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A semi-dominant allele, *niv*-525, acts *in trans* to inhibit expression of its wild-type homologue in *Antirrhinum majus*

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***Niv*-525 is a semi-dominant allele of the *nivea* locus, which encodes the enzyme chalcone synthase required for flower pigment biosynthesis in *Antirrhinum majus*. Plants heterozygous for *niv*-525 and wild-type (*Niv*$^+$) allele, have flowers with a reduced intensity and novel spatial pattern of pigmentation compared with *Niv*$^+$ homozygotes. In heterozygotes, *niv*-525 acts *in trans* to reduce the steady-state level of *nivea* transcript produced by its *Niv*$^+$ homologue and hence the quantity of chalcone synthase protein. *Niv*-525 carries an inverted duplication of 207 bp in its promoter region which has arisen following excision of the transposable element Tam 3. This structure can be explained by a model of plant transposable element excision that involves resolution of two hairpin DNA molecules. Possible mechanisms for the *trans*-acting effect of *niv*-525 and its relationship to other examples of allelic interactions, such as transvection in *Drosophila melanogaster*, are discussed.**

Key words: anti-sense RNA/chalcone synthase/dominance/transposable element/transvection

Introduction

In diploid organisms the two alleles present at a genetic locus are generally thought to be expressed independently of each other. However, there are a few cases in which the expression of an allele has been shown to be modified by its homologous allele. These types of allelic interactions are unexpected and are of interest because of their potential to reveal novel types of regulatory interactions between genes. To understand further the basis of allelic interactions we analysed an unusual allele in *Antirrhinum majus* which appears to act *in trans* on the expression of its homologue

Many alleles have been described for two different loci required for the biosynthesis of anthocyanin pigment in *A.majus*: *nivea* (*niv*), which encodes the enzyme chalcone synthase, and *pallida* (*pal*), which encoded dihydroflavonol-4-reductase (for review see Coen and Carpenter, 1986). Many of these alleles confer altered intensities or spatial patterns of flower pigmentation and carry small sequence changes which have arisen by imprecise excision of transposable elements from the *niv* and *pal* promoter regions (Coen *et al.*, 1986; Carpenter *et al.*, 1987). The sequence changes generally reduce the overall quantity of gene transcript and the alleles are recessive to wild-type. However, an exceptional allele has recently been described which appears to be semi-dominant to wild-type (Carpenter *et al.*, 1987). This allele arose when a line homozygous for *niv*-98, an unstable allele which has the transposable element Tam 3 inserted in the *niv* promoter region (Sommer *et al.*, 1985), was crossed to a line homozygous for a *Niv*$^+$ wild-type allele. With one exception, all the F_1 progeny from the cross had fully red flowers indistinguishable from wild-type (Figure 1A), as expected since they carry one dose of the *Niv*$^+$ allele. The exceptional offspring had flowers with a reduced intensity and novel spatial distribution of pigmentation, most of the colour being concentrated in the tube and around the lower lip of the flower (Figure 1B). The simplest interpretation of these results was that a dominant or semi-dominant allele of the *nivea* locus, called *niv*-525, had arisen from the unstable *niv*-98 allele.

We describe here genetic and molecular analysis of *niv*-525 and show that it corresponds to an allele of the *niv* locus which, when heterozygous with a wild-type *Niv*$^+$ allele, acts in *trans* to repress the level of transcript produced by its homologue. *Niv*-525 has a novel structure which is most easily explained as having arisen by resolution of hairpin molecules generated by excision of a transposable element. On the basis of these findings we discuss the possible mechanisms which might explain this type of allelic interaction.

Results

niv-525 is semi-dominant and acts on niv gene expression

The exceptional F_1 offspring obtained in the cross between *niv*-98 and *Niv*$^+$ lines was self-pollinated to give an F_2 progeny containing three phenotyes in a 1:2:1 ratio: plants with full red flowers (Figure 1A), plants with flowers similar to the F_1 parent (Figure 1B) and plants with very pale flowers having a similar spatial pattern of pigmentation to the F_1 parent (Figure 1C). These results suggested that the F_1 plant was heterozygous for a semi-dominant allele, *niv*-525, which segregated in the F_2. This was confirmed by crossing plants with very pale flowers (*niv*-525 homozygotes) to several wild-type lines; all progeny had the heterozygote phenotype (Figure 1B) as expected.

To determine which steps of pigment biosynthesis were affected by the *niv*-525, the pigment precursors naringenin and dihydroquercetin were fed to flowers homozygous or heterozygous for the allele. In each case additional anthocyanin was produced, suggesting that *niv*-525 affected the enzyme chalcone synthase which catalyses an early step in the biosynthetic pathway (Stickland and Harrison, 1974). The simplest explanation of this result is that *niv*-525 is an allele of the *niv* locus, which encodes chalcone synthase, and that it arose from the unstable *niv*-98 allele used in the original cross. Chalcone synthase is thought to be a multimeric enzyme, so the semi-dominance might be explained if *niv*-525 produced an altered chalcone synthase polypeptide which

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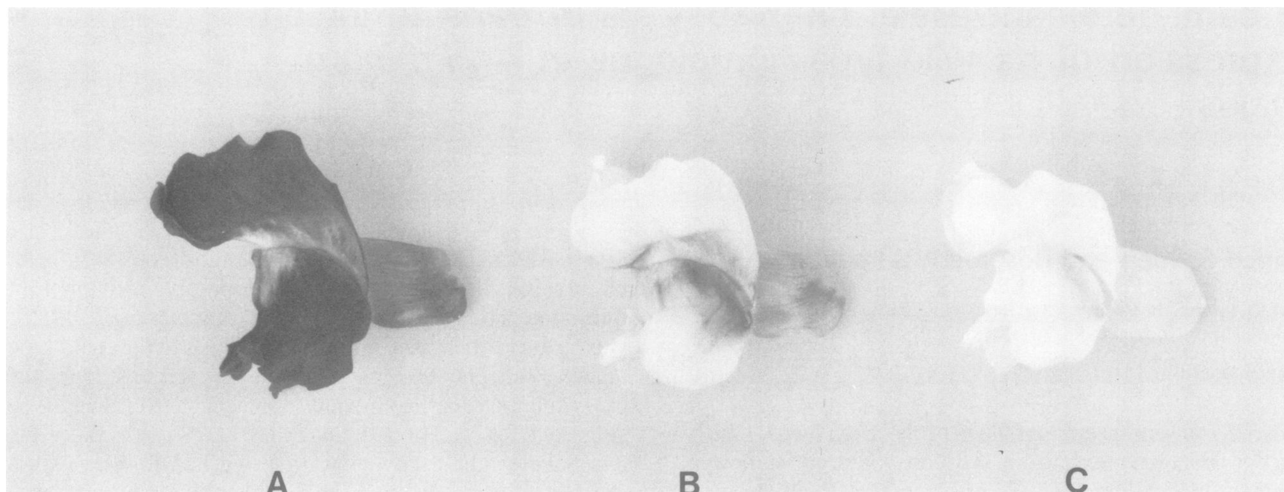

Fig. 1. Phenotypes of flowers which are (**A**) homozygous wild-type; (**B**) heterozygous for *niv*-525; (**C**) homozygous for *niv*-525.

interacted with the wild-type protein to give heteromers with reduced enzyme activity. To test this model, chalcone synthase protein from flowers of the three F_2 genotypes was analysed by immuno-blots. Chalcone synthase protein (mol. wt 42 000 daltons) could be observed in the different genotypes (Figure 2). However, flowers homozygous for *niv*-525 had much less chalcone synthase protein than homozygous Niv^+ flowers, while the heterozygote had an intermediate amount of chalcone synthase. This suggests that the different phenotypes reflect the abundance and distribution of chalcone synthase rather than a qualitative change in its protein structure.

To determine whether the observed differences in the quantity of chalcone synthase were due to altered amounts of its mRNA, poly$(A)^+$ RNA or total RNA from individual plants of the segregating F_2 population was transferred to nitrocellulose and probed with a chalcone synthase clone. The amounts of chalcone synthase transcript in the plants, relative to the wild-type homozygote, were 10−12.5% in the heterozygote and 1−2% in the homozygote for *niv*-525 (Figure 3A). If there was no interaction between the *niv*-525 and Niv^+ alleles the amount of transcript in the heterozygote should be equal to the average of the two homozygotes (50.5−51%). However, the level of transcript observed in the heterozygote (10−12.5%) is 4- to 5-fold less than the average of the two homozygote genotypes (50.5−51%). This suggests that *niv*-525 acts *in trans* in heterozygotes to reduce the level of transcript produced from Niv^+ and accounts for the semi-dominance of the allele. As a control, the same RNA was probed with a clone of the *pal* gene which encodes an enzyme acting at a later step in the pigment pathway. No reduction in *pal* transcript levels was observed in plants which carried *niv*-525 (Figure 3B), showing that it specifically reduces chalcone synthase mRNA and does not affect another enzymatic step in the pathway. The plant homozygous for *niv*-525 had about twice as much *pal* transcript as the other two plants. This difference is presumably due to the segregation of the $pal^{tubocolorata}$ allele (an allele which produces very little *pal* transcript; Coen *et al.*, 1986) in this F_2 population, so that in this experiment the plant homozygous for *niv*-525 was probably of Pal^+/Pal^+ genotype whereas the other two plants analysed were $Pal^+/pal^{-tubocolorata}$.

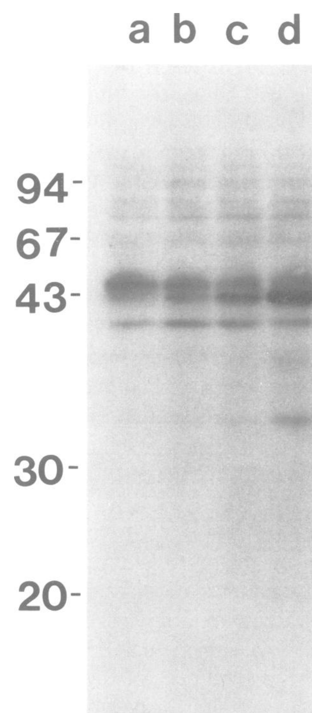

Fig. 2. Immuno-blot of total protein extracts from flowers of various genotypes incubated with chalcone synthase antibody: (**a**) stable *nivea*; (**b**) homozygous for *niv*-525; (**c**) heterozygous for *niv*-525; (**d**) homozygous wild-type. Mol. wt in kilodaltons of markers is shown on left. Several weak bands are observed in each track in addition to the band at 42 000 daltons. These bands are not *niv* locus specific since they are also present in the stable *niv* control (track **a**).

To confirm that the chalcone synthase message detected in the different genotypes varied only quantitatively and not qualitatively, the sites of transcription initiation were determined by S1 nuclease mapping. The homozygous wild-type gave three strong bands at ~144 bp, which corresponds to the previously determined chalcone synthase transcription start site (Sommer and Saedler, 1986; lane c Figure 3C). The same bands were also seen for plants either heterozygous or homozygous for *niv*-525 but at an intensity of ~12.5% or 1−2% of wild-type respectively, in agreement with the quantitative differences observed on RNA blots.

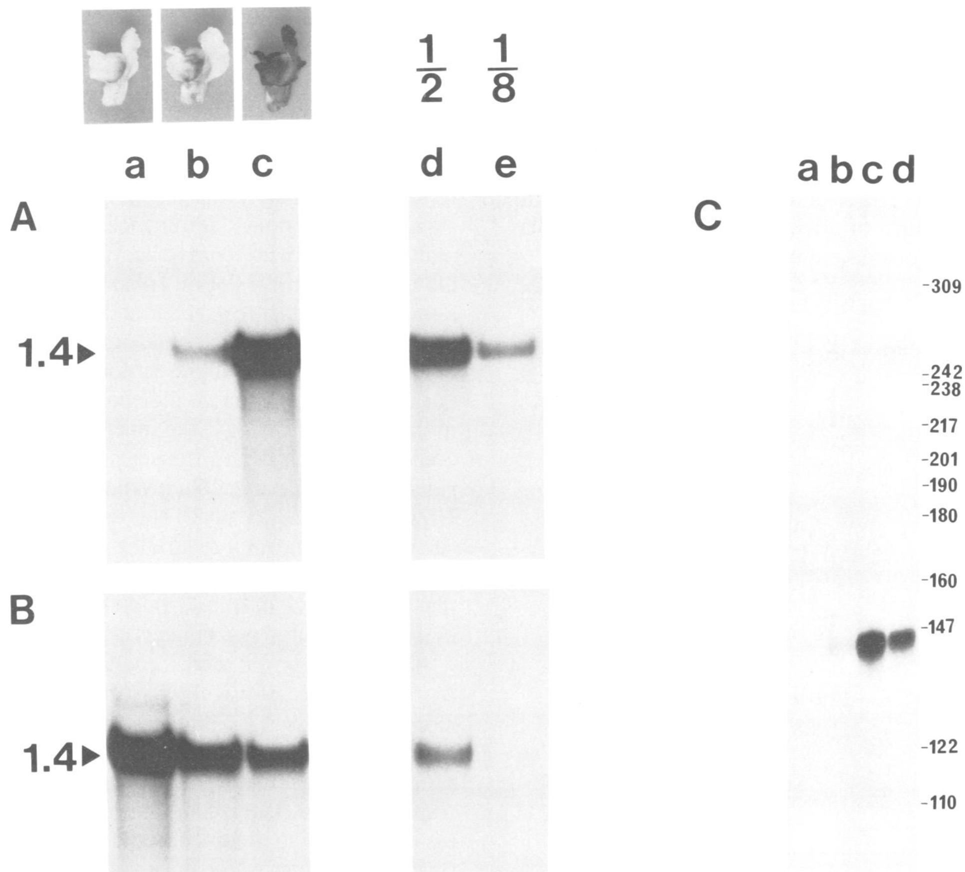

Fig. 3. Effect of *niv*-525 on expression of the *niv* and *pal* genes. RNA was from plants which were: (**a**) homozygous for *niv*-525; (**b**) heterozygous for the allele; (**c**) homozygous Niv^+. Tracks (**d**) and (**e**) show the same RNA as in (**c**) but diluted 2-fold or 8-fold respectively. RNA blots were probed with either a clone of the chalcone synthase gene (**A**) or a clone of the *pal* gene (**B**). The size of the hybridizing bands is indicated in kb. RNA was also hybridzed to a single-stranded probe from the 5′ end of the chalcone synthase gene and digested with S1 nuclease (**C**).

niv-525 has a novel structure

Several observations indicate that *niv*-525 resulted from a structural alteration of the *niv* locus. First, *niv*-525 arose from a cross involving an unstable allele, *niv*-98, which gives rise to many other *niv* alleles, presumably by rearrangements and imprecise excisions of the Tam 3 transposable element located in the *niv* promoter region of this allele (Sommer *et al.*, 1985; Carpenter *et al.*, 1987). Secondly, *niv*-525 specifically represses expression of the *niv* locus. Thirdly, *niv*-525 satisfies the classical genetic test for allelism since less pigmentation is observed in F_1 progeny when *niv*-525 homozygotes are crossed with plants homozygous for *niv* alleles (e.g. *niv*-98) than when crossed with plants homozygous for a Niv^+ allele. However, these observations do not completely rule out the possibility that *niv*-525 corresponds to a structural change in an unlinked locus which specifically *trans*-regulates the *niv*-locus. One way to test this possibility is to see if plants carrying *niv*-525 have a structural alteration of the *niv*-locus and whether this alteration segregates with the appropriate phenotypes.

DNA was extracted from individual plants of the segregating F_2 population, digested with *Eco*RI and probed with a clone or the *niv* locus. *Eco*RI cuts once in the third exon of the *niv* gene and also 3.8 kb upstream of the coding region to give a band of 5.7 kb in wild-type or 9.2 kb in *niv*-98 (the Tam 3 element is 3.5 kb long and does not contain any *Eco*RI sites; Sommer *et al.*, 1985). In addition to the 5.7-kb band, the F_2 population also contained a band of 5.9 kb which co-segregated with *niv*-525 (Figure 4). The 9.2-kb band of *niv*-98 was no longer present. Further restriction mapping of the 5.9-kb band showed that it contained an extra 200 bp compared with wild-type located at or near the site where Tam 3 is inserted in *niv*-98. This confirms the hypothesis that *niv*-525 corresponds to a new allele of the *niv*-locus, which arose from *niv*-98 (see pedigree, Figure 4).

The structure of *niv*-525 was determined by cloning the 5.9-kb *Eco*RI fragment and sequencing the region containing the additional 200 bp (Figure 5). Tam 3 in the *niv*-98 progenitor is flanked by a target duplication of 8 bp (Sommer *et al.*, 1985). The *niv*-525 allele contains no Tam 3 sequences but retains 6 bp of the upstream and 7 bp of the downstream target duplications. Between these two sequences lies a 207-bp inverted duplication which extends from the Tam 3 excision site into the first exon of the *niv* gene (Figure 5). The duplicated sequence includes ~60 bp of the 5′ non-transcribed region (containing the TATA box), the 80-bp non-translated leader and 65 bp of the coding part of the first exon.

Discussion

We have shown that a semi-dominant allele of the *niv* locus, *niv*-525, when heterozygous with a wild-type Niv^+ allele, acts *in trans* to reduce expression of its homologue to

20–25% of its normal level. In *niv*-525 homozygotes the low level of transcript observed (1–2% compared with *Niv*$^+$ homozygotes) may reflect a combination of both *cis* and *trans* effects of the *niv*-525 mutation. Structural analysis of *niv*-525 shows that it carries an inverted duplication of 207 bp where the transposable element Tam 3 was located in its progenitor allele. The structure of *niv*-525 has implications both for the nature of allelic interactions and for the mechanism of transposable element excision.

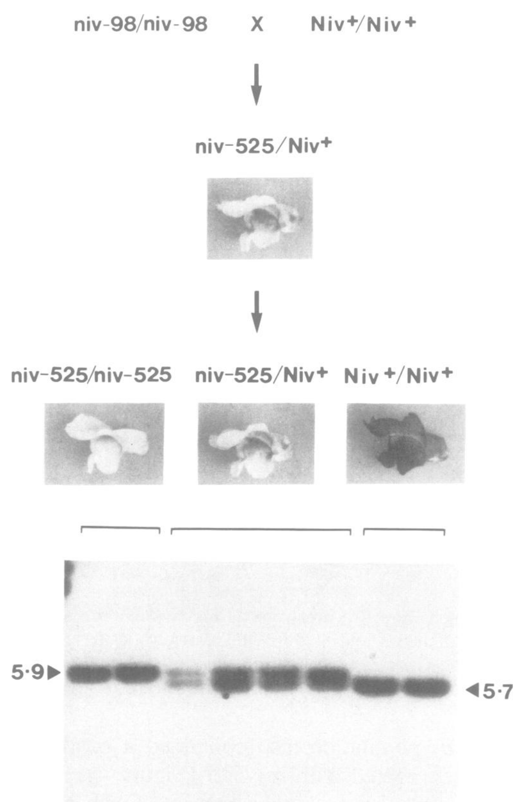

Fig. 4. Origin of *niv*-525. A Southern blot of *Eco*RI-digested DNA from eight individual plants (two of each homozygote, and four of the heterozygote) probed with a clone of chalcone synthase is shown below the appropriate phenotypes. Sizes are in kilobases.

Molecular basis of allelic interaction

Several models may be invoked to explain the ability of *niv*-525 to act *in trans* in a *niv-525/Niv*$^+$ heterozygote to reduce expression of the *Niv*$^+$ allele to 20–25% of its expected level. These fall into three general classes.

The first type of model proposes that *niv*-525 acts by removing a transcription factor required for *Niv*$^+$ expression. Transcriptional dominance of arrays of the rDNA multigene family in *Xenopus laevis* has been correlated with the number of copies of an enhancer sequence which binds a transcription factor (Reeder and Roan, 1984). It has been proposed that one nucleolus organizer is dominant over others if it has more copies of the enhancer and so titrates out most of the available molecules of transcription factor in the pool. By analogy, the inverted duplication in *niv*-525 could result in an extra transcription-factor-binding region since it includes part of the promoter region of the *niv* gene. However, it is difficult to see how a single extra binding site could significantly reduce the quantity of factor available to the *Niv*$^+$ allele unless we make the unlikely assumption that the pool size of transcription factor consists of only one or two molecules per nucleus.

A second type of model proposes that the *niv*-525 and *Niv*$^+$ alleles are physically associated during gene expression and that *niv*-525 can therefore act directly on the transcription of its homologue. This mechanism has been proposed as a possible explanation of the phenomenon of transvection in *Drosophila* (Zachar *et al.*, 1985; Pirrotta *et al.*, 1985). In the presence of the *zeste*^{1} mutation, two wild-type alleles at the *white* locus appear to inhibit each other's expression (Bingham and Zachar, 1985). The inhibition is generally much greater if the two *white*$^+$ alleles are at homologous positions on the chromosome and is thought to depend on somatic pairing of the two alleles during gene expression. A dominant mutation of *white* (w^{DZL}) has also been described which, even in the absence of the *zeste*^{1} mutation, can inhibit expression of a *white*$^+$ allele when the two alleles are at homologous chromosome positions (Bingham and Zachar, 1985). The similarity of this type of allelic interaction, particularly the properties of the dominant w^{DZL} allele, to the action of *niv*-525 suggests that inhibition by *niv*-525 may also involve physical association of alleles during gene expression. The inverted duplication of *niv*-525 could then act, either directly or through a DNA-

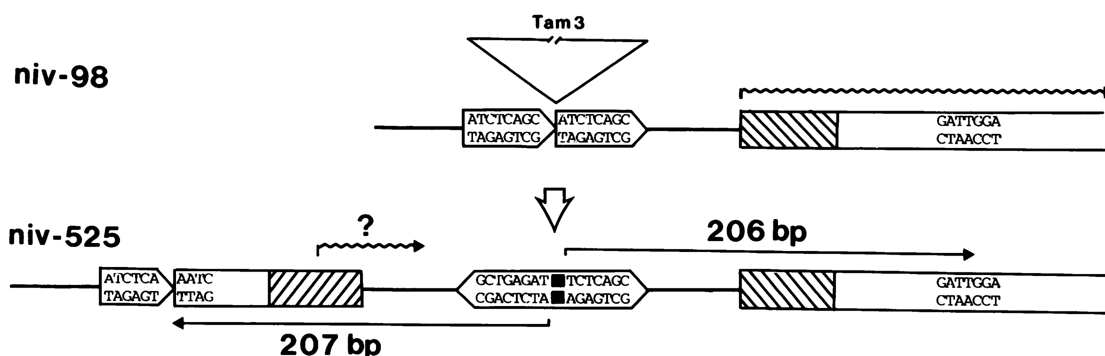

Fig. 5. Structures of *niv*-525 and its progenitor *niv*-98. The wavy arrow pointing to the right *niv*-98 indicates the normal start site and direction of *niv* transcription. The transcribed region comprises the non-translated leader (shaded section) and the protein coding region (open section; Sommer and Saedler, 1986). The sequence of breakpoints involved in the production of *niv*-525 are shown. The triangle indicates the location of Tam 3 in *niv*-98 and the boxed arrows show the 8-bp target duplications flanking Tam 3. A potential anti-sense transcript in *niv*-525 is indicated by a wavy line with a question mark above it (see Discussion).

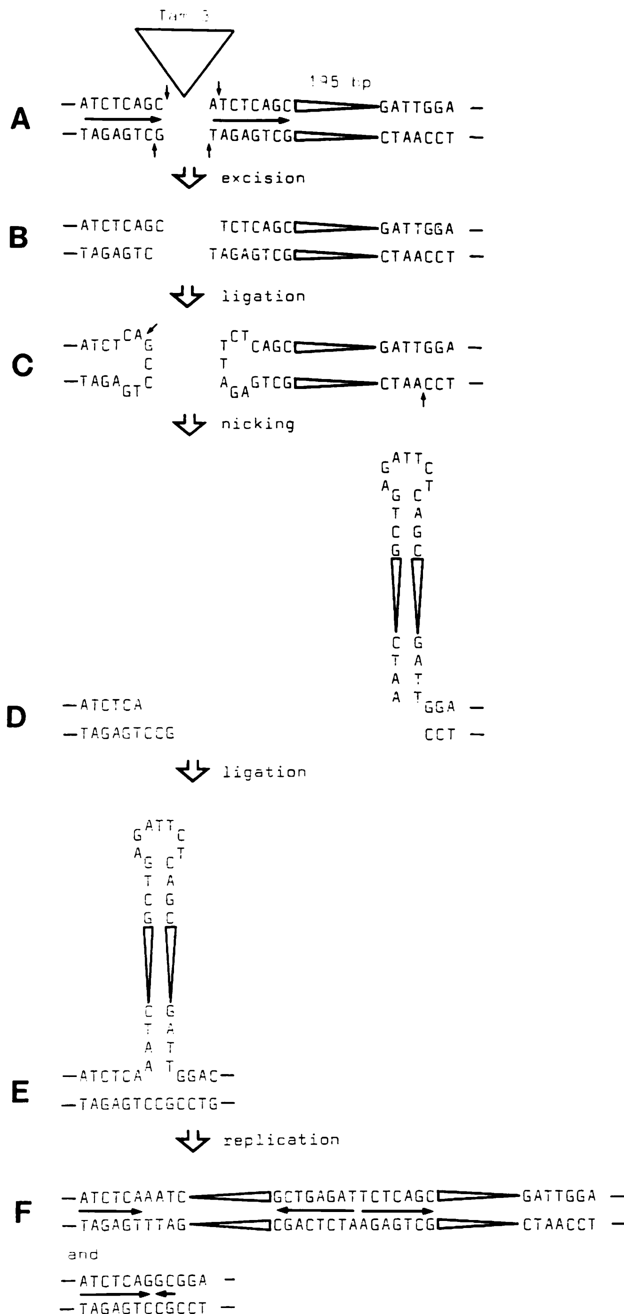

Fig. 6. Model for the origin of *niv*-525. The first steps of the excision process involve staggered single-strand cuts being made at sites flanking the element (**A**). In the case of Tam 3, these cuts are separated by 1 bp to give two DNA molecules, each with an overhang of one nucleotide (**B**) (in the example illustrated the overhang is arbitrarily shown as 3′ although a 5′ overhang is equally consistent with the data). The free ends are then ligated to form two hairpin structures (**C**) which are resolved by further single-strand cuts. In the majority of excisions these cuts occur in the single-stranded regions at the ends of the hairpins, as shown for the left hairpin. However, in the case of *niv*-525 we propose that the cut in the right hairpin occurred 207 bp from its end. Although this region is a relatively long distance along the DNA molecule from the normal site of nicking, it may have been physically close to the excision site in the particular conformation of chromatin adopted during the excision event. After nicking (**D**), the left and right DNA molecules are ligated together (**E**) and then replicated to give two reciprocal products, one with an inverted duplication of 207 bp with 1 bp deleted at the axis of symmetry (*niv*-525) and one with a deletion of ~200 bp (**F**). It is also possible that the intermediate shown in (**E**) was corrected by gene conversion before DNA replication to give only one of the products shown.

binding protein, to inhibit expression of the *Niv*$^+$ allele.

The third type of explanation for the semi-dominance of *niv*-525 is that it is due to the production of anti-sense RNA. Deletion analysis of the *Niv*$^+$ promoter has shown that a region of 1.2 kb, which lies upstream of the TATA box, is required for gene expression (Kaulen *et al.*, 1986). Apart from 15 bp this region is retained intact, upstream (to the left in Figure 5) of the inverted duplication of *niv*-525, and would be expected to drive transcription to the right if provided with a suitable TATA box. The sequence TTATAAA, identical to that of the normal TATA box, is located within the inverted duplication near the junction of the inverted leader and coding region. Transcription might therefore be driven from this new TATA box to produce an RNA with 40 bp at its 5′ end of the anti-sense strand of leader sequence (see hypothetical transcript in Figure 5). As little as 52 bp of anti-sense leader RNA has been shown to inhibit gene expression in eukaryotes, presumably by complexing with the sense RNA (Izant and Weintraub, 1985). The precise mechanism of inhibition of expression by genes producing anti-sense RNA is unclear but may involve degradation in the nucleus and/or inefficient transport from nucleus to cytoplasm of double-stranded RNA (Crowley *et al.*, 1985; Kim and Wold, 1985). In a *niv*-525/*Niv*$^+$ heterozygote, the *niv*-525 allele could produce an anti-sense RNA molecule which hybridizes to the first 40 bp of the leader RNA from the *Niv*$^+$ allele and the resulting double-stranded RNA could be degraded rapidly in the nucleus. This would account for the observed reduction of wild-type transcript in both poly(A)$^+$ and total RNA. According to this hypothesis, the anti-sense RNA transcript from *niv*-525 would have to terminate before the normal transcription start site since otherwise the RNA would snap back on itself and be unavailable to act *in trans* on transcripts from the *Niv*$^+$ allele.

One problem with the anti-sense model is that previous studies using DNA transformation indicate that inhibition by anti-sense RNA requires a 10- to 100-fold excess of DNA expressing anti-sense over DNA expressing sense RNA (Izant and Weintraub, 1985; Ecker and Davies, 1986). The *niv*-525 and *Niv*$^+$ alleles have similar upstream promoter sequences so it is unlikely that anti-sense RNA from *niv*-525 is much more abundant than sense RNA from *Niv*$^+$. Thus, anti-sense RNA produced from *niv*-525 would have to be exceptionally efficient at inhibiting sense RNA. One feature of *niv*-525 which distinguishes it from the anti-sense constructs introduced by transformation is that it is located at a chromosome position homologous to the *Niv*$^+$ allele it inhibits. If, as described above, alleles are physically associated during gene expression, a high local concentration of anti-sense RNA could be produced by *niv*-525 around the *Niv*$^+$ allele, accounting for its effectiveness as an inhibitor. An early model proposed to explain the *zeste*–*white* interaction in *D.melanogaster* also postulated that a labile untranslated RNA is produced from the 5′ region of the *white* gene which inhibits gene expression of *white* and is maintained in high local concentration around the *white* locus by somatic pairing (Jack and Judd, 1979). Perhaps, if this model is correct, the labile inhibitory RNA corresponds to an anti-sense transcript of the *white* locus initiated from a promoter located within the *white* gene. Attempts to identify anti-sense transcripts in total or poly(A)$^+$ RNA by Northern blots and S1 analysis were unsuccessful in flies carrying the *zeste* mutation (Pirrotta and Bröckl, 1984) and in plants carrying

niv-525 (our unpublished results). However, the anti-sense RNA may be labile and restricted to the nucleus so these experiments would not be expected to detect it.

The mechanism of Tam 3 excision

The *niv*-525 allele was derived from *niv*-98, which carries the transposable element Tam 3 in the *niv* promoter region. The inverted duplication in *niv*-525 forms a large palindrome and the axis of symmetry corresponds to the site where Tam 3 excised from the *niv*-98 progenitor. The palindrome is not perfect, one nucleotide being missing from the right arm (black square, Figure 5). This structure can be readily explained by a recently proposed model of plant transposable element excision that involves resolution of two hairpin DNA molecules (see Figure 6; Coen *et al.*, 1986; Peacock *et al.*, 1984). An alternative model which might account for the structure by strand-switching of DNA polymerase has been proposed by Saedler and Nevers (1985).

A notable feature of the hairpin model is that the initial single-strand cuts made at the ends of Tam 3 are staggered by 1 bp (Figure 6A). This is necessary to explain the loss of 1 bp from the centre of the inverted duplication of *niv*-525. A 1-bp staggered cut was also postulated to explain several imprecise Tam 3 excisions from the *pal* locus (Coen *et al.*, 1986; J.Almeida, R.Carpenter and E.Coen, unpublished results), suggesting that Tam 3 excision always generates hairpin structures by 1-bp staggered cuts. Most of the short inverted duplications observed following excision of the transposable elements *Ac* or *Ds* in maize have one nucleotide missing at the axis of symmetry (Sachs *et al.*, 1983; Baker *et al.*, 1986; Wessler *et al.*, 1986; Saedler and Nevers, 1985). The one exception to this rule (*adh-1*-RV2) has two nucleotides missing and may indicate occasional variability in the excision process (Sachs *et al.*, 1983). Furthermore, genetic analysis has shown that large inverted duplications can arise following *Ds* excision although the nucleotide sequence of the axis of symmetry is unknown (McClintock, 1950). These observations suggests that *Ac/Ds* elements excise in a similar manner to Tam 3, by producing 1-bp staggered cuts at the site of excision which are ligated to form hairpins. Structural similarities between *Ac* and Tam 3 have previously been noted (Sommer *et al.*, 1985) and both generally produce 8-bp target duplications, suggesting that the similar excision mechanism may reflect a common ancestry in evolution.

In contrast, the inverted duplications observed following excision of elements belonging to the *Spm/En* family of maize or the Tam 2 element of *Antirrhinum* are perfect palindromes (Schwarz-Sommer *et al.*, 1985, 1987; Hehl *et al.*, 1987; Hudson *et al.*, 1987) and can be explained by hairpins which are generated by blunt-ended DNA molecules produced by unstaggered cuts at the end of the elements. These elements share structural features (Upadhayaya *et al.*, 1985) and they all produce 3-bp target duplications but are distinct from the *Ac*/Tam3 class of elements. The particular excision mechanism involved (i.e. staggered or unstaggered cuts) therefore appears to reflect the particular class to which the element belongs.

Materials and methods

Plant material

The lines of *A.majus* and the growth conditions used were as described in Carpenter *et al.* (1987).

Protein and RNA analysis

Protein was extracted from flowers by grinding in 50 mM Tris pH 7.0 on ice with insoluble polyvinylpolypyrrolidone. Debris was removed by spinning in a microcentrifuge. Protein content was assayed using Biorad Protein reagent and 40 μg of protein for each genotype was separated by electrophoresis on a 12% polyacrylamide gel and transferred to nitrocellulose filters according to Towbin *et al.* (1979). The filters were incubated with rabbit antibody to chalcone synthase from parsley (supplied by Dr K.Hahlbrock) and stained using alkaline phosphatase conjugated with anti-rabbit IgG.

RNA was extracted from flowers according to Kloppstech and Schweiger (1979) except that total RNA was precipitated at 4°C with 2 M LiCl before application to oligo(dT)-cellulose columns. Northern blots and S1 analysis were done according to Coen *et al.* (1986). The chalcone synthase probe used in Northerns was the cDNA clone pCA1 (Sommer and Saedler, 1986) and the *pallida* probe was from pJAM501 (Coen *et al.*, 1986). The S1 probe used was a *Kpn*I M13 subclone of ~300 bp including the left copy of the inverted duplication of *niv*-525 which carries 144 bp of transcript comprising the 5' leader and part of the first exon (Sommer and Saedler, 1986).

DNA analysis

DNA was prepared from plants according to Coen *et al.* (1986) and Southern hybridizations performed according to Southern *et al.* (1975) and Wahl *et al.* (1979) using the 5.7-kb *Eco*RI fragment from pAm3 as a probe (Wienand *et al.*, 1982). DNA from a *niv*-525 homozygote was digested to completion with *Eco*RI, subjected to electrophoresis on an agarose gel and a fraction ~5.9 kb cloned into λNM1149 (Murray, 1982). Clones were screened with the 5.7-kb chalcone synthase probe (see above). The resulting clone of the *niv*-525 allele was partially digested with *Kpn*I and fragments ~300 bp were cloned into M13, mp19 and sequenced according to Sanger *et al.* (1977).

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