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Developing a transposon tagging system to isolate rust-resistance genes from flax

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Summary. A line of flax, homozygous for four genes controlling resistance to flax rust, was transformed with T-DNA vectors carrying the maize transposable elements *Ac* and *Ds* to assess whether transposition frequency would be high enough to allow transposon tagging of the resistance genes. Transposition was much less frequent in flax than in Solanaceous hosts such as tobacco, tomato and potato. Transposition frequency in callus tissue, but not in plants, was increased by modifications to the transposase gene of *Ac*. Transactivation of the excision of a *Ds* element was achieved by expressing a cDNA copy of the *Ac* transposase gene from the *Agrobacterium* T-DNA 2′ promoter. Progeny of three plants transformed with *Ac* and 15 plants transformed with *Ds* and the transposase gene, were examined for transposition occurring in the absence of selection. Transposition was observed in the descendants of only one plant which contained at least nine copies of *Ac*. Newly transposed *Ac* elements were observed in 25–30% of the progeny of some members of this family and one active *Ac* element was located 28.8 (SE = 6.3) map units from the L^6 rust-resistance gene. This family will be potentially useful in our resistance gene tagging program.

Key words: *Linum usitatissimum* – *Melampsora lini* – Rust-resistance genes – *Ac*/*Ds* gene tagging

Introduction

Major genes conditioning resistance to plant diseases are used extensively in agriculture. The genetic basis of disease resistance has been studied in many host-pathogen interactions but few studies have been as extensive as the analysis of the interaction between flax (*Linum usitatissimum* L.) and *Melampsora lini* (Ehrenb.) Lév. the flax rust fungus (reviewed by Lawrence 1988). It was the analysis of the flax–flax rust interaction that revealed the gene-for-gene relationship that exists between specific dominant resistance genes in the host plant and corresponding dominant avirulence genes in the pathogen, a relationship that has been extended to many other plant–pathogen interactions (Flor 1971).

The products of the plant resistance genes and of the pathogen avirulence genes have not been identified, and no resistance or avirulence gene has been cloned. One method for the cloning of genes without identified products uses transposable elements to mutate and tag the target gene, allowing the cloning of the gene using the DNA of the transposable element as a probe. This method is now, in theory, applicable to any plant species that can be transformed with a characterised transposable element (Baker et al. 1986).

We are investigating the potential of the transposon tagging method for isolating rust-resistance genes from flax. A line of flax called 'Forge', homozygous for four different rust-resistance genes (L^6, *M*, *N* and P^2) has been developed. The approach is to transform this line with transposable elements, to cross the transgenic plants to the cultivar 'Hoshangabad', which possesses no known rust-resistance genes, and then to screen the hybrid progeny for mutation in any of the four dominant resistance genes by inoculating them with four strains of flax rust that each detect only one of the resistance genes. Most of the hybrid progeny will be resistant to all four strains but the rare susceptible mutant progeny that have lost one of the resistance specificities may contain a tagged resistance gene (Ellis et al. 1988; Lawrence et al. 1989).

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In this paper, we describe the behaviour of the maize transposable elements *Ac* and *Ds* in flax, demonstrate the possibility of increasing transposition frequency, and assess the potential of these transposons for tagging genes controlling rust resistance.

Materials and methods

Construction of Ac/Ds vectors

Full details of the plasmid constructs used in this paper will be supplied on request. In brief, to make pKU4-2'ORF and pKU4-2'FRO (see Fig. 3) we used a cloned cDNA copy of the transposase gene of *Ac* (kindly supplied by Prof. Peter Starlinger, University of Koln) extending from a *Nae*I site (position 959 on the *Ac* map) to a *Dra*I site (position 4163). The 3'-end mRNA-processing signals were provided by the proteinase inhibitor gene, PI-II, from potato (An et al. 1989). The transposase gene and the 3'-end were cloned downstream from the 2' promoter in pKU4 (Baker et al. 1987).

A set of vectors was made where the GUS (glucoronidase) reporter gene (Jefferson et al. 1986) was used to monitor *Ac-Ds* excision (see Fig. 4 for maps). The *Ac* and *Ds* elements used in these vectors were from the P locus of maize (Lechelt et al. 1989) and were described by Finnegan et al. (1989). The GUS gene and the transposase gene were transcribed by the 1' and 2' promoters, respectively, from pKU4. The selectable marker for plant transformation was the NOS-NPT-II gene from pGV1103Neo (Hain et al. 1985). The streptomycin/spectinomycin (Sm/Sp) resistance marker used for selection in bacteria was from pGV33 (Van Haute et al. 1983). When introduced into plant cells, this set of GUS vectors provided the means to quantitate the transposition of *Ac* and so compare it to the transposition frequency of *Ds* with or without the transposase gene product.

Transfer of Ac/Ds vectors to Agrobacterium tumefaciens

The *Ac/Ds* vectors described above were all constructed on pBR322-based replicons and so could be mobilised into the *Agrobacterium* strain GV3850 (Zambryski et al. 1983) by triparental mating (Van Haute et al. 1983) and "rescued" in *Agrobacterium* by cointegration with the disarmed Ti plasmid pGV3850. The markers for selection of cointegration were kanamycin (25 μg/ml) for pKU4-2'ORF and pKU4-2'FRO, and streptomycin and spectinomycin (each at 100 μg/ml) for p1'AcGNNeo, p1'DsGN-2'ORF and p1'DsGN-2'FRO.

Plant material

Two lines of flax were used. The first, called 'Forge', is homozygous for the four rust-resistance genes, L^6, M, N and P^2. The second line was the cultivar 'Hoshangabad' (Mayo and Shepherd 1980) that contains no identified rust-resistance genes and was used as the pollen parent to produce the hybrid progeny analysed in this paper.

Transformation of flax

The transformation method has been described by Lawrence et al. (1989). In brief, cotyledons from 6-day old seedlings were treated with *Agrobacterium* and shoots were regenerated from transformed callus that developed along the cut edges of the cotyledons.

Detection of Ac/Ds transposition

(1) DNA preparation. DNA was prepared from flax as described by Taylor and Powell (1982) and 5 μg samples were analysed by Southern hybridisation as described by Peterson (1990). The analysis of excision bands in plants, or callus transformed with pKU3 and pKU4-2'ORF, was carried out as described by Baker et al. (1987).

(2) DNA probes. The 1' probe was a ClaI-AluI fragment isolated from the 667 bp AluI fragment containing the 1' and 2' promoters (Velten et al. 1984). The NPT-II probe was the *Bgl*II-*Xho*I fragment of transposon Tn5 (Jorgensen et al. 1979). When *Ac* or *Ds* was present in the NPT-II gene, the 1' probe detected a 2.3 kb band and the NPT-II probe detected a 3.6 kb band. When *Ac* or *Ds* was excised from the NPT-II gene, both probes detected a 3.0 kb excision band. The *Ac* probes were from the wx-m9 allele (Fedoroff et al. 1983) of the maize gene, waxy. The *Ac* 3'-end probe extended from the AccI site at position 4191 of *Ac* to the PstI site in the flanking DNA of the waxy gene. The 5' probe extended from the *Bam*HI site (position 181) to the *Nru*I site (position 584) of *Ac*. Both these probes also hybridised to the *Ds* elements used in this paper.

(3) Southern analysis. DNA digested with *Hin*dIII was used to identify transposed *Ac* or *Ds* elements. In addition, DNA was also analysed after digestion with *Eco*RI and with *Acc*I. These two digests were particularly informative when fragments in the *Hin*dIII digests comigrated and were not clearly resolved. If *Ac* or *Ds* transpose to a new genomic location, then both 5' and 3' plant-*Ac*-DNA junctions or T-DNA-*Ac* junctions should change. Elements were classified as transposed only when DNA on both sides of the element was different from the element's original location. Such alterations in junction fragments were detected by sequential hybridisation first with a probe from the 3' end of *Ac* and then with a probe from the 5' end of *Ac*. As a further confirmation of transposed *Ac* and *Ds* elements, the filters were sequentially hybridised to the 1' probe and the *NPT-II* probe to show that these T-DNA probes did not hybridise to the ends of the transposed elements and that the elements were no longer associated with flanking sequences of their original T-DNA locations.

Fluorimetric assay of GUS activity

GUS activity was assayed as described by Finnegan et al. (1989).

Results

Ac transposes less frequently in flax than in Solanaceous plants

In the initial experiments, *Ac* was introduced into flax by *Agrobacterium* transformation using the binary vector pBT175 (Taylor et al. 1989), which carries *Ac* and a linked selectable marker, the NPT-II gene, between the T-DNA borders. This vector does not allow selection to be applied for cells in which excision of *Ac* from the T-DNA has occurred, so plants were examined by Southern analysis to detect 'empty donor sites', copies of the T-DNA that contain no *Ac* element (Taylor et al. 1989). DNA from 35 independent primary transformants, most possessing either one or two copies of *Ac*, was examined and no evidence of *Ac* movement was detected in any of the plants. In addition, 17 progeny derived from one plant that possessed five copies of *Ac* were examined and no germinal transposition of *Ac* was observed. These

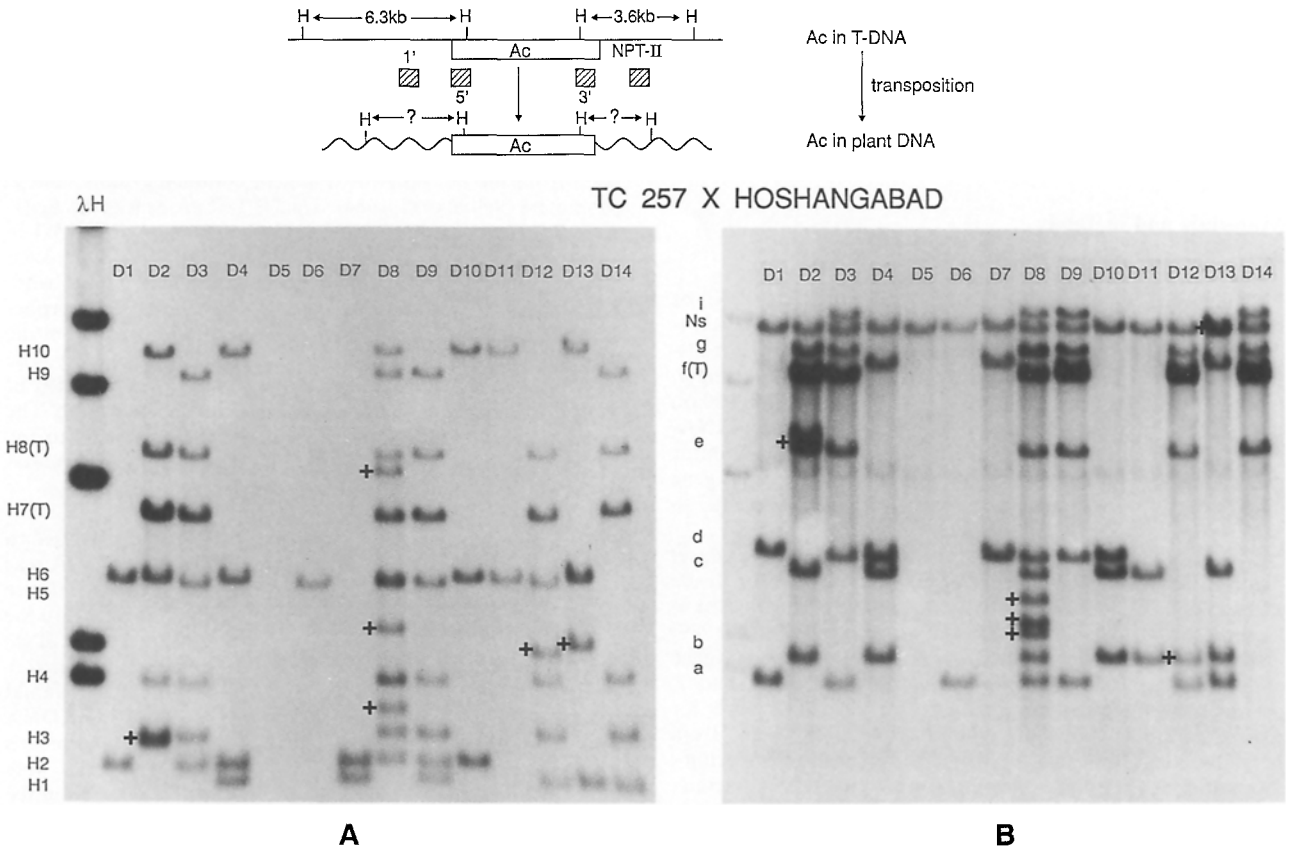


Fig. 1 A, B. Analysis of transposition of *Ac* in 14 progeny (*D1–D14*) of TC257 and 'Hoshangabad'. DNA was digested with *Hin*dIII and the filter probed sequentially with the 3′ (**A**) and 5′ (**B**) *Ac* probes and the NPT-II and 1′ promoter probes. Probe locations are indicated by *hatched boxes* on the map. *Ac* in the T-DNA gave fragments of 3.6 kb (3′ *Ac* probe) and 6.3 kb (5′ *Ac* probe) except in the case of fragment *H8* where a DNA rearrangment had occurred. Transposition of *Ac* gave rise to *Ac*-plant DNA junction fragments of unpredicted size that did not hybridise to NPT-II or 1′ promoter probes. Fragments labelled '*T*' resulted from non-transposed *Ac* elements in the T-DNA and also hybridised to the 1′ or NPT-II probes. The parental *Ac* fragments are labelled *H1–H10* (3′ probe) and *a–i* (5′ probe). All these fragments, except those marked '*T*', arose from *Ac* transposition early in the development of TC257. Fragments marked '+' are unique to one progeny plant and were not detected in the parent. These fragments resulted from later transposition in the germline of TC257 or very early in embryo development of the progeny. *Ns* indicates the non-specific hybridisation to the 5′ *Ac* probe

results are in contrast to those obtained with this and similar vectors in transgenic Solanaceous plants, like tobacco, tomato and potato, where movement of *Ac* was readily detected (Baker et al. 1986; Taylor et al. 1989; Yoder et al. 1988; Knapp et al. 1988).

Selection for Ac excision and detection of transposed Ac

Following the failure to detect *Ac* movement in flax using pBT175, flax was transformed with the vector pKU3, developed by Baker et al. (1987) and previously used to demonstrate *Ac* excision in flax callus (Roberts et al. 1990). This vector contains an NPT-II gene interrupted by an *Ac* element. Plant cells in which excision of *Ac* restores the activity of the NPT-II gene can be selected on tissue-culture medium containing kanamycin. Two transgenic plants, TC257 and TC275, were regenerated. These plants were crossed to 'Hoshangabad' and DNA from the transgenic parents, and from 14 progeny of each parent, was examined by Southern analysis. Four transposed copies of *Ac* were detected in TC275 and eight copies in TC257 and all these copies of *Ac* segregated in the progeny. The analysis of the 14 progeny of TC257 and the identification of the transposed copies of *Ac* is outlined in Fig. 1 and Table 1.

The detail of the analysis of TC257 and its progeny is as follows. The clearest interpretation of *Ac* copy number came from DNA digested with *Hin*dIII (Fig. 1). DNA of TC257 was no longer available for the preparation of this filter, but earlier analyses with DNA digested with *Eco*R1, or with *Eco*R1 and *Hin*dIII together, showed that the full complement of fragments in TC257 was represented by the sum of fragments in the progeny plants D4 and D9. The parental fragments detected by the 3′-end probe of *Ac* in the *Hin*dIII-digested DNA were labelled H1–H10 starting from the smallest fragment (Fig. 1 A). The *Hin*dIII fragments H5 and H6 co-migrated in this gel but were resolved by *Eco*R1 digestion. The *Ac* fragments H7 and H8 also hybridised to the NPT-II probe. These fragments were derived

Table 1. Co-segregation of 3′ and 5′ *Ac* fragments identifies *Ac* copies in TC257 and progeny

3′ fragment	5′ fragment	*Ac* copy
H1	g	*Ac* H1
H2	d	*Ac* H2
H3	h	*Ac* H3
H4	e	*Ac* H4
H5	a	*Ac* H5
H6	b or c[b]	*Ac* H6
H7 } T-DNA[a]	f(T-DNA)[c]	*Ac* H7[d]
H8 }		*Ac* H8[e]
H9	*i*	*Ac* H9
H10	b or c[b]	*Ac* H10

[a] Hybridises to the NPTII probe
[b] Since *Ac* fragments H6 and H10 co-segregate in all progeny it was not possible to assign the corresponding 5′-end fragments. *Ac* fragments H3 and H4 also co-segregated in the 14 progeny of TC257 analysed here but segregants from later crosses allowed assignment of corresponding 5′-end bands
[c] Hybridises to 1′ promoter probe
[d] Includes several copies of *Ac* still present in T-DNA
[e] Incomplete *Ac* in T-DNA

from non-transposed copies of *Ac* that have remained in the NPT-II gene in the T-DNA. The H7 fragment extends from the 3′ *Hin*dIII site in *Ac* to the *Hin*dIII site in the flanking T-DNA. The intensity of the hybridisation of both the 3′ *Ac* probe (Fig. 1 A) and the NPT-II probe to the H7 fragment indicated that there was more than one copy of this fragment, probably as a result of multiple insertions of the T-DNA in TC257. The *Ac* copy associated with the H8 T-DNA fragment had undergone a deletion; it did not hybridise to the 813 bp *Sph*I-fragment probe extending from position 2901 to 3714 of *Ac*.

The same filter was rehybridised with the 5′ *Ac* probe (Fig. 1 B). The hybridizing fragments were designated *a–i*, starting with the smallest fragment. The fragments associated with the T-DNA copies (detected by the 1′ promoter probe) co-migrated (fragment *f*). The fragment marked "NS", is present in non-transgenic flax and appears in varying intensity in many of our flax Southerns probed with the 5′ *Ac* probe. The function of this flax DNA, homologous to *Ac*, is not known.

By analysing the joint-segregation of the of 5′ and 3′ *Ac* fragments, it was possible to match them with ends of particular copies of *Ac* (Table 1).

This analysis identified eight copies of *Ac* (designated *Ac*H1, *Ac*H2, *Ac*H3, *Ac*H4, *Ac*H5, *Ac*H6, *Ac*H9, and *Ac*H10) that had transposed (probably soon after transformation) from the T-DNA in the germline of TC257 and, consequently, were transmitted to the progeny.

Transposition of Ac in the absence of selection

Although transposed copies of *Ac* were detected in TC257 and TC275 as a result of regenerating plants from cells that had been selected for kanamycin resistance, continuing transposition in the germline of these plants and their progeny would be necessary for tagging specific target genes. Therefore, progeny from both plants were examined for newly transposed copies of *Ac*, detected as novel (non-parental) plant-*Ac* DNA junction fragments present in progeny but absent in the parent. If equal numbers of new fragments were detected with the 3′ and 5′ probes, it was concluded that the new fragments arose by transposition of *Ac*.

No new transpositions of *Ac* were detected in the 14 progeny of TC275. However, four of the 14 progeny of TC257 had copies of *Ac* at new locations (marked "+" in lanes representing plants D2, D8, D12 and D13, Fig. 1) and of these, one plant (D8) had three non-parental copies of *Ac*. Because the intensity of the hybridisation signal of the non-parental *Ac* copies was equal to that of the parental copies, transposition must have occurred in the germline of the parent or very early in embryo development of the progeny plants. Therefore, *Ac* has remained active in the TC257 family and transposition has occurred in the absence of selection for excision.

The analysis that led to these conclusions is as follows. The non-parental *Ac* restriction fragments occurred in progeny plants D2 (one fragment), D8 (three fragments), D12 (one fragment) and D13 (one fragment), and were initially detected using the 3′ *Ac* probe (Fig. 1 A). The non-parental fragment in D2 migrates close to the H3 fragment but was clearly resolved by *Acc*I digestion. The same number of non-parental fragments were seen in D2, D8 and D13 when the 5′ *Ac* probe was used (Fig. 1 B). The fragment in D13 co-migrates with the "NS" fragment; however, the non-parental fragment is clearly darker in this track and, furthermore, was resolved by *Acc*I digestion. The picture for D12 appeared more complicated because no non-parental fragment could be seen in the *Hin*dIII-digested DNA probed with the 5′ *Ac* probe (Fig. 1 B). However, the non-parental fragment was identified as the one that migrates at the same position as fragment b. The reason for this conclusion is as follows. Fragment b is the 5′-end of either *Ac*H6 or *Ac*H10 (since *Ac*H6 and *Ac*H10 have cosegregated in all progeny, it was not possible to associate either of the 5′ fragments, *b* or *c*, with a particular 3′ fragment). Examination of *Eco*RI- or *Acc*I-digested DNA of plant D12 using the 5′ or 3′ *Ac* probes, showed that both *Ac*H6 and *Ac*H10 were absent in D12. Therefore, the fragment that migrates at the position of fragment b in plant D12 is in fact a non-parental fragment. So, for four of the 14 progeny of TC257, non-parental fragments were detected with the 3′ *Ac* probe and a corresponding number was detected with the 5′ *Ac* probe.

The TC257 line has been maintained by selfing. The copy number of *Ac* elements present in the selfed progeny has been analysed on Southern blots, and plants with high *Ac* copy number are being crossed to 'Hoshangabad' to provide hybrid seed for the screening of resistance-gene mutations. To determine whether *Ac* continues to transpose in this family, two selfed progeny of TC257, D94 and D97, were crossed to 'Hoshangabad' and 20 progeny from each cross were examined by Southern analysis. The *Ac* genotype of each parent was determined from the progeny analysis (Fig. 2). D97 contained at least 12 copies of *Ac* (some parental copies are homozygous) and six of the 20 progeny contained non-parental copies of *Ac*. In contrast, none of the 20 progeny of D94 which contained seven copies of *Ac*, but lacked

TC257
↓⊕
D94 x Hoshangabad → 20 progeny
D97 x Hoshangabad → 20 progeny (eg J457)

	Ac copy	D94	D97	J457
	Ac H10	+/+	−	−
	Ac H9	−	+/+	+/−
T-DNA copies	Ac H8*	−	+/+	+/−
T-DNA copies	Ac H7**	−	+/+	+/−
	Ac H6	+/+	−	−
	Ac H5	+/−	+/−	+/−
	Ac H4	−	+/+	+/−
	Ac H3	−	+/+	+/−
	Ac H2	+/−	+/−	+/−
	Ac H1	+/−	+/+	+/−
	Total elements	7	≥12**	≥8

* incomplete Ac
** more than one copy of Ac associated with T-DNA

Fig. 2. Analysis of *Ac* elements present in D94 and D97, selfed progeny of TC257. Both plants were crossed to 'Hoshangabad' and 20 progeny from each cross were examined by Southern TC257. The progeny analysis indicated whether the parent was (hybridization to identify *Ac* elements inherited from) homozygous (+/+) or heterozygous (+/−) for the various copies of *Ac* derived from TC257. The *Ac* genotype of the F_1 plant J457, which has been used in RFLP analysis, is also shown

the T-DNA associated copies, contained any newly transposed *Ac* elements.

Joint segregation of Ac T-DNA and rust-resistance genes

A single plant, J457, from the cross between D97 and 'Hoshangabad', was test-crossed with 'Hoshangabad' and 52 progeny were selected at random to supply a family for RFLP mapping. Five transposed copies of *Ac* (*Ac*H1, *Ac*H2, *Ac*H3, *Ac*H4, *Ac*H5, and *Ac*H9), the *Ac* copies in the T-DNA (*Ac*H7 and *Ac*H8), and four rust-resistance genes, L^6, *M*, *N*, and P^2, were segregating in this family.

The *Ac* copies, *Ac*H3, *Ac*H4 and *Ac*H9, and the T-DNA associated copies, *Ac*H7 and *Ac*H8, were tightly linked. *Ac* copies *Ac*H1, 2 and 5 segregated independently and were not linked to any T-DNA locus. Although *Ac* copies *Ac*H6 and *Ac*H10 were not present in this family, the data presented in Fig. 1 indicate that in these 14 progeny of TC257, *Ac*H6 and 10 have co-segregated and are not tightly linked to T-DNA loci. This means that five out of eight transposed *Ac* elements in TC257 are not closely linked to the original donor sites in the T-DNA.

One copy of *Ac*, *Ac*H1, was linked [28.8 (SE = 6.3) map units] to the L^6 rust-resistance gene. *Ac*H1 was capable of further transposition since it had transposed in one of the 20 progeny from the cross between D97 and 'Hoshangabad'.

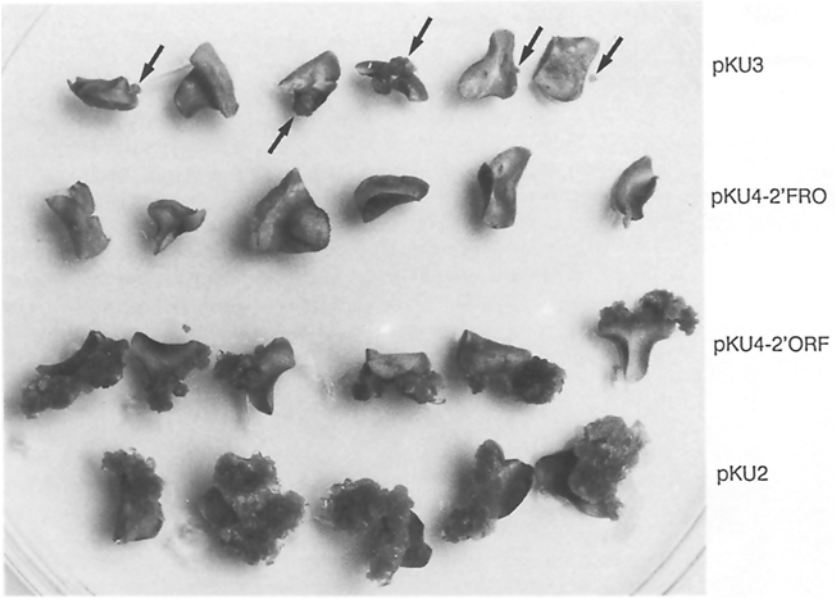
Modification of transposase expression increases excision

In an attempt to increase transposition in flax, several modifications were made to features that potentially limited the frequency of transposition of Ac in flax. For ease of carrying out the in-vitro manipulations, the modifications were incorporated into a two-element system where the transposase function was supplied in *trans* to effect the transposition of a *Ds* element. These modifications were:

(1) The use of a cDNA copy of the transposase gene to eliminate the four introns and the long untranslated leader sequence from the gene.
(2) The use of the T-DNA 2′ promoter, instead of the Ac promoter, to transcribe the transposase gene, and
(3) A 3′-end mRNA-signal from a dicot plant.

The vector pKU4-2′ORF, (Fig. 3) was derived from the *Ds* vector, pKU4 (Baker et al. 1987). The NPT-II gene is inactivated by the insertion of *Ds*; thus the transactivation of *Ds* excision could be monitored by expression of the NPT-II gene which confers resistance to kanamycin. Transformation of flax cotyledons with pKU4-2′ORF gave rise to a large increase in kanamycin-resistant callus compared to pKU3 and the control, pKU4-2′FRO, where the transposase gene had no promoter (Fig. 3). Southern analysis of plants regenerated from pKU4-2′ORF-infected tissue showed that *Ds* had transposed from the T-DNA.

To quantitate the increase in transposition provided by the two element system, a derivative of pKU4-2′ORF was made where the *Ds* element was inserted into the leader region of the GUS gene to inactivate its expression (p1′DsGN-2′ORF, Fig. 4). The excision of the transposon was then monitored by measuring the restoration of the activity of the GUS gene (Finnegan et al. 1989). As a control, p1′DsGN-2′FRO (Fig. 4), where the *Ac* transposase gene lacked a promoter, was used. A linked NPT-II gene, whose activity did not require transposition, enabled selection of transformed callus. A similar construct was made using *Ac* (p1′AcGNNeo) so that the transposition of the trans-activated *Ds* element could be compared to that of the autonomous *Ac* element. GUS activity was 13-fold higher in callus transformed with p1′DsGN-2′ORF than in callus transformed with p1′AcGNNeo (Fig. 4). In DNA prepared from callus tissue transformed with p1′AcGNNeo, p1′DsGN-2′ORF and p1′DsGN-2′FRO, a fragment resulting from excision of the transposon from the GUS gene was detected only in the callus transformed with p1′DsGN-2′ORF (Fig. 4). This was the first time we had detected an "excision band" in primary transformed flax tissue without direct selection for transposition.



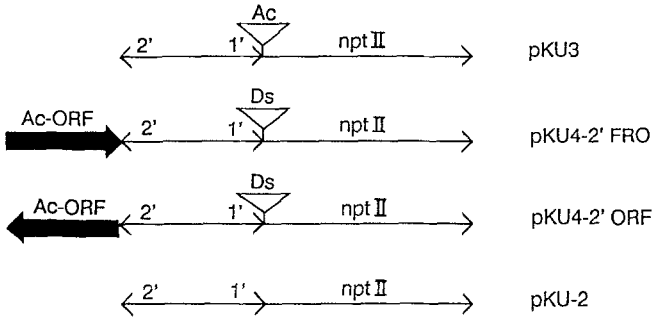


Fig. 3. Assaying excision of *Ac*/*Ds* from the NPT-II gene. Cotyledons were excised from flax seedlings and infected with *Agrobacterium* strains containing the four different transformation vectors shown on the maps (*1'* and *2'* indicate the promoters, and the cDNA copy of the transposase gene is designated *Ac-ORF*). After 2 days the explants were placed on selection medium containing kanamycin (200 mg/l). The explants were photographed 4 weeks after infection. The *arrows* indicate foci of callus resistant to kanamycin. The callusing that occurred after infection with vectors containing the interrupted NPT-II gene provides a measure of the amount of transposon excision. The amount the callusing observed with the uninterrupted vector, pKU2, indicates transformation efficiency

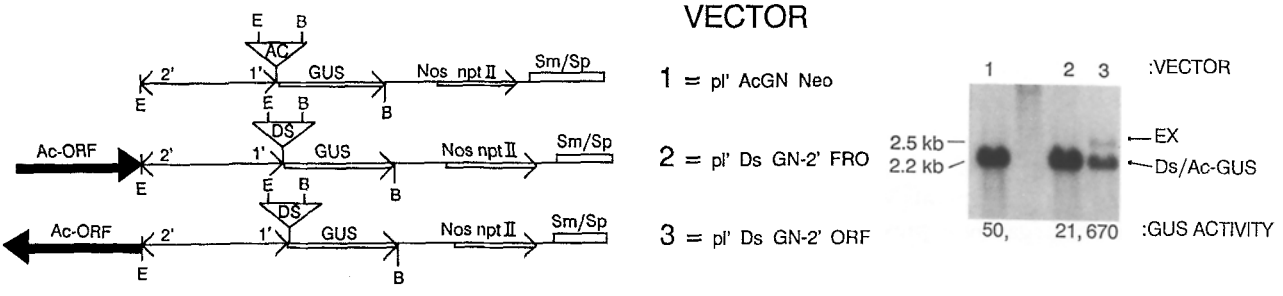


Fig. 4. Quantitation of the excision of trans-activated *Ds* using the GUS reporter gene. Flax cotyledons were transformed with the three vectors illustrated by the maps (*E* = *Eco*RI, *B* = *Bam*HI site, *Sm*/*Sp* = bacterial streptomycin/spectinomycin resistance gene, *Nosnpt II* = the NPT-II gene expressed from the nopaline synthase promoter, *GUS* specifies the glucuronidase gene's coding region and the cDNA copy of the *Ac* transposase gene is labelled *Ac-ORF*). Kanamycin-resistant callus was selected. Four weeks after transformation, GUS activity (pmoles/mg protein per min of 4-methylumbelliferone produced) was measured and DNA extracted for Southern analysis to detect excision products resulting from the transposition of *Ac* or *Ds* from the T-DNA. DNA was digested with *Eco*RI and *Bam*HI together, and probed with a DNA fragment containing the complete coding region of the GUS gene. *EX* indicates the product of excision (2.5 kb) and *Ds*/*Ac-GUS* indicates the GUS DNA interrupted by *Ac* or *Ds* (2.2 kb)

Transposition of Ds detected in primary transformants but not in their progeny

Transgenic plants were regenerated from cotyledon cells after transformation with pKU4-2'ORF and selection for excision of *Ds* from the NPT-II gene. In nine out of 15 primary transformants, 1–6 transposed copies of *Ds* were detected. The remaining seven plants contained no transposed *Ds*, suggesting that the excised *Ds* element failed to re-insert or, if it did, was lost after transposition by segregation during somatic cell division. Five primary transformants containing transposed copies of *Ds* and

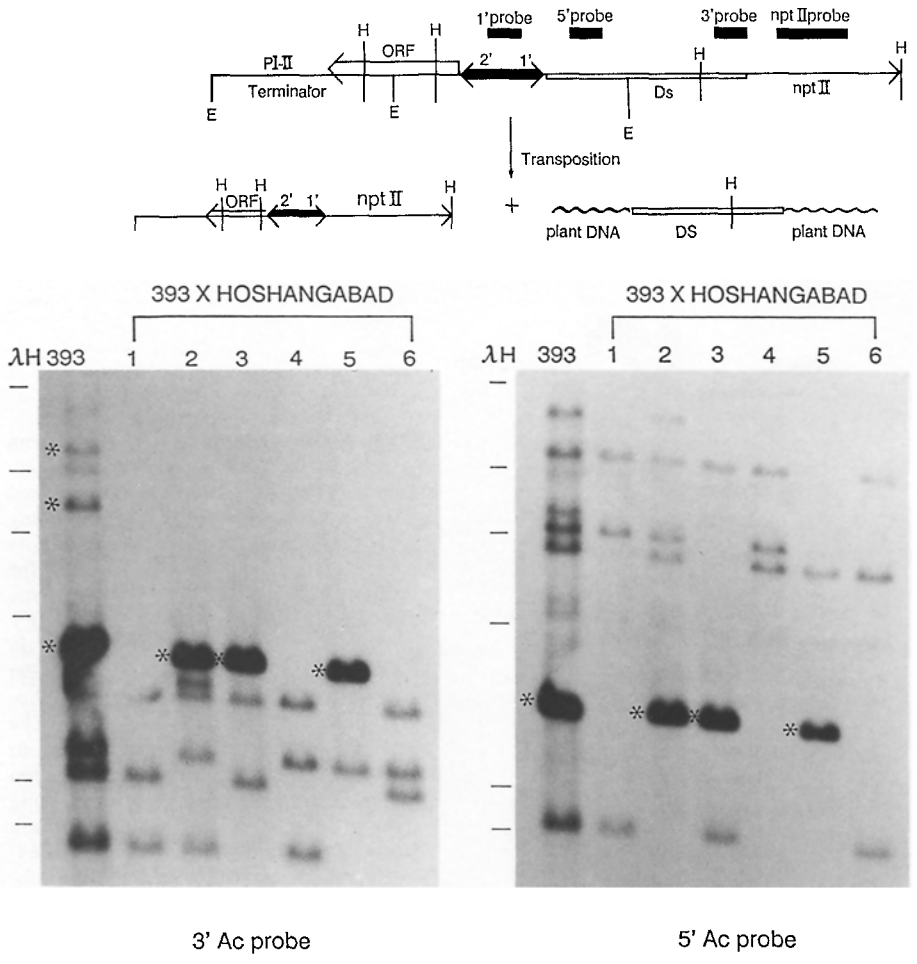


Fig. 5. Analysis of transposed *Ds* elements in plant J393, transformed with pKU4-2′ ORF, and six progeny plants. The region of the vector containing the 1′-*Ds*-NPT-II gene and the transposase gene is shown in the map together with the probe locations and expected excision product. The transposase gene (*ORF*) and its 3′-end processing region (*PI-II* Terminator) are shown. Restriction sites are, *E*=*Eco*RI, *H*=*Hin*dIII. Plant DNA was digested with *Hin*dIII. The filter was probed sequentially with the 3′ *Ac* probe, the 5′ *Ac* probe, the NPT-II probe and the 1′ promoter probe. The hybridisation pattern resulting from the 3′ and 5′ *Ac* probes are shown, and the *Ds* bands that are part of the T-DNA and also hybridise to the NPT-II probe or the 1′ promoter probe are marked *. Five *Ds* bands that did not hybridise to T-DNA probes were detected in the parent with both the 3′ and 5′ probes. These bands segregated in the progeny and were derived from transposed *Ds* elements. No new transposition events were detected in the progeny

the transposase gene were crossed to 'Hoshangabad' and a total of 36 progeny were examined for the presence of newly transposed copies of *Ds* that were not present in the parents. No transposed copies of *Ds* were observed. The results of the analysis of one of the parental plants and a family of six progeny plants where five transposed copies of *Ds* are segregating is presented in Fig. 5.

In summary, therefore, although the transactivation of *Ds* in pKU4-2′ORF by the transposase gene was effective in increasing excision of Ds in callus tissue, the frequency of transposition in germline or somatic tissue of transgenic plants was still too low to detect transposition in a sample of 36 progeny.

Discussion

Behaviour of Ac in flax

The principal findings of this study can be summarised as follows:

(1) No transposition was detected in flax plants possessing a few (1–5) copies of *Ac* that were introduced using a vector that did not allow selection for *Ac* excision. This is in contrast to the higher rates of *Ac* transposition in Solanaceous plants with the same or similar vectors.

(2) Using the pKU3 vector of Baker et al. (1987), that allows the selection of cells for resistance to kanamycin after excision of *Ac* from the leader of the NPT-II gene, *Ac* was shown to transpose in flax cells soon after primary infection.

(3) Use of the pKU3 vector resulted in one plant with high *Ac* copy number (nine or more). Some of the progeny of this plant possessed *Ac* elements at new locations, as did some of their progeny in turn. The transposition of *Ac* in these plants had occurred in the absence of selection and the intensity of hybridization of probes to the transposed copies of *Ac* was similar to that of the parental copies in the same plants. This implies that transposition occurred in the germline of the parent, or at least very early in the development of the progeny embryos.

(4) The use of a two-element system, in which a modified transposase gene (2′ promoter, transposase cDNA, dicot 3′-end) transactivated a *Ds* element, resulted in significantly increased rates of element-excision in cells soon after primary infection. Consequently, excision of *Ds* in

callus was observed even when no selection for excision had been applied. However, amongst 36 progeny of plants containing the two-element system, there was no evidence of continued *Ds* transposition. This may be due to preferential expression of the 2′ promoter in callus tissue (Langridge et al. 1989). Nevertheless, this result clearly demonstrates that the frequency of *Ac*/*Ds* transposition can be manipulated in flax by in-vitro modification of the transposase gene.

Feasibility of transposon tagging in flax

A transposon tagging system is only feasible if the transposon is active in the germline of the plant so that progeny are produced with transposons at new locations. At present, the only flax plants that satisfy this requirement are TC257 and some of its progeny. This family is characterised by having a high *Ac* copy number and, within the family, there is some evidence of a correlation between *Ac* copy number and transposition frequency. Thus TC257, with at least nine copies of *Ac*, gave four out of 14 progeny with newly transposed *Ac* elements, including one individual with three transposed elements. Plant D97 (with at least 12 copies of *Ac*), obtained by selfing TC257, gave six out of 20 progeny with *Ac* at new locations. However, a sibling plant, D94 (seven copies of *Ac*), gave 0 out of 20 progeny with *Ac* at a new location. Plant J457 (seven copies of *Ac*) gave two out of 52 progeny with transposed *Ac* elements. Another primary transformant, TC275, with at least five copies of *Ac*, gave 0 out of 14 progeny with transposed *Ac* elements. These findings suggest that it may be necessary to achieve a threshold number of *Ac* copies, perhaps about seven, before transposition occurs at a high frequency in the germline of flax. In tobacco, transposition frequency of *Ac* increases with copy number (Jones et al. 1990). However, another possibility to explain the results in flax is that one of the *Ac* elements in TC257 is highly expressed, due for example to its position in the genome, and that this element transposes and transactivates other elements. One of the T-DNA copies of *Ac* present in TC257 and D97, but absent in D94, may have this role.

The frequency of transposition in the TC257 family, where 25–30% of progeny from some crosses contain one or more newly transposed copies of *Ac*, should make gene tagging in flax feasible. Accordingly, TC257 and selfed progeny are being crossed extensively to 'Hoshangabad' to provide large numbers of hybrid seed, heterozygous at four rust-resistance loci, for the screening of tagged resistance genes. One copy of *Ac*, *Ac*H1, was located 28.8 (SE = 6.3) map units from the L^6 rust-resistance gene. Since *Ac* commonly jumps to linked locations in maize and transgenic tobacco (Greenblatt 1984; Dooner and Belachew 1989; Jones et al. 1989; Dooner et al. 1991), this linkage may be helpful in targetting the L^6 gene. In addition to using the TC257 family in tagging, further primary transformants with high *Ac* copy number are being sought and tested for *Ac* activity. Additionally, other promoters are being assessed for their ability to express the *Ac* transposase and to increase transposition in the germline of flax.

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