Somatic Inactivation and Reactivation of *Ac* Associated With Changes in Cytosine Methylation and Transposase Expression

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ABSTRACT

Several metastable *Ac* alleles at the maize *p* locus were identified that produced novel pericarp variegation patterns. From the transmission analysis of pericarp sectors, we show that *Ac* inactivation is a somatic process. *Ac* excision from *P* and transactivation of an unlinked *Ds* was delayed or absent in plants with metastable *Ac* alleles. These decreases in *Ac* activity were correlated to increases in cytosine methylation at specific sites near the start of *Ac* transcription (open reading frame a; ORFa) and at sites in some flanking *P* sequence. Reactivation of inactive alleles was accompanied by decreased methylation of *Ac* and *P* sequences. Using a competitive polymerase chain reaction assay, steady state levels of ORFa transcript were quantitatively compared among the various metastable alleles. We propose that changes in the methylation profile of *Ac* correspond to changes in *Ac* activity through the differential accumulation of *Ac* transcript.

THE action of transposable elements in plants often results in characteristic patterns of variegation reflecting the timing and frequency of transposon excisions during plant development. Both environmental (CARPENTER *et al.* 1987) and genetic factors are involved in the regulation of this transposition process (reviewed in FEDOROFF 1990). Occasionally, changes in variegation patterns are observed. In maize, these "changes in state" (MCCLINTOCK 1947–1950) can be caused by the movement of an element within a gene (WEIL *et al.* 1992), an increase or decrease in element copy number in the genome (MCCLINTOCK 1949; BRINK and NILAN 1952) or a mutational event (MCCLINTOCK 1955). These mutational events are often the result of internal rearrangements, such as deletions, which alter transposition patterns (FEDOROFF *et al.* 1983; POHLMAN *et al.* 1984; SCHIEFELBEIN *et al.* 1985; SCHWARZ-SOMMER *et al.* 1985).

In addition to stable changes of state, unstable changes in variegation patterns can occur. Termed "changes in phase," these events are often associated with novel patterns of variegation that are somatically unstable (MCCLINTOCK 1957, 1963). Changes in phase associated with a loss of activity have been described for the maize *Activator* (*Ac*) (MCCLINTOCK 1963–1965; SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987), *Suppressor-mutator* (*Spm*) (MCCLINTOCK 1957, 1958; PETERSON 1966; BANKS *et al.* 1988; FEDOROFF and BANKS 1988; BANKS and FEDOROFF 1989) and *Mutator* (*Mu*) (ROBERTSON 1983; CHANDLER and WALBOT 1986; BENNETZEN 1987; MARTIENSSEN *et al.* 1990) transposon families. Unlike stable mutations, these metastable derivatives can revert to an active form thus reestablishing variegation patterns. The loss of *Ac* function at *wx-m7* and *wx-m9*, for example, is correlated with hypermethylation of *Ac* sequences (SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987) and loss of element-encoded gene expression (KUNZE *et al.* 1987, 1988; FUßWINKEL *et al.* 1991). Furthermore, reactivation of the *Ac* element is correlated to a decrease in cytosine methylation near the start of transcription (SCHWARTZ and DENNIS 1986) and the reappearance of *Ac* transcript (KUNZE 1988). The association of methylation status with mRNA levels suggests that transcriptional changes are involved in the inactivation process.

Previous studies have suggested that the metastable changes in *Ac* activity occur somatically, but suitable genetic markers were lacking to monitor *Ac* changes during development (MCCLINTOCK 1947, 1948; SCHWARTZ and DENNIS 1986). The *p* locus of maize provides such a somatic marker, conditioning pigmentation of pericarp and glume tissues of the ear (STYLES and CESKA 1977). The pericarp shares a close lineage relationship with the megaspore mother cell allowing the detection of somatic mutations in the pericarp and the recovery of mutant *P* alleles in kernel progeny (ANDERSON and BRINK 1952; ORTON and BRINK 1966). This lineage relationship and the availability of an *Ac* insertion at the *p* locus (*P-vv*) (EMERSON 1914, 1917; BARCLAY and BRINK 1954) has facilitated numerous studies on the transposition process (GREENBLATT and BRINK 1962; GREENBLATT 1968, 1974, 1984; CHEN *et al.* 1987, 1991) and aided the development of insertional mutagenesis strategies (ORTON and BRINK 1966; ATHMA *et al.* 1992; MORENO *et al.* 1992).

In this study we recovered several metastable alleles of *Ac* and utilized the *Ac*-induced *P-vv* mutation to show that inactivation is a somatic process associated with

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TABLE 1

Genetic stocks and alleles

Allele	Phenotype	Background	Source
P-vv::A	Variegated pericarp, variegated cob	W22	EMERSON (1914)
P-vv::C	Pericarp red at tip of kernels, pink cob (see text)	W22	This study
P-vv::I	Colorless pericarp, colorless cob	W22	This study
P-wr	Colorless pericarp, colored cob	W22	Supplied by J. L. KERMICLE
P-ww	Colorless pericarp, colorless cob	W22	Supplied by J. L. KERMICLE
r-sc:m3	Colorless aleurone (−*Ac*), variegated aleurone (+*Ac*)	W22	KERMICLE (1984)
r-g	Colorless aleurone	W22	Supplied by J. L. KERMICLE
r-r	Colorless aleurone	W22	Supplied by J. L. KERMICLE
Stock	**Genotype**	**Background**	**Source**
T 55	*P-vv::A/P-vv::A, r-g/r-g*	W22	Supplied by J. L. KERMICLE
T 43	*P-wr/P-wr, r-sc:m3/r-sc:m3*	W22	Supplied by J. L. KERMICLE
T 59	*P-ww/P-ww, r-r/r-r*	W22	Supplied by J. L. KERMICLE

cytosine methylation. Metastable elements show a decrease in *Ac* activity resulting in delayed and less frequent transpositions. Those derivatives that show no activity are methylated at several sites near the start of *Ac* transcription and at some sites in flanking *P* sequence. Both the extent of cytosine methylation and steady-state levels of *Ac*-encoded mRNA are directly correlated with *Ac* activity. Reactivation of *Ac* is associated with demethylation at some, but not all sites within *Ac*, defining critical regions for *Ac* activity. Finally, the inheritance and maintenance of methylation patterns of *Ac* is discussed in a model to explain the behavior of metastable *Ac* alleles.

MATERIALS AND METHODS

Genetic analysis: The *p* locus controls the synthesis of a red flavonoid pigment in certain floral organs (STYLES and CESKA 1977). A two-letter suffix is used to denote the expression pattern of a particular allele. The *P-wr* allele, for example, conditions a colorless (w) pericarp and red (r) cob. An *Ac* at the *p* locus, first identified by EMERSON (1914, 1917) (*P-vv*), results in a variegated pericarp and variegated cob. The insertion of *Ac* disrupts *P* function rendering the pericarp colorless. Somatic excisions often restore *P* function resulting in red stripes on otherwise colorless pericarp and cob tissues. Plants heterozygous for the *P-vv* allele display a characteristic striping pattern known as medium variegated (BRINK and NILAN 1952). When an additional *Ac* element is present in the genome fewer stripes are observed in the pericarp and cob tissues resulting in a light variegated phenotype. When plants are homozygous *P-vv*, the pattern of striping differs from the light variegated response with slightly more stripes due to excisions from both *P* alleles (homozygous variegated). The *P-vv* allele used in this study was backcrossed eight generations into the W22 background (see Table 1 for a list of stocks used in this study). A *Ds* insertion at the *R-sc* gene (*r-sc:m3*) was used to monitor *Ac* activity (KERMICLE 1984). *R-sc* is an allele of the *r* locus which fully pigments the aleurone of the kernel. The *Ds* insertion (ALLEMAN and KERMICLE 1993) disrupts the *R-sc* allele resulting in a colorless aleurone in the absence of *Ac*. In the presence of an active *Ac*, *Ds* excises during endosperm development resulting in a characteristic pattern of aleurone variegation. A single active *Ac* in the triploid endosperm results in early and frequent excisions of *Ds* during endosperm development. Increasing copies of an active *Ac* element result in fewer transpositions of *Ds* (see Figure 2). Derivative *P-vv* alleles were recovered by testcrossing homozygous *P-vv/P-vv* or heterozygous *P-vv/P-wr* lines as females by the males homozygous for the *Ds*-induced *r-sc:m3* allele (tester T43).

DNA isolation and Southern hybridization analysis: Maize genomic DNA was isolated from the seventh leaf of field grown plants using published techniques (CHEN and DELLAPORTA 1993). Southern analysis was performed as previously described (MORENO *et al.* 1992). Filters were hybridized with a *P*-specific probe, OP1-5, generated by polymerase chain reaction (PCR) amplification using the plasmid pRB514 as template DNA and oligonucleotide primers OP1 and OP5 (MORENO *et al.* 1992). pRB514 contains a 3.2-kb *Sal*I fragment of *P* which includes the insertion site of *Ac* in the *P-vv* allele (MORENO *et al.* 1992). Filters were treated with 0.4 N NaOH, neutralized in 0.1 M Tris, pH 7.4, and rehybridized with an internal 1.6-kb *Hin*dIII fragment from *Ac* (AcH1.6). A genome equivalent amount of λ DNA was added to restriction digests to monitor for complete digestion; after hybridization with the *Ac*-specific probe, blots were rehybridized with radiolabeled genomic λ DNA.

Competitive PCR (cPCR) assays: A complete protocol for the cPCR assay is available upon request. Briefly, RNA was isolated from 7-day-old seedlings as previously described (GAVAZZI *et al.* 1990). From each tissue sample RNA was recovered by LiCl precipitation and DNA was also recovered from the supernatant by precipitation with EtOH. Southern analysis on DNA samples was used to confirm the methylation status of *Ac* (see RESULTS). Total RNA was treated with DNase I (1 unit/μg) before cPCR analysis. Approximately 2.0 μg of RNA were reverse transcribed in the presence of 0.1 μCi [α-^{32}P]dATP using 2 units of Superscript reverse transcriptase (BRL). Reaction products were separated from unincorporated nucleotides by Sepharose CL-6B spin chromatography (DELLAPORTA 1993) and [^{32}P]dATP incorporation determined by scintillation counting. Equal amounts of radiolabeled RNA, corresponding to approximately 1 ng of cDNA, were added to each of the PCR reactions containing known quantities of competitive template. The competitive template was derived from plasmid pYU38 (kindly supplied by A. DELONG), which contains the 3′ region of *Ac*, by digestion with *Bcl*I and *Xho*I, Klenow treatment, and ligation to generate the plasmid pYU167. pYU167 contains a deletion within *Ac* that generates a 633-bp PCR product when amplified with oAc1 and oAc2 (oAc1, 5′-GAGCCACTTGCTACATCTTCATTAT-3′; oAc2, 5′-TGTATCAATTCTATTCTAGTTGTAG-3′). This product is 74 bp larger in size than the expected *Ac* cDNA product derived from the mature open reading frame a (ORFa) message.

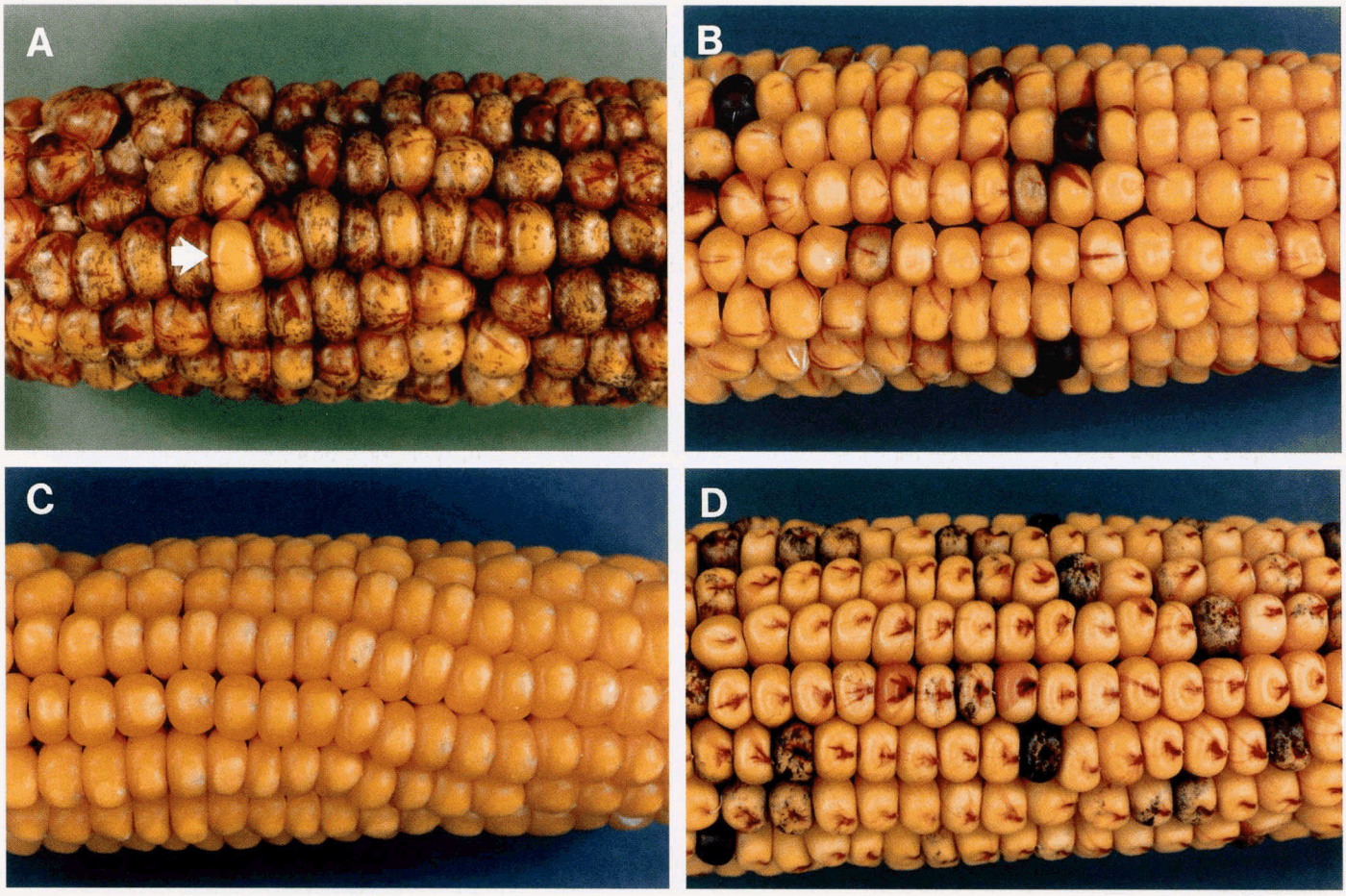

FIGURE 1.—Identification of *Ac* phase changes. (**A**) Homozygous *P-vv::A* ears were crossed by the *Ds* tester allele. Kernels were selected from F1 ears (arrow) with a very fine spotted or colorless aleurone and sown. These kernels presumably carried two *Ac* alleles; an *Ac* at its original location in *P* and a second transposed *Ac* element (*tr-Ac*) at an unknown location in the genome. Kernels with a coarse variegated aleurone were also selected as a control, possessing a single active *Ac* element present in two copies in the endosperm. (**B–D**) Progeny ears from selected near colorless kernels. (**B**) A light variegated ear (*P-vv::A/P-wr+ tr-Ac*) derived from a plant containing two active *Ac* elements. Kernels with both fully colored aleurone representing premeiotic excisions of *Ds* and kernels covered by red pericarp, show that *Ac* is active early in development. (**C**) A colorless ear showing inactivation of the *Ac* present at *P* (*P-vv::I/P-wr*). (**D**) An ear showing the cycling phenotype (*P-vv::C/P-wr*) that results in a novel variegation pattern in the pericarp with pigmentation near the silk attachment point. The apparent non-Mendelian segregation of the cycling allele in the next generation (less than 50% of the kernels are variegated) demonstrates the instability of the cycling allele.

A modified procedure of MULLIS and FALOONA (1987) was used to coamplify competitor and cDNA sequences with PCR. Samples were first denatured at 94° for 2 min, 30 sec. Amplification was then performed for 35 cycles as follows: denaturation at 94° for 45 sec, annealing at 55° for 1 min and extension at 72° for 1 min. At the end of 35 cycles, products were extended at 72° for 5 min. DNA products were visualized by ethidium bromide staining on 3% 3:1 NuSieve agarose gels and photographed with Polaroid 667 positive/negative film. Photographic negatives of the cPCR reactions were scanned with a densitometer, and integrated optical densities (IOD) of cDNA and competitor amplification products were calculated from peak areas (Millipore). cDNA values were obtained using a modified procedure of CLEMENTI *et al.* (1993) as follows. The IOD of the competitor (*C*) was corrected for its greater molar ethidium incorporation as *C*·(wild type length/competitor length) = *C*·0.833. The ratio (*Cc/W*) of corrected competitor (*Cc*) IOD to wild-type IOD (*W*) was plotted against the concentration of competitor DNA. Simple regression lines were fitted to these values (r^2 values all >9.4) and cDNA values were extrapolated when *Cc/W* = 1. Error bars show the difference between the mean and individual cDNA values in a replica experiment.

RESULTS

Identification of *P-vv* derivative alleles: The *P-vv* allele of maize conditions a variegated pericarp and cob. Stripes of red tissue in the pericarp are due to *Ac* excisions from the *P* gene during ear morphogenesis (see MATERIALS AND METHODS). Because *P-vv* contains an autonomous *Ac* element, it is also capable of transactivating unlinked, non-autonomous *Ds* elements elsewhere in the genome (BARCLAY and BRINK 1954). When females homozygous for the *Ac*-induced *P-vv* allele were crossed to males homozygous for the *Ds*-induced *r-sc:m3* allele, the majority of the ears showed the expected homozygous variegated pericarp phenotype overlying kernels with a coarse spotted aleurone (Figure 1A).

TABLE 2

Pericarp phenotypes of selected spotted kernels

Kernel selection[a]	Pericarp phenotypes of ears					Total
	Colorless	Red	Cycling	Light variegated	Medium variegated	
Coarse (2*Ac*)	1	2	0	8	149	160
Fine or colorless (0 or >4*Ac*)	12	23	7	80	17	139

[a] Selections were based on the spotting pattern in the aleurone due to *Ds* excision from the *r-sc:m3* allele. The expected *Ac* dose is shown in parentheses.

Premeiotic transpositions of *Ac* from *P* are expected to change the aleurone phenotype from coarse spotted to either colorless aleurone, if *Ac* is absent in the kernel (*P-rr*), or a finely spotted aleurone, if *Ac* copy number increases (*P-vv* + *tr-Ac*). We observed such changes in approximately 5% of the progeny kernels (Figure 1A, arrow). We examined 139 ears derived from these colorless and finely spotted kernels to identify changes in pericarp variegation patterns. In addition to the expected full red (*P-rr*) and light variegated (*P-vv* + *tr-Ac*) pericarp ears (Figure 1B), medium variegated (*P-vv*) and two unexpected phenotypes were recovered (Table 2). These medium variegated ears were most likely the result of non-concordance between the endosperm and embryo (DOONER and BELACHEW 1989). Ears with an unexpected phenotype (19/139) were of two types. Twelve out of 139 ears showed a colorless pericarp phenotype overlying progeny with colorless aleurone (Figure 1C) indicating loss of both *P* and *Ac* activity. The remaining ears (7/139) displayed a novel pattern of *P* variegation and did not display a characteristic pattern of aleurone variegation (Figure 1D).

Inactive derivatives at *P*: The colorless pericarp ears were characterized by Southern analysis. The majority of these ears (8/10) carried a rearrangement of the *p* locus accompanied by loss of *Ac* (Table 3). Further analysis indicated that 7 of the 8 rearrangements contained a large deletion with endpoints within two 5.2-kb repeats that encompass the *P* gene (data not shown). These deletions are similar to *Ac*-catalyzed deletions (*p*-Δ) previously described (ATHMA and PETERSON 1991). The eighth rearrangement was complex involving both *Ac* and *P* sequences and has not been characterized further. Two of 10 colorless pericarp ears carried *Ac* elements at *P* that were structurally indistinguishable from the parental *P-vv* by Southern analysis, yet both *Ac* and *P* activity were absent. These derivative alleles are designated inactive (*P-vv::I*) to distinguish them from the rearrangement derivatives.

Cycling derivatives at *P*: The *P* alleles producing novel variegation patterns were also examined. The pericarp phenotype associated with these alleles consisted of small red stripes often restricted to the top (crown) of the kernel (Figure 1D). When rare stripes occurred in the sides (gown) of the kernel, they were often small, indicating excision was late and infrequent during ear development. The pattern of aleurone variegation in these kernels was also different from the pattern generated by an active *Ac*. Segregation of a single active *Ac* is expected to produce a variegated aleurone in 50% of the progeny. With these elements, however, less than 50% of the kernels were variegated (see Figure 1D). Unlike the uniform pattern of variegation conditioned by an active *Ac*, those kernels with aleurone variegation often had spotting confined to regions of the aleurone (Figure 2). This variability has also been observed with metastable derivatives of the *Ac*-induced *wx-m7* (MCCLINTOCK 1964) and *wx-m9* alleles (SCHWARTZ and DENNIS 1986). In accordance with these previous studies, we designate these *P-vv* derivatives as cycling (*P-vv::Cy*). In contrast to the fairly stable patterns conditioned by active and inactive *P-vv* alleles, the highly unstable nature of the cycling alleles suggest that they represent unstable intermediates between active and inactive *Ac* elements.

The differences between active and cycling alleles were examined by comparing aleurone variegation patterns and frequencies of fully colored *R-sc* derivatives. One difference between active and cycling alleles was clearly seen in the pattern of *r-sc:m3* variegation. The active *P-vv* allele is capable of transactivating *Ds* throughout endosperm development resulting in an uniform interspersion of both large and small aleurone spots (Figure 2, A–C). The aleurones containing cycling

TABLE 3

Genotypes of colorless ears

Allele	Selection	*P* allele
P-DT91M-1	Coarse	ND[a]
P-192E-1	Light	Deletion
P-DT91R-1	Colorless	ND
P-DT91R-2	Colorless	Deletion
P-DT91R-3	Colorless	*P-vv::I*
P-DT91R-4	Colorless	Deletion
P-DT91R-5	Colorless	Deletion
P-DT91R-6	Colorless	ND
P-194C-1	Colorless	Deletion
P-201A-1	Colorless	*P-vv::I*
P-206B-1	Colorless	Deletion[b]
P-215A-1	Colorless	Deletion
P-217A-1	Colorless	Deletion

[a] ND, *P* allele not determined.

[b] The deletion breakpoints differ from the other characterized deletions.

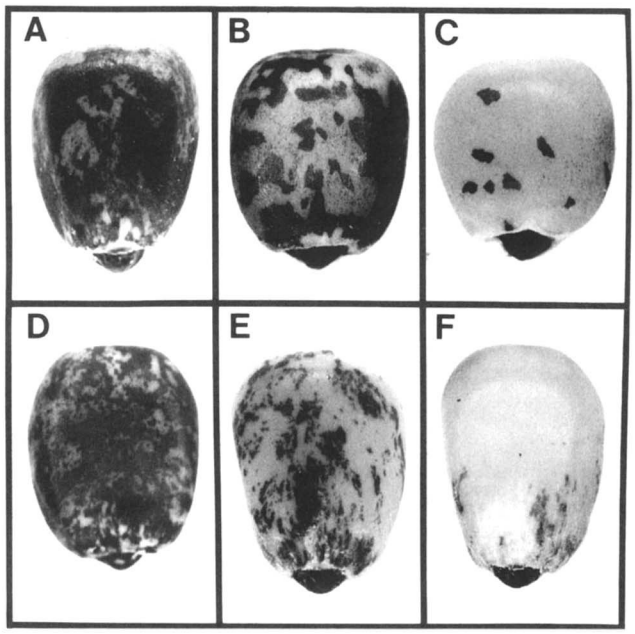

FIGURE 2.—Spotting patterns observed with active and cycling alleles of *Ac*. All kernels shown are homozygous for the *r-sc:m3* tester and are inbred into the W22 background. Pericarps have been removed to show aleurone variegation patterns. The *r-sc:m3* allele contains a *Ds* insertion at the *R-sc* locus. In the absence of *Ac*, the aleurone is colorless. When *Ac* is active, however, the pattern of aleurone variegation corresponds to the number of active copies of *Ac* present in the endosperm. Increasing copies of *Ac* result in smaller and less frequent colored spots. (**A–C**) Kernels selected from ears carrying the active *P-vv* allele transmitted through (**A**) the male (one dose), (**B**) the female (2 dose) **C**; both *i.e.*, self (3 dose). (**D–F**) Kernels selected from a selfed ear containing three copies of a *P-vv::C* allele in the endosperm. Cycling alleles display a range of variegation patterns that do not correspond to the *Ac* copy number present.

alleles, however, were often colorless or non-uniformly spotted (Figure 2, E and F). Although large regions of the aleurone were sometimes heavily pigmented, the aleurone cells were mosaic for both colored and colorless cells within this region (Figure 2D). This mosaicism is evidenced by comparing the colored areas in aleurones containing the active (Figure 2B) and cycling (Figure 2E) *P-vv* alleles. With the active allele, colored areas were uniformly pigmented with clearly defined borders (Figure 2B). With the cycling allele, colored borders in kernels were highly irregular due to the interspersion of colored and colorless cells. Thus, the dense clustering of colored cells in the aleurone actually represented several very late excision events of *Ds* at *r-sc:m3*, giving the impression of a large colored sector.

A second major difference between the derivative and active *Ac* elements was in the dosage response. Active *Ac* elements typically show a dosage response; as the number of *Ac* elements in the genome increases, transposition becomes later and less frequent during development (MCCLINTOCK 1949). This dosage response is evident in the aleurone, as *r-sc:m3* dependent variegation patterns vary with the copy number of active *Ac* alleles (Figure 2, A–C). However, the pattern of aleurone variegation was independent of *Ac* copy number in the aleurone of kernels containing cycling derivatives. Moreover, there were no discernible effects on either the timing of transposition or the frequency of transposition by varying the copy number of cycling *Ac* derivatives in the aleurone (data not shown).

A third difference between active and cycling *Ac* elements consisted of the frequency of germinal derivatives. An active *Ac* can catalyze germinal *Ds* excision at *r-sc:m3* to give self-colored kernels (*R-sc* derivatives) (ALLEMAN and KERMICLE 1993). The frequency of these self-colored kernels is a measure of the *Ac* activity prior to fertilization (*i.e.*, during or before megagametogenesis). Therefore, we determined the frequency of self-colored derivatives in plants containing an *r-sc:m3* allele and either a single active, cycling or inactive *Ac* allele (*P-vv/P-wr, r-sc:m3/r-g*). With the active *Ac* element at *P*, approximately 4% of the 2500 kernels examined were self-colored. With the cycling or inactive *P-vv* alleles, no self-colored derivatives were recovered in populations of over 5,000-kernel progeny for each allele. The failure to detect *R-sc* germinal derivatives indicates the *Ac* activity of cycling and inactive alleles is insufficient to promote germinal *Ds* transpositions.

Timing of *Ac* state changes: We were able to exploit the close lineage relationship between the pericarp and the underlying female gametophyte to monitor somatic changes at the *p* locus and test the heritability of these changes. The somatic activity of a single active *Ac* at *P* was monitored in testcrosses between females heterozygous for the *Ac* at *P* (*P-vv/P-wr*) and males homozygous for *r-sc:m3*. We screened 127 ears for pericarp sectors that were colorless (Figure 3A, arrow) or with pigmentation limited to the silk attachment point. A total of 63 such pericarp sectors were identified. Although most sectors encompassed a single kernel or less, one sector covered approximately three kernels.

To determine the heritability of the somatic change at *P*, kernels underlying these sectors were grown and testcrossed to males homozygous for *r-sc:m3*. A total of 46 ears were recovered. Due to meiotic segregation of homologs, 25 ears carried the *P-wr* homolog. Ninety-five percent (20/21) of the remaining ears showed a novel pericarp variegation pattern. Of these, eight ears displayed a pattern with frequent and small red stripes but few large red stripes. Although the pericarp phenotype was novel, the aleurone was uniformly spotted and often indistinguishable from the parental *P-vv* pattern. We refer to these derivatives as "weakened" alleles (*P-vv::W*) because the apparent diminished activity of *Ac* results in developmentally later excisions in the pericarp (Figure 3B). Seven of the 21 ears showed a colorless pericarp and probably represent either rearrangements (*e.g.*, deletions) or inactive *Ac* derivatives. Three of

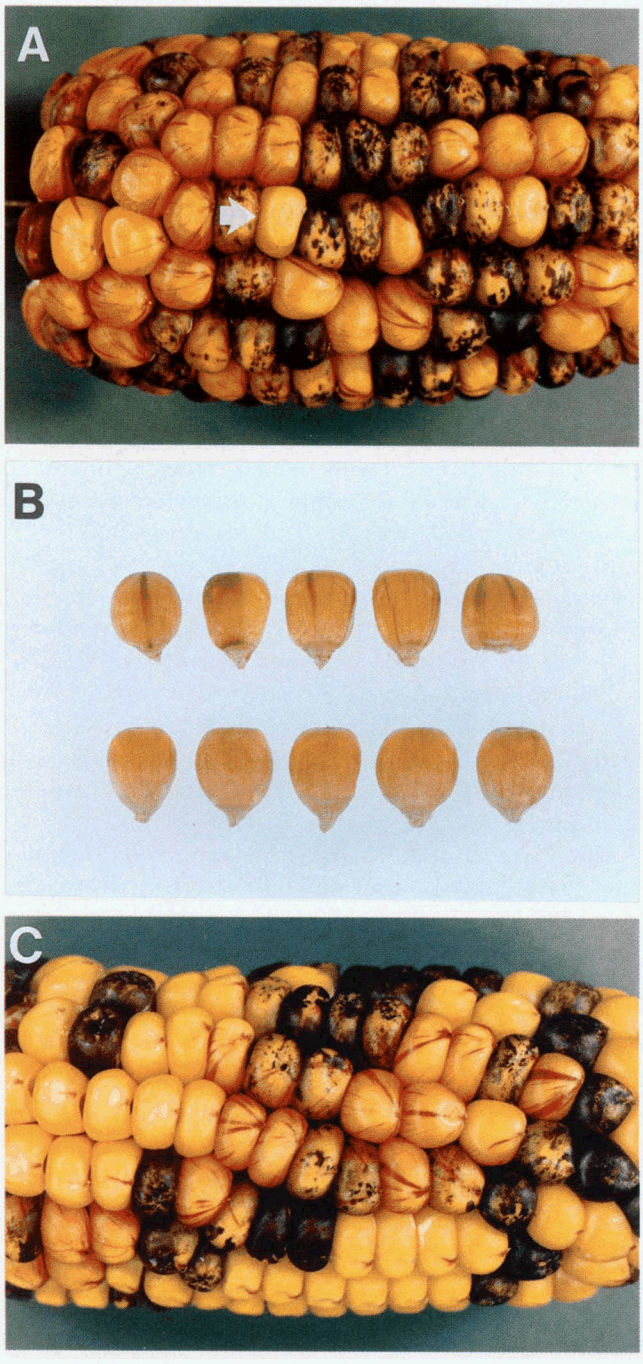

FIGURE 3.—Somatic instability of metastable alleles. (**A**) Kernels with a colorless pericarp over the germinal face (arrow) were selected from ears heterozygous for the *P-vv::A* allele the *r-sc:m3* allele. (**B**) Kernels from progeny ears with the active *P-vv::A* allele (**top**) are compared to those showing the *P-vv::W* phenotype (**bottom**). Kernels do not carry the *r-sc:m3* allele. (**C**) Sector of reactivation on an ear derived from a plant heterozygous for the *P-vv::C* allele (*P-vv::C/P-wr, r-sc:m3/r-sc:m3*).

these seven ears carried one or two kernels with a reactivated allele that showed aleurone variegation and could be classified as *P-vv::I* derivatives. Five ears were also generated with pericarp and aleurone variegation patterns characteristic of cycling *Ac* derivatives. Only active *P-vv* alleles were recovered from 100 sibling kernels showing a medium variegated pericarp and spotted aleurone (data not shown). In summary, somatic changes in *Ac* activity were identified in heterozygous *P-vv* individuals through a change in the expected pericarp variegation pattern associated with the active *Ac* allele. These changes were then transmitted to kernel progeny in most cases indicating that inactivation occurs somatically and is propagated meiotically.

Metastability of *Ac* derivatives: Cycling and inactive alleles resulted in novel variegation patterns that were often unstable both somatically and germinally. Somatic changes in variegation patterns were observed in pericarp sectors resembling either a more active or less active state of *Ac*. Plants with inactive alleles, for instance, occasionally displayed ear sectors with a cycling pattern of pericarp variegation. These sectors were infrequent and rarely larger than a few kernels in size, indicating that the change in activity occurs late in ear development. The highly unstable nature of cycling alleles often resulted in more frequent changes to more or less active forms. These changes were observed both as small and large (*i.e.*, several kernels to half an ear) sectors (Figure 3C) indicating that these metastable alleles can undergo changes in activity throughout development.

Molecular analysis of *P-vv* states: Inactive, cycling and weak alleles of *Ac* at *P* were examined by Southern analysis to determine whether or not alleles differed in their degree of cytosine methylation. We examined a 7.9-kb region of *P-vv* defined by *Sal*I sites which includes some of the *p* locus and all of the *Ac* sequence. The 7.9-kb *Sal*I fragment was detected in every allele when filters were probed with either a *P*-specific (OP1-5) (Figure 4A) or *Ac*-specific (AcH1.6) probe (data not shown), demonstrating that these *Sal*I sites were unmethylated. Because the *P*-specific probe is part of a repeat in *P*, additional bands were detected (see Figure 4) and were used to monitor for complete digestion.

Double digests with *Sal*I and several other methylation sensitive enzymes were used to monitor the methylation of sites within this 7.9-kb region. Examples of these data are shown in Figure 4, B and C, which contain the results of double digests using *Sal*I and either *Nci*I or *Pvu*I, respectively. Results from all other digests are summarized in Figure 5. In double digests with the methylation sensitive enzymes *Sal*I and *Nci*I, a predominant 640-bp fragment was seen in DNA from active and weak alleles indicating the 5′ *Nci*I site in *Ac* is unmethylated in most cells (Figure 4B, lanes 2, 7 and 8). When methylated, this 640-bp fragment is replaced by 5.9- and 6.5-kb fragments due to methylation of *Nci*I sites clustered in flanking *P* sequence. The cycling DNA (lanes 3 and 4) contained both the 640- and 6.5-kb fragments showing that the tissue contained both methylated and unmethylated forms of *Ac*, whereas the inactive DNA (lanes 5 and 6) contained a predominant 6.5-kb band

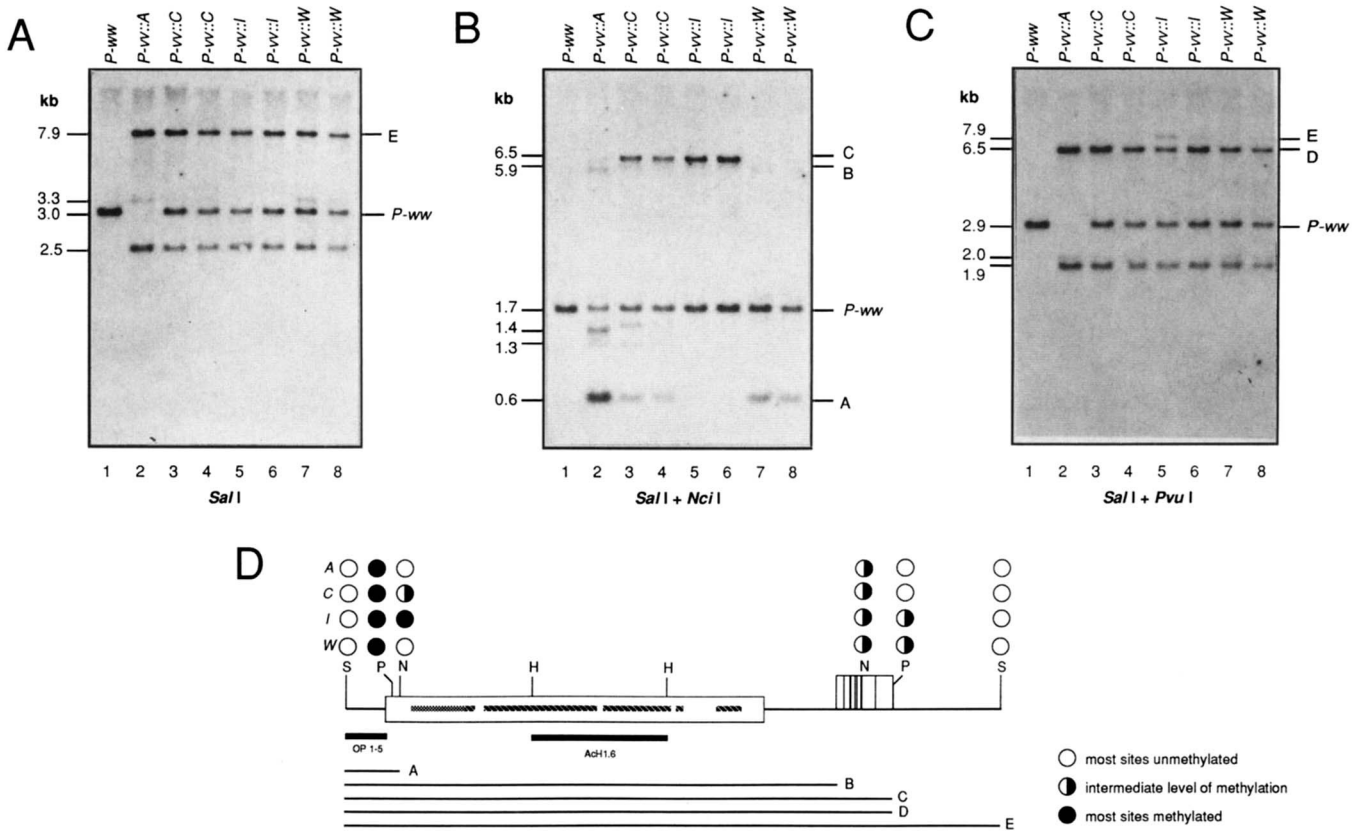

FIGURE 4.—Southern analysis of *P* alleles with methylation sensitive enzymes. DNA was digested with (**A**) *Sal*I (**B**) *Sal*I and *Nci*I or (**C**) *Sal*I and *Pvu*I, blotted and probed with OP1-5, a probe homologous to the 5′ end of the 3.3 kb *Sal*I fragment of *P*. The 2.5-kb fragment in **A**, the 1.7-kb fragment in **B** and the 1.9-kb fragment in **C** represent additional *P* homologies detected with OP1-5. The OP1-5 and AcH1.6 probes are shown in (**D**). The box represents *Ac* sequences and the thick line *P* sequences. The direction of transcription for both *P* and *Ac* is 5′ to 3′ as drawn. The hatched boxes within *Ac* represent exons and the stippled region represents leader sequences (KUNZE *et al.* 1987). Circles above the respective restriction sites summarize the methylation profile deduced from Southern analysis. Sites that could not be assayed are shown without circles. A bar is place over sites that could not be resolved in Southern analysis. Restriction enzyme abbreviations are as follows: H, *Hin*dIII; N, *Nci*I; P, *Pvu*I; S, *Sal*I.

indicating that in most cells *Ac* is methylated at the *Nci*I site. Thus, the activity of *Ac*, as monitored by variegation patterns, was correlated to the methylation status of the 5′ *Nci*I site in *Ac*.

In contrast to these results, we failed to detect cleavage in any allele at a methylation sensitive *Pvu*I site in *Ac*. A 500-bp *Sal*I-*Pvu*I fragment is expected in double digests probed with OP1-5 if the 5′ site in *Ac* is unmethylated (Figure 4C). Methylation differences were detected, however, at a flanking *Pvu* I site in *P* DNA between the active and metastable alleles. With active and cycling alleles, the sites within *P* were unmethylated in most of the DNA as indicated by the presence of a 6.5-kb band (Figure 4C, lanes 2–4). With the inactive and weak alleles, however, an additional 7.9-kb band was detected (Figure 4C, lanes 5–8) indicating that the 3′ sequences in *P* were methylated.

Ac excision from *P-vv* results in a 4.5-kb shift in fragment size. This excision fragment was detected in DNA digested with *Sal*I as a 3.3-kb fragment from the active and weak alleles (Figure 4A, lanes 2, 7 and 8) but was undetectable in DNA from the inactive alleles (lanes 5 and 6). A faint 3.3-kb band in lane 2 indicates that excisions of *Ac* are less frequent with cycling alleles relative to active or weak alleles. The excision products from cycling alleles are more apparent in the *Nci*I/*Sal*I double digests where a 1.3-kb excision band is predicted when the 5′ *Nci*I site in *P* is unmethylated (Figure 4B, lanes 2 and 3). Additional higher molecular weight bands (Figure 4B, lane 2, 1.4 kb; lane 3, 1.45 kb; lanes 7 and 8, 1.7 kb) are also detected due to differential methylation of the *Nci*I sites in flanking *P* sequence. Thus, *Ac* is capable of excision as a cycling allele, but at a lower frequency relative to an active or weak allele.

In a similar fashion, double digests were performed with the methylation-sensitive restriction enzymes *Sal*I and either *Ava*I, *Eco*RII, *Sst*II, or *Nru*I. These results are summarized in Figure 5. Typically, sites in *Ac* were more heavily methylated in the inactive alleles than in other alleles. Some sites, however, were more informative than others. For instance, the degree of methylation of the *Nci*I and *Sst*II sites within *Ac* inversely correlated with its activity. Other sites, including the 5′ most *Eco*RII and *Pvu*I in *Ac*, failed to show any

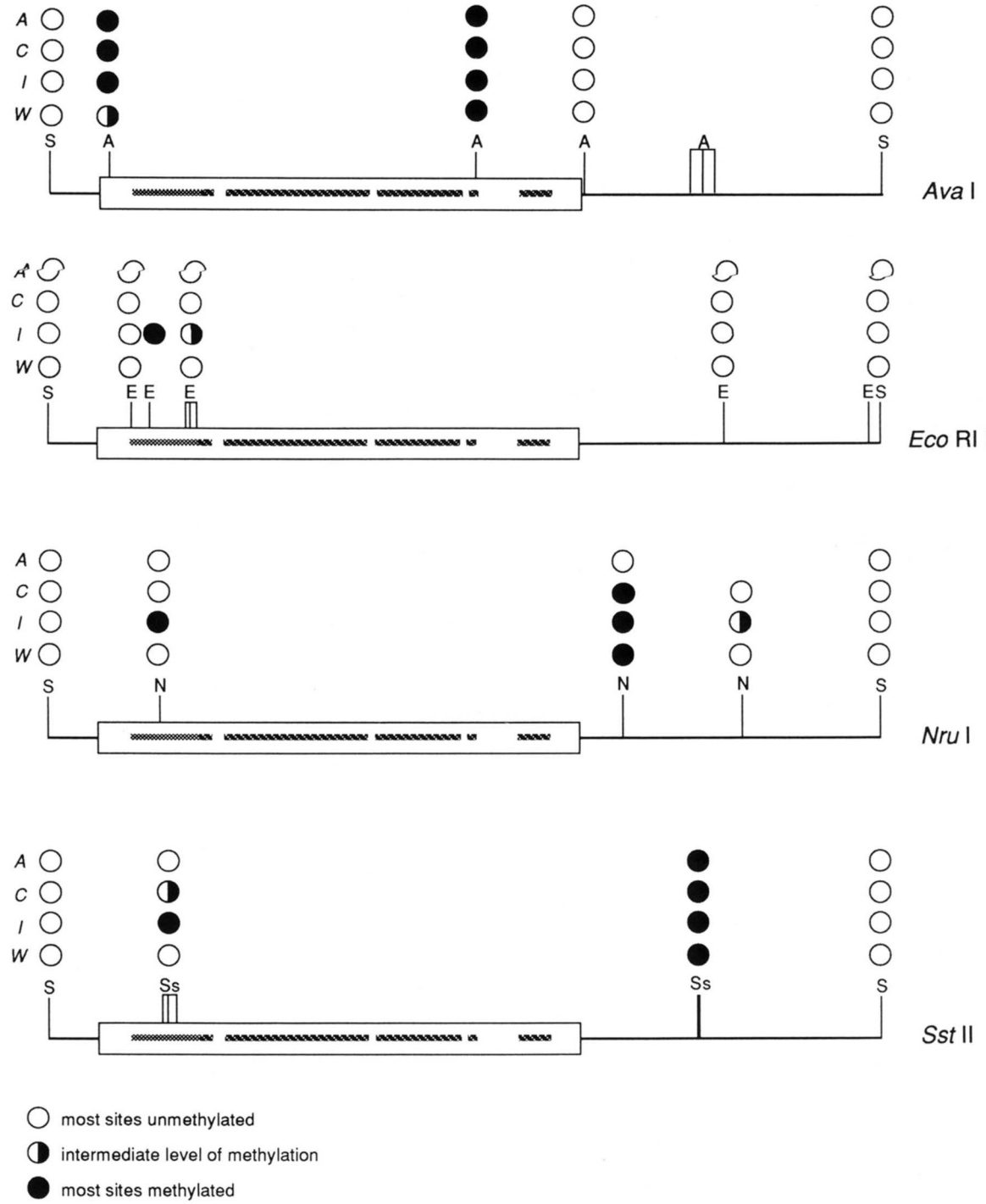

FIGURE 5.—Summary of methylation sensitive restriction digests. Double digests were performed with *Sal*I and either *Ava*I, *Eco*RII, *Nru*I or *Sst*II as described in Figure 4. The methylation profile for each allele was based on blots probed with both the *P* and *Ac* specific probes as described in MATERIALS AND METHODS. Alleles are abbreviated as follows: A, *P-vv::A/P-ww*; C, *P-vv::C/P-ww*; I, *P-vv::I/P-ww*; W, *P-vv::W/P-ww*.

methylation differences between alleles. For most sites in flanking *P* sequences, methylation differences were not detected, although differences did exist at a few sites, including a *Pvu*I and two *Nru*I sites. These data show that significant methylation differences exist between the active and metastable alleles of *Ac*. At several sites within *Ac* and a few sites within *P*, increased cytosine methylation is directly correlated with reduced *Ac* activity.

Quantitation of *Ac* transcript levels: It was previously shown that when *Ac* is in an inactive phase at *waxy* (*wx-m9*), *Ac*-encoded ORFa transcripts are not detectable by Northern analysis (KUNZE *et al.* 1988; FUßWINKEL *et al.* 1991). To investigate the relationship between ORFa levels and *Ac* activity at *P*, we examined mRNA levels of active, cycling and inactive alleles of *Ac* using

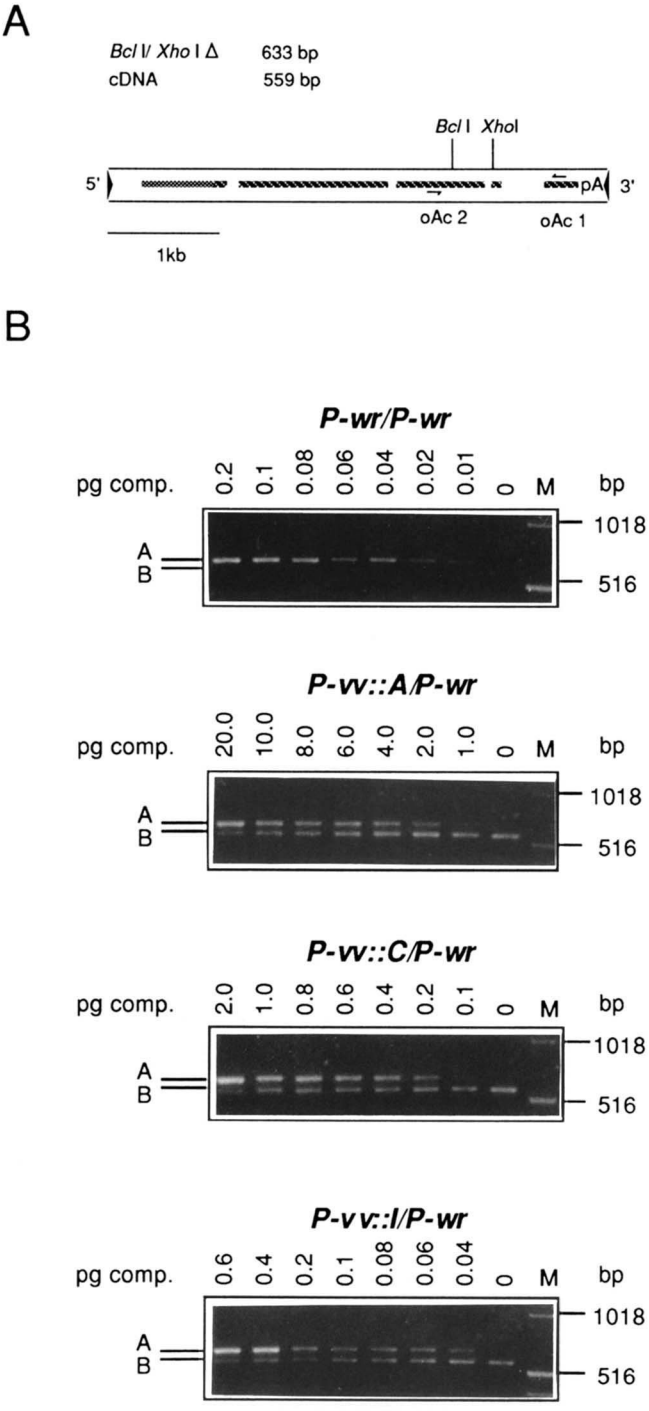

FIGURE 6.—Quantitative PCR analysis of *Ac* transcript levels. RNA was extracted and reverse transcribed as described in MATERIALS AND METHODS from seedling tissue. (**A**) diagram of *Ac* showing position of *Bcl*I and *Xho*I sites used in generating the competitor template. (**B**) Competitive PCR results. PCR reactions were visualized on agarose gels in the presence of EtBr and photographed under UV illumination. The genotype of each plant and picograms of competitor plasmid used for analysis are given above each photograph. Band **A**, representing the amplification product from the competitive template was detected at each competitor concentration but was absent when competitor was not present. Band **B**, corresponding to the cDNA amplification product was not detected when an active *Ac* is absent (*P-wr/P-wr*), but was present when plants carrying active (*P-vv::A/P-wr*), cycling (*P-vv::C/P-wr*), or inactive (*P-vv::I/P-wr*) alleles were used.

a cPCR assay. Although the assay is an indirect measure of transcript levels (GILLILAND *et al.* 1990), it is highly quantitative and extremely sensitive. As described in MATERIALS AND METHODS, a cDNA value for each allele of *Ac* was calculated to compare ORFa mRNA levels. This value represents the quantity of competitor (Figure 6A) when the cDNA and competitor are equimolar after co-amplification. Amplification products for the cPCR analysis are shown in Figure 6B. In the control RNA, 6

Quantitative PCR analysis of *Ac* transcript levels. RNA was extracted and reverse transcribed as described in MATERIALS AND METHODS from seedling tissue. (**A**) diagram of *Ac* showing position of *Bcl*I and *Xho*I sites used in generating the competitor template. (**B**) Competitive PCR results. PCR reactions were visualized on agarose gels in the presence of EtBr and photographed under UV illumination. The genotype of each plant and picograms of competitor plasmid used for analysis are given above each photograph. Band **A**, representing the amplification product from the competitive template was detected at each competitor concentration but was absent when competitor was not present. Band **B**, corresponding to the cDNA amplification product was not detected when an active *Ac* is absent (*P-wr/P-wr*), but was present when plants carrying active (*P-vv::A/P-wr*), cycling (*P-vv::C/P-wr*), or inactive (*P-vv::I/P-wr*) alleles were used.samples from a line that did not contain *Ac* (*P-wr/P-wr*), we were unable to detect ORFa message. ORFa amplification products were found in all other *Ac* containing lines including the inactive lines. In both heterozygous and homozygous individuals, decreased *Ac* activity was associated with lower levels of ORFa mRNA. By calculating cPCR values (see Figure 7) we were able to quantitate the differences in transcript levels between the alleles. Plants homozygous for an inactive allele contained only 2.8% of the ORFa present in a plant homozygous for the active allele. Similarly, a heterozygote for the inactive allele contained only 1.7% of the steady state level of *Ac*-encoded transcript present in a plant carrying a single active *Ac*. Although decreases in cDNA values for plants with cycling alleles relative to the active alleles were observed, the differences were greater between homozygous individuals than between heterozygotes. These differences could reflect the unstable nature of the cycling alleles or increased ORFa expression associated with homozygosity. Also, as shown in Figure 7, the level of *Ac*-encoded message in a plant homozygous for an active *Ac* was 1.5 times the level in a heterozygote. This result is consistent with previous studies of *Ac* that correlated an increased copy number of active *Ac* to an increase in steady state mRNA levels (FUßWINKEL *et al.* 1991).

DISCUSSION

***Ac* inactivation is a somatic process:** Several metastable alleles of *Ac* have been derived from an active element present at the *p* locus. The changes in activity

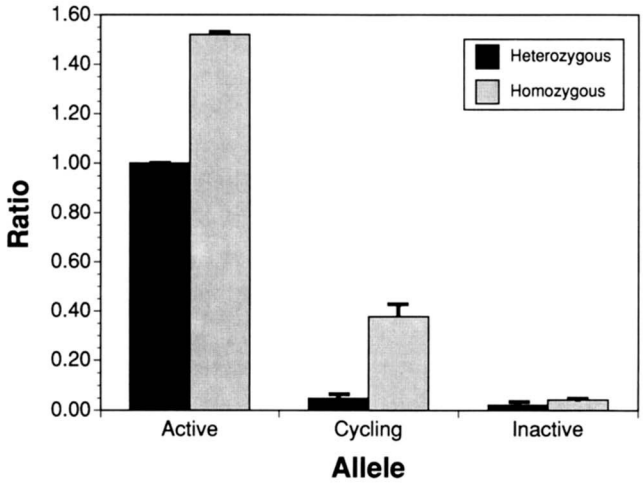

FIGURE 7.—A comparison of cDNA values from plants containing homozygous and heterozygous *P-vv::A*, *P-vv::C*, *P-vv::I* and homozygous *P-wr* alleles.

are similar to the *Ac* phase changes previously characterized at the *waxy* locus (McCLINTOCK 1964, 1965; SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987; CHOMET 1988). In this study, we show that inactivation is a somatic process by identifying changes in *Ac* activity as pericarp sectors with novel variegation patterns. The loss of *Ac* activity is associated with increased cytosine methylation and reduced ORFa (transposase) mRNA levels. Despite our ability to detect transcript, *Ac* is incapable of excision or *Ds* transactivation in the inactive lines.

The decrease in *Ac* activity associated with methylation results in a delay of transposition. Large red pericarp sectors, germinal red pericarp ears or germinal excisions of unlinked *Ds* elements were not observed with cycling or inactive alleles. These observations suggest that *Ac* is incapable of excision and *Ds* transposition before meiosis is completed in lineages that give rise to gametes. Furthermore, the pericarp phenotype associated with cycling alleles; red pericarp at the crown of the kernel, suggests that excisions of *Ac* are limited to the epidermal tissue. Clonal analysis of pericarp sectors has shown that the pericarp is comprised of both epidermal (LI) and subepidermal (LII) derived tissues (GREENBLATT 1985; DELLAPORTA *et al.* 1991). The majority of the tissue at the crown of the kernel is derived from the LI layer, while the gown of the kernel and underlying germ cells are primarily derived from the LII layer. Excisions limited to the LI layer would result in a phenotype characterized by red pericarp in tissues at the crown of the kernels and a lack of premeiotic *Ds* excisions (self-colored kernels) in progeny, a phenotype consistent with cycling alleles of *Ac*. We are currently investigating this potential tissue-specific suppression of *Ac* activity.

Cytosine methylation and *Ac* expression: The weak, cycling and inactive *Ac* derivatives are not associated with structural changes but are distinguished from active alleles and each other by the extent and degree of cytosine methylation. Previous studies of the maize transposable elements *Ac* (SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987), *Mu* (CHANDLER and WALBOT 1986; BENNETZEN *et al.* 1988; MARTIENSSEN *et al.* 1990), and *Spm* (BANKS *et al.* 1988; BANKS and FEDOROFF 1989) have also shown that metastable derivatives differ from active elements by increased DNA methylation. Furthermore, for both *Ac* and *Spm*, sequences near the start of transcription often show greater differences in the degree of methylation between active and inactive elements than do other regions of the element (SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987; BANKS *et al.* 1988; BANKS and FEDOROFF 1989). Our studies also correlate reduced transposon activity to decreases in gene expression as was shown from previous studies of *Spm* (MASSON *et al.* 1987) and *Ac* (KUNZE *et al.* 1987, 1988; FUßWINKEL *et al.* 1991). Finally, unlike previous studies that have failed to detect *Ac*-encoded transcript in seedlings with inactive alleles (KUNZE *et al.* 1987, 1988), our data indicate that methylation affects gene expression quantitatively, with the lowest levels of mRNA found in the inactive *Ac* alleles. Although it is likely that these differences reflect differences in rates of transcription (for a review see CEDAR and RAZIN 1990), we can not rule out the possibility that message stability may be affected, perhaps through the use of different initiation sites or premature transcript termination caused by methylation (BARRY *et al.* 1993).

It is not known if these decreases in ORFa expression are a direct or indirect consequence of DNA methylation. Methylation may act to inhibit transcription by blocking the binding of transcription factors directly (IGUCHI-ARIGA and SCHAFFNER 1989; PRENDERGAST and ZIFF 1991) or with proteins that bind specifically to methylated sequences, thus preventing the entry of transcriptional machinery (MEEHAN *et al.* 1989; BOYES and BIRD 1991; LEWIS *et al.* 1992). It is possible that activators of *Ac* transcription are also sensitive to methylation within *Ac*. In the CpG rich leader region that is susceptible to methylation changes, both *Ac* and *Spm* contain putative binding sites for their respective transposases (POHLMAN *et al.* 1984; PEREIRA *et al.* 1986; GIERL *et al.* 1988; KUNZE and STARLINGER 1989; FELDMAR and KUNZE 1991). This raises the intriguing possibility that transposase may activate its expression and that this regulation is sensitive to the methylation status of transposase binding sites. Reactivation of an inactive *Spm*, for example, appears to be dependent on the presence of a second active *Spm*, suggesting a role for an *Spm*-encoded product in the reactivation process (MASSON *et al.* 1987; BANKS 1988; FEDOROFF 1989). Although previous studies have indicated that the presence of an active *Ac* in the genome is insufficient for reactivation of an inactive element (McCLINTOCK 1964, 1965; SCHWARTZ and DENNIS 1986;

CHOMET *et al.* 1987), the active elements were only introduced to the inactive alleles for a single generation. As demonstrated for *Spm*, the reactivation of an inactive element may require the presence of an active element over several generations (FEDOROFF 1989).

Ac methylation may affect flanking *P* sequences: We examined several restriction sites in flanking *P* sequences for their methylation status. Although previous studies in maize have shown that increases in methylation associated with inactivation are confined to element sequences (SCHWARTZ and DENNIS 1986; CHOMET *et al.* 1987; BANKS *et al.* 1988), differences have also been identified in flanking sequences (MARTIENSSEN *et al.* 1990). We also identified a few sites in *P* sequences that were more heavily methylated in DNA from inactive alleles than in other alleles. Thus, extensive methylation of *Ac* sequences may result in a spreading of methylation into flanking DNA as demostrated in mammalian systems (TOTH *et al.* 1989, 1990). Although the phenotypic effect of increased *P* methylation has not been determined, novel patterns of *P* expression have been correlated to increased methylation of *P* sequences in studies of another mitotically unstable *P* allele (DAS and MESSING 1994).

***Ac* copy number and transposase expression:** The highly quantitative nature of the cPCR assay allowed us to accurately measure levels of *Ac*-encoded ORFa in homozygous and heterozygous individuals. Seedlings homozygous for any *Ac* allele always contained greater amounts of ORFa mRNA than in heterozygous seedlings. This result is consistent with previous studies which showed *Ac* message is more abundant in seedlings homozygous for an active *Ac* than in heterozygotes (FUßWINKEL *et al.* 1991). However, the increase does not appear to be linear with copy number and suggests that ORFa levels may be down-regulated by increasing copies of *Ac*.

Methylation may modulate the strength of the ORFa promoter: We show that the pattern of methylation is correlated with a particular metastable state of *Ac*. Increases in methylation are associated with a decrease in *Ac* activity (*i.e.*, late transposition of *Ac* from *P* and *Ds* from *R*) and a decrease in transcript levels. The strong correlation between increased methylation and decreased transcript accumulation suggests that the variegation patterns conditioned by metastable alleles of *Ac* are due to transcriptional differences among the alleles. We propose that the methylation of sequences in *Ac* near the start of transcription serve to inhibit the action of transcriptional activators. In this model (Figure 8), transcription of ORFa is achieved through an interaction of binding factors with unmethylated binding sites within *Ac*. With inactive alleles of *Ac*, a low level of transcription is maintained in most cells due to the hypermethylation of sequences near the start of transcription. Similarly, higher rates of transcription are observed in

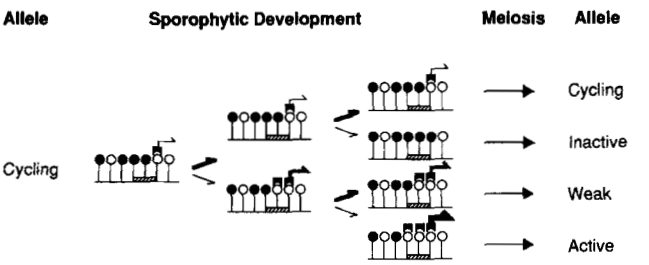

FIGURE 8.—A model for the inheritance of metastable alleles. The region shown represents *Ac* sequence at or near the start of ORFa transcription. The stippled box represents *Ac* sequence presumably recognized by transcription factors (boxes). When these sites are unmethylated (open circles), the factor(s) bind and ORFa is transcribed. Through most cell divisions (thick half arrows) the pattern of methylation is maintained. Rare changes in the pattern (thin half arrows) that are propagated through mitotic divisions may result in changes in the rate of transcription. The thickness of the arrows above the binding factors reflects the rate of transcription.

cycling, weak and active alleles as more transcription factor binding sites become available. Thus, the patterns of variegation observed with metastable alleles of *Ac* are determined by quantitative effects of methylation on ORFa transcription. Studies of *Ac* in Arabidopsis and tobacco have also suggested that transcript levels are critical in the developmental timing of transposition (DEAN *et al.* 1992; SCOFIELD *et al.* 1992; SWINBURNE *et al.* 1992). The strength of heterologous promoters fused to the ORFa cDNA determines the pattern of variegation, suggesting that transactivation of *Ds* only occurs once a threshold of *Ac* message is reached (SCOFIELD *et al.* 1992). We propose that in maize the methylation profile of *Ac* alleles quantitatively determines the rate of ORFa transcription. After a sufficient accumulation of ORFa message, a critical threshold of ORFa protein is reached and transposition may occur.

Transpositions may occur any time after this threshold is reached but the pattern of transposition is then dependent on post-translational (FUßWINKEL *et al.* 1991) controls imposed on *Ac* (*i.e.*, the negative dosage effect). For example, a cycling allele conditions a low level of transcription as a result of insufficient binding of factors required for efficient transcription (Figure 8, top). Through most cell divisions (thick arrow), the initial pattern of methylation is maintained, few sites are bound and transcription rates are low. Only late in development will sufficient transposase levels accumulate to catalyze either *Ac* or *Ds* excisions resulting in the characteristic pattern of *P* striping and aleurone variegation observed with a cycling allele. The loss of methylation at a single or a few sites allows for additional binding and increased transcription rates. As a result, the critical threshold of transposase is reached earlier and excisions can occur earlier in development. Depending on the extent of methylation loss, the patterns of variegation will resemble either weak alleles (some demethylation)

or active alleles (most sites demethylated). Similarly, an increase in the number of methylated binding sites would decrease transcription even further resulting in the generation of an inactive allele.

Although our model does not address the mechanism of inactivation, several features are apparent from our analysis. We frequently identified small (single kernel) sectors of colorless pericarp contiguous with medium variegated pericarp (see Figure 3). These sectors indicate that the inactivation process can occur rapidly, possibly within a single cell division. In addition, we show that the levels of ORFa transcript can be quantitatively related to the degree of methylation of *Ac* sequences, suggesting that methylation is directly involved in the inactivation process. However, it is not known if the methylation of *Ac* is the result of a stochastic process or a mechanism that targets repetitive sequences. The correlation of hypermethylation with inactivation in several transposable elements families suggests that a general mechanism exists to target and inactivate transposable elements. Such a mechanism may exist in maize to target duplicated sequences for inactivation perhaps through the use of cryptic transposon sequences. Although not yet identified in plants, mechanisms in *Ascobolus immersus* (GOYON and FAUGERON 1989; FAUGERON *et al.* 1990) and *Neurospora crassa* (SELKER *et al.* 1987; SELKER and GARRETT 1988) exist that specifically target and inactivate duplicated sequences endogenous to the organism. Such a mechanism in maize may also exist to regulate the potentially detrimental process of transposition.

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LITERATURE CITED

ALLEMAN, M., and J. KERMICLE, 1993 Somatic variegation and germinal mutability reflect the position of the transposable element *Dissociation* within the maize *R* gene. Genetics **135:** 189–203.

ANDERSON, R. E., and R. A. BRINK, 1952 Kernel pattern in variegated pericarp maize and the frequency of self-colored offspring. Am. J. Bot. **39:** 637–644.

ATHMA, P., and T. PETERSON, 1991 *Ac* induces homologous recombination at the maize *P* locus. Genetics **128:** 163–173.

ATHMA, P., E. GROTEWOLD and T. PETERSON, 1992 Insertional mutagenesis of the maize *P* gene by intragenic transposition of *Ac*. Genetics **131:** 199–209.

BANKS, J. A., and N. FEDOROFF, 1989 Patterns of developmental and heritable change in methylation of the *Suppressor-mutator* transposable element. Dev. Genet. **10:** 425–437.

BANKS, J., P. MASSON and N. FEDOROFF, 1988 Molecular mechanisms in the developmental regulation of the maize *Suppressor-mutator* transposable elements. Genes Dev. **2:** 1364–1380.

BARCLAY, P. C., and R. A. BRINK, 1954 The relation between *Modulator* and *Activator* in maize. Proc. Natl. Acad. Sci. USA **40:** 1118–1126.

BARRY, C., G. FAUGERON and J. ROSSIGNOL, 1993 Methylation induced premeiotically in *Ascobolus*: coextension with DNA repeat lengths and effect on transcript elongation. Proc. Natl. Acad. Sci. USA **90:** 4557–4561.

BENNETZEN, J. L., 1987 Covalent DNA modification and the regulation of *Mutator* element transposition in maize. Mol. Gen. Genet. **208:** 57–62.

BENNETZEN, J. L., W. E. BROWN and P. S. SPRINGER, 1988 The state of DNA modification within flanking maize transposable elements, pp. 237–250 in *Plant Transposable Elements*, edited by O. NELSON. Plenum Press, New York.

BOYES, J., and A. BIRD, 1991 DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell **64:** 1123–1134.

BRINK, R. A., and R. A. NILAN, 1952 The relation between light variegated and medium variegated pericarp in maize. Genetics **37:** 519–544.

CARPENTER, R., C. MARTIN and E. S. COEN, 1987 Comparison of genetic behaviour of the transposable element *Tam3* at two unlinked pigment loci in *Antirrhimum majus*. Mol. Gen. Genet. **207:** 82–89.

CEDAR, H., and A. RAZIN, 1990 DNA methylation and development. Biochim. Biophys. Acta **1049:** 1–8.

CHANDLER, V. L., and V. WALBOT, 1986 DNA modification of a maize transposable element correlates with loss of activity. Proc. Natl. Acad. Sci. USA **83:** 1767–1771.

CHEN, J., and S. L. DELLAPORTA, 1993 Urea-based plant DNA miniprep, pp. 526–527 in *The Maize Handbook*, edited by M. FREELING and V. WALBOT. Springer-Verlag, New York.

CHEN, J., I. M. GREENBLATT and S. L. DELLAPORTA, 1987 Transposition of *Ac* from the *P* locus of maize into unreplicated chromosomal sites. Genetics **117:** 109–116.

CHEN, J., I. M. GREENBLATT and S. L. DELLAPORTA, 1991 Molecular analysis of *Ac* transposition and DNA replication. Genetics **130:** 665–676.

CHOMET, P. S., 1988 Characterization of stable and metastable changes of the maize transposable element *Activator*. Ph.D. Thesis, SUNY-Stonybrook, N.Y.

CHOMET, P. S., S. R. WESSLER and S. L. DELLAPORTA, 1987 Inactivation of the maize transposable element *Activator* (*Ac*) is associated with its DNA modification. EMBO J. **6:** 295–302.

CLEMENTI, M., S. MEMZO, P. BOGNARELLI, A. MANZIN, A. VALENZA and P. E. VARALDO, 1993 Quantitative PCR and RT-PCR in virology. PCR Methods Appl. **2:** 191–196.

DAS, P., and J. MESSING, 1994 Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. Genetics **136:** 1121–1141.

DEAN, C., C. SJODIN, T. PAGE, J. JONES and C. LISTER, 1992 Behaviour of the maize transposable element *Ac* in *Arabidopsis thaliana*. Plant J. **2:** 69–81.

DELLAPORTA, S. L., 1993 Plant DNA miniprep and microprep: versions 2.1–2.3, pp. 522–525 in *The Maize Handbook*, edited by M. FREELING and V. WALBOT. Springer-Velag, New York.

DELLAPORTA, S. L., M. A. MORENO and A. DELONG, 1991 Cell lineage analysis of the maize gynoecium using *Ac*. Development Suppl. **1:** 141–147.

DOONER, H. K., and A. BELACHEW, 1989 Transposition pattern of the maize element *Ac* from the *bz-m2(Ac)* allele. Genetics **122:** 447–457.

EMERSON, R. A., 1914 The inheritance of a recurring somatic variation in variegated ears of maize. Am. Nat. **48:** 87–115.

EMERSON, R. A., 1917 Genetical studies of variegated pericarp in maize. Genetics **2:** 1–35.

FAUGERON, G., L. RHOUNIM and J. ROSSIGNOL, 1990 How does the cell count the number of ectopic copies of a gene in the premeiotic inactivation process acting in *Ascobolus immersus*. Genetics **124:** 585–591.

FEDOROFF, N., 1989 The heritable activation of cryptic *Suppressor-mutator* elements by an active element. Genetics **121:** 591–608.

FEDOROFF, N. V., 1990 Maize transposable elements, pp. 377–411 in *Mobile DNA*. American Society for Microbiology, Washington, D.C.

FEDOROFF, N. V., and J. A. BANKS, 1988 Is the *Suppressor-mutator* element controlled by a basic developmental regulatory mechanism? Genetics **120:** 559–577.

FEDOROFF, N., S. WESSLER and M. SHURE, 1983 Isolation of the transposable maize controlling elements *Ac* and *Ds*. Cell **35:** 235–242.

FELDMAR, S., and R. KUNZE, 1991 The ORFa protein, the putative transposase of maize transposable element *Ac*, has a basic DNA binding domain. EMBO J. **10:** 4003–4010.

FUßWINKEL, H., S. SCHEIN, U. COURAGE, P. STARLINGER and R. KUNZE, 1991 Detection and abundance of mRNA and protein encoded

by transposable element *Activator* (*Ac*) in maize. Mol. Gen. Genet. **225:** 186–192.

GAVAZZI, G., M. MEREGHETTI, G. CONSONNI and C. TONELLI, 1990 *Sn*, a light-dependent and tissue-specific gene of maize: the genetic basis of its instability. Genetics **125:** 193–199.

GIERL, A., S. LUTTICKE and H. SAEDLER, 1988 TnpA product encoded by the transposable element *En-1* of *Zea mays* is a DNA binding protein. EMBO J. **7:** 4045–4053.

GILLILAND, G., S. PERRIN, K. BLANCHARD and H. F. BUNN, 1990 Analysis of cytokine and mRNA and DBA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA **887:** 2725–2729.

GOYON, C., and G. FAUGERON, 1989 Targeted transformation of *Ascobolus immersus* and *de novo* methylation of the resulting duplicated DNA sequences. Mol. Cell. Biol. **9:** 2818–2827.

GREENBLATT, I. M., 1968 The mechanism of *Modulator* transposition in maize. Genetics **58:** 585–597.

GREENBLATT, I. M., 1974 Movement of *Modulator* in maize: a test of a hypothesis. Genetics **77:** 671–678.

GREENBLATT, I. M., 1984 A chromosome replication pattern deduced from pericarp phenotypes resulting from movements of the transposable element *Modulator* in maize. Genetics **108:** 471–485.

GREENBLATT, I. M., 1985 The pericarp of maize: a major tool to study transposable elements, pp. 405–417 in *Plant Genetics*, edited by M. FREELING. Alan R. Liss, New York.

GREENBLATT, I. M., and R. A. BRINK, 1962 Twin mutations in medium variegated pericarp in maize. Genetics **47:** 489–501.

IGUCHI-ARIGA, S. M. M., and W. SCHAFFNER, 1989 CpG methylation of the cAMP responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev. **3:** 612–619.

KERMICLE, J. L., 1984 Recombination between components of a mutable gene system in maize. Genetics **107:** 489–500.

KUNZE, R., and P. STARLINGER, 1989 The putative transposase of the transposable element *Ac* from *Zea mays* L. interacts with subterminal sequences of *Ac*. EMBO J. **8:** 3177–3185.

KUNZE, R., U. STOCHAJ, J. LAUFS and P. STARLINGER, 1987 Transcription of the transposable element *Activator* (*Ac*) of *Zea mays* L. EMBO J. **6:** 1555–1563.

KUNZE, R., P. STARLINGER and D. SCHWARTZ, 1988 DNA methylation of the maize transposable element *Ac* interferes with its transcription. Mol. Gen. Genet. **214:** 325–327.

LEWIS, J. D., R. R. MEEHAN, W. J. HENZEL, I. MAURER-FOGY, P. JEPPESEN *et al.*, 1992 Purification, sequence and cellular localization of a novel chromosomal protein that binds to methylated DNA. Cell **69:** 905–914.

MARTIENSSEN, R., A. BARKEN, W. C. TAYLOR and M. FREELING, 1990 Somatically heritable switches in the DNA modification of *Mu* transposable elements monitored with a suppressible mutant in maize. Genes Dev. **4:** 331–343.

MASSON, P., R. SUROSKY, J. A. KINGSBURY and N. V. FEDOROFF, 1987 Genetic and molecular analysis of the *Spm*-dependent *a-m2* alleles of the maize *a* locus. Genetics **177:** 117–137.

MCCLINTOCK, B., 1947 Cytogenetic studies of maize and *Neurospora*. Carnegie Inst. Washington Year Book **46:** 146–152.

MCCLINTOCK, B., 1948 Mutable loci in maize. Carnegie Inst. Washington Year Book **47:** 155–169.

MCCLINTOCK, B., 1949 Mutable loci in maize. Carnegie Inst. Washington Year Book **48:** 142–154.

MCCLINTOCK, B., 1950 The origin and behavior of mutable loci in maize. Proc. Nat. Acad. Sci. USA **36:** 344–355.

MCCLINTOCK, B., 1955 Controlled mutation in maize. Carnegie Inst. Washington Year Book **54:** 245–255.

MCCLINTOCK, B., 1957 Genetic and cytological studies of maize. Carnegie Inst. Washington Year Book **57:** 415–429.

MCCLINTOCK, B., 1958 The *Suppressor-mutator* system of control of gene action. Carnegie Inst. Washington Year Book **57:** 415–429.

MCCLINTOCK, B., 1963 Further studies of gene-control systems in maize. Carnegie Inst. Washington Year Book **62:** 486–493.

MCCLINTOCK, B., 1964 Aspects of gene regulation in maize. Carnegie Inst. Washington Year Book **63:** 592–602.

MCCLINTOCK, B., 1965 Components of action of the regulators *Spm* and *Ac*. Carnegie Inst. Washington Year Book **64:** 527–534.

MEEHAN, R., J. LEWIS, S. MCKAY, E. KLEINER and A. P. BIRD, 1989 Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell **58:** 499–507.

MORENO, M. A., J. CHEN, I. GREENBLATT and S. L. DELLAPORTA, 1992 Reconstitutional mutagenesis of the maize *P* gene by short-range *Ac* transpositions. Genetics **131:** 939–956.

MULLIS, K. B., and F. A. FALOONA, 1987 Specific synthesis of DNA *in vitro* via a polymerase chain reaction. Methods Enzymol. **155:** 335–350.

ORTON, E. R., and R. A. BRINK, 1966 Reconstitution of the variegated pericarp allele in maize by transposition of *Modulator* back to the *P* locus. Genetics **53:** 7–16.

PEREIRA, A., H. CUYPERS, A. GIERL, Z. SCHWARZ-SOMMER and H. SAEDLER, 1986 Molecular analysis of the *En/Spm* transposable element system of *Zea mays*. EMBO J. **5:** 835–841.

PETERSON, P. A., 1966 Phase variation of regulatory elements in maize. Genetics **54:** 249–266.

POHLMAN, R. F., N. V. FEDOROFF and J. MESSING, 1984 The nucleotide sequence of the maize controlling element *Activator*. Cell **37:** 635–643.

PRENDERGAST, G. C., and E. B. ZIFF, 1991 Methylation-sensitive sequence-specific DNA binding by the c-*myc* basic region. Science **251:** 186–189.

ROBERTSON, D. S., 1983 A possible dose-dependent inactivation of *Mutator* in maize. Mol. Gen. Genet. **191:** 86–90.

SCHIEFELBEIN, J. W., V. RABOY, N. FEDOROFF and O. E. NELSON, 1985 Deletions within a defective *Suppressor-mutator* element in maize affect the frequency and developmental timing of its excision from the *bronze* locus. Proc. Natl. Acad. Sci. USA **82:** 4783–4787.

SCHWARTZ, D., and E. DENNIS, 1986 Transposase activity of the *Ac* controlling element in maize is regulated by its degree of methylation. Mol. Gen. Genet. **205:** 476–482.

SCHWARZ-SOMMER, Z., A. GIERL, R. BERNDTGEN and H. SAEDLER, 1985 Sequence comparison of 'states' of *a1-m1* suggests a model of *Spm* (*En*) action. EMBO J. **4:** 2439–2443.

SCOFIELD, S. R., K. HARRISON, S. J. NURRISH and J. JONES, 1992 Promoter fusions to the activator transposase gene cause distinct patterns of dissociation excision in tobacco cotyledons. Plant Cell **4:** 573–582.

SELKER, E., and P. GARRETT, 1988 DNA sequence duplications trigger gene inactivation in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA **85:** 6870–6874.

SELKER, E. U., E. B. CAMBARERI, B. C. JENSEN and K. R. HAACK, 1987 Rearrangements of duplicated DNA in specialized cells of *Neurospora crassa*. Cell **51:** 741–752.

STYLES, E. D., and O. CESKA, 1977 The genetic control of flavonoid synthesis in maize. Can. J. Genet. Cytol. **19:** 289–302.

SWINBURNE, J., L. BALCELLS, S. R. SCOFIELD, J. D. G. JONES and G. COUPLAND, 1992 Elevated levels of *Activator* transposase mRNA are associated with high frequencies of *Dissociation* excision in Arabidopsis. Plant Cell **4:** 583–595.

TOTH, M., U. LICHTENBERG and W. DOERFLER, 1989 Genomic sequencing reveals a 5-methylcytosine-free domain in active promoters and the spreading of preimposed methylation patterns. Proc. Nat. Acad. Sci. USA **86:** 3728–3732.

TOTH, M., U. MULLER and W. DOERFLER, 1990 Establishment of *de novo* methylation patterns: transcription factor binding and deoxycytidine methylation at CpG and non-CpG sequences in an integrated adenovirus promoter J. Mol. Biol. **214:** 673–683.

WALBOT, V., 1992 Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43:** 49–82.

WEIL, C. F., S. MARILLONNET, B. BURR and S. R. WESSLER, 1992 Changes in state of the *Wx-m5* allele of maize are due to intragenic transposition of *Ds*. Genetics **130:** 175–185.

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