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Somaclonal variation in a maize inbred line is not associated with changes in the number or location of *Ac*-homologous sequences *

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Summary. Somaclonal variation (tissue culture-induced mutations) may result, in some instances, from the activation of transposable elements. This study was conducted to determine whether somaclonal variants in the *Zea maize* L. inbred line FR27rhm were associated with movement of the transposable element *Activator* (*Ac*). Ten variants, seven of which from genetic analyses fit a single recessive gene model and three which did not due to a low number of mutant plants, were selected for analysis. Total DNA from these and from uncultured FR27rhm seedlings were examined by Southern blot analysis using the internal 1.6-kb *Hin*dIII fragment derived from the cloned *Ac7* element as a probe. By using a restriction endonuclease which does not cut within the element, the number and distribution of the copies of *Ac*-related sequences in the FR27rhm genome could be determined. From the number of bands seen in the blots, we conclude that the FR27rhm inbred contains large numbers of *Ac*-related sequences. However, the pattern of bands seen in the ten variants and in the uncultured seedlings were identical, indicating that there had been no movement of any of the *Ac*-related sequences to cause the tissue culture-induced mutations.

Key words: Transposable elements – *Activator* – Maize – Somaclonal variation

Introduction

The culture of plant cells in vitro results in the accumulation of genetic and cytogenetic modifications in both the cultured cells and the plants regenerated from them. Such genetic variability recovered in maize (*Zea mays* L.) and other plant species regenerated from tissue culture has been termed "somaclonal variation" by Larkin and Scowcroft (1981). Larkin et al. (1985) speculated that there are likely to be several mechanisms responsible for somaclonal variation and that further studies at the molecular level are required to elucidate the exact nature of these mechanisms. One possible mechanism is the activation of transposable elements.

Interest in the possible involvement of transposable elements in somaclonal variation comes from observations that such elements can initiate a wide range of chromosomal rearrangements, such as translocations, which are also observed in vitro (Lee and Phillips 1987 a). In addition, the excision of transposable elements is usually imprecise and is associated with additions, deletions and inversions at the site of insertion (Schwarz-Sommer et al. 1985). Thus, plant transposable elements can not only mutate genes by insertion, but they can also create stable mutations upon excision. Such mutations caused by insertion and/or excision would generally be single gene, recessive and nuclear as are most of the somaclonal variants observed with maize (Edallo et al. 1981; Rice 1982; Lee and Phillips 1987 b; Zehr et al. 1987). In addition, sequences homologous to transposable elements are ubiquitous in all maize germplasms that have been examined, whether or not they contain genetically active elements (Federoff et al. 1983; Strommer et al. 1982; Bennetzen et al. 1984; Johns et al. 1985). There have been numerous instances in maize where various types of trauma to the organism or genome, such as ultraviolet light (Neuffer 1966), X-rays (Neuffer 1966), chromosome breakage (Doerschug 1973; McClintock 1984), radiation (Peterson 1960) and virus infection (Friedemann and Peterson 1982; Dellaporta et al. 1984),

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have been associated with the activation of previously silent transposable elements. Each of these treatments has been termed a "genomic stress" by McClintock (1984). McClintock also suggested that the tissue culture process, from cell or tissue isolation to callus formation and plant regeneration, must subject the plant cell to a series of traumatic experiences and thus may also be a genomic stress or shock capable of activating transposable elements.

The activation of the *Ac* element throught the tissue culture process has been shown in several instances by genetic tests of regenerated or the progeny of regenerated plants (Evola et al. 1985; Peschke et al. 1987; Dennis and Brettell 1990) and endosperm cultures (Culley 1986). The frequency of activation ranged from 0% to 80% of the regenerated plants, depending on the genetic background. It is also possible that some transposable elements are activated by one phase of the tissue culture process and become inactivated again before or during plant regeneration and thus would not be detected genetically. For example, in regenerated alfalfa plants (*Medicago sativa* L.), Grouse and Bingham (1986) discovered an unstable white-flowered mutant apparently due to a transposable element. The new allele was only slightly unstable in planta, with only 0.12% reversion back to purple flowers; however in vitro instability was much higher with 23% of the regenerated plants having purple flowers. Many of the maize transposable elements are known to cycle between an active and inactive phase during plant and kernel development (Chomet et al. 1987); elements are known in which the phase of activity depends on their location within a plant or single tissue (Federoff 1983). The transposition of one maize element was shown to vary 5–13 fold according to temperature (Peterson 1958). Thus, the activity of some transposable elements may be determined by the cellular environment (Doerschug 1973). Any relationship between somaclonal variation and transposable element activity, however, has not been established.

This study was conducted to determine if changes in the number and distribution of *Ac*-homologous sequences, which would be indicative of activation, have occurred in the genomes of ten different somaclonal variants of the maize inbred line FR27rhm (Zehr et al. 1987). These mutants were studied since the mutations (mostly single gene, recessive, nuclear) are of the type expected from the insertion and imprecise excision of a transposable element.

Materials and methods

Plant material

Seed of the inbred line FR27rhm (a B73 derivative) was obtained from Illinois Foundation Seeds, Tolono, Ill. Callus cultures of this line were initiated from a single immature embryo; media and procedures for embryogenic culture initiation, maintenance and plant regeneration have been described previously (Duncan et al. 1985). Plants were regenerated (R_0) 49–266 days after culture initiation, and their self-pollinated progeny, both the R_1 (Zehr et al. 1987) and R_2 generations, were evaluated for changes in qualitative characters. The R_2 seedlings chosen for DNA analysis were homozygous for the mutation or were from an R_2 family known to be segregating for a mutation in the case of lethal plant and male sterile since these characters are not observable at the seedling stage. Phenotypic descriptions of the mutants were given by comparison to previously described maize mutants (Neuffer et al. 1968; Neuffer and Sheridan 1980). Seven of the mutants studied possessed segregation patterns consistent with simple, recessive Mendelian inheritance, and included albino, pale green, yellow seedling, japonica, lethal plant, dwarf and male-sterile phenotypes. Three longitudinal leaf striping mutants, which included striate, yellow striate and albino with yellow stripes, segregated at a level lower than expected for a single-gene recessive character. No variants were observed among progenies derived from uncultured plants.

DNA isolation

DNA was isolated from a bulk sample of leaf tissue from three R_2 seedlings or uncultured FR27rhm seedlings by the method of Dellaporta et al. (1983). Three seedlings were necessary to obtain enough DNA from some early lethal mutants such as albino and also to insure a high probability (>98%) of including at least one plant homozygous or heterozygous for the lethal plant and male-sterile mutations. RNA was removed from the final isolated sample by lithium chloride precipitation. The DNA samples were quantified using the 3,5-diaminobenzoic acid dihydrochloride (DABA; Aldrich) fluorescence assay (Setaro and Morely 1976).

Southern blot hybridization analysis

Ten-microgram aliquots of maize DNA were digested to completion with the restriction endonuclease *Eco*RV for 15 h according to the manufacturers specifications (Bethesda Research Laboratories). The digested maize genomic DNA samples were fractionated on 0.6% agarose gels in 200 m*M* glycine, 15 m*M* NaOH and 2 m*M* EDTA (pH 9.0) at 4 V/cm for 5 h. *Hin*dIII-digested bacteriophase lambda was included in separate lanes as a reference size standard. After electrophoresis, the maize DNA was transferred to the charge-modified nylon membrane Biotrace RP (Gelman Science) by the method of Reed and Mann (1985) except that the DNA fragments were not depurinated in 0.25 *M* HCl prior to denaturation. After drying, the blots were placed in microseal bags and prewashed for 1 h at 42 °C in 1 *M* NaCl, 0.5% SDS, 50 m*M* Tris (pH 8.0) and 1 m*M* EDTA. After prewash, the blots were prehybridized for at least 1 h at 42 °C in 50% formamide, 5× SSC (0.15 *M* NaCl, 0.015 *M* sodium citrate), 20 m*M* phosphate buffer (pH 6.8), 5× Denhardt's solution (Denhardt 1966), 1% SDS and 200 µg/ml denaturated salmon sperm DNA. The blots were hybridized for 24 h at 42 °C in 50% formamide, 5× SSC, 20 m*M* phosphate buffer (pH 6.8), 1% SDS, 1× Denhardt's solution, 200 µg/ml denatured salmon sperm DNA and the denatured ^{32}P-labeled probe. The DNA probe used was the internal 1.6-kb *Hin*dIII fragment of the *Ac7* element (Muller-Neumann et al. 1984). This fragment was isolated by fractionation in low-melting temperature agarose (BRL), labeled with ^{32}P-dCTP to a specific activity of 1.7–8.5 Bq/µg according to the methods of Feinberg and Vogelstein (1983, 1984) and separated from the unincorporated nucleotides by spin-column chromatography (Maniatis et al. 1982). After hybridization, the filters were washed three times, 1 h per wash:

in 2× SSPE (0.15 *M* NaCl, 10 m*M* $NaH_2PO_4 \cdot H_2O$, 1 m*M* Na_2EDTA, pH 7.4), 0.5% SDS at 42 °C; in 2× SSPE, 0.5% SDS at 60 °C; and in 0.2× SSPE, 0.2% SDS at 60 °C.

Autoradiography was carried out on Kodak XRP-1 X-ray film at −70 °C with DuPont Cronex Lightning Plus intensifying screens.

Results and discussion

The copy number and location of transposable element sequences can be determined by examining DNA which has been digested with a restriction endonuclease that does not cut within the element itself. The sites that are cut then lie in the flanking DNA sequences that are usually unique for each element insertion. When such a digest is probed with a DNA fragment derived from the element, each insertion is seen as a fragment of unique size. The technique tests for the presence of any DNA fragment containing sequences homologous to the probe and cannot distinguish between full-sized forms of the element and deleted versions of the *Ds*-type unless the deletion has removed most or all of the sequences homologous to the probe. For the transposable element *Ac*, one such restriction endonuclease is *Eco*RV and the probe, the internal 1.6-kb *Hin*dIII fragment from the element itself. *Ac* element transposition can be inferred from the appearance of novel *Ac*-hybridized restriction fragments.

We have used this approach to examine the population of *Ac*-related sequences of the inbred FR27rhm in ten defined somaclonal variants from this inbred. These known variants were studied since one would expect the transposition probability to be highest in such variants if the two phenomenon are related. When *Eco*RV-digested DNA samples from the control seedlings was probed with the 1.6-kb internal *Hin*dIII fragment of *Ac*, several *Ac*-homologous signals could be seen ranging in size from about 5 kb to 20 kb (Fig. 1). Since the smallest of these *Ac*-homologous fragments (5 kb) is greater than that of *Ac* (4.5 kb), all of the signals could represent intact elements. At least some of these homologous but cryptic copies of *Ac*-like DNA are similar in structure to the *Ac* element. This was shown by Southern blots of FR27rhm genomic DNA probed with the 1.6-kb internal *Hin*dIII fragment, which indicated the same 1.6-kb *Hin*dIII and 2.5-kb *Pvu*II fragments as the cloned *Ac7* element (data not shown). In order to determine whether there had been any *Ac* transposition in the somaclonal variants, leaf DNA from all ten variants was analyzed in the same way. Figures 1 and 2 show the patterns seen with the *Ac* probe. In each case the control signal pattern was conserved, indicating that the variants produced in vitro are not correlated with transposition of *Ac* elements.

We do not know from these data whether the lack of *Ac* transposition in these somaclonal variants indicates

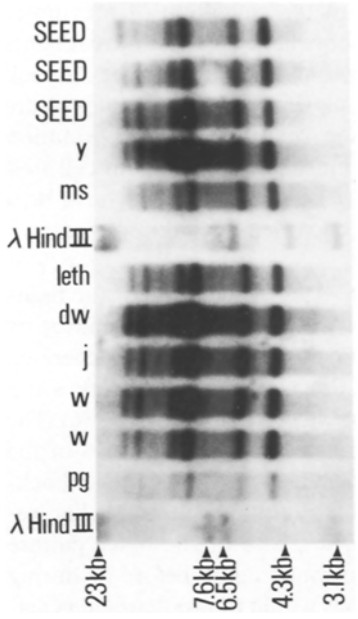

Fig. 1. Genomic blot analysis of *Ac*-homologous DNA in *Eco*RV-cut samples of FR27rhm uncultured and R_2 recessive somaclonal variant seedlings. *SEED* Uncultured FR27rhm, *y* yellow seedling, *ms* male sterile, *leth* lethal plant, *dw* dwarf, *j* japonica, *w* albino, *pg* pale green. The two albino mutants were derived from different regenerated plants. *Lanes 1* and *8* are *Hin*dIII-λ fragments as size standards

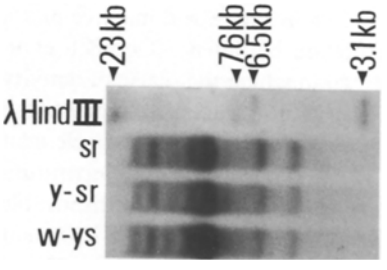

Fig. 2. Genomic blot analysis of *Ac*-homologous DNA in *Eco*RV-cut samples of FR27rhm R_2 somaclonal variants that did not fit a single recessive gene model. *sr* Striate, *y-sr* yellow striate, *w-ys* albino with yellow stripes. *Lane 4* is *Hin*dIII-λ fragments as size standards

that such events are non-existent or rare (and not associated with visible somaclonal variation) in this inbred or that the *Ac*-homologous sequences in this inbred are, in fact, incapable of movement because of deletions of or modifications to the *Ac* sequences. In another study with maize cell culture, Masterson et al. (1988) found that both the copy number and the genomic positions of sequences homologous and similar in structure to the element *Mutator* had not changed after a 40-month period for suspension-cultured cells of the maize inbred line A188. It is recognized that *Ac* is only one among a number of silent but potentially transposable elements which are present

in maize genomes that could have produced the mutations seen in FR27rhm. The discovery of homology to most transposable elements in all maize germplasm has not as yet been followed up by examination at the DNA sequence level. Chandler et al. (1988) found that the sequence of a *Mu*-homologous sequence found in the non-*Mutator* inbred line B37 was identical to the known *Mu*1.4 element; however, it is not known if this type of *Mu* element is capable of independent transposition. Thus, it is not clear how many cryptic but fully functional elements are present in the typical maize genome that are capable of activation under various circumstances. A given genetic background may contain functional copies of only certain element families.

Alternatively, the mechanisms of activation in vitro result, at the single cell level, in the activation of only certain element families or elements in a specific location in the genome. Activation of cryptic transposable elements may be a recognizable consequence of the cells's response to trauma or genomic stress (McClintock 1984), but the mechanism of response and its relationship to all of the numerous and different element families in maize is not yet clear.

Finally, although transposable elements have been shown to be activated in vitro, they may account for only a small amount of the observed mutations. Stable, single gene mutations caused by insertion and excision of transposable elements would be expected to contain short sequence duplications that usually remain after excision (Schwarz-Sommer et al. 1985). Very few tissue culture-induced mutations have been examined at the DNA sequence level. Brettell et al. (1986) and Dennis et al. (1987) identified two variants of the *adh1* gene. Both were due to single base changes of the same kind as occur following other mutagenic treatments; the frequency of nucleotide substitution observed in the *adh1* gene, 2/218 embryos cultured (a total of 1,382 regenerated plants examined), was much higher than the spontaneous mutation rate (<1 in 10^6) and thus could account for most of the somaclonal variation observed in maize.

Further studies are needed on the exact mechanism of transposable element activation and whether there is a general activation of all elements in a given cell or a more specific activation of a certain element family or an element in a certain location in the genome, on the examination of mutations produced in vitro at the DNA sequence level, and on how many of the ubiquitious sequences homologous to transposable elements represent complete elements capable of activation.

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