Maize-targeted mutagenesis: A knockout resource for maize

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We describe an efficient system for site-selected transposon mutagenesis in maize. A total of 43,776 F1 plants were generated by using Robertson’s Mutator (Mu) pollen parents and self-pollinated to establish a library of transposon-mutagenized seed. The frequency of new seed mutants was between 10^{-4} and 10^{-5} per F1 plant. As a service to the maize community, maize-targeted mutagenesis selects insertions in genes of interest from this library by using the PCR. Pedigree, knockout, sequence, phenotype, and other information is stored in a powerful interactive database (maize-targeted mutagenesis database) that enables analysis of the entire population and the handling of knockout requests. By inhibiting Mu activity in most F1 plants, we sought to reduce somatic insertions that may cause false positives selected from pooled tissue. By monitoring the remaining Mu activity in the F2, however, we demonstrate that seed phenotypes depend on it, and false positives occur in lines that appear to lack it. We conclude that more than half of all mutations arising in this population are suppressed on losing Mu activity. These results have implications for epigenetic models of inbreeding and for functional genomics.

In many organisms, genome sequencing has refined gene mapping to the nucleotide level, but the association of genetic function with nucleotide sequence remains a significant challenge (1). For example, whereas it is possible to determine gene function by allele replacement in the mouse (2, 3) this procedure is impractical in plants because of the relatively low rate of homologous recombination. An alternative strategy in Drosophila (4) and Caenorhabditis elegans (5) uses active transposons to generate populations (“libraries”) of organisms harboring new insertional mutations. Individuals carrying insertions in a particular gene of interest can then be selected by PCR with a gene-specific primer and a transposon-specific primer applied to pooled DNA samples from the population. Various pooling schemes minimize the number of DNA preparations that must be performed to identify an individual when a positive result is obtained (6–8).

In maize, two similar strategies have been used to generate collections of plants that can be screened for new insertions into genes of interest such as hcf106 (9), anl (10), or ZAG1 (11). Both schemes used the transposon Robertson’s Mutator (Mu) because of its several advantages: Mu has many copies in the genome (12, 13) and causes a high mutation rate of one new allele per 1,000–10,000 plants (14, 15). Mu elements share closely related 0.2-kb terminal inverted repeats, allowing single primers to recognize multiple elements. Further, new insertions tend to be in genomic regions (16) unlinked to the original copy (17). In these and other plant transposon collections, insertions are detected by screening DNA isolated from pooled somatic tissues of the generation preceding the archived seed stock.

One problem with this strategy is that multiple Mu autonomous elements are required to generate high levels of germinal activity, but they also generate high somatic activity (18). Somatic insertions are not transmitted to the next generation, leading to false positives. To avoid this problem, we have designed a scheme to inhibit somatic transposition genetically in the tissue from which DNA is extracted. Using a genetic inhibitor, termed Mu inhibitor, we have developed a population of 43,776 plants containing stabilized Mu insertions. The collection forms the basis for a reverse genetics facility that is openly available to the scientific community.

Materials and Methods

Transposon Insertion Site Screening. DNA samples were prepared from dried leaves by grinding the tissue to powder in a Cuisinart DLC processor, extracting 5 ml of powder with 20 ml of urea buffer plus 20 ml of phenol/chloroform, and precipitating with isopropanol (19). The pellets were resuspended in 2 ml of Tris-EDTA buffer and 2 μl of a 20-fold dilution of each sample, ~50 ng DNA, were used in PCR. Conditions for PCR were: 1× ExTaq buffer, 0.25 mM each dNTP, 1× Q buffer (a solution of betaine from Qiagen, Chatsworth, CA), 0.5 μM each primer, and 0.5 units ExTaq polymerase (PanVera, Madison, WI) per 20-μl reaction. Primers were: Mu53 (GCCTCYATTTCGTCGAATTC), Mu53s (GCCTCYATTTCGTCGAATTC), MuEnd23 (TCGTCYATAATGGCAATTATCTC), and Mu58 (CCAWSGCTCYATTTCTGT) for Mu inserted inversions and vp1–114 (GACGGCATGATGAAAGAGAA), vp1–224 (CACGAGCAACCGGGAAACA), and vp1–615 (GGCCCTGGTGAGGAAGAGTA) for the vp1 gene. Reactions consisted of 35 cycles of 94° for 30 sec, 62° for 30 sec, and 72° for 4 min or, where 72° was reduced to 62° for 4 min, and 0.5 units ExTaq polymerase (PanVera, Madison, WI) per 20-μl reaction. Primers were: Mu53 (GCCTCYATTTCGTCGAATTC), Mu53s (GCCTCYATTTCGTCGAATTC), MuEnd23 (TCGTCYATAATGGCAATTATCTC), and Mu58 (CCAWSGCTCYATTTCTGT) for Mu inserted inversions and vp1–114 (GACGGCATGATGAAAGAGAA), vp1–224 (CACGAGCAACCGGGAAACA), and vp1–615 (GGCCCTGGTGAGGAAGAGTA) for the vp1 gene. Reactions consisted of 35 cycles of 94° for 30 sec, 62° for 30 sec, and 72° for 4 min or, where Mu58 and Mu53s were used, 94° for 30 sec, 58° for 30 sec, and 72° for 4 min. Other primers were designed according to sequences submitted to the maize-targeted mutagenesis (MTM) web site (http://mtm.cshl.org/cgi-perl/newreq.cgi) by using the primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi). In a typical set of nested amplifications, an outermost primer was used with Mu58, the products were diluted 50-fold, and 2 μl was used as a template with the inner primer and Mu53s. Heritable insertions were identified by preparing DNA from 10 F2 seedlings and repeating the PCR assay.

MTM Database (mtmDB) Web Site. The mtmDB web site (http://mtm.cshl.org) is based on the Apache web server (www.apache.org) and hosts a set of Perl scripts (20) running under the embedded Perl interpreter mod_perl. The underlying database is AceDB (www.acedb.org), which communicates with the web server via the AcePerl library (21). The web interface also depends on the CGI.pm module (22) and the GD module (http://stein.cshl.org/www/software/GD).

Genetic Analysis. Ears were scored before shelling, and kernel phenotypes were scored if they segregated on the ear. Parental mutant phenotypes were exhibited by related F1 plants and were

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Abbreviations: MTM, maize-targeted mutagenesis; Mu, Mutator; mtmDB, MTM database.

†B.P.M., H.L., and E.V. contributed equally to this work.

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Table 1. Mutant and insertion frequency distributions among Mu activity classes

<table>
<thead>
<tr>
<th></th>
<th>1998a</th>
<th></th>
<th>1998b</th>
<th></th>
<th>1999</th>
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<tbody>
<tr>
<td></td>
<td>Mu on</td>
<td>Mu off</td>
<td>Mu unscorable</td>
<td>Total</td>
<td>Mu on</td>
<td>Mu off</td>
</tr>
<tr>
<td>Total ears</td>
<td>2,027</td>
<td>3,747</td>
<td>4,652</td>
<td>10,426</td>
<td></td>
<td></td>
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<tr>
<td>Kernel mutants</td>
<td>1,160</td>
<td>1,705</td>
<td>2,126</td>
<td>4,991</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency, %¹</td>
<td>57</td>
<td>46</td>
<td>46</td>
<td>48</td>
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<tr>
<td>Nonparental</td>
<td>72/192</td>
<td>170/805</td>
<td>230/942</td>
<td>472/1,939</td>
<td></td>
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<tr>
<td>Kernel mutants²</td>
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<tr>
<td>Frequency, %</td>
<td>38</td>
<td>21</td>
<td>24</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonparental vp mutants⁶</td>
<td>24</td>
<td>37</td>
<td>70</td>
<td>131</td>
<td></td>
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</tr>
<tr>
<td>Frequency, %</td>
<td>1.2</td>
<td>1.0</td>
<td>1.5</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Generic insertions⁷</td>
<td>12/2,027</td>
<td>18/3,747</td>
<td>22/5,746</td>
<td>52/11,520</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency, %</td>
<td>0.59</td>
<td>0.48</td>
<td>0.38</td>
<td>0.45</td>
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*Ears in which Mu activity could not be scored because the F₁ individual inherited the bz1 sh1 linkage group rather than the bz1-Mum9 reporter gene.
¹Frequency of indicated mutant type within each Mu subclass.
²Kernel mutants were deemed parental if scored in sibling plants at near-Mendelian ratios. The class “nonparental mutants” was defined stringently to comprise aggregate kernel data less all data derived from Mu parents that showed evidence of harboring parental mutants in any branch of the pedigree. The numerator indicates number of mutants, and the denominator indicates the subpopulation size.
³Nonparental mutants that displayed a viviparous kernel phenotype.
⁴Mu insertions into user-submitted genes recovered by PCR-based methods. Because tissue grid and ear populations do not coincide exactly, numerator indicates number of knockouts, denominator the subpopulation sizes.
⁵Adjacent frequencies that differ significantly at the 0.5% level.

Results and Discussion

Mutagenesis Scheme and Population Design. It has been previously suggested that somatic insertions of transposon Mu might be responsible for clonal homozygous mutant sectors in leaf tissue, also known as Mu stripes (14). Mu stripes can occupy as much as 1/8th leaf width (Fig. 1A) but are rarely transmitted to the next generation (data not shown). To determine whether these stripes were caused by somatic insertions, DNA was isolated from pale green stripes found in mature Mu/+/+ plants and from adjacent tissue. DNA gel blots (Fig. 1B) and amplification and sequencing (data not shown) revealed that more than half of these stripes had new Mu insertions at the hc/J106 locus. Serial dilution of sector DNA with WT DNA revealed that 1 part in 256 could be readily detected by PCR, confirming that these sectors would result in false positives and defining the maximum pool size for selection of germinal insertions (9, 10).

To avoid somatic insertions, active Mu lines (23) were crossed to a line carrying Les28, a dominant lesion mimic mutation (Fig. 1C). This line also carries dominant sectoring factors that inhibit Mu activity (24). When these plants were pollinated by using Mu-active parents, progeny initially retained Mu activity, but lost it during later development. The Les28 phenotype depends on Mu activity, so that phenotypic lesions are visible in the lower leaves where Mu is active, but not in the upper leaves where Mu has been silenced, greatly reducing the frequency of somatic insertions. Progeny of such plants usually lost activity altogether and so resembled those of Mu killer strains described by Freeling and coworkers. Because we do not know whether the factors are the same, we refer to ours as Mu inhibitor.

Each F₁ plant was given a five-digit barcode that remained associated with its tissue sample and F₂ progeny. For DNA preparations, two upper (i.e., Mu-inactive) leaves were harvested and split into halves, discarding the midvein. As clonal somatic sectors are not observed to cross the midvein, the two samples should never include the same sector, providing additional insurance against false positives. Tissue samples were dried and then arranged in 48-by-48 plant grids. Barcodes were scanned to facilitate tracking the positions of individuals within the grid. Leaf halves constituting each row and column were pooled, and DNA was prepared from the pooled tissue. Each grid of 2,304 plants was therefore represented by 96 DNA samples and an individual plant could be recognized by its unique row and column address (9). Additionally, a small leaf sample from each individual was stored in the well of a microtiter dish for later use. F₂ seed were harvested and stored. In 1998, two populations of plants were processed into grids: 11,520 were the progeny of Mu-active lines and the Mu-inhibitor line, whereas 9,216 were the progeny of Mu-active lines and the inbred line B73. These plants were arranged into nine grids. In 1999, an additional 23,040 plants were processed from Mu-active and Mu-inhibitor parents and arranged into 10 grids.

Transposon Activity and Mutant Phenotypes. To monitor Mu activity, Mu-active parents of the 1998 planting carried bz1-Mum9 (23), whereas parents of the 1999 planting carried a1-Mum2 (18), both of which spotted seeds only in the presence of Mu activity (Table 1). The Mu-inhibitor strain was the same in each case. In the 1998 planting, 35% [2,027/2,027 + 3,747)] of F₁ plants crossed with Mu inhibitor had F₂ progeny that retained Mu activity, but in the 1999 planting there were virtually none (6 of 18,971 scored). In comparison, 83% [4,300/(4,300 + 903)] of F₁ plants from crosses of Mu-active lines to B73 still had Mu activity. We conclude that the Mu-inhibitor line dominantly inactivated Mu depending on the Mu line used.

Similar phenotypes in different F₂ families from related F₁ parents were assumed to be parental mutations in the first planting (1998), and such parental mutations were avoided as much as possible in the second planting (1999). The frequency of nonparental F₂ phenotypes is shown in Table 1. For example, nine new sugary mutants were recovered. As there are six known sugary loci, this finding indicates an allele frequency of 1.5 (or...
3.4 × 10⁻⁵ per plant) and a 78% probability of recovering an insertion into any given gene. In contrast, 15 viviparous loci are known, and 173 mutants were observed, giving an allele frequency of 12.5. If other, uncharacterized genes exist that give sugary or viviparous phenotypes the frequencies may actually be lower. Allelism of the mutants was not tested, so the numbers should not appear in row and column pools.

**Molecular Selection of Insertions via PCR.** Initially, screening was attempted by using PCR between a gene-specific primer and a Mu-specific primer (9). The viviparous1 gene (vp1) was used as a test case because it is the only known viviparous mutation that also eliminates kernel pigmentation (25, 26) and a single viviparous, anthocyaninless mutant was observed in the collection. As shown in Fig. 2A, amplification of pooled DNA from the row and column corresponding to the vp1 mutation was successful, providing a unique address for the new allele. This finding indicated that new insertions could be detected, but the sensitivity was low and, at this level of pooling, 1,824 reactions would be needed to screen the entire collection. We increased the sensitivity by using sequential amplifications with nested primers and reduced the number of reactions by pooling the DNA samples. Each grid of 96 pools (48 row pools and 48 column pools) was pooled horizontally into 8 superpools, and each grid position was pooled vertically across 10 grids (Fig. 2C). The increased sensitivity provided by nested PCRs (Fig. 2C) allowed the detection of the vp1 insertion even when DNA from 576 individuals was present in a single reaction. With the information provided by horizontal and vertical pools, an individual carrying a particular insertion can theoretically be identified from among the 43,776 individuals with only 688 reactions.

Once the system was demonstrated to work for a test case, a public service was established to allow researchers to obtain insertions in genes of interest. Genomic sequences corresponding to individual genes are submitted via the mtmDB website (http://mtm.cshl.org). The service designs primers to identify insertions throughout the gene and screens the collection. For the first 72 gene sequences submitted 30 had at least one insertion (Fig. 3). Excluding insertions that may have been present in the parents, a total of 65 insertions were found for a mean frequency of 2.1 × 10⁻⁶ new insertions per gene per plant. Most insertions (92%) were found in the bz1-Mum9 population.

![Fig. 1. Scheme for selecting germinal insertions of Mu insertions. (A) Somatic transposition of Mu elements results in clonal pale green sectors of homozygous mutant tissue in hcf106/+ heterozygous plants. (B) Genomic DNA was prepared from the pale green sector shown in A (lane 2), normal tissue on either side (lanes 1 and 3), homozygous normal (lane 4), and homozygous mutant (lane 5) plants. DNA was digested with HindIII before DNA gel blot analysis using the hcf106 gene as a probe. Note the insertion of Mu DNA into the lower, WT allele. (C) Pooling scheme to identify germinal insertions. To avoid detecting somatic insertions such as those in A, Mu-active lines were first crossed to a Mu-inhibitor strain to inactivate Mu. Germinal insertions were then detected in the F₁ plants. An upper leaf from each plant was deribbed, and opposite halves were used for row and column pools. Because clonal sectors have not been observed to cross the midvein (A), somatic insertions should not appear in row and column pools.](http://www.pnas.org/content/100/20/11543.figure1)

![Fig. 2. Screening for Mu insertions in Vp1. (A) Simple PCR between a gene-specific primer and a Mu-specific primer. The products were blotted and probed with a fragment of Vp1 generated by primers vp1-114 and vp1-615. Autoradiography was 16 h. Only 48 of the 96 reactions required to screen a grid are shown. (B) Pooling strategy to reduce the number of reactions required to screen the collection. Groups of 12 rows or columns were pooled horizontally across a grid; grid positions were pooled vertically across grids. (C) Detection of the Vp1 insertion in the horizontally pooled pools. Reaction products were blotted and probed as above, except autoradiography was 2 h.](http://www.pnas.org/content/100/20/11543.figure2)
from 1998, implying a frequency of \(4.0 \times 10^{-5}\) for these grids. The difference between the 1998 and 1999 populations lies in the different Mu-active parents and underscores that germinal activity of Mu can differ substantially even if somatic activity, scored here by aleurone spotting, appears similar (17). The number of insertions detected molecularly was more consistent with the number of \(su\) alleles than \(vp\) alleles, again suggesting insertional hotspots in \(vp\). The suggestion of hotspots is supported by the apparent non-Poisson distribution of numbers of insertions per gene (Fig. 3B). Reasons for failure to recover insertions in a given gene include failure of germinal transmis-
tion through the pollen, insertional target preference, sampling bias, and incomplete screening caused by submission of partial insertions in a given gene include failure of germinal transmis-
tion to the \(F_2\) was confirmed in all cases by similar PCR analysis on DNA from 1-week-old seedlings.

Table 2. Distribution of sequenced insertions in regions of genes

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<thead>
<tr>
<th>Region</th>
<th>5' Half</th>
<th>3' Half</th>
<th>Intron</th>
<th>Exon</th>
<th>Promoter</th>
<th>3' UTR</th>
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<td></td>
<td>18</td>
<td>6</td>
<td>11</td>
<td>7</td>
<td>4</td>
<td>1</td>
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Suppression of Mutant Phenotypes. The phenotypic severity of some transposon-induced alleles can be suppressed in the presence or absence of an active transposon elsewhere in the genome (30, 31). Suppression does not involve excision of the element, but rather the regulatory interaction of transposase with insertions mostly in introns and promoters (32–35), but also in 3′ UTRs (36) (reviewed in ref. 37). We set out to measure the frequency of supressible phenotypes in MTM, by first elimi-
nating portions of the pedigrees that contained parental muta-
tions. We then investigated the occurrence of suppressible mutations in the \(F_1\) by examining the distribution of new \(F_2\) kernel mutations with respect to \(Mu\) activity. As these mutations must have arisen in the pollen of active \(Mu\) parents, \(Mu\) activity could only have been lost in the \(F_1\) plants.

The frequency of new kernel mutants failed contingency \(\chi^2\) tests for independence of \(Mu\) activity (Table 1; \(P\) values 0.00006, 0.0025, and 0.000028), but satisfied control tests for independence of \(bz1sh1\) (Table 1; \(P\) values 0.99 and 0.98). That is, fewer new mutant phenotypes were scored in the \(Mu\)-off class than in the \(Mu\)-on class for all three populations. As new mutations must have arisen in \(Mu\)-active pollen, these data suggest that many new mutations were suppressed in \(F_2\) ears that had subsequently lost \(Mu\) activity. Interestingly, the high frequency of new \(vp\) mutants was independent of both \(Mu\) activity and \(bz1\) (Table 1), as if the predicted hotspot was not suppressible (see above).

We considered the possibility that the distribution bias of visible mutants in \(Mu\)-active \(F_2\) ears reflected variation in parental \(Mu\) transposition. For example low-activity parents that gave rise to relatively few new mutants could be the source of most \(Mu\)-off progeny, whereas high-activity parents that produced many new mutants could generate most \(Mu\)-on progeny. In this scenario, \(Mu\) transposition events would be expected to show a distribution bias similar to that observed for visible phenotypes. However, the distribution of new \(Mu\) insertions detected by PCR screening in \(F_1\) plants was independent of \(Mu\) activity in the \(F_2\), indicating that the frequency of insertions per se was not responsible for the discrepancy in visible phenotypes (Table 1; \(P\) values 0.43–0.95).

Individual pollen parents contributed both to families with low \(Mu\) activity and families with high activity. When the progeny were compared it was possible to correlate activity and mutant frequency directly (Fig. 4). Newly arising phenotypes were positively correlated with \(Mu\) activity (Fig. 4, \(\alpha\), \(R = 0.71\), \(P\) value <0.01). Two sets of six 1998b families each derived from a single \(Mu\) parent crossed onto \(Mu\) inhibitor showed a similar correlation with residual \(Mu\) activity (Fig. 4, \(\bullet\), \(R = 0.85\), \(P\) value <0.05; Fig. 4, \(\bigcirc\), \(R = 0.61\), \(P\) value <0.10). These data demonstrate that mutants are detected several times more frequently in fully active \(Mu\) lines than in fully inactivated lines.

In summary, germinal transposition of \(Mu\) elements in the \(F_0\) was independent of presence of \(Mu\) activity in the \(F_2\), but visible phenotypes were not, implying the existence of a large class of suppressible mutations.

mtmDB Database. To maintain the information regarding the maize \(Mu\) population described above and manage the reverse genetics screening process, we designed and implemented a
exhaustively catalog all possible aliases for a gene, it is better to
screened. Because we do not currently make an effort to
the database to identify mutants that have already been
browsed online, and documentation on the ACeDB query
hoc queries directly to the database. The data model can be
mtmDB data model. This page allows users to pose complex
those who know the ACeDB query language and understand the
in the database, including ones that are normally not of direct
is similar to this, except that it provides access to all of the classes
as individual plants, grid addresses, and genes. The class search
based access to the biologically most relevant object classes, such
and not boolean operations. The simple search provides name-
facilitates multiple keywords or phrases via the standard and, or,
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is also available in a text-only form suitable for cutting and
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interaction retrieved dynamically from the database. Graphical information
is also available in a text-only form suitable for cutting and
and not boolean operations. The simple search provides name-
facilitates multiple keywords or phrases via the standard and, or,
The MTM project is a collection of 43,776 maize ears, each containing a unique set of Mu insertions. Phenotypic and genotypic information is accessible at mtmDB (http://mtm.cshl.org), and seeds can be ordered by any academic researcher. The research community is taking advantage of the database, and links between maize genonomic sequence and gene function can be made.

In addition to its use as a resource, MTM is the largest collection of Mu insertions to be characterized in such detail. The variety of phenotypes is typical of Mu lines, but we have demonstrated a very high proportion of suppressible mutations predicted by our analysis. Such a high frequency of suppressible insertion may account for the radical difference in fitness observed between Mu-active and -inactive lines and may have implications for inbreeding depression. SUPPRESSIBLE PHENOTYPES CAN OBSCURE THE FUNCTION OF A GENE TARGETED BY REVERSE GENETICS, BUT CAN HAVE UTILITY IN MOSAIC ANALYSIS, WHEN EXAMINING THE CELL AUTONOMY OF A MUTANT PHENOTYPE. BY SELECTING INSERTIONS IN DIFFERENT LOCATIONS WITHIN THE TARGET GENE, MTM PROMISES TO BE A VALUABLE RESOURCE IN MAIZE DEVELOPMENTAL BIOLOGY AND MAIZE GENETICS.

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