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Molecular Characterization of a Nonautonomous Transposable Element (*dTph1*) of Petunia

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An insertion sequence of 283 base pairs has been isolated from the DFR-C gene (dihydroflavonol-4-reductase) of petunia. This insert was found only in a line unstable for the *An1* locus (anthocyanin 1, located on chromosome VI) and not in fully pigmented progenitor and revertant lines or in stable white derivative lines. This implies that the *An1* locus encodes the DFR-C gene. The unstable *An1* system in the line W138 is known to be a two-element system, the autonomous element being located on chromosome I. In the presence of the autonomous element, W138 flowers exhibit a characteristic pattern of red revertant spots and sectors on a white background. In the absence of the autonomous element, the W138 allele gives rise to a stable recessive (white) phenotype. Sequence analysis of progenitor, unstable, and revertant alleles revealed *dTph1* to contain perfect terminal inverted repeats of 12 base pairs. In DFR-C, it is flanked by an 8-base pair target site duplication. Sequences homologous to *dTph1* are present in at least 50 copies in the line W138. Sequence analysis of *An1* revertant alleles indicated that excision, including removal of the target site duplication, is required for reversion to the wild-type phenotype. Derivative stable recessive alleles showed excision of *dTph1* and a rearrangement of the target site duplication. *dTph1* is the smallest transposable element described to date that is still capable of transposition. The use of *dTph1* in tagging experiments and subsequent gene isolation is discussed.

INTRODUCTION

Flavonoid synthesis in petunia has been described in great detail on both the genetic and the biochemical level (for reviews see Schram et al., 1984; Wiering and de Vlaming, 1984). The molecular analysis of this system has been initiated recently and to date the genes coding for the enzymes chalcone synthase (Koes et al., 1987), chalcone flavanone isomerase (Van Tunen et al., 1988), and dihydroflavonol-4-reductase (DFR; Beld et al., 1989) have been isolated and characterized.

A number of regulatory genes have been described that influence the expression of structural genes, especially those involved in the latter part of the pathway. The steady-state mRNA level of DFR and activity of the enzyme UDPglucose:flavonoid 3-o-glucosyltransferase are significantly reduced if one of the genes *An1*, *An2*, *An10*, or *An11* (anthocyanin-1, -2, -10, and -11) is homozygous recessive (Gerats et al., 1985; Beld et al., 1989). Molecular isolation of these genes is difficult because they have not been characterized biochemically. The use of transposable elements for tagging these genes would facilitate their molecular isolation.

Transposable elements have been isolated from a number of species (for review, see Döring and Starlinger, 1986). However, genetic analysis of phenotypes induced by transposable elements and molecular proof of their mobility is mainly restricted to two species, maize and snapdragon. Unstable alleles have been described for five petunia flavonoid genes (Cornu, 1977; Bianchi et al., 1978; Doodeman et al., 1984a; Gerats, 1985; Gerats et al., 1989). Molecular isolation of transposable elements from these genes has been slow because no clones were available for these genes.

Instability at the *An1* locus (located on chromosome VI) has been described in great detail (Bianchi et al., 1978; Doodeman et al., 1984a; Gerats et al., 1982, 1989). A two-element system has been shown to cause instability of the *An1* gene in the line W138. In the absence of the autonomous element, *an1W138* behaves as a stable recessive and gives rise to white flowers. In the presence of the autonomous element, which is located on chromosome I, *an1W138* produces a characteristic pattern of revertant spots and sectors on a white background (Wijsman, 1986). Line W138 gives rise to wild-type revertants and white-flowered derivatives at about equal frequencies of approximately 5% (Doodeman et al., 1984a).

Line W138 also gives rise to a very high number of mutant phenotypes in selfed progeny. These new phenotypes include seedling lethals, morphological mutants, and mutations in flavonoid synthesis (Doodeman et al., 1984b). The mutant loci involved in these phenotypes have been

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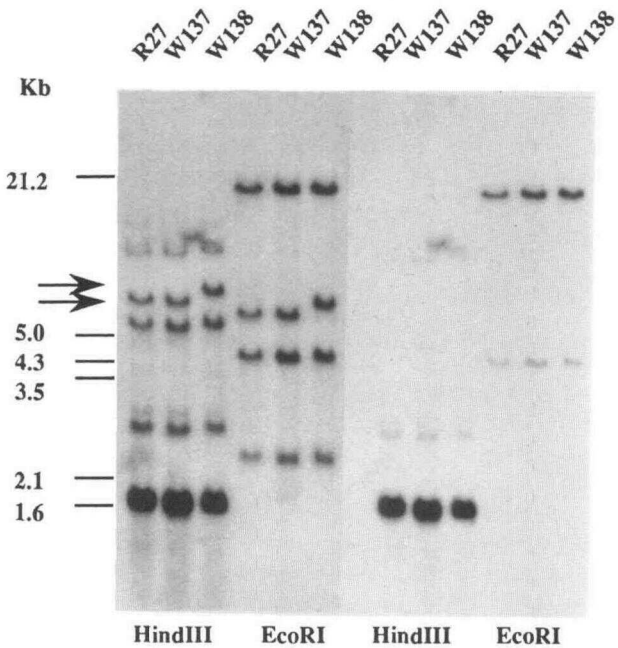

Figure 1. DNA Gel Blot Analysis of Genomic DNA of the Progenitor Line R27, the Unstable *An1* Line W138, and Its Revertant Line W137.

Genomic DNA was digested, respectively, with EcoRI and HindIII. The filter was sequentially washed under low-stringency (2 × SSC, 0.1% SDS, 60°C, left panel) and high-stringency (0.1 SSC, 0.1% SDS, 60°C, right panel) conditions after blotting and hybridization with the DFR-A cDNA probe. The band shift of around 0.3 kb in the HindIII and EcoRI digests of line W138 is indicated by the arrows (→).

located throughout the genome (Gerats et al., 1989). Six mutants exhibited a typical pattern of revertant sectors and for two of these it has been shown that instability is caused by a two-element system (Gerats et al., 1985; Wijsman, 1986). Therefore, the isolation of the element that causes instability of *an1W138* could provide an important tool for the isolation of a considerable number of interesting genes from petunia.

In a previous paper (Beld et al., 1989), we have shown that the DRF-C gene is closely linked (in genetic terms) with the *An1* locus. In this paper, it is shown that the *An1* locus coincides with the DFR-C gene. Furthermore, the isolation and characterization of the nonautonomous transposable element from the *An1* gene of the line W138 is described. Fully pigmented revertant and stable recessive alleles are analyzed, in addition to wild-type progenitor and unstable alleles. The results show that the phenotypic differences in flower color displayed by various *An1* alleles derived from the line W138 are caused by the action of this transposable element.

RESULTS

Isolation of a DFR-C (*An1*) Clone from the Unstable Line W138 and the Revertant Line W137

Although instability at the *An1* locus has been described in great detail (Gerats et al., 1989), no information on the nature of the gene product is available. In an earlier paper, we described the isolation and partial characterization of the dihydroflavonol-4-reductase genes (Beld et al., 1989). One of the DFR genes, DFR-C, appeared to be closely linked (in genetic terms) with the *An1* locus.

DNA gel blot analysis using the DFR-A cDNA as a probe revealed a hybridizing fragment in the *An1* unstable line W138 that was consistently 0.3 kb larger than the equivalent fragment from the wild-type progenitor line R27 or the revertant line W137. As shown in Figure 1, washing the blot at high stringency (0.1 × SSC, 60°C) removed the hybridization of this band, indicating that it involved the DFR-C gene, which is least homologous to the DFR-A cDNA. Given that W137 was directly derived from W138 and that W138 is separated from R27 by about 10 generations, this band shift could be attributed to the insertion of a mobile element in the DFR-C gene of the line W138.

We subsequently prepared genomic EMBL-3 libraries of the unstable line W138 and its revertant line W137. The resulting libraries of approximately 200,000 plaque-forming units were screened using DFR-A cDNA as a probe. Eleven positive clones were isolated and purified from the W137 library and six from the W138 library. These clones were digested with a number of enzymes and hybridized with the DFR-A cDNA. By washing under the same stringencies as mentioned above (Figure 1), we could confirm that one clone from each library contained DFR-C sequences (W138 λ10 and W137 λ5). The identity of the band shift was confirmed by hybridizing the DNA gel blot mentioned in Figure 1 with a subclone of W138 λ10. After washing at increasing stringencies (2 × SSC, 60°C; 0.1 × SSC, 60°C), only the bands coinciding with the DFR-C bands were retained (results not shown). From this we concluded that the W138 λ10 clone contains DFR-C sequences.

Restriction and DNA gel blot analysis of the W138 λ10 and the W137 λ5 clone revealed the presence of an insertion of around 0.3 kb in a EcoRI/SstI fragment of the W138 λ10 clone located at or near the 5′ end of the putative second exon of DFR-C, as shown in Figure 2. This size difference equaled the band shift recorded on DNA gel blots.

Sequence Analysis of the EcoRI/SstI Fragment of the W138 λ10 Clone and the W137 λ5 Clone

Sequence comparison of the 0.8-kb EcoRI/SstI fragment of the W138 λ10 clone with the 0.5-kb EcoRI/SstI fragment of the W137 λ5 demonstrated the presence of an inserted

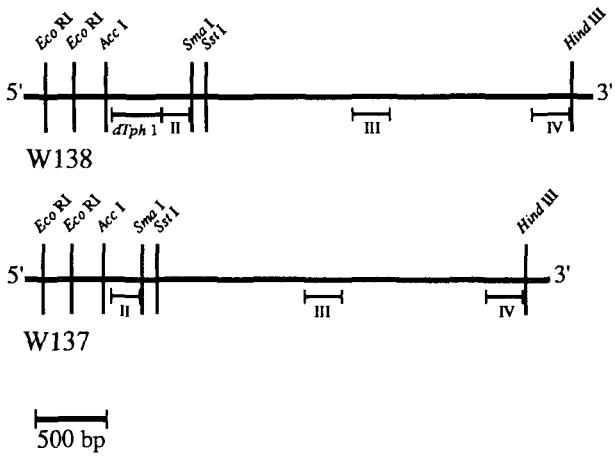

Figure 2. Restriction Endonuclease Cleavage Site Map of the W138 λ10 Clone.

The EcoRI/SstI fragment of the W138 λ10 clone is approximately 0.3 kb longer. The exons are indicated by Roman numerals beneath short horizontal lines. The exon position is based on sequence data.

fragment of 283 bp in the W138 λ10 clone. This sequence exhibited the structural characteristics of a transposable element. As Figure 3 shows, it contained perfect terminal inverted repeats of 12 bp and was flanked by a duplication of target site sequences of 8 bp.

The position of the element is at the intron-exon boundary of the putative second exon. The last nucleotide of the target site duplication induced by *dTph1* coincides with the first nucleotide of the sequences that represent the second exon in the DFR-A gene. The homology at the sequence level for the second exon of DFR-A and DFR-C is 80%, which is in the same range as for the third and fourth exon comparison.

DNA gel blot analysis indicated that sequences homologous to the first exon of DFR-A are present upstream of the insertion site of *dTph1* in the DFR-C gene (results not shown). The close linkage of DFR-C to *An1* plus the presence of the element in the unstable line W138 and its absence from both progenitor and revertant lines strongly support the view that the *An1* locus contains the DFR-C gene and that instability of W138 is due to this element. To confirm that the phenotypic differences between the progenitor, unstable, and derivative alleles are correlated with the presence or absence of *dTph1*, we performed a polymerase chain reaction (PCR) analysis for a number of these alleles.

PCR Analysis of Progenitor, Unstable, and Derivative Alleles

Using two DFR-C specific 25-mer oligonucleotides (see Methods and Figure 3), we performed a PCR analysis on genomic DNA of the unstable line W138 and several progenitor and derivative lines. The unstable line W138 usually produced a predicted fragment of around 700 bp, although a fragment of around 400 bp was sometimes visible. This result suggests that excision of *dTph1* occurs at a high frequency in leaf tissue also. The progenitor, revertant, and derivative lines produced fragments of around 400 bp, suggesting that *dTph1* is not present in the DFR-C gene of these lines, as shown in Figure 4. This was confirmed by sequence analysis of PCR products from the unstable W138 and from the progenitor R27; moreover, Figure 5 shows that three independent fully pigmented revertant alleles exhibited perfect excision of *dTph1*, including loss of the target site duplication. Sequence analysis of two stable white recessive derivative alleles revealed excision of *dTph1* and rearrangements of the target site duplication resulting in a typical transposon footprint. These molecular data confirm that the presence of *dTph1* in the DFR-C gene results in a recessive phenotype, whereas excision of the element from the DFR-C gene can restore activity of the DFR-C gene.

dTph1 Is Repetitive in the Petunia Genome

To estimate *dTph1* copy number, the EcoRI/SstI fragment of the W138 λ10 clone was used as a probe on DNA gel blots of the unstable line W138 and the Mitchell variety. The Mitchell variety (W115 in the Amsterdam collection of petunia lines) does not contain an autonomous element that is capable of activating the *dTph1* element inserted in the W138 *An1* unstable allele. DNA of these lines was digested with EcoRI and AseI. As shown in Figure 6, the element is a member of a relatively high copy number

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First nt.
  +1  GAATTCTCTT GTCAGAAGGA GAGGTTCCAA TTTAATGGCA AGAGGGTTCA
 +51  AAACTTGTTT TAAATCATAG ATGTCCAAAT CTTAGCTAGG TGTGACATCT
+101  AATATCTTTT CCACCCACTG TAATGTAGCA GTATTAGAAG TATATATTAA
+151  CATACACGAA AGATGATTGT TTAAAATTAT ATATAGATGA CAAGAGCTTT
+201  AATTTGGAAT TGTAGACAGG GGCGGAGCCA AGAATTTTAA TAAGGGGGTT
+251  CAAGAGGACA ACCGTTCAAA TATAAAAGAG GAAAAATCAA AAATATTTGT
+301  GGCAGCCCAG ATTCGATCCT GGGTGGTGAG CCCAGCATTG ACACCCCTTC
+351  GCCATTGAAC TAATGCCAAC ACTTGTTCAA AGGGGGTTCA ACACACAGTA
+401  TATATCCAAT CTAAACCAAT TTTATCACAT ATATGCAGTG TAAATTTTGC
+451  GCAAAGGGGG TTCAATTGAC ACCCCTTTGC ACCAAGTAGC TCCGCCCCTG
+501  ATTGTAGAGA ACAAGAAGAA GGTGAAACAC CTTTGGACT TGCCTAAAGC
+551  TGATACCAAT CTGACGGTAT GGAAAGCAGA CATGACAGAA GAAGGAGGTT
+601  TCGATGAAGC CATTCAAGGC TGTGAAGGTG TATTTCATGT GGCCACCCCT
+651  ATGGTTTCTG AATCCAAGGA CCCCGAGGTA GTAACTAAAT ACTTCTATCA
+701  ATCTCTGTTA AAAGGCTTTT CTGGACTCGC CCTCGGGCTC GACCCGGGCA
+751  CTAGCAAAAC GCACCGAGGT TTACGGGCGT GGCTTAGATC CGTTAGGTTA
+801  CACTTCGGGA GGCCGATTGT
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Figure 3. Nucleotide Sequence of *dTph1* in the DFR-C Gene.

The inverted repeats of *dTph1* and the CGTTT motif are underlined. The putative second exon of the DFR-C gene is indicated in italics and the primers used for PCR analysis are boxed.

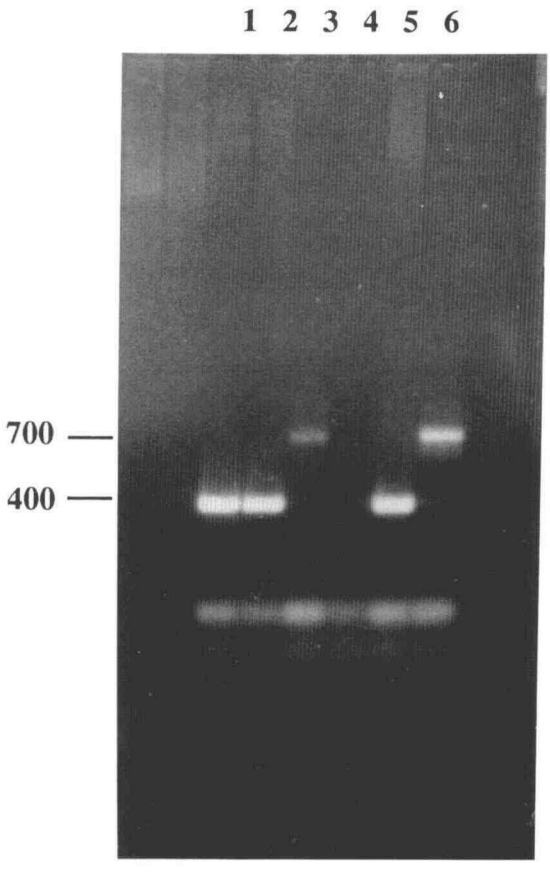

Figure 4. PCR Analysis of Genomic DNA of Unstable and Derivative Alleles of the Line W138.

The produced fragments of three independent revertant plants (lanes 1, 2, and 5), two unstable plants (lanes 3 and 6), and one stable recessive derivative plant (lane 4) are shown. The lower two bands represent the primers. Note the appearance of a revertant fragment (400 bp) in lane 6.

family. The line W138 harbors at least 50 copies of *dTph1*-related sequences, whereas the Mitchell variety has around 15 *dTph1*-related copies.

DISCUSSION

Classical genetic experiments have shown that instability of the *An1* locus in the line W138 is caused by a nonautonomous or two-element system (Wijsman, 1986). The autonomous element of this system is located on chromosome I, whereas *An1* is located on chromosome VI. The nonautonomous element appears to be a 283-bp insertion sequence that exhibits a number of characteristics typical of transposable elements (Figure 3). The element (*dTph1*) contains perfect inverted repeats of 12 bp that show homology with a number of other plant transposable elements, most notably with the *Tst1* element from potato (Köster-Töpfer et al., 1990), as shown in Table 1. Furthermore, the terminal 5 nucleotides of *dTph1* and the *Ac* and *rDt* elements of maize are identical. Although the homology between the terminal inverted repeats is evident, hardly any homology is detected between internal sequences. Deletion of the 4 terminal nucleotides at one end of *Ac* abolishes excision (Hehl and Baker, 1989), yet the 2 terminal nucleotides of *Ac* are not complementary. There are a number of elements that contain imperfect inverted repeats (see Table 1).

To our knowledge, *dTph1* is the smallest element described to date that is still capable of transposition. The elements *Tst1* and *Tpc1* are 736 bp and 927 bp, respectively, whereas the *rDt* element is 704 bp and has been shown to be capable of transposition (Hermann et al., 1988; Brown et al., 1989; Köster-Töpfer et al., 1990). *Ds* elements are structurally heterogeneous and range in size (Fedoroff, 1989), the smallest being around 400 bp (Sutton et al., 1984). The smallest *dSpm* element is 902 bp (Schiefelbein et al., 1985). Coupland et al. (1989) demonstrate that around 200 bp at each terminus are required for efficient excision of *Ac* in transgenic tobacco. No excision was observed in these experiments with constructs that retained less than 100 bp at either end.

Besides the terminal inverted repeats, subterminal regions have been shown to be involved in the excision of *En/Spm* and *Ac* (Gierl et al., 1989). The *Ac*-specific ORFa protein has been shown to bind specifically to several subterminal fragments of *Ac*, containing the hexamer motif AAACGG (Kunze and Starlinger, 1989). This motif is repeated several times in direct or inverted orientation in *Ac*. The foldback (FB4) transposable element of *Drosophila* contains multiple perfect and imperfect copies of the sequence CGTTTGCCCA near the distal ends of its terminal inverted repeats (Potter, 1982). Five nucleotides of this

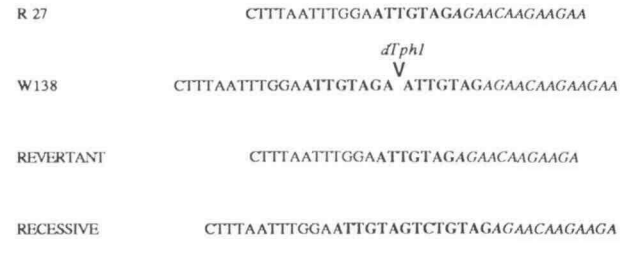

Figure 5. Nucleotide Sequence of the Region around the Insertion Place of *dTph1* in the DFR-C Gene of the Line W138 for a Number of Alleles.

The sequence was determined for the progenitor R27, the unstable W138, three independent revertant alleles, and two independent, stable white alleles.

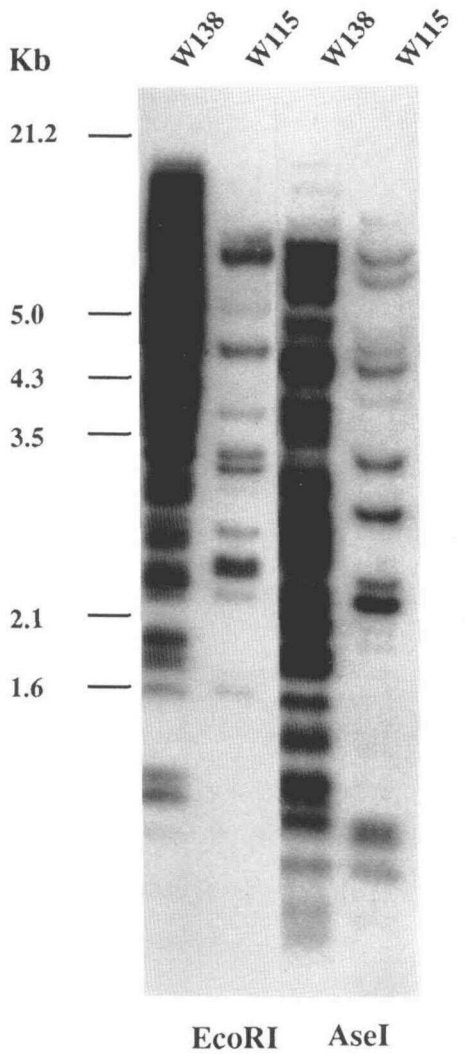

Figure 6. *dTph1* Copy Number Analysis for Two Petunia Lines.

Genomic DNA of the unstable *An1* line W138 and the Mitchell variety W115 was digested with EcoRI and AseI. The resulting filter was washed under low-stringency conditions (2 × SSC, 0.1% SSC, 60°C) after blotting and hybridization with the EcoRI/SmaI fragment of the W138 λ10 clone.

sequence are homologous to the inverted AAACGG motif, as found in *Ac* (Kunze and Starlinger, 1989). The FB4 motif is present in *dTph1* as well as in the potato element *Tst1* and the maize element *rDt*; it is found as a quite conserved copy (in 3′ to 5′ orientation) at respectively 8 bp, 11 bp, and 4 bp 5′ of the 3′-terminal inverted repeat, as shown in Table 2. Although the AAACGG/TTTGC motif is repeated twice at the 3′ end and once (CGTT) in inverse orientation at the 5′ end in *dTph1*, binding studies of these sequences with the primary product of the autonomous element are required to elucidate their significance. It has been shown that a single base pair change in the AAACGG/AACGG motif (C to G) prevents binding of the ORFa protein (Kunze and Starlinger, 1989). The analysis of transposase binding sequences of the elements that share homology between their inverted repeats might subdivide these elements in different related subfamilies. This might clarify further the evolution of transposable elements.

Like a number of transposable elements from other species, *dTph1* induces an 8-bp target site duplication (see Table 1). Sequence analysis of the PCR products of three independent revertant alleles showed the absence of *dTph1* in each case. In the majority of cases, a transposon footprint of the target site duplication is left behind upon excision of a transposable element. Surprisingly, these alleles exhibited perfect excision of *dTph1*, including the target site duplication (Figure 5). This phenomenon might be explained by the location of the element in the DFR-C gene. Sequence comparison with the DFR-A sequence indicates that the last nucleotide of the target site duplication coincides with the first nucleotide of the second exon. The analysis of two independent stable recessive derivative alleles points in the same direction (Figure 5). In these cases, *dTph1* was excised and the target site duplication was rearranged, resulting in a characteristic transposon footprint. The rearrangement is in agreement with the chromosome breakage and religation model as pre-

Table 1. 5′ to 3′ Terminal Inverted Repeats of Some Related Transposons from Different Species

Element	Repeat	Target	Species
En/Spm	CAC TAC AAG AAA A	3	*Zea mays*
Tam1	CAC TAC AAC AAA A	3	*Antirrhinum majus*
Tgm	CAC TAT TAG AAA A	3	*Glycine max*
rDt	CAG TGT TTT AAA TC	8	*Zea mays*
P	CAT GAT GAA ATA AA	8	*Drosophila melanogaster*
Hobo	CAG AGA ACT GCA	8	*Drosophila melanogaster*
Tc1	CAG TGC TGG CCA AA	8	*Caenorabdithis elegans*
dTph1	CAG GGG CGG AGC	8	*Petunia hybrida*
Tst1	CAG GGG CGT AT	8	*Solanum tuberosum*
Ac	CAG GGA TGA AA	8	*Zea mays*
Ipsr	TAG GGG TGG CAA	8	*Pisum sativum*
Tpc1	TAG GGT GTA AA	8	*Pisum crispum*
Tam3	TAA AGA TGT GAA	8	*Antirrhinum majus*

Data for the various elements are from the following references: *Spm* (Pereira et al., 1985; Masson et al., 1987), *Tam1* (Bonas et al., 1984), *Tgm1* (Vodkin, 1988), *rDt* (Brown et al., 1989), *P* (O'Hara and Rubin, 1983), *Hobo* (McGinnis et al., 1983), *Tc1* (Rosenzweig et al., 1983), *Tst1* (Köster-Töpfer et al., 1990), *Ac* (Muller-Neumann et al., 1984; Pohlman et al., 1984), *Ipsr* (Bhattacharyya et al., 1990), *Tpc1* (Hermann et al., 1988), and *Tam3* (Sommer et al., 1985). Bases that are imperfectly repeated are underlined.

Table 2. Putative Transposase Binding Motifs as Found at the 3′ Terminus of Some Transposable Elements

Element	Putative binding motif	Species
FB4	CGTTTG·CCCA	*D. melanogaster*
dTph1	CGTTTC·CCCA	*P. hybrida*
rDt	CGATTC·TCCA	*Z. mays*
Tst1	CGTTTTACCCA	*S. tuberosum*

Data for the various elements are from the following references: *FB4* (Potter, 1982), *rDt* (Brown et al., 1989), and *Tst1* (Köster-Töpfer et al., 1990). Note that the sequence for *dTph1*, *rDt*, and *Tst1* is presented in the 3′ to 5′ direction.

sented by Peacock et al. (1984) and Coen and Carpenter (1988). The rearrangement either leads to a frameshift mutation, adding 7 bp to the reading frame of the second exon, or it prevents proper splicing, leading to an unstable or nontranslatable mRNA. Derivative wild-type and recessive alleles are produced at about equal frequencies of approximately 5% (Doodeman et al., 1984a). This is surprising because one might expect a much higher frequency of recessive events. The analysis of a larger number of derivative alleles will prove whether there are other sequence compositions that give rise to a wild-type phenotype. In such derivative alleles, splicing might occur, for example, at the downstream (duplicated) splice site, leading to a wild-type mRNA. Nevertheless, the three revertant alleles that have been analyzed present an unusual event compared with excision events in general.

A number of genes have been tagged to date using the *an1W138* system. These comprise, among others, morphological genes and flavonoid genes (Gerats et al., 1989). Now that a clone of *dTph1* is isolated, we can try to isolate at least a number of these mutant genes. Furthermore, experiments aiming at the isolation of the autonomous element are in progress. Given the fact that petunia can be transformed easily, this will make petunia one of the most complete plant model systems for molecular research on gene expression and gene regulation.

METHODS

Petunia Lines

All lines used in the present investigation have been maintained as inbred stocks for at least four generations. Plants were grown under standard conditions in a greenhouse. The genotype and phenotype of the lines used in this investigation are presented in Table 3. The line R27 is the progenitor of the line W138 (see Bianchi et al., 1978). The line W115 lacks an autonomous element for this system and is, therefore, incapable of transactivating the element in the W138 unstable allele.

Ten W138 plants were selfed to obtain independent derivative alleles. From the 10 progenies we selected two white-flowered plants and three wild-type revertant plants. These plants all originated from different progenies and, thus, represent independent events. The selected plants were further inbred, and eventually homozygous lines for each of them were created. These lines were used for the PCR analysis of derivative alleles (see below).

Isolation of DNA from *Petunia hybrida*

Five grams of leaves were frozen in liquid nitrogen and homogenized with a mortar and pestle. The fine powder obtained was thawed in 15 mL of buffer [100 mM Tris, 100 mM NaCl, 20 mM EDTA, 1% (w/v) Sarkosyl, pH 8.0] and 1.5 mL of 20% SDS and left at 37°C for 2 min. The solution was extracted twice with an equal volume of phenol (saturated with 10 mM Tris, 1 mM EDTA, pH 8.0) and once with an equal volume of phenol:chloroform (1:1). The aqueous phase was precipitated with 2 volumes of 96% ethanol and left at room temperature for at least 30 min. The precipitate was centrifuged for 20 min at 4000 rpm, and the resulting pellet was dried briefly in a dessicator. The pellet was resuspended in 10 mL of 10 mM Tris, 1 mM EDTA, pH 8.0. The DNA was purified on a CsCl gradient. Miniprep DNA was isolated according to Dellaporta et al. (1983).

Genomic Clones and Subclones

Genomic DNA was extracted from the *Petunia hybrida* lines W138 and W137, partially digested with Sau3A and ligated in the phage vector λEMBL-3 according to Dean et al. (1985). Both libraries, containing 2×10^5 plaques each, were hybridized (Maniatis et al., 1982) with radioactive probes, ^{32}P-labeled by nick translation or random primer labeling (Feinberg and Vogelstein, 1983). Subcloning was performed using standard procedures and pUC and M13 vectors. All enzymes and reagents used in cloning, hybridization, and restriction analysis are commercially available.

PCR Analysis

Genomic DNA (1 μg) extracted from leaf material from different alleles was subjected to amplification with 1 unit of the DNA

Table 3. Phenotype and Genotype of the Lines Used in the Present Investigation

	Phenotype	Genotype	Autonomous Element
R27	Red	*An1/An1*	Present
W138	White, spotted	$an1^{s/p-+}/an1^{s/p-+}$	Present
W137	White, spotted[a]	$an1^{+/+}/an1^{+/+}$	Present
W115	White[b]	*An1/An1*	Absent

[a] The line W137 has white, spotted flowers because of an insertion of a transposable element in the flavonoid gene *An11*; the line is homozygous for a revertant *An1* allele.
[b] The Mitchell variety is white, due to the fact that the line is homozygous recessive for the flavonoid gene *An2*.

polymerase from *Thermus aquaticus* (Ampli Taq, Perkin-Elmer Cetus). The reaction mixture, including 1 μmol of primer cp1 (5′-CCACCCACTGTAATGCTGCAGTATT-3′) and 1 μmol of primer cp2 (3′-TGCCCGCACCGAACCTAGGCAATCGA-5′), was subjected to 25 repeated cycles of 0.2 min at 94°C, 0.2 min at 55°C, and 1 min at 72°C by using the Perkin-Elmer Cetus Thermocycler. Synthetic oligonucleotide primers, 25 bp in length, were prepared by a Du Pont oligosynthesis research facility. A PstI site was created in primer 1 and a BamHI site in primer 2 (Figure 3). As a control on contamination, samples containing both primers but no genomic DNA were assayed routinely.

Electrophoretic Analysis and Sequencing

The amplified DNAs were purified by phenol extraction and precipitation and electrophoresed through a 1.5% agarose gel in 90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3.

For cloning, the amplification primers were removed by a selective ethanol precipitation in 2.5 M NH_4OAc and 1 volume of ethanol (Kreitman and Landweber, 1989). The amplified fragments were digested with the restriction enzymes PstI and BamHI and subsequently cloned into M13mp18 for sequencing. Sequence analysis was performed using the dideoxy chain-termination method (Sanger et al., 1977).

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