face symbols and a lighter shading.

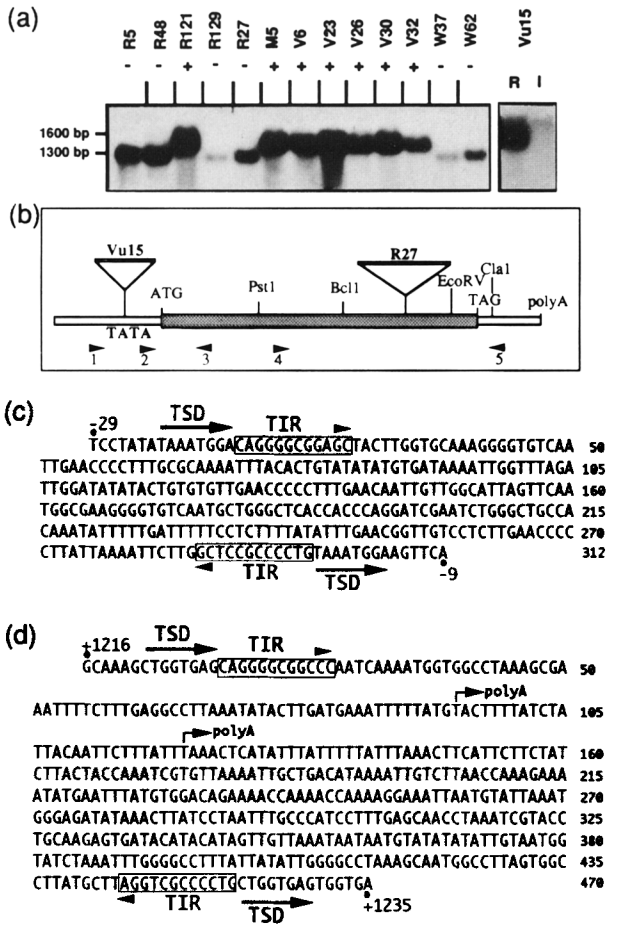

Figure 7. Analysis of mutant *rt* alleles.
(a) Left panel: gel blot analysis of RNA from the corolla limbs of *Rt*$^+$ and stably mutated *rt*$^-$ lines. The genetic status of the *Rt* locus is indicated above each lane (+ = *RtRt*, – = *rtrt*). Right panel: gel blot analysis of RNA from corolla limbs of mutable (I) and revertant flowers of the line Vu15 (R).
(b) Map illustrating the combined results of restriction, PCR and sequence analysis of the *rt*-mutable and the recessive allele. Oligonucleotides used as PCR primers are indicated by numbered arrows (for details see Experimental procedures), the triangles indicate the position of the insertion sequences in the *Rt* gene from petunia Vu15 and R27. The translational start and stop codons and four restriction sites used in cloning procedures are indicated.
(c) Nucleotide sequence of the mobile element (*dTph1-rt*) inserted in the TATA box of the *rt* gene in petunia Vu15. Numbering of the *rt* flanking sequences is as in Figure 6.
(d) Nucleotide sequence of the non-mobile insertion sequence *dTph-3* in the coding region of the *rt* gene of the line R27. The position at which a 3' RACE product terminated in a series of A residues is indicated above the sequence. TSD = target site duplication, TIR = terminal inverted repeat.

script of about 1600 bp was present in petal limbs, whereas in all *rt*$^-$ lines a shorter transcript of approximately 1300 bp was detected. We occasionally observed hybridizing bands of a larger size than 1600 bp in *rt*$^-$ mutants. The intensity of this band was always low and it varied even between different RNA preparations of the same line (from undetectable levels (Figure 7) to a maximum of about 10% of the intensity of the 1300 bp mRNA; see Discussion). To find out whether these abberant mRNAs resulted from a rearrangement in the *rt* gene, we performed a PCR analysis on genomic DNA, using primers complementary to the ends of the *rt* coding sequence (primers 1 and 5; Figure 7b). From *Rt*$^+$ lines the expected 1.5 kb fragment was amplified, whereas from all *rt*$^-$ lines an approximately 2.0 kb fragment was amplified (data not shown). This indicates that all *rt*$^-$ lines tested contain the same *rt*$^-$ allele with an insertion of about 500 bp. The *rt*$^-$ allele of line R27 was chosen for further analysis. PCR analyses with various combinations of primers showed that the insertion had occurred near the 3' end of the gene in between primers 4 and 5 (cf. Figure 7b). This region of the R27 *rt* gene was amplified by PCR, cloned and subsequently sequenced. The sequences at the ends of the insertion in the R27 *rt* gene and the flanking *rt* sequences display typical structural characteristics of transposable elements. Figure 7d shows that the inserted sequence has imperfect 12 bp inverted repeats at its termini and is flanked by 8 bp directly repeated *rt* target sequences. The 12 bp inverted repeats of the insertion element show homology to the termini of *dTph1* and to other transposons of the *Ac/Ds* family (Figure 8). However, the element does not have additional homology to *dTph1*, nor to another transposable element that was isolated recently from *P. hybrida* (*dTph2*: Van Houwelingen *et al.*, unpublished data). Therefore we named this insertion sequence *dTph3*.

To investigate how the shortened transcripts are generated by the *dTph3* insertion we cloned and sequenced two PCR products amplified from reverse transcribed R27 petal RNA via the RACE procedure (Frohman *et al.*, 1988). The sequence of both clones was

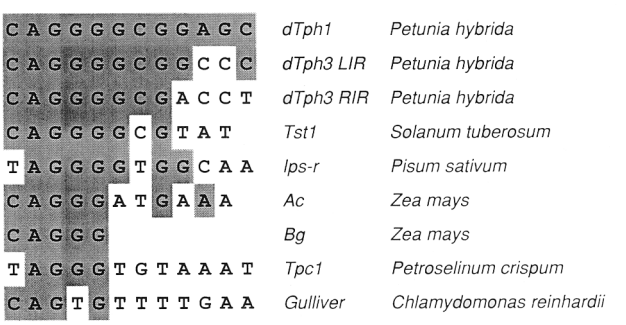

Figure 8. Terminal inverted repeats of transposable elements with an 8 bp target site.
Terminal inverted repeat sequences (left column) of various elements (middle column) of different plant species (right column) were compared with *dTph1* and displayed in order of decreasing homology. Identical nucleotides are indicated by a gray shading. For terminal inverted repeat sequences see: Gerats *et al.* (1990) and Gierl and Saedler (1992) and references therein.

colinear with the R27 genomic sequence but terminated with a stretch of A residues within the *dTph3* sequence (Figure 7d). This indicates that the *dTph3* insertion creates a novel polyadenylation signal and explains the presence of the smaller transcripts found in *rt*$^-$ lines.

Discussion

In this paper we report the cloning of seven classes of cDNA clones derived from genes that are under the control of the regulatory pigmentation genes *an1*, *an2* and *an11* from *P. hybrida*. Flowers from *an1*$^-$ lines contain little or no activity of the enzymes DFR, UF3GT, UF5GT and several anthocyanin methyltransferases, resulting in an absence of anthocyanins in the flower (Beld *et al.*, 1989; Gerats *et al.*, 1984, 1985a; Jonsson *et al.*, 1984b). Since no additional abnormalities can be visually detected in *an1*$^-$, *an2*$^-$ or *an11*$^-$ flowers it is likely that at least some, and possibly all, of the differential cDNA clones encode proteins that are involved in anthocyanin biosynthesis. For two classes of clones their involvement in anthocyanin biosynthesis has been further established. By genetic mapping (Figure 3), antisense-RNA experiments (Figure 4) and analysis of *rt* mutant alleles (Figures 5 and 6) we demonstrated that *difG* cDNAs originated from the *Rt* locus. Sequence analysis showed that *dif*A cDNAs are homologous to the *candi* gene from *A. majus* and the *A2* gene from *Z. mays* (Weiss *et al.*, 1993). Additional tests are in progress to establish the role of the other classes of differentially expressed cDNA clones in anthocyanin biosynthesis. A similar differential screening strategy was successfully used before, to isolate the *f3h* and *candi* genes from *A. majus* (Martin *et al.*, 1991).

The RT protein predicted from the V26 *rt* cDNA sequence has homology with mammalian glucuronosyl transferases and UF3GT (E.C.2.4.1.91) encoded by the *bz1* gene from maize (Figure 6b). *In vitro* mutagenesis of histo blood group A and B sugar transferases showed that single amino acid substitutions can alter the nucleotide-sugar specificity of the enzyme (Yamamoto and Hakomori, 1990), implying that it is not possible to infer the nucleotide-sugar specificity from the protein sequence alone. Three sugar-transferase enzymes are active in the anthocyanin biosynthetic pathway, UF3GT, ART, UF5GT (Figure 1). Anthocyanins in *rt*$^-$ lines from *P. hybrida* are normally 3-*O*-glucosylated but lack both the rhamnose and the 5-*O*-glucose group and are not methylated (Wiering and De Vlaming, 1973, 1984). The same holds for plants in which *rt* expression was blocked via antisense RNA (Brugliera *et al.*, 1994; this paper). In *gf*$^-$ mutants rhamnosylation is normal, but 5-*O*- glucosylation is blocked (Wiering and De Vlaming, 1973). As the levels of UF5GT enzyme activity in wild-type, *rt*$^-$ and *gf*$^-$ lines are similar (Jonsson *et al.*, 1984b) this implies (i) that 5-*O*-glucosylation is dependent on the presence of a rhamnose and an acyl group and (ii) that the *Rt* locus directly controls the addition of the rhamnose moiety and that its gene product is a rhamnosyltransferase. The molecular weight of the putative *P. hybrida* RT protein (53.4 kDa) corresponds well with that of a biochemically characterized rhamnosyl transferase (monomeric, 52 kDa) involved in production of bitter flavonoid compounds in grapefruit (*Citrus paradisi*) (Bar-Peled *et al.*, 1991).

In DNA gel blot analyses a second gene with homology to the *rt* cDNA was detected (Figure 5). In RNA gel blot analyses of RNA from *Rt*$^+$ lines, the *rt* cDNA detects a 1.6 kb mRNA whereas in lines with a stably mutated *rt*$^-$ allele only a 1.3 kb mRNA (and sometimes an RNA larger than 1.6 kb) is detected (Figure 7). This indicates that all of the 1.6 kb mRNA was transcribed from the *Rt* locus and that the second gene is expressed in petal limbs at low level, or not at all. It may be that this second gene is a silent copy of the *rt* gene, like those found for other flavonoid biosynthetic genes in *P. hybrida* (Koes *et al.*, 1989a, 1989b) or that it encodes another sugar-transferase enzyme.

In *P. hybrida* transposition has been extensively studied at the genetic level. Mutable alleles have been described for many genes among which are regulatory pigmentation genes and genes controlling plant development (Doodeman *et al.*, 1984b; Gerats *et al.*, 1989). Genetic analysis of instability at the *An1* locus of the line W138 indicated that a two-element system is involved (Huits *et al.*, unpublished data; Wijsman, 1986). At least one other family of transposable elements appears to be active in *P. hybrida*, as mutability of the *an3* gene of the line W159 depends on a different activator element (Gerats *et al.*, 1985b). Recently a small DNA insertion element (*dTph1*) with sequence characteristics of a defective transposable element was cloned from the *dfrC* gene of the *an1*-mutable line W138 (Gerats *et al.*, 1990). Although the authors stated initially that *dfrC* and *an1* are identical, subsequent experiments clearly showed that they are not (Huits *et al.*, unpublished data). This makes it difficult to assess whether the previously reported *dfrC* excision alleles (Gerats *et al.*, 1990) arose from reversions or that they represent wild-type alleles. The finding of a *dTph1* insertion in the *rt*-mutable allele of *P. hybrida* line Vu15 (re)confirms that *dTph1* is indeed a functional transposable element by all criteria: it causes a variegated phenotype in somatic tissues of the plant as well as reversions among progeny plants due to excision of the element in sporogenic tissues (Figure 5). The insertion of *dTph1* in the *rt*-mutable allele causes a reduction of the steady-state *rt* mRNA level (Figure 7a). Since the element is inserted close to the TATA box, outside the transcribed region of the gene, we assume that the inser-

tion interferes with transcription of the *rt* gene rather than causing a destabilization of the *rt* mRNA.

The insertion sequence (*dTph3*) that is present in the R27 *rt* allele has sequence characteristics of a transposable element. It has inverted repeats at the ends and it is flanked by an 8 bp directly repeated target site duplication. Even though the internal sequences have completely diverged, there is some homology between the terminal inverted repeats of *dTph1* and *dTph3*. Therefore it is possible that transposition of both elements is dependent on the same activator element. *P. hybrida* was constructed around 1830 via interspecific crosses between the white flowering *P. axillaris*, *P. parodii* group and the coloured flowering *P. violacea*, *P. inflata*, *P. integrifolia* group (Koes *et al.*, 1987; Wijsman, 1983). Genetic analysis indicated the presence of a functional *rt* gene in each of these parental species (Wijsman *et al.*, 1983) and PCR analysis showed that none of them contained the *dTph3* insertion at the *Rt* locus (unpublished data). This indicates that transposition of *dTph3* into the *rt* gene presumably occurred after the interspecific crosses took place that gave rise to *P. hybrida*. The R27 line and related lines have been extensively used in many genetic studies and among tens of thousands of progeny plants instability of the rt^- allele was never reported. This suggests that today the *dTph3* copy at the *Rt* locus can not transpose any longer. Presumably structural defects, like the non-perfect terminal inverted repeats, are the reason for immobility of this element. Nevertheless *dTph3* will be useful as a probe to clone genes that are tagged by insertion of an active transposable element from this family.

The *dTph3* insertion causes polyadenylation to occur at a new site upstream from the polyadenylation site used in a wild-type *rt* mRNA, which explains the smaller mRNA observed in rt^- lines (Figure 7). Whether or not *dTph3* causes (occasionally) premature transcription termination can however not be concluded. Occasionally we observed some larger than wild-type RNA in gel blot analyses of rt^- mutants. Brugliera *et al.* (1994) obtained similar results, but in their lines the abundance of the large RNA was much higher. As there appears to be only one stable rt^- allele present in *P. hybrida* (Brugliera *et al.*, 1994; this paper) it is unlikely that this difference is due to the different lines that were used. As the long RNA hybridizes to the 3' end of the *rt* coding region it most likely resulted from transcription through the *dTph3* element and polyadenylation at the normal *rt* polyadenylation site(s) (Brugliera *et al.*, 1994). One way to explain the variable levels of this mRNA is to assume that the (cryptic) polyadenylation in the *dTph3* sequence is relatively inefficient, taking into account that the mRNA levels (and presumably transcription) of flavonoid biosynthetic genes in plants do vary with greenhouse conditions (light intensity, temperature, daylength etc.). This would result in an increased amount of the long *rt* mRNA under high transcription conditions.

Expression of *rt* in petal limbs is under the control of three regulatory genes, *an1*, *an2* and *an11* (Figure 2). No *rt* expression is detectable in anthers from the $an4^-$ line V26 (Figure 2), whereas anthers from $An4^+$ lines accumulate high levels of *rt* mRNA (Quattrocchio *et al.*, 1993) indicating that *rt* expression in anthers is controlled by the regulatory gene *an4*. Comparison of the *rt* promoter sequence, with that of two other *an1*, *an2*, *an4*, *an11* regulated genes (*chsJ* and *dfrA*; Huits *et al.*, unpublished data; Quattrocchio *et al.*, 1993) did not, however, reveal any clear homologies that may represent *an1*, *an2* or *an11* responsive elements. Possibly these elements are too small or too diverged to be detected by sequence comparison alone. Therefore elucidation of this interaction waits for the functional analysis of *cis*-acting elements in the *an1*, *an2*, *an4* and *an11* responsive promoters and the molecular isolation of the regulatory genes. Such experiments are currently in progress.

Experimental procedures

Plant material

Petunia hybrida lines used in this study have been maintained as inbred stocks for several generations. Plants were grown under standard greenhouse conditions. For RFLP analysis a (V23×R51) F_1 hybrid was backcrossed to both parental strains and analysed for several phenotypic markers as described (Wallroth *et al.*, 1986). As R51 is rt^- and V23 is Rt^+, segregation of the *Rt* marker could be phenotypically scored in the backcross VRxR. The segregation for *RtRt* (homozygous dominant) versus *Rtrt* (heterozygous dominant plants) in the VR×V backcross was determined by selfing of each VR×V backcross plant (Gerats, unpublished data). If this yielded rt^- progeny plants, the VR×V parental plant was scored as *Rtrt* (heterozygous). If no rt^- plants were found the parent was scored as *RtRt*.

Thin layer chromatography (TLC) of flavonoids was performed as described (Wallroth *et al.*, 1986).

Construction and differential screening of cDNA libraries

A cDNA library in λZAPII was constructed using the RNAse H procedure (Gübler and Hoffmann, 1983). Starting from 2 μg of poly(A)$^+$ mRNA extracted from stage 3 flowerbuds of *P. hybrida* line V26 a library of 4 x 10^6 p.f.u. was obtained, which was used for the differential screening. A directional λGEM4 cDNA library from V26 petal RNA was prepared according to procedures of the BRL Superscript Plasmid Cloning System and screened for a full size *rt* cDNA via plaque hybridization.

For cDNA labelling 1-2 μg of poly(A)$^+$ mRNA and 1.0 μg of oligo-dT primer (Boehringer) was used to synthesize approximately 1.0 x 10^8 c.p.m. ^{32}P-labelled first strand cDNA. The labelled cDNA/RNA hybrids were purified by chromatography over Sephadex G-50 columns. Prior to hybridization the probes were denatured by boiling, followed by a 15 min incubation at

35°C with 10 μg of RNAse A and H each (Boehringer) to remove the RNA.

For differential screening about 3×10^4 p.f.u. (V26 floral cDNA library in λZAPII) were plated at 800 p.f.u./plate on to 90 mm plates and duplicate plaque lifts were taken using Hybond-N membranes (Amersham) according to the manufacturers instructions. About 20 filters were prehybridized at 42°C for 3 h in 20 ml of 50% formamide; 5 × SSPE; 5 × Denhardt's; 0.1% SDS; 100 μg ml^{-1} herring sperm DNA. For hybridization 1.0×10^8 c.p.m. ^{32}P-labelled first strand cDNA and 100 μg of poly(A) (Boehringer) were added and incubation was continued for 16–48 h at 42°C. Filters were washed in 1.0 × SSC/ 0.1% SDS at 60°C for 30 min and exposed for 3–4 days to X-AR film (Kodak). Plaques showing substantially stronger hybridization to the V26 cDNA probe than to the W162 cDNA probe were picked and retested. Inserts from selected λ clones were subcloned in pBluescript (Stratagene) via *in vivo* excision mediated by helper phage R408 (Stratagene).

Construction of antisense genes and plant transformation

The orientation of the *difG* fragment in pBluescript was determined by sequencing both ends via double strand plasmid sequencing using the universal M13-21mer primer and the M13 reverse primer. The *difG* cDNA (1.0 kb) was inserted in an antisense orientation in between a CaMV 35S-promoter and a nopaline synthase 3' flanking region in a binary T-DNA vector (Van der Krol *et al.*, 1988). This construct was mobilised into *A. tumefaciens* strain LBA 4404 by triparental mating (Ditta *et al.*, 1980) and used to transform *P. hybrida* V26 leaf disks (Horsch *et al.*, 1985). After shoot and root induction on kanamycin-containing medium transgenic plants were transferred to soil and kept in a greenhouse.

DNA analyses

All plasmid- and λ-DNA manipulations were performed using standard methods. Isolations of plant DNA and DNA gel blot analyses were performed according to previously published procedures (Beld *et al.*, 1989).

A V26 genomic library was constructed as previously described (Koes *et al.*, 1989b) except that λGEM11 (Promega) was used as a vector. Approximately 150 000 clones were screened by stringent hybridization with a ^{32}P-nick-translated *rt* cDNA probe. Positive plaques were purified to homogeneity by two rounds of plaque purification. A 4.7 kb *Hin*dIII/*Sal*I insert from one positive λ clone (λGEM11:1), containing the complete *Rt* gene, was subcloned in pBluescript vectors (Stratagene) using standard cloning procedures.

The region of the *rt* gene containing *dTph3*, was amplified from 300 ng genomic DNA, from the line R27, by polymerase chain reaction (PCR) using primers 2 and 5, cut at the internal *Cla*I and *Pst*I sites (cf. Figure 7), and cloned into *Cla*I/*Pst*I digested pBluescript. The *dTph1* insertion was cloned from the *P. hybrida* line Vu15 via amplification of the *rt* promoter region with primers 1 and 3, blunt ending with T4 DNA polymerase, phosphorylation with T4 DNA polynucleotide kinase and ligation into the *Sma*I site of pBluescript. To amplify *rt* RNA products from R27 floral RNA we used the 3' RACE procedure described by Frohman *et al.* (1988) using primer 4 and the 3' RACE primer. Amplification products were digested with *Bcl*I and *Sal*I (cut in the *rt* sequence and the 3' RACE primer respectively, see Figure 7) and ligated into *Bam*HI/*Sal*I digested pBluescript.

The primers used have the following sequences (their position in the *rt* gene is shown in Figure 7b):

primer 1: 5'-dTCATTTATGTTTCTGTTTAATATTTACC-3';
primer 2: 5'-dCGGAAAGCTTGCTCGCAGTAT-3';
primer 3: 5'-dCTGCACAAATGGACTAATATGGCC-3';
primer 4: 5'-dCACCAGCTAGTGCTGAGCTTCTC-3';.
primer 5: 5'-dACAGCCACATTCTTTCAGGCCTTG-3';
3' RACE: 5'-dGACTCGAGTCGACATCGT$_{17}$-3'

Sequencing was performed using the dideoxy-chain termination method using [^{35}S]ATP (Sänger *et al.*, 1977), or using asymmetric PCR with fluorescent M13 primers or fluorescent dideoxynucleotides and employing an Applied Biosystems DNA sequencer model 370A. If necessary nested deletions were generated using the *Exo*III/S1 method (Henikoff, 1984).

RNA analysis

Flowers were harvested from plants and frozen in liquid nitrogen and total RNA was extracted as described (Van Tunen *et al.*, 1988). Developmental stages of flowers have been described before (Van Tunen *et al.*, 1988). Poly(A)$^+$ mRNA was prepared by two passages over an affinity oligo-dT cellulose column (Pharmacia and Boehringer) and its integrity and purification factor were monitored via RNA gel blot analysis.

RNA gel blot analysis was performed according to Van Tunen *et al.* (1988), using 10 μg total RNA per sample. After electrophoretic separation in formaldehyde agarose gels, the RNA was blotted on to Hybond-N according to the suppliers protocol (Amersham). The gels were briefly stained with ethidium bromide prior to blotting to ensure equal loading and integrity of the RNA. After hybridization the blots were washed in 0.5 × SSC, 1% SDS at 60°C.

Primer extension experiments using primer 3 and 10 μg of floral RNA from the line V26 were performed as described previously (Koes *et al.*, 1989b).

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EMBL Data Library accession numbers X71059 (*P. hybrida* mRNA for rhamnosyltransferase) and X71060 (*P. hybrida* gene for rhamnosyltransferase).