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Rapid Proliferation of the Maize Transposable Element *Activator* in Transgenic Tomato

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We have found that the maize transposable element *Activator* (*Ac*) can rapidly proliferate when transformed into tomato plants. The fate of transposed *Ac* elements in self-pollinated progeny of independent transgenic tomato plants was examined by DNA gel blot hybridizations. When a single copy of *Ac* was introduced into a transformant, the number of copies usually remained low in subsequent generations. In one lineage, however, the number of *Ac* elements increased from one to more than 15 copies in only two generations. DNA gel blot analyses indicated that the amplified elements were not grossly rearranged. Amplified copies of *Ac* resided at unique sites in the genome, and segregation analysis indicated that these sites were not tightly linked at one genetic locus. Taken together, these observations indicate that the mechanism of *Ac* amplification is associated with transposition.

INTRODUCTION

In plant species, endogenous transposable elements are generally present as multicopy families. In maize and *Antirrhinum*, sequences that hybridize to endogenous transposable element probes are present at between 10 and 100 copies per genome (Geiser et al., 1982; Upadhyaya et al., 1985). The maintenance of transposable elements at high genomic copy numbers is attributed to a balance of transpositional increases and losses, copy number-dependent regulation, and selection pressures (Schwarz-Sommer et al., 1985; Syvanen, 1986; Charlesworth and Langley, 1989). The generation of high copy number families, however, is difficult to explain. If transposable elements increase in copy number as a result of transposition, minor selection pressures would eliminate the elements faster than they could duplicate, given reasonable transposition rates (Charlesworth, 1985). Charlesworth argues that for transposable elements to reach the copy number maintained by *Drosophila* P elements, transposition rates and the resulting copy number increases would need to be unusually high soon after the element invaded a genome. In later generations, copy number-dependent autoregulation mechanisms would then act to decrease transposition rates to the levels currently measured (Charlesworth, 1985). Substantial evidence has accumulated for such autoregulation of transposition in maize (McClintock, 1951), *Drosophila* (Bregliano and Kidwell, 1983), and prokaryotes (Kleckner, 1989).

A rapid increase in the genomic copy number of transposable elements has been observed in a few cases. When single *Drosophila* P elements are transformed into M cytotype strains, the number of P elements remains low for a number of generations and then suddenly increases to 20 or more copies in a single generation. The timing of the sudden increase varies, however, among the different transformed lines (Daniels et al., 1987; Scavarda and Hartl, 1987; Preston and Engels, 1989). It can be more difficult to observe the accumulation of transposable elements in plants because of the numerous transposable element-like sequences present in their genomes. In maize, the copy number of the transposable element *Mu1* in hybrids made between high and low copy parents rapidly increases to match the levels present in the high copy parent, suggesting that this element can duplicate rapidly (Alleman and Freeling, 1986). When similar experiments were conducted with *Mutator* lines that had lost their high mutation rates, the copy number decreased upon out-crossing. This suggests that copy number maintenance is linked to *Mu* activity (Alleman and Freeling, 1986; Walbot and Warren, 1988). The spontaneous increase in *Mu1* copy number levels was also observed in a few self-pollinated maize lines that were originally selected for containing a low number of *Mu1* elements; after a rapid increase in copy number to about nine to 20 copies, their copy number was maintained in subsequent generations (Hardeman and Chandler, 1989).

The discovery that the maize transposable element *Activator* (*Ac*) remains active when introduced into unrelated plant species allows one to examine the behavior of this element in virgin plant genomes (Baker et al., 1986; Van Sluys et al., 1987; Yoder et al., 1988). DNA isolated from one transformed tobacco callus hybridized more intensely to *Ac* probes than it did to probes specific for the transformed vector (Baker et al., 1986). The authors suggested that additional copies of *Ac* accumulated because *Ac*

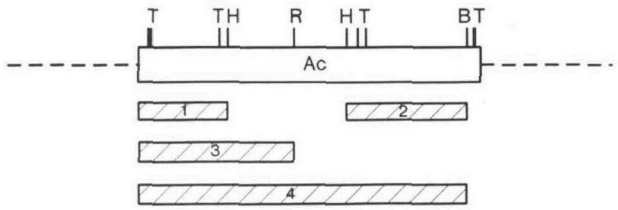

Figure 1. *Restriction Map of Ac Probes.*

The restriction map of the *Ac* element after integration into the tomato genome is given. T, TaqI; H, HindIII; R, EcoRI; B, BamHI. The shaded boxes beneath the map represent the four *Ac*-specific probes used in these experiments.

transposed into unreplicated DNA. However, because the DNA was isolated directly from transformed calli in these experiments, it is difficult to distinguish amplification events that are associated with transformation from those resulting from transposition.

To examine *Ac* behavior in a plant species that is devoid of *Ac*-hybridizing sequences, we transformed the *Ac* element into tomato. Previously, we demonstrated that *Ac* is active during the somatic development of tomato plants (Yoder et al., 1988). While studying the frequencies at which transposed *Ac*s are sexually transmitted, we found that in most plants with a single *Ac* element the copy numbers remain low in subsequent generations (Belzile et al., 1989). In one family, however, a single plant was identified in which *Ac* amplified from one to about eight copies. This was particularly intriguing because its parent contained only one copy of *Ac*. DNA gel blot analyses indicated that the amplified copies resided at unique sites in the genome and were not significantly rearranged. By examining the range and distribution of *Ac*s in R2 sibling plants, we determined that the copy numbers continued to increase in later generations as a result of both new transpositions and segregation of the preexisting insertions. These observations support the model that amplification of *Ac* is associated with transposition.

RESULTS

A Single Copy of pMAC Integrated into the Parent R0 Plant

The *Ac*-containing T-DNA vector pMAC was introduced into the tomato cultivar VF36, and mature plants were regenerated. These primary R0 transformants were self-pollinated, and their R1 seed was collected and sown. The progeny of one primary transformant, T15-01, segregated for an aberrant leaf morphology resulting in rounded leaflets; the phenotype and segregation behavior of this mutation have been described elsewhere (Yoder, 1990).

Because an interesting leaf-shape mutation was segregating in the R1 progeny of T15-01, we were interested in further characterizing the T-DNA integration event. DNA gel blot analysis of the R0 parent using T-DNA border-specific probes gave a pattern consistent with a single copy of pMAC being integrated into the genome of the R0 parent (data not shown). When a pMAC sequence internal to the T-DNA borders was used as a probe on a filter containing DNA isolated from R1 individuals, 13 out of 17 progeny contained pMAC sequences, a segregation ratio consistent with a single insertion locus (Belzile et al., 1989). Finally, when *Ac* was used as a probe on the same population, *Ac* sequences segregated in the R1 progeny as if a single copy of *Ac* were present in the primary transformant (see below). Therefore, a number of lines of evidence indicated that one copy of the donor pMAC construction integrated in the parent transformant T15-01. There was no evidence, however, that either the T-DNA or any particular *Ac* insertion segregated with the mutant phenotype (Belzile et al., 1989).

Segregation of Transposed *Ac*s in R1 Progeny

DNA was isolated from 17 R1 individuals of T15-01, digested with HindIII, transferred to a nylon membrane, and probed with the *Ac*-specific probe 1, diagrammed in Figure 1. This probe hybridizes to one end of *Ac* and produces a

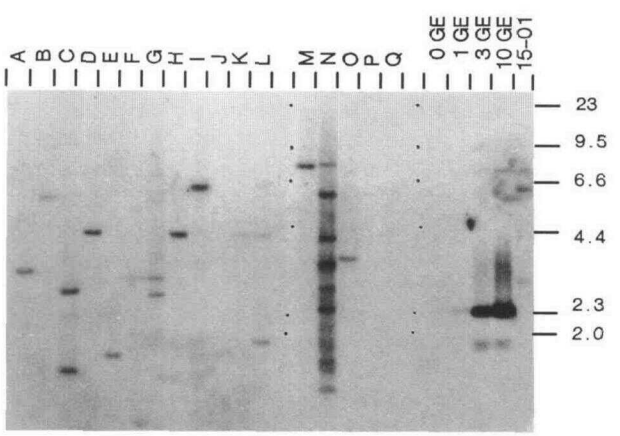

Figure 2. DNA Gel Blot Hybridization of Primary Transformant T15-01 and Its R1 Progeny.

Genomic DNA was isolated from leaves of 17 progeny obtained by selfing the primary tomato transformant T15-01. DNA was digested with HindIII and hybridized with ^{32}P-labeled *Ac*-specific probe 1 (diagrammed in Figure 1). Lanes containing DNA from the 17 R1 progeny are labeled A through Q, the lane containing DNA from the primary transformant is labeled 15-01. The four reconstruction lanes labeled 0 GE, 1 GE, 3 GE, and 10 GE contain, respectively, 0, 1, 3, and 10 genomic equivalents of pMAC mixed with VF36 DNA.

single, unique band of an unpredictable size for each transposed element. If *Ac* did not excise from the T-DNA, probing with this fragment would result in a single 2.4-kb band composed of one end of *Ac* and flanking T-DNA sequences (Belzile et al., 1989). As seen in Figure 2, there was no evidence of the 2.4-kb fragment in either the parental DNA or in any of the siblings, suggesting that *Ac* transposed early in the development of the primary transformant.

Because the primary transformant T15-01 was hemizygous for the pMAC insertion, R1 progeny should contain either zero, one, or two copies of *Ac*. *Ac* copy numbers were estimated by counting the number of *Ac*-hybridizing bands that corresponded to independent insertion sites (Nevers et al., 1986; Bennetzen et al., 1987). Of the 17 progeny examined, 13 had either one or two *Ac* insertion bands and three had none, which was consistent with the expected segregation of a single locus. Surprisingly, however, one of the progeny, plant N, had seven to 10 *Ac* insertion bands (Figure 2). Because each of the bands was of roughly the same intensity, it is likely that a single *Ac* element was present at each insertion site.

To verify that the plant with increased *Ac* copy number actually originated from T15-01 and not from an unrelated transformant, the blot was stripped of the *Ac* probe and then hybridized with a 2.1-kb fragment specific for the right border of an integrated T-DNA (Belzile et al., 1989). The same junction fragment corresponding to a single T-DNA insertion was seen in all the siblings, including the high copy plant N. This indicated that all the siblings were derived from the same primary transformant (data not shown).

Amplified Copies of *Ac* Are not Significantly Rearranged

In maize, most of the bands that hybridize to *Ac* sequences are defective derivatives of *Ac* (Fedoroff et al., 1983). To determine whether the multiple *Ac*-hybridizing sequences in plant N were internal deletions or rearrangements of *Ac*, genomic DNA from plant N was digested with Taq1 and hybridized with the *Ac*-specific probe 4. Taq1 cleaves *Ac* into three diagnostic fragments covering almost the entire element (Figure 1). In the analysis shown in Figure 3, plant N gave a banding pattern identical to that of a sibling that contained only a single copy of *Ac*. Although small changes could not be identified by this analysis, we detected no major structural rearrangements in the amplified copies of *Ac*.

Segregation of Amplified Elements in the R2 Generation

The amplified copies of *Ac* were identified in DNA isolated from leaves taken from a single R1 plant. To determine whether the amplified copies were sexually transmitted to the next generation, genomic DNA was isolated from 21 independent R2 progeny plants that were obtained by selfing the high copy plant. A DNA gel blot prepared from HindIII-digested DNA was probed consecutively with the *Ac*-specific probe 2, which hybridized to one end of *Ac*, and the *Ac*-specific probe 3, which hybridized to the other end of *Ac* and the internal 1.6-kb HindIII fragment. This allowed us to determine the *Ac* copy number by counting the number of *Ac* insertions using two independent probes and by measuring the intensity of the internal 1.6-kb fragment. To control for the amount of DNA loaded in each lane, the blot was probed a third time with a tomato chalcone synthase gene. Under these hybridization conditions, this cDNA hybridizes to a single band on a genomic

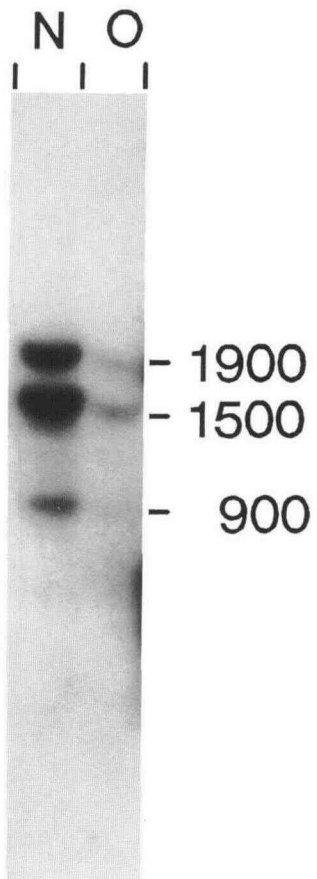

Figure 3. Taq1 Digestion of High Copy Number Plant N.

Genomic DNA from plant N, which contains about eight copies of *Ac*, and from a sibling plant O, which contains one copy of *Ac*, was digested with Taq1 and transferred to a nylon membrane. The filter was hybridized with the *Ac*-specific probe 4, which includes the entire *Ac* sequence. The three bands observed are characteristic of intact *Ac* elements.

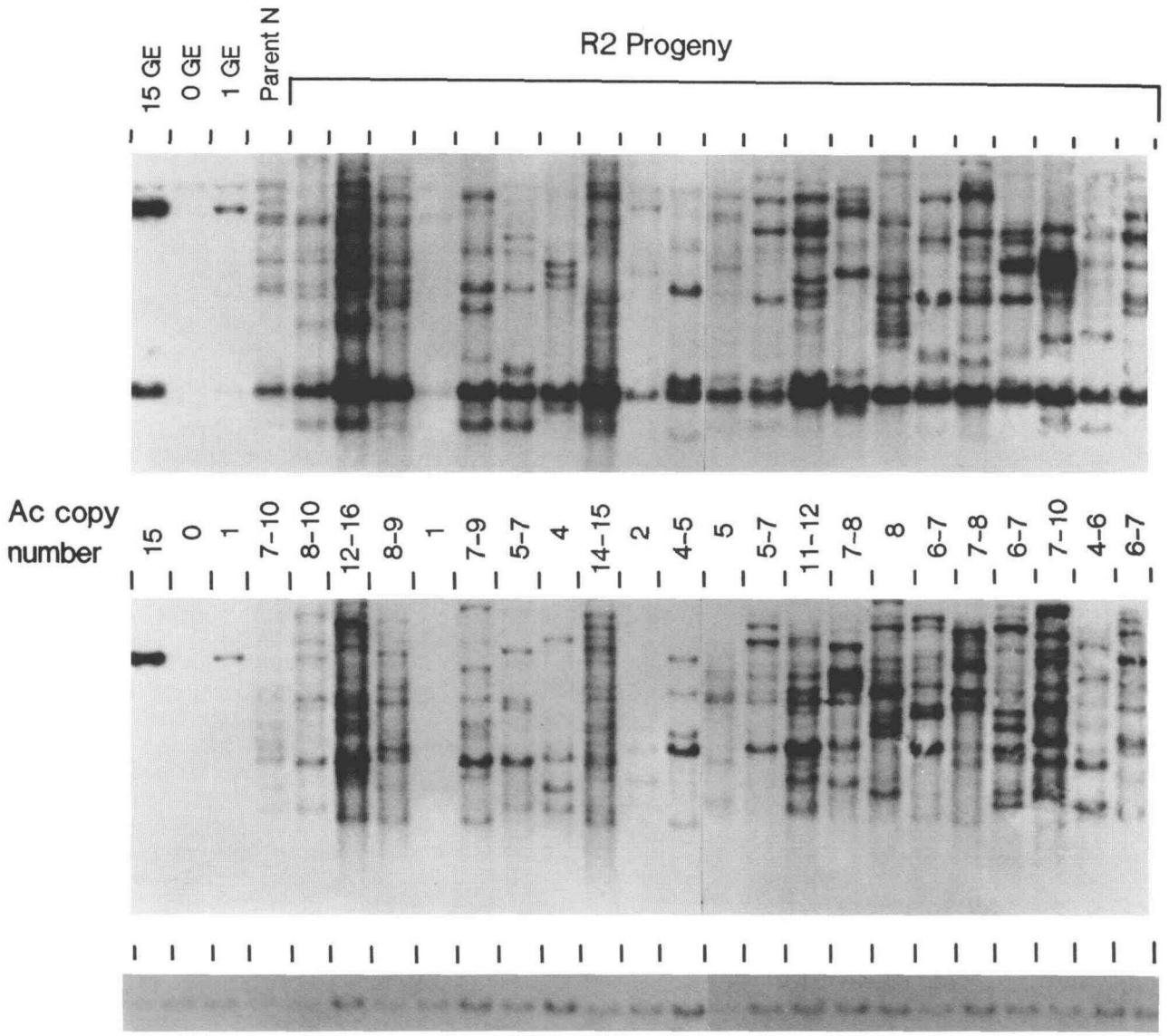

Figure 4. R2 Progeny from High Copy Number Plant N.

Genomic DNA was isolated from 21 self progeny of the high copy plant N, digested with HindIII, and probed with the *Ac*-specific probe 3. This probe hybridized to one end of *Ac* and also an internal 1.6-kb fragment. After the filter was autoradiographed and the probe removed, it was hybridized with *Ac*-specific probe 2. This probe hybridized to the other end of *Ac* and resulted in a single band for each independent *Ac* insertion. To correct the signals for variation in the amount of DNA loaded, the filters were hybridized a third time with a tomato chalcone synthase cDNA. The number of copies of *Ac* was determined by counting the bands in each lane and determining the amount of hybridization in the 1.6-kb band. The reconstruction lanes containing 0, 1, and 15 genomic equivalents of *Ac* are labeled 0 GE, 1 GE, and 15 GE, respectively. The R1 parent is labeled as parent N. The R2 progeny produced by selfing N are shown in the next 21 lanes. The *Ac* copy number determined is shown between the top two radiographs.

(Top panel) Autoradiogram after hybridization with probe 3.

(Middle panel) Autoradiogram after hybridization with probe 2.

(Bottom panel) Hybridization with the tomato chalcone synthase cDNA.

DNA gel blot (S.D. O'Neill, Y. Tong, B. Spörlein, G. Forkmann, and J.I. Yoder, manuscript submitted).

As seen in Figure 4, the number of *Ac* elements in the different R2 plants ranged between one and about 16. In plants that contained many *Ac* elements, some of the insertion bands matched those in the parent, suggesting that these bands were segregating. In addition, unique insertion bands were found in some R2 plants that were not present in their parent or siblings. This indicated that at least some of the *Ac* elements continued to transpose in the R2 generation. The further increase in copy number above that of the eight copies in the parent could, therefore, be attributed to the segregation of existing elements as well as to new elements arising through transposition.

Analysis of the R2 generation also enabled us to determine how the amplified copies from the R1 generation were segregating. If all of the duplicated copies of *Ac* were confined to a single genetic locus, the R2 progeny would be expected to contain either zero, eight, or 16 copies of *Ac*. This was clearly not the case because there were many plants with copy numbers between these values. This observation, together with the fact that each *Ac* insertion was at a unique genomic position, suggests that *Ac* amplification did not occur at a single chromosomal site.

On the other hand, if the elements were randomly scattered throughout the 24 diploid tomato chromosomes, progeny that contained only one or two copies of *Ac* would be infrequently detected. For every *Ac* insertion there would be a 50% chance that a single copy of that insertion would be transmitted to a self progeny. For a plant that contained eight *Ac* insertions the chance of transmitting only one *Ac* element to a progeny is $(0.5)^8$, or 0.004, if each copy is independently assorting. Therefore, if the eight *Ac* elements were randomly scattered among the tomato chromosomes, the frequency of obtaining a progeny plant with a single element would be less than 0.5%. The high frequency with which we identified plants containing a minimum number of elements suggested that at least some of the elements were genetically clustered.

DISCUSSION

These results demonstrate that *Ac* can rapidly amplify when introduced into a virgin genome. We observed *Ac* amplification in one out of 17 self-pollinated progeny from a primary transformant carrying a single copy of *Ac*. Because amplification of transposable elements has been observed in other systems as well, it may be a typical outcome of transposon invasion (Daniels et al., 1987; Hardeman and Chandler, 1989; Preston and Engels, 1989).

The question remains whether a mutation had occurred in either the *Ac* sequence or the tomato genome that allowed rapid amplification to occur. Such a mutation could result in the inactivation of a transposition repressor or in the enhancement of a transposition activator. Given the frequency with which transposon amplification has been observed in different species and the relatively low mutation rate in higher organisms, it is unlikely that a mutation occurred in the genome of the plant in which amplification occurred. On the other hand, maize transposable element sequences are frequently rearranged, resulting in a variety of defective elements (Starlinger et al., 1985). We are currently cloning some of the amplified *Ac* elements to determine whether they differ from wild-type elements in their nucleotide sequence.

Gene amplification is a common mechanism of overproducing a gene product either in response to drug selection or at a particular stage in development (Stark and Wahl, 1984). One possible explanation for the rapid amplification of *Ac* in tomato is that *Ac* transposed into a genomic sequence that was itself amplifying. On the basis of this model, *Ac* would be amplified passively at one chromosomal location. The segregation of amplified elements in the R2 population, however, rules out this possibility because the elements were not segregating as a single genetic locus. In addition, the *Ac* banding pattern on DNA gels was inconsistent with the amplified copies being tandemly duplicated. An extension of this hypothesis is that the amplification occurred at a single locus, and the amplified elements then transposed to new chromosomal positions. If this were the case, the *Ac* element located in the amplifying sequence should be more prevalent than *Ac* elements at other insertion sites and should be observed on DNA gel blot hybridizations as an insertion site of singularly strong intensity. Our results were also not consistent with this hypothesis because there were no predominantly strong hybridizing bands observed in either the R1 or R2 generations. This was further confirmed by the fact that the copy number determined by counting the bands accounted for the total number of elements in the genome.

A second possible explanation for the amplification of *Ac* is that it occurred during transposition. If this were the case, each amplified copy should reside at a different chromosomal location. In accordance with this hypothesis, our results indicate that the net *Ac* copy number increase was accounted for by elements at discrete chromosomal positions. In addition, the clustering of amplified elements in the R2 progeny is consistent with a transposition mechanism because transposed *Ac*s often insert at closely linked sites (Van Schaik and Brink, 1959). Therefore, the more likely hypothesis is that *Ac* amplification is associated with the process of transposition.

How can transposition increase transposable element copy number? Certain bacterial transposable elements, such as *Tn3* (Sherratt, 1989) and bacteriophage *Mu* (Pato, 1989), transpose via replicative mechanisms and duplicate during transposition. It has also been postulated that the

maize element *Mu* transposes via a replicated intermediate because of the dramatic increases in copy number observed in out-crossed progeny while parental elements continue to segregate (Alleman and Freeling, 1986). Studies of *Ac* transposition suggest, however, that these elements transpose by a conservative process that requires the excision of an element before its insertion elsewhere (Greenblatt, 1968). In a series of experiments, Greenblatt and Brink demonstrated that *Ac* can transpose from a newly replicated donor site to either its sister chromatid or to an unreplicated site on the same chromosome (Greenblatt and Brink, 1963). In either case, two copies of *Ac* are generated in one of the daughter cells upon mitotic division. A second mechanism hypothesized for copy number increases following conservative transposition is gene conversion by double-strand gap repair (Kleckner, 1989). Although double-strand gap repair processes in yeast have been documented (Szostak et al., 1983), their role in transpositional increases remains hypothetical. Certainly, either mechanism will lead to an increase in copy number over subsequent generations if it occurs in cell lineages which develop into gametes.

In maize, *Ac* transposition rates are regulated by an *Ac* copy number-dependent mechanism; increasing the dosage of *Ac* elements in a maize genome results in later and less frequent transposition (McClintock, 1951). At least some component of the repressor is encoded by the *Ac* element itself because small deletions in transposase coding sequences abolish the element's contribution to the dosage effect (Dooner et al., 1986). Interestingly, when *Ac* is introduced into tobacco, a positive dosage effect is observed and transposition is more frequent in lines homozygous for *Ac* than in heterozygous lines (Jones et al., 1989). The lack of a negative dosage response for *Ac* in tomato is also clear from its rapid amplification described here. This suggests that additional components of the maize genome, perhaps cryptic or altered *Ac* elements, are essential for dosage-dependent repression.

When single P elements are transformed into *Drosophila* M cytotype flies, lineages with an increased number of P elements can be isolated. Many of these develop P cytotype repressor, although not for a number of generations following the copy number increase (Daniels et al., 1987; Preston and Engels, 1989). To explain this phenomenon, Engels proposes that one of the amplified P elements mutates into a sequence that encodes a repressor. According to this hypothesis, it may take many generations after amplification before the correct mutation is created in an element at the proper chromosomal position to elicit the P cytotype (Engels, 1989). This hypothesis is supported by the observation that elements that repress P element transposition can be made by introducing sequence changes into the third exon of the P transposase (Engels, 1989).

P element cytoptype repression is not strictly analogous to the *Ac* dosage effect because an apparently normal *Ac* element will elicit the dosage response, whereas mutant P elements are implicated in P repression. Conceivably, aberrant transposases could be produced from a normal *Ac* element if alternative transcriptional start sequences were used or if introns were spliced abnormally; the frequency with which these would be produced would depend upon the number of *Ac* elements. Aberrant transposases may then interact with defective elements in the maize genome to form repressor molecules. The tomato lines that contain multiple copies of *Ac* afford an opportunity to investigate repressors of *Ac* transposition. We are currently using the high copy lines in crosses to transgenic tomato lines in which excision of the nonautonomous transposable element *Ds* can be assayed, and we are selecting for plants in which transposition is repressed. These will be candidates for having evolved mechanisms to attenuate transposition.

These experiments show that *Ac* elements can proliferate in virgin genomes in only a few generations. Although the amplified copies we examined were intact *Ac* elements, it seems likely that some of these may eventually undergo structural alterations typical of *Ds* sequences in maize. The timing of these changes will be investigated in subsequent generations. The rapid proliferation of autonomous elements may be the initial step in the evolution of multicopy transposable element families.

METHODS

The *Ac7* element was inserted into the *Agrobacterium tumefaciens* Ti-based vector pMON200 (Fraley et al., 1985) to make the plasmid pMAC (Yoder et al., 1988). This plasmid was then introduced into the tomato cultivar VF36 by *Agrobacterium*-mediated transformation as described previously (Yoder et al., 1988). Mature plants were regenerated and self-pollinated, and progeny seed were collected. After the convention proposed by Chaleff (1981), primary transformants are called the R0; progeny resulting from selfing R0 plants are R1.

Genomic DNA was extracted from leaf tissue by the CTAB (hexadecyltrimethylammonium) bromide method described by Bernatzky and Tanksley (1986). DNA gel blot hybridizations were conducted as previously described (Belzile et al., 1989). Before reprobing a blot with a second probe, it was stripped at 42°C in 0.4 N NaOH and then neutralized in 0.1 M Tris, pH 7.5, and 0.1% SDS. Reconstruction lanes on the gel contained known genomic equivalents of the pMAC plasmid mixed with untransformed VF36 DNA. For the reconstruction calculations, the size of the tomato genome was taken to be 7×10^8 bp per haploid genome (Galbraith et al., 1983).

The restriction fragments used as *Ac*-specific probes were derived from pJAC, an *Ac7* containing pBR322 derivative (Yoder et al., 1988), and are diagrammed in Figure 1. Probes for the analysis of T-DNA insertions have been described elsewhere (Belzile et al., 1989). The tomato chalcone synthase probe was isolated from pTCHS2 (S.D. O'Neill, Y. Tong, B. Spörlein, G. Forkmann, and J.I. Yoder, manuscript submitted). Restriction fragments to be used as probes were separated by electropho-

resis through low melting point agarose and then labeled with ^{32}P by the random primer method without prior extraction from agarose. Quantification of hybridizing bands following DNA gel blot hybridization was performed using an AMBISS radioanalytic device.

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