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

Reinhard Hehl* and Barbara Baker*

* Plant Gene Expression Center, United States Department of Agriculture, 800 Buchanan Street, Albany, California 94710
* Department of Plant Pathology, University of California, Berkeley, California 94720

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 and decrease 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) (McC1intock, 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 transposes 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 (McC1intock, 1964, 1965). This inactivation can be correlated with methylation of particular internal Pvull and HpaI 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 T-DNA vectors into tobacco. This work showed that 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 transposition in tobacco generates a 3.5-kb transcript with transcription start sites similar to those described for maize (Kunze et al., 1987; Finnegnan 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-
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 transactivatesDs transposition in tobacco, tomato, and Arabidopsis (Hehl and Baker, 1989; Lassner et al., 1988; 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 transactivation 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 tran-activation by resident or introduced Ac (Baker et al., 1986, 1987; Coupland et al., 1986; 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; Pohlan 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 pTiAc construct (Baker et al., 1988). 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 continuos Ac transposition. The parent plant R2-6 harbored two readily discernible Ac's: 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

<table>
<thead>
<tr>
<th>Progenitor Plant</th>
<th>Ac Alleles in Progenitor</th>
<th>No. of Offspring Plants Analyzed</th>
<th>No. of Offspring with Ac Alleles from Progenitor</th>
<th>No. of Offspring Plants Harboring New Ac</th>
<th>% Offspring Plants Harboring New Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>R0-1</td>
<td>Nt-1::Ac-18</td>
<td>4</td>
<td>4 - Nt-1::Ac-18</td>
<td>2</td>
<td>50%</td>
</tr>
<tr>
<td>R1-4</td>
<td>Nt-1::Ac-18</td>
<td>4</td>
<td>10 - Nt-1::Ac-18</td>
<td>8</td>
<td>83%</td>
</tr>
<tr>
<td>R2-3</td>
<td>Nt-3::Ac</td>
<td>35</td>
<td>7 - Nt-1::Ac-18</td>
<td>23%</td>
<td></td>
</tr>
<tr>
<td>R2-6</td>
<td>Nt-3::Ac</td>
<td>8</td>
<td>4 - Nt-3::Ac</td>
<td>63%</td>
<td></td>
</tr>
<tr>
<td>R2-8</td>
<td>Nt-4::Ac</td>
<td>2</td>
<td>2 - Nt-4::Ac</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>R3-5</td>
<td>Nt-1::Ac-18</td>
<td>25</td>
<td>10 - Nt-1::Ac-18</td>
<td>8</td>
<td>80%</td>
</tr>
<tr>
<td>R3-16</td>
<td>Nt-2::Ac</td>
<td>13</td>
<td>8 - Nt-1::Ac-18</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>R3-17</td>
<td>Nt-2::Ac</td>
<td>16</td>
<td>8 - Nt-1::Ac-18</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>R3-18,R3-19</td>
<td>Nt-2::Ac</td>
<td>2</td>
<td>8 - Nt-1::Ac-18</td>
<td>68%</td>
<td></td>
</tr>
</tbody>
</table>

* 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.

* One progeny of R2-6 harbored only newly transposed Ac.

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 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 were detected in 23% to 83% of the progeny obtained by selfing or outcrossing of progenitors with at least two Ac (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, 1988; 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 Pvull 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 internally, while inactive elements are resistant to cleavage and larger internally homologous Ac fragments are generated (Schwartz and Dennis, 1988; Chomet et al., 1987). The enzyme Pvull digests unmethylated Ac 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; Pohli, 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 Pvull and or HpalI 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 HpalI 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 HpalI fragment was detected exclusively in these DNAs and indicates that germinally transmitted as well as newly transposed AcS in the R2 generation remain unmethylated at internal HpalI restriction sites. DNA of three R3 plants harboring new and germinally transmitted AcS 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 Pvull or HpalI. In addition, detection of the internal 3.4-kb HpalI Ac fragment among the 25 individuals examined with this enzyme strongly suggests that none of the detectable AcS present in these plants has undergone major structural alterations of internal sequences.

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; Fidoroff, 1989). These properties of Ac transposition were examined in tobacco to determine whether or not 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 AcS 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-
(see below). It is concluded that these Acs 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, R1-1 and R2-3, R3-1 to 2; 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

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

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.

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.
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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 Ni-1::Ac-18, Ni-2::Ac, Ni-5::Ac, Ni-6::Ac, Ni-7::Ac, and Ni-8::Ac loci (Figure 3). Wild-type DNA fragments into which Ac inserted are indicated.

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

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. Ni-1 and Ni-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 Ni-1 and Ni-6 homologous fragments in tobacco (Figure 5, Ni-1 and Ni-6), the Ni-1 probes A and D (Figures 3 and 6) and the Ni-6 probe (Figure 3) were hybridized to DNA gel blots of BglII digested N. tomentosiformis and N. sylvestris DNA. The Ni-6 probe is homologous to N. tomentosiformis but not to N. sylvestris DNA (data not shown). The Ni-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 Ni-1 homologous BglII fragments in N. tabacum. Hence, Ni-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 Ni-1, 8.5-kb BglII fragment yielding the 13-kb Ni-1::Ac-18 allele of Ni-1 (Hehl and Baker, 1989; Figure 5).

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

RNA gel blot analysis of poly(A)+ RNA isolated from plants homozygous for Ni-1 (lanes 1) or homozygous for Ni-1::Ac-18 (lanes 2). Ni-1 probes (panels A and C) and an Ac probe (panel B) are shown above the restriction enzyme maps of Ni-1::Ac-18 and Ni-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 (McCintock, 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 variation (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 AcS 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 AcS. 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 AcS 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

<table>
<thead>
<tr>
<th>Ac and Ds Parent Plants</th>
<th>Total No. of Offspring Germinated</th>
<th>No. of Offspring Harboring Ac and/or Ds</th>
<th>No. of km(^{m}) Offspring</th>
<th>% of km(^{m}) Offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>R3-5/self</td>
<td>195</td>
<td>195</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ds-85/self</td>
<td>197</td>
<td>148</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R3-16 × Ds-85</td>
<td>1229</td>
<td>615</td>
<td>89</td>
<td>14%</td>
</tr>
<tr>
<td>R3-17 × Ds-85</td>
<td>489</td>
<td>122</td>
<td>5</td>
<td>4%</td>
</tr>
<tr>
<td>R3-5 × Ds-85</td>
<td>1027</td>
<td>514</td>
<td>402</td>
<td>78%</td>
</tr>
<tr>
<td>R3-18, R3-19 × Ds-85</td>
<td>564</td>
<td>282</td>
<td>247</td>
<td>86%</td>
</tr>
</tbody>
</table>

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 Sluyts 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 showed that transposed Ac 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 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 Smpt/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 transposes in a germlinal 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 (Belize 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-1B, were examined. The Nt-1 locus was shown to be transcribed and transcription was found to be dramatically altered when Ac-1B 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).

McClandt observed that increasing copies of Ac cause a delay in transposition during development of the maize endosperm and other organs of the maize plant (McClandt, 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-1B. 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 pTIAc construct and from which plant R0-1 was regenerated, was described earlier (Baker et al., 1986). The plant Ds-85, harboring pNTIII::Ds, originated from leaf disc transformation of N. tabacum cv Samsun NN with pGV3850 HPT::PKU3 (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 0.6 mL 5% sarcosyl, the suspension was incubated for 20 min at 65°C. The lysate was extracted once with 7.5 mL chloroform-isooamyl alcohol (24:1 v/v) and nucleic acids in the aqueous phase were precipitated with isopropyl alcohol. 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 (6 μg to 10 μg) was electrophoresed on agarose gels (0.8%) and transferred to nitrocellulose (Southern, 1975; Maniatis and Wahl, 1984). Hybridization was carried out as described (Hughes et al., 1978) for 24 hrs followed by a 2 x 15 min wash in 0.1 x 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 hybridization. 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 x SSC, 0.1% SDS for 2 x 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 BamH1 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 (Norrlander 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 and 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 R4-4, 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 NPTIII::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 settings of Ds-85 and RS-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.

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