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Site-Selected Transposon Mutagenesis at the *hcf106* Locus in Maize

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The *High chlorophyll fluorescence106* (*Hcf106*) gene in maize is required for chloroplast membrane biogenesis, and the *hcf106-mum1* allele is caused by the insertion of a Robertson's *Mutator Mu1* element into the promoter of the gene. Seedlings homozygous for *hcf106-mum1* are pale green and die 3 weeks after germination, but only in the presence of *Mutator* activity conferred by active, autonomous *Mu* regulatory transposons elsewhere in the genome. When *Mutator* activity is lost, the mutant phenotype is suppressed, and homozygous plants have an almost wild-type phenotype. To isolate derivative alleles at the *hcf106* locus that no longer require *Mutator* activity for phenotypic expression, we have developed a method for site-selected transposon mutagenesis in maize. This procedure, first described for *Caenorhabditis elegans* and Drosophila, involves using polymerase chain reaction (PCR) to screen pools of individuals for insertions and deletions in genes of known sequence. Pools of seedlings segregating for the progenitor allele *hcf106-mum1* were screened by PCR for insertions and deletions associated with Robertson's *Mutator*. In a 360-bp target region, two new insertions and one deletion were identified in only 700 *Mu*-active gametes screened. One of the insertions was in the progenitor *hcf106-mum1* allele and the other was in the wild-type allele, but all three new alleles were found to have breakpoints at the same nucleotide in the first intron. Unlike the *hcf106-mum1* progenitor allele, the deletion and one of the insertions conferred pale green seedling lethal phenotypes in the absence of *Mutator* activity. However, the second insertion had a weak, viable phenotype under these conditions. Although the sample size was small, our results suggest that this procedure can be used to rapidly identify transposon insertions into known genes in a single generation. Null derivative alleles can then be isolated in a second generation using the insertion as a starting point. This two-step procedure represents a powerful and simple way to "knock out" maize genes identified by sequence alone by using only a few thousand progeny from a simple cross.

INTRODUCTION

Transposable elements are versatile mutagens in microbes, plants, and animals because of their ability to disrupt genes by insertion and their ability to generate deletions, rearrangements, and point mutations once they are inserted at a given locus (McClintock, 1950; Berg and Howe, 1989). Most importantly, transpositions are typically conservative in transposon DNA sequences, providing disrupted target genes with molecular "tags." This property allows the cloning of genes defined only by the phenotypic consequences of transposon insertion, that is, by "transposon tagging" (Bingham and Judd, 1981; Fedoroff et al., 1984; Walbot, 1992). In principle, transposon tagging can be performed in reverse to identify individuals with transposon insertions in a gene that has already been cloned. These individuals can then be analyzed phenotypically to determine the function of the disrupted gene. This approach involves screening a large number of individuals for relatively rare insertion events.

The polymerase chain reaction (PCR) provides a sensitive means to identify transposon insertions via amplification of transposon and target gene fusions. In Drosophila and *Caenorhabditis elegans*, pools of animals have been screened in this way to identify rare individuals that have "site-selected" transposon insertions in a given target gene (Ballinger and Benzer, 1989; Kaiser and Goodwin, 1990; Rushforth et al., 1993). Insertions can be identified by PCR amplification using primers from transposons and target genes. Unfortunately, these insertions often fail to result in a mutant phenotype because of the removal of the transposon from the primary transcript by splicing (Rushforth et al., 1993). However, by using PCR amplification, deletions, excisions, and rearrangements (derivative alleles) can be selected from pools of siblings that carry a transposon insertion at a given locus, resulting in a "gene knockout" (Zwaal et al., 1993). This two-step procedure (insertion followed by deletion) is particularly useful in the case of homozygous lethal mutations: it allows derivative alleles to be identified in viable F_1 heterozygous individuals. In contrast, phenotypic screens must utilize F_2 families segregating nonviable homozygotes. Furthermore, if the derivative phenotype is difficult to distinguish from the progenitor allele, phenotypic screens are impractical. Given the large number of gene

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sequences emerging from genome studies, site-selected reverse genetics represents a powerful way to identify the function of genes identified by sequencing alone.

In plants, numerous families of transposable elements are suitable for site-selected transposon mutagenesis. With few exceptions, these elements can mutate genes by both insertion and subsequent imprecise excision, deletion, and other rearrangements (Fedoroff, 1989; Walbot, 1992). In maize, Robertson's *Mutator* elements are particularly suited to this procedure. Robertson's *Mutator* elements fall into at least seven classes (reviewed by Chandler and Hardeman, 1992; Bennetzen et al., 1993). Functions required for transposition are supplied in *trans* by the *Mutator* regulatory element *MuDR* (named in honor of D. Robertson), a 4.9-kb element with ~200-bp terminal inverted repeats that are shared with the other *Mu* element classes. *dMuDR* elements are derived from *MuDR* elements by internal deletion (Chomet et al., 1991; Hershberger et al., 1991; Qin et al., 1991), but most of the other classes of *Mu* elements have unrelated internal sequences. Full-length *MuDR* elements encode two genes, at least one of which is required for transposition (Hershberger et al., 1991). A number of properties make *Mu* uniquely suited for site-selected insertional mutagenesis. First, *Mu* elements can accumulate to very high copy numbers with very high forward transposition frequencies (Robertson, 1978, 1980). Second, *Mu* elements share long terminal inverted repeats that are very similar between different classes of element, providing sufficient sequence for multiple nested primers (Chandler and Hardeman, 1992). Third, germinal excision is rare, whereas transpositions and flanking deletions are very common: *Mu1* elements at the *Alcohol dehydrogenase1* and *Bronze2* loci in maize are known to generate flanking deletions resulting in null alleles at frequencies approaching 1% (Taylor and Walbot, 1985; Levy and Walbot, 1991). Such deletions are typically less than 100 bp and are thought to result from either abortive transposition or illegitimate recombination between the element and the gene. In the latter case, part of the element is lost in the deletion (Levy and Walbot, 1991).

We have used Robertson's *Mutator* elements to generate insertions and flanking deletions at the *high chlorophyll fluorescence106* (*hcf106*) locus in maize by using site-selected transposon mutagenesis. The *Hcf106* gene is required for chloroplast membrane biogenesis (Barkan et al., 1986), and the *hcf106-mum1* allele results from the insertion of a *Mu1* element in the *hcf106* promoter (Martienssen et al., 1989; Barkan and Martienssen, 1991). *hcf106-mum1* was the first *Mutator*-induced mutation in maize to be shown to be suppressible. That is to say, the mutant phenotype is suppressed, and homozygous plants are phenotypically wild type (or nearly so) when *Mutator* activity is lost (Martienssen et al., 1990). When *Mutator* activity is present (in "*Mu*-active plants"), homozygous seedlings are pale green and nonphotosynthetic, and they die 3 weeks after germination when their seed reserves are exhausted. A number of suppressible mutations at other maize loci have subsequently been described, and those loci that have been cloned all have a *Mu* element inserted in the promoter region of the suppressible allele (reviewed in Martienssen and Baron, 1994). In the case of *hcf106*, a *Mu1* element is inserted at the third of three transcription initiation sites (Barkan and Martienssen, 1991). In *Mu*-active plants, no transcripts from the locus accumulate to significant levels. However, when *Mutator* activity is lost, chimeric transcripts arise at the junction of the *Mu1* element and the *Hcf106* gene. These transcripts provide sufficient *Hcf106* gene product to rescue the mutant phenotype, resulting in viable, fertile homozygous plants that have near-normal chlorophyll pigmentation.

Homozygous mutant seedlings have occasional dark green sectors resulting from excision of the *Mu1* element from the *Hcf106* gene (Martienssen et al., 1989). They can also have larger dark green sectors resulting from somatic loss of *Mutator* activity, which is correlated with methylation of *Mu1* and *MuDR* elements in these sectors (Martienssen et al., 1990; Martienssen and Baron, 1994). Because of the frequent loss of *Mutator* activity in maize stocks carrying the original allele of *hcf106* (Martienssen and Baron, 1994), it has proven to be very difficult to perform genetic tests (e.g., double mutant studies) using this allele because phenotypic suppression greatly confuses the results. Additional biochemical analysis of the chloroplast proteins affected by the *hcf106-mum1* mutation suggests that it may be leaky: residual levels of the affected thylakoid membrane proteins accumulate in homozygous mutant plants (Barkan et al., 1986). This leakiness could be due to low levels of transcript accumulation from the mutant allele (Barkan and Martienssen, 1991). In contrast to this suppressible allele, deletions that encompass part of the coding region would not be expected to result in a functional gene product regardless of transcription from the locus. For this reason, we were interested in isolating deletion derivatives at the *hcf106* locus.

We have used PCR-based site-selected mutagenesis to isolate three new alleles at the *Hcf106* locus by transposon insertion and deletion. Two of these alleles behave genetically as loss-of-function derivatives, and the third has an almost wild-type phenotype. As in studies with Drosophila and *C. elegans*, this provides a potentially powerful method for identifying the function of cloned genes via gene knockouts and allelic series.

RESULTS

Figure 1A shows a map of the *hcf106-mum1* allele, based on cDNA and genomic sequencing (A. Baron and R. Martienssen, unpublished results). To generate null derivative alleles at the *hcf106* locus, we developed a strategy to screen for deletions that flanked the *Mu1* element in *hcf106-mum1* and encompassed the initiator ATG codon in the first exon of the gene. DNA was purified from pools of heterozygous *hcf106-mum1*/+ and +/+ seedlings and digested with TaqI, which has a unique site that overlaps the first in-frame ATG codon (Figure 1). DNA from each pool was then amplified with primers from the *Mu1* element and from the second exon of the gene, as described

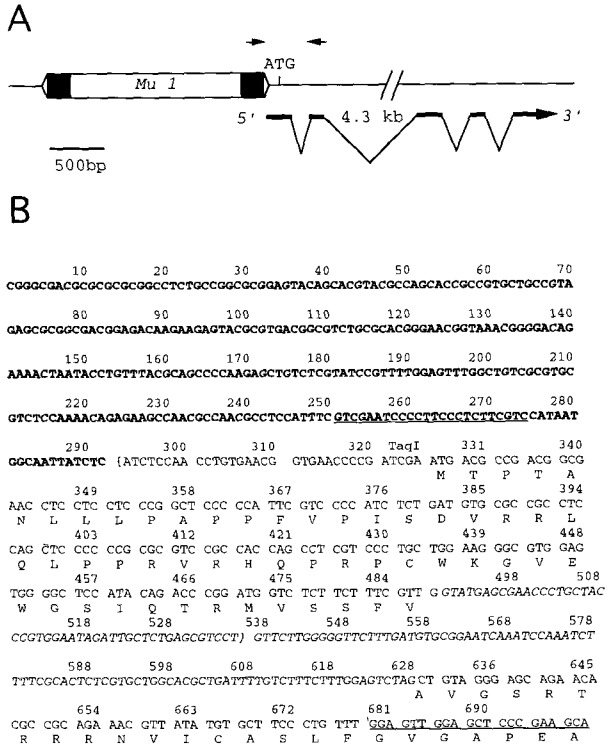

Figure 1. The *Mu1* Insertion Site at the *hcf106* Locus.

(A) A map of the *hcf106-mum1* allele is shown. The *Mu1* element is inserted at the third of three transcript initiation sites (Barkan and Martienssen, 1991). The location of the initiator ATG and the primers described in the text (arrows) are shown. The second intron of 4.3 kb is shown, but not to scale (broken line).

(B) The sequence of part of the region shown in **(A)** from the end of the *Mu1* element to the second exon of the *Hcf106* gene is shown. The sequence of the *Mu1* element is in boldface letters, and the intron sequence is in italics. Sequences corresponding and complementary to the primers used to screen seedling pools are underlined (see Methods). The 244-bp region deleted in *hcf106-mum3* is indicated by parentheses (position 293 to 536).

below. Digestion with TaqI prevents amplification of DNA from the progenitor *hcf106-mum1* allele because the primers are separated by the restriction site. Only undigested DNA that had lost the TaqI site could be amplified so that deletion derivatives could be identified among the pools of seedlings. These derivatives were expected to give amplification products of 300 bp or less, whereas residual undigested DNA from the progenitor allele was expected to give a 400-bp amplification product.

Flanking deletions that retain the original insertion are thought to arise via abortive transpositions (Levy and Walbot, 1991) so that *Mutator* activity would be required to generate deletions in *hcf106/+* parent plants. However, *Mutator* activity is readily lost from the parental stocks carrying *hcf106-mum1* (Martienssen and Baron, 1994). The pooled seedlings were therefore generated by crosses between *Mu*-active *hcf106/+* and *Mu*-inactive wild-type *+/+* plants. *Mutator* activity was monitored in the crosses by including the dominant *Les28* (*Lesion-mimic*) marker in the *Mu*-inactive *+/+* pollen parents. The *Les28* mutation requires *Mutator* activity for phenotypic expression (Martienssen and Baron, 1994). Furthermore, crosses between *Mu*-active and *Mu*-inactive lines typically result in *Mu*-active progeny (Martienssen et al., 1990; Brown and Sundaresan, 1992). Thus, only *Mu*-active *hcf106/+* parental plants produced progeny segregating for the lesion-mimic phenotype, which was otherwise suppressed in the *Mu*-inactive pollen parents. The *Les28* mutation is useful in this regard because *Mutator* activity can be monitored in the seedling (and therefore in the embryo) rather than in the aleurone layer (a derivative of the endosperm) so that heritable changes in *Mutator* activity can be recorded (Martienssen and Baron, 1994). However, in cases where *Mutator* activity is retained at high levels, it would be more convenient to use mutable anthocyanin markers expressed in the aleurone layer (Walbot, 1986; Brown and Sundaresan, 1992).

Fifteen hundred seeds were planted from six crosses that resulted in lesion-mimic progeny, but only approximately one-quarter of the resulting seedlings displayed the lesion-mimic phenotype. The *Mu*-inactive parents of these crosses were heterozygous for *Les28*, indicating that a 50% loss of activity had most likely occurred in the germ line of the *hcf106-mum1/+* parents (Martienssen and Baron, 1994), so that only 700 to 800 *Mu*-active gametes were represented by these seedlings. Seedlings were planted in three grids comprising 16 rows and 16 columns, with each position in the grid occupied by two seedlings (512 seedlings per grid), as shown in Figure 2. DNA was prepared from rows and columns by pooling 50 mg of leaf tissue from seedlings in each row and separately from an additional 50 mg of leaf tissue from seedlings in each column (32 DNA samples per grid). The pool size was chosen to avoid amplifying DNA from somatic sectors that were not germinally transmitted. We observed pale green somatic sectors on most heterozygous plants, and at least some of these resulted from transposition of *Mu* elements into the *Hcf106* gene during leaf development (R. Martienssen and A. Baron, unpublished data). These sectors are typically small (one-sixteenth of a leaf or less), but if the pool size were too large, more amplification cycles would have been required, and products corresponding to pooled somatic sectors might have confused the analysis.

DNA was amplified from each pool by PCR using a primer complementary to the DNA sequence 50 bp from the end of the *Mu1* element and a primer from the second exon of the *Hcf106* gene (Figure 1B). After we adjusted the conditions to reduce the abundance of background bands (see Methods), one position that resulted in a 200- to 300-bp PCR product was identified in each of the three grids by cross-referencing rows and columns. An example of one such position is shown in Figure 2. DNA was prepared from each of the plants at these positions, and the PCR products were amplified individually. Three candidate plants were identified, and PCR products from each were reamplified using a seminested primer complementary to the first 17 bp of the *Mu1* element; this sequence is

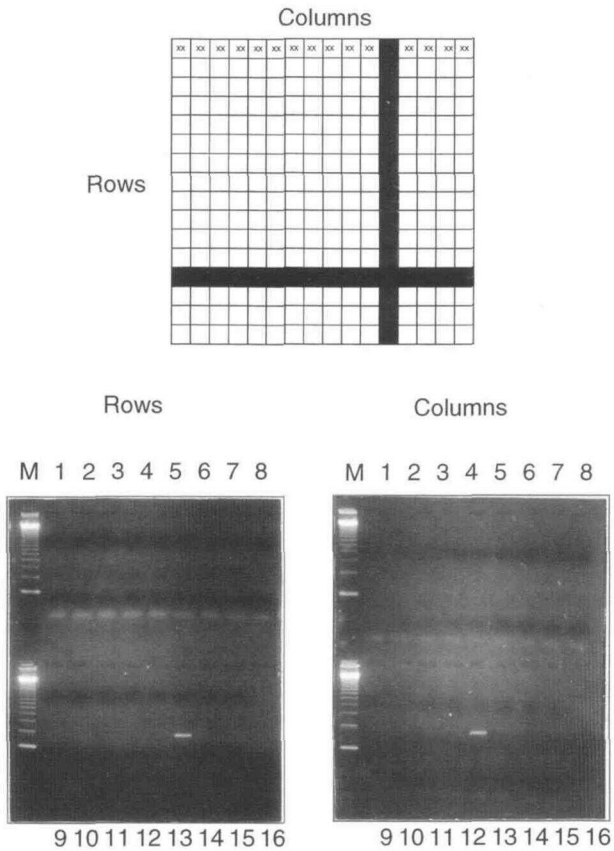

Figure 2. PCR Reactions with Pooled Samples.

Seed were germinated in a 16 × 16 grid, with two seedlings at each position in the grid as shown (x). DNA was extracted from pools of 32 seedlings from each row and each column and amplified using primers from the *Mu1* element and the second exon of the gene as described in the text. Each lane of each gel was loaded with PCR products from individual rows and columns. M indicates molecular weight marker lanes. The same derivative allele was amplified in one row and one column and was thus identified by a unique address.

known to be nearly identical in all known *Mu* elements. This primer also contained a restriction site for ease of cloning. The reamplified products were cloned and sequenced (see Methods). In each case, the sequence flanking the *Mu1* element corresponded to a breakpoint located in the first intron of the gene; this breakpoint is 244 bp downstream of the *Mu1* insertion site in the progenitor allele (Figure 1B). No other changes were observed.

The three plants resulted from three different crosses, indicating that they must represent independent derivative alleles. To confirm independence, DNA gel blotting was performed on DNA from each of the three plants using single and double digests. The blots were hybridized using a probe that encompassed the first and second exons and the first intron of the *Hcf106* gene (p106K; see Methods). An example of a DNA gel blot is shown in Figure 3, and a summary of the structure of the alleles is shown in Figure 4. Only one of the derivatives, *hcf106-mum3*, corresponded to a simple 244-bp deletion of the type envisaged by the selection scheme (Figure 4). The other two had restriction maps consistent with the insertion of new *Mu* elements at the same location in the second intron of the progenitor *hcf106-mum1* allele and in the second intron of the wild-type allele, respectively (Figure 4). Insertions were recovered because new insertions of *Mu* elements between the TaqI site and the second exon also allow amplification with the primers being used. However, the high frequency of these insertion events was unexpected (two new insertions in 700 *Mu*-active gametes).

By restriction mapping, PCR, and DNA gel blot analysis, the insertions in *hcf106-mum2* and *hcf106-mum4* were found to be *Mu1* and *dMuDR* elements, respectively. In the case of *hcf106-mum2*, digestion of genomic DNA with SstI, HindIII, and BamHI was used to show that an additional 1.4-kb insertion was present at the *hcf106-mum1* locus. Digestion with NotI revealed that this insertion had a NotI site oriented opposite to that in *hcf106-mum1* (Martienssen et al., 1989). The only *Mu* elements that have NotI sites but no SstI, HindIII, or BamHI sites are *Mu1* elements. PCR analysis confirmed that the original *Mu1* element was still present at the locus (see Methods). In the case of *hcf106-mum4*, digestion with the same enzymes indicated a 2.3-kb insertion containing two HindIII sites and two partially digested SstI sites. Reprobing of the same DNA gel blot indicated that the BamHI fragment (Figure 3) and the partial SstI fragments (data not shown) hybridized with a probe from the central portion of the *MuDR* element. This suggested that the element inserted in the *hcf106-mum4* allele might be a deletion derivative of the 4.9-kb *MuDR* element. This was

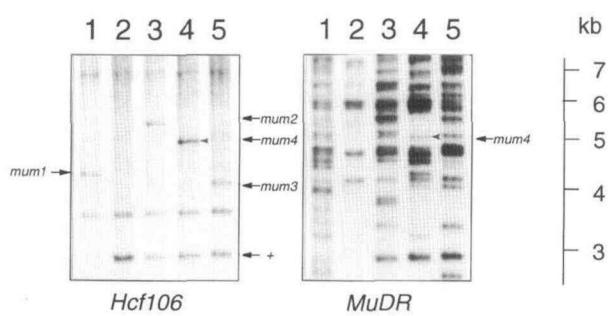

Figure 3. DNA Gel Blot Analysis of Derivative Alleles.

DNA samples from plants homozygous for *hcf106-mum1* (lanes 1) or wild-type *Hcf106* (inbred B73, lanes 2) and plants heterozygous for *hcf106-mum2* (lanes 3), *hcf106-mum4* (lanes 4), and *hcf106-mum3* (lanes 5) were digested with BamHI and subjected to DNA gel blot analysis using hybridization probes from the first two exons of the *Hcf106* locus or from the *MuDR* element. A hybridizing DNA fragment specific for each allele is marked with an arrow on the left panel. The *hcf106-mum4* allele is marked with an arrow on the right panel and with arrowheads in lanes 4. (+) marks the wild-type (B73) allele. Molecular length markers are given at right.

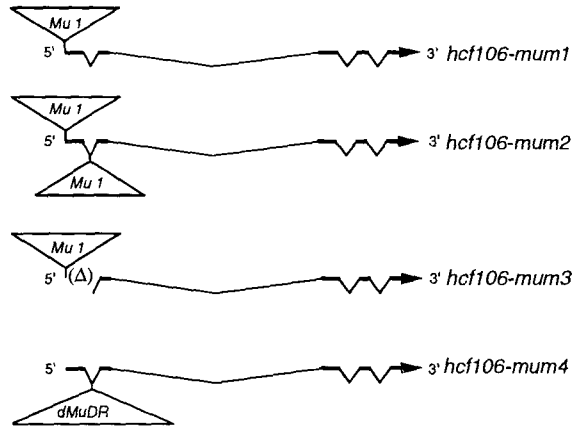

Figure 4. Schematic Maps of Progenitor *hcf106-mum1* and Derivative *hcf106-mum2*, *hcf106-mum3*, and *hcf106-mum4* Alleles.

Exon sequences are represented by horizontal lines; introns are shown as dips. The deletion in *hcf106-mum3* is shown as a triangle.

confirmed by amplification with a primer (5′-TAAGGAGCTCCA-AGCGCTGTCACC-3′) specific to the end of the *MuDR* element (Hershberger et al., 1991) and an *Hcf106* primer (5′-CCAGTG-CTTCGGGAGCTCCAACTC-3′). A 290-bp fragment was obtained confirming that the insertion was related to *MuDR* (data not shown). Digestion with HindIII alone confirmed that the *MuDR* insertion was in the wild-type (B73) allele in the case of *hcf106-mum4*. This is because a polymorphic HindIII site 4.5 kb upstream of the *Hcf106* gene in B73 is absent in *hcf106-mum1*, which has a HindIII site 10 kb upstream. In *hcf106-mum4*, HindIII fragments of 6 and 1.5 kb were observed; they could only have arisen if the progenitor allele in this case was the B73 allele (data not shown). Amplification using a primer upstream of the *Mu1* insertion site in *hcf106-mum1* (see Methods) and a primer from *Mu* gave a 300-bp product, confirming that there was no *Mu1* element at the *hcf106-mum1* insertion site in the *hcf106-mum4* allele.

Phenotypes conferred by each allele were determined by self-pollinating the heterozygous plants and planting the progeny in growth chambers. All three families segregated for unrelated white and yellow seedling lethal phenotypes found in the parent line and typical of *Mutator* lines. However, as expected, pale green lethal, high chlorophyll fluorescent *hcf106-mum2* and *hcf106-mum3* seedlings appeared in a 1:3 ratio relative to normal green siblings in these families (see following data). PCR tests and DNA gel blotting of 20 individual and 20 pooled mutant and normal seedlings confirmed that the pale green seedlings were homozygous for the new allele (data not shown). Pale green lethal seedlings were not recovered from the *hcf106-mum4* families.

Plants homozygous for the progenitor *hcf106-mum1* allele are seedling lethal only when they contain active *Mu* elements (Martienssen et al., 1990). In the absence of *Mutator* activity, functional *Hcf106* transcripts initiate within the *Mu1* element at the locus so that the mutant phenotype is suppressed and homozygous plants are viable and of near-normal pigmentation (Barkan and Martienssen, 1991). Results from a typical self-pollination of *Mu*-inactive *hcf106-mum1*/+ plants are shown in Table 1 (taken from Martienssen et al., 1990). Deletion derivative alleles would not be expected to be suppressed in this way because any *Mu*-derived transcripts would lack the first exon of the gene. To investigate whether this was the case, progeny tests were performed using the new alleles, and *Mutator* activity was monitored in the derivatives by using the *Les28* mutation, which confers a dominant lesion-mimic phenotype when *Mu* elements are active (Martienssen and Baron, 1994).

All three plants heterozygous for the original derivative alleles carried the *Les28* mutation but had lost the lesion-mimic phenotype by the seventh, fifth, and third leaves below the tassel, respectively (Martienssen and Baron, 1994). Following self-pollination and outcrossing, none of their progeny (of more than 200 examined) had the lesion-mimic phenotype. Further, when these progeny were self-pollinated, none of their progeny had the lesion-mimic phenotype either (of more than 300 examined). This suggested that these plants had lost *Mutator* activity, and this supposition was confirmed by DNA gel blotting using methylation-sensitive enzymes and *Mu1* probes. *Mu*-active plants have unmethylated *Mu1* elements, whereas *Mu*-inactive plants have mostly methylated elements (Chandler and Walbot, 1986; Bennetzen, 1987). HinfI fragments from unmethylated *Mu1*-related elements migrate at 1.7 (*Mu2*) and 1.4 kb (*Mu1*), whereas methylated elements result in a ladder of fragments depending on methylation of HinfI sites within and adjacent to the *Mu* element (Martienssen et al., 1990). Figure 5 shows that some of the original heterozygotes had predominantly unmethylated elements in the leaf from which DNA was extracted (arrows) but their progeny had predominantly methylated elements. This confirmed that their progeny had lost *Mutator* activity.

In progeny tests, plants heterozygous for *hcf106-mum2* and plants heterozygous for *hcf106-mum3* had nearly one in four mutant progeny when self-pollinated, even though they had lost *Mutator* activity (Table 1). In contrast, no mutant seedlings were observed when *Mu*-inactive *hcf106-mum1* heterozygotes were self-pollinated (Table 1). Thus, *hcf106-mum2* and *hcf106-mum3* confer a novel *Mu*-independent phenotype, as predicted by the insertion and deletion identified in each allele by site-

Table 1. *hcf106-mum2* and *hcf106-mum3* Confer a Novel, *Mu*-Independent Phenotype

Parental Genotypes	Number of Progeny	*hcf* Mutant Seedlings	*Mutator* Activity
hcf106-mum1/+ selfed	273	62	Yes
hcf106-mum1/+ selfed	293	0	No
hcf106-mum2/+ selfed	158	32	No
hcf106-mum3/+ selfed	293	67	No

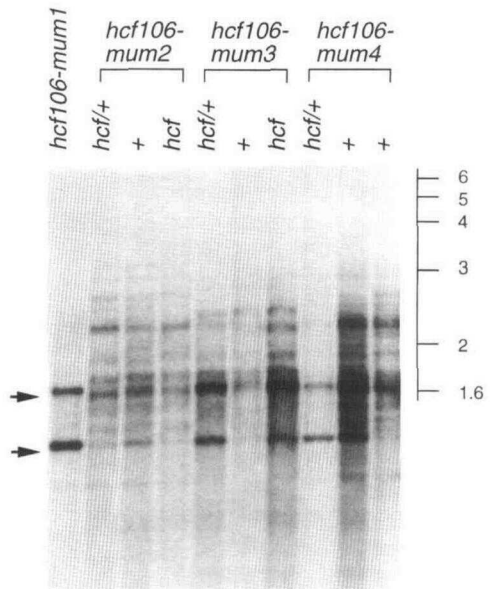

Figure 5. Methylation of *Mu1* Elements in Progenitor and Derivative Families.

DNA samples were isolated from homozygous *hcf106-mum1* seedlings from plants heterozygous for each derivative allele (*hcf*/+) and from their pooled normal (+) and mutant (*hcf*) progeny following self-pollination. No *hcf* progeny were recovered from *hcf106-mum4*. Samples were digested with HinfI and analyzed by gel blot hybridization using the *Mu1* element as a probe. Intensely hybridizing fragments at 1.4 and 1.7 kb (indicated by arrows) correspond to unmethylated *Mu1* and *Mu2* elements, respectively. Length markers at right are given in kilobases.

selected transposon mutagenesis. No pale green lethal seedlings appeared in *hcf106-mum4* families, and DNA gel blot analysis revealed that this allele confers a subtly pale green viable phenotype when homozygous in *Mu*-inactive backgrounds (data not shown). The effect of *Mutator* activity on the *hcf106-mum4* allele is under investigation.

DISCUSSION

The derivative alleles of *hcf106* described here allow the *hcf106* null phenotype to be defined morphologically and biochemically. This is important because leaky expression from the progenitor *hcf106-mum1* allele might be responsible for the variable levels of some thylakoid proteins found in mutant seedlings (Barkan et al., 1986). We are now in a position to determine thylakoid structure and protein composition in chloroplast membranes from plants devoid of Hcf106 protein: the *hcf106-mum3* allele in particular has completely lost the first exon of the gene that encodes the first two methionine codons. The new derivatives will also be useful in genetic experiments in which maintaining *Mutator* activity is impractical. This is because the phenotype conferred by the *hcf106-mum2* and *hcf106-mum3* derivatives no longer depends on *Mutator* activity, unlike the phenotype conferred by the progenitor allele *hcf106-mum1* (Martienssen et al., 1990). When *Mutator* activity is lost, somatically or germinally, *hcf106-mum1* expression is restored at the locus leading to accumulation of functional transcripts and suppression of the mutant phenotype (Barkan and Martienssen, 1991). Attempts to make double mutants and hypoploids have been greatly hampered by high-frequency loss of *Mutator* activity when the progenitor allele is introduced into non-*Mutator* lines (Martienssen and Baron, 1994). In contrast, *hcf106-mum2* and *hcf106-mum3* are mutant in the absence of *Mutator* activity, allowing multiple rounds of backcrosses into non-*Mutator* lines without loss of the mutant phenotype.

Site-selected transposon mutagenesis is a powerful method for identifying insertions and deletions in a target gene in populations of plants without phenotypic selection. In maize, Robertson's *Mutator* transposons are ideally suited for this approach because of their high copy number and conserved termini that allow a wide range of insertion alleles to be recovered using a single set of nested primers. Site-selected mutagenesis is particularly useful in the case of lethal mutations, such as *hcf106*, for which phenotypic screens are only possible in F_2 families. Another important application of this technique would be to isolate derivatives of dominant transposon-induced mutations. For example, at the *Knotted* locus in maize, the loss-of-function phenotype is not known, but there are several transposon-induced dominant alleles (Hake, 1992). By using PCR to select for derivative alleles as heterozygotes, deletions could be isolated from these dominant alleles without any prior knowledge of the mutant phenotype.

Among only 700 *Mu*-active gametes screened, two new insertions were identified at the *hcf106* locus. One was found as a second insertion (*hcf106-mum2*) in the progenitor *hcf106-mum1* allele, and the other was found as a new insertion (*hcf106-mum4*) in the wild-type allele. Thus, new insertions in a known sequence can be recovered from a small population of plants in a simple one-step screen, even when the target sequence for new insertions is extremely small (~360 bp between the TaqI site and the *Hcf106* primer). The third allele, *hcf106-mum3*, was identified as a deletion derivative of the progenitor *hcf106-mum1* allele from among the 350 *Mu*-active *hcf106-mum1* gametes recovered (the other 350 *Mu*-active gametes were +). Thus, insertions and deletions were recovered at approximately equal frequencies (1 in 350). The small target size probably accounts for the fact that all three alleles had the same breakpoint in the gene. This breakpoint must represent a favored site for *Mu* insertion. Similarly, a preferred site was observed in two of three stable derivative alleles selected phenotypically at the *Bronze2* locus in maize (Levy and Walbot, 1991), and similar numbers of deletion derivatives were recovered. Although the sample size is small, this suggests that other loci in maize are likely to exhibit a similar spectrum of insertions and deletions when selected in this way.

Two of the new *hcf106* alleles behave genetically as loss-of-function derivatives (see previous discussion). Thus, the procedure we describe can be used to identify gene knockouts in maize, whereby insertions and deletions in a given gene

can be obtained knowing only its DNA sequence. In principle, a handful of insertion alleles of a given gene can be obtained by screening a few thousand seedlings in grids using primers from the transposon and primers from the gene, as described here. Fifteen hundred seedlings (up to 3000 *Mu* gametes depending on the cross) can be screened using only 96 DNA preparations and a single round of nested PCR.

To be able to use small numbers of seedlings is very useful when the genetic background is important for the screen. However, insertions are most efficiently generated using a large, systematic collection of transposon families, as has been done in *C. elegans* (Zwaal et al., 1993). In this system, frozen animals can be maintained for later recovery after identifying new insertion alleles in pooled DNA samples. A collection of maize seed has been developed for this purpose by Pioneer Hi-Bred (Johnston, IA) using *Mu* transposable elements, and *Mu* insertions into many target genes can be routinely obtained from this collection by site-selected PCR screening of the pooled parental DNA (S. Briggs, personal communication).

Unfortunately, as in studies with *C. elegans* and Drosophila, many insertions will not result in mutant phenotypes because they disrupt introns or noncoding flanking sequences or because they are removed from the primary transcripts by splicing within the element (Kaiser and Goodwin, 1990; Ortiz and Strommer, 1990; Rushforth et al., 1993). To overcome these limitations, deletion derivatives can be identified from insertion alleles by the method described here once insertions are recovered from the primary screen. These alleles can be used to define unequivocally the effects of deleting gene activity in the plant.

Transposon insertion has numerous advantages over transgenic approaches to gene knockouts, such as gene replacement or antisense inhibition. Gene replacement strategies are still highly inefficient and can only target one gene at a time. Furthermore, derivatives cannot be selected from the initial knockouts in most gene replacement strategies. Antisense transgenic plants have been widely used to reduce gene expression; however, antisense knockouts are dominant so that lethals are very difficult or impossible to observe. In contrast, site-selected transposon insertions can target an unlimited number of genes simply by using different primer combinations. Derivative alleles are easy to identify in the next generation, and last but not least, transposon knockouts are typically recessive so that lethal mutations can be readily recovered as viable heterozygotes. Thus, this technique represents a potentially powerful method for reverse genetics (gene knockouts) in plants, just as similar methods have been powerful in animals (Zwaal et al., 1993; Greenstein et al., 1994).

METHODS

The origins and maintenance of the *high chlorophyll fluorescence106* (*hcf106*) and *Lesion-mimic* (*Les28*) mutations were as described by Barkan et al. (1986) and Martienssen and Baron (1994). *Mutator* (*Mu*)-active heterozygous *hcf106/+* parents were identified by DNA gel blot analysis or by testcrosses and progeny screens. DNA preparation was by the urea–phenol extraction method of Cone (1989), and DNA gel blotting was as described by Martienssen and Baron (1994). The hybridization probes used were from the first two exons of the *Hcf106* gene (p106K; Barkan and Martienssen, 1991) and the internal BamHI-EcoRI fragment from the *MuDR* element (a gift from V. Chandler, University of Oregon, Eugene, OR). Pooled DNA samples were digested overnight with TaqI, and polymerase chain reactions (PCRs) were performed using a primer from the end of *Mu* (5′-TCGAATCCGCTTCTCTCTTCGTCC-3′) and a primer from the second exon of the *Hcf106* gene (5′-CCAGTGCTTCGGGAGCTCCAACTC-3′). Cycling conditions are as follows: 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C, for 35 cycles in a Perkin-Elmer Cetus Thermocycler. Ten microliters from each 50-μL reaction were loaded on each lane of a mini-agarose gel containing 0.1 μg/mL ethidium bromide, and products were visualized with UV light after electrophoresis.

These conditions were established by "spiking" test reactions with dilutions of undigested DNA from the progenitor allele and determining conditions for which only spiked reactions gave a 400-bp product. Background bands were observed with single primers and primer combinations when annealing temperatures were too low or when too many cycles were used. The presence of the same band in two different cross-referenced pools was used to confirm the authenticity of amplified products. Other primers from the end of *Mu1* (5′-CAGAATTCCATAATGGCAATTATCTC-3′) and *MuDR* (5′-TAAGGAGCTCCAAGCGCTGTCACC-3′) were also used to amplify specific alleles. PCR products were digested with EcoRI and SstI and ligated into M13mp18 and M13mp19 for sequence analysis. DNA from the progenitor allele was amplified using primers from upstream (5′-GAATCAGACGGCTCAGGTTGTACC-3′) and downstream (5′-CACATCAGAGATGGGGACGAATGG-3′) of the *Mu1* insertion site in combination with *Mu1* primers under the same conditions.

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