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Studies on the introduction and mobility of the maize *Activator* element in *Arabidopsis thaliana* and *Daucus carota*

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We have co-transformed carrot (*Daucus carota*) and *Arabidopsis thaliana* with an *Agrobacterium tumefaciens* non-tumorigenic T-DNA carrying the maize transposable element *Activator* (*Ac*) and an *Agrobacterium rhizogenes* Ri T-DNA. We present evidence that the *Ac* element transposes in transformed root or root-derived callus cultures of both species. We show that fertile plants can be regenerated from transformed, root-derived callus cultures of *Arabidopsis*, demonstrating the utility of the Ri plasmid for introducing the maize *Ac* element into plants. We also present evidence that *Ac* elements that excise from the transforming T-DNA early after transformation continue to be mobile in carrot root cultures.
Key words: Activator/Arabidopsis/carrot/Ri plasmid/Ti plasmid

Introduction

Transposable elements have become valuable tools for gene isolation in eukaryotic organisms. Several strategies have been developed to enhance the usefulness of the transposon tagging technique in maize (Fedoroff *et al.*, 1984; O'Reilly *et al.*, 1985; Cone *et al.*, 1986). It has recently been reported that the maize *Activator* (*Ac*) element is mobile in tobacco, opening the possibility of using the well-characterized maize elements as transposon tags in plants other than maize (McClintock, 1951; Fedoroff, 1983; Fedoroff *et al.*, 1983; Baker *et al.*, 1986). We report here that *Ac* transposes in two additional plant species commonly used in laboratory experiments, *Arabidopsis thaliana* and *Daucus carota*.

The utility of the maize transposable element for gene cloning depends on several factors. These are the ease with which the element can be introduced into a given plant, its subsequent ability to transpose and the facility with which insertion mutations can be identified. Certain facets of these problems are addressed in the present experiments.

To broaden the range of plants into which *Ac* can be introduced, we have explored the use of *Agrobacterium rhizogenes* Ri plasmid as a biological marker of transformation for the introduction of the maize *Ac* element. *Agrobacterium rhizogenes* is the causative agent of the 'hairy root' disease that affects a variety of dicots (Riker *et al.*, 1930; Elliot, 1951; DeCleene and DeLey, 1981). The pathogenicity of *A.rhizogenes* is attributable to the transfer and integration into the plant genome of genes in the T-DNA region of the bacterium's large Ri plasmid (Chilton *et al.*, 1982; Spano *et al.*, 1982; White, 1982; Willmitzer *et al.*, 1982). Expression of the T-DNA in the transformed plant cells promotes proliferation of roots from single cells at the infection site (Petit *et al.*, 1983; Constantino *et al.*, 1984; David *et al.*, 1984). In certain species, transformed roots can be maintained in culture and can be regenerated into fertile albeit slightly abnormal plants that retain the T-DNA of the Ri plasmid (Tepfer and Tempé, 1981; Chilton *et al.*, 1982; Tepfer, 1984; Ooms *et al.*, 1985). We used an *Agrobacterium* strain carrying both an Ri plasmid and an *Ac*-containing disarmed Ti plasmid, because it was recently reported that co-transformation of plant cells occurs frequently (Depicker *et al.*, 1985; Petit *et al.*, 1986). We show that both carrot and *Arabidopsis* can be co-transformed with the T-DNAs of the *Ac*-containing Ti and Ri plasmids and that transformed *Arabidopsis* roots can be regenerated into fertile plants.

The results of recent experiments indicate that the *Ac* element excises from at least one copy of the T-DNA on which it was introduced in 25–70% of *Ac*-transformed tobacco cells (Baker *et al.*, 1987). Because there is evidence that transformed roots obtained after *A.rhizogenes* infection have a clonal origin, we used the rapidly growing transformed carrot roots to follow the fate of *Ac* elements in transformed cell lineages. We report here that the *Ac* element excised early after transformation in 28% of the carrot lines analysed. We also present evidence that the transposed *Ac* elements remain mobile in the cultured carrot roots. Similarly, more than half of the transformed *Arabidopsis* lines give evidence of *Ac* excision and transposition.

Results

Agrobacterium transformation of Arabidopsis

Arabidopsis thaliana plants were infected with *Agrobacterium tumefaciens* carrying the pTi-Ac plasmid whose structure is shown schematically in Figure 1 and the Ri plasmid pRi15834 (see Materials and methods). Roots developed at the site of inoculation within 20 days after infection (Figure 2a). No root development was observed in uninoculated, wounded controls.

Efforts were made to propagate the transformed *Arabidopsis* roots in culture. Although Ri-transformed roots of several species have been shown to grow rapidly when cultured *in vitro* (Tepfer and Tempé, 1981; Petit *et al.*, 1983), none of the roots obtained from infected *Arabidopsis* plants or stem sections have yet been propagated as roots. However, when placed on medium containing the growth hormones kinetin and naphthalene acetic acid (NAA), the roots callused rapidly (Figure 2b).

Regeneration of fertile Arabidopsis plants from transformed calli

Shoot formation commenced within several weeks on medium containing benzyladenine (BA) and NAA (Materials and methods). All of the callus cultures obtained from roots on infected plants eventually regenerated into plants (Figure 2), although some grew quite slowly and did not initiate shoot formation for several months. The morphology of the *Arabidopsis* plants regenerated from transformed roots was relatively normal, except that many of the plants were extremely small. The most common abnormalities were short internodes and short, club-shaped siliques. In addition, the siliques of transformed *Arabidopsis* plants contained fewer seeds than the siliques of normal plants.

Transformation of carrot discs

Carrot discs were inoculated with an *A.tumefaciens* strain contain-

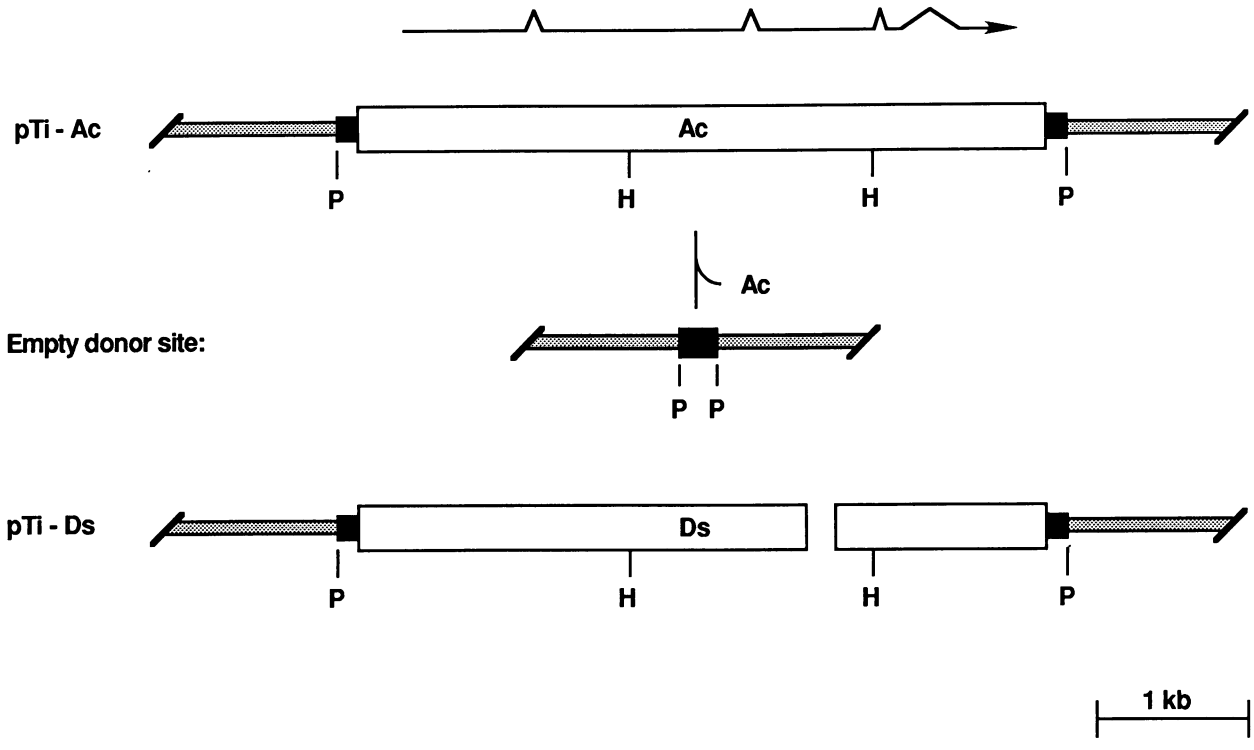

Fig. 1. A diagrammatic representation of transposable element and flanking sequences in the pTi-Ac and pTi-Ds plasmids. The open boxes represent the maize *Ac* and *Ds* elements. Filled boxes represent maize *wx* sequences flanking the element and the shaded boxes represent part of the flanking plasmid sequences in the pTi-Ac and pTi-Ds plasmids (Baker *et al.*, 1986). The arrow above the diagram represents the *Ac* transcript, with horizontal portions of the arrow corresponding to exons and the arrowhead representing the transcript's 3′ end (Kunze *et al.*, 1987). The gap in the *Ds* element represents a 194-bp sequence that was deleted from the *Ac* element, giving the transposition-defective *Ds* element (McClintock, 1963; Fedoroff, 1983). The location of *Pst*I (P) sites flanking the element and the *Hin*dIII (H) sites within the element are shown.

ing both pRi15834 and the pTi-Ac plasmid or the pTi-Ds plasmid (Figure 1; Materials and methods). Roots that developed on the inoculated surface were subcultured by propagating a 1−2 cm terminal section of the main root. DNA extracted from root masses after 3−5 subcultures were used to assess the transposition activity of the introduced *Ac* and *Ds* elements.

Co-transformation by Ti and Ri T-DNAs

Transformation by the Ri T-DNA was initially judged by the appearance of roots at the site of inoculation in *Arabidopsis* and the formation of roots capable of rapid growth on hormone-free medium in carrot. Roots or root-derived callus cultures were subsequently tested for nopaline, a marker of the *Ac*- and *Ds*-T-DNAs. As shown in Table I, all of the *Arabidopsis* calli were nopaline-positive. The presence of the T-DNA of the pRi15834 plasmid cannot be detected as reliably by opine markers as can the *Ac*-T-DNA, because the opine biosynthetic genes and those involved in root proliferation are on separate T-DNA segments and are not always co-transferred during transformation (Jouanin, 1984; De Paolis *et al.*, 1985). The presence of Ri T-DNA was therefore confirmed by hybridization analysis of DNA isolated from root-derived calli of *Arabidopsis*. All of the transformed lines contain sequences homologous to the T-DNA of the Ri plasmid (Table I).

A majority (77%) of the 92 carrot root cultures tested were nopaline-positive (Table I). All of the nopaline-positive transformants were subsequently shown to contain the *Ac* or *Ds* element introduced on the Ti plasmid, indicating that the nopaline synthase gene provides a reliable marker for introduction of the closely linked *Ac* element on the T-DNA of the pTi-Ac plasmid. Of the nopaline-positive roots, 24 were tested for the presence of Ri T-DNA sequences by blot hybridization and all were found to contain them (Table I). Hence the co-transformation frequency in the carrot cultures was at least 77% and may well have been higher, since transformed root cultures not expressing the nopaline synthase gene were not tested for the presence of the *Ac*-T-DNA.

Transposition of Ac in Arabidopsis

To determine whether the *Ac* element is present in root-derived *Arabidopsis* calli, DNA extracted from calli was probed with the complete *Ac* element (see Materials and methods). A 4.8-kb *Pst*I fragment homologous to the *Ac* element is present in DNA from each of the transformed *Arabidopsis* calli (Figure 3a). The same 4.8-kb *Pst*I *Ac*-containing fragment can be detected in *Arabidopsis* DNA using a probe consisting only of the maize *wx* sequence flanking the element (Figure 3b). Thus the nopaline-positive, root-derived *Arabidopsis* calli contain the *Ac* element introduced on the T-DNA (Figure 1). DNA from untransformed *Arabidopsis* plants shows no homology to either maize DNA fragment (data not shown).

Initial evidence that the *Ac* element transposes in *Arabidopsis* was of two types. First, several of the DNA samples display new *Pst*I fragments with homology to *Ac* that have a lower mobility than the introduced 4.8-kb *Pst*I fragment (Figure 3a). Some of these fragments are homologous to the *Ac* probe, but not the *wx* probe (Figure 3a and b). The most prominent of these are marked by dots in Figure 3a. Other fragments are homologous to both probes and may represent T-DNA copies in which some of the *Pst*I sites flanking the element are resistant to digestion by vir-

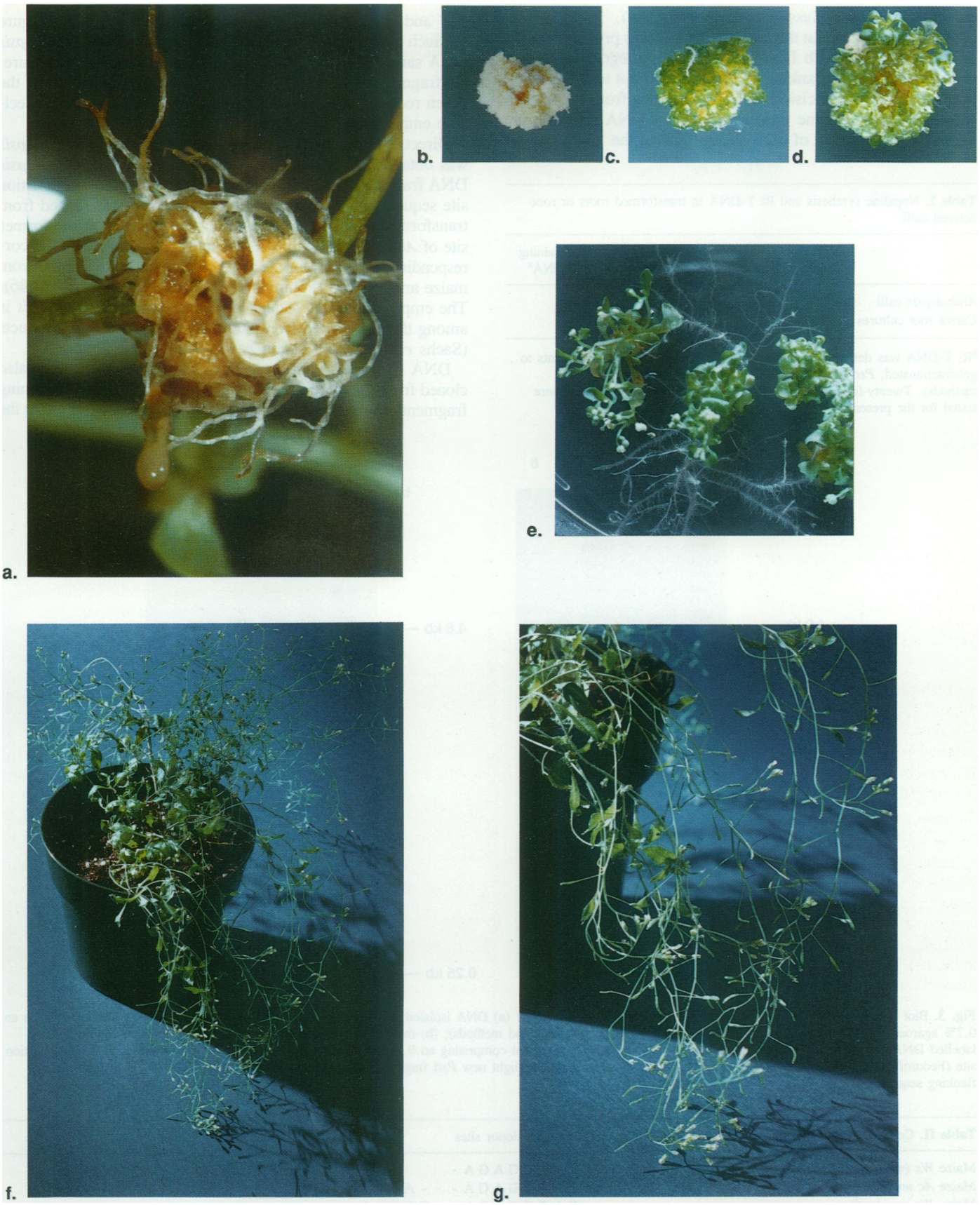

Fig. 2. Stages in the regeneration of *Arabidopsis* plants from transformed roots: (**a**) roots developing at the site of inoculation; (**b**) unpigmented callus; (**c** and **d**) development of shoots on calli; (**e**) rooted shoots; (**f**) regenerated *Arabidopsis* plant; (**g**) floral shoots.

tue of methylation (Kessler and Holtke, 1986).

Further evidence that the element excises was provided by the detection of a 0.25-kb DNA fragment homologous to the *wx* sequence originally flanking the element but not to the element itself (Figure 3b). Excision of the *Ac* element from its original insertion site within the flanking maize *wx* DNA should leave a small *Pst*I fragment of 0.25 kb, designated the 'empty donor site' and homologous to the *wx* insertion site sequence (Figure 1). Such a fragment is readily visualized in two of the *Arabidopsis* DNA samples shown in Figure 3b. Upon prolonged exposure, the fragment can also be detected in lane 6 of Figure 3b. Of the seven root-derived *Arabidopsis* calli analysed, four had detectable empty donor site fragments.

Direct evidence that the *Ac* element transposes in *Arabidopsis* was obtained by analysing the structure of cloned *Arabidopsis* DNA fragments with homology to *Ac* and the maize *wx* insertion site sequence. An empty donor site fragment was cloned from transformed *Arabidopsis* DNA and the sequence at the former site of *Ac* insertion is shown in Table II, together with the corresponding sequences for the same empty donor site isolated from maize and tobacco (Pohlman *et al.*, 1984; Baker *et al.*, 1986). The empty donor site sequence recovered from *Arabidopsis* is among those expected from other *Ac* and *Ds* excision products (Sachs *et al.*, 1983; Sutton *et al.*, 1984).

DNA fragments with homology to the *Ac* element were also cloned from transformed *Arabidopsis* callus DNA. *Ac*-containing fragments were identified that showed no homology to either the

Table I. Nopaline synthesis and Ri T-DNA in transformed roots or root-derived calli

	Number tested	Nopaline positive	% nopaline positive	% containing Ri T-DNA[a]
Arabidopsis calli	7	7	100	100
Carrot root cultures	92	71	77	100

[a]Ri T-DNA was detected by hybridizing ^{32}P-labelled T-DNA fragments to gel-fractionated, *Pst*I-digested callus or root DNA (see Materials and methods). Twenty-four of the nopaline-positive carrot root cultures were tested for the presence of Ri T-DNA.

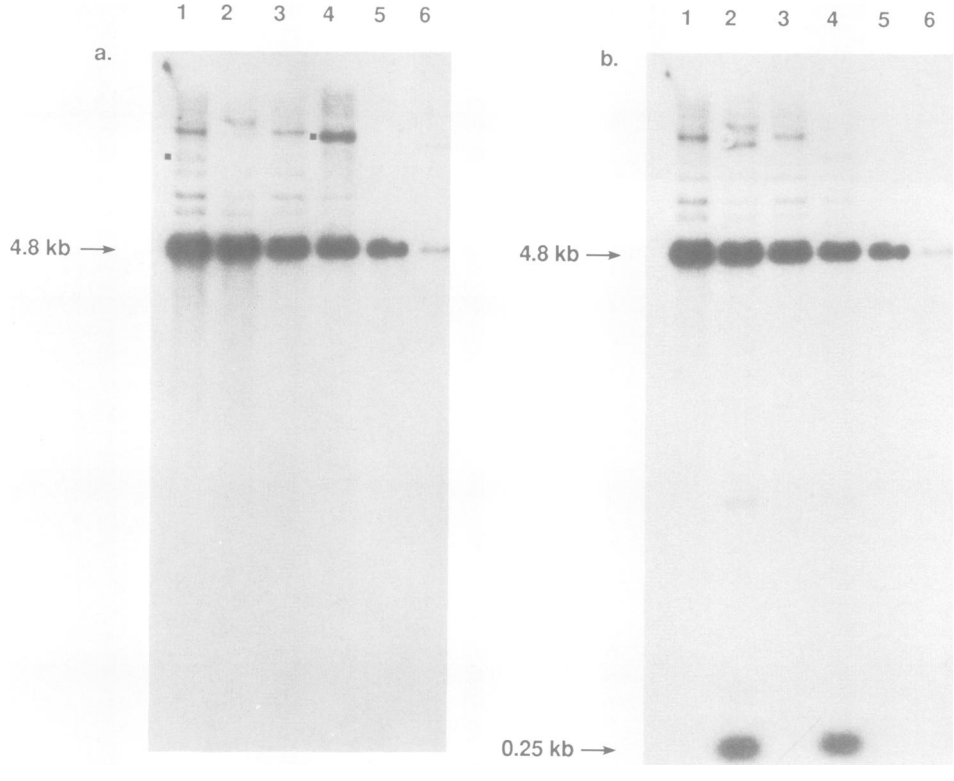

Fig. 3. Blot hybridization analysis of transformed *Arabidopsis* callus DNA: (**a**) DNA isolated from *Arabidopsis* calli was digested with *Pst*I, fractionated on an 0.7% agarose gel, and probed with ^{32}P-labelled *Ac* DNA (see Materials and methods); (**b**) the membrane used in (a) was heated to remove hybridized, labelled DNA and reprobed with a ^{32}P-labelled 'empty donor site' fragment comprising an 0.25-kb fragment of the *wx* locus corresponding to the *Ac* insertion site (Fedoroff *et al.*, 1983; Pohlman *et al.*, 1984). The squares in (a) highlight new *Pst*I fragments that are homologous to *Ac*, but not the original *wx* flanking sequence.

Table II. Comparison of *wx* sequences at the *Ac* insertion site and empty donor sites

Maize *Wx* (wild-type)[a]	- C A T G G A G A -
Maize *Ac wx-m9*[b]	- C A T G G A G A - - - A c - - - - C A T G G A G A -
Maize *Wx* revertant[b]	- C A T G G A G A - - T G G A G A -
Tobacco empty donor site 1[c]	- C A T G G A G A - - T G G A G A -
Tobacco empty donor site 2[c]	- C A T G G A G T G A T G G A G A -
Arabidopsis empty donor site	- C A T G G A _ T G A T G G A G A -

From [a]Klosgen *et al.* (1986); [b]Pohlman *et al.* (1984); [c]Baker *et al.* (1986).

wx or plasmid sequences that flanked the element in the input pTi-Ac plasmid (data not shown). The cloned fragments did, however, hybridize to *Arabidopsis* DNA, indicating that the cloned fragments comprise *Ac* elements inserted at new sites in the *Arabidopsis* genome (Figure 4). A cloned DNA fragment comprising part of the *Ac* element and the flanking sequence is homologous to a single 4.5-kb *Pst*I fragment in untransformed *Arabidopsis* DNA and to both the 4.5-kb *Pst*I fragment and the *Ac*-containing 4.8-kb *Pst*I fragment of the T-DNA in the genomic DNA of the transformed callus. The DNA from the transformed, root-derived callus contains additional fragments homologous to the probe. One of these may correspond to the 9.1-kb *Pst*I fragment containing the transposed *Ac* element, although the fragment containing the transposed element is much less abundant than the fragment lacking the insertion. These observations indicate that the *Ac* element both excises and transposes in *Arabidopsis*.

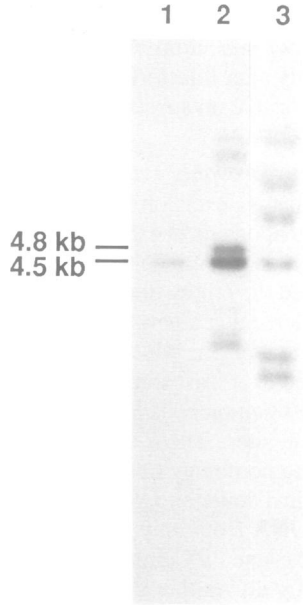

Fig. 4. *Arabidopsis* DNA sequences flanking newly transposed *Ac* elements. DNA extracted from untransformed *Arabidopsis* plants (**lane 1**) or *Ac*-T-DNA-transformed *Arabidopsis* callus (**lane 2**) was digested with *Pst*I, fractionated and hybridized to ^{32}P-labelled probe DNA as described in the legend to Figure 3. The probe was a 6.5-kb *Eco*RI fragment of genomic DNA cloned from the *Ac*-T-DNA-transformed callus line (**lane 2**). It comprises 2 kb of the *Ac* element and 4.5 kb of the contiguous flanking sequence. The cloned fragment was initially identified as one containing a transposed *Ac* element by the absence of homology to the *wx* and plasmid sequences that flank the element on the input T-DNA. **Lane 3** contains labelled bacteriophage λ DNA cut with *Hin*dIII.

Evidence for Ac transposition in transformed carrot root cultures

DNA was extracted from transformed carrot roots representing successive subcultures of a single transformed root and examined for the presence of *Ac*- and *wx*-homologous sequences (Figure 5). A 4.8-kb *Pst*I fragment with homology to the *Ac* element was detectable in all of the DNAs from *Ac*-transformed carrot root cultures, indicating the presence and persistence of the *Ac* element at its original insertion site on the T-DNA. The *Ds* element of the *Ds*-T-DNA differs in structure from the *Ac* element of the *Ac*-T-DNA by a 194-bp internal deletion (Figure 1; Fedoroff *et al.*, 1983; Pohlman *et al.*, 1984). The expected 4.6-kb *Pst*I fragment with homology to the *Ac* element is present in all of the DNAs isolated from nopaline-positive carrot root cultures initially co-transformed by the *Ds*-T-DNA.

Evidence that *Ac* excises in the transformed carrot root cultures is provided by the detection of the 0.25-kb empty donor site fragment homologous to the maize *wx* probe (Figure 6). That excision of *Ac* depends on an element-encoded gene product is further indicated by the detection of the 0.25-kb *wx* empty donor site fragment only in DNAs from *Ac*-T-DNA-transformed roots and not in DNAs from *Ds*-T-DNA-transformed roots (Figure 6). Five of 18 independent *Ac*-T-DNA-transformed root cultures anlaysed showed clear evidence of *Ac* excision, and none of the 14 *Ds*-T-DNA-transformed root cultures gave such evidence. There are prominent new *Pst*I fragments with homology to the

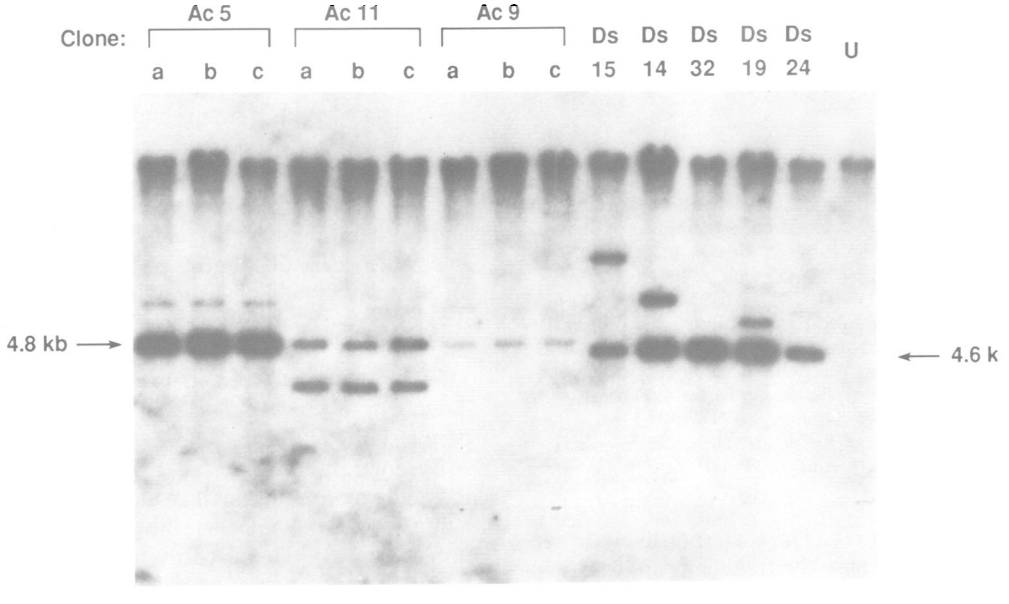

Fig. 5. *Ac* sequences in DNA from transformed carrot root cultures. DNA was digested with *Pst*I, fractionated and hybridized with ^{32}P-labelled *Ac* DNA as described in the legend to Figure 3. Samples designated **a**, **b** and **c** represent DNAs isolated from the same transformed clone on successive subcultures. *Ac*5, -9 and -11 are the designations of three independent lines from carrots transformed with *Ac*-T-DNA, while Ds14, -15, -19, -24 and -32 represent DNA isolated from a single subculture each of five independent carrot root lines transformed with Ds-T-DNA. The lane marked U contained a *Pst*I digest of untransformed carrot DNA.

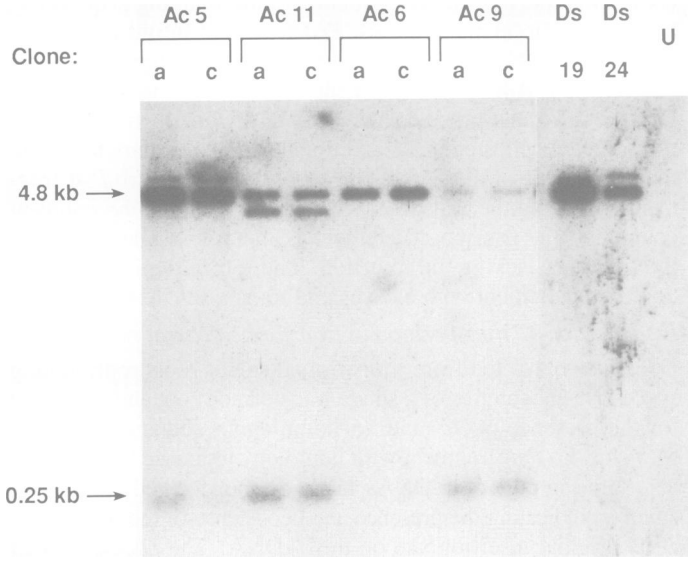

Fig. 6. Empty donor sites in DNA from transformed carrot root cultures. The sources and designations of the DNA samples, as well as the analytical procedures are the same as described in Figure 5, except that the probe was the 0.25-kb 'empty donor site' fragment used in Figure 3b.

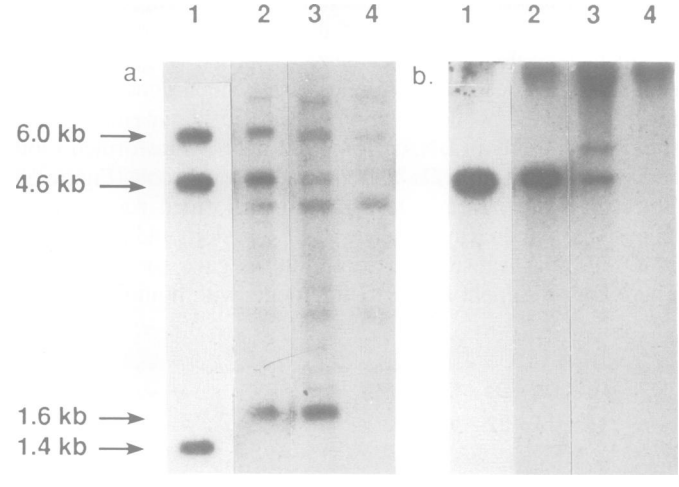

Fig. 7. *Ac*-homologous sequences in *Hin*dIII- and *Pst*I-digested DNA from transformed and untransformed carrot root cultures. Digestion, fractionation and hybridization of DNA samples was carried out as described in Figure 3, except that *Hin*dIII was used in (**a**) and *Pst*I was used in (**b**). *Ac*-homologous sequences were detected by hybridization to ^{32}P-labelled *Ac* DNA. The sources of DNA were: (**1**) a *Ds*-T-DNA-transformed carrot root line (*Ds*32); (**2**) an *Ac*-T-DNA-transformed root line in which no empty donor site fragment was detected (*Ac*18); (**3**) an *Ac*-T-DNA-transformed root line in which an empty donor site fragment was detected (*Ac*9); (**4**) untransformed carrot tissue. It should be noted that the 4.6-kb *Pst*I fragment containing the *Ds* element on the input T-DNA (part b) is coincidentally the same length as one of the *Ac* and *Ds* border fragments obtained upon digestion with *Hin*dIII (part a).

element in several of the cultures. These are not likely to represent transposed elements because the fragments are also homologous to the *wx* sequences flanking the element on the input pTi-Ac or pTi-Ds plasmid (Figures 5 and 6).

Ac excises early after transformation

Each of the *Ac*-containing cultures that show evidence of excision has a characteristic abundance of *Ac*-containing and empty donor sites. Identical patterns are observed with DNA samples isolated from roots derived from successive subcultures of the main root (Figures 5 and 6, cf. lanes a, b and c). Since each subculture was initiated from a small root fragment, excision events occurring during propagation should have had the opportunity of being represented in a sizeable clone of cells. The identity of the hybridization patterns with *Ac* and *wx* probes in the DNAs extracted from successive root subcultures indicates that most excision events occurred early after transformation and that the *Ac* element in the T-DNA was stable on subsequent subculture of the transformed roots.

Excised Ac elements probably remain mobile

Despite evidence that *Ac* excision in carrot root cultures occurs early after transformation and not thereafter, there are indications that the excised elements continue to move. If the early excision events that generated the empty donor sites were associated with reinsertion of the element at a new site with no subsequent transposition, then the manner in which the roots were propagated should have resulted in at least some clones with prominent new restriction fragments homologous to the element and corresponding to new insertion sites. These should be readily identifiable as fragments lacking homology to the *wx* sequences flanking the element at the original donor site. Such a fragment was observed in *Pst*I digests of DNA from only one culture, and that is illustrated in Figure 7b, lane 3. Commonly, however, *Pst*I digests of DNA from root cultures that give evidence of transposition yield a smear of *Ac*-homologous, high-molecular-weight DNA fragments that are not homologous to the original *wx* flanking sequences (Figures 5 and 6). There is more high-molecular-weight, *Ac*-homologous DNA in *Pst*I digests of DNA from cultures that show transposition (Figure 7b, lane 3) than in those from *Ac*-transformed cultures that do not show transposition (Figure 7b, lane 2), but some hybridization of *Ac* to high-molecular-weight *Pst*I fragments is observed even in DNA from transformed carrot roots that do not contain *Ac* (Figure 7b, lane 4).

Evidence that there are transposed *Ac* elements in DNA from root cultures in which *Ac* has excised is provided by the hybridization pattern of DNA digested with an enzyme that cuts within the element. As shown diagramatically in Figure 1, *Hin*dIII cuts twice within the element, generating an internal fragment of 1.6 kb. The adjacent *Hin*dIII sites on the input pTi-Ac plasmid are 2.5 and 4.8 kb from the ends of the element, so that the *Ac*-T-DNA gives two additional *Ac*-homologous *Hin*dIII border fragments of 4.6 and 6.1 kb (Figure 7a, lane 2). If no transposition has occurred, then the relative abundance of the internal and border fragments should be equivalent and their relative intensities in *Hin*dIII digests of transformed DNA hybridized to an *Ac* probe should be roughly equivalent because *Hin*dIII subdivides the *Ac* element into three approximately equal parts (Figure 1). This expectation is realized for DNA isolated from *Ds*-T-DNA-transformed roots (Figure 7a, lane 1) and those *Ac*-T-DNA-transformed roots that give no evidence of an empty donor site (Figure 7a, lane 2).

By contrast, the internal 1.6-kb *Hin*dIII fragment of *Ac* is markedly more abundant than the border fragments in DNA from a transformed cell line that shows evidence of transposition (Figure 7a, lane 3). The disparity in the abundance of internal and border fragments may be even greater than it appears in lane 3 of Figure 7a because the untransformed *Arabidopsis* DNA used in lane 4 exhibits weakly cross-hybridizing fragments that almost co-migrate with the *Ac* border fragments of the input T-DNA construct. There are also several *Ac*-homologous *Hin*dIII fragments that are unique to the transformed root DNA and a smear

of hybridizing material larger than the 1.6-kb internal *Hin*dIII fragment. This pattern of hybridization indicates that many of the *Ac* elements have new border fragments, but that there are few border fragments present in high enough abundance to give a discrete band. It is probable, therefore, that the *Ac* elements that have excised from the T-DNA continue to transpose.

Discussion

Introducing Ac by co-transformation

In initial experiments to assess the ability of the *Ac* element to transpose in plants other than maize, the *Ac* element was introduced into tobacco protoplasts using a non-tumorigenic derivative of *A.tumefaciens* Ti plasmid (Baker *et al.*, 1986). More recently, *Ac*-Ti plasmids have been developed which permit the use of a drug to select transformed cells in which the *Ac* element has excised from the T-DNA (Baker *et al.*, 1987). Because not all plants regenerate as readily from protoplasts or tissue fragments as tobacco and because the utility of drug-resistance genes as markers of T-DNA transformation also varies in different species, the methods developed for tobacco may not be widely useful for introducing and using the maize *Ac* element as a transposon tag for gene isolation (Herrera-Estrella *et al.*, 1983; Hain *et al.*, 1985; Czernilofsky *et al.*, 1986).

In order to extend the range of plants into which the *Ac* element can be introduced, we have explored the use of the Ri plasmid of *A.rhizogenes* as a biological marker of transformation. Genes borne on the Ri T-DNA are responsible for the differentiation of transformed cells into roots in a variety of dicots (Birot *et al.*, 1987). Unlike cells transformed with the Ti plasmid of *A. tumefaciens*, Ri-transformed roots can generally be regenerated into fertile, albeit slightly abnormal plants. The design of the present experiments was further based on the recent observations that *Agrobacterium* strains containing two plasmids with T-DNAs frequently give rise to plant cells co-transformed by both T-DNAs (Depicker *et al.*, 1985; Petit *et al.*, 1986; Simpson *et al.*, 1986). We used an *Agrobacterium* strain containing both an Ri plasmid and a non-tumorigenic Ti plasmid carrying the maize *Ac* or *Ds* element and a nopaline synthase gene. We observed that the T-DNAs of the two plasmids were co-transferred at a high frequency from the infecting *Agrobacterium* strain to both *Arabidopsis* and carrot cells.

It has already been reported that Ri-transformed carrot roots can be regenerated into fertile plants (Chilton *et al.*, 1982; David *et al.*, 1984). We have demonstrated here that callus cultures derived from Ri-transformed *Arabidopsis* roots can also be regenerated into fertile plants. Because the *Ac* element was on a separate plasmid from the Ri plasmid, it is possible that the Ri- and *Ac*-T-DNAs have inserted on different chromosomes and will segregate in subsequent generations, permitting the recovery of progeny plants devoid of the Ri genes responsible for the growth abnormalities of the 'hairy root' phenotype (Tepfer, 1984). We are presently exploring this possibility. We have demonstrated that expression of the 'hairy root' T-DNA of the Ri plasmid can be used as a biological marker for the introduction of the maize *Ac* element in the T-DNA region of a separate Ti plasmid. This procedure may prove useful for plants that are not readily regenerated from protoplasts or transformed by Ti plasmids with drug-resistance markers.

Ac transposition in Arabidopsis

We have demonstrated that the maize *Ac* element transposes in *Arabidopsis* callus cultures. More than half of the transformed root-derived *Arabidopsis* calli showed clear evidence of *Ac* excision, as judged by the appearance of an empty donor site fragment homologous to the maize *wx* sequences that initially flanked the element. Because some cultures that contained no detectable empty donor site fragment nonetheless show new large DNA fragments homologous to the *Ac* element, but not the flanking *wx* sequences of the input construct, the fraction of cultures in which the *Ac* element has excised is probably higher than that estimated by detection of empty donor sites, possibly due to segregation of cell lineages containing the donor site during callus culture.

Sequence analysis of an empty donor site fragment cloned from the DNA of a transformed *Arabidopsis* callus revealed the sequence of the former insertion site to be similar to one previously obtained in tobacco (Baker *et al.*, 1986). The 8-bp duplication generated upon insertion of the *Ac* element was retained, except for deletion of a single nucleotide, and the two nucleotides immediately adjacent to the former insertion site, an A and a C residue, were replaced by a T and a G residue respectively. Similar deletions and transversions are commonly observed at former *Ac* and *Ds* insertion sites in maize (Sachs *et al.*, 1983; Sutton *et al.*, 1984; Döring and Starlinger, 1986). Moreover, several DNA fragments with homology to *Ac* were cloned from two of the transformed *Arabidopsis* callus lines and shown to contain *Arabidopsis* genomic sequences, but to have no homology to the sequences that originally flanked the element on the input Ti plasmid. We conclude that the maize *Ac* element both excises and transposes in *Arabidopsis*. The small genome size and rapid reproductive cycle of *Arabidopsis* should facilitate assessing the utility of *Ac* as a transposon tag (Redei, 1975; Meyerowitz and Pruitt, 1985).

Evidence for transposition of Ac in untransformed carrot root cultures

The results of blot hybridization experiments provide evidence that the *Ac* element excises from its original insertion site in carrot roots, while the internally deleted *Ds* element does not. Because the *Ds* element used in the present experiments is known to be transposition-defective in maize (McClintock, 1963; Fedoroff *et al.*, 1983), we conclude that an *Ac*-encoded gene product is required for excision. This, in turn, implies that the *Ac* element is expressed in carrot cells, as it is in *Arabidopsis* and tobacco cells (Baker *et al.*, 1986; Kunze *et al.*, 1987).

The fraction of transformed cultures giving evidence of *Ac* excision is similar in the present experiments with Ri-transformed carrot roots and in tobacco cultures (Baker *et al.*, 1987). Among the *Ac*-T-DNA-containing carrot root line, 28% gave evidence of *Ac* excision. However, unrearranged *Ac*-T-DNA copies were more abundant than empty donor sites in most of the carrot root lines analysed. Moreover, we found the pattern of *Ac*-homologous fragments, as well as the relative abundance of empty donor sites, to be the same through several subcultures. We infer from these observations that the *Ac* element excised from a small fraction of the integrated *Ac*-T-DNA copies and that excision occurred early after transformation. Had excision continued, we would expect to have observed a diminution in the abundance of *Ac*-containing donor sites and an accumulation of empty donor sites with time and we did not. Although the *Arabidopsis* calli were not analysed with equivalent precision, the results obtained with DNA extracted after different periods of culture gave results comparable with those obtained with carrot root cultures. That is, major changes over time in the relative abundance of the empty donor sites and the unaltered T-DNA construct were not observed (M.A.Van Sluys, unpublished work).

At the same time, there is reason to believe that the *Ac* elements that excised early after transformation continue to transpose. In genomic DNA from cultures showing *Ac* excision, internal element fragments are over-represented in genomic DNA relative to element border fragments, suggesting that different copies of the element have different border sequences. This in turn suggests continuing transposition of the *Ac* element in carrot root cultures. We do not presently understand the difference in the apparent mobility of *Ac* elements that excise early and those that do not. However, high continued mobility of introduced *Ac* elements has been noted in tobacco callus and appears to decrease upon regeneration of the cultured cells into plants (B.Baker, personal communication). Since the carrot root cultures used in the present experiments were propagated without exogenous growth hormones and consist of differentiated tissues, high element mobility cannot be attributed to conditions that promote growth of plant cells as undifferentiated callus.

Control of transposition frequency is an essential factor in the design of useful transposons for gene tagging and cloning. Too high a transposition frequency is undesirable because continued element transposition and multiplication complicate molecular characterization and cloning of a gene with a new insertion mutation, while very low transposition frequencies make the isolation of insertion mutations a formidable task. Efforts are being directed to the design of plant transposon systems in which transposition can be controlled.

Materials and methods

Bacterial strains and plasmids

The Ti plasmids carrying the maize *Ac* and *Ds* elements are designated pTi-Ac and pTi-Ds and have been described previously (Baker *et al.*, 1986). The Ri plasmid, designated pRi15834, was introduced by conjugation into *A.tumefaciens* C58C1 carrying either the pTi-Ac or pTi-Ds plasmid. Ri-containing exconjugants were isolated based on their ability to grow on opines as a sole carbon source, as described by Petit *et al.* (1986).

Two cloned maize DNA fragments were used to probe DNA from pTi-Ac- and pTi-Ds-transformed plant tissue. These were the complete *Ac*-containing *Pst*I fragment comprising the 4.6-kb *Ac* element and the 249-bp flanking *wx* locus sequence and a 247-bp fragment of the *wx* locus cloned from a maize *Wx* revertant of the original *Ac* insertion mutation (Fedoroff *et al.*, 1983; Pohlman *et al.*, 1984). The former is designated the *Ac* probe and the latter, the *wx* probe. The 0.25-kb maize *wx* fragment is also referred to as the 'empty donor site'.

Transformation

Surface-sterilized *A.thaliana* (cvar. Bensheim, kindly provided by M.Jacobs) seeds were germinated and grown axenically on Murashige and Skoog (MS) minimal medium (Gibco) supplemented with 30 g/l sucrose and solidified with 0.8% agar (MS30). Plants were infected when ~1 month old by one of two procedures. Floral stems on intact plants were decapitated or wounded with a scalpel dipped in a dense, fresh bacterial culture. Alternatively, flower stem sections were cut, dipped into a dense bacterial culture and inverted into medium solidified with agar. Roots developed at the site of inoculation after 10–20 days.

Carrots used for transformation were obtained from the local market, sterilized by immersion in a commercial preparation of sodium hypochlorite, peeled, sliced and placed with the lower surface up on MS minimal medium solidified with 0.8% agar. A dense bacterial culture from a freshly grown plate was applied to the exposed surface with a loop. Roots developed at the site of inoculation after 10–14 days.

Arabidopsis root culture and plant regeneration

Roots developing at the site of inoculation were removed carefully to avoid bacterial contamination and placed on a callusing medium consisting of commercial MS30 medium supplemented with 1 mg/l each of calcium pantothenate, nicotinic acid, pyridoxin and thiamine, 5 μg/l of biotin (MS30v) and containing kinetin and NAA, each at 2×10^{-5} M, and solidified with 0.8% agar. Calli formed along the length of each root within 2 weeks and were transferred to a shoot-inducing medium (MS30v–0.8% agar, 1 mg/l BA and 0.1 mg/l NAA, 0.5 mg/ml carbenicillin). The calli turned green and produced rosettes within 1 month for the most rapidly growing cultures and within 3 months for the cultures with the lowest growth rate. The antibiotic carbenicillin was included in the shooting medium because its use promotes shoot regeneration in *Arabidopsis* (N.Fedoroff, unpublished work). Rosettes were transferred to an elongation medium (MS30v–0.8% agar, 0.1 mg/l BA, 0.5 mg/l NAA, 0.1 mg/l giberellic acid and 0.5 mg/g carbenicillin). After 2–3 weeks, elongated shoots were transferred to a rooting medium (MS30v–0.8% agar, 2×10^{-7} M NAA). Roots developed rapidly on half or more of the shoots. Cultures were maintained in an illuminated incubator at 24–25°C with an 8-h illumination period.

Shoots with well-developed roots were transferred to sterile vermiculite moistened with half-strength MS medium containing 0.5% sucrose and 2×10^{-5} M NAA. The cultures were maintained in a closed container in continuous light at ambient laboratory temperatures (24–26°C). Shoots that showed good growth were subsequently rinsed and transferred to sterile vermiculite moistened with sterile Hoagland's solution, initially in a closed container to maintain high humidity. After about 1 week, the humidity was reduced by progressively opening the container over a period of several days and the shoots were transferred to soil. Flowering was often observed even before root formation, but good seed set was obtained only after transfer of the plants to soil at ambient humidity levels, largely because anthers did not dehisce in the humid environment of the closed container. Seeds were collected, dried at ambient temperature for several days and maintained for 4 days at 4°C prior to germination.

Carrot root cultures

Roots developing on carrot discs inoculated with the *A.tumefaciens* C58C1 containing the pRi15834 plasmid and either the pTi-Ac or the pTi-Ds plasmid were transferred to MS30v–0.8% agar containing 0.5 mg/ml cephalosporin to inhibit bacterial growth and subcultured onto the same medium without antibiotic as soon as root growth was sufficient to permit transfer of bacteria-free root segments. When the roots had grown to cover the surface of a standard Petri dish, a 1–2 cm section of the main root was subcultured onto the same medium and the rest of the root mass was transferred to a large culture dish (150 mm × 25 mm) containing 50–70 ml of MS30v medium without agar. Cultures were incubated at ambient temperatures and light levels, then harvested by freezing in liquid nitrogen for DNA extraction when the wet wt of the root mass was 5–15 g. Subculturing was repeated as described above 3–5 times prior to analysis of the extracted DNA. Root segments were taken for nopaline assays to detect the presence of the pTi-Ac and pTi-Ds plasmids at the first subculture.

Nopaline assay

Small fragments of callus, root, shoot or leaf tissue were submerged in 100 μl of water in an Eppendorf tube and the tubes were incubated in a boiling water bath for 10 min. The aqueous extract was removed, dried *in vacuo* and the residue was redissolved in 1 μl of H_2O/10 mg wet wt of tissue. The extract was spotted on Whatman 3MM chromatography paper, and the paper was then wetted with 3% formic acid–6% acetic acid buffer (pH 1.9) and subjected to electrophoresis in the same buffer at 40 V/cm for 15 min. Nopaline purchased from Sigma was used as an electrophoretic marker. Opines were visualized by dipping the dried paper twice into a freshly prepared solution of ethanol containing 2% NaOH and 20 μg/ml phenanthrene quinone (Sigma), drying the paper between applications of the stain. The dried paper was photographed under short-wave UV illumination.

DNA extraction and blot hybridization

DNA was extracted from *Arabidopsis* callus cultures and carrot roots as described by Fedoroff (1985). Restriction endonuclease digests were done under conditions specified by the enzyme suppliers (Boehringer-Mannheim, Bethesda Research Laboratories, New England Biolabs), using 2–5 units of enzyme/μg of plant DNA. DNA digests were fractionated on 0.8% agarose gels, transferred to nylon membrane (Genescreen, NEN Research Products) and hybridized with ^{32}P-labelled *Ac* or *wx* probes labelled to a sp. act. of 1×10^9 c.p.m./μg using a random primer labelling kit and [^{32}P]dCTP, as specified by the supplier (Amersham). Hybridization was carried out as specified by the NEN Research Products for Genescreen nylon membrane filters.

Cloning and nucleotide sequence analysis

Arabidopsis genomic DNA was digested to completion with either *Bam*HI or *Bgl*II, ligated to purified arms of *Bam*HI-digested lambda *EMBL4*, packaged and propagated in *Escherichia coli* strain K803 as described by Fedoroff (1985). The recombinant phage libraries were screened with the *wx* probe, then rescreened with the *Ac* probe, to identify clones containing T-DNA fragments from which the *Ac* element had excised and newly transposed *Ac* elements. Recombinant phage were grown and the DNA extracted by minor modifications of the methods described by Maniatis *et al.* (1982). Phage containing empty donor sites were identified by the presence of an 0.25-kb *Pst*I fragment homologous to the *wx* probe. The empty donor site fragment was subcultured into Bluescript phage (Stratagene) and sequenced by the dideoxy sequencing procedure of Sanger *et al.* (1977).

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References

Baker,B., Schell,J., Lorz,H. and Fedoroff,N. (1985) *Proc. Natl. Acad. Sci. USA*, **83**, 4844–4848.

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

Birot,A.M., Bouchez,D., Casse-Delbart,F., Durand-Tardif,M., Jouanin,L., Paritot,V., Robaglia,C., Tepfer,D., Tepfer,M., Tourneur,J. and Vilaine,F. (1987) *Plant Physiol. Biochem.*, **7**, 323–335.

Chilton,M.D., Tepfer,D.A., Petit,A., David,C., Casse-Delbart,F. and Tempé,J. (1982) *Nature*, **295**, 432–434.

Cone,K.C., Burr,F.A. and Burr,B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9631–9635.

Constantino,P., Spano,L., Pomponi,M., Benvenuto,E. and Ancora,G. (1984) *J. Mol. Appl. Gen.*, **2**, 465–470.

Czernilofsky,A.P., Hain,R., Baker,B. and Wirtz,U. (1986) *DNA*, **5**, 473–482.

David,C., Chilton,M.-D. and Tempé,J. (1984) *Biotechnology*, **2**, 73–76.

DeCleene,M. and DeLey,J. (1981) *Bot. Rev.*, **47**, 148–194.

DePaolis,A., Mauro,M.L., Pomporri,M., Cardarelli,M., Spano,L. and Constantino,P. (1985) *Plasmid*, **13**, 1–7.

Depicker,A., Herman,L., Jacobs,A., Schell,J. and Van Montagu,M. (1985) *Mol. Gen. Genet.*, **201**, 477–484.

Döring,H.-P. and Starlinger,P. (1986) *Annu. Rev. Genet.*, **20**, 175–200.

Elliot,C. (1951) *Manual of Bacterial Plant Pathogens*. 2nd revised edition. Chronica Botanica, Waltham, MA, p. 186.

Fedoroff,N. (1983) In Shapiro,J. (ed.), *Mobile Genetic Elements*. Academic Press, New York, pp. 1–63.

Fedoroff,N. (1985) In Setlow,J. and Hollaender,A. (eds) *Genetic Engineering: Principles and Methods*. Plenum Publishing Corporation, New York, Vol. 7, pp. 115–134.

Fedoroff,N., Wessler,S. and Shure,M. (1983) *Cell*, **35**, 243–251.

Fedoroff,N., Furtek,D. and Nelson,O. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3825–3829.

Hain,R., Stabel,P., Czernilofsky,A.P., Steinbiss,H.H., Herrera-Estrella,L. and Schell,J. (1985) *Mol. Gen. Genet.*, **199**, 166–168.

Herrera-Estrella,L., DeBlock,M., Messens,E., Hernalsteens,J.-P., Van Montagu, M. and Schell,J. (1983) *EMBO J.*, **2**, 987–995.

Jouanin,L. (1984) *Plasmid*, **12**, 91–102.

Kessler,C. and Holtke,H.-J. (1986) *Gene*, **47**, 1–153.

Klösgen,R.B., Gierl,A., Schwarz-Sommer,Zs. and Saedler,H. (1986) *EMBO J.*, **5**, 237–244.

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

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) In *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 80–85.

McClintock,B. (1951) *Cold Spring Harbor Symp. Quant. Biol.*, **16**, 13–47.,

McClintock,B. (1963) *Carnegie Institute of Washington Year Book*, **62**, 486–493.

Meyerowitz,E.M. and Pruitt,R.E. (1985) *Science*, **229**, 1214–1218.

Ooms,G., Karp,A., Burrel,M.M., Twell,D. and Roberts,Y. (1985) *Theor. Appl. Genet.*, **70**, 440–446.

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

Petit,A., David,C., Dahl,G.A., Ellis,J.G., Guyon,P., Casse-Delbart,F. and Tempé,J. (1983) *Mol. Gen. Genet.*, **190**, 204–214.

Petit,A., Berkaloff,A. and Tempé,J. (1986) *Mol. Gen. Genet.*, **202**, 388–393.

Pohlman,R.F., Fedoroff,N.V. and Messing,J. (1984) *Cell*, **37**, 635–643.

Redei,G.P. (1975) *Annu. Rev. Genet.*, **9**, 111–127.

Riker,A.J., Banfield,W.M., Wright,W.H., Keitt,G.W. and Sagen,H.E. (1930) *J. Agric. Res. (Washington)*, **41**, 507–540.

Sachs,M.M., Peacock,W.J., Dennis,E.S. and Gerlach,E.L. (1983) *Maydica*, **28**, 289–303.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Simpson,R.B., Spielmann,A., Margossian,L. and McKnight,T.D. (1986) *Plant Mol. Biol.*, **6**, 403–415.

Spano,L., Pomponi,M., Constantino,P., Van Slogteren,G.M.S. and Tempé,J. (1982) *Plant Mol. Biol.*, **1**, 291–300.

Sutton,W.D., Gerlach,W.L., Schwartz,D. and Peacock,W.J. (1984) *Science*, **223**, 1265–1268.

Tepfer,D. (1984) *Cell*, **37**, 959–967.

Tepfer,D. and Tempé,J. (1981) *C.R. Hebd Séances Acad. Sci. Paris, Ser, III*, **292**, 153–156.

White,F.F. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3193–3197.

Willmitzer,L., Sanches-Serrano,J., Buschfeld,E. and Schell,J. (1982) *Mol. Gen. Genet.*, **186**, 16–22.

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