Epigenetic Interactions among Three dTph1 Transposons in Two Homologous Chromosomes Activate a New Excision-Repair Mechanism in Petunia

Adèle van Houwelingen,1 Erik Souer, Jos Mol, and Ronald Koes2

Department of Genetics, Institute for Molecular Biological Sciences, Vrije Universiteit, BioCentrum Amsterdam, de Boelelaan 1087, 1081 HV, Amsterdam, The Netherlands

Unstable anthocyanin3 (an3) alleles of petunia with insertions of the Activator/Dissociation-like transposon dTph1 fall into two classes that differ in their genetic behavior. Excision of the (single) dTph1 insertion from class 1 an3 alleles results in the formation of a footprint, similar to the “classical” mechanism observed for excisions of maize and snapdragon transposons. By contrast, dTph1 excision and gap repair in class 2 an3 alleles occurs via a newly discovered mechanism that does not generate a footprint at the empty donor site. This novel mechanism depends on the presence of two additional dTph1 elements: one located in cis, 30 bp upstream of the an3 translation start in the same an3 allele, and a homologous copy, which is located in trans in the homologous an3 allele. Absence of the latter dTph1 element causes a heritable suppression of dTph1 excision-repair from the homologous an3 allele by the novel mechanism, which to some extent resembles paramutation. Thus, an epigenetic interaction among three dTph1 copies activates a novel recombination mechanism that eliminates a transposon insertion.

INTRODUCTION

The best characterized class of transposons comprises elements with short terminal inverted repeats that transpose via a “cut-and-paste” mechanism. This class includes, for example, Tn7 and Tn10 of Escherichia coli, Activator (Ac), Dissociation (Ds), and Enhancer/Suppressor-Mutator (En/Spm) elements from maize, various Tam elements from snapdragon, P and Hobo elements from Drosophila, and various families of Tc elements from Caenorhabditis elegans. In addition, an increasing amount of evidence indicates that the intervening sequences, which are excised from genes encoding immunoglobulins and T-cell receptors to join variable (V), diversity (D), and joining (J) segments, originate from a cut-and-paste transposon (Agrawel et al., 1998; Hiom et al., 1998; reviewed in Roth and Craig, 1998).

The mobility of these transposable elements is controlled by a variety of mechanisms, all of which regulate expression of the recombinase enzyme(s) involved. For instance, in mammals, V(D)J joining is restricted to certain lymphoid cells, because other cell types do not express the proteins RAG1 and RAG2 that initiate the joining reaction (Oetinger et al., 1990). In Drosophila, differential splicing of the P transposase mRNA yields either a functional transposase (in the germ line) or a truncated version of the transposase (in the soma) that represses transposase gene transcription and transposition. Certain deletion derivatives of P repress transposition, presumably by a similar mechanism (reviewed in Engels, 1996). Also, epigenetic mechanisms contribute to the regulation of transposition frequency. The variegated flower color specified by the Tam2 insertion allele nivea-44 of snapdragon is inherited in a non-Mendelian manner, suggesting that one or more epigenetic factors regulate Tam2 activity (Hudson et al., 1987; Krebbers et al., 1987). In maize, the heritable inactivation of Ac and Spm elements is associated with increased methylation and reduced transposase expression (Banks et al., 1988; Brutnell and Dellaporta, 1994). At low frequency, the introduction of an active transposon copy can result in demethylation and reactivation of the repressed element (Banks et al., 1988; Fedoroff, 1989; Schwartz, 1989; Brutnell and Dellaporta, 1994; Brutnell et al., 1997). In the case of Spm, this interaction is mediated by the TNPA protein, which is expressed from the active Spm and binds, activates, and demethylates the promoter of the inactive Spm element (Schläppi et al., 1994).

Recent evidence shows that transposition and V(D)J joining, as well as replication of certain bacteriophages and retroviral integration, rely on the same basic chemistry for the breaking and joining of DNA segments. Hydrolytic nicking of DNA strands (in which H2O acts as the nucleophile) generates free 3’ hydroxyl groups, which by nucleophilic attack

---

1 Current address: Centre for Plant Reproduction Research (CPRO-DLO), Wageningen, The Netherlands.
2 To whom correspondence should be addressed. E-mail koes@bio.vu.nl; fax 31-20-4447155.
engage in transesterification reactions and joining to the target DNA (reviewed in Craig, 1995; Roth and Craig, 1998). The differences in the products that are produced by distinct DNA transfer reactions depend on the site(s) and the strand(s) at which donor and target DNAs are cleaved. Presumably, because their free 3’ OH ends attack the top and bottom strand of the target DNA at a slightly different position, each transposon duplicates a characteristic number of nucleotides of the target site during integration. For instance, integration of P or Ac and related elements produces 8-bp target site duplications, whereas those produced by En/Spm and related transposons are 3 bp in length.

By analyzing (phenotypic) reversions of unstable transposon insertion alleles, one can monitor the excisions of these transposons. Strikingly, P, Ac, and Tam insertion alleles appear to revert by different mechanisms in their cognate hosts (reviewed in Rommens et al., 1993). This may be due to differences in the excision mechanism or in the mechanism that repairs the double strand excision-induced break.

The excision of a plant transposon usually results in the complete removal of transposon sequences and the formation of a so-called footprint (reviewed in Kunze et al., 1997). Generally, such footprints consist of remainders of the target site duplication. These are often separated by inverted copies of the target site. Typically, footprints created by Ac-like transposons lack at least one nucleotide in the axis of symmetry, whereas those created by transposons of the CACTA family (e.g., Spm) generally produce perfect palindromes. To explain these features, Coen et al. (1986) proposed that Ac-like and CACTA transposons excise by a 1-bp staggered and a blunt-ended cut, respectively, and that the DNA ends at the excision break form hairpin structures. After subsequent resolution of the hairpin by a second nick, the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created. By using in vitro systems, it was shown that hairpin molecules indeed attack the ends are ligated and a footprint is created.

Engels et al. (1990) showed that the (germinal) reversion frequency of unstable Drosophila alleles with P insertions depends on the structure of the allele on the homologous chromosome and proposed that excision gaps are repaired by a gene conversion–like mechanism, called DSB repair, that uses the homologous chromosome or sister chromatid as a template. The demonstration that marked sequences could be transferred from the homologous allele or a transgene into the empty donor site provided direct support for the DSB repair model (Gloor et al., 1991; Johnson-Schlitz and Engels, 1993). DSB repair also has been implicated in the repair of the gaps created by Tc1 and Tc3 elements in C. elegans germ line cells (Plasterk, 1991), even though these transposons are unrelated to P. Although the role of DSB repair in transposon excision in plants has not been directly investigated, some transposition events have been explained by invoking DSB repair. For instance, Mutator (Mu) elements of maize are thought to transpose by a (quasi)replicative mechanism in germinal cells, and the structure of Mu excision products fits with a DSB repair model (Lisch et al., 1995; Hsia and Schnable, 1996; Mathern and Hake, 1997). Even though reversion of Ac insertion alleles in maize does not display homolog dependency (Baran et al., 1992), it has been suggested that some excision products result from DSB repair (Chen et al., 1992; Rommens et al., 1993).

We recently generated by random transposon mutagenesis a series of mutant alleles for the anthocyanin3 (an3) locus of petunia (van Houwelingen et al., 1998). This locus contains the gene coding for the enzyme flavanone 3β-hydroxylase (F3H), which is required for the synthesis of anthocyanin pigments in the flower. Consequently, stable an3 mutants have nearly white flowers, whereas unstable an3 mutants have nearly white flowers with colored revertant sectors and spots. Molecular analysis showed that the unstable an3 alleles contained one or two insertions of either of the petunia transposons dTph1 or dTph2. Both of these transposons are members of the Ac superfamily, because they duplicate 8 bp of target sequence when integrated and because the sequences of their terminal inverted repeats are similar to those of Ac and related transposons (Gerats et al., 1990; Kroon et al., 1994; van Houwelingen et al., 1998). Furthermore, the footprints left behind after dTph1 and dTph2 excision are similar to those found for other members of the Ac superfamily (Souver et al., 1996, 1998; de Vetten et al., 1997; Alfenito et al., 1998; this study). Both dTph1 and dTph2 are nonautonomous transposons because they are too small (284 and 177 bp, respectively) to encode their own transposase. Genetic mapping showed that transposition of dTph1 requires the immobile Actl locus that may encode either the transposase or a host factor involved in transposition (Huils et al., 1994).

In this study, we show that an3 alleles with a single transposon insertion (dTph1 or dTph2) behave like unstable transposon insertion alleles in maize and snapdragon. Surprisingly, we found that the four unstable an3 alleles harboring two dTph1 elements revert preferentially via a novel mechanism. This mechanism relies on (epigenetic) interactions among three dTph1 copies on the two homologous chromosomes and results in elimination of a dTph1 insertion without leaving a footprint.

RESULTS

Analysis of Excision Alleles

Figures 1A and 1B summarize the flower phenotypes and the structures of mutant an3 alleles. The transposon insertion alleles are all genetically unstable and undergo somatic and germinal transitions to new stable derivatives with activities ranging from wild type to null. Polymerase chain reac-
PCR analyses showed that these transitions correlated, with a few exceptions (see below), with excision of the transposon. To study the excision mechanism, we isolated and sequenced a series of such excision products. For one set of an3 alleles (termed class 1), we found that the wild-type sequence was rarely restored after excision of dTph1 or dTph2 and that a so-called transposon footprint was usually generated (Figure 2A). A partial reversion allele (called W29FT) in which dTph1 had excised from an3-W29 was identified by phenotype in progeny from self-pollinated W29/W29 plants. Sequence analysis showed that W29FT contained a footprint that consisted of remainders of the duplicated 8-bp target site separated by inverted repetitions of part of the target site (Figure 2A). However, phenotypic selection of excision alleles may favor the recovery of certain excision alleles (those that partially or fully restore gene activity) over others. To avoid such a bias, we analyzed PCR-amplified somatic excision products from plants homozygous for the dTph1 insertion alleles W29, W2018, W2303, W2140, W2298, V2221, and W138A. In all somatic excision products, transposon footprints were found that in structure resembled that in W29FT (Figure 2A).

Excisions of dTph2 from the an3-GSm allele created footprints that were very similar to the dTph1-induced footprints (Figure 2A). In one case, we recovered a perfectly restored wild-type sequence. However, we believe that this is a rare

---

Figure 1. Schematic Showing the Structure and Genetic Relation of an3 Alleles.
(A) The structure and pedigree of an3 alleles isolated in the W138 genetic background.
(B) The structure of an3 alleles isolated in various other genetic backgrounds.

Black boxes represent exon sequences. Positions of transposons are indicated by triangles; the arrows indicate their direction. The flowers show the phenotype for each allele when homozygous. Black, An3+ (colored flowers); spotted, an3mt (flowers with red/pink spots on white background); white, an3− (nearly white flowers). The presence of a footprint is indicated with FT; the wavy line in S206 represents DNA sequences that were fused to the an3 locus by a large chromosomal rearrangement (a translocation or inversion). Deletions in the an3 locus are indicated by empty regions flanked by parentheses. Note that W138A harbors a dTph1 insertion (open triangle) 30 bp upstream of the translation start, which does not affect pigmentation of the flower on its own. The unstable alleles R134, S205, S857, and X2092 are derived from W138A and harbor a second dTph1 insertion (shaded triangle), downstream of the ATG, that is responsible for the mutant phenotype. an3 alleles derived from an3-W138B have a single dTph1 insertion. Details on the structural analysis of the transposon insertion alleles and the stable recessive alleles W62, S206, and W1006 are documented elsewhere (van Houwelingen et al., 1998). The alleles W29FT, V512, T3463, and W2018FT were isolated during this work.
Figure 2. Excision Products Produced by Class 1 and Class 2 an3 Alleles.

(A) Sequence analysis of PCR-amplified excision products generated by the transposon in class 1 an3 alleles. For each an3 allele, the position of the insertion is indicated by an arrow, and the direction is as given in Figure 1. The target site duplications flanking the transposon are underlined. Excision alleles are indicated with "s" (somatic excision) or "g" (germinal excision). Numbers in parentheses indicate the number of times that excision alleles with the given sequence were found. Black dots indicate missing base pairs of the target site duplication after excision of the element. For comparison, the wild-type sequences (wt) surrounding the insertion sites are also shown.

(B) Sequence analysis of PCR-amplified excision products generated by excision of the downstream dTph1 element in class 2 an3 alleles.

(C) Sequence analysis of PCR-amplified excision products generated by excision of the upstream dTph1 element in a class 2 an3 allele. The germinal excision allele X2330 derived from R134, V512, and T3463 derived from S857, and V2307 derived from S857.

(D) Analysis of PCR-amplified excision products of class 1 and class 2 an3 alleles on 6% polyacrylamide gels. Excision products of the class 1 allele W2018 are compared with those produced by the downstream element of the class 2 alleles S857, R134, S205, and X2092, and the upstream element of the class 2 allele R134. Homozygotes (hom) as well as hemizygotes over the W62 deletion allele are examined for each an3 allele, and the corresponding phenotypes are indicated above the lanes as follows: m, an3^- phenotype (spotted flower); (-), an3^- phenotype (white flower); and (+), An3^+ phenotype (colored flower). The upper part of the gel shows the products that contain the dTph1 insertion; the lower part shows the excision products. The excision products with wild-type size are marked WT, whereas the size of longer products is indicated with +6, +7, or +8 (bp).
excision product, because it was isolated from a somatically reverted branch (phenotypic selection for wild-type activity), whereas crosses showed that (germinal) excision of \textit{dTph2} from an3-GSm virtually always results in stably inactivated an3 alleles and rarely in An3+ revertant alleles (data not shown).

The an3 alleles S205, R134, S857, and X2092 behaved quite differently and were therefore collectively designated as class 2 alleles. These four alleles all contain two \textit{dTph1} elements at the an3 locus (Figure 1). In general, self-pollinating homozygotes of any of these class 2 alleles yields ~50% of progeny with an an3\textsuperscript{mut} phenotype similar to the parental line and ~50% with an An3\textsuperscript{+} revertant phenotype (data not shown). In progenies obtained by selfing R134/R134 or S205/S205 plants, we found at a low frequency (1 to 5%) with a very low spotting frequency (weakly an3\textsuperscript{mut}) that were often difficult to distinguish from a stable an3\textsuperscript{+} phenotype. PCR analysis showed that such plants were always homozygous for the presence of the downstream \textit{dTph1} element but could be either heterozygous or homozygous for the presence of the upstream element (Figure 1A; see hereafter for a detailed analysis). Because too few progeny were grown, we do not know if X2092/X2092 or S857/S857 also produce weak an3\textsuperscript{mut} progeny after self-pollination.

PCR experiments showed that in the An3\textsuperscript{+} reversion alleles, the \textit{dTph1} element upstream of the ATG remained present, whereas the \textit{dTph1} copy downstream of the ATG was invariably lost. Sequencing of four S205-derived, two R134-derived, and one S857-derived (independent) germinal revertant alleles showed that the wild-type sequence had been perfectly restored at the empty donor sites in all seven cases. In another 24 somatic excision products recovered without phenotypic selection by PCR amplification from homozygous S205/S205, R134/R134, S857/S857, or X2092/X2092 plants, we again found a perfect restoration of the wild-type sequence (Figure 2B).

These data indicate that elimination of the single \textit{dTph1} element in a class 1 an3 allele or the upstream \textit{dTph1} element in a class 2 an3 allele involves a ("classical") mechanism similar to that observed for transposons in snapdragon and maize. The downstream element of a class 2 an3 allele, however, appears to be preferentially eliminated by a different ("novel") mechanism that yields products without a footprint.

### Reversion of Class 2 Alleles Is Suppressed in Hemizygotes

Testcrosses between unstable an3 mutants and lines harboring the W62 deletion allele revealed yet another difference between unstable class 1 and class 2 an3 alleles.

Croses between homozygotes for an unstable class 1 an3 allele and the W62 allele yielded predominantly progeny with spotted (an3\textsuperscript{mut}) flowers (Table 1). The spotting frequency of such flowers always corresponded with that of the parental class 1 allele in the homozygous condition (Figures 3A and 3C). PCR analysis showed these an3\textsuperscript{mut} progeny plants contained the parental class 1 an3 allele, including the \textit{dTph1} element. an3-W62 is a complete deletion, which implies that reversion of a class 1 allele is independent of an3 sequences.

#### Table 1. Genotypes and Phenotypes Produced by Crossing Unstable and Stable Recessive an3 Mutants

<table>
<thead>
<tr>
<th>an3\textsuperscript{mut} Parent</th>
<th>W62</th>
<th>S206</th>
<th>W1006</th>
<th>W2018FT</th>
<th>W29FT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>An3\textsuperscript{+}</td>
<td>an3\textsuperscript{m}</td>
<td>an3\textsuperscript{+}</td>
<td>an3\textsuperscript{m}</td>
<td>an3\textsuperscript{+}</td>
</tr>
<tr>
<td>W2018</td>
<td>0</td>
<td>62</td>
<td>6</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>W2298</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>W2303</td>
<td>0</td>
<td>9</td>
<td>8</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>W29</td>
<td>29</td>
<td>63</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>W2140</td>
<td>1</td>
<td>17</td>
<td>0</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>W2258</td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>GSm</td>
<td></td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>S205</td>
<td>155</td>
<td>0</td>
<td>166</td>
<td>46</td>
<td>0</td>
</tr>
<tr>
<td>S857</td>
<td>23</td>
<td>0</td>
<td>13</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>R134</td>
<td>49</td>
<td>0</td>
<td>38</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>X2092</td>
<td>30</td>
<td>21</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All parental plants used were homozygous for the specific allele.

\textsuperscript{b}For each phenotypic class, the number of progeny is given. Boldface numbers denote progeny harboring the unstable parental allele. The other numbers indicate progeny with derived excision alleles. A minus sign indicates that no data are available.

\textsuperscript{c}Plant lacking the upstream \textit{dTph1} element from S857: for details, see Table 2, family V2307, and accompanying text.
on the homologous chromosome. Other plants in the same progenies had evenly colored (An3\textsuperscript{+}) or nearly white flowers without spots (an3\textsuperscript{-}) (Figure 3B). PCR analysis showed that such plants always lacked the dtph\textsubscript{1} insertion in the class 1 allele, apparently due to an excision event in germinal cells of the parent. Depending on the sequence of the footprint and its position in the an3 gene, such excision alleles have either a wild-type (An3\textsuperscript{+}) or a stable mutant (an3\textsuperscript{-}) phenotype.

Surprisingly, crosses between homozygotes for the three class 2 alleles, S857, R134, or S205, and W62 yielded only An3\textsuperscript{+} and an3\textsuperscript{-} progeny (Figure 3F); the expected an3\textsuperscript{mut} phenotype was missing (Table 1). PCR analyses showed that the An3\textsuperscript{+} plants lacked the downstream dtph\textsubscript{1} element of the class 2 allele, presumably as a result of germinal excision, whereas the an3\textsuperscript{-} plants contained the parental class 2 allele, including both dtph\textsubscript{1} copies. We did not find an3\textsuperscript{-} progeny containing an excision derivative of the class 2 allele with a footprint (Table 1), consistent with the finding that these excisions preferentially result in restoration of the

![Figure 3. Phenotypes of Flowers with Different Combinations of an3 Alleles.](image-url)

(A) Flower of an W2140/W2140 plant showing an an3\textsuperscript{mut} phenotype.
(B) Flower of an An3\textsuperscript{+} plant harboring an3-W62 and an An3\textsuperscript{+} revertant allele derived from an3-W2140.
(C) Flower of a W2140/W62 plant showing an an3\textsuperscript{mut} phenotype.
(D) Flower of a W2140/W29FT showing an an3\textsuperscript{mut} phenotype.
(E) Flower of a homozygote for the S857 allele (class 2) showing an an3\textsuperscript{mut} phenotype.
(F) Flower of an S857/W62 heterozygote with an an3\textsuperscript{-} phenotype due to suppression of S857 instability.
(G) Flower of an S857/W29FT heterozygous plant from family V2307 (see Table 2) with an an3\textsuperscript{-} phenotype.
(H) Flower of a V2307 sibling plant with an an3\textsuperscript{mut} phenotype that is heterozygous for W29FT and a derivative allele of S857 that lacks the upstream dtph\textsubscript{1} copy.
(I) Flower of an S205/S205 plant showing an an3\textsuperscript{mut} phenotype.
(J) Flower of an S205*/V512 plant showing an an3\textsuperscript{-} phenotype.
(K) Flower of an S205*/S205* plant showing an an3\textsuperscript{-} phenotype.
(L) Flower of an S205*/S205 plant showing an an3\textsuperscript{mut} phenotype.
wild-type sequence (Figure 2B). The absence of revertant spots in hemizygotes for an3-W62 and a class 2 an3 allele indicates that in this F1 background, reversions of the class 2 allele are somehow suppressed (cf. the flowers in Figures 3E and 3F).

The suppressed somatic reversion frequencies might be explained by (1) a decreased frequency of dTph1 excision or (2) an alteration in the spectrum of excision products (to products that do not restore gene activity). To discriminate between these possibilities, we separated the complete array of PCR-amplified somatic excision products from class 1 and class 2 homozygotes and hemizygotes on high (1-bp) resolution gels. Using controls, we performed similar PCRs with plants harboring (stable) wild-type (Figure 2D, lanes 1, 5, 9, 13, 17, and 21) or germinal excision alleles (lanes 4, 8, 12, 16, and 20).

Figure 2D shows that the class 1 an3 allele W2018 generates predominantly excision products that are 7 bp longer than is the wild-type sequence (cf. lanes 1 and 2), which is consistent with the presence of footprints (cf. Figures 2A and 2D). When we used a cloned W2018 DNA fragment as a template, these products were not detected, confirming that they result from in vivo transposon excisions and not from PCR artifacts (data not shown). In W2018/W62 hemizygotes, no gross alterations in the spectrum of W2018-derived excision products were observed (Figure 2D, lane 3; note that W62 is a complete deletion that does not generate PCR products). An an3– sibling from the same cross yielded only the an3 fragment with a 7-bp footprint, whereas the dTph1-containing an3 fragment was not detectable (Figure 2D, lane 4), indicating that dTph1 had excised in germinal tissue of the an3-W2018 parental plant. Similar results were obtained for other class 1 an3 alleles (W29, W2258, W2303, G5m, and W138A; data not shown).

In homozygotes of the class 2 alleles S857, R134, and S205, excisions of the downstream element preferentially yielded products of a wild-type size (consistent with the absence of a footprint), although a small amount of slightly larger sized products was detectable (Figure 2D, lanes 6, 10, and 14). However, when the same alleles were hemizygous over the W62 deletion, the wild-type-sized products were no longer detectable, whereas the larger excision products remained (Figure 2D, lane 7) or slightly increased in abundance (lane 11). The absence of wild-type excision products in these hemizygotes made it possible to clone and sequence some of the larger excision products. For R134/W62 and S857/W62 plants, this revealed that they contain a footprint similar to those in excision products of class 1 alleles, although a small amount of slightly larger products were detected (Figure 2D, lane 10). Thus, the absence of wild-type excision products in W2092/W62 hemizygotes is accompanied by a relatively strong increase in (reversion) products with a footprint, which can account for the relatively high reversion frequency seen in W2092 hemizygous flowers.

We also examined excisions produced by the upstream element of the class 2 allele R134. Figure 2D shows that excisions of this dTph1 copy yielded products that were longer than the wild-type sequence both in R134/R134 homozygotes (lane 22) and in R134/W62 hemizygotes (lane 23). Apparently, excisions of this dTph1 element preferentially result in the formation of footprints independent of the homologous an3 allele, similar to excision of the dTph1 element in class 1 alleles.

We conclude from these data that in homozygotes, the downstream dTph1 element of a class 2 allele can be eliminated by either of two mechanisms. One mechanism, which is used infrequently, resembles the classical mechanism by which class 1 alleles revert, because it yields typical footprints and is not suppressed in a cross with the line W62.

**Figure 4.** Excision Products Generated by Class 2 an3 Alleles in a Hemizygous Condition.

Sequence analysis of PCR-amplified somatic excision products produced by the downstream element of a class 2 an3 allele in a hemizygous condition. Sequences of the wild-type allele, the class 2 allele harboring the dTph1 insertion, and the excision products are as given in Figures 2A to 2C.
However, class 2 alleles revert preferentially by way of a second, novel mechanism that restores the wild-type sequence and becomes suppressed after a cross with W62.

**Instability of Class 2 an3 Alleles Is Homolog Dependent**

The petunia lines W40 and W39 also contain the W62 deletion allele, but they are in a different genetic background. Crosses between homozygotes of class 2 alleles and W40 or W39 also produced progeny in which reversions of the S205, S857, and R134 alleles were suppressed, making it unlikely that suppression was caused by an unknown gene in the background of these lines (data not shown). To test whether the observed suppression of class 2 reversions was due to the complete absence of an3 sequences in line W62, we examined whether stable recessive an3 alleles that retained different parts of the an3 gene would cause suppression of class 2 reversions. Therefore, we crossed homozygotes for class 1 and 2 unstable alleles with homozygotes for the alleles S206 and W1006. Both of these an3 alleles were isolated in the W138 background and contain relatively large chromosome rearrangements (inversion/translocation and a deletion, respectively), with breakpoints in the an3 locus (Figure 1; van Houwelingen et al., 1998). These crosses produced progeny that were essentially similar to those produced by the crosses with W62 (Table 1). Each progeny plant was analyzed using PCR to determine which harbored the original class 1 or class 2 unstable an3 allele and which harbored excision derivatives.

Table 1 shows that plants harboring a class 1 an3 allele heterozygous with either S206 or W1006 have spotted flowers (an3mut). By contrast, flowers that harbored one of the class 2 alleles S857, R134, or S205 heterozygous with either S206 or W1006 were white (an3-), indicating that reversions of the class 2 alleles were suppressed. Analysis of the PCR-amplified excision products produced by the downstream dTpH1 copy of S857 and R134 on high-resolution gels showed that excision products with a wild-type size were strongly reduced in heterozygotes with S206 (data not shown), similar to the results described above for S857 and R134 hemizygosites. The fourth class 2 allele, X2092, specified an an3mut phenotype when heterozygous with W1006 or S206, similar to the X2092/W62 hemizygosites described above.

Next, we crossed class 2 and class 1 alleles with plants harboring the W2018FT or W29FT alleles of an3. The two latter alleles were isolated as germinal excision derivatives of W2018 and W29, respectively, and only contain small sequence alterations (footprints) in the otherwise intact an3 gene (Figures 1 and 2A). The results of these crosses (Table 1) showed that W29FT and W2018FT also were capable of suppressing reversions of S857, R134, and S205 in heterozygotes (see the example in Figure 3G) but not of X2092 or any of the class 1 alleles (see the example in Figure 3D). These suppressed reversion frequencies were extended to germinal tissues because no An3+ revertants could be recovered in subsequent progeny of these plants (see below).

Because many of the S205/S206 and S857/S206 progeny were homozygous for the unstable an1-W138 allele, we were able to examine whether reversions at another locus also were suppressed in the same plants. The an1 locus controls transcription of a set of anthocyanin biosynthetic genes (Quattrrochio et al., 1993), and the unstable an1-W138 allele contains a single 284-bp dTpH1 insertion that is eliminated by the classical mechanism, because footprints are generated upon its excision (C. Spelt and R. Koes, unpublished data). Because none of the an3- alleles blocks anthocyanin synthesis completely (even W62/W62 flowers are very pale rather than completely white), we still were able to observe phenotypic reversions of the an1-W138 allele (an1- cells are completely white), even in an3- plants. These observations were confirmed by PCR experiments, which also showed that the dTpH1 copy in an1-W138 was still excising in somatic and germinal tissues of plants in which reversions of S205, R134, and S857 were suppressed. Additional crosses (data not shown) demonstrated that the lines harboring W62 and S206 did not suppress the reversions of the rt-Vu15 allele, which harbors a dTpH1 insertion in the rhamnosylation at three (rt) locus (Kroon et al., 1994).

In summary, these data show that any stable recessive an3 allele can suppress reversions of a class 2 allele in trans-heterozygotes, making it unlikely that this suppression is due to the absence of an3 sequences alone. Furthermore, these data show that the observed suppression is specific for class 2 an3 alleles, because instability of an1, rt, and class 1 an3 alleles is not suppressed in the same crosses.

**Loss of the Upstream dTpH1 from a Class 2 Allele**

**Results in a Novel Allele That Behaves as a Class 1 Allele**

The four class 2 alleles all harbor two dTpH1 elements, suggesting that this structure may be responsible for their unusual behavior. If true, this predicts that loss of the upstream dTpH1 copy would change the genetic behavior of a class 2 allele into that of a class 1 allele. In five cases, our crosses yielded progeny with an exceptional phenotype that suggested that the original class 2 allele had indeed changed into a new allele with a class 1 behavior (Table 2). These plants were examined by PCR to determine the structure of the an3 allele(s).

Family V2307, obtained from a cross between S857/S857 and W29FT/W29FT parents, consisted of 17 An3+ plants (germinat revertants that had lost the downstream dTpH1 element of S857; data not shown), 15 an3- plants (suppression of S857 reversions; see Figure 3G), and one plant with an3mut flowers (Figure 3H). Sequence analysis of PCR products amplified from the single an3mut plant showed that the upstream dTpH1 element of S857 had germinally excised, creating a 7-bp footprint (see Figure 2C, allele V2307),
whereas in the transposon sequences and the insertion site of the downstream element, no changes were observed (data not shown).

A similar phenomenon was seen in family X2330, originating from a cross between R134/R134 and W2018FT/W62 plants. This family consisted of 13 wild-type revertants (An3mut; resulting from excision of the downstream element of an3-R134), 22 plants with white flowers (an3-, due to suppression of R134 instability by the W2018FT or W62 allele), and one plant with spotted flowers (an3mut). Sequence analysis of the PCR-amplified an3 allele in this plant showed that the upstream dTph1 element of R134 had excised, creating a 7-bp footprint (see Figure 2C, X2330). No alterations were found in the transposon sequences or the insertion site of the downstream element (data not shown).

Family V1519 was generated by crossing S205/S205 and W62/W62 parents at a time when we were still unaware of the upstream dTph1 copy in the S205 allele. PCR analysis showed that the six An3mut plants contained a germinal excision derivative of S205 that lacked the downstream dTph1 copy, whereas this element was still present in the 21 progeny plants that were scored as an3-. However, hindsight shows that one of these 21 must have displayed a low number of very small revertant spots (similar to the V512/W62 and T3463/W62 plants described below) that we did not notice at that time. After we had discovered the upstream dTph1 copy, we reanalyzed the DNA samples from V1519 and found this element to be present in all progeny, except for one plant that had initially been (mis)scored as an3-. We then germinated the seeds of this plant and obtained an3mut progeny (data not shown), whereas self-pollinating S205/W62 siblings from the V1519 family gave only an3- progeny (see below).

In two progenies obtained by self-pollination of S205/S205 plants, heterozygous plants were identified (by PCR) that harbored S205 and a derived excision allele lacking the upstream dTph1 element (alleles T3463 and V512; see Figures 1 and 5). Sequence analysis showed that T3463 and V512 contained a footprint at the former insertion site of this dTph1 element (Figure 2C, an3-T3462 and an3-V512), whereas no changes were observed in the sequence or insertion site of the downstream element (data not shown). When homozygous, the reversion frequency of the T3463 and V512 alleles was so low that it was barely detectable in the W138 (an1mut) genetic background in which they were maintained. Therefore, V512 and T3463 are depicted as nearly stable recessive alleles in Figures 1 and 5. Electrophoretic analysis of the complete spectrum of excision products showed that excision of the downstream element from S205 yielded, in the large majority of cases, a product of wild-type size and, at a much lower frequency, products with a footprint (Figure 6A, lanes 2 and 4). In V512/V512 homozygotes, the wild-type-sized excision products were strongly diminished, but the excision products with a footprint were still detectable (Figure 6A, lane 5). The fraction of footprints (±3, ±6, or ±9 bp) that restore the reading frame and potentially allow reversion makes up a small fraction of the complete collection of footprints generated, thus explaining the low reversion frequency in V512/V512 homozygotes. This indicates that V512 now behaves as a typical class 1 allele.

To test whether T3463 and V512 displayed other features associated with class 1 alleles, we crossed T3463/S205 and V512/S205 heterozygotes to W62/W62 homozygotes. In both cases, half of the progeny had weakly unstable (an3mut) flowers (one to five small spots per flower, representing a low number of late reversions), whereas the flowers of the other half had no revertant spots at all and were scored an3- (Table 2, families Z2345 and Z2046, respectively). PCR analysis showed that all S205/W62 progeny had an3- flowers, indicating suppression of S205 reversions. The progeny with weakly unstable an3mut flowers were genotypically V512/W62 or T3463/W62. Similar results were obtained when the V512/S205 plant was crossed to an an3-W29FT homozygote (Table 2, family Z2045). Apparently, the low reversion frequencies of V512 and T3463 (which are relatively easily detected in this An1- genetic background) are not suppressed by the W62 or W29FP allele, whereas the high

| Table 2. Conversion of Class 2 in Class 1 an3 Alleles |
|-----------------|-----------------|-----------------|
| Family          | Parents          | Progeny with Phenotypea          |
| An3-           | an3-            | an3mut          |
| V2307          | S857/S857 × W29FT/W29FT | 17 | 15 | 1*  |
| X2330          | R134/R134 × W2018FT/W62 | 13 | 22 | 1*  |
| Z2046          | V512/S205 × W62/W62 | 0  | 25 | 21* |
| Z2045          | V512/S205 × W29FT/W29FT | 0  | 21 | 17* |
| Z2345          | T3463/S205 × W62/W62 | 0  | 6  | 6a  |
| V1519          | S205/S205 × W62/W62 | 6  | 20 | 1b  |

aBoldface numbers denote plants that harbor the stabilized class 2 allele. The asterisks indicate plants in which the upstream dTph1 copy of the corresponding class 2 an3 allele is absent.
bInstability was noticed in selfed progeny of this plant.
reversion frequency of S205 is completely suppressed. However, when we compared the complete spectrum of PCR-amplified excision products in S205, V512, and T3463 hemizygotestes, we did not detect consistent differences that could account for the differing an3- and weak an3mut phenotypes (Figure 6A, lanes 6 to 9), most likely because the V512- or T3463-derived reversion products made up too small a fraction of the total amount of PCR products that were recovered in these assays.

Taken together, these data show that loss of the upstream dTph1 element of a class 2 allele results in a new allele that genetically behaves as a class 1 allele, because (1) the frac-

---

**Figure 5.** Genetic Analysis of the Suppression of an3-S205 Instability.

Genetic analysis of the suppression of an3-S205 by the derivative alleles an3-T3463 and an3-V512 is shown in the form of a pedigree. Each progeny (boldface numbers) resulted from a single pollination of parents with known genotypes, as indicated by the arrows. x denotes a self-fertilization; xSS denotes an outcross to unstable S205/S205. For each family, the flower phenotypes are shown as in Figures 1 and 3. For simplicity, the low numbers of revertant spots specified by an3-T3463 and an3-V512 are ignored and the corresponding phenotypes are indicated as white. For each phenotypic class, the genotype (determined by PCR) of several randomly selected individuals is shown in italics; for each genotype, the number of plants is given in parentheses. The different alleles are abbreviated as follows: S, S205; S*, S205* (suppressed reversions); T, T3463; V, V512; (+), revertant allele that lost the downstream dTph1 copy of S205.
tion of excision derivatives with wild-type sequence is strongly diminished and (2) it specifies spotted (an3mut) flowers when heterozygous over a stable recessive an3 allele.

Reactivation of Suppressed Class 2 Alleles

If the suppressed reversion of class 2 alleles in trans-heterozygotes is imposed by the stable recessive an3 allele on the homologous chromosome, one would expect that the reversions are restored when the class 2 allele is made homozygous again. Therefore, we self-pollinated heterozygous plants harboring various combinations of the class 2 alleles S857, R134, and S205 and the stable recessive alleles W62, S206, and W1006 and analyzed the progeny (Table 3). Among a total of 777 progeny plants resulting from 24 different self-pollinations, no An31 plants were found. This indicated that reversions of the class 2 alleles in the heterozygous parent were inhibited in germinal tissues of the parental plants, as they are in their somatic tissues.

Only 24 of these 777 progeny plants displayed some somatic An3+ reversions (i.e., spots). In all of these cases, an3 instability appeared to be irregular, because we found on the same plant flowers with an3mut and an3" phenotypes. Furthermore, the number of revertant spots on an3mut flowers was usually lower than the number on flowers of the parental class 2 homozygous lines. PCR analysis confirmed that all the plants bearing an3mut flowers were indeed homozygous for the class 2 allele. However, the large majority of class 2 homozygotes (identified by PCR) remained phenotypically an3" and did not show any An3+ reversions. This suggested that some unknown factor prevented the reactivation of instability in most (~85%) of the class 2 allele homozygotes.

Suppression of an3-S205 Reversions Has Epigenetic Features

To gain further insight into the mechanism that prevents reversion of a class 2 allele, we focused on the interactions between the S205 allele and the derived alleles T3463 and V512 (see Figures 1 and 2C for their structures). We selected these alleles for the following reasons. First, all three are in the same genetic background (W138). Second, suppression of S205 reversions is very stable in progeny (Table 3). Third, the reversion frequency of T3463 and V512 is very low, enabling us to detect the relatively high reversion frequency of a S205 allele in heterozygotes against a very low background of V512- or T3463-derived reversions. In fact, the V512 and T3463 reversion frequencies are completely negligible compared with those of S205 (see Figures 3I to 3L); therefore, the V512 and T3463 flower phenotypes are, for simplicity, indicated as “white” (an3") in Figures 1 and 5.

The V512 allele was identified in family V512 in a heterozygous (V512/S205) plant. This heterozygote had white flowers (an3"), indicating that V512 suppressed reversions of the S205 allele on the homologous chromosome (cf. Figures 3I and 3J). Hereafter, we mark such a suppressed allele with an asterisk (S205*) to differentiate it from a frequently reverting S205 allele, even though we have not been able to detect structural differences between S205 and S205* (epi)alleles by using PCR or sequencing. The V512/V512 and V512/S205*

Figure 6. Spectrum of Excision Allele Products Generated by an3-S205 and Derived (Epi)Alleles.

(A) PCR-amplified excision products produced by the downstream dTph1 element of S205, the derived alleles V512 and T3463, and the epiallele S205* were separated on a 6% polyacrylamide gel. Homozygotes as well as heterozygotes over an3-W62 were examined for each an3 allele. Lanes 1 and 2 represent a short exposure of lanes 3 and 4.

(B) Excision products produced by the downstream dTph1 of R134 were analyzed as in (A). The top part of the gel shows the products that contain the dTph1 insertion; at bottom are the excision products. Major excision products with wild-type size or footprints are marked by WT and +7 or +8 (bp), respectively.
progeny obtained by self-pollinating this plant (family Z2043) had an3− flowers, as expected. Also, the S205*/S205* homozygote was an3− (Figure 3K), indicating that reversions were not reactivated after the S205* allele was made homozygous again. When the same V512/S205* parent was outcrossed to an S205/S205 sibling with an an3mut phenotype, the progeny segregated for An3−, an3mut, and an3+ phenotypes (family Z2044). PCR analysis showed that the An3+ plants were heterozygous for the downstream dThp1 insertion, presumably due to a germinal reversion in the S205/S205 parent. The an3− progeny were genotypically V512/S205*, indicating that the V512 allele again prevented reversions of the newly introduced S205 allele (thus marked with an asterisk in Figure 5). The an3mut progeny (see Figure 3L for an example) all appeared to be genotypically S205*/S205.

Crosses with a V512/S205* plant from family Z2043 gave the same result. After self-pollination, the S205*/S205* homozygotes were an3− (family Z2355), whereas outcrosses to an S205/S205 plant yielded S205*/S205 progeny with an an3mut phenotype (family Z2356). We also selected a V512/S205* plant from family Z2044, in which suppression of S205 reversion was established by an independent event. Again, self-pollinations yielded only an3− progeny (family Z2357), whereas outcrosses to an S205/S205 plant with an an3mut phenotype yielded an3mut and An3− progeny (Z2358). The An3− progeny were all heterozygous for the downstream dThp1 insertion, presumably due to a germinal reversion in the S205/S205 parent.

The T3463 allele has the same structure as V512 (Figures 1 and 2C) but arose independently. In a heterozygote, T3463 also suppressed reversions of S205 (thus marked with an asterisk in Figure 5, family T3463), and this suppressed state was maintained when the S205* allele was made homozygous again by selfing (family Z2051). Interestingly, two other plants in the T3463 family displayed an an3− phenotype. PCR analysis showed that they were both homozygous for an an3 allele that was indistinguishable in structure from S205, except that somatic excision products were markedly decreased (data not shown). These suppressed S205* alleles behaved in further crosses in a manner identical to the suppressed S205* alleles described above. Thus, repeated self-fertilizations yielded only an3− progeny, even after three generations (families V2308, V2310, and progenies thereof), whereas outcrosses to homozygotes for an unstable S205 allele produced an3mut progeny (families V2309 and V2311). The simplest explanation for these results is that the T3463 allele arose in germinal cells of the parental plant, leading to the suppression of reversions of the S205 allele on the homologous chromosome and maintenance of this state in the gametes and progeny originating therefrom (family T3463).

It is possible that in the S205*/S205 heterozygous plants produced by the various outcrosses, both alleles contributed to the spotted an3mut phenotype (implying that S205* had converted back to S205) or that only S205 contributed to the revertant spots, whereas S205* remained in its stabilized state. Molecular analyses of the S205*/S205* heterozygotes themselves cannot distinguish between these possibilities, because excision products of either of the two alleles would have the same structure. Instead, we self-pollinated S205*/S205* plants from three different families to examine whether the stabilized S205* could be recovered in the progeny. All resulting families (A2433 and data not shown) contained a high number of an3− plants, suggesting that the instability of the S205* allele had not been reactivated in the S205*/S205 parent.

Similar to the crosses described above, 1 to 5% of the progeny resulting from self-fertilization of frequently revert- ing R134/R134 plants exhibited a reversion frequency that was so low (i.e., weakly an3mut) that it was difficult to distinguish an an3− phenotype. PCR analysis of two such plants showed they were both homozygous for an an3 allele that was indistinguishable from R134, except that the amounts of somatic excision products were markedly decreased (data not shown). This suggests that, in addition to S205, R134 also could change into a “stabilized” (epi)allele, which we designated R134*, analogous to the (epi)allele S205*.

To analyze why the reversion frequencies became so strongly suppressed in S205* and R134* heterozygotes and homozygotes, we analyzed the spectrum of excision products produced by the downstream element. Figure 6A shows that in S205*/V512, the fraction of wild-type-sized excision products was markedly reduced when compared with S205/S205 homozygotes (cf. lanes 10 and 11 with lanes 2 and 4) and was of an essentially similar composition as that produced by V512/V512 homozygotes (lane 5) or V512/W62 hemizygotes (lane 7). Because in S205*/S205* homozygotes again a similar pattern of excision products was found (Figure 6A, lanes 12 and 13), we assume that S205* and V512 contribute similarly to the collection of excision products found in heterozygotes. These data indicate that the essential difference between S205 and its derived epiallele S205* is that the latter produces many fewer wild-

### Table 3. Reactivation of Repressed an3 Unstable Alleles

<table>
<thead>
<tr>
<th>Parent Used for Self-Fertilization</th>
<th>No. of Distinct Parents Tested</th>
<th>Progeny with Phenotypea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>an3+</td>
</tr>
<tr>
<td>an3-R134/an3-W1006</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>an3-R134/an3-W62</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>an3-S857/an3-W62</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>an3-S857/an3-S206</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>an3-S205/an3-W62</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>an3-S205/an3-S206</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>0</td>
</tr>
</tbody>
</table>

aPlants in which only some flowers showed an3 instability were scored an3mut.
A Novel Mechanism for Transposon Elimination

Class 1 an3 Alleles Revert by a Classical Mechanism

The genetic behavior of class 1 unstable an3 alleles, harboring a single dTph1 or dTph2 insertion, is very similar to that of unstable alleles in other plants (Figure 7A). Excisions of Ac and Ds elements result, in the large majority of cases, in complete removal of transposon sequences and the formation of a footprint (Baran et al., 1992; Kunze et al., 1997). We refer to this mechanism of transposon excision and subsequent break repair as the classical mechanism. Most likely, the DSBs that excise the transposon occur by hairpin formation at the ends of the flanking DNA (Coen et al., 1986; Kennedy et al., 1998), but little is known about the mechanism by which the break in the chromosome is repaired. At a low frequency, Ac/Ds insertions produce excision alleles that lack a footprint, and it has been suggested that these may be formed by a distinct mechanism (Scott et al., 1996).

The simple class 1 an3 alleles, harboring a single dTph1 or dTph2 insertion, behaved very similarly. In the majority of cases, transposon excision resulted in the formation of a footprint, whereas at a low frequency, excision alleles without a footprint could be found, especially when excision products were phenotypically selected (Figure 2A). Similar results were obtained with dTph1 insertions at four other petunia loci (Souer et al., 1996, 1998; de Vetten et al., 1997; Alfenito et al., 1998). Furthermore, the footprints in maize, snapdragon, and petunia excision alleles all have a similar structure, consisting of remainders of the target site duplication that are often separated by inverted repetitions of the target site. Similar to other Ac-like transposons in plants, but unlike CACTA elements (Coen et al., 1986), dTph1 and dTph2 generate footprints that lack one nucleotide in the inverted repeat (Figure 2A; see also Souer et al., 1996, 1998; de Vetten et al., 1997; Alfenito et al., 1998).

DISCUSSION

It is by now widely accepted that the activity of a gene is not determined by its sequence alone and that additional, epigenetic factors (e.g., changes in methylation or chromatin structure) can heritably modulate its expression (discussed in Martienssen and Richards, 1995; Riggs and Porter, 1996; Henikoff and Matzke, 1997; Lewin, 1998). The activity of transposon-encoded transposase genes can be subject to epigenetic regulation, as are immobile cellular genes. This regulation can be visualized by the presence or absence of revertant spots (reviewed in Fedoroff et al., 1995; Kunze et al., 1997). Here, we provide evidence that epigenetic mechanisms may also affect a DNA recombination mechanism involved in transposon excision and/or break repair. We show that a dTph1 transposon can be eliminated from the host gene by either one (classical) or the other (novel) recombination mechanism, and that the latter mechanism depends on a partially epigenetic interaction between at least three dTph1 copies in two homologous chromosomes, as summarized in Figure 7.
Our results indicate that reversion of class 1 alleles is largely insensitive to the structure of the an3 allele on the homologous chromosome. The absence of an3 sequences on the homologous chromosome (as in hemizygotes with an3-W62) did not reduce the spotting frequency or cause alterations in the spectrum of excision products (Figure 2D). We also did not observe upregulation of the spotting frequency when intact an3 sequences were present opposite the insertion sites (as in heterozygotes harboring the S206 or W29FT allele). Thus, gene conversion mechanisms, like DSB repair, play at most a minor role in reversion of class 1 an3 alleles.

Although a single dTph1 insertion may in principle generate an array of different footprints, our data indicate that certain footprints are preferred over others. For instance, excision of dTph1 from W2018 preferentially results in a 7-bp footprint (Figures 2A and 2D) that causes a frameshift, consistent with the observation that most excisions result in a stable an3 allele (Table 1; data not shown). Yet, the revertant spots on W2018 flowers show that different footprints, which restore an3 function, also can occur but at a lower frequency. Analysis of a large number of Ac and Ds excisions from different positions at the maize waxy locus or Arabidopsis transgene(s) revealed a similar preference for certain footprints over others, which is determined at least in part by DNA sequences immediately flanking the transposon insertion (Scott et al., 1996; Rinehart et al., 1997). When a footprint is formed, its effect on the phenotype is also dependent on the position in the host gene; +7-bp footprints are tolerated in an intron but not in the coding sequence. Thus, two distinct phenomena, both dependent on the insertion site of the transposon, determine the actual reversion frequency, which can explain why different class 1 an3 alleles display such enormously different reversion frequencies.

Class 2 an3 Alleles Revert by a Novel Mechanism

Four unstable an3 alleles, designated class 2, appear to revert by an unusual (novel) mechanism that differs from the classical reversion mechanism of class 1 alleles in several aspects (Figure 7B). First, the germinal reversion frequency of all class 2 alleles is very high (30 to 50%) compared with that of any of the class 1 alleles (Table 1). Second, in the large majority of excision products the wild-type sequence is restored and products with a footprint are very rare (Figure 2), explaining why the reversion frequencies of class 2 alleles vary so little with the insertion site. Third, the reversion frequency of class 2 alleles in germinal and somatic tissues depends on an interaction with the an3 allele on the homologous chromosome (Table 1 and Figures 2, 3, and 5).

A homolog-dependent reversion frequency is the hallmark of the DSB repair mechanism by which transposon-excision gaps are repaired in the germ line of Drosophila and C. elegans (Engels et al., 1990; Plasterk, 1991). If reversion of a class 2 an3 allele would involve DSB repair, the reversion frequency should depend on the an3 sequences in the homologous an3 allele, but this is apparently not the case. First, all stable recessive an3 alleles, regardless of which an3 sequences they contain, inhibit reversion of the class 2 an3 allele in the homologous chromosome (Table 1). Second, the position of the mutation in the stable an3 allele, relative to the two dTph1 insertions in the class 2 an3 allele, does not seem to matter. For instance, in W29FT/S205 heterozygotes, the dTph1 copies are on either side relative to the lesion in W29FT, whereas in W29FT/S857 or W29FT/R134, both dTph1 copies are upstream of the footprint. Yet, both configurations result in suppressed reversion frequencies, whereas a DSB repair model would have predicted an increase. Third, in PCR assays designed to detect the conversion of marked sequences (e.g., the Stul restriction site created by the footprint in W29FT) from a stable recessive an3 allele into the class 2 allele on the homologous chromosome, we could not detect gene conversion products (A. van Houwelingen and R. Koes, unpublished results). Although these data do not exclude the possibility that reversion of class 2 an3 alleles requires an3 sequences in the homologous chromosome, they do show that an3 sequences alone are not sufficient. In fact, our data show that the class 2 allele senses a dTph1 copy in the an3 locus of the homologous chromosome (see below). None of these characteristics fits with a DSB repair model, implying that class 2 an3 alleles revert by a different, novel mechanism.

Analysis of Ds excisions from six different positions in the maize waxy (wx) gene showed that one allele (wx-C28) produced predominantly excision alleles with a wild-type sequence (~66% of the excisions) rather than a footprint (~33% of the excisions), and this was taken as an indication that the two types of product were generated by two distinct mechanisms (Scott et al., 1996). It is possible that the wx-C28 allele reverts by the same novel mechanism as the class 2 an3 alleles described here. It will be interesting to determine if wx-C28 contains, analogous to the class 2 an3 alleles, a second Ds element at or near the wx locus and whether its reversion frequency is dependent on the wx allele in the homologous chromosome.

Reversion of a Class 2 an3 Allele Requires an Interaction among at Least Three dTph1 Elements

As outlined above, the reversion of class 2 an3 alleles occurs by a novel mechanism that is distinct from the mechanisms of transposon excision and break repair discussed above. As a first step toward unraveling this mechanism, it is important to identify which components are involved and how they are involved. Figure 7B shows a generalized structure of the an3 locus in a class 2 homozygote and summarizes the genetic interactions between different dTph1 copies that appear essential for the elimination of one dTph1 copy (copy II) by the novel mechanism.
The key feature that distinguishes class 2 an3 alleles from class 1 alleles is the presence of a second dTph1 insertion (copy I; Figure 7) that by itself does not affect the flower phenotype (although it does reduce the amount of an3 transcripts; A. van Houwelingen and R. Koes, unpublished data). Several observations indicate that the elimination of dTph1 copy II by the novel mechanism indeed depends on the presence of copy I in the same allele. First, excision of dTph1 copy I from S205 yields novel alleles (V512 and T3463) with a barely detectable reversion frequency because they preferentially generate excision products with a footprint (Figure 6). Second, the spectrum of excision products does not show any major alterations when the V512 allele is in a homozygous or hemizygous condition (Figure 6). Third, excision of dTph1 copy I from three different class 2 alleles results in new alleles that specify an unstable (an3mut) phenotype when heterozygous over a stable recessive an3 allele (Figure 3H and Table 2). These data indicate that upon loss of dTph1 element Ia (Figure 7), the class 2 allele changes its reversion behavior into that of a class 1 allele, which implies that dTph1 copy I is required for the element downstream in the same allele (dTph1 copy II) to be eliminated by the novel mechanism.

Strikingly, the interaction between dTph1 copies I and II is not symmetrical. dTph1 element I appears to be preferentially removed from a class II allele by the classical mechanism, because excisions result independently in footprints from the structure of the an3 allele on the other chromosome (Figures 2C and 2D). Possibly, the directionality of this interaction is specified by the relative orientations of both dTph1 copies, but because the relative orientation of dTph1 copies is the same in all four class 2 an3 alleles (Figure 1), we could not verify that experimentally.

Although the presence of dTph1 copy I is necessary, it is clearly not sufficient to eliminate the downstream copy II by the novel mechanism, because sequences in the homologous an3 allele also are required. The one common feature shared by all an3 alleles that suppress reversions of a homologous class 2 allele in trans is the absence of dTph1 element Ia, whereas the only an3 allele that does not provoke the suppression, S205*, does contain element Ia. The effect of dTph1 copy Ia on the reversion frequency of the homologous allele is most clearly seen in plants in which the S205(*) allele is heterozygous over V512 or T3463. In these heterozygotes, both the reversion frequency and the amount of wild-type-sized excision products are markedly decreased when compared with those in S205/S205 or S205(*) reversion by the novel mechanism. This implies that the absence of dTph1 copy Ia (as in V512 and T3463) does not affect only the elimination of the element downstream in the same allele (copy Ia) but also that of the downstream element (II) in the homologous S205(*) allele. Thus, elimination of dTph1 element II somehow requires the presence of dTph1 copy Ia on the homologous chromosome. However, we cannot conclude from our data whether this interaction is direct or indirect, for instance, via dTph1 copy I (Figure 7).

To test whether dTph1 copy II also senses dTph1 copy Ia on the homologous chromosome, one needs to examine the instability of a class 2 allele in a heterozygote harboring an allele like an3-W138A or a reversion allele. The difficulty is that in such a plant, the reversion products produced by the class 2 alleles cannot be discerned (either by PCR or by phenotype) from the an3 allele (W138A or R27) on the other chromosome.

**Suppression of Class 2 Allele Reversions Fits the Genetic Definition of Paramutation**

The phenomenon that one allele induces a directed and heritable alteration of the other allele in a heterozygote has been termed paramutation (Brink et al., 1968). In plants, several examples of paramutation are known, all of which concern the silencing of an endogenous gene (Hollick et al., 1997). Paramutation at the r, b, and pl loci of maize, all encoding regulators of the anthocyanin pathway, differs in several aspects, which suggests that distinct molecular mechanisms may fit the operational (genetic) definition of paramutation. For instance, paramutation at the (complex) r locus requires the presence of repeated gene sequences and results in an increased methylation of the paramutated allele (Kermicle et al., 1995). By contrast, paramutation of b and pl does not require gene repeats, and no alterations in the methylation pattern of paramutated b alleles could be detected (Hollick et al., 1995; Patterson et al., 1995).

Our data indicate that the interaction between dTph1 copies II and Ia has an epigenetic character and that the inactivation of the novel transposon excision repair mechanism by which a class 2 predominantly reverts fits the (genetic) definition of paramutation. When dTph1 copy Ia is absent, the elimination of dTph1 copy II from the homologous allele by the novel mechanism is suppressed and the class 2 allele undergoes a transition to a different “state” (essentially becoming a class 1 allele) that is heritable over at least three generations (Figures 2, 4, 5, and 6). How (methylation or otherwise?) and where (in dTph1 and/or an3 sequences?) the suppressed state of a class 2 allele is remembered remain to be determined.

The epigenetic suppression of the novel transposon excision-repair mechanism differs in several important aspects from the previously described examples of paramutation or epigenetic inactivation of transposons. First, the target process is not transcription of a transposase gene or an immobile gene but one or more steps in a DNA recombination process. Second, the epigenetic suppression specifically blocks elimination of the transposon by the novel mechanism, without blocking elimination by a second independent mechanism (the classical mechanism). Third, unlike paramutated r, b, or pl (epi)alleles, a paramutated (epi)allele such as S205* does not change the behavior of a frequently reverting class 2 allele on the homologous chromosome.
METHODS

Genetic Analyses and Plant Material

The isolation and the molecular analysis of the unstable anthocyanin3 (an3) alleles shown in Figure 1 have been described elsewhere (van Houwelingen et al., 1998). The an3-W62 allele was maintained in three different Petunia hybrida lines (W62, W39, and W40). We used line W62 as a parent for crosses unless stated otherwise. Most an3 alleles were isolated in line W138. This line also contains an unstable allele (an1-W138) at the an1 locus. Some unstable an3 alleles were maintained in an an1<sup>W62</sup> unstable background, whereas other alleles were maintained in an an1<sup>-</sup> revertant background. It was easy to distinguish instability of an1 and an3 because an1 controls pigmentation in a cell-autonomous manner and produces revertant spots with a sharp border, whereas an3 acts non-cell-autonomously and produces revertant spots with a fuzzy border (Koes et al., 1995; van Houwelingen et al., 1998). Because none of the mutant an3 alleles blocks pigmentation completely (an3 mutants have very pale flowers rather than pure white), an1 instability was still detectable in an3<sup>-</sup> flowers.

All petunia plants were grown in a single greenhouse that was essentially free of pollinating insects. The plants used for this study were grown side by side with a wide array of other petunia lines, in use for other studies, that harbor mutations in various genes controlling the development and pigmentation of flowers and inflorescences. For crosses, we selected closed flower buds, which were still virgin because anthesis had not yet occurred; we then opened the corolla with a longitudinal cut, removed the anthers, and transferred pollen from another (open) flower of the same (self-pollination) or another (outcross) plant to the pistill. Subsequently, the fertilized flower was reclosed by wrapping a small rope around the corolla. Under these conditions, the opportunity for pollen contamination is virtually nonexistent.

For virtually every cross, we analyzed some of the progeny by polymerase chain reaction (PCR) to establish the an3 genotype and, when necessary, the an1 genotype. In case it was unclear whether two dTph1 elements detected at the an3 locus of transheterozygotes were in the same allele or in the two different alleles, the plant was considered to represent instability of an1 and an3.

DNA Methodology

Plant genomic DNA was extracted from leaves as described previously (Souer et al., 1995). PCR analysis of the an3 locus was performed with primers complementary to appropriate regions of the an3 locus, as described elsewhere (van Houwelingen et al., 1998).

Somatic excision products were isolated by PCR amplification of an3 fragments from homozygous plants with specific unstable an3 alleles and cloned in pBluescript SK<sup>+</sup> or SK<sup>-</sup> (Stratagene, La Jolla, CA). Recombinants that lacked transposon sequences were considered to originate from somatic excisions. Cloned excision products that had the same sequence were considered only to represent independent excision events when they had been amplified from different plants.

All sequencing was conducted by asymmetric PCR with fluorescent M13 primers, employing a DNA sequencer (model 370A; Applied Biosystems, Foster City, CA).

Analysis of Excision Products on Sequencing Gels

To analyze the full spectrum of excision products generated by a dTph1 insertion, we performed a PCR reaction with primers flanking the transposon insertion. Amplification products were purified with Magic PCR Preps DNA Purification System (Promega) to remove excess primers. Subsequently, six (linear) amplification cycles were performed using 2 ng of purified fragment as a template and a single-nested primer that had been end labeled with phosphorus-32. The radiolabeled products were separated on 6% polyacrylamide (sequencing) gels, using sequence ladders of DNA fragments with a known sequence as a size marker. For each an3 allele, this analysis was repeated with DNA preparations isolated from different plants to ensure that major excision products did not result from an occasional (early) excision event that had produced a large sector.

For the different an3 alleles, the following primer combinations were used: an3-1, an3-12, and 32P-labeled an3-5 for an3-W2018; an3-2, an3-5, and 32P-labeled an3-14 for an3-S857; an3-1, an3-12, and 32P-labeled an3-5 for an3-R134; an3-11, an3-12, and 32P-labeled an3-6 for an3-S205; an3-13, an3-6, and 32P-labeled an3-8 for an3-X2092; an3-4, an3-14, and 32P-labeled an3-3 for the up-stream element in an3-R134.

The sequences of the primers are as follows: an3-5, 5'-CCGGATCTCTAGATGGCTAGAGAC-3'; an3-6, 5'-CCGGATCTCTCTCCAAACCCTATAG-3'; an3-11, 5'-GGGCAAGACAGACTCTCATGAT-G-3'; an3-13, 5'-CTCCACATTGCTCCGAG-3'; and an3-8, 5'-GGATCTCTTTATTACGTGTTTGCGG-3'. The sequences of the other primers have been described elsewhere (van Houwelingen et al., 1998).

ACKNOWLEDGMENTS

We thank Toon Stuitje and Maike Stam for critical comments on the manuscript. We are grateful to Daisy Kloos for her assistance in the genetic experiments, to Pieter Hoogeveen and Martina Meesters for their care of the plants, and to Wim Bergenhenegouwen and Fred Schuurof for photography. This research was supported by The Netherlands Technology Foundation (STW), with financial aid from The Netherlands Organization for the Advancement of Research (NWO).

Received December 7, 1998; accepted April 12, 1999.

REFERENCES


