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Excision of the maize transposable element *Ac* in flax callus

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ABSTRACT

The frequency and fidelity of *Ac* transposition, and that of its non-autonomous derivative *Ds*, were investigated in flax callus. Flax (*Linum usitatissimum* var. *Antares*) hypocotyls were transformed with *Agrobacterium* Ti plasmid vectors containing the *Ac* or *Ds* element inserted within the untranslated leader sequence of a chimaeric neomycin phosphotransferase II gene. Kanamycin resistant tissues were produced as a result of excision of *Ac* in around 35% of the total number of *Ac*-containing transformants. In contrast, no excision was observed from transformants containing the *Ds* element. Whilst *Ac* appears to have excised completely from T-DNAs, little evidence was found to infer reintegration of the *Ac* element into the genome.

ABBREVIATIONS

NPT-II/*npt-II*, Neomycin phosphotransferase II; kb, Kilobasepairs; bp, basepairs; MSO, Murashige and Skoog medium; NAA, naphthalene acetic acid; BAP, 6-benzylaminopurine.

INTRODUCTION.

The isolation of genes with unknown products cannot be achieved easily using classical cloning methods. Most genes conferring disease or pest resistance fall into this category and are of obvious scientific and commercial interest. One method of cloning such genes is by transposon tagging, the process of producing insertion mutants using transposable elements in order to identify a gene. This is a strategy which has been employed successfully in maize and *Antirrhinum* (Fedoroff *et al.* 1984; O'Reilly *et al.* 1985; Martin *et al.* 1985; Theres *et al.* 1987). Endogenous transposable elements were used as gene tags in these species, but since detailed knowledge of transposons is limited to maize and *Antirrhinum*, it would be useful if a well characterised element could be used in other plants. The maize element *Ac* ("*Ac*tivator") is a good candidate, since *Ac* has previously been shown to be active in tobacco (Baker *et al.* 1986), *Arabidopsis* and carrot (Van Sluys *et al.* 1987), potato (Knapp *et al.* 1988) and tomato (Yoder *et al.* 1988). *Ac* is thus recognised as being highly autonomous, in that the single protein it encodes, (Kunze *et al.* 1987), is sufficient for transposition in a variety of chromosomal backgrounds. Thus it appears likely that *Ac* may function in many monocotyledonous and dicotyledonous plants. Flax has several attributes which makes it amenable to a gene tagging programme. Firstly, the transformation and tissue culture of this species have already been described, (Basiran *et al.* 1987) and are simple procedures. Furthermore, the small genome of flax (n = 0.7pg) and the availability of haploid plants from twinning embryos (Rajhathy 1976) ease constraints in genetic and molecular analysis. A suitable target for a heterologous transposon tagging experiment is the family of genes responsible for resistance to the rust fungus *Melampsora lini*, which is a pathogen of flax. Around 30 separate alleles for resistance are known and have been well studied genetically (see Ellis *et al.* 1988). With a view to developing a tagging programme, we have investigated the behaviour of *Ac* in flax using the phenotypic excision assay system developed by Baker *et al.* (1987).

MATERIALS AND METHODS.

Bacterial Strains and Plasmids

The *E. coli* plasmid pKU2 contains the *npt-II* gene under the control of the *pl'* promoter (Velten *et al.* 1984), which confers constitutive kanamycin resistance in plants. The *Ac* element, isolated as a 4.6 kb fragment, was inserted into the untranslated leader sequence of the *npt-II* gene in pKU2 to produce the plasmid pKU3. In this plasmid, expression of NPT-II is prevented by the presence of the *Ac* element. A similar *npt-II* gene interupted by an artificial *Ds* element was created by the deletion of a central 1.6 kb *Hin*dIII fragment of *Ac* in pKU3. This deletion removes a large section of the *Ac* transposase coding region; the resulting plasmid was named pKU4. These *E. coli* plasmids were recombined into a derivative of the disarmed Ti plasmid pGV3850, (Zambryski *et al.* 1983), pGV3850HPT, which carries a hygromycin resistance gene on the T-DNA, and were conjugated into *Agrobacterium tumefaciens* C58C1rif. These plasmids were constucted by, and gratefully received as a gift from Professor Peter Starlinger and Dr. George Coupland, (see Baker *et al.* (1987) for details and *Fig. 1* for structures).

Transformation of Flax Hypocotyls.

1 to 2 mm sterile hypocotyl sections were transformed with *Agrobacterium* following the method previously described by Basiran *et al.* (1987). Following inoculation, explants were transferred onto the callusing medium, MSD4x2, (MSO salts with the addition of 30mg/ml sucrose, 0.1 mg/ml NAA and 1.0 mg/ml BAP), containing 200μg/ml cefotaxime, which prevents growth of *Agrobacterium*, or 200μg/ml cefotaxime plus either 30μg/ml hygromycin or 200μg/ml kanamycin. Resistant callus was maintained by

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subcloning ten random 0.5cm pieces onto 20mls of MSD4x2 medium in 9cm petri dishes with appropriate antibiotics.

NPT-II Assays

Tissues were mass screened using a rapid dot assay, as described by McDonnell *et al.* (1987). A selection of NPT-II positive tissues were assayed using the non-denaturing polyacrylamide gel assay described by Reiss *et al.* (1984).

Southern Analysis

DNA was extracted with CTAB (cetyl triethylammonium bromide) by a method described in Draper *et al.* (1988), except that DNA was precipitated with -20°C isopropanol. 5-10μg of DNA were digested overnight with the appropriate restriction endonucleases, and the resulting fragments separated on 0.8% agarose gels. The DNA was transferred onto Hybond-N filters (Amersham) and hybridised according to a method based on that originally described by Southern (1975). Probes were prepared in 0.6% low gelling temperature agarose and random primer labelled, (Feinberg and Vogelstein 1984), to a specific activity of 1×10^8 - 1×10^9 cpm/μg of DNA. The *Ac* probe was isolated as a 4.6 kb *Bss*HII fragment from pJAC, (received from Peter Starlinger), which includes 60bp of maize *waxy* DNA. The *p1'* promoter probe was isolated from pOP4434 (Velten *et al.* 1984) as a 0.5 kb *Eco*RI/*Bam*HI fragment.

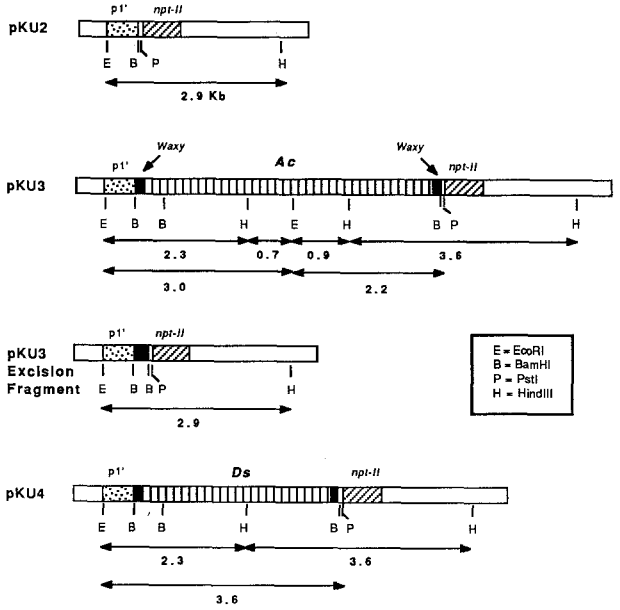

Figure 1. Structures of the pKU plasmids and derived structure following *Ac* excision in transformed plants. Restriction fragment sizes are indicated in kilobases.

RESULTS

Transformation and Tissue Culture

Inoculated and uninoculated hypocotyls were transferred onto normal MSD4x2 medium, and onto medium including the various selective agents. Hygromycin selects for transformants, and kanamycin for transposon excision events. Callus growth began on many control explants as early as 7 days after inoculation. Two or three days later, explants inoculated with *Agrobacteria* harbouring pGV3850HPT::pKU2, pKU3 and pKU4 began to produce callus on media containing hygromycin. In the presence of kanamycin, callus production was significantly delayed, and the high degree of swelling of the hypocotyls observed with other explants was reduced. After 20-30 days following inoculation, callus growth had progressed to a point in all cases where differences in the responses to selection of the explants was apparent, (*Fig. 2*). These differences are summarised in Table 1.

SELECTION	UNINOC-ULATED		pKU2		pKU3		pKU4	
Cefotaxime	20	20	20	20	20	20	20	20
Cefotaxime + Hygromycin	20	0	40	40	127	127	63	63
Cefotaxime + Kanamycin	40	0	50	50	287	102	85	0

TABLE 1. Numbers of plated hypocotyls and regenerating calli.

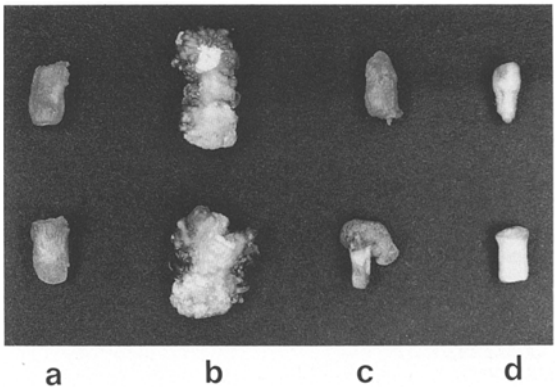

Figure 2. Representative hypocotyl explants transformed with pGV3850HPT::pKU plasmids after 30 days in culture in the presence of kanamycin. a. untransformed, b. pKU2, c. pKU3, d. pKU4.

Hygromycin and kanamycin resistant calli were subcloned on selection medium and cultured for 3 months before bulking up for protein and DNA extraction for analysis. In all cases the subcloned calli grew vigorously in the presence of selective antibiotics.

NPT-II Activity

Twenty eight pKU3 kanamycin resistant lines derived from independent explants were subjected to the rapid dot assay of McDonnell *et al.* (1987) and all exhibited NPT-II activity. The levels of activity were similar to that of protein extracts from pKU2-transformed callus. A number of these positive lines were then used to perform *in situ* polyacrylamide gel assays, which give information about the size of the NPT-II protein. The samples were run on a gel including a bacterial NPT-II extract and an extract from pKU2 callus. All pKU3 NPT-II proteins co-migrated with the bacterial and pKU2 callus proteins on gels, (*Fig. 3*). Specific activities were generally slightly lower than the pKU2 reconstruction. NPT-II activity was not detected in hygromycin resistant pKU4 calli in similar assays.

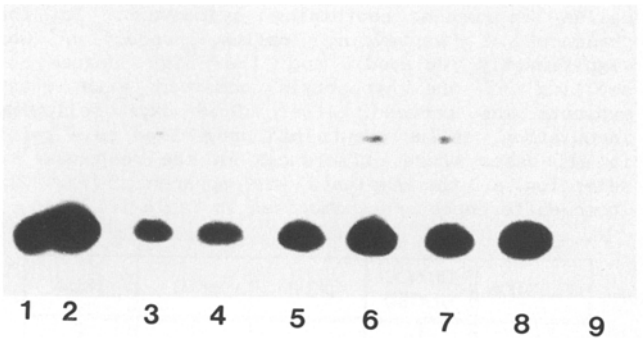

Figure 3. Neomycin phosphotransferase II assay of transformed calli. Lane 1, Bacterial extract; Lanes 2 and 8, pKU2 transformants; Lanes 3-7, pKU3 transformants; Lane 9, untransformed callus.

Southern Analysis

More detailed information about the transposition of *Ac* was sought using Southern analysis. In order to investigate excision from the T-DNA, DNA extracts from pKU3-transformed lines were digested with *Eco*RI plus *Hin*dIII to produce fragments characteristic of intact T-DNA or the reconstituted *npt-II* gene. The pl´-containing fragment expected from T-DNA where *Ac* has excised is 2.9 kb, whilst an intact T-DNA produces a 2.3 kb fragment, (see *Fig. 1*). Hybridisations with the *pl´* probe showed the presence of both fragments in DNA from all 9 pKU3-transformed lines tested (*Fig. 4*). DNA was also digested with *Eco*RI plus *Pst*I and Southern blots of these fragments probed with the *Ac* sequence in an effort to detect re-inserted *Ac* elements. However, we found no conclusive evidence for the reinsertion of excised *Ac* elements into the genome of any transformants.

DISCUSSION

Frequency of Generation of Kanamycin Resistant Calli.

Uninoculated hypocotyls failed to callus on media containing hygromycin or kanamycin, indicating that selection was efficient, whilst the rapid growth of callus from untransformed explants in the presence of cefotaxime shows that this agent does not adversely affect the growth of flax tissues. The selection systems we employed therefore enable an estimation of the frequency of transposon excision as reported by the restoration of kanamycin resistance. For this purpose, we have interpreted the 100% kanamycin resistant callus production by pKU2-inoculated hypocotyls as a 100% transformation efficiency. Thus, transformation efficiency in this context is the proportion of inoculated hypocotyls producing callus expressing a T-DNA encoded resistance gene. Whilst realising that the cut end of a hypocotyl will present a number of cells competent for transformation by *Agrobacteria*, we observed that in all cases, only one individual callus ever arose (from a localised region) of such a wounded surface. Furthermore, previous T-DNA border analysis on flax callus regenerated from hypocotyls shows that in the majority of cases, calli are not chimaeric, and presumably arise from a single transformed infected cell, (Basiran 1988). Thus, we propose that the 102 of 287 pKU3-inoculated hypocotyls producing kanamycin resistant callus represent approximately 35% of the number of transformants. In the original work describing the use of these plasmids in assessing *Ac* transposition frequency in tobacco, Baker *et al.* (1987) transformed protoplasts and regenerated calli under selection. This system gives a more accurate estimate of the relative numbers of kanamycin resistant cells produced by pKU2, pKU3 and pKU4 transformation. Their figure of 25% frequency of *Ac* excision is comparable to our result of 35%. Knapp *et al.* (1988) also used these plasmids in potato and found excision frequencies as high as 50% in shoots derived from inoculated leaf discs. Our results also confirm the finding of the above work that the artificial *Ds* element in pKU4 is stable in transgenic tissues.

Kanamycin Resistance is due to NPT-II Activity.

The NPT-II assays showed that in all cases, kanamycin resistance was a result of the expression of the neomycin phosphotransferase II enzyme, which is not present in untransformed flax. This NPT-II activity in pKU3 calli is expected as a result of reconstitution of the *pl´-npt-II* gene to a pKU2-like form following *Ac* excision, (see *Fig 1*). The two genes will not be identical, due to the presence in the pKU3 plasmid of the 60 bp of *waxy* DNA in the untranslated leader. However, since this contains no ATG codons, this difference is unlikely to affect NPT-II synthesis. That all NPT-II enzymes from transgenic calli comigrate with bacterial NPT-II shows that they have similar charge and size characteristics to the native protein. Thus it is unlikely that the enzymes assayed had suffered any amino acid deletions or additions as a result of T-DNA rearrangements or incomplete excision.

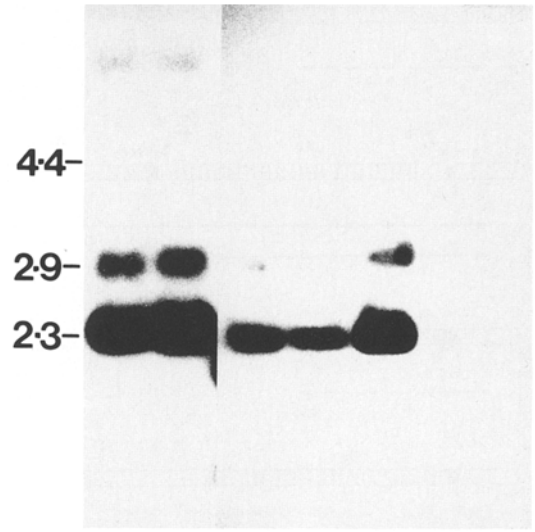

Figure 4. Southern blot of DNA from five pKU3-transformed callus lines digested with *Eco*RI + *Hin*dIII and probed with pl´ promoter. (Fragment sizes are indicated in kilobases.) All DNAs showed a 2.9 kb band, but a fainter band (4^{th} lane) was lost during photographic reproduction.

Ac Excises in Flax.

The Southern analyses provided information about the DNA constitution of the cells forming the kanamycin resistant calli in which *Ac* excisions were presumed to have taken place. The *pl´* probed *Eco*RI+*Hin*dIII digests were designed to show either a single band at 2.3 kb representing intact T-DNA, or a single 2.9 kb band representing the reconstituted *pl´-npt-II* gene. However, it was found that all nine tested calli contained both restriction fragments, suggesting the presence of more than one T-DNA insertion in DNA isolated from each line. The data also indicated the presence of more intact T-DNAs than reconstituted *npt-II* genes, a situation

similar to that reported by Knapp *et al.* (1988), using pKU3 in potato. It is unlikely that non-clonal calli are responsible for these observations for reasons outlined above; in addition the number of unaltered T-DNAs detected in Southern blots would infer a high content of kanamycin sensitive cells in a chimaeric callus. However, kanamycin resistance was selected for immediately, and sensitive cells cannot be cross-detoxified by NPT-II from resistant cells. It is more probable that calli do consist of clonal cells but possess more than one T-DNA insertion per genome. In this case a single *Ac* excision event would lead to conversion to kanamycin resistance, and Southern analysis would show both intact and altered T-DNAs. Such multiple T-DNA insertions are a commonly reported phenomenon in tissues transformed by engineered Ti plasmids. This explanation may also be supported by the NPT-II assay, which shows a higher NPT-II specific activity in pKU2 protein than in pKU3 extracts. Multiple insertions of *pl'-npt-II* in pKU2 could lead to a higher level of synthesis than a single, or fewer, reconstituted genes in pKU3 transformants. That only the two expected bands were seen in the Southerns demonstrates that gross T-DNA rearrangements did not occur, and confirms the implication of NPT-II assays that *Ac* excises as a complete 4.6kb element.

Considering these findings, it is interesting that we found no reinserted elements. Reintegration of *Ac* has been reported in all species previously studied (Baker *et al.* 1987, Van Sluys *et al.* 1987, Yoder *et al.* 1988, Knapp *et al.* 1988), implying that flax may be unusual in this respect.

REFERENCES.

Baker B , Schell J , Lörz H , Fedoroff N (1986) *Proc. Nat. Acad. Sci. USA* 83:4844-4848

Baker B , Coupland G , Fedoroff N , Starlinger P , Schell J (1987) *EMBO J.* 6:1547-1554

Basiran N , Armitage P , Scott R J , Draper J (1987) *Plant Cell Reports* 6:396-399

Basiran N (1988) PhD. Thesis.

Draper J , Scott R J , Armitage P , Walden R , Ed. (1988) *Plant Genetic Transformation and Gene Expression.* Blackwell Scientific Publications.

Ellis J G , Lawrence G J , Peacock W J , Pryor A J (1988) *Ann. Rev. Phytopathol.* 26:245-263

Fedoroff N , Furtek D , Nelson O (1984) *Proc. Nat. Acad. Sci. USA* 81:3825-3829

Feinberg A P , Vogelstein B (1984) *Anal. Biochem.* 137:266-267

Knapp S , Coupland G , Uhrig H , Starlinger P , Salamini F (1988) *Mol. Gen. Genet.* 213:285-290

Kunze R , Stochaj U , Laufs J , Starlinger P (1987) *EMBO J.* 6:1555:1563

Martin C , Carpenter R , Sommer H , Saedler H , Coen E S (1985) *EMBO J.* 4:1625-1630

McDonnell R E , Clark R D , Smith W A , Hinchee M A (1987) *Plant Mol. Biol. Reporter* 5:380-386

O'Reilly C , Shepherd N S , Pereira A , Schwarz-Sommer Zs , Bertram I , Robertson D S , Peterson P A , Saedler H (1985) *EMBO J.* 4:877-882

Rajhathay T (1976) *Z. Pflanzenzüchtg.* 76:1-10

Reiss B , Sprengel R , Will H , Schaller H (1984) *Gene* 30:211-218

Southern E M (1975) *J. Mol. Biol.* 98:503-517

Theres N , Scheele T , Starlinger P (1987) *Mol. Gen. Genet.* 209:193-197

Van Sluys M A , Tempe J , Fedoroff N (1987) *EMBO J.* 6:3881-3889

Velten J , Velten L , Hain R , Schell J (1984) *EMBO J.* 3:2723-2730

Yoder J , Palys J , Alpert K , Lassner M (1988) *Mol. Gen. Genet.* 213:291-296

Zambryski P , Joos H , Genetello C , Leemans J , van Montagu M , Schell J (1983) *EMBO J.* 2:2143-2150