Theor Appl Genet (1989) 77:383–393

Molecular characterization of *Mutator* systems in maize embryogenic callus cultures indicates *Mu* element activity in vitro

M. G. James and J. Stadler

Department of Genetics, Iowa State University, Ames, IA 50011, USA

Received July 15, 1988; Accepted November 15, 1988
Communicated by F. Salamini

Summary. Active *Mutator* lines of maize (*Zea mays L.*) are characterized by their ability to generate new mutants at a high rate and by somatic instability at *Mutator*-induced mutant alleles. Mutagenically active lines with fewer than ten *Mu* elements per diploid genome have not been observed. Alteration of *Mutator* activity has been shown to correlate with the state of modification of *Hin*fI restiction sites that lie within inverted terminal repeats of *Mu* elements. To determine whether active *Mutator* systems can be established and maintained in culture, copy number and modification state of *Mu* elements were investigated in embryogenic callus lines derived from F_1s of crosses of active *Mutator* stock with the inbred lines A188 and H99. All callus lines studied maintain high *Mu*-element copy numbers, and more than half show a continued lack of modification at the *Mu* element *Hin*fI sites; thus, parameters associated with mutagenic activity in planta are present in some, but not all, callus lines. *Mutator* activity was then tested directly by restriction fragment analysis of subclonal populations from A188/*Mu*^{2} and H99/*Mu*^{2} embryonic cultures. Novel *Mu*-homologous restriction fragments occurred in 38% of the subpopulations which contained unmodified *Mu* elements, but not in control cultures containing modified, genetically inactive *Mu* elements. We conclude that *Mu* elements from active *Mutator* parents can remain transpositionally active in embryogenic cell culture. Active *Mutator* cell lines may be useful for the production of mutations in vitro.

Key words: Transposable elements – *Mutator* – Embryogenic callus culture – Maize

Introduction

In 1978, Robertson described a line of maize characterized by the ability to generate new mutants at a high frequency, 30–50 times the spontaneous level (Robertson 1978). This *Mutator* system was first characterized molecularly by the isolation of a 1376 base-pair (bp) element from an unstable *Mutator*-induced allele of *Adh1* (Bennetzen et al. 1984). Sequence analysis showed that the element, designated *Mu*1, has transposon-like features which include inverted terminal repeats of 213 and 215 bp flanked by 9-bp host sequence duplications (Barker et al. 1984). Hybridization of genomic DNA from *Mutator* lines with *Mu*1 reveals a family of elements of different lengths that are similar in structure and share homology in the terminal repeats (Barker et al. 1984; Walbot et al. 1985; Taylor et al. 1986; Chen et al. 1987; Talbert et al. 1988). Related sequences of this type, collectively termed *Mu* elements, are typically found in 10–70 copies in *Mutator* stocks (Bennetzen 1984). The best-characterized elements, *Mu*1 and *Mu*1.7, are thought to be incapable of autonomous transposition.

There is evidence that *Mu* element transposition occurs by replication (Alleman and Freeling 1986) and that it is developmentally rather than chronologically triggered, taking place either late in the development of the germline (meiotically or postmeiotically) (Robertson 1980, 1981a, 1984) or early in the development of the embryo (Robertson 1985, 1986, 1988). Transpositional activity is retained in 90% of outcross progeny (Robertson 1978) but is typically lost after two to four generations of intercrossing (Robertson 1983). About 40% of all newly induced mutants are somatically unstable (Robertson, personal communication). This instability yields wild-type revertant sectors, due to excision of the *Mu* element from the mutant allele (Taylor and Walbot

1987). Because it is not known whether transposition and excision are related, total *Mutator* activity is usually measured by two criteria: (1) the induction of new mutants, stemming from *Mu* element transposition; (2) somatic mutability of mutant alleles, representing *Mu* element excision events.

Alteration of *Mutator* somatic activity correlates with the state of modification of *Hin*fI restriction sites that lie within the inverted repeats of *Mu* elements. *Mu* elements of *Mutator*-induced mutant alleles that have become somatically stable by intercrossing have been shown to have modified *Hin*fI sites, while these *Hin*fI sites remain unmodified in *Mu* elements of lines showing continued somatic mutability (Chandler and Walbot 1986). Bennetzen and coworkers found that lines with extensive *Mu* element modification had also lost mutagenic activity in subsequent generations (Bennetzen et al. 1987). Mutagenically active *Mutator* lines with fewer than ten *Mu* elements per diploid genome have not been observed (Bennetzen et al. 1987), and stable maintenance of a high *Mu* element copy number from one generation to another is thought to be a feature of an active *Mutator* system (Walbot and Warren 1988).

Research to date with the *Mutator* system has centered primarily on the analysis of differentiated maize tissues in planta, including leaves, seedlings, immature ears, and kernels. However, tissue culture can provide an important extension of studies of certain in planta phenomena. In vitro methods have been used in a limited way to analyze the activity of the *Activator* and *Enhancer* transposable element systems in maize endosperm cultures (Reddy and Peterson 1977; Culley 1986), and more extensively in the analysis of transgenic expression of *Ac* activity in tobacco (Baker et al. 1986). For this study, Type II embryogenic callus lines containing families of *Mu* elements were established from F_1 embryos of crosses of active *Mutator* lines with two maize inbreds. Type II callus lines of maize (Armstrong and Green 1985) are reliably fast-growing, allowing for continued analysis of an expandable pool of tissue which is genotypically identical except for random rearrangements induced by somaclonal variation.

We report the characterization of *Mu* element copy number and *Hin*fI site modification in 13 independently derived embryogenic callus lines. In addition, we present molecular data indicating transposition of *Mu* elements in vitro.

Materials and methods

Seed stocks. *Mutator* stocks were generously supplied by D. S. Robertson, Iowa State University. First-generation *Mutator* stock is designated Mu^2, the progeny of a cross between two standard *Mu* lines (Robertson 1982). Mu^2 lines were used as active *Mutator* parent stock because of their expected high mutation frequency. *Mu* lines intercrossed for four or five generations (Mu^{16} and Mu^{32}) were used as presumptive inactive *Mu* parent lines (Robertson 1983). A188 seed was a gift from R. L. Phillips, University of Minnesota, and H99 seed was a gift from W. A. Russell, Iowa State University.

Crosses. Crosses of maize inbred lines A188 and H99 with *Mutator* lines and the Type II embryogenic cultures derived from each cross are listed in Table 1. Inbreds A188 and H99 were chosen as F_1 parents because of the previously demonstrated ability of the immature embryos of these lines to form embryogenic callus (Green and Phillips 1975; Hodges et al. 1986). Mu^2 parental lines were also outcrossed to standard lines for subsequent tests of *Mutator* activity (Robertson 1978).

Culture initiation and maintenance. Cultures of 9- to 12-day embryos (approximately 1–2 mm in length) were initiated by standard methods (Green and Phillips 1975) on N6 basal medium (Chu et al. 1975) supplemented with 2% (w/v) sucrose, 0.74 mg/l 2,4-dichlorophenoxyacetic acid (2,4D), 6 m*M* asparagine, 5.5 m*M* myoinositol, 12 m*M* proline, and 0.3% Gelrite (Kelco). One month after culture initiation, the proline concentration was reduced to 6 m*M*. Cultures were incubated at 28 °C in the dark. After 2 weeks, all embryos were moved without selection to fresh medium. Type II embryogenic callus (Armstrong and Green 1985) was thereafter selected and propagated biweekly, and the remaining tissue from each line was saved and stored at −20 °C (Fig. 1).

DNA isolation and Southern blot analysis. Genomic DNA was isolated from frozen embryogenic callus tissue by the method of Rivin et al. (1982). After purification by ultracentrifugation, DNA concentrations were determined spectrophotometrically. Restriction enzymes were obtained from Bethesda Research Laboratories and New England Biolabs and were used according to the suppliers' instructions. Approximately 5 µg of high-molecular-weight DNA was digested with 3–5 units of restriction enzyme/µg DNA and subjected to electrophoresis through 0.8% agarose gels. UV-nicked DNA fragments were transferred to Genetran (Plasco) by the procedure of Southern (1975). After baking for 2 h at 80 °C under vacuum and washing for 2 h at 65 °C in 0.1 × SSC, 0.1% SDS, hybridizations were carried out in 50% formamide, 5 × Denhardt's, 3 × SSC, 100 µg/ml denatured salmon sperm DNA, 1% SDS, 5% dextran sulfate, and $\sim 2 \times 10^7$ cpm ^{32}P-labeled DNA at 42 °C for 36 h. Filters were washed in 50% formamide, 5 × SSC, 0.2% SDS for 30 min at 42 °C, then washed three times for 45 min each in 0.1 × SSC, 0.1% SDS at 65 °C with agitation and exposed to Kodak XAR-5 film with a Lightening Plus intensifying screen (DuPont) at −70 °C for 2–5 days. Filters were prepared for rehybridization by removal of probe in washings at 95 °C in 0.1 × SSC, 0.1% SDS.

Probes. The *Mu*1 probe used was a 959 base pair *Mlu*I fragment isolated from pJMA4, which contains a clone of the entire *Mu*1 element. This fragment contains 944 base pairs of the internal portion of the *Mu*1 element and 15 base pairs from one of the inverted terminal repeats (Fig. 2d). To check for complete digestion of DNA samples, blots were stripped and rehybridized to a 9-kb maize ribosomal tandem repeat probe isolated from pZmR1 by digestion with *Eco*RI. pZmR1 was kindly supplied by D. Grant, Pioneer Hi-bred International. Probes were labeled with ^{32}P by the random hexamer (Pharmacia) primer reaction (Feinberg and Vogelstein 1983).

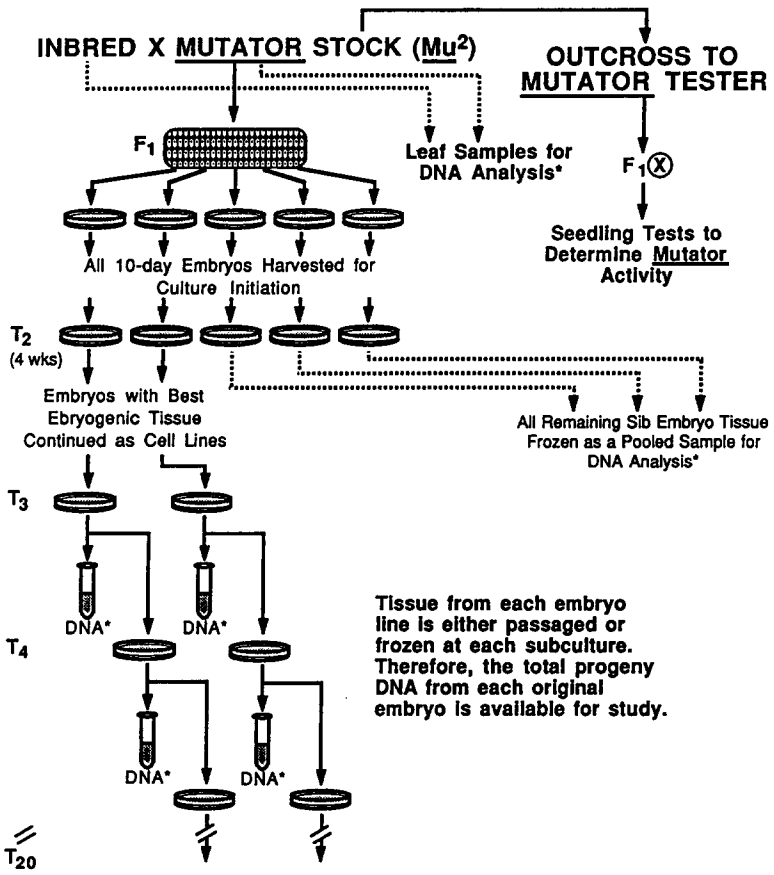

Fig. 1. Scheme for propagation of embryogenic cell populations and collection of samples for molecular analysis. Inbred A188 or H99 plants were crossed with *Mutator* stock as described in Table 1. Embryos harvested at approximately the 10-day stage from F_1 progeny ears were initiated in culture on N6 medium supplemented as described (Materials and methods). Embryos with the best Type II embryogenic callus were continued as callus lines at the second transfer (T_2) while nonselected embryos were pooled and stored at −20 °C for subsequent molecular analysis. All tissue from each selected callus line was either transferred to fresh medium or stored at −20 °C at each subculture. Leaves from the 4- to 7-leaf stage of the plant or immature second ears were used for molecular analysis of inbred and *Mutator* parental plants. All *Mutator* parents were outcrossed to standard lines. The F_2 progeny of these crosses were used in the test for *Mutator* activity as described (Results)

Table 1. *Mu*-element *Hin*fI-site modification in F_1 hybrid callus cultures

Cross	New mutant frequency[a]	*Mu* Parent modification state[b]	F_1 ear	Modification of pooled sib embryos[b]	Embryo line	Modification state[b] (~6 mo. samples)
A188/*Mu*²						
3579-3 × 021-1	7%	na	3579-3	na	3579-3A	−
3579-16 × 021-1	7%	na	3579-16	na	3579-16C	−
646-8 × 207-8	12%	−	646-8	±	646-8B	−
					646-8C	±
					646-8D	+
643-9 × 863-8	30%	−	643-9	−	643-9B	−
H99/*Mu*²						
607-4 × 606-3	na	na	607-4	na	607-4A	+
					607-4B	−
					607-4C	−
647-8 × 845-6	4%	−	647-8	±	647-8A	±
					647-8B	±
657-2 × 846-3	0	na	657-2	+	657-2A	+
					657-2C	+
H99/*Mu*[16 or 32]						
666-2 × 640-8	na	na	666-2	+	666-2A	+
666-4 × 640-9	na	na	666-4	+	666-4A	+
666-6 × 840-5	na	na	666-6	+	666-6A	+

[a] Percent new seedling mutants in F_2 seeds (Robertson 1981b)

[b] + all *Mu* elements in this sample were modified at the *Hin*fI sites; ± some of the *Mu* elements were modified and some were unmodified at the *Hin*fI sites; − none of the *Mu* elements were modified at the *Hin*fI sites; na data not available

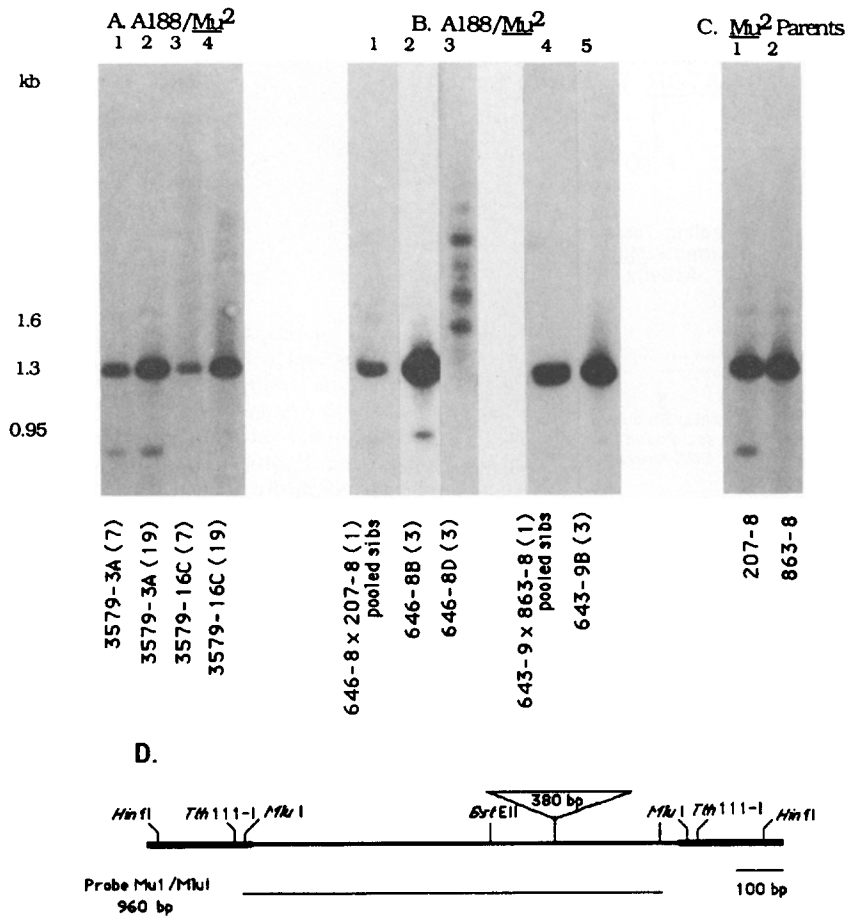

Fig. 2A–D. *Hin*fI digestions of genomic DNA from A188/*Mu*^{2} embryogenic cultures and *Mu*^{2} parent plants (number in parentheses indicates months in culture). DNA samples (5–8 μg) were digested with *Hin*fI, electrophoresed through 0.8% agarose gels, transferred to a nylon membrane, and hybridized with the fragment of *Mu*1 (*Mu*/*Mlu*I) shown on the restriction map **D**. **A** DNA from tissue samples of A188/*Mu*^{2} callus line 3579-3A, isolated after 7 months and 19 months in culture, was digested with *Hin*fI (*lanes 1* and *2*); DNA from tissue samples of line 3579-16C, taken at the same time points, was similarly digested (*lanes 3* and *4*). **B** *Hin*fI digestions of DNA from tissue samples of pooled sib embryos (pooled at T_2 and T_3) of the crosses 646-8 × 207-8 (*lane 1*) and 643-9 × 863-8 (*lane 5*); *Hin*fI digestions of DNA from individual embryogenic callus lines 646-8B (*lane 2*), 646-8D (*lane 3*), and 643-9B (*lane 5*). **C** DNA from immature leaf samples of *Mutator* parent plants 207-8 (*lane 1*) and 863-8 (*lane 2*) was digested with *Hin*fI

Results

Experimental design: scheme for propagation of embryogenic cell populations and collection of samples for molecular analysis

Progeny embryos from each inbred/*Mutator* F_1 ear were cultured (T_0) and examined after 4 weeks (T_2) and 6 weeks (T_3) for the appearance of Type II embryogenic callus. At T_2 and T_3, tissues from embryos with the best Type II embryogenic morphology were preserved and propagated as separate embryogenic callus lines, whereas all other sib embryos and nonembryogenic tissues were pooled and stored at –20 °C (Fig. 1). Beginning with T_4, the best Type II callus from each individual line was transferred and propagated, and nonselected tissues were stored separately at –20 °C for subsequent molecular analysis. In this manner, all tissue descending from each embryo population was saved in the fresh or frozen state. DNA isolated from sib embryos pooled at 4 and 6 weeks (T_2 and T_3) was analyzed molecularly to give the earliest possible characterization of *Mu* elements in an embryogenic callus culture.

To determine the effects of culturing on *Mu* element copy number and modification state, it was necessary to make comparisons with parental plant controls. Leaf samples taken at the four- to seven-leaf stage from *Mutator* parents were used for molecular analysis of *Mu* element copy number and *Hin*fI-site modification (Table 1). In addition, all *Mu*^{2} parent plants were tested genetically for *Mutator* activity (Robertson 1981 b). In this test, *Mu*^{2} plants were outcrossed to a standard line, and at least 50 F_1 seeds were planted and self-pollinated in the next growing season. Approximately 50 seeds from each F_2 ear were then planted in a sand bench and screened for seedling mutants. The observed mutation frequency (percentage of F_1 plants segregating for seedling mutants observed) is used as a measure of *Mutator* activity. By this criteria, *Mutator* parent plants 207-8, 863-8, and 647-8 were mutagenically active. In contrast, *Mutator* parent plant 657-2 generated no new seedling mutants (Table 1).

The number of *Mu*1-hybridizing sequences (endogenous *Mu* elements) in each inbred parent line was determined by analysis of genomic DNA isolated from leaf samples of A188 and H99 plants at the four- to seven-leaf stage. A188 was found to have two resident *Mu*-hybridizing sequences and, H99, one (data not shown).

Six A188/*Mu*^{2}, seven H99/*Mu*^{2}, one H99/*Mu*^{32}, and two H99/*Mu*^{16} embryogenic lines were chosen for molecular analysis because of their excellent Type II callus phenotype and fast growth.

Mu element copy number

Determination of *Mu* element copy number in these F_1 embryogenic callus lines was made in two ways. First, copy number reconstructions of 10, 20, and 50 copies of *Mu* elements were established from the *Mu*1-containing plasmid pJMA4. For callus lines with unmodified *Mu* elements, visual density comparisons were then made between these copy reconstructions and the 1.3-kb *Mu* 1 element bands generated by digestion with *Hin*fI and hybridization with the *Mu* probe. Similar comparisons were also made by digesting DNA from callus cultures with modified *Mu* elements with the enzyme *Tth*111I, another enzyme for which there are restriction sites in the *Mu* element inverted repeats (Fig. 2D). Although there is no published information on the specific effect of 5-methyl-deoxycytosine on cleavage by *Tth*111I, otherwise-modified *Mu* elements have been cleaved with this enzyme (Chandler and Walbot 1986). These methods allow crude estimates of copy number for both unmodified and modified *Mu* elements. Second, because the *Mu* elements are normally dispersed throughout the genome, digestion of DNA with enzymes that cleave outside the *Mu* 1 elements yields a ladder of discrete bands upon hybridization with *Mu* 1. A count of the number of these *Mu*1-hybridizing bands provides a more accurate assessment of copy number. With these two methods, we determined that all A188/*Mutator* and H99/*Mutator* embryogenic callus lines used in this study have approximately 15–25 copies of randomly dispersed *Mu*1-hybridizing elements.

Modification state of Mu elements in F_1 embryogenic callus lines

The most common type of modified nucleoside in plant DNA is 5-methyl-deoxycytosine (Grierson 1977). In maize, approximately 25% of the deoxycytosine residues are methylated, primarily at CpG or CpNpG sites (Hake and Walbot 1980). Although there are conflicting reports on the methylation sensitivity of *Hin*fI (McClelland and Nelson 1985; Gruenbaum et al. 1981), Chandler and Walbot (1986) suggest that the modification seen at *Mu* element *Hin*fI sites is deoxycytosine methylation.

Digestion of DNA with *Hin*fI and subsequent hybridization with a *Mu* 1 probe result in discrete 1.3-kb and 1.6-kb bands which represent the *Mu* 1 and *Mu* 1.7 elements, respectively, when the *Mu* element *Hin*fI sites are unmodified. However, if the cytosine residues within the *Hin*fI recognition sequences are methylated, the enzyme will fail to cleave the DNA at those sites. In this case, hybridization with the *Mu* 1 probe yields a ladder of bands larger than 1.3 kb because of *Hin*fI digestion at unmodified restriction sites external to the *Mu* elements. When such modification was observed, DNA from the same line was digested with *Tth*111I. The absence of higher molecular weight bands homologous to *Mu* 1 upon digestion with *Tth*111I indicates that the ladder of bands seen with *Hin*fI digestion is due to *Mu* element modification, rather than the presence of larger sized *Mu*1-homologous elements in the genome.

The state of *Mu* element *Hin*fI modification varies in the 13 F_1 cultures examined (Table 1). Among the six A188-derived embryogenic callus lines, one culture (646-8D, Fig. 2B, lane 3) has *Mu* elements which are uniformly modified at these restriction sites, one has a mixed population of *Mu* elements with both modified and unmodified *Hin*fI sites (646-8C, data not shown), and four have only *Mu* elements which are unmodified at the *Hin*fI sites (3579-3A and 3579-16C, Fig. 2A; 643-9B and 646-8B, Fig. 2B). Similar variation occurs when H99 is the contributing inbred parent (Fig. 3A and B). Although the sample size is small, these data indicate that the genetic contribution of neither inbred parent is sufficient to provide strong, uniform regulation of the state of *Hin*fI modification in F_1 hybrid cultures.

As expected, those callus lines generated by crossing H99 with inactive *Mutator* parents (*Mu*^{16} and *Mu*^{32}) show complete *Hin*fI modification of *Mu* elements, as seen in outcrosses of *Mutator* lines previously made inactive by intercrossing (Bennetzen 1987) (Fig. 3C, lanes 1–3).

Mu element modification state is stable over time in culture

Two A188/*Mutator* callus lines (3579-3A and 3579-16C) have been in continuous culture for almost 2 years. For line 3579-3A, comparison of samples taken after 7 and 19 months in culture showed that complete absence of *Hin*fI modification of *Mu* elements persisted (Fig. 2A, lanes 1 and 2). Similar examination of line 3579-16C after 7 and 19 months also indicated stability of modification state; however, the appearance of two faint, high molecular weight bands in the later sample indicated that some modification of *Mu* elements may have taken place after many months of growth in vitro (Fig. 2A, lanes 3 and 4).

Mu element modification in sibling H99/*Mu*^{2} lines (607-4A and 607-4B) was also examined after 3, 6, and 9 months of culturing. At all three time points, the *Mu* elements in line 607-4B remained uniformly unmodified (Fig. 3A, lanes 1–3), whereas sibling line 607-4A retained approximately 24 copies of *Mu* elements modified at *Hin*fI (Fig. 3A, lanes 4–6).

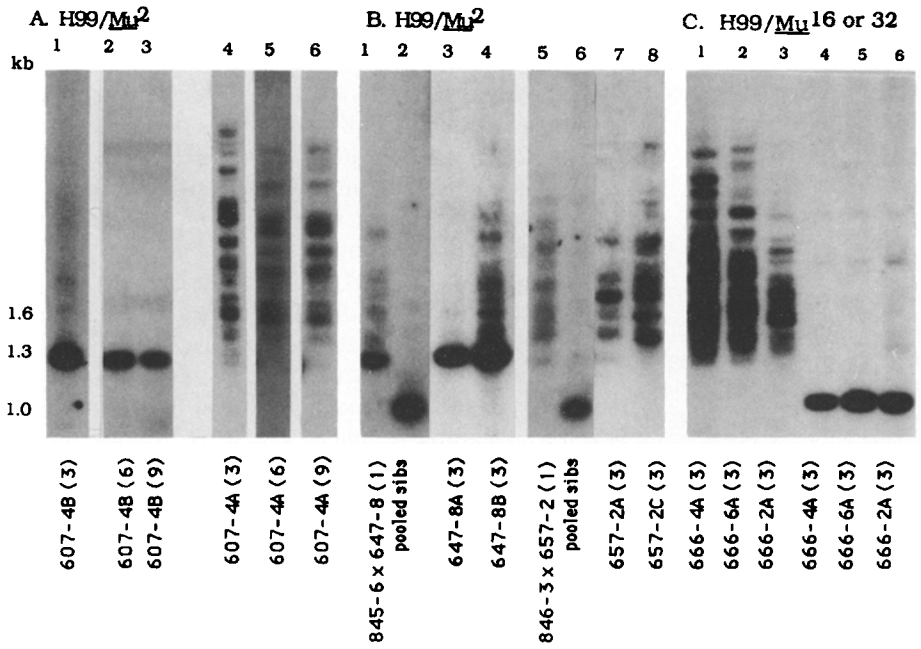

Fig. 3A–C. *Hin*fI and *Tth*111I digestions of genomic DNA from H99/*Mutator* embryogenic callus cultures (number in parentheses indicates months in culture). DNA samples (5–8 μg) were digested with *Hin*fI or *Tth*111I and treated as described in Fig. 2. **A** *Lanes 1–3* show *Hin*fI digests of DNA from tissue of line 607-4B isolated after 3, 6, and 9 months in culture. *Lanes 4–6* show *Hin*fI digests of DNA from tissue samples of sibling line 607-4A after 3, 6, and 9 months in culture. **B** *Hin*fI and *Tth*111I digests of DNA from pooled sib embryos from the crosses 845-6 × 647-8 (*lanes 1* and *2*, respectively), and 846-3 × 657-2 (*lanes 5* and *6*, respectively); *Hin*fI digests of descendent H99/Mu^2 embryogenic callus lines (sibling lines 647-8A and 647-8B in *lanes 3* and *4*; sibling lines 657-2A and 657-2C in *lanes 7* and *8*). **C** *Hin*fI and *Tth*111I digests of DNA from H99/Mu^{16} callus lines 666-4A (*lanes 1* and *4*, respectively) and 666-2A (*lanes 3* and *6*, respectively), and H99/ Mu^{32} line 666-6A (*lanes 2* and *5*, respectively)

The basic stability of the initial state of modification in almost all lines examined suggests that continued culturing does not generally reverse modification patterns that were established early in culture.

Comparison of Mu element modification in parent plants and F_1 cultures

The experimental plan used in this study allowed comparisons of *Mu* element modification and mutagenic activity in the Mu^2 parent with the modification state of *Mu* elements in the pooled F_1 sib embryos (T_2 and T_3) as well as with individual callus lines established from the same ear (Table 1). Embryos derived from crosses involving Mu^2 parents with significant mutagenic activity, and/or families of *Mu* elements unmodified at the *Hin*fI sites, do give rise to callus cultures with stably inherited, unmodified *Mu* elements; for example, line 643-9A (Table 1). This parallels the observation that *Mu* elements of F_1 plants in the first outcrossed generation remain unmodified (Bennetzen 1987). In two instances, however, there is discordance between the state of *Mu* element modification in the Mu^2 parent and the descendant callus cultures. In line 646-8D and in sibling lines 647-8A and B (Table 1), mutagenically active Mu^2 parents with unmodified *Mu* elements gave rise to F_1 embryogenic calli with modified elements. When this unexpected modification occurred, it was detected in pooled sib embryos in culture as early as 38 days after fertilization (28 days after explant).

New Mu-hybridizing restriction fragments in subclonal populations of A188/Mu^2 and H99/Mu^2 callus lines

New transposon insertions in planta are detected by the appearance of a new, usually mutable, phenotype. In cell culture, when such phenotypic evidence is absent, *Mu* element transposition must be inferred from the appearance of novel *Mu*-hybridizing restriction fragments. As a result of our procedures, in which selected tissues are propagated biweekly and nonselected tissues are stored for molecular analysis (Fig. 1), inoculum for each new stock transfer often includes less than 10% of the cell population in later passages, and in early passages (T_2 and T_3), often much less than 1%. This culture method causes population "bottlenecking", so that random events which occur in only a few cells may be lost (Cavalli-Sforza and Bodmer 1971; Holliday et al. 1977).

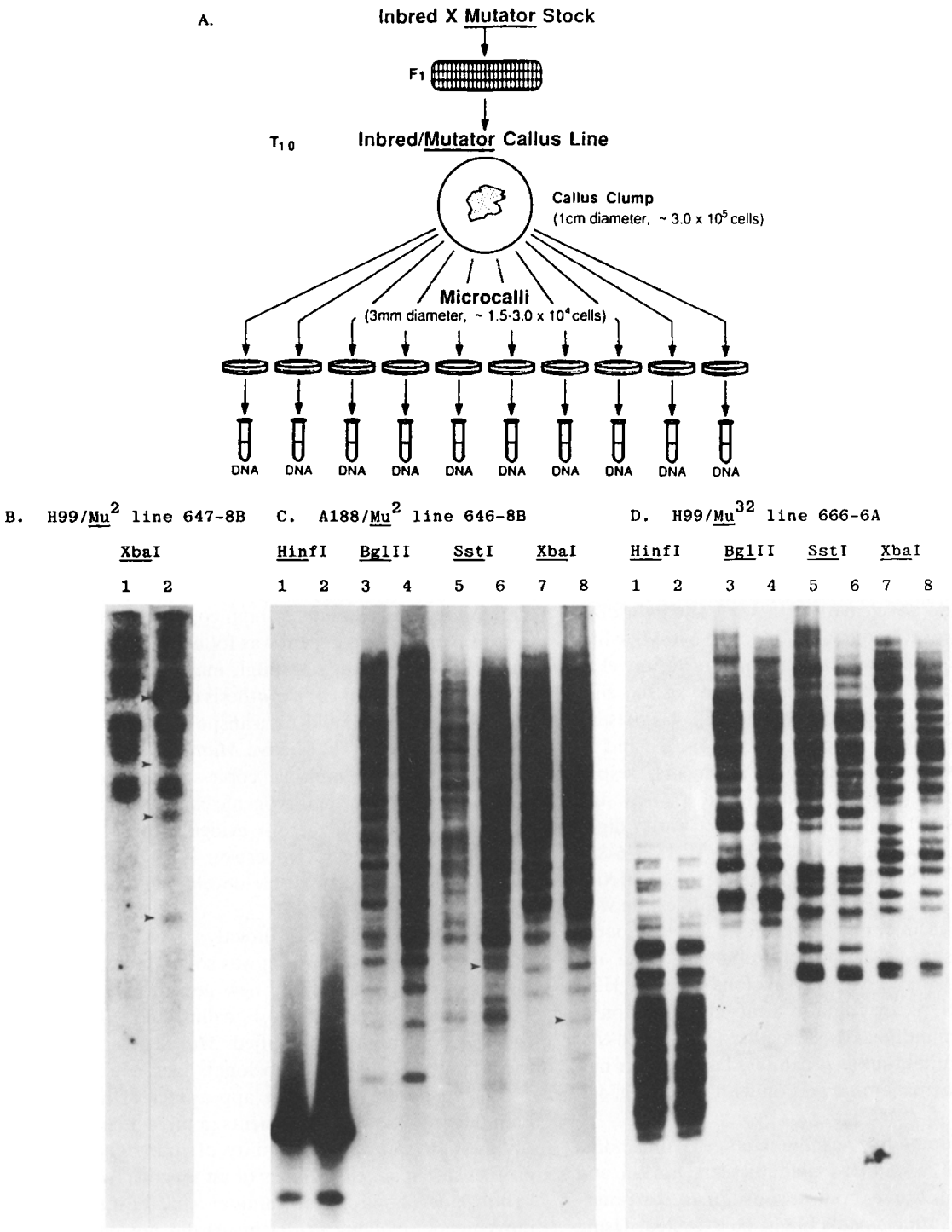

Fig. 4. **A** Scheme for the propagation of embryogenic callus subclonal lines. After 2.5 months in culture (T_{10}), one callus clump ($\sim$1 cm diameter, containing $\sim 3 \times 10^5$ cells) was subdivided into ten microcalli. Each 3-mm diameter microcallus contained $\sim 3 \times 10^4$ cells. Tissue from each microcallus was propagated until at least 10 g of tissue could be harvested (4 weeks). DNA was isolated from all tissue harvested from each line and digested in single digests with *Hin*fI, *Bgl*II, *Sst*I, and *Xba*I. **B** Comparison of *Xba*I digests of two subclonal lines from H99/Mu^2 callus line 647-8B upon hybridization with the *Mu*1/*Mlu*I probe. *Arrows* denote novel restriction fragments in one subclonal line (*lane 2*) ($\sim$9.5 kb, 5.9 kb, 3.1 kb, and 2.9 kb). **C** Comparison of digests of two subclonal lines from A188/Mu^2 646-8B callus line (subline 646-8B1 in *lanes 1, 3, 5,* and *7*; subline 646-8B2 in *lanes 2, 4, 6,* and *8*). The *Mu*1/*Mlu*I probe was used for hybridization. *Arrows* denote novel restriction fragments in subline 646-8B2 upon digestion with *Sst*I ($\sim$2.75 kb) and *Xba*I ($\sim$2.15 kb) (*lanes 6* and *8*). **C** Two subclonal lines from H99/Mu^{32} 666-6A are similarly compared using the same probe (subline 666-6A1 in *lanes 1, 3, 5,* and *7*; subline 666-6A2 in *lanes 2, 4, 6,* and *8*). *Lanes 1* and *2* show that *Mu* elements are completely modified at the *Hin*fI sites. There are no alterations in the hybridization profiles of these subclonal lines

Thus, a de novo transposition event which occurred in one cell and was transmitted to only a few cell descendants in the 2 weeks between subculture would have a 90% probability of being excluded from transfer by exile to storage. In this instance, it is likely that the low copy number of newly generated restriction fragment polymorphisms in stored tissues would be beneath the limits of detection by Southern analysis.

To avoid the stochastic problems caused by bottlenecking, subclonal populations of A188/*Mu*^{2}, H99/*Mu*^{2}, and H99/*Mu*^{32} callus lines were established and grown without selection or storage of tissue (Fig. 4A). A small (1-cm) callus clump from a young culture (T_{10}, 20 weeks) exhibiting a good Type II embryogenic phenotype was subdivided into ten microcalli, each 3 mm in diameter (with approximately $1.5-3 \times 10^4$ cells). All progeny callus in each of these subclonal lines was cultured and propagated until 10 to 17 g of tissue could be harvested. During this time, each microcallus line underwent at least 11 population doublings to produce about 3×10^7 cells. DNA isolated from each of these terminal samples was digested in single digests with *Bgl*II, *Sst*I, and *Xba*I, enzymes which do not cleave within *Mu*1, and hybridized with the *Mu*1 probe. In the A188/*Mu*^{2} line 646-8B, which contains unmodified *Mu* elements, one to four novel restriction fragments were detected in three of the eight subclonal lines examined. To illustrate, Fig. 4C presents a comparison of two subclonal lines (646-8B1 and 2), without and with new restriction fragments, respectively. In 646-8B2 (Fig. 4C, lanes 6 and 8), new *Mu*-homologous restriction fragments were seen after digestion with *Sst*I and *Xba*I, but not with *Bgl*II. In 646-8B1 (Fig. 4C, lanes 3, 5 and 7), the patterns of restriction fragments seen after *Sst*I, *Xba*I, and *Bgl*II digestion were identical to those in four other sister subclonal populations which showed no novel fragments (data not shown). Similar analysis of the subclonal lines of H99/*Mu*^{2} line 647-8B, which contains a mixed population of modified and unmodified *Mu* elements, also showed that three of eight of these sublines exhibited one to four new restriction fragments upon digestion with *Xba*I (Fig. 4B, lanes 1 and 2).

Because random chromosomal changes (somaclonal variation) occur in cultured plant tissues (Larkin and Scowcroft 1981), some nontranspositional rearrangements involving *Mu* elements might be expected. Using Southern analysis, these chromosomal alterations might also be detected as *Mu* restriction fragment polymorphisms. To investigate this possibility, subcultures of a callus line with an inactive *Mutator* system were established as described. In these sublines, new *Mu*-hybridizing restriction fragments would be expected to occur because of somaclonal variation rather than *Mu* element transposition. Gel blot analysis of the DNA of ten subclonal lines from H99/*Mu*^{32} (a fifth-generation intercrossed line with *Mu* elements modified at the *Hin*fI sites) showed no change in the *Mu1* hybridization profiles. Genomic DNAs were also digested with *Sst*I, *Xba*I, and *Bgl*II, as well as with *Hin*fI, which acts as an external-cleaving enzyme when *Mu* elements are modified. Examples of this uniformity of *Mu* insertion pattern are shown in Fig. 4D. Such stability indicates that rearrangements of *Mu*-homologous fragments due to somaclonal variation probably did not occur in these subclonal lines during the 11 population doublings tested.

Discussion

The establishment and characterization of independent maize embryogenic cultures with potentially active *Mutator* systems is described. These cell lines were derived from F_1 progeny of mutagenically active *Mu*^{2} plants crossed with inbred lines capable of producing embryogenic callus. In these cultures, transmission of unmodified *Mu* elements in high copy number from the parent plants to the F_1 calli was followed by stable transmission of these traits through many rounds of tissue transfer. Support for the hypothesis that the *Mu* elements in these cultures should be transpositionally active lies first in the fact that, like active *Mutator* systems in planta, they contained multiple copies of unmodified *Mu* elements. Also, since embryogenic callus lines arise from early tissue in which there is evidence for *Mu* element transposition in planta (Robertson 1985, 1986, 1988), it was felt that these compatible developmental influences might persist in culture.

To test this hypothesis directly, molecular evidence for *Mu* element transposition was sought in experiments designed to fix and amplify new genomic insertions in small subpopulations of two F_1 callus lines (646-8B and 647-8B) containing unmodified *Mu* elements. Within each family of parallel subclonal lines, transposition events were indicated by the appearance of novel *Mu*-homologous restriction fragments in three of eight of the sublines. In contrast, uniformity of four hybridization profiles in ten subclonal lines of an inactive *Mu*/H99 F_1 hybrid line showed that random chromosomal rearrangements involving *Mu* elements did not occur. This lack of rearrangement in the latter control cultures suggests that the novel bands seen in the A188/*Mu*^{2} and H99/*Mu*^{2} subclonal lines represent the transposition of *Mu* elements to new positions in the genome during culture.

An alternative explanation for the origin of the novel *Mu*-hybridizing restriction fragments in A188/*Mu*^{2} and H99/*Mu*^{2} subclonal lines is that the progenitor tissue of the embryogenic cell line may have been multicellular

rather than unicellular in origin, and that this tissue might therefore have been mosaic for *Mu* insertion patterns. Possible mosaicism for genomic *Mu* insertion pattern within each F_1 embryogenic culture established from a small amount of embryonic tissue is made unlikely, however, by the described method of culture in early tissue transfers. Severe bottlenecking not only tends to eliminate low level de novo events but also contributes to homogeneity in the remaining cell population. Also, founder effect (Cavalli-Sforza and Bodmer 1971) in successive subcultures preceding T_{10} should have revealed latent mosaicism. Southern analysis of all tissues before T_{10} (4 g in five time samples), however, showed that none of the novel restriction fragments seen in the sublines could be detected in the DNA of these earlier tissues (data not shown). The observed absence of *Mu* restriction pattern variations in these early cell generations further indicates that the experiments with nontruncated subcultures have allowed retention and detection of new *Mu*-hybridizing fragments which occurred spontaneously during their growth.

The correlation between absence of *Mu* element modification and *Mutator* activity observed in planta (Chandler and Walbot 1986) was also demonstrated in vitro by the finding that only lines containing unmodified *Mu* elements exhibited new restriction fragments (Fig. 4). The role of modification in the regulation of *Mutator* activity is not clearly understood. For example, it is not known whether methylation results in the loss of *Mutator* activity or, conversely, whether an inactive *Mutator* system allows methylation. Bennetzen (1987) suggests that sequences within *Mu*1-like elements may be genetically active and that modification may block transcription of factors necessary for transposition. Alternatively, he suggests that the modification of *Mu* elements may interfere with their ability to interact appropriately with a trans-acting transposase.

Because it has been shown that progeny from outcrosses of *Mutator* stock have *Mu* elements unmodified at the *Hin*fI sites (Bennetzen 1987), it was expected that all of the embryogenic cultures established from the F_1 progeny of an outcross between an active *Mutator* line with H99 or A188 would contain similarly unmodified *Mu* elements. The finding that many of the *Mu* elements in approximately half of the cultures from active Mu^2 parents were modified at the *Hin*fI sites is surprising and suggests that this change may be due to conditions related to the establishment of cultures from maize embyos. It is known that the culturing of plant tissues results in increased genetic variability (Larkin and Scowcroft 1981). Genetic and cytogenetic changes such as aneuploidy, chromosomal rearrangement, and increased frequency of recombination have been well documented (review, Larkin et al. 1985), and more subtle molecular effects have also been characterized. For example, Brown and Lorz (1986) report that generalized changes in methylation of genomic DNA are triggered by tissue culture stress, and they suggest that these methylation changes may contribute to somaclonal variation. Also, some still uncharacterized conditions of plant cell culture are known to activate previously silent controlling elements. Peschke and Phillips (1987) report the induction of *Ac* activity in somaclones regenerated from embryogenic cultures derived from parent plants with cryptic *Ac*, and Groose and Bingham (1986) describe the in vitro activation of an unstable somaclonal mutant for flower color in alfalfa.

Our findings indicate that modification of the *Mu* element *Hin*fI sites, when seen, was an event which probably occurred before 4 weeks in culture and that the state of transposon modification was not altered further during the succeeding year of cell growth. This suggests that an initial shock, caused by the explant of the embryo and/or its transfer to the in vitro environment, may have triggered the modification events. The in vitro generation of somatic mutability in alfalfa (Groose and Bingham 1986) also occurred at the onset of the culturing process. An alternative interpretation is that modification of the *Mu* element *Hin*fI sites in these cultures may have resulted from the developmental stage of the tissue cultured, rather than from the culturing process per se. Research with mammalian cells has shown that most cell types are capable of methylating CpG sequences but that early embryonic cells in culture are particularly active in this respect (review, Bird 1986). Bird (1986) and others speculate that the de novo methylating activity in cells may be higher than usual in the embryo or that the methylation may be the consequence of inactivity of certain genes at this stage.

It is interesting to note that five of the eight F_1 cell lines obtained from crosses of Mu^2 parents which were genetically positive for mutagenic activity (and contained unmodified *Mu* elements when tested) had complements of *Mu* elements which were either entirely unmodified or, conversely, entirely modified. This indicates that the factor(s) regulating modification in vitro acted in those instances to produce a cell-wide event. Although the three remaining cultures from active Mu^2 parents show a mixture of modified and unmodified elements, this may only reflect a mixed cell population rather than a mixture of states of modification within individual cells. The segregation of *Mu*-element modification states between F_1 cultures, including those from the same inbred background or the same ear (Table 1), suggests either the segregation of a regulator from the Mu^2 hybrid parent or the operation of subtle environmental influences upon the early development of individual immature embryos before explant.

Active *Mutator* systems in maize culture have at least two useful applications. First, it should be possible to

take advantage of the mutagenic activity of the *Mutator* system previously demonstrated in planta. Mobile *Mu* elements in these cell lines may be very effective as mutagens and subsequent gene tags in the production of culture-selectable maize mutants. Mutants of maize cell physiology will strongly complement the extensive genetic knowledge of this organism derived from mutant genes obtained in the field. Also, additional studies of the *Mutator* system per se, especially the effects of environmental stress on regulation of *Mu* element modification and activity, will be possible with these cell cultures.

Acknowledgements. We are grateful to D. S. Robertson for his advice and for his assistance in the planting of stocks. We also thank C. Matthews and M. Leonard for expert technical assistance. This is journal paper No. J-12840 of the Iowa Agriculture and Home Economics Experiment Station, Ames/IA, Project No. 2722.

References

Alleman M, Freeling M (1986) The *Mu* transposable elements of maize: Evidence for transposition and copy number regulation during development. Genetics 112:107–119

Armstrong CL, Green CE (1985) Establishment and maintenance of friable, embryogenic maize callus and the involvement of L-proline. Planta 164:207–214

Baker B, Schell J, Lorz H, Fedoroff N (1986) Transposition of the maize controlling element "Activator" in tobacco. Proc Natl Acad Sci USA 83:4844–4848

Barker RF, Thompson DV, Talbot DR, Swanson JA, Bennetzen JL (1984) Nucleotide sequence of the maize transposable element *Mu*1. Nucleic Acids Res 12:5955–5966

Bennetzen JL (1984) Transposable element *Mu*1 is found in multiple copies only in Robertson's Mutator maize lines. J Mol Appl Genet 2:519–524

Bennetzen JL (1987) Covalent DNA modification and the regulation of *Mutator* element transposition in maize. Mol Gen Genet 208:45–51

Bennetzen JL, Swanson JA, Taylor WC, Freeling M (1984) DNA insertion in the first intron of maize *Adh1* affects message levels: Cloning of progenitor and mutant *Adh1* alleles. Proc Natl Acad Sci USA 18:4125–4128

Bennetzen JL, Cresse A, Brown WE, Lee L (1987) Molecular cloning of maize genes by transposon tagging with *Mutator*. UCLA Symp Mol Cell Biol (in press)

Bird AP (1986) CpG-rich islands and the function of DNA methylation. Nature 321:209–213

Brown PTH, Lorz H (1986) Molecular changes and possible origins of somaclonal variation. In: Semal J (ed) Somaclonal variations and crop improvement. Martinus Nijhoff, Boston, pp 148–159

Cavalli-Sforza L, Bodmer W (1971) Genetics of human populations. Freeman, San Francisco, pp 416–418

Chandler VL, Walbot V (1986) DNA modification of a maize transposable element correlates with loss of activity. Proc Natl Acad Sci USA 83:1767–1771

Chen C-H, Oishi KK, Kloeckener-Gruissem B, Freeling M (1987) Organ-specific expression of maize *Adh1l* is altered after a *Mu* transposon insertion. Genetics 116:469–477

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Culley DE (1986) Evidence for the activation of a cryptic transposable element *Ac* in maize endosperm cultures. In: VI Intl Congr Plant Tissue Cell Culture Abstr. University of Minnesota, Minneapolis, p 220

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Green CE, Phillips RL (1975) Plant regeneration from tissue cultures of maize. Crop Sci 15:117–121

Grierson D (1977) The nucleus and the organization and transcription of nuclear DNA. In. Smith H (ed) The molecular biology of plant cells, vol 14. Blackwell, Oxford, pp 213–255

Groose RW, Bingham ET (1986) An unstable anthocyanin mutation recovered from tissue culture of alfalfa (*Medicago sativa*). Plant Cell Rep 5:104–110

Gruenbaum Y, Cedar H, Razin A (1981) Restriction enzyme digestion of hemimethylated DNA. Nucleic Acids Res 9:2509–2515

Hake S, Walbot V (1980) The genome of *Zea mays*. It's organization and homology to related grasses. Chromosoma 79:251–270

Hodges TK, Kamo KK, Imbrie C, Becwar MR (1986) Genotype specificity of somatic embryogenesis and regeneration in maize. Biotechnol 4:219–223

Holliday R, Huschtscha LI, Tarrant GM, Kirkwood TBL (1977) Testing the commitment theory of cellular aging. Science 198:366–372

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of genetic variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Larkin PJ, Brettel RIS, Ryan SA, Davies PA, Pallotta MA, Scowcroft WR (1985) Somaclonal variation: Impact on plant biology and breeding strategies. In: Zaitlin M, Day P, Hollaender A (eds) Biotechnology in plant science. Academic Press, New York, pp 83–100

McClelland M, Nelson M (1985) The effect of site specific methylation on restriction endonuclease digestion. Nucleic Acids Res Suppl 13:201–207

Peschke VM, Phillips RL (1987) Discovery of *Ac* activity among progeny of tissue culture-derived maize plants. Science 238:804–807

Reddy AR, Peterson PA (1977) Callus initiation from waxy endosperm of various genotypes of maize. Maydica 22:125–130

Rivin CJ, Zimmer EA, Walbot V (1982) Isolation of DNA and RNA recombinants from maize. In: Sheridan WF (ed) Maize for biological research. Plant Molecular Biology Assoc, Charlottesville/VA, pp 161–164

Robertson DS (1978) Characterization of a mutator system in maize. Mutat Res 51:21–28

Robertson DS (1980) The timing of *Mu* activity in maize. Genetics 94:969–978

Robertson DS (1981 a) Mutator activity in maize. Timing of its activation in ontogeny. Science 213:1515–1517

Robertson DS (1981 b) Tests of two models for the transmission of the *Mu* mutator in maize. Mol Gen Genet 183:51–53

Robertson DS (1983) A possible dose-dependent inactivation of Mutator (*Mu*) in maize. Mol Gen Genet 191:86–90

Robertson DS (1984) Putative forward mutation frequencies at the *y1* and *wx* loci in the presence of *Mu*. Maize Genet Coop Newsl 58:11–12

Robertson DS (1985) Evidence for *Mutator* activity in the male gametophyte. Maize Genet Coop Newsl 59:14–15

Robertson DS (1986) New information on the timing of *Mu*'s mutator activity. Maize Genet Coop Newsl 60:12–14

Robertson DS (1987) Mendelian ratios in crosses of mutable *Mutator*-induced *a1* mutants. Maize Genet Coop Newsl 61:11–13

Robertson DS (1988) Additional evidence that *Mu* Mutator activity can occur during the development of the embryo. Maize Genet Coop Newsl 62:21

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–518

Talbert LE, Kelly AJ, Chandler VL (1988) Characterization of a class of *Mu* elements present in normal lines. Maize Genet Coop Newsl 62:59

Taylor LP, Walbot V (1987) Isolation and characterization of a 1.7 kb transposable element from a *Mutator* line of maize. Genetics 117:297–307

Taylor LP, Chandler VL, Walbot V (1986) Insertion of 1.4 kb and 1.7 kb *Mu* elements into the *bronze1* gene of *Zea mays* L. Maydica 31:31–45

Walbot V, Warren C (1988) Regulation of *Mu* element copy number in maize lines with an active or inactive *Mutator* transposable element system. Mol Gen Genet 211:27–34

Walbot V, Chandler VL, Taylor LP (1985) Alterations in the mutator transposable element family of *Zea mays*. In: Freeling M (ed) Plant genetics, UCLA Symposium on Molecular and Cellular Biology, vol 35. Alan Liss, New York, pp 333–342