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Phenotypic assay for excision of the maize controlling element *Ac* in tobacco

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We describe a phenotypic assay designed to detect excision of the maize controlling element *Ac* from a selectable marker gene, neomycin phosphotransferase II (NPT II). An NPT II gene which expresses kanamycin resistance in tobacco cells, and contains a unique restriction enzyme site in the untranslated leader region, was constructed. *Ac*, or a defective *Ac* element (*Ac*Δ), was inserted into the leader region of this gene. The transposon insertions inactivated the NPT II gene as determined by transient NPT II expression assays. The three plasmids were inserted into the T DNA of *Agrobacterium tumefaciens* Ti plasmid vectors, and transferred to tobacco protoplasts. The transformed protoplasts were selected with 100 or 200 μg/ml kanamycin. Protoplasts transformed by the NPT II gene interrupted by *Ac* formed ~25% as many calli resistant to 100 or 200 μg/ml kanamycin as protoplasts transformed by the uninterrupted NPT II gene. Protoplasts transformed by the NPT II gene interrupted by *Ac*Δ did not form any calli resistant to 200 μg/ml of kanamycin when transformed under similar conditions. Southern blot hybridization analyses of seven kanamycin-resistant calli or plants obtained after transformation by the NPT II gene interrupted by *Ac* revealed that in all cases *Ac* had excised, restoring the structure of the NPT II gene. This assay is therefore useful to monitor the activity of a transposable element such as *Ac* and to define the regions of this element involved in transposition activity.

Key words: Ac-transposition/tobacco transformation/gene tagging/transposon insertion

Introduction

The maize (*Zea mays*) transposon *Ac* was among the first transposable elements to be described (McClintock, 1951). Two *Ac* insertions at the *Wx* locus have been cloned and their nucleotide sequence determined (Fedoroff *et al.*, 1983; Behrens *et al.*, 1984; Pohlman *et al.*, 1984; Müller-Neumann *et al.*, 1984). These experiments revealed that in common with other transposable elements, *Ac* duplicates a short sequence (8 bp) at the site of insertion, has an 11-bp terminal inverted repetition and contains long open reading frames.

In addition to promoting its own transposition, *Ac* encodes functions required for the transposition of *Ds* elements (McClintock, 1951; for review, see Fedoroff, 1983). All *Ds* elements isolated so far contain 11-bp terminal inverted repeats similar or identical to those of *Ac*. However, the internal sequences of *Ds* elements vary (for review, see Döring and Starlinger, 1984). One class are simple internal deletions of *Ac* (Fedoroff *et al.*, 1983; Döring *et al.*, 1984). A second class contains several hundred base pairs at each end which are homologous to *Ac* whereas the internal sequences are unrelated (Merkelbach *et al.*, 1986). *Ds* elements of a third class share the 11-bp inverted repeats and short adjacent internal sequences with *Ac* (Sutton *et al.*, 1984). These three classes of *Ds* elements transpose only when *Ac* is present in the genome.

Ac transposes in cells of *Nicotiana tabacum* (Baker *et al.*, 1986). This was demonstrated by introducing *Ac* into tobacco cells using a Ti plasmid vector and showing that the element can excise from its original location in the T-DNA and integrate elsewhere in the tobacco genome. The occurrence of typical *Ac* footprints (Sachs *et al.*, 1983; Pohlman *et al.*, 1984; Saedler and Nevers, 1985; Schwarz-Sommer *et al.*, 1985; Baker *et al.*, 1986) suggested that excision of *Ac* in tobacco occurred by a mechanism similar to that in maize. The ability to introduce foreign DNA into tobacco makes it an attractive system in which to study the sequences required for the activity of *Ac*. However, a disadvantage of this system is the lack of a phenotypic assay for *Ac* excisions. In maize the genetic studies of *Ac* have been greatly

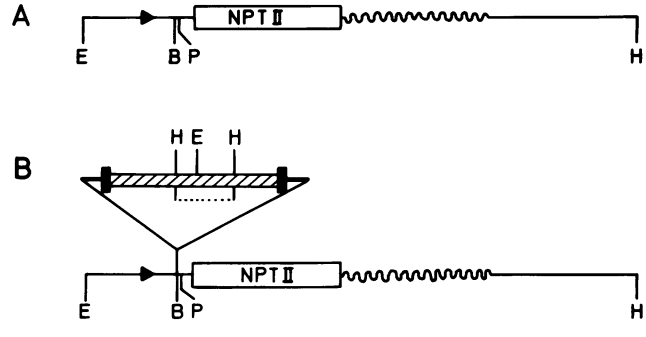

Fig. 1. Partial restriction enzyme map of the region encoding the NPT II gene of plasmid pKU2 and derivatives. (**A**) pKU2. The 1′ promoter of octopine Ti plasmid TR-DNA is located at least 100 bp downstream from the *Eco*RI restriction enzyme site. The transcription initiation site, indicated by the solid arrow, is located ~50 bp upstream from the *Bam*HI restriction enzyme site. The translation initiation codon of the NPT II gene is located 30 bp downstream from the *Bam*HI restriction enzyme site. The NPT II gene coding sequences are represented by the open box. The waved line denotes 3′ untranslated sequences and includes the polyadenylation signal from the octopine synthase gene. (**B**) pKU3 and pKU27 are *Ac* insertion derivatives cloned at the *Bam*HI site of pKU2. The orientation of the 4.5-kb *Ac* insert (hatched boxed area) in pKU3 is such that the long open reading frame of *Ac* reads from left to right (Kunze and Starlinger, 1986). The element was cloned in the opposite orientation in pKU27. The *Ac* element is drawn to 1/5 scale of that of the NPT II gene plasmid sequences. The element contains 11-bp terminal inverted repeats indicated by the solid rectangles (not to scale). The 60 bp of maize *Wx* sequence that flank the element are indicated by the thicker solid lines adjacent to the inverted repeats (not to scale). pKU4 and pKU11 were constructed by deletion of the internal 1.6-kb *Hin*dIII fragment of the *Ac* element of pKU3 and pKU27, respectively. The sequences deleted in pKU4 and pKU11 are indicated by the dotted line drawn below the *Ac* element. Restriction enzymes were abbreviated as follows: *Eco*RI, E; *Bam*HI, B; *Pst*I, P, and *Hin*dIII, H.

facilitated by the use of easily visualized endosperm markers, which allowed *Ac* insertions and excisions to be scored. We therefore designed a phenotypic assay which could monitor *Ac* excisions in tobacco. An NPT II gene whose expression is prevented by the insertion of *Ac* was constructed *in vitro*. After its introduction into tobacco, *Ac* can excise, resulting in NPT II gene expression and consequently in kanamycin-resistant tobacco cells. The frequency of *Ac* excision can, therefore, be monitored by the frequency with which kanamycin-resistant calli appear after transformation.

Results

Plasmid constructions

To clone the *Ac* element within the region coding for the untranslated leader of the NPT II gene, it was first necessary to construct an appropriate gene fusion containing a restriction endonuclease cleavage site within the untranslated leader. This was achieved by replacing the nopaline synthase promoter, which expresses the NPT II gene in pLGV1103neo (Hain *et al.*, 1985), with the 1′ promoter of octopine TR-DNA (Velten *et al.*, 1984). The resulting plasmid, pKU2, contains a unqiue *Bam*HI site within the leader sequence (Figure 1A).

In the *wx-m7* allele of maize, *Ac* is flanked by nearby *Bss*HII restriction endonuclease cleavage sites (Behrens *et al.*, 1984; Müller-Neumann *et al.*, 1984; Klösgen *et al.*, 1986). Cleavage with this enzyme yields a 4.6-kb fragment which contains *Ac* plus 60 bp of the *wx* locus. This fragment was inserted in both orientations into the *Bam*HI site of pKU2 to produce the plasmids pKU3 and pKU27 (Figure 1B). It was reasoned that the insertion of this 4.6-kb fragment would inactivate the NPT II gene, and that the excision of *Ac*, leaving only the 60-bp fragment of *wx* DNA in the untranslated leader, would restore NPT II activity and resistance to kanamycin.

Plasmids pKU3 and pKU27 contain the entire NPT II coding sequence separated from the 1′ promoter by the insertion of *Ac*, and were expected to produce kanamycin-resistant calli by specific excision of *Ac*. In order to determine the frequency with which kanamycin-resistant calli could be produced by a mechanism other than *Ac*-controlled excision, the internal *Hin*dIII fragment of *Ac* was deleted from pKU3 and pKU27, resulting in plasmids pKU4 and pKU11 (Figure 1B). This *Hin*dIII fragment contains the 194 bp which were deleted in the inactive *Ds 9* element (Fedoroff *et al.*, 1983; Pohlman *et al.*, 1984), therefore the *Ac* deletion derivatives present in pKU4 and pKU11 should not be capable of transposon-encoded excision from the NPT II gene.

Ac insertion within the untranslated leader of the NPT II gene prevents expression of the gene

To determine whether *Ac* insertion did indeed inactivate the NPT II gene, the ability of pKU3 and pKU4 to promote NPT II expression was determined in a transient expression assay (see Materials and methods).

As expected, pKU2 and pLGV1103neo which contain fusions of plant promoters to the NPT II coding sequence were able to promote NPT II expression in tobacco protoplasts (Figure 2, lanes 1 and 4; Velten *et al.*, 1984; Czernilofsky *et al.*, 1986a,b). However, neither pKU3 nor pKU4, which contain, respectively, a complete *Ac* and *Ac*Δ element in the untranslated leader, were able to promote NPT II expression in this transient assay (Figure 2, lanes 2 and 3).

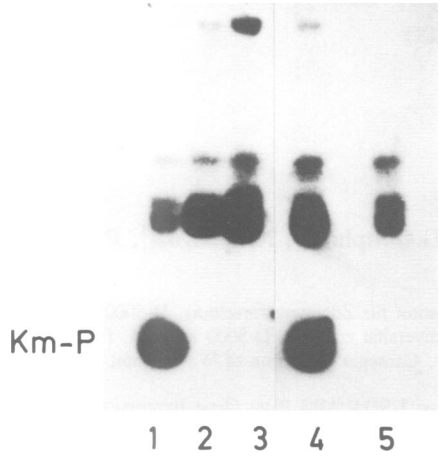

Fig. 2. Transient NPT II enzyme activity in tobacco protoplasts. NPT II enzyme activity expressed in tobacco protoplasts 2 days after transformation with *E. coli* plasmids. The position of the kanamycin phosphate is indicated. Each lane represents the measurement of NPT II activity in 1×10^5 protoplasts transformed with 10 μg of plasmid DNA: pKU2, **lane 1**; pKU3, **lane 2**; pKU4, **lane 3**; pLGV1103neo, **lane 4**; and control with no DNA, **lane 5.**

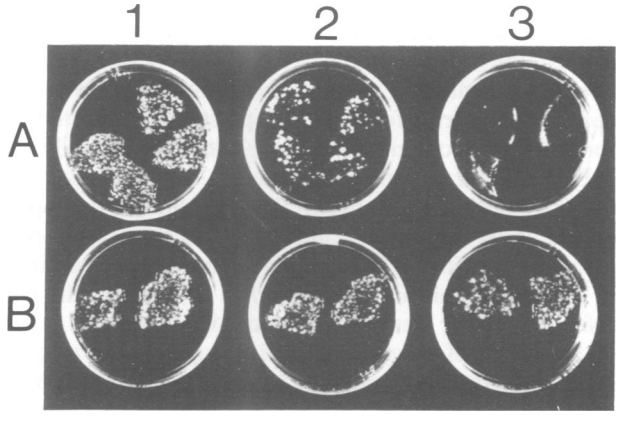

Fig. 3. Co-cultivation experiment 5 (Table I) of tobacco protoplasts with *Agrobacterium* strains containing Ti plasmid recombinants (**1**) pGV3850HPT::pKU2, (**2**) pGV3850HPT::pKU3 and (**3**) pGV3850HPT::pKU4 in (**A**) and (**B**). Approximately 2.25×10^5 protoplasts were co-cultivated with each *Agrobacterium* strain. (**A**) Calli resistant to both kanamycin (200 μg/ml) and hygromycin (15 μg/ml); (**B**) calli resistant to hygromycin alone (15 μg/ml). Antibiotics were applied 10 days after infection and calli are visualized after 6 weeks.

Ac excision in stably transformed tobacco cells restores NPT II gene expression

The plasmids described above (pKU2, pKU3, pKU4, pKU27, pKU11; Figure 1) were transferred to *Agrobacterium tumefaciens* containing Ti plasmid vectors (see Materials and methods). These strains were used in eight independent co-cultivation experiments, one of which is shown in Figure 3, and the results of all these experiments are summarized in Table I.

In experiments numbered 1–4 (Table I) *A. tumefaciens* strains harboring pGV3850 recombinants of the above plasmids were used for transformation. After co-cultivation with *A. tumefaciens* containing pGV3850::pKU2, ~1% of the total number of proto-

Table I. Number of kanamycin-resistant colonies derived from tobacco protoplasts infected with *Agrobacterium tumefaciens* strains

Experiment	Ti plasmid vector	Number of infected protoplasts	Km (μg/ml)	pKU2		pKU3		pKU4	
				A	B	A	B	A	B
1	pGV3850	2.1×10^4	100	185	(100%)	50	(27%)	17	(9%)
2	pGV3850	2.1×10^4	100	78	(100%)	16	(21%)	0	(0)
3	pGV3850	2.1×10^4	100	140	(100%)	27	(19%)	4	(2.5%)
4	pGV3850	3.15×10^4	200	274	(100%)	70	(25.5%)	0	(0)
5	pGV3850HPT	5.6×10^4	200	224	(100%)	91	(40%)	0	(0)
6	pGV3850HPT	1.3×10^5	200	140	(100%)	51	(36.4%)	0	(0)
7	pGV3850HPT	1.3×10^5	200	120	(100%)	42	(35%)	0	(0)
8	pGV3850HPT	1.3×10^5	200	197	(100%)	26	(13%)	0	(0)

A = total number of colonies that grew after selection with kanamycin (Km). For experiments 1 and 3 colonies were counted that continued to grow after transfer from bead culture to solidifed MS agar medium containing 100 μg/ml kanamycin. The others were counted directly in bead cultures.
B = the number of kanamycin-resistant transformants expressed as a percentage of the pGV3850::pKU2 number in each experiment.

plasts originally exposed to the bacteria produced kanamycin-resistant calli. This confirmed that the 1′ promoter − NPT II gene fusion present in pKU2 allows selection of stably transformed calli.

In the co-cultivation experiments numbered 5−8 (Table I) the *A. tumefaciens* containing recombinants of the above plasmids and pGV3850HPT, a Ti plasmid vector carrying a hygromycin phosphotransferase gene (HPT), active in plant cells, were used for transformation. The frequency of transformation could therefore be determined by selecting a portion of the protoplasts with hygromycin (Figure 3B). The frequency of transformation by each *A. tumefaciens* strain was similar in these co-cultivation experiments regardless of the expression of the NPT II gene (Figure 3).

The NPT II gene disrupted by *Ac* was transferred to tobacco via *A. tumefaciens* (pGV3850::pKU3 or pGV3850HPT::pKU3) and produced kanamycin-resistant calli at ~25% (average of all experiments in Table I) of the frequency found with pGV3850::pKU2 or pGV3850HPT::pKU2. These data suggested that 25% of all transformed cells gave rise, after only 10−12 days of growth, to microcalli which had sustained an *Ac* excision from at least one of the several *Ac*-carrying NPT II genes present in most transformed cells, leading to reactivation of the NPT II gene. Co-cultivation experiments with pGV3850::pKU27 gave results similar to those obtained with pGV3850::pKU3. These data indicated that the frequency of excision of *Ac* was independent of the element's orientation with respect to the NPT II gene promoter sequences (data not shown). The levels of NPT II activity in kanamycin-resistant calli obtained after transformation with either pGV3850::pKU2 or pGV3850::pKU3 were approximately equivalent (Figure 4, lanes 1−4).

To determine the frequency of *Ac* excision at times later than 10−12 days following transformation, hygromycin-resistant calli (Table I, experiment 5) were grown for 3 or 4 months after initiation of hygromycin selection and were then challenged for 1 month with kanamycin. The results are presented in Table II. The number of hygromycin-resistant pGV3850HPT::pKU3 transformants that were also kanamycin resistant was 70% of the number of doubly resistant pGV3850HPT::pKU2 transformants. Thus the frequency of *Ac* excision measured 3−4 months after transformation was ~1.7 times the frequency measured after 10−12 days of unchallenged growth.

Co-cultivation with *A. tumefaciens* pGV3850::pKU4, pGV3850HPT::pKU4 or pGV3850::pKU11, which contain the

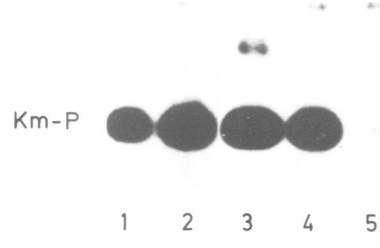

Fig. 4. Neomycin phosphotransferase II assay of kanamycin-resistant tobacco calli. **Lane 1**, pGV3850::pKU2-transformed tissue (100 μg/ml kanamycin); **lane 2**, pGV3850::pKU2-tranformed tissue (200 μg/ml kanamycin); **lanes 3** and **4**, pGV3850::pKU3-transformed tissue (200 μg/ml); **lane 5**, pGV3850::pKU4-transformed tissue (100 μg/ml kanamycin). The approximate concentration of protein (mg/ml), determined by comparison to BSA standards, was the following for lanes 1−5: 0.25, 0.25, 1.0, 0.25, 0.5.

NPT II gene inactivated by *AcΔ*, produced no kanamycin-resistant calli when selected on 200 μg/ml kanamycin under the conditions described in Table I. After selection on 100 μg/ml of kanamycin (Table I) or by using higher densities of infected protoplasts ($>2.2 \times 10^5$ infected protoplasts/plate) in the co-cultivation experiments, a few kanamycin-resistant calli were observed after transformation with pGV3850::pKU4 or pGV3850HPT::pKU4 (Figure 3, and see Materials and methods). One kanamycin-resistant pGV3850HPT::pKU4 callus was found in the experiment described in Table II. As can be seen in Figure 5 the NPT II gene in these kanamycin-resistant calli still carried the *AcΔ* insert. The low NPT II enzyme activity detected in these calli (Figure 4, lane 5) is therefore not due to *AcΔ* excision.

NPT II gene structure is restored after Ac excision

Analysis of DNA from transformed callus tissue was performed in order to confirm that kanamycin resistance of calli transformed with pGV3850::pKU3 was a consequence of *Ac* excision. We analyzed DNA from three pGV3850::pKU2-, four pGV3850::pKU3- and five pGV3850::pKU4-transformed kanamycin-resistant callus lines which were initially selected with, and grown in the presence of, 100 μg/ml kanamycin. DNA was also analyzed from one pGV3850::pKU2 and three pGV3850::pKU3 kanamycin-resistant plants regenerated in the presence of 100 or 200 μg/ml kanamycin.

Table II. Frequency of *Ac* excision measured 3–4 months after transformation

	Transformed calli		
	pGV3850HPT::pKU2	pGV3850HPT::pKU3	pGV3850HPT::pKU4
Total Hy^R calli tested	100	100	100
Number of calli surviving hygromycin selection (a)	99	96	89
Number of calli surviving kanamycin selection (b)	78	53	1
Percent of Km^R calli	78.79%	55.2%	1.2%
Percent of Km^R calli normalized to pGV3850HPT::pKU2 value	100%	70%	1.4%

Two equal portions of tissue were taken from 100 individual 3- to 4-month-old hygromycin-resistant (Hy^R) calli and placed on: (a) media containing hygromycin (15 μg/ml); (b) media containing both hygromycin (15 μg/ml) and kanamycin (Km) (200 μg/ml). Surviving calli were scored 1 month after transfer of tissue.

DNA isolated from transformed tobacco tissue was digested with restriction enzymes which produce DNA fragments characteristic of originally constructed, *Ac* interrupted or reconstituted NPT II gene structures. The DNA fragments used as hybridization probes (p1′ probe, Tn5 probe and *Ac* probe) were comprised of sequences which would distinguish the three different NPT II gene fragments (see Figure 5D and Materials and methods). DNA isolated from the *A. tumefaciens* strains was analyzed in parallel with transformed tobacco DNAs.

The NPT II coding region and 5′ and 3′ flanking sequences are present on an *Eco*RI–*Hin*dIII T-DNA fragment (2.9 kb) in pGV3850::pKU2 Ti plasmid DNA and in all four tobacco DNAs transformed with this *Agrobacterium* strain. The 2.9-kb fragment hybridizes to the p1′ probe (Figure 5, panel A, lanes 1 and 4) and Tn5 probe (data not shown). The 5′ flanking leader region of the wild-type NPT II gene is present on an *Eco*RI–*Pst*I restriction enzyme fragment (0.5 kb) in both *A. tumefaciens* and transformed tobacco DNA and is detected by hybridization to p1′ probe (Figure 5B, lanes 1 and 4 and Figure 5D, lane 1).

After insertion of *Ac* or *Ac*Δ into the leader region of the NPT II gene the *Eco*RI–*Hin*dIII and *Eco*RI–*Pst*I restriction fragments are interrupted by *Ac* sequences, so that *Eco*RI–*Hin*dIII and *Eco*RI–*Pst*I digestion of pGV3850::pKU3 and pGV3850::pKU4 *Agrobacterium* DNA yield fragments comprised of leader sequences (Figure 5A and B, lanes 5 and 6, Figure 5D, lanes 2 and 4) and *Ac* sequences (Figure 5C, lane 3; Figure 5D, lanes 2 and 4). Excision of *Ac* in transformed cells is expected to yield DNA fragments of size and composition similar to that found for pGV3850::pKU2 *Agrobacterium* and transformed tobacco DNA but the fragments containing the untranslated leader are expected to be slightly larger due to the 60 bp of *wx* sequence that were cloned with the element, and are expected to remain in the leader region after excision (Figure 5D, lane 3). The reconstituted NPT II gene fragments were indeed observed in the DNA of all seven kanamycin-resistant lines obtained after transformation with pGV3850::pKU3 and an example of the results of p1′ probe hybridization to the DNA of one callus line is shown in Figure 5A and B, lanes 2.

The results of hybridization with p1′ probe to *Eco*RI–*Hin*dIII digestions of all callus and plant DNAs transformed with pGV3850::pKU3 revealed that the reconstituted NPT II gene fragment (3.0 kb) was of greater intensity than the *Ac* interrupted fragment (2.3 kb). The ratio of the 3.0-kb fragment to the 2.3-kb fragment was ~5:1, as determined by densitometer scanning, for all callus DNAs. This suggests that *Ac* excised from a majority of the T-DNAs in the transformed kanamycin-resistant tissues (Figure 5A, lane 2).

None of the pGV3850::pKU4-transformed tobacco DNAs contained the fragments expected if the *Ac*Δ had excised from the leader of the NPT II gene. In three callus lines *Ac*Δ and surrounding T DNA sequences were arranged as originally constructed (Figure 5D, lane 4) and an example of one of these is shown in Figure 5A and B, lanes 3. In two lines the T-DNA and *Ac*Δ sequences appeared rearranged. However, as all NPT II gene fragments were linked to *Ac* sequences, neither line showed evidence for excision of the element (data not shown).

Similar analyses were performed using DNA isolated from two pGV3850::pKU2-, three pGV3850::pKU3- and two pGV3850::pKU4-transformed nopaline-positive callus lines (Table I, experiment 2) that had not been selected on kanamycin in order to determine if selection with kanamycin had indeed 'enriched' for a population of cells in which *Ac* had excised from the NPT II gene (see Materials and methods). The results of analysis of DNA isolated from the pGV3850::pKU2 and pGV3850::pKU4 unselected transformants were similar to those described above for the kanamycin-resistant tissues (data not shown).

The three pGV3850::pKU3 transformants contained the *Eco*RI–*Hin*dIII NPT II gene fragment characteristic of *Ac* excision. However, the ratio of the intensities, as determined by densitometer scanning, of hybridization of p1′ probe to the reconstituted (3.0 kb) and *Ac* interrupted (2.3 kb) NPT II gene fragments was different in each of the callus lines examined. In one line the ratio of the 3.0-kb fragment to the 2.3-kb fragment was ~10:1 (data not shown), similar to that described above for the kanamycin-selected calli (Figure 5A, lane 2). In another line the reconstituted and interrupted NPT II gene fragments were of similar intensity and the ratio was determined to be 1:1. In the third line the ratio of the 3.0-kb fragment to the 2.3-kb fragment was ~0.05:1 (data not shown).

These results differ from those described above for kanamycin-resistant callus lines, where in all cases the intensity of the reconstituted 3.0-kb fragment exceeded that of the 2.3-kb fragment. These data suggest that kanamycin selection had indeed 'enriched' for a population of cells in which *Ac* had excised from the NPT II gene.

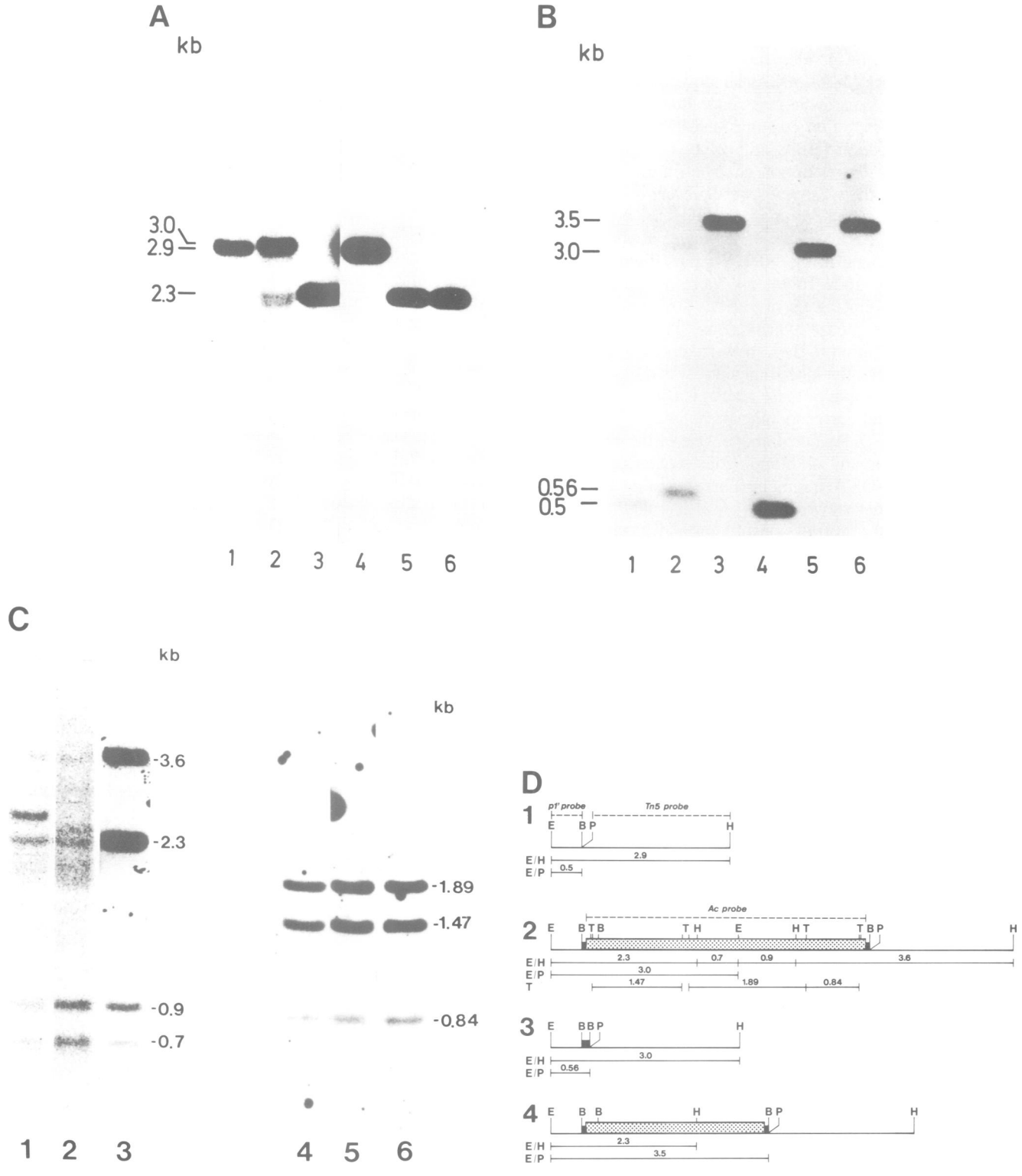

Fig. 5. Southern blot analysis of DNA isolated from transformed lines of tobacco callus and total DNA isolated from *A. tumefaciens*. Tobacco DNA was isolated from individually propagated callus lines derived from transformed protoplasts initially selected with 100 μg/ml kanamycin. Total *Agrobacterium* DNA was isolated from overnight cultures. Ten micrograms of tobacco DNA or 5 ng of *Agrobacterium* DNA were digested with restriction enzymes. **Panel A**, tobacco DNA, **lanes 1–3**, and *Agrobacterium*, **lanes 4–6**, were digested with *Eco*RI and *Hin*dIII and hybridized to pl′ probe; lanes 1, 2 and 3 contain tobacco DNA transformed with pGV3850::pKU2, pGV3850::pKU3 and pGV3850::pKU4, respectively, and lanes 4, 5 and 6 contain *A. tumefaciens* DNA harboring pGV3850::pKU2, pGV3850::pKU3 and pGV3850::pKU4, respectively. **Panel B**, DNA samples and hybridization probe as in panel A, but DNAs digested with *Eco*RI and *Pst*I. **Panel C, lanes 1** and **4** contain tobacco DNA isolated from one callus line; **lanes 2** and **5** contain tobacco DNA isolated from another independently derived callus line; **lanes 3** and **6** contain *A. tumefaciens* DNA harboring pGV3850::pKU3; lanes 1–3 were digested with *Eco*RI and *Hin*dIII and lanes 4–6 with *Taq*I. The size of the restriction enzyme fragments is given in kilobase pairs (kb). **Panel D**, schematic summary of the results of Southern blot hybridization with pl′, Tn5 and *Ac* probes to tobacco transformed with *A. tumefaciens* harboring the following Ti plasmids: (**1**) pGV3850::pKU2; (**2**) pGV3850::pKU3; (**3**) pGV3850::pKU3 with *Ac* excised; (**4**) pGV3850::pKU4. Hybridization probes are indicated by the dashed lines. Abbrevations are as in Figure 1, and T indicates *Taq*I. Numbers indicate the size of restriction enzyme fragments in kb.

Ac excision is accompanied by integration of elements elsewhere in the tobacco genome

To determine if excised *Ac* elements had integrated elsewhere in the genome of pGV3850::pKU3 kanamycin-resistant lines, DNA isolated from kanamycin-resistant callus and plant tissue was digested with restriction enzymes and hybridized with *Ac* probe. The hybridization results of *Eco*RI−*Hin*dIII digestions showed that internal *Ac* restriction enzyme fragments (0.9 and 0.7 kb) produced by these enzymes were present in all four calli and two of the three plants, but that flanking *Ac*−NPT II gene fragments (3.6 and 2.3 kb) were of far lower abundance than expected if *Ac* remained in the NPT II gene (Figure 5D, lane 2). New *Ac* homologous fragments were visualized in six transformants as several bands of various sizes and intensities or as a smear of less intense hybridization. The overall pattern of *Ac* probe hybridization to these new fragments, presumably new *Ac*−tobacco DNA flanking fragments, was different in each transformant. An example of these results is shown for two different callus lines and corresponding *A. tumefaciens* control in Figure 5C, lanes 1−3. As noted above, one of the regenerated plants did not contain any *Ac* homologous sequences, although it did contain the T-DNA fragments corresponding to an excision product (data not shown).

We investigated the overall constitution and integrity of the transposed *Ac* elements in the kanamycin-resistant callus lines by digestion of callus DNAs with *Taq*I. There are seven *Taq*I sites within the *Ac* element (Pohlman *et al.*, 1984; Müller-Neumann *et al.*, 1984). These are distributed such that three major internal fragments are produced (1.89, 1.47 and 0.84 kb), and comprise 92% of the *Ac* element (Figure 5D, lane 2). The relative intensities and sizes of these three fragments were similar after *Ac* hybridization in all callus DNAs to those of the corresponding *Agrobacterium* DNA. An example of the results obtained with two callus DNAs and the corresponding *A. tumefaciens* DNA is shown in Figure 5C, lanes 4−6. These data indicate that the structure of *Ac* elements in these lines (the majority of which are transposed) is, for the most part, unperturbed.

Discussion

We have described a semi-quantitative method for determining transposition activity of the maize transposable element *Ac* in heterologous plant hosts. This method relies upon *Ac* excision restoring the activity of a selectable gene expressed from a plant promoter. These experiments differ from the original demonstration of *Ac* transposition in tobacco (Baker *et al.*, 1986) in that *Ac* excisions can be monitored phenotypically, that the frequency of *Ac* excisions in the first 12 days after transformation can be measured, and that challenge with kanamycin allows the selection of a plant or callus derived from cells in which an *Ac* excision event has taken place.

Insertion of *Ac* into the untranslated leader of the NPT II gene inactivated the expression of the gene, as demonstrated by transient NPT II expression assays. It was, however, assumed that after *Ac* excision the activity of the gene would not be affected by the 60 bp of *waxy* gene sequence remaining in the leader. The original NPT II gene fusion contained a leader of 80 bp, whereas that produced by *Ac* excision was expected to be ~140 bp. The increase in size of the leader region was indeed observed in the genomic DNA of all kanamycin-resistant pGV3850::pKU3 transformants examined. In both the original NPT II gene and the restored NPT II gene following *Ac* excision, translation should initiate at the first codon of the open reading frame of the NPT II gene, as neither of the leader regions contain ATG triplets (Rogers *et al.*, 1985; Klosgen *et al.*, 1986). Indeed, gene activity was restored after *Ac* excision, since kanamycin-resistant calli were reproducibly selected in the presence of 200 μg/ml kanamycin after transformation with the plasmid containing *Ac*. However, at concentrations of 600 μg/ml no calli transformed with the *Ac* construction survived, although the wild-type gene did encode resistance to 600 μg/ml kanamycin though the number of kanamycin-resistant colonies was smaller (data not shown). This suggests that the longer leader present after *Ac* excision reduced expression of the gene, perhaps by reducing the stability, or efficiency of translation of the mRNA. Alternatively, a microcallus in which *Ac* has excised in only a fraction of the cells may be more susceptible to high concentrations of kanamycin than a microcallus expressing the NPT II enzyme of the wild-type gene in every cell.

The excision of *Ac* in tobacco, scored by resistance to kanamycin and confirmed by analysis of the restored NPT II gene structure, was dependent on the intact *Ac* element. The *Ac* deletion derivative present in pKU4 does not contain the 1.6-kb internal *Hin*dIII fragment. This fragment contains part of the long open reading frame of *Ac* (Müller-Neumann *et al.*, 1984; Pohlman *et al.*, 1984). This *Ac* derivative was incapable of autonomous excision as measured by selection with 200 μg/ml kanamycin. The few calli transformed with the defective *Ac* that survived selection with 100 μg/ml kanamycin did not contain any DNA fragments representative of a DNA molecule resulting from specific excision of the defective *Ac* element.

In a total of eight experiments the number of kanamycin-resistant calli detected with the construction containing *Ac* in the leader varied from 13 to 40% of that found with the wild-type NPT II gene when selection was applied 10−12 days following co-cultivation. In one experiment the frequency of excision of *Ac* in 3- to 4-month-old transformed calli was found to be 70%, which represents an increase in the excision frequency of 75% when compared to the frequency after 10−12 days. These data indicate that *Ac* excision continues for >2 weeks after its introduction into the tobacco cell. These values imply a high excision frequency of *Ac*, and are in accord with previous observations (Baker *et al.*, 1986). The high excision frequency of *Ac* could not have been the result of increased transcription of the *Ac* element from the adjacent 1′ promoter since high frequencies of excision were measured with *Ac* inserted in either orientation with respect to this promoter.

Several plant genes have been cloned by first isolating a mutated gene carrying an inserted transposon by using the previously cloned transposable element as a probe to isolate the element and flanking DNA, and then utilizing this flanking DNA as a probe to isolate the gene from a wild-type line (Fedoroff *et al.*, 1984; O'Reilly *et al.*, 1985; Martin *et al.*, 1985). This procedure has been termed gene tagging (Bingham *et al.*, 1981). Its use has so far been limited to those few plant species which contain genetically and molecularly well characterized transposable elements. Transformation of *Ac* into plant species not already containing a characterized transposable element should extend the usefulness of the gene tagging strategy. The *Ac* excision assay we describe here would allow any plant susceptible to DNA transformation, and to selection with kanamycin, to be readily checked to determine whether *Ac* is active in that particular plant. We report here that excision of *Ac* is usually accompanied by integration of the element elsewhere in the genome, and therefore selection with kanamycin will not only

enrich for a population of cells in which *Ac* has excised but also for cells in which *Ac* is integrated at new locations.

This assay should also be useful in determining whether *in vitro* constructed derivatives of *Ac* are capable of excision, thereby defining the regions of *Ac* required for this activity.

Materials and methods

Bacterial strains

A. tumefaciens strain C58C1 *rif* containing the pGV3850 Ti plasmid (Zambryski *et al.*, 1983; Joos *et al.*, 1983) or the pGV3850HPT Ti plasmid. (Kreuzaler, unpublished results) were recombined with *Escherichia coli* plasmids pKU2, pKU3, pKU4, pKU11 and pKU27 (Van Haute *et al.*, 1983). The pGV3850HPT plasmid carries a chimeric hygromycin phosphotransferase resistance gene (Van den Elzen *et al.*, 1985) within the T DNA region and confers hygromycin resistance to transformed cells. The resulting Ti plasmids were designated pGV3850::pKU2, pGV3850::pKU3, pGV3850::pKU4, pGV3850::pKU11, pGV3850::pKU27, pGV3850HPT::pKU2, pGV3850HPT::pKU3 and pGV3850HPT::pKU4. The structure of all Ti plasmids was checked by Southern blot hybridization. *A. tumefaciens* strains containing the above plasmids were used to transform tobacco protoplasts.

Construction of E. coli plasmids

pKU2 was constructed by substitution of the 410-bp *Eco*RI−*Bcl*I fragment containing the pNOS promoter of pLGV1103neo (Czernilofsky *et al.*, 1986b) with the 506-bp *Eco*RI−*Hin*dIII fragment containing the p1′ promoter of TR-DNA of pOP4434 (Velten *et al.*, 1984).

The 4623-bp *Bss*HII fragment containing the entire *Ac* element flanked by 60 bp of *wx* sequence was isolated from the *wx-m7* allele of maize (Behrens *et al.*, 1984). The ends of this fragment were filled in with the Klenow fragment of DNA polymerase and ligated to *Bam*HI-digested, filled in pKU2 DNA. The *Ac* fragment was inserted in both orientations to generate plasmids pKU3 and pKU27.

pKU4 and pKU11 were generated from pKU3 and pKU27, respectively. pKU3 and pKU27 plasmids were digested with *Hin*dIII and re-ligated. Resulting plasmids were screened for those which had lost the internal *Hin*dIII fragment of *Ac*.

Transformation of tobacco protoplasts by direct DNA uptake

Protoplasts isolated from *Nicotiana tabacum* cv. Petit Havana SR1 plantlets were transformed by the calcium nitrate−PEG6000 fusion technique described by Wirtz *et al.* (1987).

Ten micrograms of plasmid DNAs were used to transform 1×10^5 protoplasts. After transformation cells were washed, resuspended in K3 medium (Hain *et al.*, 1985) and placed at 25°C. Cells were collected after 48 h and prepared for NPT II enzyme assays (transient NPT II expression assay).

Transformation of tobacco protoplasts by co-cultivation with A. tumefaciens

A modified co-cultivation system (Marton *et al.*, 1979; Hain *et al.*, 1985) was used to transform SR1 tobacco protoplasts. Protoplasts were prepared and infected for 3−4 days with *Agrobacterium* strains containing plasmids pGV3850::pKU2, pGV3850::pKU3, pGV3850::pKU4, pGV3850::pKU27, pGV3850::pKU11 and pGV3850 or corresponding pGV3850HPT strains. Varying numbers of cells infected with each experimental *A. tumefaciens* strain were dispensed into individual dishes. The final concentration of infected tobacco cells was equalized in each dish by addition of pGV3850- or pGV3850HPT-infected cells.

After 7−8 days cells were embedded in agarose (Shillito *et al.*, 1983) and selection of transformants using 100 or 200 μg/ml kanamycin and/or 15 μg/ml hygromycin was initiated. In all co-cultivation experiments 12.5 or 25% of each embedded culture was grown without addition of antibiotics. In co-cultivation experiments 5−8 (Table I) 12.5 or 25% of the embedded cultures were grown in the presence of hygromycin (15 μg/ml). Medium containing antibiotics was replenished weekly. After 5−6 weeks colonies resistant to kanamycin and/or hygromycin were counted. Resistant callus colonies were transferred to solidified MS medium containing naphthaleneacetic acid at 1 mg/l, kinetin at 0.2 mg/l, kanamycin at 100 or 200 μg/ml and hygromycin at 15 μg/ml. Individual calli of bead culture (Table I, experiment 2) grown in the absence of kanamycin were screened for the presence of nopaline (Aerts *et al.*, 1979). Nopaline-positive transformants were propagated further without addition of antibiotics. All callus tissue was propagated for extraction of DNA, and/or for NPT II assays. Transformed plants were regenerated as described by Hain *et al.* (1985).

In experiments 4 and 5 (Table I) protoplasts transformed with pGV3850::pKU4 and pGV3850HPT::pKU4, respectively, did yield kanamycin-resistant calli (eight calli, experiment 4; two calli, experiment 5; Figure 3) when 2.5×10^5 and 2.25×10^5 protoplasts, respectively, were plated. These figures represent 1.0 and 0.3% of the number of pGV3850::pKU2 and pGV3850HPT::pKU2 transformants in experiments 4 and 5, respectively.

Preparation and analysis of DNA isolated from tobacco tissue

DNA was prepared as described (Czernilofsky *et al.*, 1986a), digested with restriction enzymes, separated by electrophoresis on 1% agarose gels, transferred to nitrocellulose, and hybridized with radioactively labeled DNA probes. Probes were produced by nick-translation to a specific activity of 1×10^8 c.p.m./μg. Purified DNA fragments used to generate the probes were the 506-bp *Eco*RI−*Hin*dIII fragment of pOP4434 (p1′ probe; Figure 5D; Velten *et al.*, 1984), the 1.6-kb *Pst*I fragment of pLGV1103neo (Tn5 probe; Figure 5D, lane 1; Czernilofsky *et al.*, 1986b) and the 4.8-kb fragment of pAc2 (Fedoroff *et al.*, 1983) from *wx-m9* (*Ac* probe; Figure 5D, lane 3). Radioautographs were scanned by a densitometer.

Neomycin phosphotransferase II assay

The *in situ* enzymatic gel assay for neomycin phosphotransferase II (NPT II) activity was performed as described (Reiss *et al.*, 1984). Relative protein amounts for the experiments shown in Figure 4 were determined according to Sedmak and Grossburg (1977). The transient NPT II expression assay shown in Figure 2 was peformed 2 days after direct DNA transfer to 1×10^5 protoplasts.

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