

Use of the Transposon *Ac* as a Gene-Searching Engine in the Maize Genome

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We show here that, although genes constitute only a small percentage of the maize genome, it is possible to identify them phenotypically as *Ac* receptor sites. Simple and efficient *Ac* transposition assays based on the well-studied endosperm markers *bz* and *wx* were used to generate a collection of >1300 independent *Ac* transposants. The majority of transposed *Ac* elements are linked to either the *bz* or the *wx* donor loci on chromosome 9. A few of the insertions produce obvious visible phenotypes, but most of them do not, suggesting that these populations will be more useful for reverse genetics than for forward transposon mutagenesis. An inverse polymerase chain reaction method was adapted for the isolation of DNA adjacent to the transposed *Ac* elements (*tac* sites). Most *Ac* insertions were into unique DNA. By sequencing *tac* sites and comparing the sequences to existing databases, insertions were identified in a number of putative maize genes. The expression of most of these genes was confirmed by RNA gel blot analysis. We report here the isolation and characterization of the first 46 *tac* sites from the two insertion libraries.

INTRODUCTION

The maize genome is made up largely of repetitive DNA (Hake and Walbot, 1980), of which a significant fraction consists of methylated retrotransposons arranged in clusters (SanMiguel et al., 1996). Genes likely make up <5% of the total nuclear genome and are found in hypomethylated CpG islands (Antequera and Bird, 1988; Bennetzen et al., 1994). This also is the genomic component into which the transposon *Activator* (*Ac*) inserts (Dellaporta and Chomet, 1985; Chen et al., 1987). One can take advantage of this property of *Ac* to identify genes phenotypically as *Ac* receptor sites, hereafter termed *tac* sites (for transposed *Ac* sites). Genes then would be isolated and sequenced as the DNA adjacent to *Ac*. An advantage of this approach to maize functional genomics is that, in addition to a sequence that can be compared with the existing databases, it generates an insertion library that can be screened for subtle mutant phenotypes, particularly after learning where the genes are expressed. Many genes are expected to have minor effects or to be induced only under special conditions and would be

missed from a conventional transposon mutagenesis screen designed to identify gross changes in phenotype (Thatcher et al., 1998).

There is ample evidence that other maize transposons, such as *Mu* and *Spm/En*, also insert preferentially into hypomethylated DNA (Chandler and Walbot, 1986; Cone et al., 1986; Schmidt et al., 1987; Bennetzen et al., 1988). Furthermore, maize genes and transposons occupy an isochore of DNA with an extremely narrow G-C content that constitutes only 10 to 20% of the genome (Carels et al., 1995). Thus, in general, transposons can be used to search for genes within the bulk of repetitive DNA. This concept served as the basis for projects that generated large pools of genomic DNAs from tens of thousands of plants carrying *Mutator* elements at multiple locations in the genome (Das and Martienssen, 1995; Meeley and Briggs, 1995). From these populations, popularly known as *Mu* gene machines, several mutations have been identified in genes for which a DNA sequence was available (Bensen et al., 1995; Frey et al., 1997). This resource has enabled investigators to proceed from sequence to mutant very rapidly. Most mutations recovered from the *Mu* gene machines, as well as from regular *Mu* transposon mutagenesis experiments, carry the nonautonomous, but highly abundant, *Mu1* element (Bennetzen, 1996).

Ac insertion libraries represent a complementary approach to the *Mu* gene machine. As an insertion, *Ac* has several advantages: (1) *Ac* transpositions can be selected phenotypically. Thus, it is possible to use convenient seed

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phenotypes to assemble a large collection of independent genomic transpositions of *Ac* (*trAc*s) with little effort. (2) *Ac* has a dominant *trans*-acting phenotype, namely, the capacity to *trans*-activate the excision of a defective *Ds* (*Dissociation*) element from a reporter allele located elsewhere. Therefore, *trAc*s can be identified in heterozygous conditions and then rendered homozygous to screen for mutant phenotypes. (3) *Ac*'s dominant phenotype permits the quick and simple analysis of cosegregation between a *trAc* and a new mutation, information that is useful to the investigator in deciding on the next experimental step. (4) *Ac*'s visible phenotype allows the mapping of a *trAc* insertion in the genome independently of the molecular mapping of its adjacent sequence. (5) *Ac* tends to transpose to sites that are linked closely to the donor site (Van Schaik and Brink, 1959; Greenblatt, 1984; Dooner and Belachew, 1989). This property of *Ac* can be used to "saturate" defined regions of the genome with insertions. Placing *Ac* in launching pads at multiple regularly spaced locations would enable selective targeting of the entire genome, as was done in *Arabidopsis* in the work that led to the directed targeting of the *FAE1* gene (James et al., 1995). (6) The number of sequences in the genome that are homologous with the central part of the *Ac* element is low (four to six) (Fedoroff et al., 1983). Furthermore, most of those sequences are methylated, whereas *Ac* is not (Schwartz and Dennis, 1986; Chomet et al., 1987). These two features make it feasible to devise polymerase chain reaction (PCR) strategies for the isolation of DNA adjacent to *Ac* using *Ac* primers.

This discussion should serve to illustrate why the *Mu* and *Ac* approaches to functional genomic analysis in maize are complementary. The power of both strategies lies in their ability to define gene function through gene disruption. In the former, mutagenesis relies on the mobilization of high-copy-number, nonautonomous *Mu1* elements that do not transpose locally (Lisch et al., 1995), and in the latter, it relies on the regional mobilization of the single-copy, autonomous *Ac* element that targets nearby sites preferentially.

We have generated a large *Ac* insertion library consisting of 1225 independent transpositions from one launching platform, the *wx-m7(Ac)* mutable allele in 9S. Only 61 of the 1225 *Ac* transposants yielded clear mutant phenotypes, of which 6 cosegregated with *Ac*. Hence, factors other than *Ac* were responsible for most of the mutations, suggesting that these populations will be more useful for reverse genetics than for forward mutagenesis. As expected, most transposed elements were linked to the *wx* donor locus. We have begun to isolate and sequence the DNA adjacent to the *trAc* elements from this and an additional, smaller insertion library generated previously from the *bz-m2(Ac)* allele (Dooner and Belachew, 1989; Dooner et al., 1994). We report here the characterization of the first 46 *tac* sites isolated from these libraries. All *Ac* insertion sites isolated to date correspond to low-copy DNA, and more than half have homology with either known genes or expressed sequence tags (ESTs) in the sequence databases. The expression of a

subset of *tac* sites, including some lacking homology with any known sequence, was confirmed by RNA gel blot analysis.

RESULTS

Generation and Genetic Characterization of an *Ac* Insertion Library from *wx-m7(Ac)*

We have developed an efficient single-seed selection scheme to identify germinal transpositions of *Ac* from the *wx-m7(Ac)* mutable allele. Figure 1 outlines the genetic derivation of the transposants. The system relies on the coupling of two easily scored endosperm markers, *wx* and *bz*, to monitor *Ac* transposition in the female germline. *wx-m7(Ac)* is a highly mutable allele described by McClintock (1964). It reverts to *Wx'* in the germline, with frequencies on the order of a few percent. The 4.6-kb *Ac* element is inserted upstream of the *Wx* coding sequence (Muller-Neumann et al., 1984; Klosgen et al., 1986), in a region of the *Wx* gene in which excision footprints do not seem to interfere with gene function. The reporter allele *bz-m2(D1)* produces a heavily spotted aleurone phenotype in the presence of *Ac* and a solid-bronze phenotype in its absence. The other two alleles, *bz-R* and *wx*, are stable mutations. In ears of *bz-R wx-m7(Ac)/bz-m2(D1) wx* heterozygotes pollinated with *bz-m2(D1) wx*, germinal transpositions of *Ac* can be selected as single exceptional kernels with a nonwaxy, spotted (*Wx'* *bz-m*) phenotype among a majority of kernels with a waxy or waxy mutable phenotype.

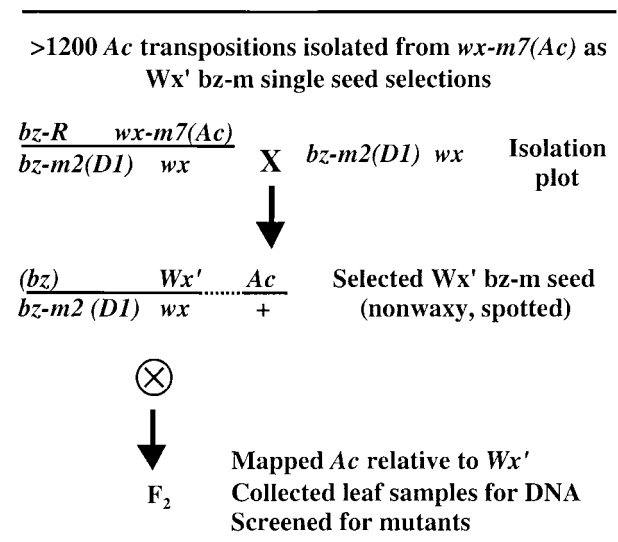


Figure 1. Derivation of an *Ac* Insertion Library from the *wx-m7(Ac)* Mutable Allele.

A collection of 1225 independent *Ac* transposants was generated, and all of the *trAc* sites were mapped relative to the *wx* donor locus by two-point crosses. The distribution of *Ac* receptor sites is shown in Figure 2. As has been observed at other maize loci (Greenblatt, 1984; Dooner and Belachew, 1989), the majority of *trAc* elements (63%) are linked to the donor locus. Of the 1225 families produced by selfing the primary transposants, 61 (or ~5%) segregated for obviously visible mutants. Table 1 lists the obvious phenotypes identified either in the ear of the primary transposants or in field screens of their progeny conducted at different stages of development. The mutations were typical of those recovered in other mutagenized populations and affected various parts of the plant. Segregating mutant seed phenotypes included defective kernels, dull endosperms, and empty pericarps. Several F2 families in the nursery segregated seedling phenotypes, such as pale green, luteus, and albino, which often were lethal. Some of the adult plant phenotypes observed were zebra cross-bands, wrinkled leaves, yellow green, and wilted. Cosegregation analysis of the *trAc* and the new mutant revealed that only a minority (six, or ~10%) may be tagged by *Ac*. At least two of them are tagged by a *Ds* element, judging from the somatic and germinal reversions that occur only in the presence of *Ac*.

Although this collection of 1225 transposants is of potential utility for gene isolation by conventional transposon tagging, our primary objective is to isolate the DNA adjacent to the *trAc* element (*tac* sites), which in most cases will correspond to genes, independent of whether the *trAc* confers a mutant phenotype. We also are isolating *tac* sites from a smaller collection of 102 *trAc*s derived from the *bz-m2(Ac)* mutable allele (Dooner and Belachew, 1989). All of the *trAc*s in this set were mapped relative to the *bz* donor locus in chromosome 9 (Dooner and Belachew, 1989; Dooner et al., 1994), and those that were unlinked to *bz* were assigned subsequently to chromosomes on the basis of their linkage to reciprocal translocation break points (Dooner et al., 1994). The latter group of *trAc* stocks could serve as starting points for localized transposon mutagenesis from chromosomes other than 9 (Dooner, 1995). All of the stocks described in this report have been deposited in the Maize Stock Center (Urbana, IL).

Isolation of *tac* Sites

The potential utility of a maize *Ac* insertion library for functional genomics will depend on the ease with which *tac* sites can be isolated. In transgenic dicots, in which the introduced *Ac* usually is present in one or two copies, *tac* sites can be isolated readily by PCR approaches, such as inverse PCR (IPCR) (Whitham et al., 1994; James et al., 1995) and thermal asymmetric interlaced PCR (TAIL-PCR) (Tsugeki et al., 1996). Maize, on the other hand, has several endogenous *Ac*-homologous sequences (Fedoroff et al., 1983), which render *tac* site isolation by PCR-based methods

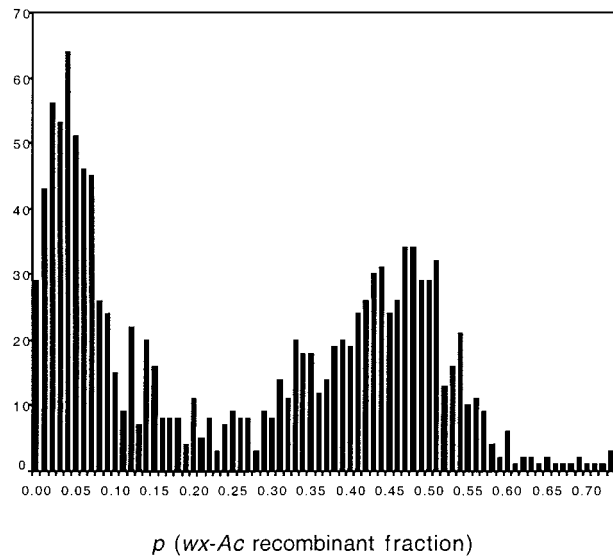


Figure 2. Distribution of 1225 *trAc*s among *Wx'* Revertants from *wx-m7(Ac)*.

The genetic distance between the *wx* donor locus and a *trAc* is plotted against the number of *trAc*s for each genetic distance. Genetic distance is expressed as *p*, the fraction of recombinants between *wx* and *Ac*. The distribution of *p* values is bimodal. The left cluster corresponds to receptor sites closely linked to *wx* on 9S, and the right cluster corresponds to unlinked receptor sites.

much more difficult. Although IPCR has been used to isolate an *Spm*-adjacent sequence in maize (Earp et al., 1990), this method has not been used widely because usually too many nonspecific sequences are amplified. To minimize the amplification of unwanted sequences, we have introduced a gel-purification step before performing IPCR. Although somewhat laborious, the method works well and, to date, it has enabled us to clone 42 *tac* sites corresponding to germinal transpositions of *Ac*. This is the largest number of DNA sequences adjacent to confirmed germinal transpositions of an autonomous and therefore phenotypically traceable transposable element yet isolated in maize.

First, we examined diagnostic DNA gel blots of primary transposants or their progeny to identify unique *Ac*-hybridizing bands of a size appropriate for IPCR amplification of the DNA flanking *Ac* (5 to 9 kb). An internal control in these blots is provided by the *bz-m2(D1)* allele, which, like *trAc*, is present in heterozygous condition and hybridizes to the *Ac* probe. An example of such a blot is shown in Figure 3A. Then, we cut out the corresponding fragment from a preparative gel and performed IPCR to amplify the *tac* site. We digested genomic DNA with either methylation-sensitive (*Pst*I, *Sal*I) or methylation-insensitive (*Bgl*II, *Sst*I, *Nco*I, *Eco*RI) enzymes. Except for *Eco*RI, none of these enzymes cut within *Ac*, so our IPCR products should contain sequences adjacent

Table 1. Segregating Mutant Phenotypes

Mutant Phenotype	No.	Cosegregation with Ac
Seed		
Dull	2	
Defective kernel (small endosperm, germless)	15	
Anthocyanin low	1	
Abnormal kernel, low anthocyanin	1	
Abnormal kernel (small kernel, with embryo)	4	
Lethal embryo (normal endosperm, germless)	3	2
Inverted kernel	1	
Paper-thin kernel	3	
New <i>Wx-m</i>	1	1
Sugary	1	
Seedling		
Pale green lethal	4	
Pale green	2	
Luteus	3	
Plant		
Short, pale green, wilted	2	1
Adherent upper whorls	1	1
Ear semisterility	3	
Pale green, shorter	6	
Yellow green	1	
Ramosa tassel	1	
Abnormal leaf blades	1	
Stunted, necrotic	1	
Wrinkled leaf	1	
White stripes	1	
Zebra	1	
Pollen		
Reduced male transmission	1	1
Total	61	6

to both sides of *Ac*. Thus, we can determine immediately after sequencing if the *Ac* ends are flanked by the 8-bp duplication expected of a new *Ac* insertion. Most of the cloned *tac* sites were chosen on the basis of the feasibility of amplifying them by IPCR.

To confirm that we had isolated a true *tac* site, we compared the DNAs of transposants. If a true *tac* site has been isolated, then the transposant contributing the amplified sequence that is used as a probe should have a band that is 4.6 kb larger than the band in the other transposants. That is precisely the pattern seen in Figure 3B, where transposant 905.29 contributed both the probe and the DNA in the second lane. Confirmation DNA gel blots probed with the putative *tac* sites sometimes failed to reveal the expected polymorphism, suggesting that the isolated DNA most likely corresponds to either a preexisting *Ds* insertion or a somatic *Ac* transposition. The repeated recovery of clones with the same sequences from different transposants suggests that most of these sequences represent preexisting *Ds* insertions

in our stocks. Remarkably, almost all of the probes tested detected only one or two bands, showing that our IPCR strategy leads to the amplification of single- or low-copy DNA.

Characterization of *tac* Sites

Table 2 lists the 46 *tac* sites that have been sequenced and confirmed by DNA gel blot analysis to date. Most (41 of 46) detected either one or two bands in genomic DNA gel blots, and 54% (25 of 46) had homology with sequences in the public databases (BLAST search of GenBank databases). *Ac* elements in the 900 series (sites 1 to 39) are derived from *wx-m7(Ac)*. They were mapped relative to the *wx* donor locus and, in the case of *Ac902.33*, by the recombinant inbred method (Burr et al., 1988). *Ac* elements with a four-digit designation (sites 40 to 46) are derived from *bz-m2(Ac)*. They were mapped relative to the *bz* donor locus (Dooner and Belachew, 1989; Dooner et al., 1994) and, in the case of *Ac8193* and *Ac8194*, by the *wx* reciprocal translocation method (Dooner et al., 1994). Reflecting the location of the *wx* and *bz* donor loci, a large number of the cloned *tac* sites reside on 9S.

Several *tac* sites have been identified that have homology with other plant genes, mostly from *Arabidopsis* and rice. Among them are genes encoding proteins of predicted function, such as ring zinc finger proteins, Ser/Thr kinases, and a DNA-3-methyladenine glycosidase, as well as hypothetical proteins of unknown function. As indicated above, although several mutants have been identified, only a minority of them appear to be tagged by *Ac* on the basis of genetic cosegregation data. Two interesting examples of novel mutations apparently created by *trAc*s are the lethal embryo mutations that resulted from the insertion of *Ac902.33* (Table 2, site 5) in a gene homologous with the rice chloroplast ribosomal protein S9 gene and *Ac921.20* (Table 2, site 35) in a gene homologous with the rice Arg decarboxylase gene.

Five of the *tac* sites isolated from the collection of *trAc*s from *bz-m2(Ac)* are very close to *bz* on 9S (Table 2, sites 40 to 44). These *tac* sites have been studied in greater detail because they have helped to identify genes in the gene-rich *bz* region (Fu et al., 2001). Two *trAc*s (*Ac6087* and *Ac6067*) landed in *stc1*, a sesquiterpene cyclase gene that is located immediately distal to *bz* (Table 2, sites 41 and 42). This gene is involved in the tritrophic interaction, a fascinating system of indirect defense that enables maize to protect itself from herbivorous insects by attracting parasites of the herbivore (Turlings et al., 1990; Farmer, 1997). *stc1* is induced by volicitin, a compound produced in the salivary glands of beet army worms that forage on maize leaves (Shen et al., 2000). Both *Ac* insertions are null: the *stc1* gene fails to be induced in response to volicitin and, as a consequence, a specific sesquiterpene is missing from the mixture of volicitin-elicited volatiles in the mutant seedlings. These are good examples of *Ac* insertions whose nonobvious phenotype could be investigated in detail after isolating the corresponding *tac* sites and identifying the gene in which *Ac* had inserted. Two

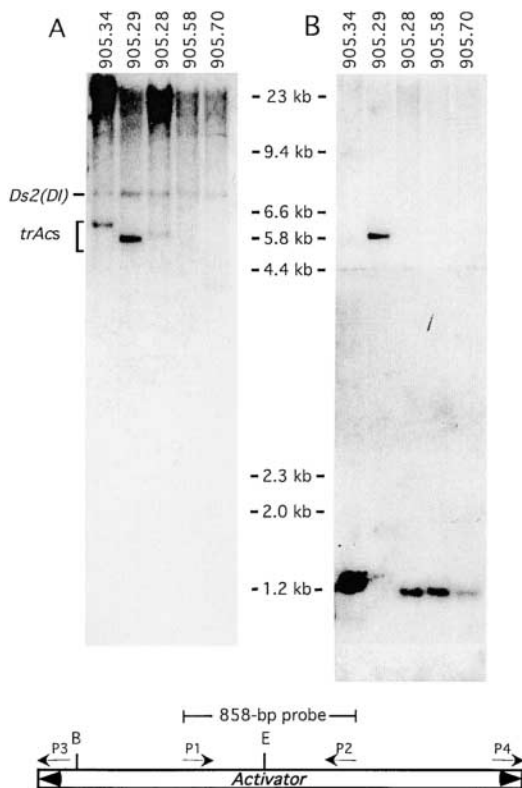


Figure 3. DNA Gel Blot Analysis of Ac Insertion Sites.

Each lane on the gel corresponds to DNA from an individual transposant digested with PstI.

(A) Diagnostic hybridization with the 858-bp Ac probe shown below. New Ac-hybridizing bands are detected in transposants 905.34, 905.29, and 905.28.

(B) Confirmation hybridization of the same membrane with the IPCR product from transposant 905.29 amplified with primers P3 and P4 (putative *tac*_{905.29} site). The probe detects a 4.6-kb larger band only in the lane containing DNA from transposant 905.29.

B, BamHI; E, EcoRI.

other *trAc*s (*Ac*₆₀₅₈ and *Ac*₇₀₇₇) have tagged sequences with no homology in the public sequence databases (Table 2, sites 43 and 44). We have shown that these are genes by isolating cDNA clones for both *tac* sites from a tassel cDNA library (Fu et al., 2001; W. Park and H.K. Dooner, unpublished data). Thus, *tac* sites can be used to define plant genes that are not found in the sequence databases even after the Arabidopsis genome sequencing project has been largely completed (Arabidopsis Genome Initiative, 2000).

Expression of *tac* Sites

The expression of all *tac* sites listed in Table 2 was analyzed by RNA gel blotting. RNA from the following maize tissues was included in the analysis: developing endosperms and em-

bryos (2 to 5 weeks); seedling shoots (1 week); juvenile roots (4 weeks); adult roots, leaves, and stems (10 weeks); immature tassels from four developmental stages (4 to 15 cm, 20 to 27 cm, 29 to 42 cm, and 42 to 50 cm); and immature ears from two developmental stages (3 and 8 cm). RNA samples from special tissue sources also were included in some analyses, for example, from seedling leaves treated with the insect elicitor volicitin (Shen et al., 2000) and from juvenile roots treated with NaCl. Figure 4 shows representative RNA gel blots for four different *tac* sites. The results from all RNA gel blots are summarized in Table 2 (Expression Data), which lists the tissue(s) giving the strongest signals for each *tac* site.

A critical issue in this type of expression analysis is whether the signal detected by the *tac* probe can be attributed specifically to the putative gene defined by the Ac insertion. This is particularly relevant when the gene in question encodes a protein belonging to a family of related proteins. Therefore, we distinguish two types of *tac* sites: those that give single bands in high-stringency confirmation DNA gel blots, and those that give more than one band. Among the 46 *tac* sites, 31 (67%) gave a positive signal with RNA collected from at least one tissue. Of these 31, 6 gave more than one band in DNA gel blots, making the results of the RNA gel blot analysis ambiguous. For one of these six (*tac*₇₀₇₇; see above), we have obtained cDNA evidence of expression. The remaining expressed *tac* sites gave single bands in high-stringency DNA gel blots, indicating that the signal detected in RNA gel blots is specific. Therefore, at least half of the sequences tagged by Ac are expressed.

One way to confirm the specificity of the signal detected by a *tac* site is to use RNA from a deletion mutation as a negative control. We have done so for the five *tac* sites located in the vicinity of *bz* (*tac*₂₀₉₄, *tac*₆₀₆₇, *tac*₆₀₈₇, *tac*₆₀₅₈, and *tac*₇₀₇₇) and have verified that the signal detected in RNA gel blots by probes that gave single bands in DNA gel blot analysis was, in fact, *tac* site specific (Fu et al., 2001; B. Shen, D. Lu, and H.K. Dooner, unpublished data). The only *tac* site in the *bz* region that hybridized to RNA from both the wild type and the *sh-bz-X2* deletion control was *tac*₇₀₇₇, which consistently detected two bands in DNA gel blots. Although this is a powerful genetic approach, it is limited to those regions of the genome that are homozygous viable when deleted, an infrequent occurrence in maize. A second way to verify signal specificity is to monitor gene expression in Ac homozygous individuals, of which most presumably are mutated for the gene in question. If the signal is specific to the *tac* site being tested, such individuals should show either highly reduced transcript levels or, occasionally, an altered transcript size from Ac-mediated improper splicing (Wessler, 1988). Because the survey nature of our project precludes this type of exhaustive RNA analysis, we have not performed tests with mutant RNAs.

An alternative means to determine that a particular *tac* site is expressed is to establish that the predicted 3' untranslated region of a cDNA or an EST matches the *tac* site genomic sequence. In addition to the *tac*₆₀₅₈ and *tac*₇₀₇₇

Table 2. Characterization of Isolated *tac* Sites

No.	<i>tac</i> Site	Enzyme ^a	Sequence Homology	E Value	Expression Data	Location	Copy No.
1	<i>tac901.04</i>	SstI	Arabidopsis cytochrome P450	5e ⁻⁴⁰	Undetected	9: 13 cM from <i>wx</i> ^b	1
2	<i>tac902.05</i>	Sall	tRNA-gly (GCG)	2e ⁻³³	Constitutive	9: 29 cM from <i>wx</i>	1
3	<i>tac902.07</i>	Sall	Arabidopsis ring zinc finger	1e ⁻¹⁷	Seedling; tassel; 3-week-old embryo	9: 18 cM from <i>wx</i>	1
4	<i>tac902.33</i>	SacI	Rice plastid S9 ribosomal protein	6e ⁻⁸⁶	Endosperm; ear; tassel	1L, close to <i>telo</i>	1
5	<i>tac903.05</i>	SstI	No homology		Wounded or salt-treated seedlings	Unlinked to <i>wx</i>	1
6	<i>tac903.06</i>	SstI	Arabidopsis auxin-inducible protein	4e ⁻⁶	Etiolated seedlings; light repressed	Unlinked to <i>wx</i>	1
7	<i>tac905.05</i>	EcoRII	No homology		Undetected	9S: 7 cM from <i>wx</i>	1
8	<i>tac905.29</i>	PstI	Arabidopsis Ser Thr kinase	7e ⁻¹¹	Tassel; roots	9S: 9 cM from <i>wx</i>	1
9	<i>tac907.25</i>	PstI	Arabidopsis sugar transporter	1e ⁻⁸	Roots	Unlinked to <i>wx</i>	1
10	<i>tac907.49</i>	PstI	No homology		Undetected	Unlinked to <i>wx</i>	1
11	<i>tac907.51</i>	NcoI	Arabidopsis short-chain ADH ^c	3e ⁻³³	Higher in older tissues	Unlinked to <i>wx</i>	1
12	<i>tac907.54</i>	PstI	Arabidopsis hypothetical protein	7e ⁻²⁵	Immature ear; 2-week-old seed	Unlinked to <i>wx</i>	1
13	<i>tac909.06</i>	EcoRI	Tobacco hypothetical protein	1e ⁻⁵	Undetected	Unlinked to <i>wx</i>	4-5
14	<i>tac911.14</i>	EcoRI	Arabidopsis DNA-3-methyladenine glycosidase	3e ⁻²⁷	Mature leaves; silk	Unlinked to <i>wx</i>	1
15	<i>tac911.16</i>	PstI	No homology		Undetected	Unlinked to <i>wx</i>	1
16	<i>tac911.17</i>	PstI	No homology		Undetected	Unlinked to <i>wx</i>	1
17	<i>tac911.22</i>	PstI	Maize early embryo EST	5e ⁻¹⁰	Tassel (3 to 8 cm); stem	Unlinked to <i>wx</i>	1
18	<i>tac911.28</i>	PstI	Arabidopsis Ser Thr kinase	2e ⁻⁷	Endosperm; seedlings	Unlinked to <i>wx</i>	3
19	<i>tac911.30</i>	PstI	No homology		Undetected	Unlinked to <i>wx</i>	1
20	<i>tac913.01</i>	PstI	No homology		Roots: salt induced; seed	9S: 8 cM from <i>wx</i>	1
21	<i>tac913.09</i>	PstI	Arabidopsis hypothetical protein	8e ⁻⁶	Tassel (3 to 8 cm); seedling shoot	9S: 10 cM from <i>wx</i>	2
22	<i>tac913.14</i>	PstI	No homology		Endosperm; seedling shoot; root	9S: 12 cM from <i>wx</i>	1
23	<i>tac914.07</i>	PstI	No homology		Undetected	Unlinked to <i>wx</i>	1
24	<i>tac914.11</i>	PstI	No homology		Undetected	9S: 16 cM from <i>wx</i>	1
25	<i>tac914.13</i>	BglII	No homology		Undetected	9S: 21 cM from <i>wx</i>	1
26	<i>tac914.23</i>	PstI	Maize <i>wx:Ac</i> to intron 4	7e ⁻⁸²	Endosperm; pollen	9S: <i>wx</i>	1
27	<i>tac917.09</i>	NcoI	No homology		Immature ear	9S: 4 cM from <i>wx</i>	4
28	<i>tac917.12</i>	EcoRI	Arabidopsis NAM-like protein	3e ⁻⁵	Etiolated seedlings	9S: 14 cM from <i>wx</i>	1
29	<i>tac917.27</i>	PstI	Arabidopsis auxin efflux carrier protein	1e ⁻⁵	Tassel; seedling; silk	9S: 12 cM from <i>wx</i>	1
30	<i>tac917.29</i>	PstI	No homology		Seedling shoot and root; 3-week-old embryo	9S: 8 cM from <i>wx</i>	1
31	<i>tac918.02</i>	PstI	No homology		Undetected	Unlinked to <i>wx</i>	1
32	<i>tac919.27</i>	PstI	Arabidopsis Pro-rich protein	2e ⁻⁵	Tassel; stem; silk	9: 35 cM from <i>wx</i>	1
33	<i>tac919.39</i>	BglII	Barley SERK1 Leu-rich repeat protein	1e ⁻¹⁴	Undetected	9: 37 cM from <i>wx</i>	1
34	<i>tac919.41</i>	BglII	No homology		Etiolated seedlings	9: 32 cM from <i>wx</i>	1
35	<i>tac921.20</i>	EcoRI	Rice Arg decarboxylase	4e ⁻⁵	Tassel; leaf	9S: 3 cM from <i>wx</i>	1
36	<i>tac923.08</i>	EcoRI	No homology		3-week-old embryo	Unlinked to <i>wx</i>	2
37	<i>tac923.13</i>	EcoRI	No homology		Undetected	Unlinked to <i>wx</i>	1
38	<i>tac923.17</i>	EcoRI	No homology		Immature seed; tassel	Unlinked to <i>wx</i>	1
39	<i>tac929.20</i>	EcoRI	Arabidopsis unknown protein	2e ⁻¹⁵	Undetected	9S: 9 cM from <i>wx</i>	1
40	<i>tac2094</i>	λ	Arabidopsis MAPK kinase ^d	e ⁻¹¹³	Root; ear	9S: <1 cM from <i>bz</i>	4
41	<i>tac6067</i>	λ	Maize sesquiterpene cyclase	0	Leaves, volicitin induced	9S: <1 cM from <i>bz</i>	1
42	<i>tac6087</i>	λ	Maize sesquiterpene cyclase	0	Leaves, volicitin induced	9S: <1 cM from <i>bz</i>	1
43	<i>tac6058</i>	PCR	No homology		Endosperm; tassel	9S: <1 cM from <i>bz</i>	1
44	<i>tac7077</i>	EcoRII	No homology		Constitutive	9S: <1 cM from <i>bz</i>	2
45	<i>tac8193</i>	PstI	No homology		Undetected	5: 4 cM from T5-9c	1
46	<i>tac8194</i>	PstI	Rice zinc finger protein	2e ⁻¹²	Undetected	7: 3 cM from T7-9a	1

^a Restriction enzyme used to digest genomic DNA; four *tac* sites were isolated by either cloning in λ or direct PCR.

^b cM, centimorgan.

^c ADH, alcohol dehydrogenase.

^d MAPK, mitogen-activated protein kinase.

cDNAs described above, we have isolated cDNAs corresponding to *Ac902.33* and *Ac921.20*, the two *Acs* that apparently resulted in embryo-lethal mutations. Again, because of the nature of our work, we have limited this type of analysis to only a few *tac* sites.

Overall, the data on DNA homology, copy number, and expression presented in Table 2 show that *Ac* inserts preferentially into genes and that *Ac* is, in fact, an excellent gene-searching engine in the highly repetitive maize genome. There are two interesting correlates in Table 2 that are worth noting. Most *tac* sites having homology with sequences in the databases are expressed in some tissue (20 of 25). On the other hand, 52% of *tac* sites lacking homology with sequences in the databases failed to give signals in RNA gel blots (11 of 21). These may represent *Ac* insertions in genes that are expressed at very low levels or only under special conditions. Alternately, they could represent *Ac* insertions in intergenic regions or long introns.

DISCUSSION

Single-Kernel Selection of *Ac* Transposants

Many schemes that monitor the transposition of *Ac* using *Ds* reporters have been described in maize and other plants. The most efficient schemes combine *Ac* and *Ds* mutable alleles that are expressed in the kernel (e.g., in the pericarp and aleurone; Dellaporta and Moreno, 1994) or seedling (Jones et al., 1989; Sundaresan et al., 1995), permitting the ready screen of a large number of individuals. In this study, we have taken advantage of the excellent endosperm genetics of maize to develop a simple assay that detects the germinal transpositions of *Ac*. The system uses the two easily scored endosperm markers *wx* and *bz* to monitor *Ac* transposition in either the female or the male germline. We use the *Ac* parent as female because this makes it possible to identify premeiotic transpositions as clonal sectors in the ear. If the *Ac* parent is used as male, premeiotic sectors are randomized upon pollen shedding, making clonal information much more difficult to obtain. As shown in Figure 1, transpositions of *Ac* that occur at or near meiosis in the *Ac* female parent can be selected as individual kernels having a unique nonparental phenotype. No attempt was made in this study to select for either linked or unlinked transpositions. However, stocks carrying appropriate *Ac* and *Ds* mutable alleles flanked by endosperm markers also can be used to identify transpositions of *Ac* specifically to unlinked sites as exceptional single kernels in an ear (Dooner et al., 1994).

Ac as a Gene Searcher

We have shown here that in maize the transposon *Ac* inserts preferentially into genes. This was suspected from previous

work that had established that maize genes reside in hypomethylated CpG islands (Antequera and Bird, 1988; Bennetzen et al., 1994) and that *Ac* transposes preferentially into hypomethylated DNA (Chen et al., 1987). By isolating and sequencing the DNA adjacent to transposed *Ac* elements (*tac* sites), comparing their sequences to the nucleic acid databases, examining their copy numbers in DNA gel blots, and analyzing their expression by RNA gel blot analysis, we have confirmed that most *Ac* receptor sites in maize correspond to genes. Significantly, no matches to retrotransposons—sequences that make up a large percentage of the repetitive DNA in the maize genome (SanMiguel et al., 1996)—have been detected, supporting the notion that sites of *Ac* visitation tend to correspond to unique DNA. Another maize transposon, the miniature inverted repeat transposable element *Heartbreaker*, displays a similar insertion site preference (Zhang et al., 2000), in agreement with the finding that miniature inverted repeat transposable elements and retrotransposons occupy different domains in the maize genome (Tikhonov et al., 1999).

Our observations that all *tac* sites isolated to date are present in one or a few copies in the genome and that many *tac* sites are homologous with known genes and show tissue-specific patterns of expression suggest that *Ac* is a reliable gene searcher in the highly repetitive maize genome. In fact, we have used *Ac* to identify new putative genes. Some of the *tac* sites have no homology with any sequence in the databases, yet they are transcribed in specific tissues, suggesting that they are genes (Table 2, *tac913.01*, *tac913.14*, *tac6058*, and *tac7077*). For the latter two sites, we have isolated full-length cDNAs and defined exon-intron junctions (Fu et al., 2001; W. Park, unpublished data). Therefore, homology with a *tac* site sequence may turn out to be a useful gene-recognition criterion in maize.

Maize class II transposons (i.e., those that excise during transposition) also are being used to locate genes in Arabidopsis and rice, species with much smaller genomes and a much lower abundance of retrotransposons than maize (Arabidopsis Genome Initiative, 2000; Le et al., 2000; Turcotte et al., 2001). Two types of transposant populations have been generated: those containing selected transpositions of either *Ds* or *dSpm* to a site unlinked to the donor locus (Parinov et al., 1999; Tissier et al., 1999), and those containing unselected transpositions of *Ac*, *Ds*, or *dSpmII*, a majority of which will be linked to the donor locus (Dubois et al., 1998; Wisman et al., 1998; Chin et al., 1999; Enoki et al., 1999; Ito et al., 1999; Speulman et al., 1999). Many sequences adjacent to the transposed elements have been isolated from these populations, and a large number of them show similarity to gene sequences in the databases. Thus, in these species, maize transposons also transpose preferentially into genes and can be used as efficient tools for functional genomics (Martienssen, 1998). Yet, it is in maize, with its high content of repetitive DNA, that the value of excisive transposons as gene searchers is clearest. Maize functional genomics projects using transposons are being

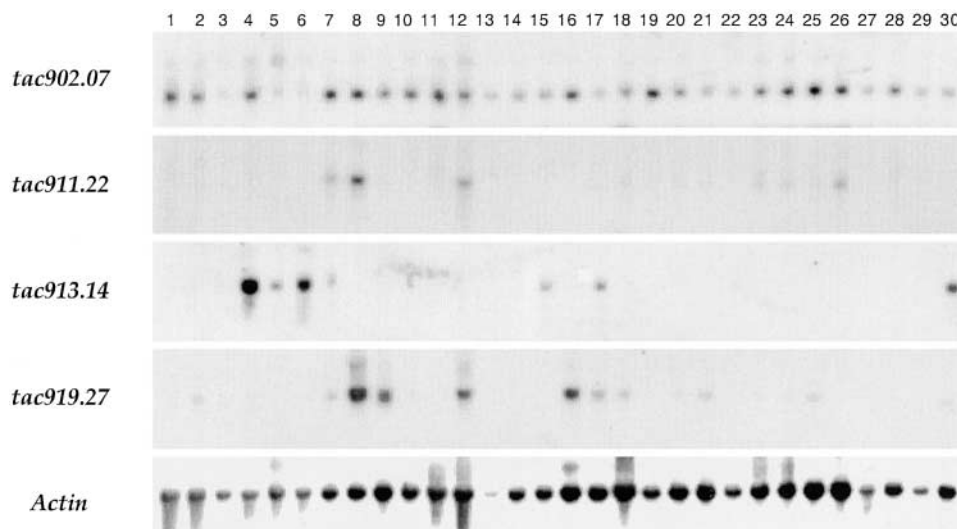


Figure 4. RNA Gel Blot Analysis of Several *tac* Sites.

Total RNA from a variety of tissues was hybridized with the *tac* site probes indicated at left. Lanes 1 to 4, seed at various developmental stages (1, 1 DAP; 2, 5 DAP; 3, 7 DAP; 4, 21 DAP); lane 5, 14-DAP endosperm; lane 6, 35-DAP endosperm; lane 7, 21-DAP embryo; lanes 8 to 11, tassels at different developmental stages (lane 8, 3.8 cm; lane 9, 20 to 27 cm; lane 10, 30 to 33 cm; lane 11, 44 cm) at anthesis; lane 12, adult stem; lane 13, adult leaf; lane 14, adult root; lane 15, juvenile root; lane 16, silk from 8-cm ear; lane 17, 6-day-old seedling shoot; lane 18, 8-day-old seedling shoot; lane 19, 14-day-old seedling shoot, cut and incubated for 2 hr in water; lanes 20 to 24, 14-day-old seedling shoots incubated in different solutions (lane 20, 0.2 M NaCl for 48 hr; lane 21, 2% Suc for 48 hr; lane 22, water for 48 hr; lane 23, 20 mM KCl for 8 hr; lane 24, 20 mM KCl for 4 hr, followed by water for 4 hr); lane 25, 10-day-old etiolated seedling shoot after 8-hr light exposure; lane 26, 10-day-old etiolated seedling shoot in continuous darkness; lanes 27 to 29, 2-cm leaf segments from 14-day-old seedlings incubated for 7 hr under different treatments (lane 27, water in light; lane 28, water in darkness; lane 29, 7% hydrogen peroxide in light); and lane 30, 6-day-old seedling root.

pursued by several groups of investigators (summaries are available at <http://plantgenome.sdsc.edu/projects.html>). The transposons being used in these projects are either *Ac* or *Mutator*, but all of the strategies rely directly or indirectly on the propensity of maize class II transposons to insert preferentially into genes.

We do not know at present if *Ac* occasionally transposes into repetitive retrotransposon DNA in maize. Because these sequences are highly methylated (Burr et al., 1988; Bennetzen et al., 1994; Rabinowicz et al., 1999), it is likely that if such a transposition occurred, *Ac* would become methylated and inactivated (Schwartz and Dennis, 1986; Chomet et al., 1987). In fact, a methylated and inactive "cryptic" *Ac* element has been isolated from repetitive, methylated DNA (Leu et al., 1992). Yet, this element was present at the same position in several unrelated inbred lines, indicating that an old transposition event placed the cryptic *Ac* in its present location in the genome. Transposed *Ac* elements identified by virtue of their activity may represent a selected set, namely, transpositions into genes. No *tac* sites with homology with retrotransposons were recovered in the present study, although both methylation-sensitive and methylation-insensitive enzymes were used to identify IPCR-clonable *tac* sites. The fraction of *tac* sites having homology with se-

quences in the databases was similar whether the genomic DNA was digested with a methylation-sensitive (0.48) or a methylation-insensitive (0.58) enzyme. This suggests that there is no bias in using methylation-sensitive enzymes to identify IPCR-clonable bands. Digesting genomic DNA with methylation-sensitive enzymes is preferable because the number of *Ac*-hybridizing bands is reduced considerably (Chen et al., 1987). In heterologous systems, the insertion of class II maize elements in repetitive DNA occurs rarely. In the one study that reported that *dSpm* could insert in repetitive DNA, including retrotransposons, the methylation status of those sequences was not analyzed (Tissier et al., 1999).

Positioning of *Ac* Insertions within Genes

A recent analysis of *Ds*-insertion lines in *Arabidopsis* revealed a preference for mobilized *Ds* elements to reinsert close to the 5' ends of genes (Parinov et al., 1999). When the distance between the site of insertion and the start codon was plotted for 232 *Ds* transpositions, a strong preference was found for insertion close to the 5' end of the gene. A similar study in *Arabidopsis* analyzed transposed *En/Spm* elements in 48,000 plant lines, of which ~80% re-

sult from independent transpositions of a *dSpm* element (Tissier et al., 1999). The authors reported no preference for the 5' ends of genes, although they did notice a preference for genic DNA. Specifically, they analyzed the flanking sequences from 1200 independent insertions and found that 70% of the insertions were into coding regions.

We have performed a similar analysis with our independent *Ac* insertions. However, we are limited by the current state of maize sequencing. In cases in which maize genomic and either cDNA or EST sequences were available, we used that information to determine the position of the *Ac* insertion in the gene. We were able to do this for *Ac902.33*, *Ac914.23*, *Ac921.20*, *Ac2094*, *Ac6058*, *Ac6067*, *Ac6087*, and *Ac7077*. Otherwise, we used the most similar sequence in the database and made the assumption that exon size and number would be conserved across plant species. Given the sequence information available at present, we determined either the codon position of the *Ac* insertion in the coding region or the number of nucleotides away from the beginning or the end of translation. Flanking sequences that had no homology with sequences in the public databases or had homology with an EST for which no genomic sequence is available were ignored. *Ac6058* and *Ac7077* were considered because, although they have no homology with database sequences, we have complete genomic and cDNA

sequences as well as RNA gel blot expression data for both of them.

Our data, summarized in Table 3, show that there is no preference for *Ac* to insert close to the ATG start codon. In fact, the majority of our insertions appear to occur in the middle of genes. Of the insertions for which a genic position was determined, 75% were in exonic DNA. Interestingly, the insertions tend to occur near the ends of exons, because 50% were within one of the two terminal fifths of the exon.

Mutants in *Ac* Stocks

New mutants with obvious visible phenotypes segregated in ~5% of the F2 families from the primary *Ac* transposants. The dominant *trans*-acting effect of *Ac* on the excision of *Ds* from the reporter allele *bz-m2(D1)* allowed us to test for cosegregation between *trAc* and the new mutation in each F2 family. We found that *Ac* cosegregated with a mutation in just a handful of cases. The *tac* sites in the two *Ac*-cosegregating mutations with the most dramatic phenotype, embryo lethality, have been isolated. They correspond to *Ac* insertions in genes encoding proteins with homology with the rice chloroplast ribosomal protein S9 and Arg decarboxylase. Currently, we are attempting to obtain germinal

Table 3. *Ac* Location within Genes

<i>trAc</i>	Gene Homology	<i>Ac</i> Location	Position in Gene
<i>Ac2094</i>	Maize MAPK kinase ^a	5' UTR ^b	35 nucleotides upstream of ATG
<i>Ac6058</i>	Maize cDNA, no database homology	Codon 87	Middle of 2nd exon
<i>Ac6067</i>	Maize sesquiterpene cyclase	Codon 45	Middle of 1st exon
<i>Ac6087</i>	Maize sesquiterpene cyclase	5' UTR	104 nucleotides upstream of ATG
<i>Ac7077</i>	Maize cDNA, no database homology	5' UTR	175 nucleotides upstream of ATG
<i>Ac8194</i>	Arabidopsis zinc finger protein	Codon 314	5' end of 3rd exon
<i>Ac901.04</i>	Arabidopsis cytochrome P450	Codon 102	Middle of last exon
<i>Ac902.05</i>	Arabidopsis tRNA-gly (GCG)	Intergenic space	50 nucleotides 3' of terminator
<i>Ac902.07</i>	Arabidopsis ring zinc finger protein	Codon 171,	Middle of 1st exon
<i>Ac902.33</i>	Maize plastid S9 ribosomal protein	Codon 184	3' end of last exon
<i>Ac905.29</i>	Arabidopsis Ser Thr kinase	Codon 540	3' end of 3rd exon
<i>Ac907.25</i>	Arabidopsis sugar transporter	Codon 14	5' end of 1st exon
<i>Ac907.51</i>	Arabidopsis short-chain ADH ^c	Codon 80	5' end of 1st exon
<i>Ac907.54</i>	Arabidopsis hypothetical protein	5' UTR	59 nucleotides upstream of ATG
<i>Ac911.14</i>	Arabidopsis DNA-3-methyladenine glycosidase	Codon 266	3' end of 4th exon
<i>Ac911.28</i>	Arabidopsis Ser Thr kinase	Codon 434	Middle of only exon
<i>Ac913.09</i>	Arabidopsis hypothetical protein	Codon 544	3' end of last exon (4th)
<i>Ac914.23</i>	Maize starch synthase	Codon 301	3' end of 4th exon
<i>Ac917.12</i>	Arabidopsis NAM1-like protein	Codon 191	Middle of last exon
<i>Ac917.27</i>	Arabidopsis PIN1 auxin efflux carrier protein	Codon 305	3' end of 1st exon
<i>Ac919.27</i>	Arabidopsis Pro-rich protein	Intergenic space	366 nucleotides after stop codon
<i>Ac919.39</i>	Barley Leu-rich repeat protein	Codon 220	3' end of only exon
<i>Ac921.20</i>	Rice Arg decarboxylase	Codon 580	3' end of only exon
<i>Ac929.20</i>	Arabidopsis unknown protein	Codon 2229	Middle of 11th exon

^a MAPK, mitogen-activated protein kinase.

^b UTR, untranslated region.

^c ADH, alcohol dehydrogenase.

revertants of these mutants to confirm that they are, in fact, tagged by *Ac*. Isolating revertants from lethal mutants is not a trivial task, but it is facilitated by our ability to identify heterozygotes on the basis of *Ac*'s dominant phenotype.

Transposed *Ds* elements represent a likely source of the new mutations that do not cosegregate with *Ac*. In total, there are ~40 to 50 copies of the short 0.4-kb *Ds1* elements and the larger *Ds* elements in the maize genome (Dennis et al., 1988). The short *Ds1* elements have internal sequences completely unrelated to *Ac*, whereas the larger *Ds* elements arise from internal deletions of *Ac*. Possibly, both types of elements contribute to the spectrum of mutants recovered in *Ac* stocks. Mutants tagged by both classes of *Ds* elements have been reported at a diversity of loci (summarized by Neuffer et al., 1997). If new mutations are caused by *Ds* elements, they should be unstable in the presence of *Ac*. However, somatic instability is not always easy to detect, particularly if the size of the somatic sectors is small. We have detected somatic instability of a pale green seedling mutant, a sugary endosperm mutant, and a zebra-striped plant mutant and have shown that the instability of the latter two is dependent on an *Ac* element located elsewhere in the genome. On the other hand, we have not seen somatic instability of mutant phenotypes, such as luteus and albino seedlings, in which somatic reversion should have been readily detectable. It is possible, therefore, that some of the mutants recovered are not tagged by either *Ac* or *Ds*.

Some of the mutations could be spontaneous point mutations, but the overall mutation frequency for obvious visible phenotypes (5%) is much higher than has been observed in similar experiments. For instance, Neuffer (1978) reported a spontaneous overall mutation frequency of 0.7%, which is much lower than what we observed. Insertions that do not belong to the *Ac-Ds* family are other possible sources of mutations. The recovery of mutants harboring insertions other than those known to be present in a particular stock is common in transposon mutagenesis experiments (Schmidt et al., 1987; Schnable et al., 1989; Johal and Briggs, 1992; Michel et al., 1995; Tacke et al., 1995). However, such mutants usually show somatic instability. Finally, there exists the possibility that some of the stable mutants arise from two-step transpositions of *Ac* that create a transposon footprint at the first site of insertion. Although this possibility often is raised as a concern by researchers who espouse two-element systems for transposon mutagenesis, there are no data to support it. Furthermore, given the preference of *Ac* to transpose to closely linked sites, one would expect that the mutations in our study frequently would be linked to the *trAc* element, but they are not. Thus, we do not believe that the latter mechanism plays a major role in the generation of mutations that are not tagged by *Ac*.

Given the fact that *Ac* transposes preferentially into genes, it might seem paradoxical that so few of the *trAc*s cause obvious mutant phenotypes. The cumulative evidence for the large-scale duplication, triplication, and even greater amplification of segments of the maize genome

(Helentjaris et al., 1988; Gaut and Doebley, 1994; Gaut, 2001) suggests that a possible reason for the dearth of mutant phenotypes is genetic redundancy. However, redundancy in maize rarely is complete. The evolved differences in function between duplicate genes can range from slight quantitative differences of expression in certain tissues (*orp1* and *orp2*; Wright et al., 1992) to completely nonoverlapping spatial expression patterns (*sh2* and *agp1*; Giroux and Hannah, 1994). A collection of lines carrying *Ac* insertions in different, partially sequenced genes will help investigators to screen for subtle mutant phenotypes, particularly after obtaining information on where in the plant the mutated gene is expressed. It also will make it possible to identify mutations lacking obvious visible phenotypes (Shen et al., 2000) or mutations in genes with minor quantitative effects that would be missed from conventional transposon mutagenesis screens designed to identify gross changes in phenotype.

METHODS

Description of Maize Stocks

All of the stocks used in this study were in the common genetic background of the maize (*Zea mays*) inbred line W22. *wx-m7(Ac)* is an unstable *wx* allele described by McClintock (1964). It arose by insertion of the 4.6-kb *Ac* element in the 5' untranslated region of the *Wx* gene (Muller-Neumann et al., 1984; Klosgen et al., 1986). *bz-m2(Ac)* is an unstable *bz* mutation that arose from the insertion of *Ac* in the second exon of the *bz* gene (McClintock, 1955; Ralston et al., 1988). *bz-m2(D1)* is the first derivative from *bz-m2(Ac)* isolated by McClintock (1962). It harbors a 3.3-kb internally deleted *Ds* element at the same position as *Ac* in *bz-m2(Ac)* (Dooner et al., 1986). *bz-R* and *wx* are stable recessive mutations.

Derivation of an *Ac* Insertion Library from *wx-m7(Ac)*

bz-R wx-m7(Ac)/bz-m2(D1) wx heterozygotes were pollinated with *bz-m2(D1) wx* in a large hand-pollinated isolation plot. In the resulting test cross ears, which will have predominantly waxy, bronze kernels, germinal transpositions of *Ac* can be selected as exceptional kernels that appear shiny and spotted (*Wx' bz-m*). From 1185 ears, more than 2500 independent *Wx' bz-m* kernels were selected. These occurred as separate kernels in the ears, indicative of meiotic or post-meiotic *Ac* transposition events. Very few ear sectors contained multiple adjacent *Wx' bz-m* kernels, which are indicative of premeiotic events. Of the single-kernel selections, approximately half were heritable; that is, they had resulted from *Ac* transposition events that were included in both endosperm and embryo. The other half had noncorresponding genotypes in the endosperm and embryo and, most likely, resulted from gametophytic *Ac* transposition events that were recovered only in the endosperm. Kernels with nonconcordant embryos and endosperms are a common occurrence in selection schemes for germinal transpositions of other transposable elements.

Selfed ears were harvested from 1225 confirmed primary transposants, and rough estimates of the *wx-Ac* linkage were obtained for

each transposant by counting the number of spotted and nonspotted kernels among the waxy class, the majority of which would have received at least one copy of the *bz-m2(D1)* reporter allele. The recombinant fraction p was estimated as $1 - \sqrt{(a_2/n)}$ where a_1 is the number of bz-m wx kernels, a_2 is the number of bz wx kernels, and $n = a_1 + a_2$. Errors in the estimate of p in such two-point mapping arise from excisions of Ac without reinsertion and from transpositions of Ac to unlinked sites. The latter source is significant only for very closely linked receptor sites and results in a slight displacement of the modal p value away from 0. Ac was found to be unlinked to the wx donor locus (the null hypothesis tested) in $\sim 37\%$ of transposants and was found to be linked in the remaining transposants. This crude distribution is not very different from what has been observed at other loci (Greenblatt, 1984; Dooner and Belachew, 1989).

Nucleic Acid Extraction, Blotting, and Hybridization

Leaf DNA was isolated by a urea extraction procedure (Greene et al., 1994). Total RNA was extracted using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Rockville, MD). DNA concentration was established by comparing aliquots of sample DNA with 1 μ g of λ DNA on an agarose gel stained with ethidium bromide. RNA concentration was determined by comparing samples with standards on ethidium bromide-stained gels and UV light spectrophotometry. Restriction enzyme-digested DNA (10 μ g for methylation sensitive, 8 μ g for methylation insensitive) and total RNA (30 μ g) were resolved on 0.8 and 1.2% agarose gels, respectively, and then transferred to Hybond N⁺ membranes (Amersham Pharmacia, Piscataway, NJ). ³²P-labeled probes were generated with Ready-To-Go DNA labeling beads (Amersham Pharmacia). The probe used to detect Ac-hybridizing bands was a polymerase chain reaction (PCR)-generated fragment from the middle of Ac. The primers used to generate this probe were Ac2047 and Ac2905R (P1 and P2, respectively, in Figure 3). The probe used for RNA gel blot analysis and *tac* site confirmation was a PCR-generated fragment corresponding to the complete inverse PCR (IPCR) product with minimal Ac ends. The primers used to create this probe were Ac24R and Ac4542 (P3 and P4, respectively, in Figure 3). These primers generate a probe containing 48 nucleotides of Ac, which are insufficient to reveal any additional bands in blots. Hybridization and membrane washing followed established protocols (Church and Gilbert, 1984). The sequences of the primers described above are as follows: Ac2047 (P1), 5'-GGATTGATGATGATTGGTCTCC-3'; Ac2905R (P2), 5'-TTGTACCTAGGGTCAAGGAAGCAT-3'; Ac24R (P3), 5'-TTTCCATCCTACTTTTCATCCCTG-3'; and Ac4542 (P4), 5'-CGTTACCGACCGTTTTCATCCCTA-3'.

tac Site Amplification and Sequencing

Independent *tac* sites were identified in diagnostic DNA gel blots as restriction fragment length polymorphisms when hybridized with the internal Ac probe. Fragments <9 kb were selected as likely substrates for IPCR amplification. Thirty micrograms of genomic DNA then was digested with the appropriate enzyme and separated by gel electrophoresis as described above. The region containing the band of interest was excised and purified using a QiaQuick Gel Extraction Column (Qiagen, Valencia, CA). The purified fragment was diluted to 2 ng/ μ L and circularized using T4 DNA Ligase (New England Biolabs, Beverly, MA). The ligation reaction was extracted once with phenol:chloroform (1:1) and precipitated with 0.1 vol NaOAc and 2 vol

ethanol. The precipitation reaction was centrifuged for 20 min at 14,000g, and then the pellet was washed with 70% ethanol. The pellet was vacuum dried and resuspended in 50 μ L of deionized water.

IPCR was performed according to the protocol of QiaTaq (Qiagen). An aliquot of the ligated DNA sample recovered from fragment purification was used as a template for IPCR. The reaction consisted of 1 \times PCR buffer, 1 \times Q buffer (Qiagen), 1 mM total deoxynucleotide triphosphates, 400 nmol of each primer, and 2 units of rTth polymerase (Perkin-Elmer). The reaction was denatured at 95°C for 3 min, cooled to 80°C, and held for the addition of polymerase. The reaction continued with 37 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 62°C, and 4 min of extension at 72°C. The last 20 cycles autoextended the extension time by 20 sec per cycle. The first pair of primers, designated Ac2578R and Ac4372, often produced a very faint band when electrophoresed on an agarose gel. The products from the first reaction were diluted 10-fold, and then 1 μ L was used for the nested reaction. This produced a total 500-fold dilution of template DNA for the nested amplification.

The nested amplification was performed as described above with the following changes. The polymerase used was QiaTaq polymerase (Qiagen) to facilitate T/A cloning. The primers used were Ac100R and Ac4473. The sequences of the four oligonucleotide primers used in the nested PCR were as follows: Ac2578R, 5'-GACAAACATACCTGCGAGGATCAC-3'; Ac4372, 5'-ACCGAACAATAACCGGTTCCG-3'; Ac100R, 5'-ATATCCCGTTTCCGTTCCGTTTC-3'; and Ac4473, 5'-TCCCGTTTTCCGTTTCCGTC-3'. After the nested reaction, a clear, sharp band was visible. The band of interest was excised from a gel and purified with a BIO101 glass bead kit (Q-Bio, Carlsbad, CA). The PCR product then was cloned into a pGEM-EasyT vector (Promega, Madison, WI) and sequenced on an ABI377 DNA sequencer (Applied Biosystems, Foster City, CA). Fragments were analyzed using the Lasergene99 software suite (DNASar, Madison, WI). Ac genomic DNA junctions were identified, and the flanking genomic DNA was used as a query against the various GenBank databases.

Accession Numbers

The *tac* sites listed in Table 2 have been submitted to GenBank under the following accession numbers: *tac901.04*, AY065586; *tac902.05*, AH011332, AY065594, AY065595; *tac902.07*, AY065576; *tac902.33*, AH011329, AY065572, AY065573; *tac903.05*, AH011338, AY065606, AY065607; *tac903.06*, AH011337, AY065604, AY065605; *tac905.5*, AF338121; *tac905.29*, AH011322, AY065560, AY065561; *tac907.25*, AH011327, AY065568, AY065569; *tac907.49*, AH011328, AY065570, AY065571; *tac907.51*, AH011334, AY065598, AY065599; *tac907.54*, AH011343, AY065616, AY065617; *tac909.06*, AF338125; *tac911.14*, AY065584; *tac911.16*, AY065587; *tac911.17*, AY065589; *tac911.22*, AH011336, AY065602, AY065603; *tac911.28*, AH011323, AY065558, AY065559; *tac911.30*, AH011325, AY065564, AY065565; *tac913.01*, AH011330, AY065590, AY065591; *tac913.09*, AH011340, AY065610, AY065611; *tac913.14*, AH011333, AY065596, AY065597; *tac914.07*, AH011324, AY065562, AY065563; *tac914.11*, AY065577; *tac914.13*, AY065585; *tac914.23*, AH011342, AY065614, AY065615; *tac917.09*, AH011331, AY065592, AY065593; *tac917.12*, AY065578; *tac917.27*, AH011326, AY065566, AY065567; *tac917.29*, AY065588; *tac918.02*, AH011335, AY065600, AY065601; *tac919.27*, AY065579; *tac919.39*, AH011339, AY065608, AY065609; *tac919.41*, AH011341, AY065612, AY065613; *tac921.20*, AF338139; *tac923.08*, AY065582; *tac923.13*, AY065580; *tac923.17*, AY065581; *tac929.20*, AY065583; *tac2094*,

AF338110; *tac6067*, AF338112; *tac6087*, AF338113; *tac6058*, AF338111; *tac7077*, AF338114; *tac8193*, AY065574; *tac8194*, AY065575.

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