Mol Gen Genet (1988) 213:291–296

MGG

Ac transposition in transgenic tomato plants

John I. Yoder[1], Joe Palys[1], Kevin Alpert[2], and Michael Lassner[1]

[1] Department of Vegetable Crops, University of California-Davis, Davis, CA 95616, USA
[2] Campbell Institute of Research and Technology, Route 1, Box 1314, Davis, CA 95616, USA

Summary. As an initial step towards developing a transposon mutagenesis system in tomato, the maize transposable element *Ac* was transformed into tomato plants via *Agrobacterium tumefaciens*. Southern analysis of leaf tissue indicated that in nine out of eleven transgenic plants, *Ac* excised from the T-DNA and reintegrated into new chromosomal locations. The comparison of *Ac* banding patterns in different leaves of the same primary transformant provided evidence for transposition during later stages of transgenic plant development. There was no evidence of *Ds* mobilization in tomato transformants.

Key words: Transposon-tagging – *Lycopersicon esculentum* – *Ac* element – Gene transfer

Introduction

Lycopersicon esculentum (tomato) is an excellent model system for plant genetics. The tomato genetic map is saturated with a large number of morphological, biochemical and DNA restriction site polymorphisms (Tanksley et al. 1987). Of additional interest to molecular geneticists, the tomato is amenable to manipulation in tissue culture and can be transformed by *Agrobacterium*-mediated gene transfer (McCormick et al. 1986; Koornneef et al. 1986; Fillatti et al. 1987). Unfortunately, most of the tomato mutant germplasm is inaccessible to molecular analysis because there are no characterized transposable elements useful for transposon tagging.

Transposon tagging is a powerful technique for isolating genes which encode unknown gene products (Bingham et al. 1981; Shepherd 1987). A number of genes have been cloned from maize and *Antirrhinum* using a transposable element as a molecular tag (Fedoroff et al. 1984; O'Reilly et al. 1985; Martin et al. 1985; Theres et al. 1987). In each successful case of transposon tagging to date, the mutagenic element has been endogenous to the host plant. For plants, such as tomato, which contain no characterized transposons, a potential approach is to transform a known transposable element into the desired plant host.

The maize transposable element *Ac* has been shown to transpose in tobacco cells, *Arabidopsis* calli, and carrot root cultures (Baker et al. 1986; Van Sluys et al. 1987). Using a biological excision assay for *Ac* in tobacco cells, it was determined that *Ac* excised from at least one copy of the T-DNA in 25%–70% of transformed calli (Baker et al. 1987). Using the presence of an empty donor site band as a criterion for *Ac* excision in *Arabidopsis* calli, *Ac* was found to undergo excision in 4 out of 7 calli examined (Van Sluys et al. 1987). By a similar criterion, the same authors detected evidence of *Ac* excision in 5 out of 18 independently transformed carrot root cultures. Southern analysis of continuously cultured carrot roots indicated that under these conditions, *Ac* appeared to excise early from the T-DNA after transformation. While some elements appeared to remain active, most were stabilized in subsequent subcultures (Van Sluys et al. 1987).

With the goal of developing a transposon tagging system in tomato, we have characterized the behavior of *Ac* in transgenic tomato plants. We have detected evidence of both *Ac* excision and transposition in leaf tissue of regenerated, transgenic tomatoes. By examining DNA isolated from different leaves of the same primary transformant, we have shown that transposition continues in the later stages of plant development. These results suggest that *Ac* may prove to be a useful element for transposon tagging in tomato.

Materials and methods

DNA constructions. A lambda clone containing the *Ac7* element and flanking *wx* sequences (Behrens et al. 1984) was digested with *Bgl*II and subcloned into the *Bam*HI site of pUC13. This intermediate vector was digested with *Sal*I and *Pst*I, and the 6 kb fragment containing *Ac* was cloned into the *Xho*I site of the Ti-based vector pMON200 (Fraley et al. 1985). The resulting construction was called pMAC. A restriction map of the transforming portion of pMAC is shown in Fig. 1.

The vector pDS203 is a derivative of pMON200 that contains the *Ds1* element together with flanking maize *Adh1* sequences (Sutton et al. 1984). It was prepared by blunt-end cloning the 750 bp *Hin*dIII-*Bam*HI fragment of pDS2.A (generously provided by W. Gerlach, CSIRO, Australia) into the *Eco*RI site of pMON200. A map of this construction is shown in Fig. 2.

These two chimeric plasmids were introduced into *Agrobacterium tumefaciens* strain GV3111 by triparental mating using the helper plasmid pRK2013 to supply mobilization functions. Selection for recombination of the plasmids into

Offprint requests to: J.I. Yoder

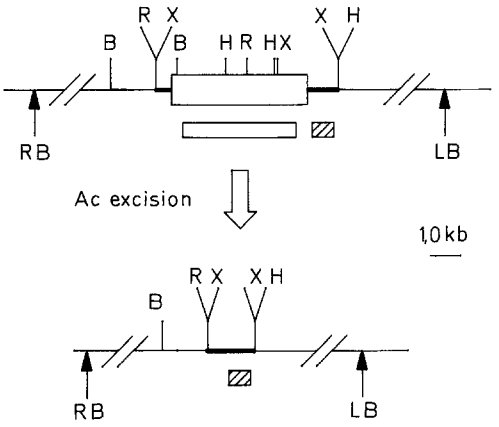

Fig. 1. pMAC restriction map and predicted excision map. The structure of pMAC integrated into transgenic plants is shown. The diagram *below* the arrow indicates the predicted restriction map if *Ac* excises from the donor plasmid. The *Ac7* element is shown as a *box*, flanking *wx* sequences are shown as a *heavy line*, Ti sequences are shown as a *thin line*. B, *Bam*HI; R, *Eco*RI; X, *Xba*; H, *Hin*dIII; RB, T-DNA right border; LB, T-DNA left border. The *Ac* probe is shown as an *open box*, the *wx*-specific probe is shown as a *hatched box*

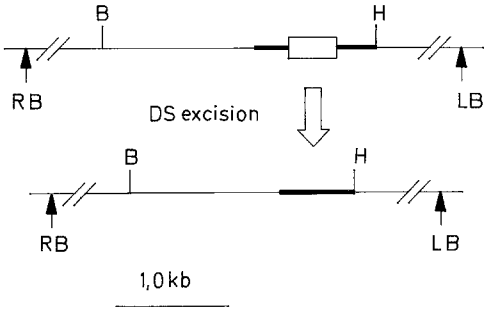

Fig. 2. pDS203 restriction map and predicted excision map. The structure of the clone containing *Ds1* is given. *Below* the arrow is the predicted restriction map if *Ds* excises in tomato. The *open box* is the *Ds* element, the *bold line* represents maize *Adh1* sequences, the *thin line* represents T-DNA sequences. B, *Bam*HI; H, *Hin*dIII; RB, T-DNA right border; LB, T-DNA left border

the avirulent Ti plasmid pTiB6S3-SE was as described (Fraley et al. 1985).

pJAC-D contains a "clipped wing" *Ac* element in pBR322. One terminal inverted repeat of *Ac7* as well as flanking *wx* sequences were removed by exonuclease III and nuclease S1 treatment. A 4.3 kb *Ac*-specific sequence was obtained by digestion with *Cla*I and *Pvu*I. The plasmid pWx1.2 contains 1.2 kb of *wx* DNA, 400 bp of which are present in pMAC (Fig. 1). The 1.2 kb *wx* sequence was released by digestion with *Eco*RI and *Hin*dIII.

Leaf disk transformation of tomato. The cultivar VF36 was transformed with pMAC and pDS203 by an adaptation of published transformation procedures (Koornneef et al. 1986; Fillatti et al. 1987). Seeds were surface sterilized for 1 h in 50% commercial bleach and germinated in MSSV medium (Fillatti et al. 1987). Four- to seven-day-old cotyledons were excised and placed onto freshly prepared tobacco feeder plates, prepared by decanting 1–2 ml of tobacco cells in suspension culture onto 2Z medium (Thomas and Pratt 1981). After 48 h the cotyledons were immersed for 5 min in an overnight culture of *Agrobacterium* diluted to an OD_{600} of 0.1. They were then blotted dry and replaced onto the feeder plates. After 24 h the explants were plated onto 2Z medium supplemented with 350 mg/l carbenicillin and 100 mg/l kanamycin sulfate. Excised shoots were rooted in medium containing 50 mg/l kanamycin. In order to ensure that each transformant was derived independently, only one kanamycin resistant seedling was propagated per explant.

Southern hybridization. Genomic DNA was isolated from young leaves of primary transformants by CsCl purification as described (Fischer and Goldberg 1982). Ten micrograms of genomic DNA were digested to completion with the appropriate enzymes, electrophoresed in 0.8% agarose, and transferred onto ZetaProbe membrane filters (BioRad). The hybridizations and washings were conducted as described by the manufacturer except that 10% dextran sulfate was included in the hybridization buffer to accelerate hybridization (Wahl et al. 1979). For reprobing, the probe was stripped from the filters by washing twice in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.1% pyrophosphate and 1.0% SDS at 95° C for 15 min. The stripped filters were then autoradiographed for at least 24 h to ensure that all the probe was removed.

DNA for the *Ac* probe was isolated as a 4.3 kb *Cla*I-*Pvu*I fragment from pJAC-D. The 1.2 kb *wx* fragment was isolated from the pWX1.2 plasmid following digestion with *Eco*RI and *Hin*dIII. Both fragments were separated from the plasmid sequences by two purifications through agarose gels. Following electroelution and purification on an NACS column (BRL), the insert DNA was labeled with ^{32}P by nick-translation (Maniatis et al. 1982).

Results

Ac *excision in transgenic tomatoes*

Genomic DNA was isolated from leaf tissue of 12 independent kanamycin-resistant regenerants and digested with either *Xba*I, *Eco*RI and *Hin*dIII, or *Bam*HI and *Hin*dIII. Southern blots were prepared from each digest and probed with the 1.2 kb *wx*-specific probe. A transformant which contained at least one copy of T-DNA with a non-transposed *Ac* would have a hybridizing band of 2.1 kb in the *Xba*I digest and of 2.4 kb in both the *Eco*RI-*Hin*dIII and *Bam*HI-*Hin*dIII double digests. The presence of these resident bands would indicate that at least one of the transformed elements had not excised. As seen in Figs. 3b, 4b and 5b, these bands were present in 7 of the 12 transformants (plants 25, 24, 8, 14, 18, 22, and 6). There was no hybridization of the *wx* probe to untransformed VF36 genomic DNA.

If *Ac* had excised from the T-DNA, additional *wx*-specific bands corresponding to the empty donor site would be expected. The predicted sizes for an empty donor site fragment for our *Ac* construction are 1.4 kb for the *Xba*I digest, 1.5 kb for the *Eco*RI-*Hin*dIII double digest, and 3.0 kb for the *Bam*HI-*Hin*dIII double digest. When the blots were probed with the *wx*-specific fragment, 9 of the regenerants (plants 25, 10, 12, 8, 14, 22, 6, and 29) contained a *wx*-specific band of the size predicted for an empty donor site (Figs. 3b, 4b, and 5b). These bands are marked on

the figures with a triangle. The intensity of an empty donor site band compared to a band resulting from a non-excised *Ac* is an estimate of the proportion of cell lineages in which an excision event had occurred. As predicted, the relative intensities of empty donor site bands in different digests was constant for a given transformant.

Evidence for the reinsertion of Ac

The same blots were probed with the 4.3 kb *Ac*-specific probe. Both the *Eco*RI-*Hin*dIII and the *Bam*HI-*Hin*dIII digestions resulted in restriction fragments that were entirely internal to *Ac*. *Ac* at any location in the transformant genome would produce bands of 0.7 and 0.9 kb for the *Eco*RI-*Hin*dIII digest and a doublet of 1.6 kb for the *Bam*HI-*Hin*dIII digest. The intensity of the internal fragment bands is an indication of the number of elements present in the transformant genome. By these criteria, 10 of the transformants contained from 1 to 10 copies of *Ac* (plants 25, 10, 24, 8, 14, 18, 22, 6, 29, and 13) (Figs. 4a and 5a).

In 9 of 10 *Ac* transformants (plants 25, 10, 8, 14, 18, 22, 6, 29, and 13), there were additional bands of various sizes which hybridized to *Ac* but not to *wx* (Figs. 3a, 4a and 5a). Up to 8 discrete new bands were observed in some of the transformants. It is highly improabable that these new bands resulted from incomplete digestion: each transformant gave a different banding pattern when probed with *Ac*, the bands were *Ac*-specific, and no evidence of partial digestion was observed when a *wx* probe was used. Each transformant that contained new *Ac*-specific bands also contained a *wx*-specific empty donor site. This suggested that transposition of *Ac* required prior excision from the T-DNA.

Three *Ac* transformants (plants 22, 29 and 13) contained bands which hybridized to both *Ac* and *wx* probes. It has been documented that multiple T-DNA insertions can result in aberrant T-DNA restriction digests (Zambryski et al. 1983; Spielmann and Simpson 1986; Jorgensen et al. 1987). Alternatively, these may have arisen from aberrant transposition events.

Transformant 12 did not contain any detectable *Ac* hybridizing sequences on any of the three blots. Also, there was no evidence of unexcised *Ac* when using a *wx*-specific probe. DNA from this plant did however contain an empty donor site band. The element in this transformant was apparently lost subsequent to an early excision event. Regenerant 34 contained neither *wx* nor *Ac* sequences and was either not transformed or had obtained a severely deleted T-DNA.

DS is not mobilized in tomato

We similarly analyzed 8 primary tomato transformants carrying pDS203, a pMON200 construction bearing *DS1*. Transformant DNA was digested with *Hin*dIII and *Bam*HI and probed with a 750 bp fragment containing both the *Ds1* element and flanking *Adh1* sequences. With this combination of enzymes and probe, an integrated *Ds1* element would yield a 2.1 kb band (Fig. 2). If *Ds* excised from the donor plasmid, an empty donor site fragment of 1.7 kb would be apparent. Seven of the transformants contained the 2.1 kb fragment expected for a successful pDS203 transformation (Fig. 6), the remaining plant was not transformed. None of the transformants contained a 1.7 kb empty donor site band, indicating that *Ds* did not excise in transgenic tomatoes. One pDS203 transformant (lane 2) had an additional band of 2.8 kb, which was not consistent with *Ds* transposition. This band probably resulted from a complex T-DNA integration event, since digestion of the DNA from this plant with other enzymes demonstrated that it had undergone multiple T-DNA insertions (data not shown).

Transposition of Ac *occurred in somatic tissue of whole plants*

There was considerable variation in the intensity of different *Ac*-specific bands within a transformant. This indicated that different insertion events were not equally represented in the sampled tissue, suggesting that transposition occurred during the somatic development of the plant. The intensity of the insertion band would be indicative of the size of the somatic sector containing a particular insertion event. In order to determine whether transposition occurred in later developing tissue, DNA was isolated from different leaves of the same primary transformant. The DNA was then digested with *Xba*I and probed with the 4.3 kb *Ac* probe.

The results are shown in Fig. 7. Lanes A, B and C were loaded with DNA isolated from increasingly younger leaves; hence, DNA from lane A was isolated from the bottom of the plant, DNA in lane C was from the top. Many of the *Ac*-specific bands remained constant between the different leaf samples, indicating that these insertion events were stable over the developmental stages examined. There were, however, also differences in the *Ac* banding pattern in the different leaves. This indicated that *Ac* transposition occurred during the development of the plant.

Discussion

These experiments indicated that *Ac* transposes in transgenic tomato plants. We determined, by the presence of a *wx*-specific band of the size predicted for an empty donor site, that *Ac* excised from the donor T-DNA in 9 out of 11 primary transformants. Most of these transformants also contained bands that were indicative of a non-excised *Ac* element being present. There are two explanations for this observation. The tissue sampled may have been a mixture of cells with *Ac* excised in some but not others. Alternatively, the plant may have been transformed with multiple copies of *Ac*, of which only some were active. Since we know that some transformants contained multiple inserts and also that transposition occurred in later stages of plant development, neither of these mechanisms can be excluded.

A few of the transformants had an intense empty donor site band but no resident plasmid band. Since we cannot say when in the cell lineage the excision event occurred, it is impossible to determine whether this is due to a single early excision or to frequent late events. In any case, this is a higher excision frequency than has been previously reported (Baker et al. 1987; Van Sluys et al. 1987). Since we prepared DNA from the transgenic plants about 7 months after transformation, the high frequency may be simply a result of allowing more time for excision to occur. Alternatively, the high activity may reflect some property of either the pMAC construction or of the tomato plants.

Using an *Ac*-specific probe, a number of new bands of unpredictable sizes were observed. New *Ac*-specific

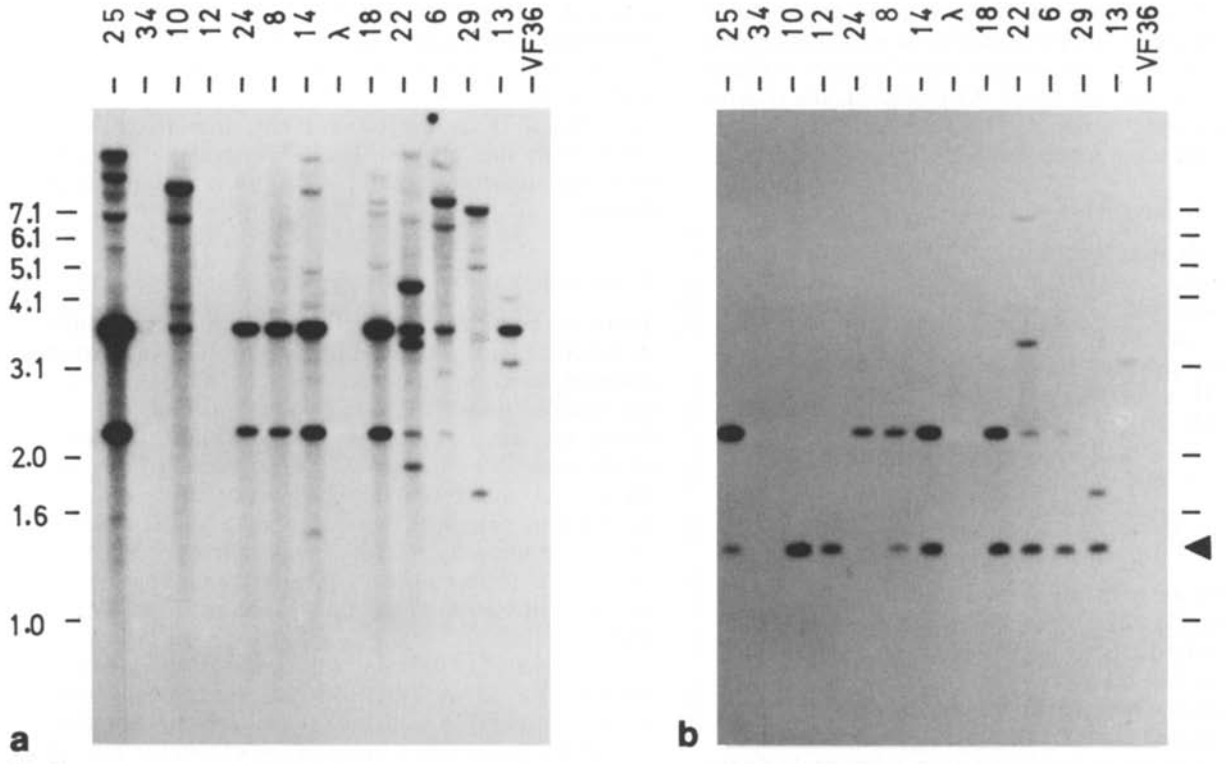

Fig. 3

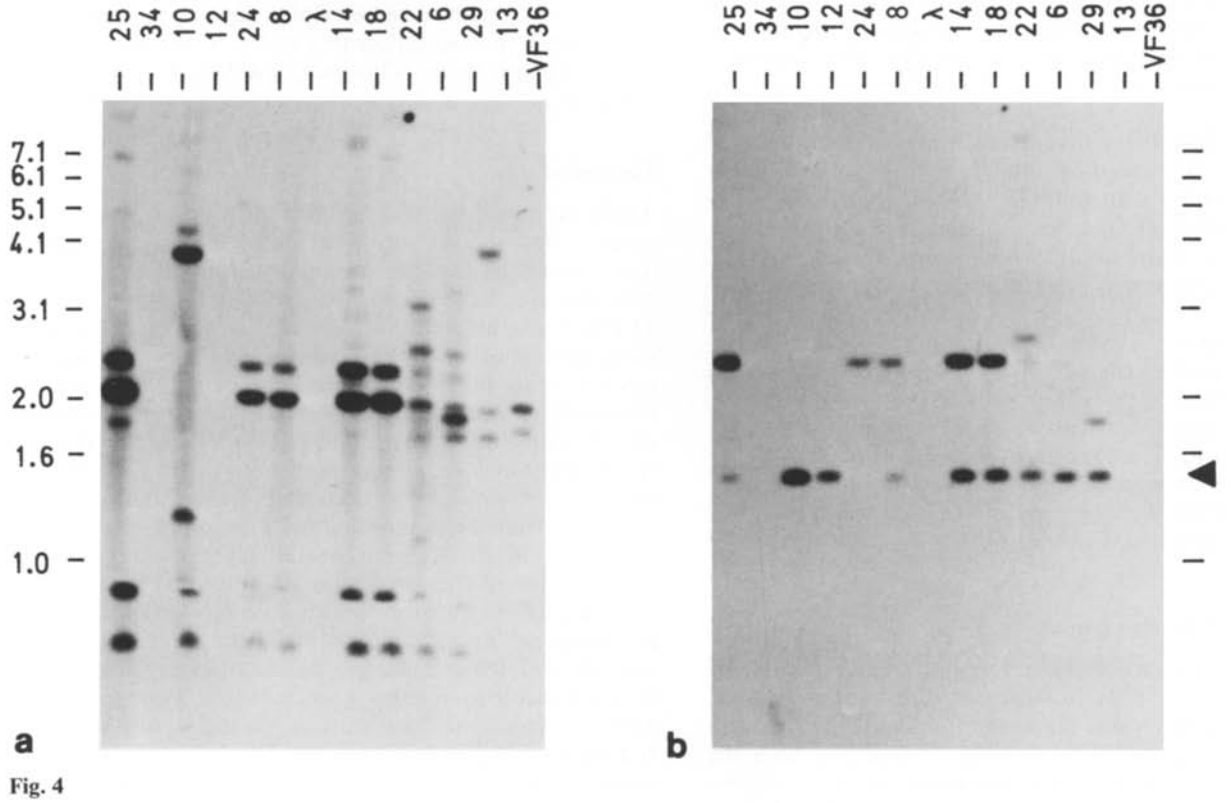

Fig. 4

Figs. 3–5. Southern analysis of pMAC transformants. Genomic DNA was isolated from 12 independent kanamycin-resistant regenerants and cleaved with *Xba*I (Fig. 3), *Hin*dIII and *Eco*RI (Fig. 4) or *Hin*dIII and *Bam*HI (Fig. 5). The filters were then hybridized with the 4.3 kb *Ac* probe (Figs. 3a, 4a, and 5a). Bands which correspond to a non-transposed *Ac* element are 2.1 and 3.8 kb for the *Xba*I digest, 2.0 and 2.4 for the *Hin*dIII-*Eco*RI double digest, and 2.4 and 1.6 kb for the *Hin*dIII-*Bam*HI double digest. Following autoradiography, the filters were stripped as described in the Materials and methods and probed with the 1.2 kb *wx*-specific probe (Figs. 3b, 4b, and 5b). The band corresponding to the empty donor site using the *wx* probe is indicated by the *triangle*. Transformant plant numbers are shown above the lanes; the numbers at the left side are the sizes of DNA markers (kb)

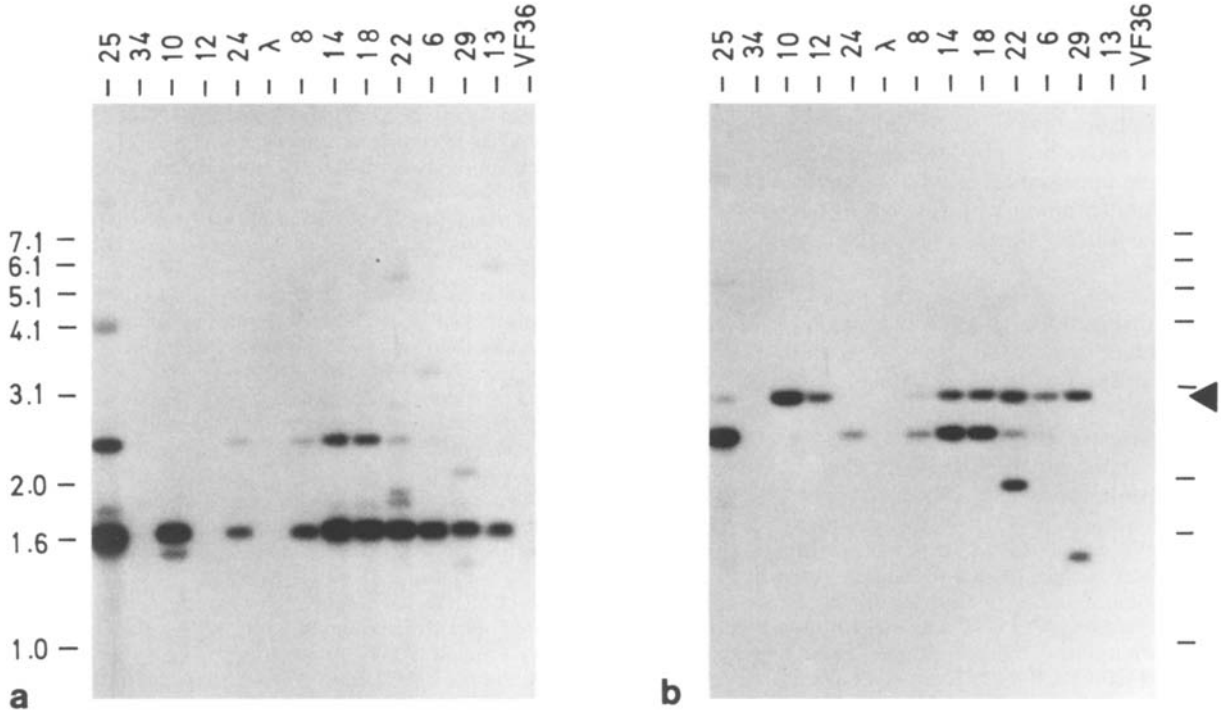

Fig. 5

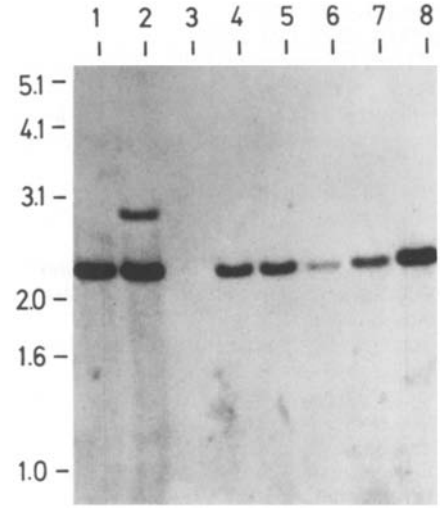

Fig. 6. Southern analysis of pDs203 transformants. Genomic DNA from 8 independent regenerants from a transformation with pDS203 was digested with *Hin*dIII and *Bam*HI, blotted and probed with the probe containing both *Ds1* and flanking *Adh1* sequences. If *Ds* excised, an empty donor site would be predicted at 1.7 kb

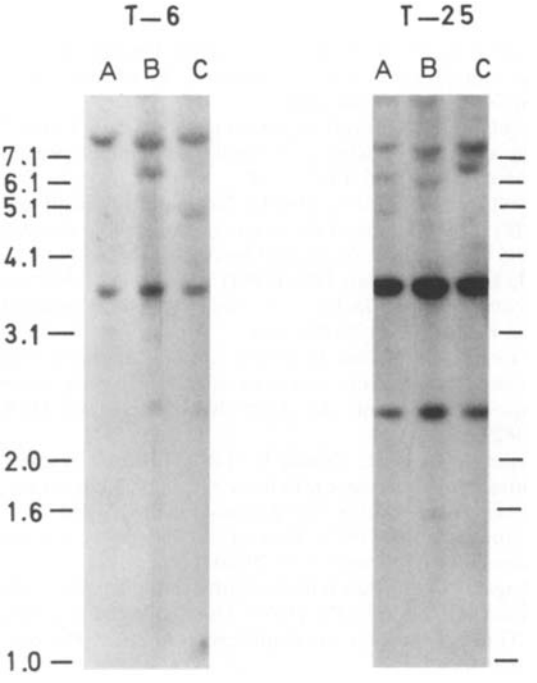

Fig. 7. Southern analysis of different leaves of two pMAC transformants. DNA was isolated from three increasingly younger individual leaves of transformants 6 and 25. Following digestion with *Xba* and blotting onto ZetaProbe membrane filters, the Southern blot was probed with the 4.3 kb *Ac*-specific probe. Lane A, DNA from a bottom leaf; lane B, DNA from a middle leaf; lane C, DNA from a leaf at the top of the plant

bands were observed only in those transformants which contained an empty donor site band. In some of the plants (25, 10, 24, 8, 14, 18, and 6) there was a good correlation between the intensity of the revertant fragment and the number and intensity of new *Ac* fragments. This suggested that excision of *Ac* was concomitant with its reinsertion into a new chromosomal location. In other transformants, there were fewer *Ac* bands than expected for the observed excision frequency. This was most striking for transformant 12 in which an intense empty donor site fragment was present but there was no evidence of *Ac* sequences. Late transposition events would result in integration events not detectable by hybridization because they would be present in low molar amounts. Alternatively, *Ac* elements may be lost after excision. This could result from a faulty transposition mech-

anism or by segregation of *Ac* following transposition to a sister chromatid.

It has been reported that *Ac* is mobilized in heterologous systems early after transformation and tends to stabilize in later stages (Van Sluys et al. 1987). We obtained direct evidence that *Ac* is active at later developmental stages in tomato. Because the appearance of new *Ac*-specific bands in young leaves is concomitant with the loss of bands from older tissue, it is most likely that the same *Ac* element continues to transpose.

When *Ds* is transformed into tomato plants, there is no evidence of transposition. This has also been observed in other plants (Baker et al. 1986; Van Sluys et al. 1987). This is further confirmation that transposition of *Ac* required sequences present in the intact element, presumably for correct expression of the *Ac* transcript (Kunze et al. 1987). The genetic *trans* activation of these elements in tomato is currently being evaluated.

Acknowledgements. We would like to acknowledge the excellent technical assistance of F. Belzile, R. Khush, and P. Peterson. We also thank R. Michelmore and S. O'Neill for critical discussions on this project. The vector pMON200 was generously provided by the Monsanto Corporation. This research was funded by grants from USDA-CSRGO (86-CRCR-1-1991) and the California Fresh Market Tomato Advisory Board.

References

Baker B, Schell J, Loerz H, Fedoroff N (1986) Transposition of the maize controlling element "*Activator*" in tobacco. Proc Natl Acad Sci USA 83:4844–4848

Baker B, Coupland G, Fedoroff N, Starlinger P, Schell J (1987) Phenotypic assay for excision of the maize controlling element *Ac* in tobacco. EMBO J 6:1547–1554

Behrens U, Fedoroff N, Laird A, Mueller-Neumann M, Starlinger P, Yoder JI (1984) Cloning of the *Zea mays* controlling element *Ac* from the *wx-m7* allele. Mol Gen Gent 194:346–347

Bingham PM, Levis R, Rubin GM (1981) Cloning of DNA sequences from the *white* locus of *D. melanogaster* by a novel and general method. Cell 25:693–704

Fedoroff N, Furtek D, Nelson O (1984) Cloning of the *bronze* locus in maize by a simple and generalized procedure using the transposable element *Ac*. Proc Natl Acad Sci USA 81:3825–3829

Fillatti JJ, Kiser J, Rose R, Comai L (1987) Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio/Technology 5:726–730

Fischer RL, Goldberg RB (1982) Structure and flanking regions of soybean seed protein genes. Cell 29:651–660

Fraley RT, Rogers SG, Horsch RB, Eichholtz DA, Flick JS, Fink CL, Hoffman NL, Sanders PR (1985) The SEV system: a new disarmed Ti plasmid vector for plant transformation. Biotechnology 3:629–635

Jorgensen R, Snyder C, Jones J (1987) T-DNA is organized predominantly in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol Gen Genet 207:471–477

Koornneef M, Hanhart C, Jongsma M, Toma I, Weide R, Zabel P, Hille J (1986) Breeding of a tomato genotype readily accessible to genetic manipulation. Plant Sci 45:201–208

Kunze R, Stochaj U, Laufs J, Starlinger P (1987) Transcription of the transposable element *Activator* (*Ac*) of *Zea mays* L. EMBO J 6:1555–1563

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: A laboratory manual. Press, Cold Spring Harbor Laboratory, New York

Martin C, Carpenter R, Sommer H, Saedler H, Coen ES (1985) Molecular analysis of instability in flower pigmentation of *Antirrhinum majus*, following isolation of the *pallida* locus by transposon tagging. EMBO J 4:1625–1630

McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disk transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Reports 5:81–84

O'Reilly C, Shepherd NS, Pereira A, Schwarz-Sommer Zs, Bertram I, Robertson DS, Peterson PA, Saedler H (1985) Molecular cloning of the *a1* locus of *Zea mays* using the transposable elements *En* and *Mu1*. EMBO J 4:877–882

Shepherd NS (1987) Transposable elements and gene-tagging. In: Shaw C (ed) Plant molecular biology: A practical approach. IRL Press, London

Spielmann A, Simpson RB (1986) T-DNA structure in transgenic tobacco plants with multiple independent integration sites. Mol Gen Gent 205:34–41

Sutton WD, Gerlach WL, Schwartz D, Peacock WJ (1984) Molecular analysis of *Ds* controlling element mutations at the *Adh1* locus of maize. Science 223:1265–1268

Tanksley SD, Mutschler MA, Rick CM (1987) Linkage map of tomato. In: O'Brien SJ (ed) Genetic maps. Cold Spring Harbor Laboratories, New York, pp 655–669

Theres K, Scheele T, Starlinger P (1987) Cloning of the *Bz2* locus of *Zea mays* using the transposable element *Ds* as a gene tag. Mol Gen Gent 209:193–197

Thomas BR, Pratt D (1981) Efficient hybridization between *Lycopersicon esculentum* and *L. peruvianum* via embryo callus. Theor Appl Genet 59:215–219

Van Sluys MA, Tempe J, Fedoroff N (1987) Studies on the introduction and mobility of the maize *Activator* element in *Arabidopsis thaliana* and *Daucus carota*. EMBO J 6:3881–3889

Wahl GM, Stern M, Stark GR (1979) Efficient transfer of larger DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc Natl Acad Sci USA 76:3683–3687

Zambryski P, Joos H, Genetelllo C, Leemans J, Montagu M, Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without the alteration of their normal regeneration capacity. EMBO J 2:2143–2150

Communicated by H. Saedler

Received February 17, 1988