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Genetic and molecular analysis of tissue-culture-derived *Ac* elements

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Summary. Our previous experiments on maize (*Zea mays* L.) plants regenerated from tissue culture revealed genetic activity characteristic of the transposable element *Activator* (*Ac*) in the progeny of 2–3% of the plants tested, despite the lack of *Ac* activity in the progenitor plants. The objective of the present study was to determine whether the presence of *Ac* activity in tissue-culture-derived plants was associated with changes in the number or structure of *Ac*-homologous DNA sequences. Families segregating for *Ac* activity were obtained by crossing plants heterozygous for *Ac* activity onto *Ac*-responsive tester plants. A DNA probe derived from a previously isolated *Ac* sequence was used to examine the *Ac*-homologous sequences within individual progeny seedlings of segregating families and noncultured control materials. All plants tested had six or more *Ac*-homologous DNA sequences, regardless of whether *Ac* activity was present. In the segregating progeny of one tissue-culture-derived plant, a 30-kb *Ac*-homologous SstI restriction fragment and a 10-kb *Ac*-homologous BglII restriction fragment were found to cosegregate with *Ac* activity. We propose that these fragments contained a previously silent *Ac* sequence that had been activated during tissue culture. Although one or more *Ac* sequences were often hypomethylated at internal PvuII and HpaII sites in plants with *Ac* activity, hypomethylation was not a prerequisite for activity. Reduced methylation at these sites may have been a result rather than a cause of *Ac* activity.

Key words: *Zea mays* L. – Somaclonal variation – *Activator* (*Ac*) – Methylation – Tissue culture

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Introduction

One of the many genetic effects of plant tissue culture ("somaclonal variation"; Larkin and Scowcroft 1981) is the activation of previously silent transposable elements (Evola et al. 1984, 1985; Groose and Bingham 1986; Peschke et al. 1987; Peschke and Phillips 1991). McClintock (1978, 1984) proposed that transposable elements become active as a response to "genomic stress". The classic source of "genomic stress" is chromosome breakage; numerous reports have documented the activation of transposable elements in this way (McClintock 1950; Peterson 1953; Neuffer 1966; Bianchi et al. 1969; Doerschug 1973; Rhoades and Dempsey 1982; Walbot 1988). While it is possible that the chromosome breakage frequently observed in tissue-culture-derived plants (review: Lee and Phillips 1988) may be responsible for transposable element activity, other mechanisms cannot be excluded.

One of the elements found to become active during tissue culture is the autonomous (self-transposing) maize transposable element *Activator* (*Ac*) (McClintock 1950; reviewed in Dennis et al. 1988). This activity was detected (Peschke et al. 1987) by crossing the progeny of 301 regenerated plants from 94 embryo-derived cell lines with tester plants containing the related element *Dissociation* (*Ds*) linked to easily scored genetic markers (see "Materials and methods"). Based on approximately 1,200 scored testcrosses, progeny of 11 regenerated plants from three embryo cell lines were identified as potentially containing *Ac* activity. A number of additional genetic tests confirmed that ten plants from two of the three embryo cell lines did have *Ac* activity. Thirty-three tests of plants from control kernels (grown on the same ears from which the embryos were explanted) were negative, indicating that *Ac* activity was not present before tissue culture. In addition, the two cell lines that had produced plants with

confirmed *Ac* activity also produced a number of plants without *Ac* activity, providing further evidence that *Ac* activity was not present in the initial explant source but arose later, during callus growth or plant regeneration. The occurrence of *Ac* activity in nine regenerated plants from one of the cell lines is strong evidence of activation during the callus phase (Peschke et al. 1987).

Maize lines without transposable element activity nearly always contain element-homologous DNA sequences (Fedoroff et al. 1983; Johns et al. 1985; Cone et al. 1986; Chandler et al. 1988). For example, Fedoroff et al. (1983) demonstrated the presence of four to eight sequences hybridizing to the center (transcribed region) of *Ac* in maize lines without *Ac* activity, as well as the presence of about 40 copies of sequences hybridizing to the ends of *Ac* (i.e., *Ds*-like sequences). It is presumed that at least some of these sequences are capable of being "activated" under appropriate conditions.

Several researchers who have studied *Ac* elements that cycle between activity and inactivity have observed that the "phase" of these *Ac*'s (i.e., active versus inactive) is correlated with methylation at specific restriction enzyme sites (Schwartz and Dennis 1986; Chomet et al. 1987; Schwartz 1989). In a cycling version of *Ac* at the *waxy* locus (*wx-m7::Ac*), the two PvuII sites were unmethylated in at least a portion of the cells when the *Ac* was active. In contrast, both PvuII sites were methylated when the *Ac* was inactive (Chomet et al. 1987). The activity of another cycling *Ac* element at *waxy* (*wx-m9::Ac*) was found to be similarly correlated with hypomethylation at PvuII and HpaII sites, albeit in a more complex fashion (Schwartz and Dennis 1986). Schwartz (1989) has observed a strong correlation between *Ac* activity and hypomethylation at the single BamHI site located in the 5′ end of *Ac*. In addition, the BamHI site in a particular *Ds* element is unmethylated when an active *Ac* element is present in the genome, but fully methylated in the absence of *Ac* activity. It is suggested that the lack of *Ds* methylation in the presence of *Ac* activity is due to a conformational change in *Ds* caused by binding of the *Ac* gene product, making it inaccessible to methylase. The correlation between hypomethylation and element activity has also been observed for other maize transposable elements (*Mu:* Chandler and Walbot 1986; Bennetzen 1987; *Spm:* Banks et al. 1988; Fedoroff and Banks 1988).

The objective of the present study was to examine the molecular basis of the *Ac* genetic activity detected in tissue-culture-derived maize plants. Southern hybridization patterns of sibling plants with and without *Ac* activity were compared, in order to correlate *Ac* genetic activity with the presence of a specific *Ac*-homologous DNA fragment. In addition, the methylation of *Ac*-homologous DNA sequences within these segregating families was tested.

Materials and methods

Source of tissue-culture-derived plant materials

The plant materials used in the present study are the progeny of regenerated plants identified in previous experiments (Peschke et al. 1987) as containing *Ac* activity produced during tissue culture. These regenerated plants came from two different embryo cell lines, designated 4-41 and 1-42 by Lee (1984). The embryos used to initiate the cell lines had been produced by sib pollinations within F_2 rows of the cross (A188 × Oh43 *ms13*). Nine regenerated plants from line 4-41 had been shown to contain *Ac* activity; six of these are represented in the present study. Only one regenerated plant from line 1-42 had tested positive for *Ac* activity. Since it was possible that the *Ac* elements in two different regenerated plants (even from the same cell line) would not be identical, records of the precise "donor" regenerated plant were maintained for all experiments, using the original numbers assigned to these plants (Lee 1984). The two ancestors of cell lines 4-41 and 1-42, the inbred A188 and the backcross-derived line Oh43 *ms13*, were included in many of the analyses.

Families segregating for *Ac* activity were obtained from testcrosses of plants heterozygous for *Ac* activity onto the *C Ds* tester stock previously described (Peschke et al. 1987). This line has a chromosome-breaking *Ds* element close to the centromere of chromosome *9S;* distal to *Ds* is a dominant *C* allele, which is necessary for aleurone pigmentation. In contrast, the tissue-culture-derived materials were recessive at *C*. Testcross kernels that do not receive an active *Ac* will be fully colored (genotype *C C c*). Kernels that receive a dose of *Ac* activity will be variegated, due to the transposition of *Ds* and consequent loss of the dominant *C* alleles in some cells. A testcross of a plant heterozygous for a single, active *Ac* element will normally produce an ear segregating approximately 1 : 1 for variegated (+*Ac*) and nonvariegated (−*Ac*) seeds (Fig. 1). Variegated and nonvariegated seeds from each testcross were sorted using a dissecting microscope and then planted separately. Seeds that were ambiguous or unusual were not included.

DNA analysis

Total DNA was isolated from individual, 3-week-old maize seedlings (Shure et al. 1983). Approximately 7 µg DNA was digested with a threefold excess of one of the restriction enzymes

Fig. 1. Two kernels from an ear produced from a testcross of a plant heterozygous for *Ac* activity, segregating approximately 1 : 1 for nonvariegated (*left*) and variegated (*right*) kernels. Such testcross ears provided the starting material for many of the experiments in the present study

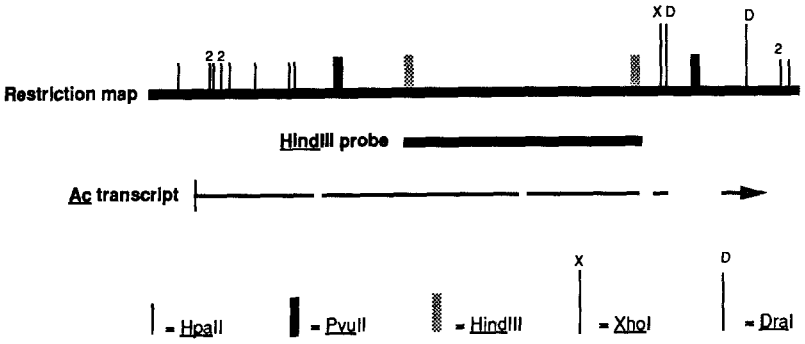

Fig. 2. Schematic drawing of the *Ac* transposable element, based on the published sequences of *Ac7* (Muller-Neumann et al. 1984) and *Ac9* (Pohlman et al. 1984). Regions of *Ac* spanned by the most abundant transcript and the 1.6-kb HindIII probe are indicated. A "2" above an enzyme designation indicates that two closely spaced sites for that enzyme are present

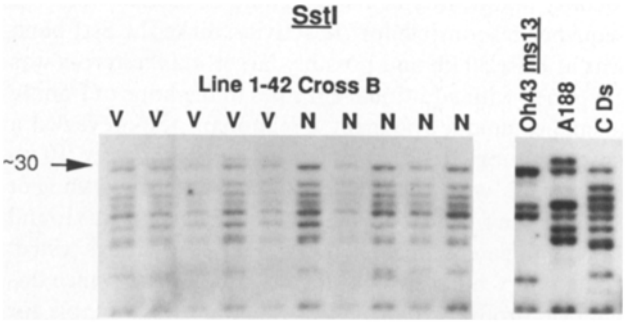

Fig. 3. Illustration of the 30-kb SstI fragment which cosegregates with *Ac* activity in this family (cross B, line 1–42). In this and the following figures, "V" and "N" indicate seedlings grown from variegated (+*Ac*) and nonvariegated (−*Ac*) seeds, respectively. Note that the 30-kb band is approximately the same molecular weight as one of the cryptic *Ac*-homologous sequences in A188, which is included in the figure for comparison

(Bethesda Research Laboratories) described below, for 12–24 h. Digested DNA was loaded onto agarose gels (0.6–0.8%) in 1 × TBE buffer and fractionated using no higher than 40 V. Lambda DNA cut with one or more enzymes was included in every gel to provide standards for molecular weight determinations. The gels were soaked with agitation in 0.25 *N* HCl for approximately 30 min, rinsed with water, and blotted onto Zetabind (AMF Products) nylon membrane using 0.4 *M* NaOH (Reed and Mann 1985) for 18–24 h. Southern hybridization was performed as described by Benner et al. (1989). The filters were exposed to X-Omat X-ray film, with one Lightning Plus intensifying screen, at −70 °C.

The probe was a 1.6-kb HindIII fragment which spans the center of the *Ac* element (Fig. 2), derived from the full-length clone of the *Ac9* element isolated by Fedoroff et al. (1983) and provided to us by D. Culley (Washington State University, Pullman). This center HindIII fragment detects genomic sequences that resemble an intact *Ac* element. The insert was isolated from the vector by cleavage with HindIII, electrophoresis, and isolation from the agarose by repeated freezing and thawing. Approximately 100 ng of isolated insert was labelled to a specific activity of 1×10^9 cpm/μg in an 8-h reaction, using the "random primer" method (Feinberg and Vogelstein 1983).

Restriction enzymes were chosen for each experiment based on knowledge of restriction sites in *Ac9* (Fig. 2). For the cosegregation analyses, enzymes (SstI, BglII, EcoRV) were used that have no sites within the published sequence of *Ac*. These enzymes are either insensitive to methylation or do not have CG or CNG in their recognition sequence (most plant DNA methylation occurs in CG and CNG sequences; Gruenbaum et al. 1981). They were also used to examine noncultured control materials for the presence of *Ac*-homologous sequences. The number of bands produced with such enzymes should approximate the number of *Ac*-like sequences within the genome. Several methylation-sensitive enzymes cutting outside of *Ac* (PstI, SmaI, SalI) were also included in some experiments, as were the enzymes DraI (methylation-insensitive; two sites in *Ac*) and XhoI (methylation-sensitive; one site within *Ac*).

The enzymes PvuII and HpaII were used in tests for methylation within *Ac* (Schwartz and Dennis 1986; Chomet et al. 1987). *Ac9* has two PvuII sites separated by 2.5 kb. If both PvuII sites of a similar *Ac* element are unmethylated, the probe will detect a 2.5 kb fragment since it spans a portion of *Ac* between the two PvuII sites (Fig. 2). If one or both PvuII sites are methylated, the PvuII fragment detected by the probe will be greater than 2.5 kb. Similarly, the smallest detectable HpaII fragment is 3.4 kb based on the *Ac9* sequence; this will occur if the two innermost HpaII sites are unmethylated (Fig. 2).

Results

Examination of Ac-homologous sequences in noncultured control materials

The lines A188, Oh43 *ms13*, and the *C Ds* tester were examined for *Ac*-homologous sequences using the 1.6-kb HindIII fragment from *Ac*. Digests with SstI revealed different patterns of six to ten *Ac*-homologous sequences in each line (Fig. 3); digests with BglII produced 9–15 bands per line (Fig. 4). The pattern of bands observed for a given line/enzyme combination was consistent over repeated testing. These results were similar to those of Fedoroff et al. (1983), who observed four to ten bands using this same probe and several other inbreds without *Ac* activity.

Cosegregation of an Ac-homologous sequence with Ac activity

To determine whether *Ac* activity in plants from line 4-41 could be associated with an *Ac*-homologous sequence, testcross seed from a plant heterozygous for *Ac* activity (approximately 1 : 1 variegated to nonvariegated seed; see Fig. 1) was sorted and planted. DNA was isolated from four plants (two *Ac*+, two *Ac*–) and cleaved with five

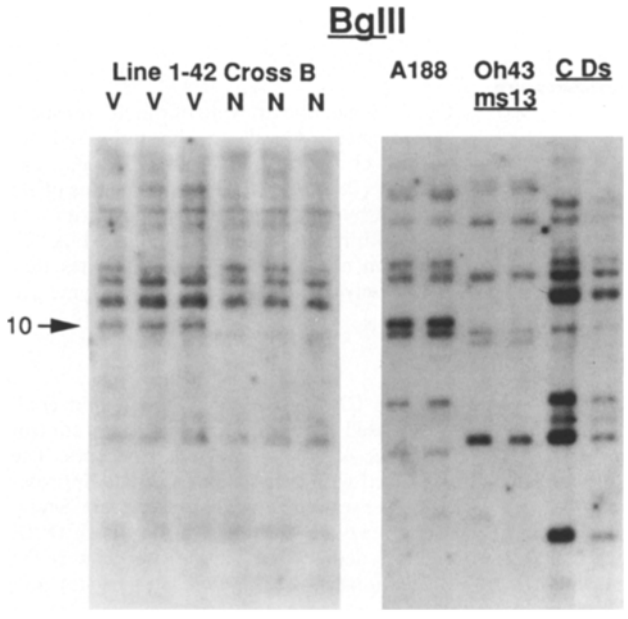

Fig. 4. Illustration of the 10-kb BglII fragment which cosegregates with *Ac* activity (cross B, line 1–42). A 10-kb band cosegregated with *Ac* activity in 38 out of 38 seedlings tested. This band is approximately the same molecular weight as one of the cryptic *Ac*-homologous sequences in A188, which is included in the figure for comparison

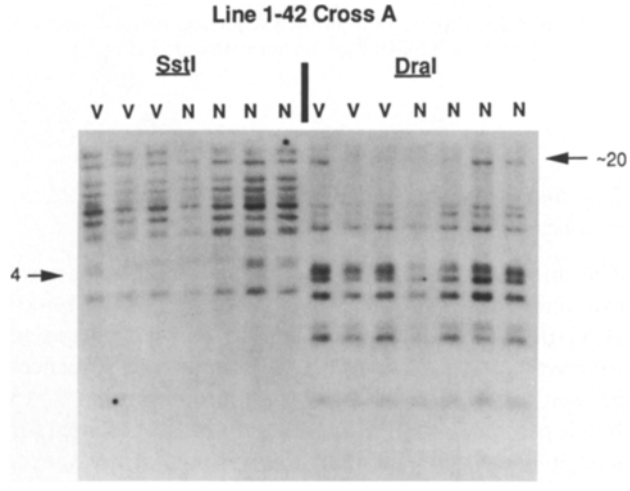

Fig. 5. Tests of seedlings from line 1–42, cross A. *Arrows* indicate a 4-kb SstI fragment and a 20-kb DraI fragment which segregate independent of *Ac* activity. No band cosegregated with *Ac* activity. The 20-kb DraI band appeared to be linked to the 4-kb SstI band (they are either both present or both absent in the same seedling) and is likely to represent the same *Ac*-homologous sequence

different restriction enzymes (SstI, BglII, SmaI, PstI, SalI) for analysis with the *Ac* probe. All four plants produced identical patterns (data not shown). For the first testcross sampled from line 1-42, two *Ac*-homologous DNA sequences segregated, but neither one was associated with *Ac* activity (Fig. 5).

Analysis of a testcross ear produced from a second progeny plant from line 1-42 showed the presence of an SstI cosegregating band of at least 30 kb in length (Fig. 3). This band was found to cosegregate with *Ac* activity in 33 out of 33 plants tested; the DNA of 19/19 plants with *Ac* activity had the fragment, while 14/14 without *Ac* activity did not. One other SstI band (independent of *Ac* activity) of ca. 4 kb also segregated in this testcross. These data show that the 30-kb band is tightly linked to *Ac* genetic activity and possibly contains the DNA sequence responsible for *Ac* activity.

A rationale for the cosegregation experiments was to identify unique fragments that could be used to clone the sequence responsible for *Ac* activity. Since the SstI band was at least 30 kb and possibly larger, this testcross was examined with additional enzymes in the hope of finding a smaller unique fragment. Preliminary tests revealed a cosegregating BglII fragment of approximately 10 kb (Fig. 4), as well as an EcoRV fragment of 5.5 kb (not shown). On further testing, the BglII fragment was found to cosegregate with *Ac* activity in all 38 seedlings tested. The EcoRV band was not investigated further, since this enzyme produces blunt ends which are less suitable for cloning. Experiments to clone the 10-kb BglII sequence are in progress.

Tests for methylation of Ac-homologous sequences

Seedlings from segregating testcross ears were used to compare methylation of *Ac* sequences in plants with and without *Ac* activity. Tests of line 4-41 included progeny of six regenerated plants; it is probable that the *Ac* activity in these six plants arose from a single event during tissue culture. DNA from 7–12 seedlings representing each regenerated plant was cleaved with PvuII and analyzed with the *Ac* probe. A subset of these seedlings (three to nine per family) was also tested with HpaII.

Despite the presumed common origin of *Ac* activity in the six regenerated plants, each set of progeny appears to have a different pattern of methylation within the *Ac*-like sequences (Fig. 6A–D). In general, even plants without *Ac* activity have a faint *Ac*-hybridizing PvuII fragment of 2.5 kb that was not apparent in A188 or Oh43 *ms13* control plants (Fig. 6D).

The progeny of line 4-41 plant no. 34 (Lee 1984) apparently segregate for differences in intensity of hybridization to the 2.5 kb PvuII fragment (Fig. 6A). Although there is at least a faint band in all lanes, it is consistently darker when *Ac* activity is present. This does not appear to be explained by a segregation of one versus two copies of a faint band (one cryptic, one active), since the darker band is more than twice as intense as the lighter ones. A comparison of the higher-molecular-weight bands in each lane indicates that loading differences would not account for the differences in intensity of

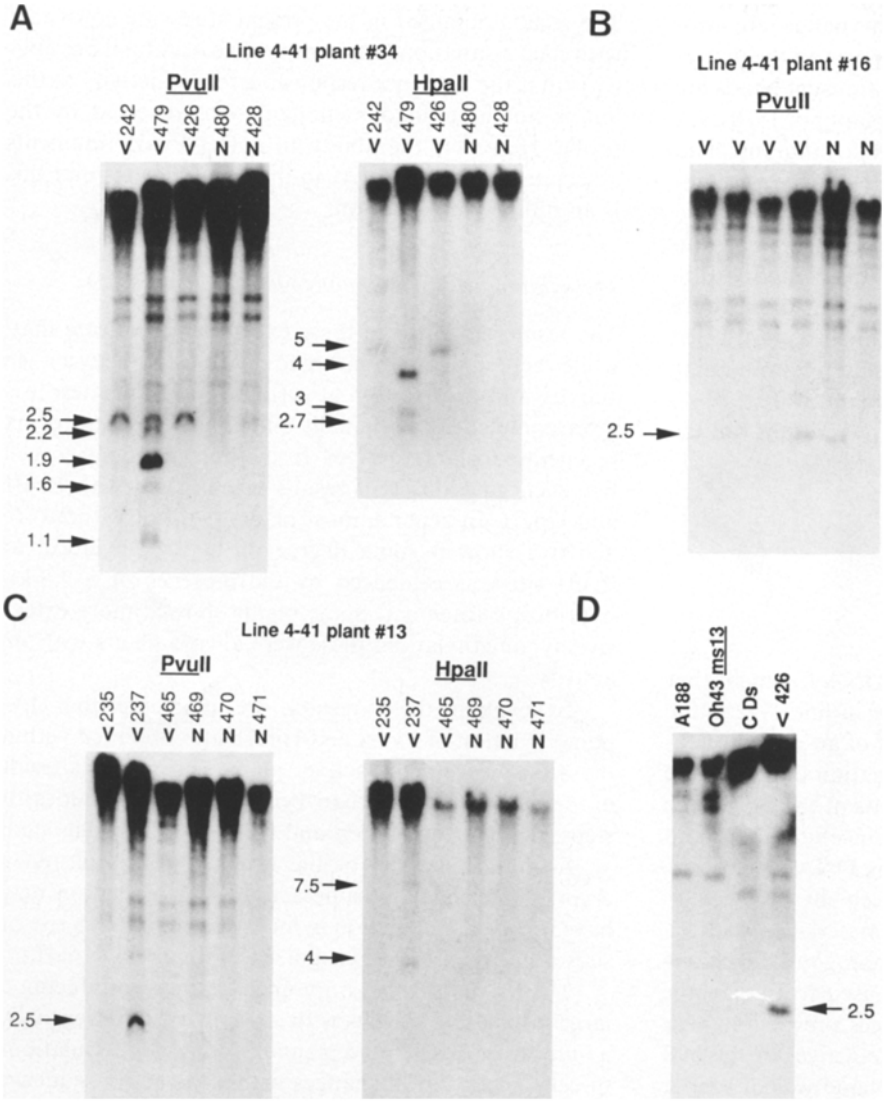

Fig. 6A–D. Tests of methylation within *Ac*-homologous sequences in tissue-culture-derived plants and noncultured controls. **A** Methylation within *Ac*-homologous sequences in a testcross of a plant descended from line 4-41 plant no. 34. A prominent 2.5-kb PvuII fragment is observed in plants with *Ac* activity, while only a faint band is seen in plants without *Ac*. Testing with HpaII also shows hypomethylation of *Ac*-homologous sequences in plants with *Ac* activity, as indicated by the bands of approximately 5 kb (plants no. 242 and 426) and smaller (plant no. 479). **B** Methylation tests (using PvuII) of a progeny plant descended from line 4-41 plant no. 16. No differences are observed in plants with and without *Ac* activity. Digests with HpaII gave only high-molecular-weight fragments (not shown). **C** Methylation tests of a progeny plant descended from line 4-41 plant no. 13. One *Ac*-containing seedling (no. 237) shows hypomethylation when tested with either PvuII or HpaII. All of the other plants appear to have similar methylation, regardless of whether or not they have *Ac* activity. **D** Test of noncultured control materials and *Ds* tester stock with PvuII. The *last lane* is a DNA sample with *Ac* activity, which was shown in several experiments to have a dark 2.5-kb PvuII band (see no. 426, Fig. 6A). Digests of control with HpaII gave only high-molecular-weight fragments (not shown)

the 2.5-kb band. In addition, a number of intense bands ranging from 1 to 2 kb are visible in some plants with *Ac* activity. When plants from this family were tested with HpaII, a band of approximately 5 kb could be detected in three plants with *Ac* activity; two of these are pictured. A fourth *Ac*-containing plant (no. 479) gave several bands below 4 kb; this same plant had shown multiple bands (2.5 kb and below) when tested with PvuII (Fig. 6A). Plants without *Ac* activity gave no low-molecular-weight HpaII bands.

In contrast to these patterns, no segregating PvuII or HpaII fragments of any size were observed in progeny of line 4-41 plant no. 16 (Fig. 6B); any differences in band intensities can be explained by loading differences.

The behavior of the remaining four families falls between these two extremes. In some *Ac*-containing plants

in each family, low-molecular-weight bands are produced with one or both enzymes. However, in the majority of plants (both *Ac*+ and *Ac*–) no unusual bands are seen. For example, in the progeny of plant no. 13, a dark 2.5-kb PvuII fragment is seen in one *Ac*-containing plant but in none of the others, regardless of whether they contained *Ac* activity (Fig. 6C).

Several progeny plants from line 1-42 (with and without *Ac* activity) were examined using HpaII and PvuII in preliminary tests. No low-molecular-weight bands were found, therefore methylation in this line was not tested further. It is interesting to note that the *Ac*-containing plants which apparently had no hypomethylated *Ac* sequences had previously given positive results in the cosegregation tests with SstI and BglII.

Discussion

Cosegregation analysis

The presence of an *Ac*-homologous DNA fragment that cosegregates with *Ac* genetic activity in line 1-42 is the first evidence for the molecular basis of an element activated by tissue culture. The cosegregation data indicate that the sequence is within 5 map units of the *Ac* genetic activity, and possibly much closer. Since no other band appears to be a likely candidate, this DNA sequence is presumed to contain the active *Ac* element.

Analysis of noncultured control materials revealed a large number of inactive, *Ac*-homologous DNA sequences present in the progenitors used to initiate the tissue cultures; one of these sequences presumably was altered in such a way that it became active. In the SstI and BglII digests, the cosegregating band was of similar molecular weight to one of the bands in A188, indicating that the element might have been derived from that particular cryptic sequence from the A188 parent. This result would require that the element had not transposed from its original location.

It is not known why no cosegregating bands were observed in plants from line 4-41 when enzymes cutting outside of *Ac* were used. The most likely explanation is insufficient sampling (of families or enzymes); however, additional testing may not have helped. For example, if the active *Ac* sequence and a cryptic copy are in identical positions on homologous chromosomes, cosegregation analysis will not reveal the active sequence, unless the active *Ac* transposes or is separated from the cryptic homologous copy by crossing to an unrelated line. Another possibility is that the element transposes so frequently that it is rarely in the same place twice. In either of the above situations, cosegregation might be detected when examining methylation of internal restriction sites (e.g., PvuII) but not when using flanking enzyme sites. The results obtained in the present study are consistent with such a situation but do not prove it. A final possibility is that the sequence responsible for *Ac* activity in this line is not homologous enough to be detected by the probe. However, the ability to detect PvuII fragments associated with *Ac* activity in this line indicates that this is an unlikely explanation.

Methylation within Ac-homologous sequences

The results obtained in these experiments indicate that, while there appears to be some relationship between *Ac* activity and methylation at certain restriction sites, it is more complex than originally anticipated. Progeny of six regenerated plants derived from the same embryo cell line each gave different results when tested with PvuII and HpaII. In general, most plants (with or without *Ac* activity) showed some degree of hypomethylation at PvuII sites, as evidenced by the presence of a 2.5-kb hybridizing fragment. Some plants showed more extensive hypomethylation; these were always plants with *Ac* activity.

To explain these results, we postulate that hypomethylation of PvuII and HpaII sites observed within the *Ac* sequence(s) of "active" plants occurred as a result of *Ac* activity, rather than being its cause. A series of steps involving activation and methylation is suggested.

1. In a single cell of the original tissue culture, a cryptic *Ac* element became active. This activation may have involved a reduction in methylation, but at a site or sites other than those recognized by PvuII or HpaII.

2. This cell divided a number of times, producing a large population of cells with *Ac* activity and eventually a number of regenerated plants. In some subpopulations of cells, methylation changes within the active sequence took place; this could occur during the callus phase, during plant regeneration, or even in progeny plants. If changes occurred during the development of plant floral structures or gamete formation, they would be detected in the progeny in unusual ratios.

3. In some plants, the methylation of additional cryptic *Ac*-homologous sequences was reduced by the presence of *Ac* activity. This would account for the presence of PvuII fragments smaller than 2.5 kb in some plants with *Ac* activity (e.g., Fig. 6a, plant no. 479). It is suggested that these small bands represent *Ds*-like elements, i.e., homologous to *Ac* but containing various-sized deletions of the center. Whether or not any of these sequences is now able to transpose (either autonomously or nonautonomously) is unknown.

The well-established correlation between hypomethylation and transposable element activity does not prove a simple cause and effect relationship, and in some cases argues against it. For example, *Mu*-homologous sequences are unmethylated in active *Mutator* lines, but

highly methylated in inactive lines (Chandler and Walbot 1986; Bennetzen et al. 1988; Walbot et al. 1988). However, these *Mu* elements are believed to be the nonautonomous members of a multi-element system and too small to encode a functional gene product themselves. It appears in this case that the lack of methylation results from the gene activity of a separate autonomous element (possibly the *Cy* element; Schnable and Peterson 1989) rather than regulating the *Mu* elements per se (Walbot et al. 1988). Similarly, methylation in the BamHI site of a particular *Ds* element was found to be correlated with the presence or absence of an active *Ac* element in the genome (Schwartz 1989). Gierl et al. (1988) have suggested that the *Spm* "transposase" protects active autonomous elements, as well as similar nonautonomous elements, from becoming methylated by binding to them. McClintock (1958, 1959, 1971) noted that the presence of one active *Spm* element in the genome could cause the transient activation of others. DNA analysis has revealed that the methylation of inactive *Spm* sequences in the genome is reduced by the presence of an active element. In some cases, these inactive sequences become genetically active, depending to a large extent on their initial degree of methylation (Banks et al. 1988; Fedoroff 1989).

Methylation changes resulting from tissue culture

At the time our studies on the activation of transposable elements in tissue culture were initiated, a great deal had already been learned about the effects of tissue culture on the genome, and efforts had been turned toward a study of the mechanisms involved. Unfortunately, since that time, little more has become known about the processes that eventually lead to genetic and cytological abnormality in tissue cultures and regenerated plants.

Recent work has focused on the role of methylation changes in generating somaclonal variation. In studies of the total DNA methylation status of regenerated maize plants (estimated by digesting DNA with HpaII and MspI and examining the degree of cutting in ethidium-bromide-stained gels), heritable changes (both increases and decreases) were observed in approximately 16% of the regenerated plants when compared to controls (Brown and Lorz 1986; Brown 1989). Southern blots probed with cloned functional gene sequences (e.g., *waxy*) also revealed altered methylation; phenotypically normal plants often showed abnormal methylation and vice versa (Brown 1989). However, none of the probes represented genes obviously related to the abnormal phenotypes. Similar results have been obtained (Phillips et al. 1990) using both random and identified (e.g., Adh1) cloned sequences as probes; in general, however, methylation was found to be reduced relative to noncultured controls.

Culley (1986) found that *Ac* sequences in endosperm callus cultures were greatly hypomethylated when compared to plant tissue of the same genotype. It was thought that this might also be true for immature embryo-derived callus, and that transposable element activity in regenerated plants could result from extensive demethylation in callus, followed by lack of proper remethylation when plants were regenerated. Our studies do not support this hypothesis, since levels of methylation in A188 plant and callus DNA were found to be similar, both within and around *Ac*-homologous sequences (V.M. Peschke, unpublished observations) and in terms of overall 5-methyl-C levels measured using HPLC (S. Kaeppler, personal communication). The methylation of *Mutator*-homologous sequences in callus initiated from both active and inactive *Mutator* lines was generally stable, although occasionally the unmethylated *Mu* sequences in callus from active lines became methylated (James and Stadler 1989; Planckaert and Walbot 1989).

Despite the accumulating evidence that tissue culture can cause significant changes in DNA methylation, in addition to its other seemingly diverse effects, the basis for these alterations is still unknown. One possibility is that tissue-culture-induced methylation changes, as well as chromosome breakage events, are a result of cell cycle perturbations (Phillips et al. 1990; Phillips and Peschke 1988). While much of the data obtained using regenerated plants is consistent with this hypothesis, testing it would require analysis of small numbers of cells within the callus. The ability to assess genetic stability at an early stage would also be invaluable in designing culture systems to either increase or decrease variability, depending on the needs of the researcher.

The experiments described in this report have provided additional information regarding one aspect of tissue-culture-induced genetic change, the activation of transposable elements. At this point, there is little evidence for a direct role of transposable elements in generating somaclonal variation (Peschke and Phillips 1991). However, the presence of active transposable elements is certain to contribute to at least some degree of genetic variation and instability. In addition, changes occurring within and around newly-active transposable element sequences may be symptomatic of alterations occurring throughout the genome. As such, continued study of the activation and behavior of transposable elements in vitro may lead to further insights on the nature of tissue-culture-induced genetic variability.

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