The Plant Cell, Vol. 2, 709-721, August 1990 © 1990 American Society of Plant Physiologists

Properties of the Maize Transposable Element *Activator* in Transgenic Tobacco Plants: A Versatile Inter-Species Genetic Tool

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The maize controlling element *Activator* (*Ac*) transposes autonomously from an integrated T-DNA vector to new sites in the genomes of tobacco and other heterologous plant species. Here we demonstrate that critical functions required for transposition of *Ac* in maize are conserved in tobacco and that *Ac* transposes at high frequency for at least five generations. *Ac* structure and terminal sequences are conserved upon transposition and a characteristic 8-bp duplication of target sequences is generated upon integration. *Ac* remains unmethylated, transcriptionally active, and capable to *trans*-activate transposition of the nonautonomous *Dissociation* (*Ds*) element throughout several generations. In tobacco, as in maize, *Ac* transposes adjacent to low copy or unique DNA, and transcriptional analysis of unique target DNA provides evidence that an *Ac* element transposed into a gene. In maize, increasing copies of *Ac* delay the timing and reduce the frequency of early transposition of *Ac* and *Ds*. In tobacco, increasing copies of *Ac* correlate with an increased frequency of *Ds trans*-activation. These data firmly establish that the *Ac* and *Ds* transposable elements are versatile genetic tools well suited for use as insertional mutagens and demonstrate that thorough investigation of mechanism and regulation of transposition is facilitated in heterologous settings.

INTRODUCTION

The maize transposable element *Activator* (*Ac*) was first described by Barbara McClintock, who showed that it transposes autonomously and *trans*-activates members of a second class of related elements designated *Dissociation* (*Ds*) (McClintock, 1947, 1950). The isolation of *Ac* and *Ds* elements was followed by extensive molecular studies (for a recent review, see Fedoroff, 1989). *Ac* is 4.65 kb in length with 11-bp imperfect terminal inverted repeats and generates an 8-bp duplication of target sequences upon insertion. *Ac* transposase is encoded by one major transcript of 3.5 kb. In contrast, *Ds* elements are a heterogeneous class of nonautonomous transposable elements ranging from simple internal deletions of *Ac* to those that share little more sequence similarity with *Ac* than the terminal inverted repeats. Integration of *Ac* and *Ds* into genes normally interferes with gene expression, a feature that has been utilized for the tagging and subsequent cloning of several maize genes (Fedoroff et al., 1984; Chen et al., 1987; Theres et al., 1987; Hake et al., 1989).

A variety of studies have shown that *Ac* transposition is regulated in maize. McClintock observed "changes in phase," a reversible inactivation of *Ac* during plant development (McClintock, 1964, 1965). This inactivation can be correlated with methylation of particular internal PvuII and HpaII sites of *Ac* (Schwartz and Dennis, 1986; Chomet et al., 1987) and absence of detectable 3.5-kb *Ac* RNA transcript (Kunze et al. 1988). A second factor influencing *Ac* activity is its copy number. Increasing numbers of *Ac* cause a delay of transposition during development and an apparent decrease in early transposition frequency (McClintock, 1948, 1951).

Systematic molecular study of transposable elements in maize has been impeded by inefficient transformation and regeneration techniques. In contrast, the facility with which tobacco and other dicot species are transformed, regenerated, and propagated makes them appropriate choices for molecular genetic analysis of *Ac*.

Toward these ends, *Ac* and *Ds* were introduced on Ti-plasmid vectors into tobacco. This work showed that only *Ac* transposes autonomously, while *Ds* transposition requires *trans*-activation by resident *Ac* or a subsequently introduced *Ac* or *Ac* cDNA (Baker et al., 1986, 1987; Coupland et al., 1988; Hehl and Baker, 1989). *Ac* transcription in tobacco generates a 3.5-kb transcript with transcription start sites similar to those described for maize (Kunze et al., 1987; Finnegan et al., 1988). The introduction of *Ac* into tobacco and development of a phenotypic assay for excision (Baker et al., 1986, 1987) permitted initial inves-

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tigations to delineate the sequences required in *cis* and *trans* for excision (Coupland et al., 1988, 1989).

Demonstration of *Ac* transposition in tobacco, *Arabidopsis*, carrot, tomato, and potato suggested that this transposable element and its derivatives may function as insertional mutagens in those plants (Baker et al., 1986; Van Sluys et al., 1987; Knapp et al., 1988; Yoder et al., 1988). However, development of efficient transposon tagging strategies will require comprehensive data about *Ac* activity in heterologous plants. Parameters such as the frequency of transposition, variation in transposition rate between generations, the distances elements transpose, and insertion site specificity all bear directly on the utility of *Ac* as an insertional mutagen in heterologous species. It has been shown that *Ac* transposes in 13% to 40% of transformed regenerating tobacco protoplasts (Baker et al., 1987), a value comparable with transposition frequencies measured in transformed regenerating potato shoots (50%, Knapp et al., 1988). *Ac* remains active and continues to transpose from the T-DNA vector in one subsequent generation of tobacco and two subsequent generations of tomato and *Arabidopsis* (Belzile et al., 1989; Schmidt and Willmitzer, 1989; Taylor et al., 1989). In tomato, approximately 30% of progeny of primary transformants inherit a transposed *Ac* from their parent. Transposed *Ac* continues to transpose for at least three generations of tomato (Belzile et al., 1989). Transposed *Ac* elements are transmitted to subsequent generations in tobacco and tomato (Baker et al., 1988; Belzile et al., 1989), and *Ac* transactivates *Ds* transposition in tobacco, tomato, and *Arabidopsis* (Hehl and Baker, 1989; Lassner et al., 1989; Masterson et al., 1989). Although the transpositional behavior of *Ac* and *Ds* from the T-DNA vector is well characterized, little is known about the properties of transposed elements. Only one transposed element, *Ac-18*, has been cloned and characterized in detail. It sustained a 4-bp terminal deletion and is apparently stable, although able to *trans*-activate *Ds* transposition (Hehl and Baker, 1989).

The work presented here focuses on molecular genetic analysis of transposed *Ac* elements. All *Ac*s present in the tobacco line examined transposed away from the T-DNA. One conclusion of the results is that characteristic features defining active *Ac* elements in maize are retained in transgenic tobacco. *Ac* is shown to transpose for five generations, a result attributed to observations that structure and terminal sequences of *Ac* are conserved. *Ac* remains unmethylated, transcriptionally active, and capable of inducing *Ds* transposition. *Ac* integrates into low and unique copy DNA in both tobacco and maize and, significantly, alters transcription of at least one tobacco gene.

In contrast to maize, we do not observe a negative dosage effect of *Ac* in tobacco. Instead, there is a positive correlation between *Ac* copy number and apparent *trans*-activation frequency of *Ds*. Fundamental properties of *Ac* transposition are shared between maize and tobacco, yet the absence of negative dosage in tobacco represents a significant difference in *Ac* activity between the two species.

This work provides a framework for the molecular genetic investigation of mechanistic and regulatory features of *Ac* transposition and establishes the utility of *Ac* and *Ds* as genetic tools for insertional mutagenesis and for the study of genome organization in heterologous species.

RESULTS

Ac Continues To Transpose

Ac and *Ds* elements were independently introduced on Ti plasmid vectors into regenerating tobacco protoplasts. *Ac* was shown to transpose autonomously, while *Ds* transposition required *trans*-activation by resident or introduced *Ac* (Baker et al., 1986, 1987; Coupland et al., 1988; Hehl and Baker, 1989). Figure 1 shows that transposed *Ac* elements detected in a primary transgenic parent plant, R0-1, continue to transpose for four subsequent generations, R1-R4, and that newly transposed *Ac* elements are germinally transmitted from parent to progeny (see also Table 1). Generations R1-R3 were obtained by selfing individual R0, R1, and R2 parents, and the R4 progeny were generated by crossing R3 transgenics to tobacco free of *Ac*. DNA gel blots of EcoRI-BglII digested genomic DNA from individual plants of each generation were hybridized with an internal *Ac* HindIII fragment probe (Figure 1). BglII does not digest within *Ac*, while EcoRI cleaves once to yield two fragments corresponding to each element [(Figure 1)(Fedoroff et al., 1983; Pohlman et al., 1984)]. Transposed *Ac*s in the R0-1 DNA are identified by the appearance of new, *Ac*-specific restriction enzyme fragments distinct in size from those characteristic of *Ac* at its original T-DNA location [(Figure 1, R0-1)(Baker et al., 1986, 1988; Hehl and Baker, 1989)]. Newly transposed *Ac*s in subsequent generations are identified by the appearance of novel *Ac* fragments arising in progeny plant DNAs as compared with parent DNAs. Germinally transmitted *Ac*s are identified by inheritance of pairs of *Ac* homologous fragments among sibling offspring. The results of DNA gel blot hybridizations identifying new and germinally transmitted *Ac*s are summarized in Table 1.

The progenitor R0-1 plant harbored several transposed *Ac* elements that are identified by the presence of at least five pairs of novel, *Ac* homologous genomic DNA fragments (Figure 1, R0-1). One progeny plant of R0-1, R1-4, harbored a germinally transmitted element, *Nt-1::Ac-18*, and an apparent newly transposed *Ac*, *Nt-3::Ac* (Figure 1, compare R0-1 and R1-4; Table 1). *Nt-1* and *Nt-3* are designations for two different integration sites of *Ac*. The element and flanking genomic DNA at *Nt-1::Ac-18* were cloned, restriction enzyme mapped, and partially sequenced. The results confirmed that the germinally trans-

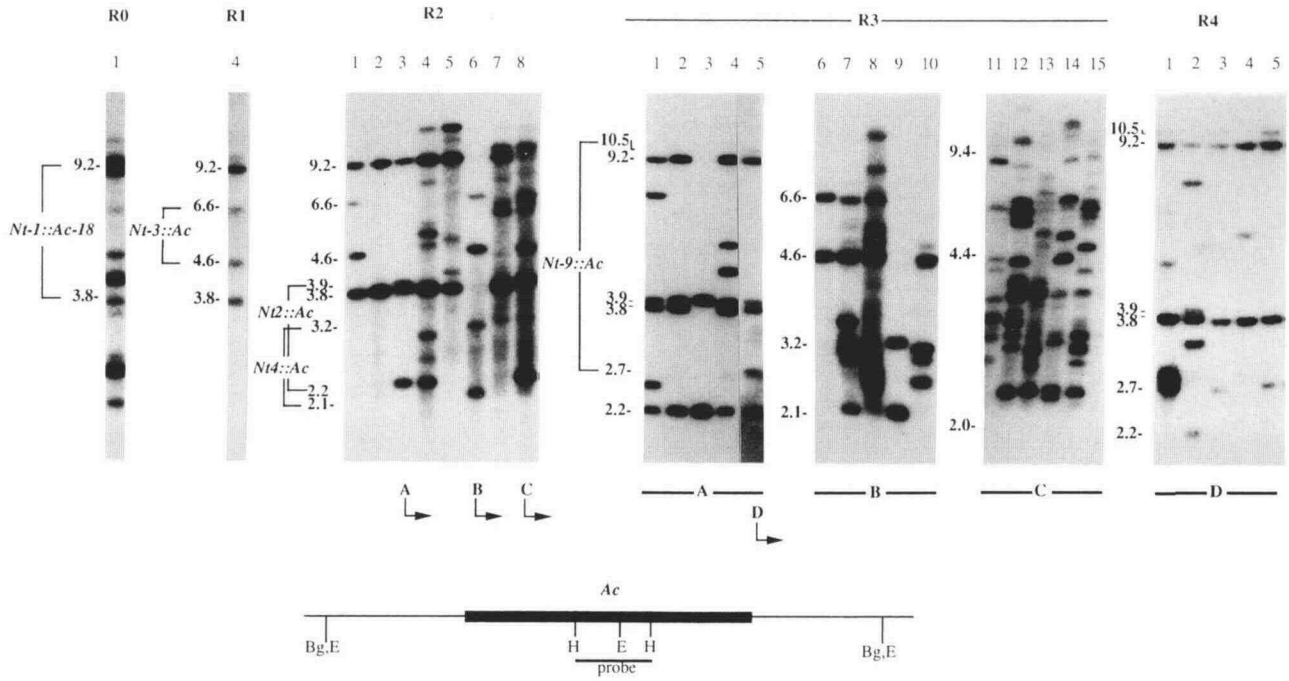

Figure 1. DNA Gel Blot Analysis of *Ac* Transposition in Five Generations of Transgenic Tobacco.

DNA gel blot hybridizations of EcoRI/BglII digested DNA isolated from individual R0, R1, R2, R3, and R4 plants with the 1.6-kb *Ac* internal HindIII fragment probe. The R0-1 transgenic plant was regenerated from tobacco protoplasts transformed with the pTi*Ac* construct (Baker et al., 1986). R1-4 and R2-1 to R2-8 are selfed progeny of R0-1 and R1-4, respectively. Plants R3-1 to R3-5, R3-6 to R3-10, and R3-11 to R3-15 are selfed progeny of R2-3 (A), R2-6 (B), and R2-8 (C), respectively. Plants R4-1 to R4-5 are F1 progeny of R3-5 (D) crossed to tobacco void of *Ac*. Restriction fragments representing *Nt-1::Ac-18* (9.2 and 3.8 kb), *Nt-2::Ac* (3.9 and 2.2 kb), *Nt-3::Ac* (6.6 and 4.6 kb), *Nt-4::Ac* (3.2 and 2.1 kb), and *Nt-9::Ac* (10.5 and 2.7 kb) are indicated.

mitted 9.2-kb and 3.8-kb fragments, visualized in DNA gel blots, indeed represent a transposed *Ac* (Hehl and Baker, 1989). Further analysis of this *Ac* indicated that it sustained a 4-bp terminal deletion and is stable; no new *Ac* fragments are observed in progeny that harbor only this element (Hehl and Baker, 1989). *Nt-3::Ac*, a newly transposed *Ac* in plant R1-4, is characterized by two novel EcoRI/BglII DNA fragments of 6.6 kb and 4.6 kb (Figure 1, R1-4; Table 1). Only the 4.6-kb fragment is visualized as a new fragment in DNA of R1-4 due to comigration of a similarly sized 6.6-kb *Ac* fragment in R0-1 DNA. *Nt-1::Ac-18* and *Nt-3::Ac* are germinally transmitted from parent R1-4 to eight and six out of 12 selfed R2 progeny, respectively, and the results of DNA gel blot hybridization of eight progeny DNAs are shown in Figure 1 [Figure 1, compare R1-4 with R2-1, 2, 3, 4, 5, and 7 (*Nt-1::Ac-18*) and with R2-1, 6, and 8 (*Nt-3::Ac*); Table 1]. Two progeny plants, R2-1 and R2-2, harbor exclusively those elements, *Nt-1::Ac-18* (R2-1 and R2-2) and *Nt-3::Ac* (R2-1), detected in the parent R1-4 (Figure 1, compare R1-4 with R2-1 and R2-2).

Among the 12 R2 selfed progeny of R1-4 examined, 10 show new *Ac* homologous fragments distinct in size from those detected in the R1-4 parent (Figure 1; compare R1-4 with R2-3, 4, 5, 6, 7, and 8 and data not shown). Plant R2-3 harbors the germinally transmitted *Nt-1::Ac-18* as well as a newly transposed *Ac*, *Nt-2::Ac*, characterized by 3.9-kb and 2.2-kb fragments (Figure 1, compare R1-4 with R2-3; Table 1). *Nt-2::Ac* was cloned and restriction enzyme mapped and the two new fragments of plant R2-3 verified to correspond to a new *Ac* transposition (see below). *Nt-2::Ac* was germinally transmitted to 28 out of 35 R3 progeny, and Figure 1 shows the results of analysis of five progeny DNAs (Figure 1, compare R2-3 with R3-1, 2, 3, 4, and 5; Table 1). Among the 35 R3 progeny of plant R2-3, eight contained new *Ac* fragments (Figure 1, compare R2-3 with R3-1, 4, and 5 and data not shown; Table 1). Because the parent plant, R2-3, harbored only one element, *Nt-2::Ac*, in addition to the stable *Nt-1::Ac-18* (Figure 1, R2-3), these new *Ac* insertions likely arose by *Ac* transposition from the *Nt-2::Ac* locus.

The selfed offspring plants of R2-6 and R2-8 (Figure 1 and Table 1) provided additional evidence for continuous *Ac* transposition. The parent plant R2-6 harbored two readily discernible *Acs:* one, the germinally transmitted *Nt-3::Ac* (6.6 kb and 4.6 kb) and a newly transposed

Table 1. Germinal Transmission and Transposition of *Ac* in Transgenic Tobacco Plants

Progenitor Plant	*Ac* Alleles in Progenitor	No. of Offspring Plants Analyzed	No. of Offspring with *Ac* Alleles from Progenitor	No. of Offspring Plants Harboring New *Ac*	% Offspring Plants Harboring New *Ac*
R0-1	*Nt-1::Ac-18* ND > 2[a]	4	4 *Nt-1::Ac-18*	2	50%
R1-4	*Nt-1::Ac-18* *Nt-3::Ac*	12	6 *Nt-1::Ac-18* 4 *Nt-3::Ac* 2 *Nt-1::Ac-18;Nt-3::Ac*	10	83%
R2-3	*Nt-1::Ac-18* *Nt-2::Ac*	35	7 *Nt-1::Ac-18* 7 *Nt-2::Ac* 21 *Nt-1::Ac-18;Nt-2::Ac*	8	23%
R2-6	*Nt-3::Ac* *Nt-4::Ac*	8[b]	4 *Nt-3::Ac* 2 *Nt-4::Ac* 1 *Nt-3::Ac/Nt-4::Ac*	5	63%
R2-8	ND > 2	10	ND	8	80%
R3-5	*Nt-1::Ac-18* *Nt-2::Ac* *Nt-9::Ac*	25	6 *Nt-1::Ac-18* 13 *Nt-1::Ac-18;Nt-2::Ac* 6 *Nt-1::Ac-18;Nt-9::Ac*	17	68%
R3-16	*Nt-1::Ac-18*	–	– –	–	–
R3-17	*Nt-2::Ac*	29	16 *Nt-2::Ac*	0	–
R3-18,R3-19	ND > 2	–	– –	–	–

[a] Plant R0-1 harbors, in addition to *Ac-18*, at least two *Ac* elements that are indicated here as ND > 2. Plants R2-8, R3-18, and R3-19 harbor abundant *Ac* elements and numbers are similarly specified.
[b] One progeny of R2-6 harbored only newly transposed *Ac*s.

element *Nt-4::Ac* with fragment sizes of 3.2 kb and 2.1 kb (Figure 1, R2-6; Table 1). Both elements were germinally transmitted to approximately half of the eight R2-6 offspring examined (Figure 1, compare R2-6 with R3-6, 7, 8, 9, and 10 and data not shown; Table 1). Five R3 offspring had new *Ac* fragments distinct from those of the R2-6 parent (Figure 1, compare R2-6 with R3-7, 8, and 10 and data not shown; Table 1). In contrast to plants R2-3 and R2-6, DNA of plant R2-8 exhibited a complex pattern of *Ac* fragments, and in DNA of eight out of 10 of its R3 offspring, new fragments were detected (Figure 1, compare R2-8 with R3-11, 12, 13, 14, and 15 and data not shown; Table 1).

R4 plants were obtained by crossing plants free of *Ac* to two R3 plants, R3-17, an identical sibling to R3-3 carrying *Nt-2::Ac* (Figure 1 and see below), and R3-5, harboring *Nt-1::Ac-18*, *Nt-2::Ac*, and *Nt-9::Ac* (Figure 1, R3-5). *Nt-2::Ac* was germinally transmitted to over half of the progeny of plant R3-17 (data not shown), but no new *Ac*s were detected among them (Table 1). Among 25 R4 (F1) plants derived from R3-5, six harbored the germinally transmitted *Nt-9::Ac* and 17 displayed fragments at previously unoccupied target sites. (Figure 1, compare R3-5 to R4-1, 2, and 5 and data not shown; Table 1).

These data establish that *Ac* maintains the genetic capacity to transpose throughout five generations of transgenic tobacco. Newly transposed *Ac*s were detected in 23% to 83% of the progeny obtained by selfing or outcrossing of progenitors with at least two *Ac*s (Table 1). Four identified mobile elements, *Nt-2, 3, 4*, and *9::Ac*, arising in different parent plants were transmitted to sibling offspring (Table 1). It is concluded that the new pairs of *Ac* fragments arising in DNA of individual plants of each generation represent germinal or germinal-like transpositions of *Ac*.

Ac Sequences Remain Unmethylated

A correlation between reversible inactivation of *Ac* and methylation of subterminal element sequences was described in maize (Schwartz and Dennis, 1986; Chomet et al., 1987). Additionally, the characteristic 3.5-kb *Ac* transcript is not detected in maize lines carrying genetically inactive and methylated *Ac* (Kunze et al., 1987, 1988). HpaII and PvuII are two enzymes, among several, whose ability to cleave DNA is sensitive to methylation of C residues within the di- and trinucleotide CpG and CpNpG of their recognition sequence. In maize, these enzymes cleave genetically active *Ac*s internally, while inactive elements are resistant to cleavage and larger internally homologous *Ac* fragments are generated (Schwartz and Dennis, 1986; Chomet et al., 1987). The enzyme PvuII digests unmethylated *Ac*s twice, generating an internal 2.5-kb fragment, whereas HpaII cleaves *Ac* multiple times, yielding a characteristic internal 3.4-kb fragment (Müller-Neumann et al., 1984; Pohlmann et al., 1984).

*Ac*s present in the five tobacco generations described

above are apparently active and would be predicted to display methylation-sensitive restriction enzyme digestion patterns not unlike those of active elements in maize. DNA gel blots of PvuII and or HpaII digested R0, R1, R2, R3, and R4 DNAs were hybridized to the internal *Ac* HindIII probe. No DNA fragments larger than the internal 2.5-kb PvuII fragment were detected in the R0 and four R1 plant DNAs (one of these was R1-4, Figure 1), showing that those sites in these *Ac* elements were unmethylated (data not shown). Methylation of *Ac* in the R2 generation was assessed similarly by HpaII digestion of DNA from R1-4 and the same 12 R2 offspring described above in Table 1 and Figure 1. Figure 2 shows that the 3.4-kb internal HpaII fragment was detected exclusively in these DNAs and indicates that germinally transmitted as well as newly transposed *Ac*s in the R2 generation remain unmethylated at internal HpaII restriction sites. DNA of three R3 plants harboring new and germinally transmitted *Ac*s as well as nine R4 (F1) offspring of one R3 were similarly analyzed and equivalent results obtained (data not shown). No methylation of *Ac* sequences was observed among a total number of 29 individuals of five generations of tobacco using the enzymes PvuII or HpaII. In addition, detection of the internal 3.4-kb HpaII *Ac* fragment among the 25 individuals examined with this enzyme strongly suggests that none of the detectable *Ac*s present in these plants has undergone major structural alterations of internal sequences.

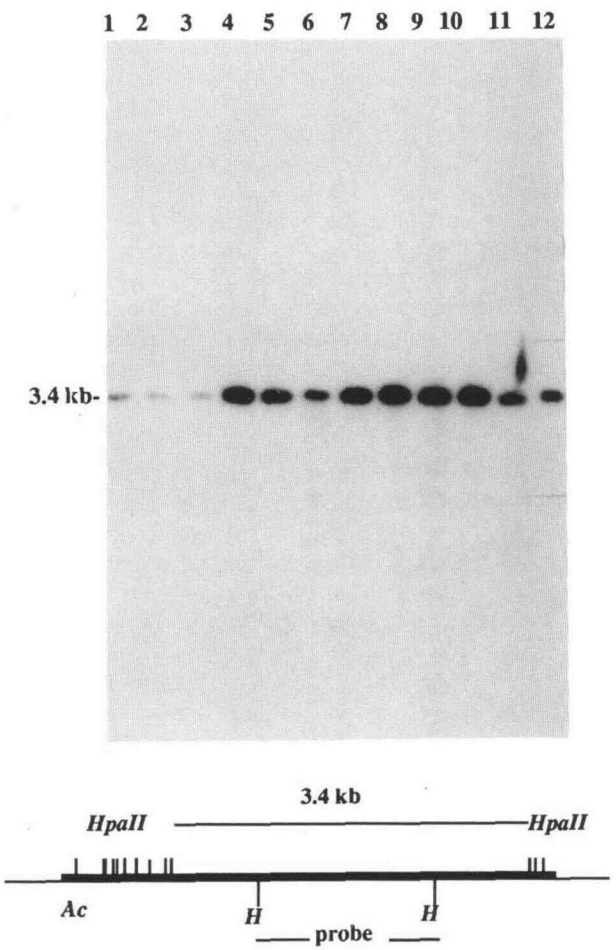

Figure 2. *Ac* Elements Are Unmethylated.

DNA gel blot hybridization of HpaII digested genomic DNAs isolated from plants R2-1 to R2-12 (R2-1 to R2-8 are shown in Figure 1) with the internal *Ac* HindIII fragment probe. The HpaII sites and the internal 3.4-kb HpaII fragment of *Ac* are indicated.

The Mechanism of *Ac* Integration in Tobacco and Maize Is Similar

In maize, *Ac* structure and sequence are conserved upon integration and like other transposable elements, *Ac* generates a short characteristic duplication of target sequences upon insertion (Müller-Neumann et al., 1984; Pohlmann et al., 1984; Fedoroff, 1989). These properties of *Ac* transposition were examined in tobacco to determine whether they are conserved in a heterologous species. Previously, we reported the isolation and partial characterization of one *Ac*, *Nt-1::Ac-18* (Hehl and Baker, 1989). Here, five additional transposed *Ac*s were isolated from lambda genomic libraries. All elements and their flanking DNA were characterized by restriction enzyme mapping. Figure 3 shows that the deduced maps indicated that all six of these elements retained original *Ac* structure and that each clone corresponded to a different integration event. Five elements and flanking genomic DNA, designated *Nt-1::Ac-18*, *Nt-5::Ac*, *Nt-6::Ac*, *Nt-7::Ac*, and *Nt-8::Ac*, were isolated from plant R1-4, while *Nt-2::Ac* was cloned from plant R2-3 (Figures 1 and 3). *Nt-1::Ac-18* was represented eight times in the genomic library of plant R1-4 (Hehl and Baker, 1989), while the other four, *Nt-5::Ac*, *Nt-6::Ac*, *Nt-7::Ac*, and *Nt-8::Ac*, were uniquely represented. These four elements were not detected in our original analysis of plant R1-4 (see Figure 1). Moreover, no *Ac* fragments corresponding in size to those of the lambda clones were detected in side by side comparisons of genomic and cloned lambda DNA or in genomic DNA of R2 offspring of plant R1-4 (data not shown).

To ascertain whether lambda clones corresponding to *Nt-5,-6*, *-7*, and *-8::Ac* were generated by rearrangement during the cloning procedure, *Ac*-adjacent DNA fragments were hybridized to DNA gel blots of normal tobacco DNA. The *Nt-5*, *-6*, *-7*, and *-8 Ac*-adjacent DNA probes detected target tobacco DNA fragments about 4.6 kb smaller than the corresponding lambda clones harboring *Ac* (see below). Furthermore, the 2.4-kb target fragment of *Ac* in *Nt-6::Ac* (Figure 3) was cloned, restriction enzyme mapped, and partially sequenced. Comparison of the re-

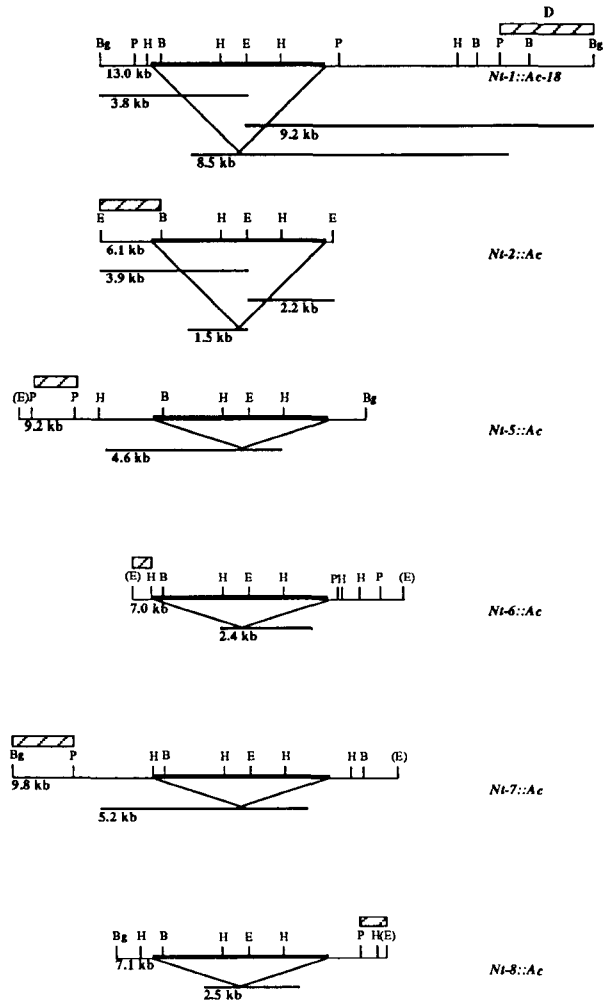

Figure 3. Restriction Enzyme Map of Six Transposed *Ac* Elements and Adjacent Tobacco DNA.

Restriction enzyme maps of *Nt-1::Ac-18*, *Nt-2::Ac*, *Nt-5::Ac*, *Nt-6::Ac*, *Nt-7::Ac*, and *Nt-8::Ac* cloned in phage lambda. The filled bars and adjacent lines represent *Ac* and flanking tobacco DNA, respectively. Genomic DNA fragments bearing *Nt-1::Ac-18* and *Nt-2::Ac* are indicated by thicker lines immediately below the *Nt-1::Ac-18* and *Nt-2::Ac* loci. *Ac* insertion sites within wild-type BglII or EcoRI/BglII target fragments (see Figure 5) are indicated. DNA fragments employed in DNA and RNA gel blot hybridizations (Figures 5 and 6) are indicated by the hatched boxes above each map. Restriction enzymes are abbreviated: BamHI (B), BglII (Bg), EcoRI (E), HindIII (H), and PstI (P). The EcoRI sites in parentheses are present either in genomic DNA or in the EMBL 4 polylinker. The sizes of the *Ac* loci, corresponding genomic DNA fragments, and wild-type loci are given in kilobase pairs.

striction enzyme maps and partial sequence data of the *Nt-6* target fragment and the *Ac* containing clone, *Nt-6::Ac*, indicated that the two clones are identical with the exception of the *Ac* insertion and host duplication in *Nt-6::Ac* (see below). It is concluded that these *Ac*s represent somatically transposing elements present in the genomes of cells in a fraction of the tissue analyzed. *Nt-3::Ac* was not represented in the lambda genomic library. In contrast, cloned tobacco loci *Nt-1::Ac-18* and *Nt-2::Ac*, present on 9.2-kb and 3.8-kb and 3.9-kb and 2.2-kb EcoRI/BglII fragments, respectively, were detected both in the genomic DNA from which they were isolated and in progeny DNAs (Figure 1, R1-4 and R2-3, R3-1 to 5; Figure 3; and data not shown).

Sequence analysis of the *Ac* termini and flanking tobacco DNA of *Nt-6::Ac* and *Nt-7::Ac* indicated that the termini are intact, and Figure 4 shows that each element is immediately flanked by an 8-bp duplication of target DNA. Approximately 180 bp of 5′ and 100 bp of 3′ *Ac* termini as well as approximately 250 bp of flanking genomic DNA were sequenced for each locus (data not shown). To verify that one of the 8-bp duplications resulted from *Ac* insertion, a 2.4-kb EcoRI/BglII target fragment of *Ac* at *Nt-6* (Figure 3) was cloned and approximately 250 bp surrounding and including the site of integration was sequenced. A single copy of the 8-bp sequence was detected in the target fragment, indicating that the duplication was induced upon integration (Figure 4 and data not shown). No other differences between sequences surrounding the target site in *Nt-6* and sequences adjacent to *Ac* in *Nt-6::Ac* were detected. Conservation of *Ac* structure and terminal sequences as well as duplication of 8 bps of target integration sequences are characteristic features of *Ac* transposition in maize. These features are retained in tobacco, suggesting that the mechanism of *Ac* integration in tobacco and maize is similar.

Ac Integrates Adjacent to Low Copy DNA and Alters Transcription of Target DNA

The complexity of *Ac* target sequences and possible insertion of *Ac* into transcribed regions were investigated. DNA fragments flanking *Ac* were isolated from each of the

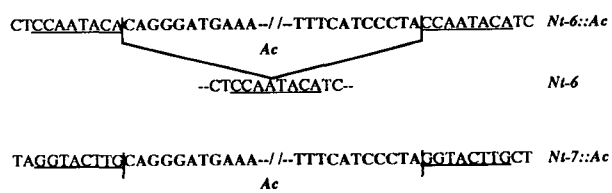

Figure 4. DNA Sequence of *Ac* Termini and One *Ac* Target Site.

DNA sequence of the *Ac* 11-bp imperfect terminal repeats at *Nt-6::Ac* and *Nt-7::Ac* and adjacent tobacco DNA. The 8-bp duplication flanking each *Ac* is underlined. The sequence of the *Ac* integration site of *Nt-6* is given below *Nt-6::Ac* and the 8 bp duplicated in *Nt-6::Ac* are underlined.

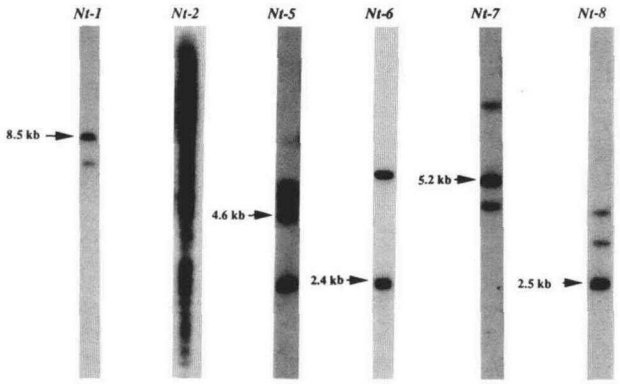

Figure 5. DNA Gel Blot Analysis of *Ac* Target Sites.

DNA gel blots of normal tobacco DNA, digested with BglII (lanes 1 and 2) or EcoRI/BglII (lanes 3, 4, 5, and 6), and hybridized to tobacco DNA restriction fragments that flank the cloned *Ac* elements of *Nt-1::Ac-18*, *Nt-2::Ac*, *Nt-5::Ac*, *Nt-6::Ac*, *Nt-7::Ac*, and *Nt-8::Ac* loci (Figure 3). Wild-type DNA fragments into which *Ac* inserted are indicated.

six *Nt::Ac* lambda clones and used as probes to DNA gel blots of BglII or EcoRI/BglII digested tobacco genomic DNA. Figure 5 shows that the *Nt-1* and *Nt-6* probes hybridized to two tobacco fragments, whereas *Nt-5*, *Nt-7*, and *Nt-8* probes each detected three to five fragments. The *Nt-2* probe detected many fragments, indicating that this probe recognizes repetitive tobacco DNA. All probes, with the exception of *Nt-2*, detected fragments of the size predicted for the target DNA. These fragments are indicated in Figure 5 and are 4.6 kb smaller than the corresponding *Nt::Ac* alleles. (Compare fragment sizes indicated in Figures 3 and 5.)

The genome of *Nicotiana tabacum* is amphidiploid, composed of the two diploid genomes of *N. sylvestris* and *N. tomentosiformis* (Gray et al., 1974). Hence, recessive mutations resulting from insertion of *Ac* into tobacco genes are impossible to detect. However, the effects of *Ac* insertion on transcription of a given locus are possible to assess when *Ac* target probes detect a specific transcript distinguishable, for example, by size from transcripts made from the wild-type loci and other homologous loci. Transcriptional analysis is facilitated when *Ac* target DNA is homologous to low numbers of genomic DNA fragments. *Nt-1* and *Nt-6* probes were chosen to investigate transcription of target tobacco loci as they detect only two DNA fragments in tobacco (Figure 5).

To determine which progenitor *Nicotiana* species gave rise to the two *Nt-1* and *Nt-6* homologous fragments in tobacco (Figure 5, *Nt-1* and *Nt-6*), the *Nt-1* probes A and D (Figures 3 and 6) and the *Nt-6* probe (Figure 3) were hybridized to DNA gel blots of BglII digested *N. tomintosiformis* and *N. sylvestris* DNA. The *Nt-6* probe is homologous to *N. tomentosiformis* but not to *N. sylvestris* DNA (data not shown). The *Nt-1* probes A and D hybridized to single BglII fragments of *N. sylvestris* and *N. tomentosiformis* DNA, and each fragment corresponded in size to one of the two *Nt-1* homologous BglII fragments in *N. tabacum*. Hence, *Nt-1* is unique DNA represented twice in the amphidiploid tobacco (Hehl and Baker, 1989; data not shown). It has been shown previously that *Ac* integrated within the *N. tomentosiformis Nt-1*, 8.5-kb BglII fragment yielding the 13-kb *Nt-1::Ac-18* allele of *Nt-1* (Hehl and Baker, 1989; Figure 5).

To ascertain whether *Nt-1* and *Nt-6* are transcribed regions of tobacco, *Nt-1* probe A (Figure 6, probe A) was hybridized to RNA gel blots of poly(A)$^+$ RNA of *N. tabacum*,

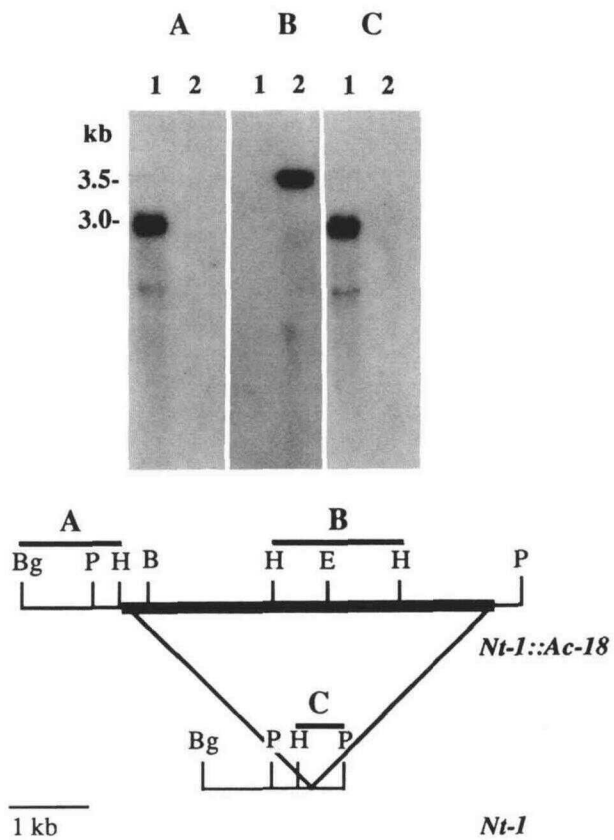

Figure 6. RNA Gel Blot Analysis of *Nt-1::Ac-18*- and *Nt-1*-Specific Transcripts.

RNA gel blot analysis of poly(A)$^+$ RNA isolated from plants homozygous for *Nt-1* (lanes 1) or homozygous for *Nt-1::Ac-18* (lanes 2). *Nt-1* probes (panels A and C) and an *Ac* probe (panel B) are shown above the restriction enzyme maps of *Nt-1::Ac-18* and *Nt-1*. The filled bar, lines, and *Ac-18* insertion site are as in Figure 3. Restriction enzymes are abbreviated as in Figure 3. The sizes of RNA transcripts are indicated in kilobases.

N. sylvestris, and *N. tomentosiformis* and probe *Nt-6* to RNA of *N. tabacum*. *Nt-1* probe A, containing sequences immediately adjacent to the *Ac* target site, detected an RNA transcript in *N. tomentosiformis* and *N. tabacum* but not *N. sylvestris* (Figure 6; data not shown). The *Nt-6* probe failed to detect an RNA transcript in *N. tabacum* (data not shown). *Nt-1* probes A and C were used to investigate the impact of *Ac-18* insertion on transcription of *Nt-1*.

Figure 6 shows RNA gel blots of poly(A)$^+$ RNA from plants homozygous for *Nt-1* or for *Nt-1::Ac-18* that were hybridized to *Nt-1* probes A and C as well as to the *Ac* internal probe B. *Nt-1* A and *Nt-1* C probes detected a 3-kb transcript in RNA isolated from plants homozygous for *Nt-1* (Figure 6, probes A and C, lanes 1) but detected no transcript in RNA of *Nt-1::Ac-18* homozygotes (Figure 6, probes A and C, lanes 2). The *Ac* probe detected a characteristic 3.5-kb mRNA only in the plant harboring *Ac-18* (Figure 6, probe B, lane 2). These results are consistent with the conclusion that *Ac-18* insertion alters transcription of the *Nt-1* locus, and demonstrate that *Ac* has transposed into transcribed tobacco DNA: hence, into a gene.

A Positive Correlation between *Ac* Copy Number and Frequency of *Ds trans*-Activation

Ac activity in maize is subject to control, and increasing numbers of the element cause a delay of transposition during development and an apparent decrease in early transposition frequency. The delay is visualized as a decrease in numbers of large revertant sectors in the endosperm or other tissue where excision is being monitored (McClintock, 1948, 1951). Recently, Jones et al. (1989) reported that the negative *Ac* dosage effect found in maize is not observed in tobacco. The excision frequency of *Ac* from an introduced *SPT::Ac* allele is higher in plants homozygous for *SPT::Ac* than those heterozygous for *SPT::Ac*. These results suggest a positive dosage effect of the *SPT::Ac* allele on the level of variegation (Jones et al., 1989).

To determine whether *Ac* copy number regulates the timing and/or frequency of *Ds trans*-activation in tobacco, plants bearing one, two, or more *Ac*s were crossed to plants harboring *Ds* inserted into the marker gene neomycin phosphotransferase II (NPTII) (Baker et al., 1987). Early and/or frequent *Ac*-induced somatic transposition of *Ds* from NPTII reconstitutes a functional gene and renders F1 seedlings kanamycin resistant, a phenotype readily scored in tobacco (Hehl and Baker, 1989). The frequency of *Ds* excision is measured as the percentage of F1 seedlings, bearing both *Ac* and *Ds*, that germinate and grow in the presence of kanamycin (Hehl and Baker, 1989). The frequency of somatic *Ds trans*-activation was measured to be as high as 90% in F1 progeny derived from crosses between *NPTII::Ds* and *Ac-18* transgenic plants (Hehl and Baker, 1989). The effect of *Ac* copy number on frequency and/or timing of *Ds* excision was assessed in this study by comparing the percentage of kanamycin-resistant F1 derived from crosses between *NPTII::Ds* and *Ac* plants that bear one, two, or more *Ac*s. Genetic segregation and DNA gel blot analysis were performed to evaluate the percent of the F1 progeny that inherited *NPTII::Ds* and *Ac* as well as the number of copies of *Ac* harbored by progeny of each population (see Methods).

The *Ds* parent plant, *Ds-85*, heterozygous for a single *NPTII::Ds* locus and previously characterized with respect to frequency of *Ds trans*-activation by *Ac-18* (Hehl and Baker 1989), was crossed to plant R3-16, homozygous for *Nt-1::Ac-18*, plant R3-17, an identical sibling to R3-3, heterozygous for *Nt-2::Ac*, and to plant R3-5, homozygous for *Ac-18* and heterozygous for two additional elements *Nt-2::Ac* and *Nt-9::Ac* (Figure 1). The proportion of F1 progeny harboring both *Ac* and *Ds* that resulted from R3-16, R3-17, and R3-5 crossed to *Ds-85* was 50%, 25%, and 50%, respectively. The number of *Ac*s transmitted to F1 progeny was determined to be 1 from both R3-16 and R3-17 and approximately 2.5 from R3-5 (see Methods).

Seed obtained from crosses between *Ac* and *NPTII::Ds* plants and from selfings of each parent were germinated on media containing 400 μg/mL kanamycin (Methods). The results of the F1 kanamycin selection are displayed in Table 2. No kanamycin-resistant, selfed offspring were obtained from plants harboring only *Ac* or *NPTII::Ds* (Table 2, R3-5/self and *Ds-85*/self). *Nt-1::Ac-18* and *Nt-2::Ac* independently *trans*-activated *Ds* to excise at frequencies of 14% and 4%, respectively, when F1 seed was propagated on 400 μg/mL kanamycin (Table 2, R3-16 × *Ds-85* and R3-17 × *Ds-85*). The proportion of kanamycin-resistant F1 plants on 400 μg/mL kanamycin increased significantly to 78% when at least two copies of *Ac trans*-activated *Ds* (Table 2, R3-5 × *Ds-85*). This result is consistent with the notion that higher copy numbers of *Ac* lead to earlier or more frequent *trans*-activation of *Ds*. Similar high *trans*-activation frequencies were also obtained using two *Ac* parent plants derived by selfing of plant R2-8 (see Figure 1, R3-11 to 15). These *Ac* plants (R3-18 and 19, Table 1) did not contain the stable *Nt-1::Ac-18* but harbored more than two independently segregating elements, as judged from the results of *Ac* probe hybridization to DNA gel blots of R2-8 parent and progeny DNAs (see Figure 1, R3-11 to 15 and data not shown). The frequency of kanamycin-resistant offspring in crosses between these multicopy *Ac* plants and the *NPTII::Ds* plant was approximately 86% on 400 μg/mL kanamycin (Table 2, R3-18, R3-19 × *Ds-85*). This result indicates that the positive correlation between *Ac* copy number and *Ds* transposition frequency in tobacco is not only a feature of the stable *Nt-1::Ac-18* in conjunction with *Nt-2::Ac* and/or *Nt-9::Ac* but can be generalized to include the numerous active elements found among progeny of plant R2-8.

Table 2. Percentage of Kanamycin-Resistant Offspring Plants from Crosses between *Ac* and *NPTII::Ds* Plants

Ac and *Ds* Parent Plants	Total No. of Offspring Germinated	No. of Offspring Harboring *Ac* and/or *Ds*	No. of kn^{res} Offspring	% of kn^{res} Offspring
R3-5/self	195	195	–	–
Ds-85/self	197	148	–	–
R3-16 × *Ds*-85	1229	615	89	14%
R3-17 × *Ds*-85	489	122	5	4%
R3-5 × *Ds*-85	1027	514	402	78%
R3-18,R3-19 × *Ds*-85	564	282	247	86%

DISCUSSION

Ac was shown previously to transpose from an integrated T-DNA vector in primary transgenic tobacco, tomato, *Arabidopsis*, carrot, and potato (Baker et al., 1986, 1987; Van Sluys et al., 1987; Knapp et al., 1988; Yoder et al., 1988). *Ac* remains active and continues to transpose from the T-DNA vector in one subsequent generation of tobacco and two subsequent generations of tomato and *Arabidopsis* (Belzile et al., 1989; Schmidt and Willmitzer, 1989; Taylor et al., 1989). In tomato, approximately 30% of progeny of primary transformants inherit a transposed *Ac* from their parent. Transposed *Ac* continues to transpose for at least three generations of tomato (Belzile et al., 1989). Assessment of *Ac* transposition in progeny of transgenic tobacco and tomato and *Arabidopsis* has relied on the appearance of new *Ac*-homologous DNA fragments in DNA gel blots of genomic DNA isolated from primary transgenics and their progeny. Evidence that new *Ac*-homologous DNA fragments represent transposed *Ac* in progeny plants is provided by the work presented here. Several *Ac*-homologous DNAs were cloned from tobacco progeny, restriction enzyme mapped, and partially sequenced. We show that transposed *Ac*s in a selfed R1 progeny are structurally intact and reside at different chromosomal locations. Furthermore, we demonstrate that *Ac* transposes into low copy and transcribed DNA and that increased copies of *Ac* correlate with an increased frequency of *Ds trans*-activation.

Partial sequence analysis of two transposed *Ac*s revealed that at least 100 bp at the *Ac* termini are conserved upon integration. These 100 bp are part of sequences required in *cis* for transposition (Coupland et al., 1988, 1989). It may be inferred that many of the elements observed in the five progeny generations have conserved terminal sequences because many new transpositions are scored in each generation.

Conservation of *trans*-acting functions of *Ac*, demonstrated by continued transposition of *Ac* for five generations, was verified independently by efficient *trans*-activation of *Ds* transposition in F1 progeny obtained by crossing R3 plants carrying one or more *Ac*s with plants harboring *NPTII::Ds*.

A characteristic feature of *Ac* transposition in maize is the induction of an 8-bp duplication of target sequences upon integration (Müller-Neumann et al., 1984; Pohlman et al., 1984). Here it is shown that *Ac* induces an 8-bp duplication of target sequences upon integration in tobacco, which demonstrates that the mechanism of *Ac* integration in tobacco and maize is similar. A similar result was obtained for the maize *Spm/En* element that induces a 3-bp duplication of target sequences in maize, tobacco, and potato (Frey et al., 1989; Masson and Fedoroff, 1989; Pereira and Saedler, 1989).

In addition to a description of the properties of *Ac* transposition, this study provides assessment of the utility and effectiveness of *Ac* and *Ds* as genetic tools in heterologous species. The frequency of sustained germinal transposition and the complexity of chromosomal DNA into which *Ac* integrates bear directly on the suitability of *Ac* for mutagenesis and chromosome mapping. Germinal excision of *Ac* in maize is usually accompanied by integration of the element elsewhere in the genome, often to genetically linked sites (Greenblatt, 1984). This study reports the proportion of plants harboring germinally transposed *Ac*s integrated at different chromosomal locations throughout five generations. The proportion of selfed or out-crossed progeny harboring newly transposed *Ac* elements varied from 23% to 83% when parents carried at least two *Ac*s. The lack of germinal transposition activity of *Nt-2::Ac*, observed in progeny of plant R3-17 carrying this element alone, may result from an inherent low transposition frequency of a single element, its residence in repetitive DNA, and/or a mutation that specifically alters its activity. Genetic transmission of four specific elements, *Nt-2, 3, 4*, and *9::Ac*, to sibling progeny demonstrated the germinal nature of the different integration events described in Figure 1 and summarized in Table 1. The frequency of germinal transposition of an *Ac* derivative from a single T-DNA locus, *SPT*, was reported to be 1% to 9% with integration accompanying germinal excision in approximately half of the progeny examined (Jones et al., 1989). Although our data regarding the frequency of germinal *Ac* integration cannot be directly compared with the frequency of germinal excision in maize, estimated to be 1% to 17% (Greenblatt, 1968), or that of an *Ac* derivative

in a single tobacco generation, the percentages of progeny harboring new *Ac*s reported here signify that *Ac* transposes in a germinal fashion at frequencies sufficient to generate numerous independent insertions. The frequency of *Ac* transposition reported here (23% to 83%) appears to be higher than that reported previously in tobacco (1% to 9%; Jones et al., 1989) and *Arabidopsis* (0.2% to 0.5%; Schmidt and Willmitzer, 1989) and appears to be similar to that reported in tomato (Belzile et al., 1989). The lower frequency of *Ac* transposition in tobacco reported previously, 1% to 9% (Jones et al., 1989), may have resulted from the in vitro alteration introduced into 3′ subterminal sequences of *Ac* affecting sequences required in *cis* for transposition (Jones et al., 1990). The low frequency of *Ac* transposition in *Arabidopsis* compared with tobacco seeks molecular explanation. Host factors required for *Ac* transposition may vary significantly between tobacco and *Arabidopsis*, and/or the level of *Ac*-transposase expression may differ between the two species.

In maize, *Ac* often transposes to genetically active and hypomethylated regions of the genome (Chen et al., 1987). In this report, six elements and flanking genomic DNA of tobacco were characterized, and all but one was shown to be integrated into or adjacent to low copy or unique DNA. To assess the possibility of *Ac* transposition into a gene, genomic sequences flanking two transposed *Ac*s, *Nt-6::Ac* and *Nt-1:: Ac-18*, were examined. The *Nt-1* locus was shown to be transcribed and transcription was found to be dramatically altered when *Ac-18* was integrated at *Nt-1*, suggesting that *Ac* transposes into a gene.

Methylation and dosage, properties known to correlate with negative regulation of *Ac* activity in maize, were not detected in tobacco. Reversible inactivation of *Ac* activity in maize is correlated with methylation of element sequences in subterminal regions and the absence of detectable steady-state levels of the *Ac* transcript (Schwartz and Dennis, 1986; Chomet et al., 1987; Kunze et al., 1988). Cycling *Ac*s in maize arise rarely de novo in genetic stocks harboring mutable *Ac* alleles and are recognized in kernels as loss and recovery of a visually scored mutable phenotype (Schwartz and Dennis, 1986; Chomet et al., 1987; S.L. Dellaporta, personal communication). Recently, Taylor et al. (1989) showed that *Ac* elements in four different R0 tobacco transformants are unmethylated. To assess the methylation of many transposed *Ac*s and to determine whether methylation of *Ac* occurs gradually in subsequent generations, we analyzed methylation of subterminal regions of transposed *Ac* in plants from four successive tobacco generations. The absence of detectable methylation of terminal domain sequences of numerous *Ac*s in 29 transgenic plants correlates with its continued activity and suggests that, unlike the Tam3 element of *Antirrhinum majus*, *Ac* does not have the tendency to become methylated when introduced into tobacco (Martin et al., 1989).

McClintock observed that increasing copies of *Ac* cause a delay in transposition during development of the maize endosperm and other organs of the maize plant (McClintock, 1948, 1951). The positive correlation between *Ac* copy number and frequency and/or timing of *Ds* transposition observed here for tobacco represents a significant difference in the regulation of transposition of *Ac* between the two species. Our results on the dosage effect of *Ac* in tobacco were obtained by examining the effect of increasing *Ac* copy numbers on *Ds trans*-activation frequency and are consistent with those of Jones (Jones et al., 1989), who reported a positive effect on excision frequency of *Ac* from an *SPT* gene when two loci versus one T-DNA locus bearing *SPT::Ac* were present in transgenic tobacco. The positive *Ac* dosage in tobacco was observed with either an in vitro modified *Ac* (Jones et al., 1989, 1990) or here with an *Ac* that has sustained a 4-bp terminal deletion, *Nt-1::Ac-18*. We also demonstrated that the positive effect of increased copy number on *Ds trans*-activation is observed with plants bearing "wild-type" *Ac*.

The molecular basis of regulation of transposition in maize is unknown and explanations for the absence of inhibition of early and/or frequent transposition in tobacco by increasing copies of *Ac* is speculative. The positive effect of *Ac* copy number on timing and/or frequency of transposition observed here in tobacco could be accounted for by one or more of the following: (1) the absence or lack of specificity of dosage-mediating factors in tobacco, (2) the presence of tobacco factors that suppress action of dosage-mediating factors, or (3) a difference in expression, subsequent processing, or modification of *Ac* products that normally mediate dosage.

The capacity of *Ac* and *Ds* to function as insertional mutagens and as mobile chromosome markers in heterologous species is documented by this work. Further assessment of the utility of *Ac* and *Ds* is best accomplished using genetically and physically mapped diploid plant species in which the properties ascribed here to *Ac* in tobacco have also been demonstrated.

METHODS

Transgenic Plants

Transformed *Nicotiana tabacum* cv SRI callus, harboring the pTI*Ac* construct and from which plant R0-1 was regenerated, was described earlier (Baker et al., 1986). The plant *Ds-85*, harboring *NPTII::Ds*, originated from leaf disc transformation of *N. tabacum* cv Samsun NN with pGV3850 HPT::pKU4 (Baker et al., 1987). Leaf disc transformations were performed according to Horsch et al. (1985) using a modified procedure (Hehl and Baker, 1989).

Isolation of Genomic DNA

Genomic plant DNA was prepared as reported by Bernatzky and Tanksley (1986) using a modified procedure (S. Tanksley, per-

sonal communication): 2 to 10 g of leaf tissue was frozen in liquid nitrogen, homogenized, and transferred to 50-mL centrifuge tubes. After addition of 20 mL extraction buffer (0.35 M Sorbitol, 0.1 M Tris, 5 mM EDTA, 20 mM Na-bisulfite, pH 8.0), the tissue was thawed and centrifuged at 3500 rpm in a Beckman TJ-6 table-top centrifuge. The supernatant was discarded and the pellet was resuspended in 2 mL extraction buffer. After addition of 2 mL nuclear lysis buffer (20 mM Tris, 50 mM EDTA, 2 M NaCl, 2% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide, pH 7.5) and 0.8 mL 5% sarcosyl, the suspension was incubated for 20 min at 65°C. The lysate was extracted once with 7.5 mL chloroform-isoamyl-alcohol (24:1 v/v) and nucleic acids in the aqueous phase were precipitated with isopropylalcohol. Nucleic acids were recovered by centrifugation and resuspended in TE buffer. Genomic DNA was further purified on CsCl-ethidium bromide gradients (Maniatis et al., 1982).

DNA and RNA Gel Blot Analysis

Digested genomic DNA (8 μg to 10 μg) was electrophoresed on agarose gels (0.8%) and transferred to nitrocellulose (Southern, 1975; Meinkoth and Wahl, 1984). Hybridization was carried out as described (Hughes et al., 1978) for 24 hrs followed by a 2 × 15 min wash in 0.1 × SSC, 0.1% SDS at 50°C. DNA fragments were radioactively labeled (Feinberg and Vogelstein, 1983) using a commercially available kit (Amersham).

Preparation and purification of poly(A)$^+$ RNA were performed as described (Schwarz-Sommer et al., 1984). Four μg of poly(A)$^+$ RNA was fractionated on 0.8% or 1.2% agarose gels containing formaldehyde. After electrophoresis the RNA was transferred to nitrocellulose and hybridized using those conditions described for DNA gel blot hybridizations. To verify that similar amounts of poly(A)$^+$ RNA were loaded in each lane of the gel, filters were rehybridized to a soybean actin probe (kindly provided by R. Meagher). Following the hybridization with the actin probe, filters were washed in 2 × SSC, 0.1% SDS for 2 × 15 min at 50°C.

Cloning of Genomic Plant DNA

Cloning of the *Nt-1::Ac-18* and *Nt-1* alleles from plant R1-4 (Figure 1) has been described (Hehl and Baker, 1989). To isolate new *Ac* clones, the same recombinant library used for isolation of *Nt-1::Ac-18* and *Nt-1* was differentially hybridized to the 1.6-kb *Ac* internal HindIII fragment (Figure 1) and a 2.6-kb PstI/BglII fragment specific for *Nt-1::Ac-18* and *Nt-1* (probe D, Figure 3). Four clones were isolated that hybridized only to the *Ac* specific probe (*Nt-5::Ac*, *Nt-6::Ac*, *Nt-7::Ac*, and *Nt-8::Ac*). The target DNA fragment of *Ac* at *Nt-6::Ac* was cloned from the same BglII library using a DNA fragment adjacent to *Ac* from clone *Nt-6::Ac* as a probe (Figure 3). Two clones were identified to harbor the 2.4-kb target fragment of *Ac* at *Nt-6::Ac* (Figures 3 and 5).

Nt-2::Ac was isolated from a BglII genomic library of plant R2-3 (Figure 1). BglII digested DNA from plant R2-3 was cloned into the BamHI site of EMBL4 (Frischauf et al., 1983) according to standard procedures (Maniatis et al., 1982). *Nt-2::Ac* and *Nt-1::Ac-18* recombinant phage were distinguished as described above. Two *Nt-2::Ac* phage clones with characteristic 3.9-kb and 2.2-kb *Ac* fragments were identified (Figures 1 and 3). *Ac*-containing fragments were subcloned into pUC19 (Yanisch-Perron et al., 1985) and subjected to restriction enzyme analysis as well as DNA gel blot hybridizations according to standard procedures (Maniatis et al., 1982).

DNA Sequence Analysis

Sequence analysis of DNA fragments was performed according to Sanger et al. (1977). Appropriate DNA fragments harboring 180-bp 5′ and 100-bp 3′ terminal *Ac* sequences of *Nt-6::Ac* and *Nt-7::Ac* as well as approximately 250-bp flanking tobacco DNA were subcloned into M13mp18 and mp19 (Norrander et al., 1983; Yanisch-Perron et al., 1985). To sequence the target site of *Ac* at *Nt-6::Ac*, a 246-bp HindIII/PstI fragment from the 2.4-kb EcoRI/BglII target fragment (Figures 3 and 5) was subcloned into M13mp18 and mp19. Sequence analysis was performed using a commercially available sequencing kit (Amersham).

Segregation Analysis

The genotypes with respect to *Ac* or *Ds* of transgenic tobacco plants R3-16, R3-17, and R3-5 (*Ac*) and *Ds-85* (*Ds*) were determined as follows:

The *Ac* parent plants R3-16, R3-17, and R3-5 (Figure 1), used in crosses to *Ds-85*, were shown to harbor either *Nt-1::Ac-18* (R3-16; Table 1), *Nt-2::Ac* (R3-17; Table 1), or *Nt-1::Ac-18*, *Nt-2::Ac*, and *Nt-9::Ac* (R3-5; Figure 1, Table 1) by DNA gel blot hybridization. To determine whether plants R3-16 and R3-5 were homozygous or heterozygous for *Nt-1::Ac-18*, a unique DNA fragment adjacent to *Ac-18* at *Nt-1::Ac-18* was used as a probe in DNA gel blot hybridizations of BglII digested DNA isolated from both plants. This probe distinguishes *Nt-1::Ac-18* homozygotes from heterozygotes (Hehl and Baker, 1989), and in both plants only the 13-kb *Nt-1::Ac-18* fragment (Figure 3) was detected, indicating that both plants were homozygous for *Nt-1::Ac-18* (data not shown). The homozygous or heterozygous state of the *Nt-2::Ac* allele in R3-17 and that of *Nt-2::Ac* and *Nt-9::Ac* in R3-5 were determined by DNA gel blot hybridization of DNA isolated from R-4, F1 progeny produced by *Ds-85* and R3-17 or R3-5 crosses. F1 progeny were propagated under nonselective conditions and hybridization performed using the internal *Ac* HindIII fragment probe. The results, summarized in Table 1, showed that among 29 offspring plants of R3-17, 16 harbored *Nt-2::Ac*. It was concluded that plant R3-17 is heterozygous for *Nt-2::Ac*. Similar results were obtained for R3-5. Among 25 R3-5 offspring, 13 harbored *Nt-2::Ac*, indicating that R3-5 is heterozygous for *Nt-2::Ac* (Table 1). The *Nt-9::Ac* allele of R3-5 was germinally transmitted to six out of 25 F1 offspring, suggesting that R3-5 is heterozygous for *Nt-9::Ac* (Table 1). Among the 25 F1, R3-5, offspring analyzed, 17 showed new *Ac* homologous fragments not present in the R3-5 progenitor (Table 1). No more than four *Ac* elements were detected in any one F1 progeny of R3-5. The approximate copy number of *Ac* per plant was determined by dividing the total number of *Ac* elements detected in all 25 F1 plants by 25. The approximate copy number of *Ac* was determined to be 2.5 *Ac* elements per plant. Plant *Ds-85* was found to be heterozygous for a single *NPTII::Ds* locus by genetic segregation and analysis of a T-DNA encoded gene product, nopaline synthase (Aerts et al., 1979), among progeny as described earlier (Hehl and Baker, 1989).

Selection of Kanamycin-Resistant F1 Offspring

F1 seeds obtained from crosses between *Ds-85* and *Ac* bearing plants and seed from selfings of *Ds-85* and R3-5 were germinated directly on MS media and MS media containing 400 μg/mL kanamycin (Hehl and Baker, 1989). At least 95% of all seeds germinated on nonselective media. Kanamycin-resistant F1 plants were scored 4 weeks after germination and incubation at 26°C. The total number of seeds germinated on 400 μg/mL kanamycin is summarized in Table 2. All seeds were collected from at least two independent crosses.

ACKNOWLEDGMENTS

We thank Jeff Schell and Nina Fedoroff for inspiring this work; Candace Waddell and Brian Osborne for critical discussions and review of the manuscript. This research was supported by U.S. Department of Agriculture CRIS No. 5335-22230-002-00D.

Received April 19, 1990; revised June 5, 1990.

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