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Phenotypic effects of short-range and aberrant transposition in *Antirrhinum majus*

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Abstract

We describe two novel ways in which changes in gene expression in *Antirrhinum majus* may arise as a consequence of the Tam3 transposition mechanism. One involves excision of Tam3 from the *nivea* gene promoter and insertion of two new Tam3 copies 3.4 kb and 2.1 kb away, on either side of the excision site. One of the new insertions is in the *nivea* coding region and completely blocks production of an active gene product. This allele probably arose by a symmetrical double transposition, following chromosome replication. The second case involves a small deletion at one end of Tam3 in the *pallida* gene, flanked by a sequence typical of a Tam3 excision footprint. This suggests that the end of Tam3 was cleaved at an early step in an attempted transposition and re-ligated back to its original flanking sequence. The alteration restores some expression to the *pallida* gene, suggesting that the ends of the intact Tam3 element contain components which can actively inhibit gene expression. The implications of these findings for the mechanism of Tam3 transposition and for the effects of Tam3 on host gene expression are discussed.

Introduction

Many unstable alleles conferring variegated phenotypes in plants are due to transposable elements inserted in genetic loci required for pigment biosynthesis. An interesting property of these unstable alleles is that they can give rise to series of derivative alleles conferring altered intensities of pigmentation (reviewed in [18, 20, 3]. Many of the derivatives confer stable phenotypes and have arisen by imprecise excision of transposable elements from the host genes, altering functionally important gene sequences [27, 5]. Other derivatives show altered pigment intensities but still confer variegated phenotypes. This suggests that they retain the transposable element but that its interaction with the host gene is altered. Some of these alleles may arise from structural alterations within the transposable element, as has been shown for a derivative of the *al* locus in maize [26]. It has also been suggested that these derivatives might result from changes in the position of the transposable element in the gene [19]. To investigate further how such alleles are produced, we have studied two derivatives in *Antirrhinum majus* which have comparable but opposite effects on host gene expression when compared to their progenitors.

The *nivea* (*niv*) locus encodes the enzyme chalcone synthase required for the production of red anthocyanin pigment in flowers [32, 30]. The *nivea*^{recurrens}-98 (*niv*-98) allele carries the transposable element Tam3 in the *niv* promoter and gives flowers having a palely pigmented background against which can be seen spots or sites of full red pigmentation [28] (Fig. 1). The pale background indicates that the Tam3 insertion reduces, but does not completely block, *niv* gene expression and the full red spots show that normal gene expression can be restored by excision of Tam3 during flower development. The *niv*-98 allele gave rise to an unstable derivative, *niv*-540, which has a lower rate of Tam3 excision and confers no background pigmentation [1] (Fig. 1). This phenotype suggests that Tam3 has been retained within the gene, but that it now fully prevents chalcone synthase expression unless it excises.

The second allele studied was derived from *pallida*^{recurrens}-2 (*pal*-2) which carries Tam3 in the promoter region of a gene encoding the enzyme dihydroflavonol-4-reductase, required at a late stage in the anthocyanin biosynthetic pathway [4, 15]. The *pal*-2 allele gives a phenotype in which full red sites, produced by excision of Tam3, are seen against an ivory background indicating that Tam3 prevents *pal* expression unless it excises (Fig. 1). The unstable allele *pal*-510 arose from *pal*-2 and gives a phenotype with a reduced frequency of full red sites on a pale red background [5] (Fig. 1). The background pigmentation of *pal*-510 indicates that Tam3 no longer completely prevents *pal* gene expression. The *pal* and *niv* derivative alleles therefore illus-

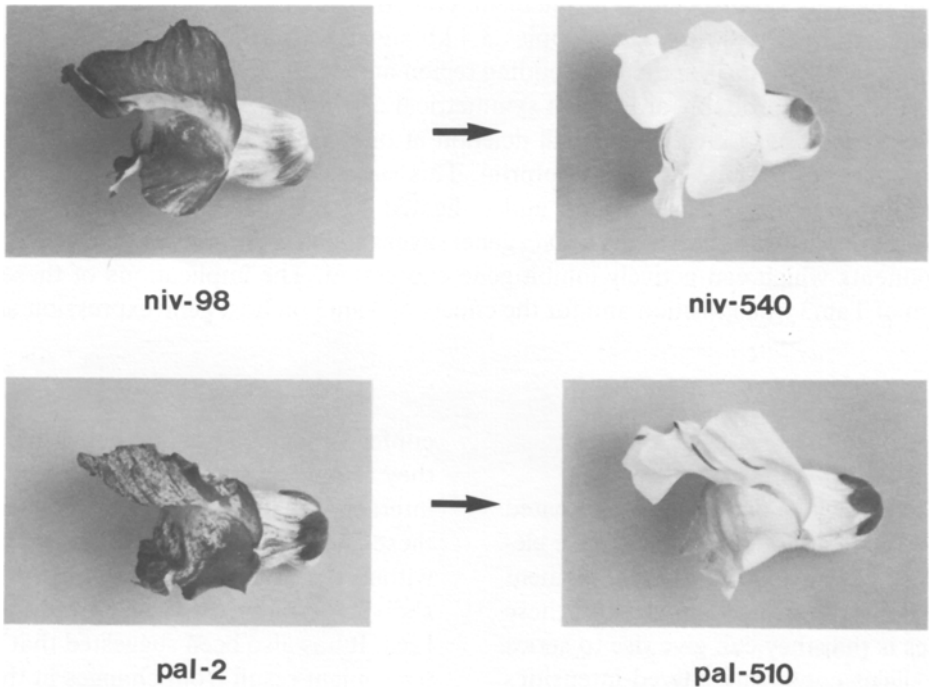

Fig. 1. Flower phenotypes conferred by the *pal* and *niv* alleles. The *niv*-98 allele produces a high frequency of full red sites (represented by dark regions on these black-and-white photographs) on a paler background, although the intensity of the background colour makes it difficult to distinguish the sites with the naked eye. The *niv*-98 flower also shows pale red and white sites, presumably caused by imprecise excision of Tam3 [16, 27]. The *niv*-540 allele produces a low frequency of full red sites on a white background. In *pal*-2, a high frequency of full red spots are produced on a colourless background, though the high frequency makes it difficult to determine the lack of background pigmentation with the naked eye. The *pal*-510 allele produces a very low frequency of sites on a palely pigmented background.

trate comparable but opposite phenotypic changes when compared to their progenitors: the *niv*-540 derivative abolishes background gene expression whereas *pal*-510 restores it.

We show here that *niv*-540 carries two copies of Tam3 which most probably arose by short-range Tam3 transposition following replication at the donor site. *Pal*-510 has a small structural change at one end of Tam3, probably as a result of aberrant transposition. The changes in the phenotypes produced by these alleles shows how diversity can arise as a consequence of different aspects of the Tam3 transposition mechanism.

Materials and methods

Antirrhinum *strains*

The *pal*-2 allele has been maintained in an inbred line for many generations [12]. The *pal*-510 allele was first detected in progeny from plants of this line grown at 15 °C [5]. The *niv*-98 allele arose by integration of Tam3 into the chalcone synthase gene [28]. The *niv*-540 allele was detected amongst progeny of a cross between the line carrying the *niv*-98 allele and line JI.45, which is homozygous for the null allele *niv*-45, and was recovered as the homozygote in the subsequent generation [1]. Line JI.45, homozygous for the *niv*-45 allele, was obtained as the commercial variety 'Snowman' marketed by Suttons Seeds and inbred for many generations.

DNA manipulation

Plant DNA was isolated as previously described [5]. Phage and plasmid DNA isolation was carried out as described ([34] and [28] respectively). Southern hybridisation was performed as previously described [28, 31, 33].

Cloning and sequencing of plant DNA

The *pal*-510 allele and both *Eco* RI fragments of the *niv*-540 allele were cloned into λNM1149 [17]. Genomic DNA was digested with *Eco* RI and separated on agarose gels. Size fractions around 7.8 kb for *pal*-510, and 9.2 kb and 4.0 kb for the upstream and downstream fragments of *niv*-540 were isolated by freezing strips of the gel and expelling the liquid as the gel thawed. DNA was ligated into *Eco* RI-digested λNM1149, and phages were screened on a C600, *HflA*$^+$ strain using either a *pal*-specific fragment of the pJAM4 probe [15] or the *niv* probe pcA1 kindly provided by H. Sommer [30]. The presence of Tam3 was confirmed by screening with the Tam3-specific fragment of pJAm4. Regions sequenced were subcloned into M13 mp18 and mp19 vectors for sequencing [23].

RNA analysis

RNA was extracted from flower buds and analysed on northern blots as previously described [15]. Single-stranded probe preparation and S1 digestion were carried out as described previously [5], using a *Nae* I-*Nru* I fragment of the wild-type *Pal*$^+$-501 allele which extends from within the first exon to 12 bp upstream of the Tam3 target site.

Results

niv-*540 contains two new copies of Tam3*

The structure of the *niv*-540 allele was initially investigated by Southern hybridizations. The wild-type chalcone synthase gene is contained within two *Eco* RI fragments of genomic DNA: a 5.7 kb upstream fragment containing the *niv* promoter, the first two exons and part of the third exon, and a 0.5 kb downstream fragment carrying the remainder of the third exon and 3′ untranslated region [30] (Fig. 2C). The progenitor of *niv*-540, *niv*^{rec}-98, contains the 3.5 kb Tam3 element within the promoter and, because Tam3 contains no *Eco* RI sites, it produces an upstream fragment 3.5 kb larger than wild-type (9.2 kb compared to 5.7 kb) in Southern hybridizations

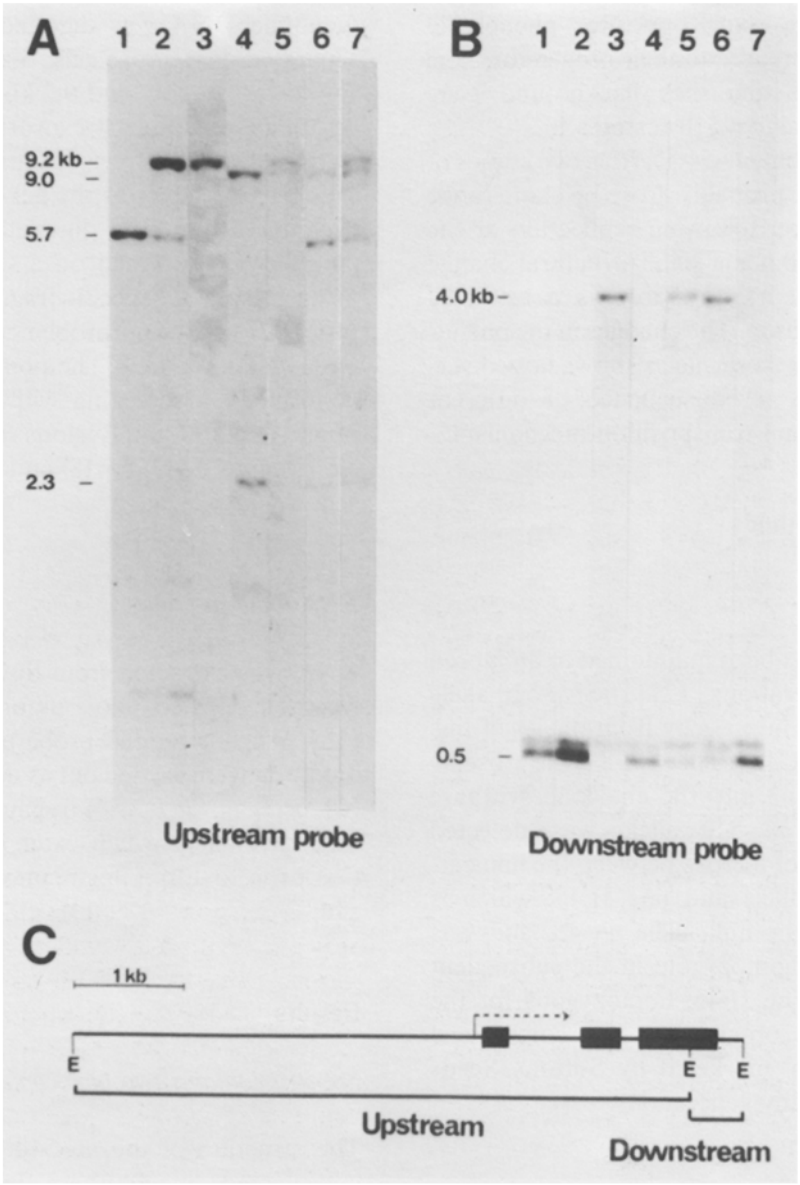

Fig. 2. Southern hybridisation of *Eco* RI-digested DNA from different *niv* genotypes probed with the upstream (A) and the downstream (B) *Eco* RI fragments of the chalcone synthase gene. The genotypes are: 1, *Niv*$^+$/*Niv*$^+$; 2, *niv*-98/*niv*-98; 3, *niv*-540/*niv*-540; 4, *niv*-45/*niv*-45; 5, *niv*-540/*niv*-45; 6, a derivative of *niv*-540 having lost the upstream copy of Tam3, heterozygous with *niv*-45, showing an unstable phenotype similar to that of the *niv*-540 allele (see Fig. 4B); 7, a derivative of *niv*-540 having lost the downstream Tam3 insertion, showing a full red phenotype (see Fig. 4C). The structure of the alleles derived from *niv*-540 can be determined in heterozygotes with *niv*-45 from this Southern hybridisation. With the upstream probe, the *niv*-45 allele produces *Eco* RI fragments of 9.0 kb and 2.3 kb as seen in all the *niv*-45 heterozygotes. If the *niv*-540 derivative retains its upstream Tam3 insertion, then it produces an additional 9.2 kb fragment. If the insertion has been lost, then the additional fragment is 5.7 kb long. With the downstream probe, *niv*-45 yields an *Eco* RI fragment of 0.5 kb, as does a *niv*-540 derivative which has lost its downstream insertion. An additional band of 4.0 kb is therefore seen only in heterozygotes carrying a *niv*-540 derivative which retains its downstream insertion of Tam3. The band of 5.7 kb seen in lanes 2 and 7 is presumably produced by somatic excision of Tam3 from the upstream *Eco* RI fragment in the tissue from which DNA was extracted. Differences in the relative intensity of the somatic band can be attributed to segregation of *Stabiliser*, a semi-dominant allele which specifically reduces Tam3 excision [1]. *St* is present in the JI.45 progenitor of the line JI.540, but not in the JI.98 parent. Additional fainter bands are produced by hybridisation to homologous sequences not from the *niv* locus. C: The origin of the upstream and downstream probes. Thick lines indicate exons of the chalcone synthase gene, and the dotted arrow the start and direction of transcription. *Eco* RI (E) sites are shown.

with the upstream probe [28] (Fig. 2A). In the *niv*-540 allele, both the upstream and downstream *Eco* RI fragments were 3.5 kb larger than in the wild type (9.2 kb and 4.0 kb), suggesting that the allele contained two insertions of Tam3, one located in each of the *Eco* RI fragments. These two fragments were cloned from size-fractionated DNA of a plant heterozygous for the *niv*-540 and *niv*-45 alleles into the *Eco* RI site of λNM1149. *niv*-45 is a stable null allele which can be recognised in Southern hybridisations because it produces *Eco* RI fragments of 9.0 kb and 2.3 kb with the upstream probe and 0.5 kb with the downstream probe [14] (Fig. 2A, B). Size fractionation of the DNA therefore ensured that only the fragments unique to the *niv*-540 allele were cloned. These were identified by hybridisation with the chalcone synthase probe, pcA1, which spans the internal *Eco* RI site of the gene [30]. All positive plaques also hybridised with a clone of Tam3, pJAM4 [5], confirming that Tam3 was present in both of the *Eco* RI fragments. DNA fragments containing the junctions between Tam3 and flanking sequences were subcloned into M13 mp18 and mp19 vectors for sequencing.

The upstream Tam3 insertion was found to be 3.4 kb upstream from its original position in the promoter of *niv*-98, and the second insertion 2.15 kb downstream within the final exon of the gene. The two new copies of Tam3 each had generated target duplications of 8 bp which showed no strong sequence homology with each other or with previously described Tam3 target duplications [5, 28] (Fig. 3). Both Tam3 copies were in the same orientation as the copy of Tam3 in the progenitor allele, *niv*-98. Sequencing of the excision site of Tam3 in the promoter revealed that imprecise excision of the element had occurred. A single nucleotide was missing from the ends of both copies of the target duplication and two additional nucleotides (GC) were found at the excision site (Fig. 3). Short inverted duplications, adjacent to target sites with the terminal nucleotide deleted, are typical products of plant transposable element excision [22, 4].

The downstream Tam3 copy is responsible for the niv-*540 phenotype*

In order to determine which copy of Tam3 produced the phenotype of the *niv*-540 allele, it was necessary to produce alleles from *niv*-540 which carried only one of the Tam3 copies. To enable the phenotypic effects of one dose of each allele to be studied, plants were produced which carried the alleles heterozygous with the stable null allele *niv*-45. Out of 123 progeny grown from self-pollinated *niv*-540/*niv*-45 heterozygotes, 67 had flowers similar to the unstable *niv*-540 phenotype, 7 had full red flowers and 49 had stable white flowers. The stable white-flowered plants presumably include *niv*-45 homozygotes and plants carrying stable null alleles produced by imprecise excision of the Tam3 copy in the exon of *niv*-540. DNA was extracted from a sample of plants with full red or unstable phenotypes, digested with *Eco* RI, transferred to nitrocellulose and probed

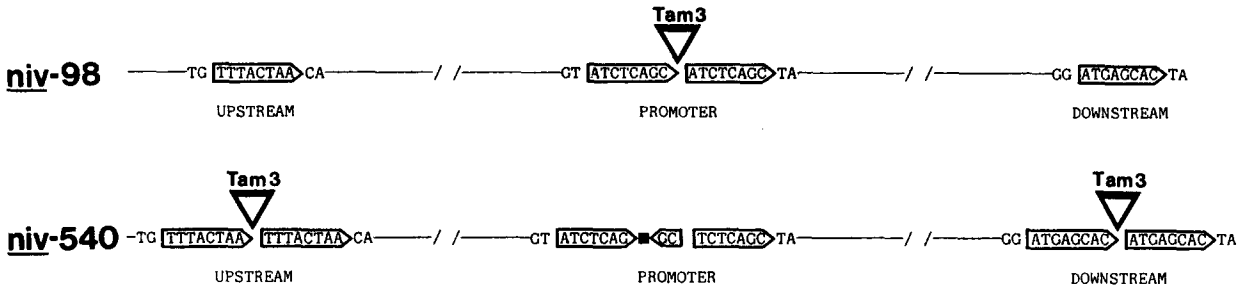

Fig. 3. Sequences of the upstream, promoter and downstream sites of Tam3 integration in the *niv*-98 and *niv*-540 alleles. The Tam3 target sites are indicated by boxed arrows. A solid square indicates the single nucleotide missing from the symmetry axis of the short inverted duplication in *niv*-540. Open triangles represent Tam3.

with the upstream and downstream chalcone synthase fragments. This enabled the structure of the alleles derived from *niv*-540 to be determined (see legend to Fig. 2 for details). Ten plants (seven unstable, three full red) were found to be heterozygous for *niv*-45 and an allele from *niv*-540. All seven plants with unstable phenotypes contained the downstream Tam3 insertion and three of these also had the upstream insertion (Fig. 2, Lane 5, Fig. 4A). In the remaining four unstable plants, the upstream insertion was not present, presumably as a consequence of Tam3 excision in the germinal tissue of the parent (Fig. 2, Lane 6, Fig. 4B). Thus, only the downstream Tam3 copy, within the final exon, was responsible for the unstable phenotype conditioned by the *niv*-540 allele. Two of the three full red progeny had lost the downstream Tam3 copy but retained the upstream copy (Fig. 2, Lane 7, Fig. 4C). These plants showed occasional white sites on their red flowers, indicating that the upstream copy was causing occasional inactivation of the *niv* gene. This may either be a consequence of large deletions induced by the upstream Tam3 copy [16] or of short-range transpositions of the remaining Tam3 copy into the *niv* gene [11]. The remaining full red plant had lost both copies of Tam3 (Fig. 4D).

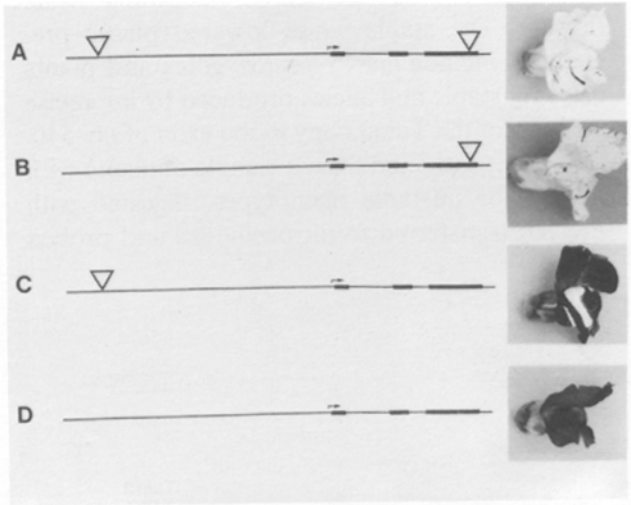

Fig. 4. Alleles and phenotypes derived from *niv*-540. Thick lines indicate exons of the *niv* gene and arrows pointing to the right show the transcription start-site in wild-type. The open triangles represent Tam3. The flowers shown come from plants carrying the illustrated allele heterozygous with *niv*-45 (a stable null allele).

The pal-*510 allele has a small alteration at one end of Tam3*

The *pal*-510 allele had been found to have an indistinguishable restriction map from its progenitor, *pal*-2 [5]. To determine if a small sequence alteration was responsible for the *pal*-510 phenotype, DNA was extracted from a *pal*-510 homozygote and the allele cloned into the *Eco* RI site of λNM1149. The junctions between Tam3 and its flanking DNA were cloned in M13 mp18 and mp19 vectors and sequenced. The sequence at the end of Tam3 further upstream from the *pal* coding sequence was the same as that found in the progenitor (Fig. 5). However, a small sequence change was found at the end of the element nearer to the gene. The terminal 3 bp of Tam3 was found to be deleted and 1 bp was also missing from the end of the right target duplication (Fig. 5). An extra nucleotide (A) was found inserted between these two deletions. This nucleotide may have resulted from an inverted duplication of the first nucleotide (T) of the right target duplication in the progenitor (TACCC).

The background expression from pal-*510 initiates from the normal* pal *promoter*

In the *pal*-2 allele, the full red sites resulting from excision of Tam3 are seen against an ivory background which indicates that Tam3 prevents

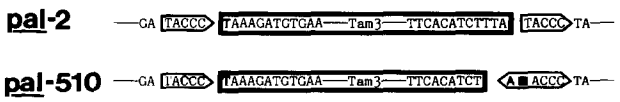

Fig. 5. Sequence at the ends of Tam3 in *pal*-2 and *pal*-510. Tam3 sequences are enclosed in thick lines and only the terminal inverted repeats of Tam3 are shown in detail. The target site duplications are enclosed in boxed arrows and a solid square indicates the single nucleotide missing from the symmetry axis of the short inverted duplication in *pal*-510.

expression of *pal* unless it excises. This was confirmed by showing that lines in which Tam3 excision from *pal*-2 is repressed by factors in the genetic background contain no detectable *pal* transcript [5]. In *pal*-510 the full red spots appear against a pale red background, suggesting that Tam3 no longer fully prevents *pal* expression. One possible explanation for this might be that the alteration around the end of Tam3 in the *pal*-510 allele allows transcription initiated within the element to read through into the *pal* coding sequence. In order to investigate this possibility, transcription of the *pal*-510 allele was determined using RNA blots and S1 nuclease mapping. Poly(A)$^+$ RNA from *Pal*$^+$ and *pal*-510 alleles was transferred to nitrocellulose and probed with a *pal* clone. Both alleles gave a normal size *pal* transcript of about 1.5 kb [15], although the quantity of transcript from *pal*-510 was much less than that from *Pal*$^+$. To test if the transcripts had the same startsite, poly(A)$^+$ RNA from *pal*-510 and *Pal*$^+$ homozygotes was used to protect a single-stranded probe of *Pal*$^+$ DNA. The probe spanned the sites of transcription initiation in the *Pal*$^+$ gene and extended beyond the point of Tam3 integration in the *pal*-2 allele, although it contained no Tam3 sequences. Any transcript initiated within Tam3 was expected to give a protected fragment of 249 bp, starting from the point of Tam3 integration and extending downstream towards the *pal* gene. The RNA from *Pal*$^+$ plants gave three protected fragments two of which, at about 141 bp and 180 bp, correspond to the two previously described transcription startsites located 70 bp and 105 bp downstream from the Tam3 integration site [5] (Fig. 6). The third fragment at 164 bp was not well resolved in previous experiments and was attributed to degradation of the 180 bp fragment [5]. It is more likely that it corresponds to a third transcription startsite since there is a suitable TATA box (TATAAA) located 33 bp upstream from it. The only protected fragments detected in *pal*-510 were at 141 bp and 164 bp, corresponding to the two major wild-type sites of initiation (Fig. 6). The experiments were not sensitive enough to determine whether the 180 bp fragment was also present. No protected fragment was seen at 249 bp, indicating that the background expression from the *pal*-510 allele is not produced by transcription initiated within Tam3 and running into the *pal* gene.

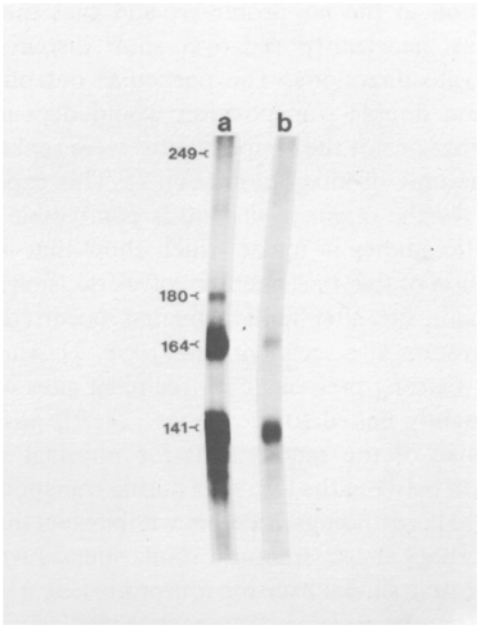

Fig. 6. S1-mapping of transcripts from (a) *Pal*$^+$ and (b) *pal*-510 alleles. Sizes of fragments protected from S1 nuclease are indicated in bp.

Discussion

Allelic derivatives are a consequence of the Tam3 transposition mechanism

The production of the *niv*-540 allele from *niv*-98 involved imprecise excision of Tam3 from the promoter and integration of two copies of the element, one 3.4 kb upstream, the other 2.1 kb downstream of the original site. The two insertions probably arose from a single event because only the downstream insertion was selected for due to its phenotypic effect on flower colour. It is unlikely that an independent upstream insertion occurred by chance since short-range transpositions of 3–4 kb are rare events [16]. The proximity of both Tam3 copies to a Tam3 excision site suggests that Tam3 was replicated at its original

position in the *niv* promoter, and that the two copies then transposed over short distances in opposite directions. The particular outcome of such a double transposition would depend on whether or not the recipient sites were replicated at the time of integration (Fig. 7). This explanation for the origin of *niv*-540 is compatible with genetic studies in maize which show that transposition of the *Ac* element involves excision from a donor site after replication has occurred and integration at a recipient site [10, 8, 2]. There is also a strong preference for recipient sites which are tightly linked to the donor [24, 9], possibly because of the requirement for physical association between the two sites during transposition [21]. These findings, together with the fact that *Ac* and Tam3 share structural similarities as well as generating similar excision footprints [29, 6], suggest that these elements transpose by a very similar mechanism. However, in contrast to transposition of *Ac* which usually involves only one copy of the newly replicated element, both copies of Tam3 probably transposed in the case of *niv*-540.

When compared to its progenitor, *pal*-510 has a 3 bp deletion at the right end (gene-proximal) of Tam3 flanked by a sequence typical of a Tam3 excision footprint [22, 4] (Fig. 5). This suggests that *pal*-510 arose by an attempted Tam3 transposition in which only the right end of the element

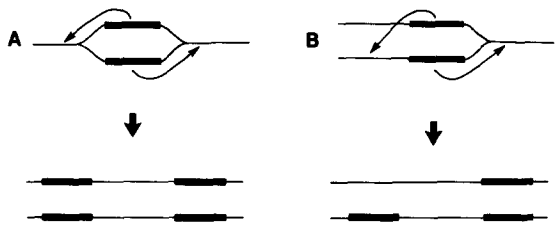

Fig. 7. Models to explain the origin of *niv*-540. Each line represents a double strand of DNA and the thick solid lines represent Tam3. Both models propose that after passage of a replication fork the two copies of Tam3 both transpose to new sites. In model A both Tam3 copies transpose into unreplicated DNA. In model B, one Tam3 copy transposes into unreplicated DNA while the other copy transposes into replicated DNA of the sister chromatid. The resulting chromatids after completion of replication are shown. In all cases the original site occupied by the element has been vacated.

was cleaved and subsequently re-ligated back to the donor site. The 3 bp deletion may have been a result of exonuclease digestion of the Tam3 end while it was free from the donor site, or of endonucleolytic cleavage during the process of religation. A similar deletion at one end of Tam1 has been described [13], suggesting that cleavage and re-integration of one end may be a common event. This may reflect a mechanism of transposition in which cleavage at one end of the element allows the free end to invade a recipient site [21]. Alterations in element structure have also been described for *Ac* and *En/Spm* elements in maize, although these generally involve internal deletions of the elements rather than deletions at one terminus [26, 25, 7].

Effects of Tam3 on gene expression

The absence of background pigmentation and the low frequency of full red sites produced by the *niv*-540 allele have a relatively simple explanation. The downstream copy of Tam3 is within the translated region of the gene and should therefore prevent production of a transcript encoding a functional protein. Furthermore, only precise excisions of Tam3 will restore the ability to produce the wild-type *niv* gene product. This explains the low frequency of full red sites seen on *niv*-540 flowers compared to its *niv*-98 progenitor which carries Tam3 in a region of the promoter known to be tolerant of small sequences alterations [27].

The 3 bp deletion at the gene-proximal end of Tam3 in *pal*-510 restores some expression to the *pal* gene. No readthrough transcript from Tam3 into the *pal* gene could be detected in *pal*-510, indicating that the 3 bp deletion relieves inhibition by the intact Tam3 rather than allowing gene activation from within the element. The deletion might reduce the affinity of the end of Tam3 for binding transposition proteins, so allowing increased access of transcription factors to the *pal* promoter. This would account for both the background gene expression, and the reduced frequency of Tam3 excision. One difficulty with this model is that in the *niv*-98 allele, Tam3 is found

in a comparable position to that in *pal*-2, yet it allows background gene expression. The difference between these alleles may reflect dissimilar promoter structures of the *niv* and *pal* genes, or it could be a consequence of the opposite orientations of Tam3 with respect to transcription in these alleles.

The *niv*-540 and *pal*-510 alleles show how changed levels of gene expression can arise from transposable element insertions by short-range or aberrant transposition. Together with imprecise excision, these processes explain how abundant phenotypic diversity can arise as a consequence of different aspects of the transposition mechanism.

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