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Distribution of *Activator* (*Ac*) Throughout the Maize Genome for Use in Regional Mutagenesis

Judith M. Kolkman,*,[1] Liza J. Conrad,*,†,[1] Phyllis R. Farmer,* Kristine Hardeman,‡ Kevin R. Ahern,* Paul E. Lewis,* Ruairidh J. H. Sawers,* Sara Lebejko,‡ Paul Chomet‡ and Thomas P. Brutnell*,[2]

**Boyce Thompson Institute, Cornell University, Ithaca, New York 14853,* †*Department of Plant Breeding, Cornell University, Ithaca, New York 14853 and* ‡*Monsanto/Mystic Research, Mystic, Connecticut 06355*

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ABSTRACT

A collection of *Activator* (*Ac*)-containing, near-isogenic W22 inbred lines has been generated for use in regional mutagenesis experiments. Each line is homozygous for a single, precisely positioned *Ac* element and the *Ds* reporter, *r1-sc:m3*. Through classical and molecular genetic techniques, 158 transposed *Ac* elements (*tr-Acs*) were distributed throughout the maize genome and 41 were precisely placed on the linkage map utilizing multiple recombinant inbred populations. Several PCR techniques were utilized to amplify DNA fragments flanking *tr-Ac* insertions up to 8 kb in length. Sequencing and database searches of flanking DNA revealed that the majority of insertions are in hypomethylated, low- or single-copy sequences, indicating an insertion site preference for genic sequences in the genome. However, a number of *Ac* transposition events were to highly repetitive sequences in the genome. We present evidence that suggests *Ac* expression is regulated by genomic context resulting in subtle variations in *Ac*-mediated excision patterns. These *tr-Ac* lines can be utilized to isolate genes with unknown function, to conduct fine-scale genetic mapping experiments, and to generate novel allelic diversity in applied breeding programs.

TRANSPOSON tagging is a powerful tool for gene isolation and characterization (Fedoroff *et al.* 1984). It has been used extensively in a number of plant species including Arabidopsis and rice, where large collections of modified *Ds* elements have been generated and precisely positioned throughout their genomes (Muskett *et al.* 2003; Zhu *et al.* 2003; Kim *et al.* 2004; Kolesnik *et al.* 2004; Kuromori *et al.* 2004). In Arabidopsis and rice, *Ac* and *Ds* have been used to isolate genes (Bancroft *et al.* 1993; James *et al.* 1995; Bhatt *et al.* 1996; Meissner *et al.* 1999), define promoter and enhancer elements (Sundaresan *et al.* 1995; Chin *et al.* 1999; Greco *et al.* 2003; Jin *et al.* 2004; Wu *et al.* 2003), and create genetic mosaics for clonal analysis (Peng and Harberd 1997; Jenik and Irish 2001).

Although *Ac/Ds* transposon tagging has proven to be a valuable tool in many plant species, two features of *Ac/Ds* have restricted their utility in large-scale mutagenesis programs in maize (Walbot 2000; Brutnell 2002). For one, *Ac/Ds* elements transpose at rates 50- to 100-fold lower than those of the prolific *Mutator* family of transposable elements (Walbot 2000). This has precluded the use of *Ac* in reverse genetic programs that exploit a high forward mutation rate to generate a large collection of transposon insertions in many genes with relatively few plants. A second feature of *Ac/Ds* elements that has restricted their use in gene-tagging experiments is their propensity for transposition to linked sites in the genome (Van Schaik and Brink 1959; Greenblatt 1984; Dooner and Belachew 1989). In studies of *Ac* transposition at the *p1* and *bz1* loci, ~60% of transpositions were to genetically linked sites and the majority of these transpositions were to regions within 10 cM of the donor element (Greenblatt 1984; Dooner and Belachew 1989). Thus, the utility of *Ac/Ds* as insertional mutagens is limited by the proximity of the transposon to a target locus.

Despite the current limitations of *Ac* for genome-wide mutagenesis, several molecular and genetic characteristics make the *Ac/Ds* system an extremely attractive resource. *Ac* elements are maintained at a relatively low copy number in the genome, facilitating molecular characterization (Fedoroff *et al.* 1983). In maize, increasing copies of *Ac* result in a developmental delay of *Ac* and *Ds* transposition (McClintock 1951). Therefore, the copy number of *Ac* elements can be monitored using many well-characterized *Ds* elements as reporters of *Ac* dosage, including several in genes for anthocyanin and starch biosynthesis (McClintock 1955; Dooner and Kermicle 1971). The ability to monitor increases in *Ac* copy number also provides a powerful tool in gene-

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[1]These authors contributed equally to this work.

[2]*Corresponding author:* Boyce Thompson Institute, Cornell University, 1 Tower Rd., Ithaca, NY 14853. E-mail: tpb8@cornell.edu

mapping experiments (Greenblatt 1984; Van Schaik and Brink 1959; Dooner and Belachew 1989). Another feature of *Ac/Ds* that is extremely useful for gene tagging is a high frequency of somatic excision. Revertant alleles can be used to confirm the identity of an *Ac*-tagged allele, thereby alleviating the need for transgenic complementation or the recovery of multiple insertion alleles (Schultes *et al.* 1996; Schauser *et al.* 1999). Furthermore, somatic transposition events can be exploited to obtain additional gene sequence (Singh *et al.* 2003). Imprecise *Ac* or *Ds* excision events also have the potential to generate "footprints" resulting in stable alleles that create novel proteins with altered activities (Wessler *et al.* 1986; Giroux *et al.* 1996).

Although a few *Ac* elements have been precisely positioned in the maize genome, the utility of *Ac/Ds* would be greatly improved if elements were distributed at 10- to 20-cM intervals and positioned on physical and genetic maps. *Ac* insertions at the *p1* (Emerson 1917; Athma *et al.* 1992), *bz1* (McClintock 1955; Ralston *et al.* 1988), and *wx1* (McClintock 1964; Klosgen *et al.* 1986) loci have been utilized as platforms for random mutagenesis (Dellaporta and Moreno 1994; Cowperthwaite *et al.* 2002). However, only a few genes have been cloned using closely linked *Ac* or *Ds* elements as donor loci in regional mutagenesis experiments (Dellaporta *et al.* 1988; Hake *et al.* 1989; DeLong *et al.* 1993; Colasanti *et al.* 1998; Shen *et al.* 2000; Singh *et al.* 2003). Through the precise placement of *Ac* elements throughout the maize genome, it should be possible to greatly expand the scope of *Ac* mutagenesis programs in maize.

In this study, we have utilized genetic procedures to distribute ~158 *Ac* elements throughout the maize genome. DNA sequence flanking 69 of these elements has been cloned and used to position 41 of the *Ac* elements on one of three publicly available recombinant inbred populations (Burr *et al.* 1988; Lee *et al.* 2002). *Ac* elements have been mapped to all 10 of the maize chromosomes and are primarily located in single- or low-copy genomic sequence. However, insertions into retrotransposons and other repetitive elements were also identified. Characterization of the *Ac*-mediated variegation patterns revealed that genomic context may influence *Ac* activity. These near-isogenic lines along with the cloning methodologies presented here offer powerful resources for efficient *Ac*-based mutagenesis programs in maize.

MATERIALS AND METHODS

Description of maize stocks: All stocks were maintained in a color-converted W22 inbred (Dooner and Kermicle 1971; Kermicle 1984). Approximately 400 primary transpositions were generated from either *P1-vv* or *bti97156::Ac*. The maize *p1* locus controls flavonoid production in floral tissues including pericarp and glumes (Styles and Ceska 1977). *P1-vv* confers a variegated pericarp and cob due to an unstable *Ac* insertion in a *P1-RR* allele of the *p1* locus (Lechelt *et al.* 1989). *bti97156::Ac* is located on chromosome 5 bin 5.04 (Singh *et al.* 2003). The *Ds* tester line used was *r1-sc:m3*, which contains a *Ds* insertion in the *r1* locus (Alleman and Kermicle 1993) and renders the kernel colorless. *Ac*-mediated excisions of *Ds* restore *R* function, resulting in sectors of purple aleurone. The size and number of sectors reflect the copy number of *Ac*. In general, increasing copies of *Ac* result in fewer and smaller sectors (McClintock 1951). When four copies of *Ac* are present in the triploid endosperm, *Ds* excisions are rare or absent, resulting in a few small colored sectors or a colorless aleurone, respectively. When one copy of the *r-sc:m3* reporter is present in the endosperm (see Figure 1), the frequency of colored sectors is reduced, but the average size of colored sectors is similar to what is observed when two or three copies of the *Ds* reporter allele *r-sc:m3* are present. Thus, our selection for finely spotted and colorless kernels in F_1 testcross progeny ensures that we detect all transposition events that contribute to dosage. An average transposition frequency of 2–4% was calculated from a total of ~12,400 kernels generated from 10 different *Ac* lines.

Identification of newly transposed *Ac* elements: To identify transposed *Ac* elements (*tr-Acs*), genomic DNA from ~1 g of pooled leaf tissue was isolated from 5–10 individuals homozygous for the *tr-Ac*. DNA pools were made from two families that carried the same *tr-Ac* to ensure that the *Ac*-containing band was heritable. DNA extraction was performed as described by Chen and Dellaporta (1994). Restriction enzyme digests were performed according to the manufacturer's recommendation using 3–5 μg of genomic DNA in a 20-μl total reaction volume (Promega, Madison, WI). Digests were fractionated overnight on 0.8% standard electrophoresis-grade Low EEO agarose (Fisher Scientific, Fair Lawn, NJ) gels containing ethidium bromide in Tris-acetate EDTA (TAE) buffer along with digoxygenin (DIG)-labeled Roche Ladder VII (Roche Applied Science, Indianapolis, IN). The digests were then transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Filters were hybridized sequentially with the internal 700 bp (Ac700) and 900 bp (Ac900) *Eco*RI-*Hin*dIII fragments of *Ac*. DIG-labeled DNA fragments were synthesized according to the manufacturer's recommendations, using the DIG Probe Synthesis kit (Roche Applied Science) with *Ac*-specific primers TBp38 and TBp39 for Ac700 and TBp40 and TBp41 for Ac900 fragments (Table 1). Hybridizations were performed as described (Sawers *et al.* 2002) and the filters were imaged using the Kodak Image Station 440 CR chemiluminescence detection system (Eastman Kodak, Rochester, NY). All of the lines were examined using the restriction enzyme *Eco*RI, and in some cases additional digestions were performed with the methylation-sensitive enzymes *Pst*I, *Sal*I, or *Bam*HI.

Amplification of *Ac* flanking sequences: Several methods were employed to obtain sequences flanking *tr-Acs*, including a modified amplification of insertion mutagenized sites protocol (AIMS; Frey *et al.* 1998) and three inverse PCR (IPCR) methods (detailed protocols are available at http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_currentmap.html). For all protocols, a 200-μl restriction digest was performed according to the manufacturer's recommendations (Promega) using 15 μg of DNA. The digest was fractionated overnight on an agarose gel as described above. DNA from the appropriately sized region was gel extracted and purified using the GeneCleanIII kit (Qbiogene, Vista, CA), following the protocol provided by the manufacturer. For all three IPCR techniques, ~20 ng of the purified product was self-ligated using T4 DNA ligase in a 50-μl reaction (Promega). The ligation reactions were used as template DNA for one of the following methods. All PCR reactions were performed

TABLE 1

PCR primer sequences

First-round PCR primers	Protocol[a]	Second-round PCR primers	Protocol[a]
		Inverse PCR	
Ac10: TGAACTTGGTTGCAAAGGATG GCTTG	iPCR-2a, iPCR-3a	Ac11: GGGTTCGAAATCGATCGGG ATAAAACT	iPCR-2a
Ac14: TCCACTCCTCGGCTTTAGGA CAAATTG	iPCR-2b, iPCR-3b	Ac12: GCAGGAACAATTGAGAAA ATCAAAGCG	iPCR-2a
Ac18: ACGAAACGGGATCATCCCGAT TAAAAAC	iPCR-2a, iPCR-3a, iPCR-3c	Ac15: AGGTATTTTACCGACCGTT ACCGACCG	iPCR-2b
JGp2: CCGGTTCCCGTCCGATTTCG	iPCR-1b, iPCR-1c	Ac16: AATTGAGACAAACATACCTG CGAGGA	iPCR-2b
JGp3: ACCCGACCGGATCGTATCGG	iPCR-2b, iPCR-3b, iPCR-3c	JGp3: ACCCGACCGGATCGTA TCGG	iPCR-1b, iPCR-1c
TBp35: GTCGGGAAACTAGCTCT ACCG	iPCR-1a, iPCR-1c	TBp32: CAAACATACCTGCGAGGA TCAC	iPCR-1b
TBp42: GGCTGTAATTGCAGGAAC AATTG	iPCR-1a	TBp34: ACCTCGGGTTCGAAATC GATCGG	iPCR-1a, iPCR-1c
TBp43: GAATTTATAATGATGACATG TACAAC	iPCR-1b	TBp37: TAATGAAGTGTGCTAGTG AATGTG	iPCR-1a
Ac700 fragment	Protocol[b]	Ac900 fragment	Protocol[b]
		Probe synthesis and PCR verification	
Ac700 (5′): TTTCCCATCCTACTTTCA TCCCTG	PCR verification	Ac900 (3′): CGTTACCGACCGTTTT CATCCCTA	PCR verification, probe synthesis
TBp38: AAGCTTCATTTGTCAATAAT CATG	Ac700 probe	TBp40: GAATTCAACCTATTTGAT GTTGAG	Ac900 probe
TBp39: CATCTAGTTGAGACATCATA TGAG	Ac700 probe	TBp41: CAACAATCTCCGAACCAA GACG	Ac900 probe

[a] *Ac*-specific primers for iPCR-1, iPCR-2, and iPCR-3 are listed; primers used to amplify sequences flanking (a) 5′ (*Bam*HI) end of *Ac* of *Eco*RI-digested fragments, (b) 3′ end of *Ac* of *Eco*RI-digested fragments, or (c) both ends of *Ac* of *Pst*I-digested fragments are shown.

[b] *Ac*-specific primers for use in PCR verification protocol and for probe synthesis for use in DNA blot analysis.

using Mastercycle Gradient PCR machines (Eppendorf, Westbury, NY).

iPCR-1: A standard IPCR protocol (Ochman *et al.* 1988) using two rounds of PCR with nested primers (Table 1) was executed to amplify flanking sequences up to ~2 kb in size. Approximately 20 ng of ligation products was added to a 50-μl reaction mix containing 2 μl DMSO (Fisher Scientific), 2.5 units *Taq* (Promega), 0.2 mm dNTPs (Promega), 0.5 μm primers, and 1× Promega Buffer with $MgCl_2$. The DNA was denatured at 94° for 2 min, followed by 30 cycles of 94° for 30 sec, 57° for 30 sec, and 72° for 1 min (+ 1 min for every kilobase of flanking sequence) and one cycle of 72° for 10 min. Products from the first round of iPCR-1 were diluted in water (1:200), and 1 μl of the dilution was used for the second round of PCR with nested primers (Table 1), using the same cycling program as round one.

iPCR-2: This method is a modified touchdown PCR protocol (McPherson and Møller 2000), using two rounds of PCR with nested primers (Table 1). Fifty microliters of 1× TE pH 8.0 was added to the ligation reaction and placed at 70° for 10 min. The samples were purified using the QIAGEN (Valencia, CA) nucleotide removal kit and resuspended in 50 μl of 1× TE pH 8. Approximately 0.5 ng of purified ligation product was added to a 50-μl reaction mix containing 2 μl DMSO (Fisher Scientific), 2.5 units *Taq* (Promega), 0.2 mm dNTPs (Promega), 0.2 μm primers, and 1× Promega Buffer containing 15 mm $MgCl_2$. The PCR reaction was denatured at 96° for 3 min; followed by 15 cycles of 94° for 45 sec, 72° for 1 min less 1° per cycle, and 72° for up to 5 min (1 min per kilobase); followed by 16 cycles of 94° for 45 sec, 58° for 1 min, and 72° for up to 5 min with a final cycle of 72° for 10 min. Products from the first round of IPCR were diluted (1:50), and 10 μl of dilution was used for the second round of PCR with nested primers (Table 1), using the same cycling protocol as round one.

iPCR-3: A long-range IPCR protocol (Barnes 1994) was used to amplify fragments with flanking sequences that were up to 8.0 kb. Approximately 3 ng of the ligated DNA template was added to a 20-μl PCR mix containing 1× buffer, 2 mm $MgSO_4$, 0.3 mm dNTPs, 0.8 μl DMSO, 0.5 mm beteine, 0.3 μm primers, 0.5 units of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA), and dH_2O to a final volume of 20 μl. Reaction conditions included 1 cycle of 94° for 4 min; followed by 10 cycles of 94° for 10 sec, 58° for 1 min, and 68° for up to 12 min (1 min per kilobase); 25 cycles of 94° for 10 sec, 58° for 1 min, and 68° for up to 12 min + 10 sec per cycle; and 1 cycle of 72° for 20 min. Only one round of inverse long-range PCR was performed with the *Ac* primers listed in Table 1.

AIMS: The AIMS protocol used in this study was based on the method developed by Frey *et al.* (1998) with several modifications. Following a 200- to 300-ng genomic DNA diges-

tion, adaptors with one of four selective bases were ligated to the ends of the fragment. After 12 cycles of PCR with a biotinylated primer complementary to one of two *Ac* end fragments, the products were bound to Streptavidin-coated magnetic beads to enrich for PCR products containing the *Ac* end. Sequences flanking the *Ac* insertion were amplified using a nested *Ac* end primer and an adaptor primer.

Cloning *Ac* flanking sequences: Following PCR amplification, products were separated on a 0.8% agarose gel and purified using the QIAquick gel extraction kit (QIAGEN). DNA was cloned into either pGEM-T Easy (Promega) or the TOPO TA cloning kit for sequencing (Invitrogen) and sequenced as previously described (Singh *et al.* 2003).

Verification of cloned flanking sequences: For each flanking sequence, a primer pair was designed to the corresponding *Ac* end and the flanking sequence using PrimerSelect (DNASTAR, Madison, WI) or Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/) (see Table 1 for *Ac* end primers and http://bti.cornell.edu/Brutnell_lab2/BMGG_home.html for sequences of flanking primers). Primers were selected to yield a 150- to 300-bp product. PCR conditions were as described above for iPCR-1 and amplification products were fractionated on a 1% agarose gel. DNA blot analysis was performed as described above using DIG-labeled DNA fragments generated using primers designed for PCR verification.

Recombinant inbred mapping: Panels of filters were created that contained parental DNA samples from the IBM94 (B73 × Mo17; Lee *et al.* 2002) and BNL96 (CM37 × T232 and Co159 × Tx303; Burr *et al.* 1988) populations. Each parental DNA sample was digested with *Eco*RI, *Hin*dIII, *Eco*RV, *Sac*I, *Pst*I, *Bgl*II, *Bam*HI, and *Xho*I and hybridized with DIG-labeled *Ac* flanking sequences described above to identify RFLPs. Progeny blots containing DNA from 94 recombinant inbred (RI) lines of the IBM94 population, 47 lines of the CM37 × T232 population, or 41 lines of the Co159 × Tx303 population were used to score segregation. When polymorphisms existed between B73 and Mo17, the IBM94 population was scored because this population is now serving as a framework for multiple maize genomics projects (Sanchez-Villeda *et al.* 2003). However, we also utilized the BNL96 populations to place nine *Ac* elements. All map position data can be obtained at our project website (http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_currentmap.html), as well as at the Maize Genetics and Genomics Database website (MaizeGDB; http://www.maizegdb.org). To access the map position data, the complete *Ac* name (*e.g.*, *mon00150::Ac*) should be used as a search term on the MaizeGDB website. The coordinate position for each *Ac* will be listed relative to markers on the IBM RI populations.

Genomic analysis of sequences flanking *tr-Acs*: The sequences flanking *tr-Acs* were submitted to the NCBI for TBLASTX analysis to the NR, EST, GSS, HTGS, and dbSTS databases. Sequence scores were compared for most significant hit ($e < 10^{-15}$) and putative function (Tables 2 and 3). A chi-square goodness-of-fit was used to examine distribution of *Ac* elements throughout the genome. Each of the *Ac* flanking sequences was also submitted to MaizeGDB for assembly into genome annotations of GSS assemblies (http://www.plantgdb.org/prj/AcDsTagging/).

Accession numbers: The sequences flanking the *tr-Ac* (Table 2) have been submitted to GenBank under the following accession numbers: bti00191, AY559204; bti00207, AY618476; bti00209, AY559195; bti00226, AY559177; bti00245, AY559181; bti00252, AY559208; bti00257, AY559233; bti95004, AY559216; bti95006, AY559192; bti95076, AY559207; bti99224, AY559201; mon02901, AY559179; mon03068, AY618479; mon03073, AY559213; mon03077, AY618478; mon03080, AY618477; mon03082, AY559210; mon00004, AY559178; mon00012, AY559231; mon00030, AY559197; mon00038, AY559229; mon00044, AY559219; mon00060, AY559220; mon00068, AY559191; mon00072, AY618471; mon00084, AY618474; mon00092, AY559212; mon00098, AY559226; mon00106, AY559176; mon00108, AY559206; mon00110, AY559183; mon00126, AY559211; mon00152, AY559217; mon00178, AY559203; mon00186, AY559221; mon00192, AY559223; mon00200, AY559202; mon00212, AY559232; mon00218, AY559185; mon00236, AY559188; mon00238, AY559194; bti00190, AY559199; bti00194, AY559209; bti00220, AY559218; bti00225, AY559205; bti00228, AY618475; bti00238, AY559172; bti00242, AY559198; bti00256, AY559227; bti99221, AY618472; mon00002, AY559182; mon00020, AY559174; mon00028, AY559200; mon00042, AY559180; mon00054, AY559184; mon00058, AY559230; mon00066, AY559186; mon00070, AY559193; mon00080, AY559196; mon00102, AY559173; mon00128, AY618473; mon00132, AY559189; mon00160, AY559190; mon00166, AY559225; mon00168, AY559214; mon00194, AY559234; mon00204, AY559228; mon00210, AY559187; and mon00240, AY559224.

RESULTS

Distribution of *Ac* elements: *Ac* elements were distributed throughout the maize genome utilizing the genetic scheme illustrated in Figure 1. An estimated 400 *Ac* transpositions (*tr-Acs*) were selected from two loci, *P1-vv* (Emerson 1917) located on chromosome 1 (1.03) and *bti97156::Ac* located on chromosome 5 (5.04; Singh *et al.* 2003). Transpositions were selected from testcross progeny obtained by crossing plants homozygous for the donor *Ac* as females with pollen from a reporter line containing a *Ds* insertion at the *r1* locus, *r1-sc:m3* (see materials and methods). As illustrated in Figure 1A, the majority of testcross progeny inherit a single active *Ac* element through the female gametophyte. This results in two copies of *Ac* in cells of the triploid endosperm corresponding to large and frequent sectors of colored aleurone (coarsely spotted). However, *tr-Acs* that are inherited with the donor element result in an increased copy number of *Ac* elements in the endosperm tissue and a dramatic delay in the timing of *Ds* excision (McClintock 1951). In fact, the presence of four *Ac* copies in the endosperm often conditions a completely colorless or very finely spotted aleurone (Figure 1B, white arrow). These "near-colorless" kernels were observed at a frequency of ~2–4% on testcross ears homozygous for *Ac* insertions in W22 (see materials and methods). As the endosperm and embryo tissues share a common gametophytic lineage, selection of near-colorless aleurone was expected to enrich for *Ac* insertions that were transmitted to both endosperm and embryo tissues.

To genetically map *tr-Ac* elements relative to the donor *Ac*, near-colorless selections were testcrossed to the *Ds* reporter (Figure 2A). If a *tr-Ac* shows complete linkage to the donor *Ac*, then kernels inheriting the parental chromosome with two copies of *Ac* are expected to condition a colorless or near-colorless aleurone, whereas kernels that inherited the nonrecombinant chromo-

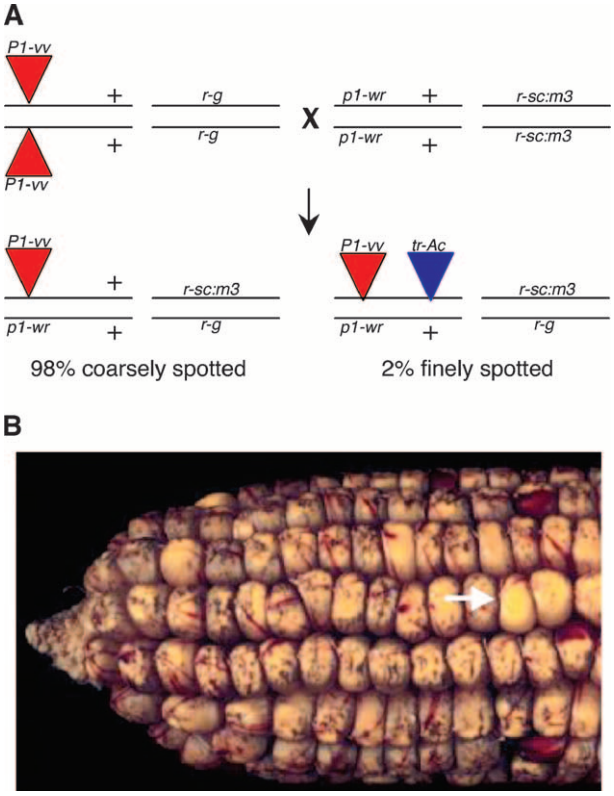

FIGURE 1.—Generation and selection of *tr-Acs*. (A) Lines homozygous for an *Ac* insertion (*P1-vv* illustrated) were crossed to a *Ds* tester reporter line containing the *r1-sc:m3* allele. Approximately 98% of the progeny kernels inherit the original "donor" *Ac*, resulting in coarsely spotted kernels. Transpositions inherited with the donor *Ac* result in an increase in *Ac* copy number in ~2% of the progeny and finely spotted or colorless kernels. (B) Typical F_1 ear showing *p1* locus variegation (red stripes) and aleurone variegation (purple sectors) mediated by *Ac* or *Ds* excision events, respectively. Transmission of two *Ac* elements through the female gametophyte results in four copies of *Ac* in the triploid endosperm and a finely spotted or colorless aleurone (white arrow in B).

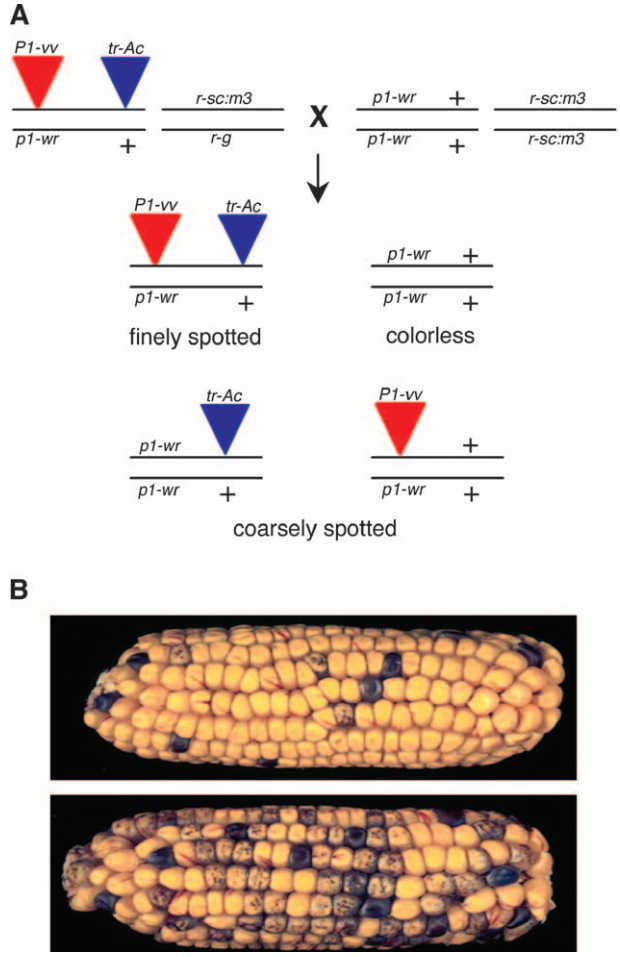

FIGURE 2.—Genetic mapping and segregation of novel *tr-Ac*. (A) Finely spotted kernels from F_1 ears (Figure 1) are testcrossed to the *Ds* tester (*r1-sc:m3*) to map the *tr-Ac* relative to the donor *Ac* element. The resulting ear will contain kernels that are doubly hemizygous for both *Ac* elements, hemizygous for one *Ac* element, or that do not contain any *Ac* insertions (*r1* alleles not shown in schematic). (B) Crosses in which the *tr-Ac* is linked to the donor *Ac* will result in few coarsely spotted kernels (top ear). If the *tr-Ac* is unlinked to the donor *Ac*, approximately one-half of the kernels will be coarsely spotted (bottom ear).

some are completely colorless due to the absence of *Ac* activity. However, recombination between the donor *Ac* and the *tr-Ac* results in a decrease in *Ac* copy number and a coarsely spotted aleurone. Thus, by simply counting the number of kernels with coarsely spotted aleurone and dividing by the total number of spotted and near-colorless kernels, this two-point linkage test can be used to approximate the genetic distance between *Ac* elements. Examples of ears segregating linked and unlinked *Ac* elements are shown in Figure 2B. The presence of kernels with fully colored aleurone is indicative of premeiotic or gametophytic excision of the *Ds* reporter from *r1-sc:m3* and thus they were excluded from the estimates of linkage. It is important to note that this two-point testcross is a rough estimate of genetic distance. *Ac* excision or transposition to unlinked sites may also result in coarsely spotted kernels and the overestimation of genetic distance. Nevertheless, it is likely that the linkage estimates are accurate to within 2–4 cM, corresponding to the transposition frequency of *Ac* (SINGH *et al.* 2003).

To select *tr-Acs* unlinked to the donor *Ac*, coarsely spotted kernels were chosen from 158 testcross ears in which the donor and *tr-Ac* were determined to be genetically unlinked. Ten coarsely spotted kernels were selected from each of the testcross ears and these plants were self-pollinated to generate ears that would contain either the segregating donor *Ac* or the *tr-Ac*. When *P1-vv* was used as the donor *Ac*, ~50% of the ears inherited the parental *Ac* and were easily discernible as *P1-vv* conditions variegated cob and pericarp tissues. Variegated ears were discarded and a single *p1-wr* ear was selected to represent the unlinked *tr-Ac*. However, the parental line *bti97156::Ac* does not condition any obvi-

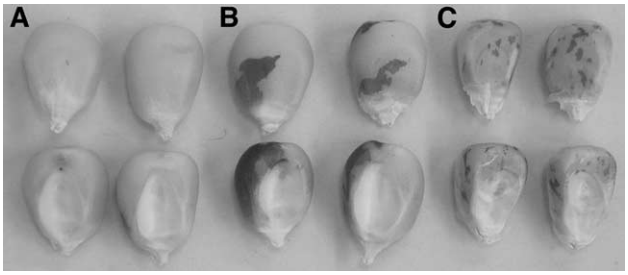

FIGURE 3.—Variation in *Ds*-mediated variegation pattern. Kernels homozygous for three independent *Ac* insertions, (A) *mon00044::Ac*, (B) *mon00060::Ac*, (C) *mon03073::Ac*, display different patterns of aleurone variegation. All kernels are homozygous for the *Ds* reporter at *r1* (*r1-sc:m3*) and are maintained in the W22 inbred.

ous mutant phenotype and could be distinguished from the *tr-Ac* only by using a molecular assay. Leaf tissue was collected and DNA was digested with the methylation-insensitive restriction enzyme *Eco*RI. DNA blot analysis was performed as described in MATERIALS AND METHODS to identify plants that carried either *bti97156::Ac* or the unlinked *tr-Ac*. Plants that inherited *bti97156::Ac* carried a 2.7-kb *Eco*RI fragment that was detected using the internal 700-bp *Eco*RI-*Hin*dIII fragment of *Ac* and were discarded. Families that did not contain this band were assumed to carry the unlinked transposition events. All self-pollinated ears were examined for the presence of the *p1-wr* allele and an aleurone variegation pattern that was consistent with the segregation of a single active *Ac* element. One representative ear from each of the 158 families that met the above criteria was chosen and kernels with few sectors were selected as representing kernels homozygous for a single *Ac* insertion (Figure 3). These plants were self-pollinated and seed from 3–5 ears from each line was collected.

As mentioned above, the selection for transposition was based on the negative dosage effect, whereby increasing copies of *Ac* results in a delay in the timing of *Ds* or *Ac* excision events in maize. This dominant inhibition of transposition is likely to be mediated in part by post-translational control of *Ac* activity (HEINLEIN 1996), but the mechanism remains poorly understood. Close observation of aleurone tissues revealed a continuum of variegation patterns conditioned by independent *tr-Ac* insertions. At one extreme, the aleurone variegation pattern consisted of a few large colored sectors and no fine spots due to *Ds* excision events that occurred early but not late in endosperm development. In other lines, a finely spotted aleurone variegation pattern was observed, suggesting a suppression of *Ac* activity early but not late in endosperm development. Figure 3 shows representative kernels homozygous for each of three different *Ac* insertions. Although the *Ac* copy number is the same in each kernel, there are clear differences in the *Ds*-mediated aleurone spotting patterns. In each case, triploid endosperm cells carry three copies of a single active *Ac*, yet the timing of *Ds* excision varies among lines. As these lines have been maintained in a uniform genetic background and are homozygous for the *Ds* reporter gene, *r1-sc:m3*, the variation is unlikely to be attributable to segregating modifier loci. This variation may be attributable to spontaneous alterations of transposon activity associated with changes in DNA methylation (BRUTNELL and DELLAPORTA 1994; BRUTNELL *et al.* 1997). Alternatively, it is possible that *cis* effects corresponding to the proximity of *tr-Ac* to enhancer elements or heterochromatic regions of the genome mediate these differences in variegation pattern. Similar position-dependent *Ac* variegation patterns have been reported in maize (CHOMET 1988), suggesting that the weak promoter activity of *Ac* is modulated to some extent by the chromosomal context of the element. It is important to note that if a *tr-Ac* did not contribute to the negative dosage effect, it would have escaped our visual screen that relied on reduced kernel variegation. That is, "silenced" *tr-Acs* inherited with the donor *Ac* would be phenotypically indistinguishable from kernels that inherited the donor *Ac* alone or would have resulted in ears that lacked any *Ac* activity if the silenced *tr-Ac* cosegregated with the empty donor site. In either case, these potential *Ac* inactivation events would not have been recovered.

Isolation and characterization of sequences flanking active *Ac* elements: The two-point testcross mapping described above enabled selection for 158 *Ac* transpositions that were genetically unlinked to donor elements. To precisely determine the location of each *tr-Ac*, we developed a two-step procedure to first identify the *tr-Ac* through DNA blot analysis and then amplify the flanking DNA for cloning, using several PCR protocols. DNA was first extracted from pooled seedling tissues and DNA blot analysis was performed as described in MATERIALS AND METHODS. Both methylation-sensitive and -insensitive restriction enzymes were used to increase the likelihood of detecting an *Ac*-containing band. *Ac*-containing bands not detected in either progenitor line were candidates for representing the *tr-Ac* (Figure 4). Use of the methylation-insensitive enzyme *Eco*RI resulted in the fractionation of both heavily methylated cryptic *Ac* elements (CHOMET *et al.* 1987) and hypomethylated active *Ac* elements (Figure 4). However, because the lines are highly inbred and the cryptic *Ac* elements are by definition incapable of transposition, they can be easily distinguished from the active *Ac* bands as those that are common among all *Ac* lines. DNA blot analysis was performed on progeny from two ears for each of the 158 *tr-Acs*. A putative *Ac* insertion that was present in only one of the two families was considered likely to represent either a novel transposition event or a somatic transposition event and the corresponding ears were discarded (Figure 4, lane 1). Lines that carried an *Ac*-containing restriction fragment present in two families but not in either progenitor were advanced for cloning, sequenc-

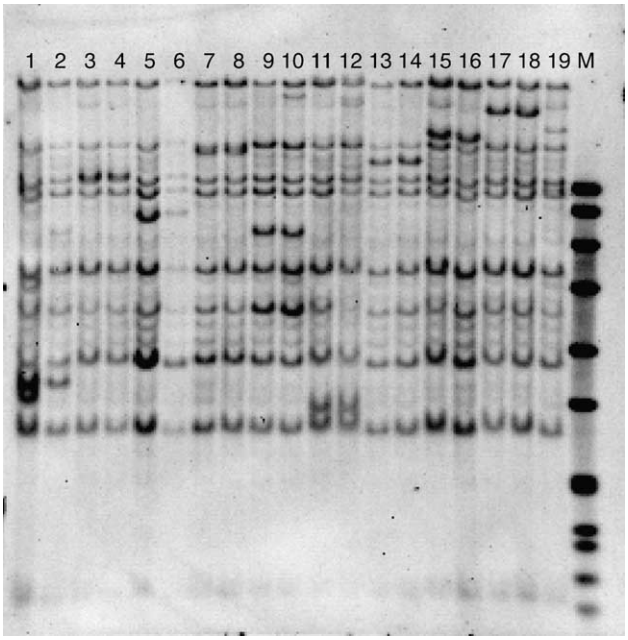

Figure 4.—Identification of novel *tr-Ac* elements in DNA blot analysis. DNA blot analysis was performed with genomic DNA from pooled seedlings derived from two independent ears that each carry the same *tr-Ac* (lanes 1–18; *e.g.*, lanes 1 and 2 are expected to carry the same *tr-Ac*, lanes 3 and 4 are expected to carry another *tr-Ac*), *P1-vv* (lane 19), and DIG-labeled DNA VII ladder (lane 20; Roche). DNA was digested with *Eco*RI and hybridized to the internal 700-bp *Eco*RI/*Hin*dIII fragment of *Ac*. The active, germinally inherited *tr-Acs* are detected as novel bands present only in the two lines harboring the *Ac* insertion.

ing, and mapping (*e.g.*, Figure 4, lanes 9 and 10). Of the 158 *tr-Acs*, a novel *Ac* fragment was identified in 114 *tr-Ac* lines. We then attempted to clone and sequence the DNA flanking each of these unique *Ac* insertions through one of several PCR-based methods.

To clone *Ac* flanking sequences, several PCR-based methods were utilized over the course of this project (detailed protocols available at http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_currentmap.html). All strategies relied on first detecting an *Ac*-containing restriction fragment in DNA blot analysis. Ultimately, two methods were deemed highly robust and used to clone the majority of *Ac*-flanking sequences that varied in size from 200 bp to 8 kb (see materials and methods). A modified AIMS protocol was used to amplify the flanking sequence from 12 *tr-Acs* (Frey *et al.* 1998), but was expensive and labor intensive, whereas iPCR-1 was an unreliable method. We now routinely use iPCR-2 to amplify sequences from 200 bp to 4 kb of sequence flanking *Ac*. iPCR-3 has been used to amplify sequences up to ~8.0 kb in size. Following PCR amplification, DNA fragments were cloned and sequenced (see materials and methods).

As mentioned above, cryptic *Ac* and *Ds* elements with high sequence similarity to active elements are distributed throughout the maize genome (Fedoroff *et al.* 1983; Leu *et al.* 1992). In addition, somatic transposition of *Ac* will create a population of sequences that flank active *Ac* elements, but that are not inherited. Thus, a PCR verification method was developed to discriminate between sequences flanking active and either *Ds* or cryptic *Ac* elements and to distinguish between somatic and germinally transmitted events. Examples of the verification PCR assay are shown in Figure 5B. Regions flanking each *tr-Ac* were sequenced and used to design a *tr-Ac* flanking sequence-specific primer (see materials and methods). PCR reactions were performed with this *tr-Ac* flanking sequence primer and an *Ac* end primer using DNA derived from progenitor lines in addition to DNA pools derived from two families carrying the same *tr-Ac*. Amplification products from the progenitor lines would indicate that sequences flanking either a *Ds* or cryptic *Ac* element were cloned. Forty-five of 114 cloned fragments were determined to be flanking cryptic elements on the basis of this assay and were not characterized further. Amplification of a band of the predicted size in one of the two progeny samples and the absence of bands in the progenitor lines indicated that a somatic transposition event had been recovered. Two flanking sequences were scored as somatic transposition events on the basis of this assay. Only when PCR products of the appropriate size were amplified from both progeny samples and a band was absent from the progenitor lanes was a *tr-Ac* considered to be an active, germinally heritable element (as shown in Figure 5B). Sixty-nine *tr-Acs* from the 114 *tr-Acs* identified by DNA blot fulfilled these criteria and were considered likely to represent novel and heritable *tr-Acs*.

To confirm the PCR results, DNA blot analysis was also performed using the DNA flanking sequences as molecular probes. DNA blot analysis was performed using a DIG-labeled flanking sequence probe (see materials and methods). As shown in Figure 5A (left), a shift in RFLP size between DNA from progenitor plants (Figure 5A, lanes 3 and 4) and from plants carrying the *bti00245::Ac* (Figure 5A, lanes 1 and 2) is observed that corresponds to the introduction of an *Eco*RI restriction site from the *Ac* element. This result is consistent with the PCR verification result and indicates that the cloned DNA flanks an active, germinally transmitted *Ac* element. The presence of a single band in DNA blot analysis also indicates that *bti00245::Ac* had inserted into a single-copy region of the maize genome.

Surprisingly, sequences flanking several *tr-Acs* that were verified by the PCR assay were highly repetitive in the maize genome, even though the corresponding PCR amplicon was used as the DNA blot probe. An example of one such hybridization result is shown in Figure 5A (right). Although the PCR verification (Figure 5B, right) indicates that *bti00194::Ac* is a *tr-Ac*, the intervening DNA between the *Ac* end and the flanking sequence primer site is present in multiple copies in the maize genome,

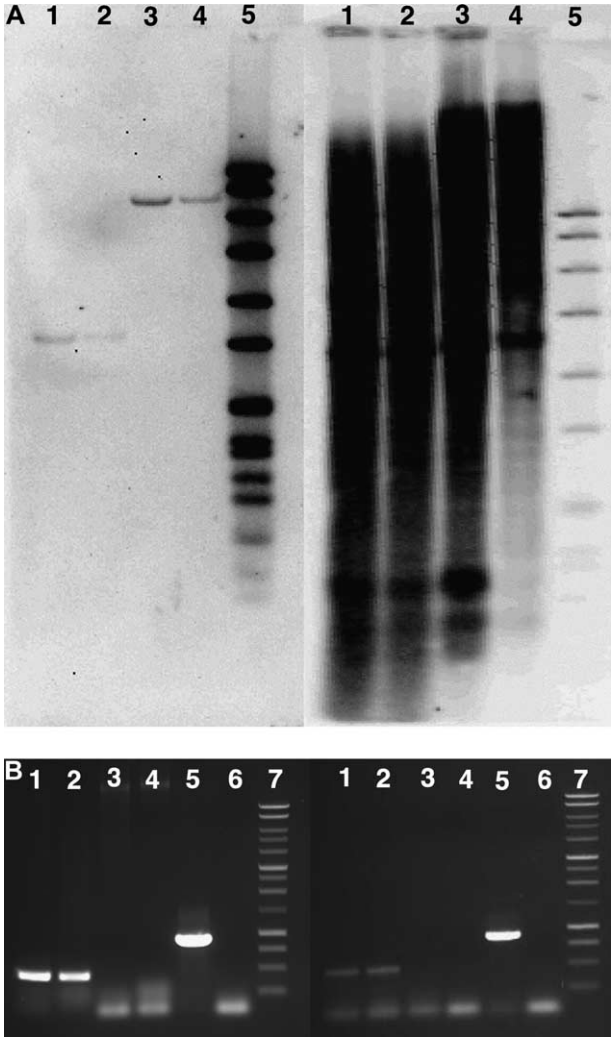

FIGURE 5.—Verification analysis. (A) DNA blot verification. Genomic DNA was isolated from plants carrying *bti00245::Ac* (left, lanes 1 and 2) or *bti00194::Ac* (right, lanes 1 and 2) and from progenitor *Ac*-containing *P1-vv* (lanes 3) and the *Ds* reporter plants (lanes 4). Lane 5 contains DIG-labeled DNA VII ladder (Roche). DNA was digested with *Eco*RI and fractionated on 0.8% agarose gels. DNA blot analysis was performed with a DIG-labeled flanking sequence probe of 443 bp (*bti00245::Ac*) or 432 bp (*bti00194::Ac*) generated using the *Ac*-end primer and flanking sequence-specific primer pairs [DIG-labeled VII ladder (lane 5)]. (B) PCR verification for the presence of *bti00245::Ac* (left) and *bti00194::Ac* (right). Verification PCR was performed using DNA from plants that carried the *tr-Ac* element (lanes 1 and 2), the progenitor *P1-vv* allele (lane 3), and the *r1-sc:m3* allele (lane 4). An *Ac*-end primer was used with a gene-specific primer to amplify fragments of 443 and 432 bp from *bti00245::Ac* and *bti00194::Ac*, respectively. Lanes 5 and 6 represent positive (plasmid template) and negative (no DNA) controls; lane 7 contains a 1-kb DNA ladder (Roche).

as indicated by the smear in DNA blot analysis. Despite our ability to amplify sequences flanking 69 active elements, ~41% (28) contained DNA sequences that were too repetitive to map using conventional RFLP analysis. Because it was not possible to obtain an independent confirmation through DNA blot analysis, these flanking sequences were not placed on genetic maps. Thus, all *Ac* flanking sequences that were mapped, as described below, were confirmed as flanking an active *Ac* element through two independent assays, a PCR verification test and DNA blot analysis.

Mapping and distribution of *Ac* elements: As precisely defined map positions are essential to effectively utilizing *Ac* in gene-tagging experiments, one of three RI populations was utilized to map the *tr-Ac* insertion sites (see MATERIALS AND METHODS). The vast majority of *tr-Ac* flanking sequences have been placed on an intermated B73 × Mo17 population that provides ~0.5-cM resolution (LEE *et al.* 2002). However, 9 of the 41 *tr-Ac* lines were mapped relative to markers developed for the BNL96 population (BURR *et al.* 1988). All *tr-Ac* elements were positioned using the same DIG-labeled flanking sequence probes that were used to show the element was active and heritable in DNA blot analysis. Map bin locations for all 41 *tr-Ac* lines are listed in Table 2. The precise location of the *tr-Ac* lines with reference to specific markers can be found on the project website (http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_currentmap.html) or at MaizeGDB (http://www.maizegdb.org).

In summary, ~400 *tr-Acs* were selected from two donor loci, of which 158 *tr-Acs* were determined to be unlinked to their donor *Ac*. Through DNA blot analysis, we identified RFLPs for 114 of the 158 *tr-Acs* that were of an appropriate size for cloning, using one of three PCR techniques. Sixty-nine fragments were cloned, sequenced, and used to develop PCR primer pairs to confirm their identity as germinal transposition events. Twenty-eight sequences contained elements that were too repetitive to position using RFLP analysis and 41 were represented in the genome as single- or low-copy DNA (Table 2). These 41 *Ac* flanking sequences were positioned on one of three recombinant inbred mapping populations. Homozygous stocks of each of these lines have been propagated and have been made available through the Maize Genetics Stock Center (http://www.maizegdb.org/stock.php).

A composite map showing the position of each of the mapped *tr-Acs* is shown in Figure 6. The *tr-Acs* are shaded to reflect the donor *Ac* from which each was derived. One-third of the *tr-Acs* were derived from *P1-vv* (Figure 6, open arrowheads), whereas two-thirds of the *tr-Acs* were derived from *bti97156::Ac* (Figure 6, solid arrowheads). Insertions were placed on all 10 chromosomes.

A previous study had shown that *Ac* transpositions unlinked to the *bz1* locus often transposed to physically linked sites (*i.e.*, the opposite arm of the same chromosome; DOONER *et al.* 1994). To examine potential *Ac* insertion site biases in this study, we examined the distribution of 39 of the 41 *tr-Ac*s among the 100 defined bins of the maize genome. Two *Ac* elements were genetically mapped as linked to the donor *Ac* and were not

TABLE 2

Description of cloned *tr-Acs*, including the most significant TBLASTX homology to GenBank

No.	*tr-Ac*	GenBank accession	Bin location	Restriction enzyme	Cloning method	Fragment size (kb)	*Zea mays* Accession[a]	*E*-value	Library source[b]
				Mapped *tr-Ac*					
1	bti00191	AY559204	2.02	*Pst*I	iPCR-1	6.1	CC630662	e^{-113}	MF
2	bti00207	AY618476	4.07/4.08	*Eco*RI (Ac900)	iPCR-1	4.6	BZ788264	0.0	High CoT
3	bti00209	AY559195	4.06	*Eco*RI (Ac900)	iPCR-1	2.7	CG379427	$3e^{-88}$	MF
4	bti00226	AY559177	6.00	*Eco*RI (Ac900)	iPCR-1	4.1	CC384514	$3e^{-45}$	High CoT
5	bti00245	AY559181	4.08	*Eco*RI (Ac700)	iPCR-1	3.1	CC368252	e^{-104}	High CoT
6	bti00252	AY559208	1.04/1.05	*Pst*I	iPCR-1	5.4	CG111052	$2e^{-80}$	High CoT
7	bti00257	AY559233	1.05	*Eco*RI (Ac900)	iPCR-1	3.1	CG319773	e^{-148}	MF
8	bti95004	AY559216	1.02/1.03	*Eco*RI (Ac900)	iPCR-1	3.8	CG178263	$6e^{-75}$	High CoT
9	bti95006	AY559192	1.03	*Eco*RI (Ac900)	iPCR-1	3.8	BZ819698	e^{-116}	High CoT
10	bti95076	AY559207	6.05	*Eco*RI (Ac900)	iPCR-1	3.0	CG007394	e^{-118}	UF
11	bti99224	AY559201	10.01/10.02	*Eco*RI/*Kpn*I	iPCR-1	2.0	CC637600	$6e^{-76}$	MF
12	mon02901	AY559179	6.04	*Eco*RI (Ac900)	iPCR-1	3.2	BZ532959	e^{-118}	MF
13	mon03068	AY618479	7.02	*Eco*RI (Ac900)	iPCR-1	3.2	CC617664	e^{-175}	MF
14	mon03073	AY559213	4.06	*Eco*RI (Ac900)	iPCR-1	3.4	CC415995	$4e^{-80}$	High CoT
15	mon03077	AY618478	1.01	*Eco*RI (Ac900)	iPCR-1	3.4	CF633631	e^{-122}	cDNA
16	mon03080	AY618477	1.02	*Eco*RI (Ac700)	iPCR-2	3.1	CG159118	$3e^{-75}$	High CoT
17	mon03082	AY559210	5.08/5.09	*Eco*RI (Ac700)	iPCR-1	4.0	CC649598	e^{-180}	MF
18	mon00004	AY559178	9.06/9.07	*Eco*RI (Ac900)	iPCR-1	3.0	CC613709	e^{-134}	MF
19	mon00012	AY559231	6.01	*Eco*RI (Ac700)	iPCR-1	3.6	BH895218	$2e^{-99}$	*Mu*
20	mon00030	AY559197	5.03/5.04	*Eco*RI (Ac900)	iPCR-2	3.8	CG129443	$4e^{-55}$	High CoT
21	mon00038	AY559229	6.01/6.02	*Eco*RI (Ac900)	iPCR-2	6.2	CG164865	e^{-161}	High CoT
22	mon00044	AY559219	5.06	*Eco*RI (Ac900)	iPCR-1	3.0	CC626710	e^{-170}	MF
23	mon00060	AY559220	7.04	*Pst*I	AIMS	6.3	CC624521	$5e^{-18}$	MF
24	mon00068	AY559191	1.05	*Pst*I	AIMS	5.1	CC698616	e^{-125}	MF
25	mon00072	AY618471	7.03	*Eco*RI (Ac700)	iPCR-3	6.1	CG436891	$3e^{-77}$	MF
26	mon00084	AY618474	2.08	*Eco*RI (Ac700)	iPCR-3	6.1	—	—	—
27	mon00092	AY559212	9.07/9.08	*Eco*RI (Ac700)	iPCR-2	4.6	CG130353	e^{-132}	High CoT
28	mon00098	AY559226	7.04	*Eco*RI (Ac700)	iPCR-1	2.9	CG187250	$6e^{-24}$	High CoT
29	mon00106	AY559176	1.03	*Eco*RI (Ac900)	iPCR-3	8.0	CC607430	e^{-174}	MF
30	mon00108	AY559206	8.07	*Pst*I	AIMS	6.3	CC709951	$3e^{-38}$	MF
31	mon00110	AY559183	8.02	*Eco*RI (Ac900)	AIMS	2.8	CC711298	$5e^{-88}$	MF
32	mon00126	AY559211	4.02/4.03	*Pst*I	AIMS	5.0	CG375880	$1e^{-61}$	MF
33	mon00152	AY559217	5.04/5.05	*Eco*RI (Ac900)	iPCR-3	10.0	BZ637224	e^{-117}	MF
34	mon00178	AY559203	3.05	*Eco*RI (Ac900)	iPCR-2	3.4	CG045942	$6e^{-42}$	High CoT
35	mon00186	AY559221	1.06/1.07	*Eco*RI (Ac700)	iPCR-3	5.7	CC804870	$1e^{-37}$	UF
36	mon00192	AY559223	1.03	*Eco*RI (Ac700)	iPCR-3	7.4	CC435538	$1e^{-29}$	High CoT
37	mon00200	AY559202	1.10/1.11	*Eco*RI (Ac900)	iPCR-3	9.5	CC011003	$6e^{-48}$	High CoT
38	mon00212	AY559232	5.01	*Pst*I	AIMS	5.4	CC375028	e^{-141}	High CoT
39	mon00218	AY559185	10.07	*Eco*RI (Ac700)	iPCR-2	5.5	CC718212	$8e^{-93}$	MF
40	mon00236	AY559188	9.03	*Eco*RI (Ac700)	iPCR-3	7.4	CC441960	$2e^{-71}$	High CoT
41	mon00238	AY559194	5.06	*Eco*RI (Ac900)	AIMS	5.2	CC614740	$7e^{-66}$	MF
				Repetitive *tr-Ac*					
42	bti00190	AY559199	Unknown	*Eco*RI (Ac900)	iPCR-1	3.1	CC328152	e^{-153}	MF
43	bti00194	AY559209	Unknown	*Eco*RI (Ac900)	iPCR-1	2.8	CG291158	e^{-151}	MF
44	bti00220	AY559218	Unknown	*Eco*RI (Ac700)	iPCR-1	4.7	CC646925	e^{-169}	MF
45	bti00225	AY559205	Unknown	*Eco*RI (Ac700)	iPCR-1	3.7	—	—	—
46	bti00228	AY618475	Unknown	*Pst*I	iPCR-1	6.8	CG018677	e^{-156}	MF
47	bti00238	AY559172	Unknown	*Eco*RI (Ac900)	iPCR-1	3.5	CG311429	0.0	MF
48	bti00242	AY559198	Unknown	*Eco*RI (Ac700)	iPCR-1	3.0	CC808672	$2e^{-36}$	BAC
49	bti00256	AY559227	Unknown	*Eco*RI (Ac900)	iPCR-1	3.7	CC735442	$1e^{-44}$	MF
50	bti99221	AY618472	Unknown	*Eco*RI (Ac900)	iPCR-1	2.7	CC890275	$2e^{-72}$	BAC
51	mon00002	AY559182	Unknown	*Eco*RI (Ac700)	iPCR-2	5.2	CG308013	$1e^{-76}$	MF
52	mon00020	AY559174	Unknown	*Eco*RI (Ac900)	AIMS	3.0	CC004576	$2e^{-48}$	Genomic
53	mon00028	AY559200	Unknown	*Eco*RI (Ac900)	iPCR-2	4.4	CG222531	$5e^{-71}$	MF
54	mon00042	AY559180	Unknown	*Eco*RI (Ac900)	iPCR-2	2.4	—	—	—

(*continued*)

TABLE 2

(Continued)

No.	*tr-Ac*	GenBank accession	Bin location	Restriction enzyme	Cloning method	Fragment size (kb)	*Zea mays* Accession[a]	*E*-value	Library source[b]
				Repetitive *tr-Ac*					
55	mon00054	AY559184	Unknown	*Bam*HI	AIMS	5.2	CG031998	$4e^{-47}$	High CoT
56	mon00058	AY559230	Unknown	*Pst*I	AIMS	5.0	CC445930	$2e^{-19}$	High CoT
57	mon00066	AY559186	Unknown	*Eco*RI (Ac900)	iPCR-2	4.2	CG240822	$1e^{-34}$	MF
58	mon00070	AY559193	Unknown	*Eco*RI (Ac900)	iPCR-1	2.7	CC756012	$4e^{-25}$	cDNA
59	mon00080	AY559196	Unknown	*Eco*RI (Ac900)	iPCR-2	4.0	BZ974435	$3e^{-57}$	High CoT
60	mon00102	AY559173	Unknown	*Eco*RI (Ac900)	iPCR-1	2.8	CG371472	e^{-111}	MF
61	mon00128	AY618473	Unknown	*Eco*RI (Ac900)	iPCR-3	3.3	BZ809673	e^{-139}	High CoT
62	mon00132	AY559189	Unknown	*Eco*RI (Ac900)	iPCR-3	10.0	CC428556	e^{-103}	High CoT
63	mon00160	AY559190	Unknown	*Eco*RI (Ac700)	iPCR-3	7.0	BH408973	$3e^{-85}$	*Mu*
64	mon00166	AY559225	Unknown	*Pst*I	iPCR-3	8.4	BZ801148	e^{-100}	High CoT
65	mon00168	AY559214	Unknown	*Eco*RI (Ac900)	iPCR-2	3.0	CC696403	$2e^{-71}$	MF
66	mon00194	AY559234	Unknown	*Eco*RI (Ac700)	iPCR-3	6.2	CG178282	$2e^{-79}$	High CoT
67	mon00204	AY559228	Unknown	*Eco*RI (Ac900)	AIMS	3.6	CC357240	e^{-111}	High CoT
68	mon00210	AY559187	Unknown	*Eco*RI (Ac900)	iPCR-2	3.3	BZ828425	$2e^{-70}$	High CoT
69	mon00240	AY559224	Unknown	*Pst*I	iPCR-3	8.6	BZ817976	$3e^{-51}$	High CoT

[a] Most significant homologies identified in TBLASTX searches of public databases.
[b] Methylation filtration (MF), unfiltered (UF), and high-CoT libraries; rescue *Mu* flanking sequence libraries (*Mu*); or GenBank database.

included in this analysis. A chi-square test revealed that the distribution of *Ac* elements was consistent with a random (Poisson) distribution ($\chi^2 = 0.17$; $P = 0.919$) and suggests that there is no strong regional insertion site bias for *Ac* transposition from the two donor *Acs*. However, due to our small sample size only an extremely strong bias would be detectable. Nevertheless, these results suggest that a high-density distribution of *Ac* throughout the maize genome is feasible using relatively few donor *Acs*.

***Ac* inserts preferentially into hypomethylated sequences in the maize genome:** The flanking sequences of all 69 heritable active *tr-Ac* elements were analyzed for sequence homology through searches of GenBank. All flanking sequences, with the exception of three, *bti00225::Ac*, *mon00042::Ac*, and *mon00084::Ac*, identified a highly significant ($e < 10^{-15}$) homology to database sequences (Table 2). The majority of sequences with the highest similarity to DNA flanking the 41 mapped *tr-Acs* were evenly distributed between those found in methylation-filtered (19 sequences) and high-CoT (17 sequences) libraries (Rabinowicz *et al.* 1999; Yuan *et al.* 2003). Four of the mapped *tr-Acs* inserted in DNA sequenced from an unfiltered library, a *Mu* library,

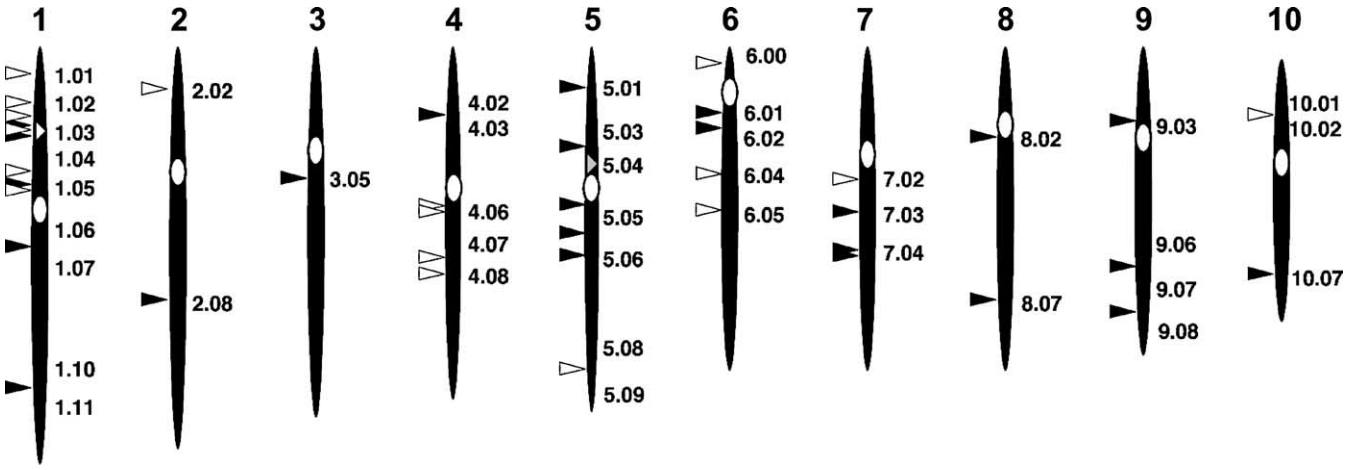

Figure 6.—Schematic of maize chromosomes showing the approximate bin locations of the *tr-Ac* elements. The locations of the two original donor *Acs* used to develop new *tr-Acs* are highlighted by the small open arrowhead and bin location (*P1-vv*) at 1.03 or the small shaded arrowhead and bin location (*bti97156::Ac*) at 5.04. The *tr-Acs* are distributed on each of the 10 chromosomes and are indicated as either open (*P1-vv*, donor *Ac*) or solid arrowheads (*bti97156::Ac*, donor *Ac*).

or a cDNA library, and a single mapped *Ac* flanking sequence did not match any published sequence ($e < 10^{-15}$; Table 2). Surprisingly, of the 28 flanking sequences that were too repetitive to map by RFLP analysis, 11 were highly similar to sequences present in methylation-filtered libraries and 10 were most similar to sequences present in high-CoT libraries. Five sequences were distributed among several libraries as shown in Table 2 and 2 sequences had no strong sequence similarity to GenBank entries. These results are consistent with previous findings indicating *Ac* has a strong insertion site bias for hypomethylated, low-copy sequences in the genome (Cowperthwaite *et al.* 2002). However, our findings suggest that highly repetitive stretches of DNA can be imbedded in these low-copy or hypomethylated islands and that repetitive regions serve as suitable targets for *Ac* insertion in this context.

BLAST searches were also performed of the GenBank nr and est databases to identify potential *Ac* insertions into genes of known or unknown function. Of the 41 mapped flanking sequences surveyed, 4 inserted into sequences with strong similarity to genes of known function and 9 were to genes of unknown function (Table 3). The high number of hits to genes with unknown function is reflective of the relatively uncharacterized gene space in maize. GenBank searches of the 28 *tr-Acs* flanking sequences that were too repetitive to map resulted in 22 significant hits to sequences in the nr database. These included 7 sequences that show high similarity to maize retroelement sequences. Interestingly, 5 of these sequences were also present in the methylation-filtered and high-CoT libraries (see Tables 2 and 3). These results suggests that hypomethylated and low-copy sequences are preferred targets for *Ac* insertion, but that short stretches of highly repetitive sequence within these hypomethylated clusters are also suitable targets for *Ac* insertion.

DISCUSSION

Development of *Ac* insertional mutagenesis in maize: Through combined classical and molecular genetic approaches we have distributed 158 *Ac* elements throughout the maize genome. DNA flanking 69 of these elements has been cloned and sequenced and 41 *tr-Acs* have been placed on one of three recombinant inbred populations. These lines are publicly available (http://w3.aces.uiuc.edu/maize-coop/) and have been developed to facilitate the implementation of *Ac*-based regional mutagenesis programs in maize. Each *Ac* line is homozygous for a unique *tr-Ac* insertion that can be molecularly tracked using both PCR and DNA blot assays and monitored phenotypically with a *Ds* reporter gene *(r1-sc:m3)*. Detailed molecular and genetic characteristics for each *tr-Ac* insertion can be found at http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_currentmap.html

Several gene-tagging strategies have been described in maize to disrupt and clone target genes using *Ac* (Brutnell and Conrad 2003; Dellaporta and Moreno 1994). Because of the low germinal transposition frequency of *Ac*, these strategies exploit reporter genes to either monitor *Ac* or *Ds* excision events from a donor locus or detect an increase in *Ac* copy number. These strategies also exploit the tendency of *Ac/Ds* elements to insert at closely linked sites in the genome. This tendency of *Ac* to insert at physically and genetically closely linked sites has been demonstrated at several loci throughout the maize genome (McClintock 1948; Greenblatt 1984; Dooner and Belachew 1989). Due to this general property of *Ac*, the 41 *tr-Ac* lines described here are ideally suited to clone and characterize genes that are closely linked to these donor *Acs*. To demonstrate the utility of these lines, we have recently cloned and characterized multiple *Ac*-induced alleles of the *Vp7* gene using an *Ac* donor that mapped to within 4 cM of the *Vp7* gene (Singh *et al.* 2003).

Ac insertions into a multigene family member or the disruption of a single gene controlling a multigenic trait will likely lead to subtle mutant phenotypes that may be detected only in a highly uniform population. Therefore, an extremely important feature of these materials is that they have been maintained in the inbred W22. The uniformity of the genetic materials also facilitates the molecular cloning of *Ac* flanking sequences as both methylation-sensitive and methylation-insensitive restriction enzymes can be readily utilized to identify novel *Ac* insertions in the genome (Figure 4). Unlike many lines that harbor highly prolific *Mutator* elements (May *et al.* 2003), the mutational load of these *Ac* lines is relatively low due to the infrequent transposition of *Ac* and *Ds* elements. Another important feature of these lines is that they exploit endogenous transposable elements, utilizing classical genetic techniques to mobilize and map insertions. Thus, the growth and propagation of these nontransgenic lines does not necessitate the need for an APHIS permit and compliance with the associated regulations for ensuring genetic isolation of transgenes (http://www.aphis.usda.gov/brs/perstds.html). Thus, these lines can be easily distributed and utilized by researchers in the United States and abroad, facilitating their use in the public research domain.

In addition to developing the genetic resources for regional mutagenesis in maize, we have described two PCR techniques to facilitate the cloning and characterization of *Ac*-induced mutations. Both techniques exploit the use of an agarose gel purification step to fractionate active *Ac* insertions from cryptic elements. Nevertheless, despite this purification step and one or two rounds of PCR amplification using two or four *Ac*-specific primers, several sequences flanking cryptic elements were amplified. Sequences flanking cryptic elements were isolated when both methylation-sensitive and -insensitive restriction enzymes were used, sug-

TABLE 3

Putative function of *Ac* flanking sequences and insertion site location (in parentheses), if known

tr-Ac	Database	*E*-value	Accession	Putative function
			Mapped *tr-Acs*	
bti00207	nr	$5e^{-25}$	AF466202	Maize putative pol protein gene
bti00209	est	$3e^{-22}$	AU067887	Rice unknown gene
bti00226	nr	$2e^{-43}$	AF413200	Maize *d8* gene (promotor and 5′-UTR region)
bti00245	est	$6e^{-46}$	BQ163759	Maize unknown gene
mon03077	est	e^{-122}	CF633631	Maize unknown gene
mon03082	nr	$2e^{-76}$	AY105664	Maize unknown gene
mon00012	nr	$5e^{-69}$	AY105294	Maize unknown gene
mon00044	nr	$1e^{-59}$	NM_122227	Arabidopsis serine carboxypeptidase-related gene (coding sequence)
mon00060	nr	$9e^{-18}$	CA619151	Wheat unknown gene
mon00110	nr	$8e^{-45}$	AF100769	Maize receptor-like kinase gene (5′-UTR region)
mon00200	est	$2e^{-23}$	BF728928	Maize unknown gene
mon00212	est	$5e^{-37}$	AW231322	Maize unknown gene
mon00218	est	$4e^{-40}$	CF244390	Maize unknown gene
			Repetitive *tr-Acs*	
bti00190	nr	e^{-151}	AF466203	Maize gypsy-type retrotransposon RIRE2
bti00194	nr	$3e^{-15}$	AY105224	Maize unknown gene
bti00220	nr	e^{-110}	NM_105440	Arabidopsis leucine-rich repeat family protein (coding sequence)
bti00228	nr	e^{-106}	AP003022	Pseudogene, transposable element Txlc protein 2 (putative coding sequence)
bti00242	nr	$1e^{-25}$	AF448416	Maize retrotransposon Xilon2 LTR in *bz* genomic region
bti00256	nr	$7e^{-32}$	D63956	Maize *gmlip15* gene (upstream of TATA box)
mon00002	nr	$1e^{-54}$	AP002869	Rice putative polygalacturonase *PG2* gene (coding sequence)
mon00020	nr	$2e^{-48}$	AF546189	Maize repetitive region in 19-kD zein gene family
mon00028	nr	$1e^{-29}$	AP004693	Rice putative dioscorin class A precursor (coding sequence)
mon00054	nr	$2e^{-31}$	AY109416	Maize unknown gene
mon00066	nr	$2e^{-30}$	AF464738	Maize copia-type pol polyprotein
mon00070	nr	$1e^{-18}$	AF050455	Maize gypsy/Ty3-type retrotransposon Tekay LTR
mon00102	nr	$2e^{-63}$	AC027037	Rice putative cellulose synthase (coding sequence)
mon00128	nr	$1e^{-60}$	U57899	Maize repressor-like protein (*in1*) gene
mon00132	est	$5e^{-55}$	AI939783	Maize unknown gene
mon00160	nr	$3e^{-50}$	AP005245	Rice putative DNA-damage-repair toleration protein (coding sequence)
mon00168	nr	$7e^{-17}$	AY144442	Sorghum: located between Kaema-2 retrotransposon and MITE
mon00194	nr	$1e^{-15}$	AF010283	Sorghum: next to solo LTR of retroelement Leviathan
mon00204	est	$6e^{-46}$	BG901272	Maize unknown gene
mon00210	nr	$4e^{-40}$	AC116033??	Maize repetitive region in 19-kD zein gene family
mon00240	nr	$7e^{-32}$	L22344	Maize *globulin-1* gene (promoter region)

gesting that some percentage of the inactive *Ac* derivatives are found in hypomethylated regions of the genome. Although methylation-sensitive enzymes were more effective for cloning flanking sequences linked to active *Ac* elements, only ~15% of the fragments cloned could be fractionated with these enzymes, while 85% could be visualized using methylation-insensitive enzymes. Thus, we prefer the use of methylation-insensitive enzymes to identify and clone sequences flanking *Ac* elements in maize.

The differences between the two favored PCR techniques utilized lie in the use of high-fidelity *Taq* polymerase (iPCR-3) or a low-cost *Taq* polymerase (iPCR-2) and one (iPCR-3) *vs.* two (iPCR-2) rounds of PCR amplification. We found that the incorporation of a high-fidelity *Taq* and long-range protocol enabled the cloning of sequences up to 8.0 kb from the donor locus. The use of a relatively nonprocessive *Taq* (Promega) proved satisfactory to amplify flanking sequences of up to 4.0 kb. Importantly, both techniques can be used to efficiently clone sequences flanking most *Ac* insertions in the maize genome and thus are effective for recovering *Ac*-flanking sequences in gene-tagging experiments.

***Ac* insertion site preferences:** Previous studies of *Ac* transposition in maize have shown that *Ac* transposes during the replication of mitotic chromosomes (Brink and Nilan 1952; Greenblatt and Brink 1962) into

hypomethylated regions of the maize genome (Chen *et al.* 1987). In a recent survey of 46 *Ac* insertion sites, Dooner and colleagues found that all *Ac* transposition events occurred to low- or single-copy regions of the maize genome (Cowperthwaite *et al.* 2002). However, as reported here, a number of *Ac* insertions were located in repetitive sequences. A number of possibilities may explain this apparent discrepancy. For one, most of the *Ac*-flanking sequences cloned in this study were identified using *Eco*RI, a methylation-insensitive restriction enzyme that rarely discriminates between the hypomethylated low-copy sequences in the genome and the hypermethylated repetitive sequences in the genome. The majority of *Ac*-flanking sequences characterized in previous studies were identified using methylation-sensitive restriction enzymes that would enrich for *Ac* insertions in single- or low-copy sequences in the genome. In addition, we utilized a two-step approach to first verify that sequences flanking an *Ac* element were indeed flanking a heritable, active element and then determined if the sequences were repetitive. In previous studies, sequences that were highly repetitive in DNA blot verification may have been discarded as representing sequences flanking cryptic *Ac* elements, thus biasing the population for low- or single-copy insertions.

Our finding that *Ac* occasionally inserts into repetitive elements in the genome that are present in high-CoT and MF libraries suggests that at least a portion of the highly repetitive sequences in the maize genome is hypomethylated and thus a target for *Ac* insertion. Indeed, our recent characterization of the phytochrome gene family in maize has shown that a Ty3-like insertion is present in the second intron of *PhyB2* (Sheehan *et al.* 2004) and miniature inverted repeat transposable elements (MITES) are often located throughout genic regions (Tikhonov *et al.* 1999; Zhang *et al.* 2000; Fu *et al.* 2001). *Ac* insertions into retroposons or MITES located within genes could explain why some of the *Ac* flanking sequences (Table 3) contained repetitive DNA despite a preference for hypomethylated regions of the genome. Despite our inability to position these *Ac* elements on a genetic map using RFLP analysis, placement to a physical map may be possible as additional maize genomic sequences are assembled.

Variation in *Ac* dosage effects: One of the surprising findings was the degree of variation in *Ac*-mediated *Ds* variegation patterns. Kernel variegation patterns ranged from nearly colorless to showing relatively large and frequent excision events (Figure 3). As all *Ac* elements in this study were monitored using the same *Ds* reporter in near-isogenic lines, it is unlikely that the variation observed is due to variation at the *r1-sc:m3* locus or segregating modifier loci. Previous studies of *Ac*-mediated variegation patterns have indicated that methylation plays an important role in determining the timing of *Ac* or *Ds* excision in metastable *Ac* alleles, corresponding to altered transcriptional activities of *Ac* (Brutnell and Dellaporta 1994). Furthermore, *Ac* derivatives such as *Ac-st1* (McClintock 1948) and *Ac-st2* (Brutnell *et al.* 1997) that are structurally identical to *Ac* but display dosage-independent (*Ac-st1*) or positive dosage effects (*Ac-st2*) are also altered in their transcriptional profiles relative to active *Ac* elements (Brutnell 1995). Thus, it is likely that the variation in *Ac*-mediated excision patterns is due to small changes in the transcriptional activity of *Ac* across the genome. As the *Ac* element itself contains weak promoter sequences (Kunze *et al.* 1987; Fusswinkel *et al.* 1991), and both *Ac* and *Ds* excision is extremely sensitive to small (less than twofold) changes in *Ac* transcript levels (Brutnell and Dellaporta 1994), these lines may serve as an ideal resource for probing *cis*-acting elements that control gene expression throughout the maize genome.

***Ac*-based gene-tagging programs in maize:** The distribution of *tr-Ac* elements on all 10 chromosomes of maize represents an important first step toward the development of an *Ac*-based insertional mutagenesis program in maize. To further distribute *Ac* elements throughout the genome, transposition events have been generated from each linkage group and/or arm from a subset of the 41 "donor" *Ac* lines and are being precisely positioned on the IBM94 population (http://bti.cornell.edu/Brutnell_lab2/Projects/Tagging/BMGG_pro_currentmap.html). The ultimate goal of this program is to distribute *Ac* elements at 10–20 cM throughout the maize genome. As genome sequencing efforts proceed (Palmer *et al.* 2003; Whitelaw *et al.* 2003), it is likely that *tr-Acs* will be rapidly placed on physical maps using *Ac* flanking sequences (*e.g.*, http://www.plantgdb.org/prj/AcDsTagging/). A complete genome sequence of maize will also greatly aid in the development of efficient *Ac*-based gene-tagging strategies (Brutnell and Conrad 2003) to identify candidate genes that are tightly linked to *Ac* insertions and thus serve as ideal targets for regional mutagenesis (Fu *et al.* 2001). Ultimately, the development of an *Ac*-based forward genetics program to identify genes on the basis of phenotype will greatly complement ongoing reverse genetic programs in maize that exploit gene sequence data to identify *Mutator* insertions in candidate genes (Brutnell 2002).

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