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Mu Element-Generated Gene Conversions in Maize Attenuate the Dominant Knotted Phenotype

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ABSTRACT

The *knotted1* gene was first defined by dominant mutations that affect leaf morphology. The original allele, *Kn1-O,* results from a 17-kb tandem duplication. *Mutator* (*Mu*) insertions near the junction of the two repeats suppress the leaf phenotype to different degrees depending on the position of the insertion. The *Mu* insertions also increase the frequency of recombination at *Kn1-O* to create derivative alleles in which the *Mu* element and one copy of the repeat are lost. These derivatives are normal in appearance. Here we describe two derivatives that retained the tandem duplication but gained insertions of 1.7 and 3 kb in length in place of the *Mu* element. In each case, the inserted DNA is a sequence that normally flanks the distal repeat unit. Thus, each derivative consists of a tandem duplication in which the repeat unit has been extended at its distal end by the length of the new insertion. The 1.7-kb insertion dampens the phenotype, as did the original *Mu* insertion, whereas the 3-kb insertion completely suppresses the knotted phenotype. We propose that gene conversion, stimulated by the double-strand break of the *Mu* excision, gave rise to these derivatives.

THE normal maize leaf consists of three parts, a distal blade that is broad and flat, a narrow sheath that wraps around the stem, and a ligule/auricle region where the blade and sheath join (SHARMAN 1942). In the dominant *Knotted1* (*Kn1*) mutants, cells along the veins of the blade have sheath, ligule, and/or auricle characteristics (FREELING and HAKE 1985; SINHA and HAKE 1994). Hollow protuberances, or knots, form along the lateral veins in the mutant leaf blade. The knots can be found evenly dispersed across the veins in some alleles or clustered near the midvein in others. Sporadic patches of ectopic ligule can be found in pairs bordering the sheath-like cells. Ectopic expression of the *kn1* homeobox gene (VOLLBRECHT *et al.* 1991) in cells along the lateral veins of leaf blades has been correlated with the dominant mutant phenotype (SMITH *et al.* 1992; JACKSON *et al.* 1994).

The first *Kn1* mutation identified, *Kn1-O,* is highly penetrant and expressive (BRYAN and SASS 1941; GELINAS *et al.* 1969; FREELING and HAKE 1985). In addition to the presence of knots and ectopic ligule, *Kn1-O* plants lack a normal ligule. *Kn1-O* results from a tandem duplication of 17 kb (VEIT *et al.* 1990) that includes the entire coding region, 400 bp of 5′ sequences and 10 kb of sequences 3′ of the coding region (LOWE *et al.* 1992). *Kn1-O* differs from the other dominant *Kn1* mutations that result from the insertion of transposable elements into introns (VOLLBRECHT *et al.* 1989; HAKE 1992; GREEN *et al.* 1994). A number of other dominant mutations have recently been described that also affect the maize leaf such as *Rough sheath1, Liguleless3, Liguleless4* (FREELING 1992; BECRAFT and FREELING 1994; FOWLER and FREELING 1996).

The insertion of *Mutator* (*Mu*) elements into *Kn1-O* modulates the dominant leaf phenotype. *Mu* elements, first identified genetically by ROBERTSON (1978), are characterized by a high forward mutation rate. The family of *Mu* elements consists of approximately eight nonautonomous members and an autonomous element, *MuDR* (reviewed by CHANDLER and HARDEMAN 1992). All *Mu* elements contain conserved 200-bp inverted repeats. *Mu* activity is conditioned by the presence of *MuDR* (CHOMET *et al.* 1991; HERSHBERGER *et al.* 1991; QIN *et al.* 1991); in its absence, nonautonomous elements are incapable of excision and are methylated (CHANDLER and WALBOT 1986; BENNETZEN 1987). The effect of *Mu* elements on *Kn1-O* depends on their position in the gene and *Mu* activity (VEIT *et al.* 1990; LOWE *et al.* 1992). *Kn1-O174* (*174*) contains a *Mu1* insertion 120 bp 5′ of the start of transcription in the proximal repeat (Figure 1). Plants carrying the *174* allele display a mild knotted phenotype in which the first leaf always shows ligule displacement and other leaves have occasional knots or ligule displacement. *Kn1-O169* (*169*) results from a *Mu8* insertion in the same position as the *Mu1* insertion in *174.* These plants show a milder phenotype with at least one knot or some ligule displacement on each plant. Both of these insertions are *Mu*-suppressible; in the presence of *Mu* activity, the plants display the mild knotted phenotype, and in the

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absence of *Mu* activity, the plants are as knotted as the original *Kn1-O* mutation. In contrast, *Kn1-O167* (*167*), which results from a *Mu6/7* insertion in the distal repeat of *Kn1-O*, displays only a rare knot, with or without *Mu* activity (LOWE *et al.* 1992).

The presence of active *Mu* element insertions increases the instability of the *Kn1-O* tandem duplication 100- to 2000-fold (LOWE *et al.* 1992). Derivatives that have lost one copy of the duplication are normal in appearance. Since no flanking markers are exchanged, the recombination event leading to loss of one of the repeats is either sister chromatid exchange, intrachromatid exchange or gene conversion. The last model is attractive due to the fact that gene conversion has been demonstrated to function in the gap repair of *P*-element excision in Drosophila (ENGELS *et al.* 1990) and may also occur during *Mu* element excision in maize (LISCH *et al.* 1995).

In this article we describe two derivatives of *174* that maintain the tandem duplication but have lost the *Mu* element. In place of the *Mu* element is the insertion of 1.7 or 3 kb of DNA. We determined that the insertions are sequences normally found immediately flanking the distal repeat. A model involving gene conversion is used to explain the production of these derivatives. In addition, the derivatives highlight the sequences responsible for the dominant mutant phenotype.

MATERIALS AND METHODS

Generation of derivatives: An individual of the following genotype *174 adh1-F6/kn1+ adh1-F* was self-pollinated. The *adh1* genotypes of kernels from a single ear (No. 385-3) were determined (FREELING and SCHWARTZ 1973) and kernels carrying the linked *adh1-F6* allele (1 map unit distance) either in homozygous or heterozygous condition were planted in 1990 and again in 1994. *174* conditions a mild knotted phenotype that is visible on the first or last leaves. Of the 10 plants in the first planting (family 1168), four appeared normal, one was very knotted, and the remaining five had a mild knotted phenotype. One of the normal homozygous individuals was outcrossed to an inbred line that carried the *adh1-F* allele. All progeny were normal in appearance and *adh1-F/adh1-F6* (families 1351 and 1806). From DNA gel blot analysis of these progeny, we determined that two different events had occurred to produce the normal-appearing *adh1-F6* parent. Half the progeny were segregating DNA polymorphisms consistent with loss of one repeat (LOWE *et al.* 1992) and half were segregating for a new polymorphism as will be discussed. The normal-appearing derivative associated with a new polymorphism was named *174a*. In the second planting (family 2688), five of the 18 heterozygous and homozygous plants appeared normal, two had a strong knotted phenotype and the remaining nine were mildly knotted. DNA from a number of the individuals was analyzed by DNA gel blot analysis using *kn1* probes. One mildly knotted *adh1-F6/adh1-F6* individual had a new polymorphism (unlike that of *174a*) in addition to the polymorphism normally associated with *174*. This individual was outcrossed to the Berkeley Slow line that carries an *adh1-S* allele and progeny segregating with a new polymorphism were identified by DNA blots. This derivative was named *174c*. An *adh1-F6/adh1-S* individual was self-pollinated and two different homozygotes outcrossed. No. 3474 was crossed twice to inbred Berkeley slow, and No. 3479 was crossed to an active *Mu* line, and then crossed to Berkeley slow. We surmise that the recombination events leading to *174a* and *174c* occurred in gametes of the *174/kn1+* parent (385-3).

Molecular biology methods: DNA and RNA were extracted and blotted as previously described (GREENE *et al.* 1994). Tissue for RNA extractions was obtained from 2-week-old seedlings by first removing the oldest three leaves. The 2 cm of tissue above the meristem (but clearly not including the meristem) were considered the leaf fraction (L) and the tissue below this fraction, which includes the meristem, stem and youngest leaf primordia were considered the meristem-enriched fraction (M). Tissue from four to six individuals was collected for each sample. *Mu* activity was determined by digestion with *Hin*fI and hybridization with *Mu1* and *Mu8* probes (GREENE *et al.* 1994). The probes were made radioactive using the Multiprime DNA labeling system of Amersham. The 9.4-kb *Eco*RI fragment of *174a* was cloned into EMBL4 (Stratagene). Sequencing reactions were performed using the Dye Terminator Sequencing Kit of Perkin Elmer, run on an ABI373 automatic sequencing machine and analyzed using the Sequencher program.

PCR: PCR was used with the primers SH1 and JM1 to amplify genomic fragments in *174c*, *174*, and *174a*. Conditions were as follows: 1 min at 94°, 1 min at 55°, and 2 min at 72° for 30 cycles using Promega reaction buffer, *Taq* polymerase and 1 μg of genomic DNA. PCR products were electrophoresed on an agarose gel and the gel stained with ethidium bromide. PCR was also used to amplify genomic fragments in the flanking region of different *kn1* alleles. Primers J6B and RKn1L were used to amplify DNA from the inbred B73 and individuals homozygous for the following genotypes: *Kn1-O*, *174*, *kn1+* and *174c*. Conditions were 1 min at 94°, 1 min at 59°, 3 min at 72° for 35 cycles. One milliliter of the reaction was used to reamplify a shorter fragment with primers J13B and SH4 for 1 min at 94°, 1 min at 59°, 2 min at 72° for 35 cycles. The fragments were purified from an agarose gel for sequencing.

The sequences of the primers are as follows: SH1: 5′-ATG TCCAGGATGTGAGCG-3′; JM1: 5′-AGCACTAAACCGATA GGG-3′; J6B: 5′-GCTCTACAGCCAGACAACCA-3′; J13B: 5′-CACACCAGCTCTGATACGTT-3′; SH4: 5′-CTGGAGGCCAA TGCAGCTGCCT-3′; RKNL1: 5′-GGGAAGGAGAGGGAAAG GAGAAGG-3′.

Phenotypic analysis: Ten or more plants from *174a*, *174c*, *174*, *Kn1-O* and the inbred Berkeley Slow were grown in a greenhouse under similar conditions and examined at every leaf for knots and ligule disturbances. Fewer *Kn1-O* plants were examined since they have been examined in large numbers over the years and are known to have a consistently severe phenotype (VEIT *et al.* 1990; LOWE *et al.* 1992).

Probes: The ubiquitin probe, a 700-bp *Pst*I/*Sac*I fragment from pskUBI, was from P. QUAIL (University of California, Berkeley) (CHRISTENSEN *et al.* 1992). The *Mu8* probe is a 500-bp *Pst*I to *Pvu*II internal fragment (FLEENOR *et al.* 1990), and the *Mu1* probe is from pAB5 (CHANDLER *et al.* 1986). The entire *kn1* cDNA, pOC5 (VOLLBRECHT *et al.* 1991) was used as a probe for RNA blot hybridization. Probe A is from BV204, a 1-kb *Sac*I-*Eco*RV fragment that spans the junction of the two repeats in *Kn1-O* (VEIT *et al.* 1990). Probe B [also known as B3 (VEIT *et al.* 1990)] is a 430-bp *Bam*HI-*Sal*I fragment located in the distal flanking sequences.

RESULTS

Sequences outside the tandem duplication are inserted near the site of *Mu* element excision in deriva-

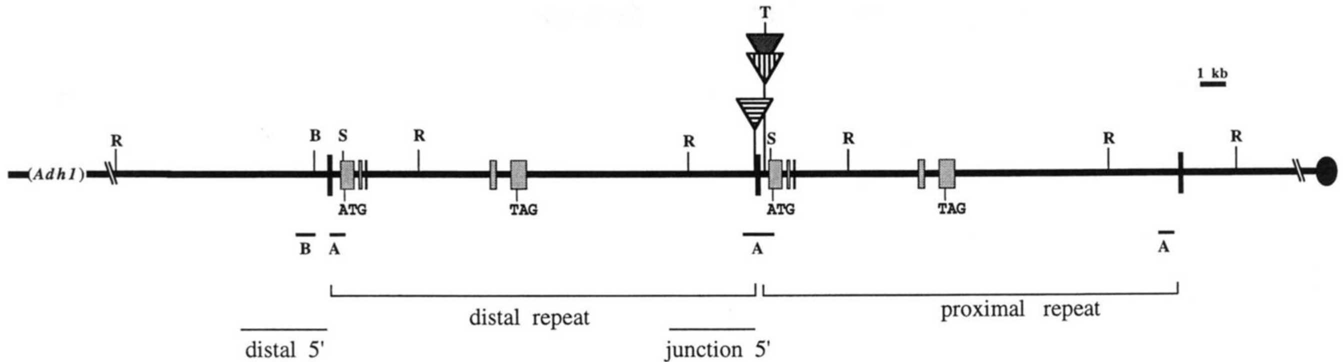

FIGURE 1.—Map of *Kn1-O* and *Mu* insertion alleles. *Kn1-O* is a tandem duplication of 17 kb. We have oriented the repeats by the relative position to the centromere shown as a black circle to the right (LOWE *et al.* 1992). Both the distal and proximal repeats are designated. A *Mu1* and a *Mu8* insertion, indicated by triangles, are in the same position in the proximal repeat of *174* and *169;* the *Mu6/7* insertion in *167* is in the distal repeat (VEIT *et al.* 1990; LOWE *et al.* 1992). Exons are indicated as shaded boxes. All *Eco*RI restriction sites (R) are shown, but only one *Bam*HI (B) and *Sac*I (S) site are shown to orient Figure 4. The *Bst*EII (T) site in the *Mu1* element of *174* is shown. Probes used in DNA gel blot analysis are shown as A and B.

tives of *174*: *Kn1-O174* (*174*) has a mild knotted phenotype that is easy to score in the seedling or adult leaves. We previously documented two different events that alter this phenotype; methylated *Mu1* elements result in a more severe knotted phenotype, similar to *Kn1-O,* and loss of one of the repeats from recombination produces normal-appearing plants (LOWE *et al.* 1992). We recovered two additional derivatives, *174a* and *174c,* that alter the phenotype of *174.* These derivatives arose from a self-pollination of a *174 adh1-F6/kn1+ adh1-F* heterozygote (MATERIALS AND METHODS). *174a* was normal in phenotype whereas *174c* was knotted but milder than *174* (see below).

DNA samples from progeny of *174a* and *174c* were analyzed to determine if molecular changes at *174* could account for the diminution of the knotted phenotype. *Kn1-O* consists of a tandem repeat of 17 kb. The *Mu1* element of *174* is located in the proximal repeat, 302 bp from the junction of the repeat and 120 bp 5′ of the start of transcription (Figure 1). *Kn1-O* DNA, digested with *Eco*RI and hybridized to probe A, produces three fragments due to the tandem duplication, a 13-kb band that extends outside the distal repeat, a 5.3-kb band that extends outside the proximal repeat, and a 6.3-kb band that includes the junction (Figure 2A). *174, 174a* and *174c* all contain the 13- and 5.3-kb bands, however, different sized fragments have replaced the 6.3-kb fragment that spans the junction. *174* contains a 7.6-kb band, which accounts for the 1.3-kb *Mu1* element in the 6.3-kb junction fragment; *174c* has an 8.0-kb band; and *174a* has a 9.4-kb band (Figure 2A). *kn1+* individuals contain the 13-kb band, similar to *Kn1-O* and its derivatives, but are polymorphic in the proximal region and contain a 5.0-kb band instead of the 5.3-kb band. Individuals that are heterozygous with *kn1+* also have the 5.0-kb band (Figure 2A, lanes 2 and 4).

DNA samples were digested with enzymes that cleave within *Mu1* to determine if the *Mu1* element was present in *174a* or *174c.* Following digestion with *Eco*RI and *Bst*EII, the 7.6-kb band of *174* was replaced by two smaller bands, whereas the 8-kb band of *174c* and the 9.4-kb band of *174a* were unaltered (Figure 2B). These results suggest that either the *Mu1* element is absent or that the *Mu1* element has been altered such that the *Bst*EII site is no longer present. PCR experiments to amplify a fragment in *174a* or *174c* using a *Mu* end primer consistently failed whereas the control experiment using *174* DNA successfully amplified products (data not shown).

To determine the cause of the restriction fragment size changes in *174a,* we cloned the 9.4-kb *Eco*RI fragment and named it p174a. Sequence comparisons of p174a were made with *Kn1-O,* the progenitor of *174.* Sequence analysis showed colinearity with *Kn1-O* across the tandem duplication junction in a 5′ to 3′ direction until 30 bp 3′ of the position where the *Mu1* element had resided (Figure 3). At this point, the sequence diverged. Sequence comparison in a 3′ to 5′ direction also showed colinearity with *Kn1-O;* however, at the position of the tandem duplication junction, the p174a sequence was colinear with sequence outside the tandem duplication, flanking the distal repeat (Figure 3). A diagram of the rearrangement is presented in Figure 4.

We hypothesized that *174c* might result from a rearrangement similar to the one that produced *174a* since both *174a* and *174c* alter the knotted phenotype and arose from the same parents. Primers, such as JM1 and SH1, were chosen that would not amplify DNA unless such a rearrangement occurred (Figure 4). These primers amplified products in *174a* and *174c* DNA, but not *174* (data not shown). One of the products from *174c* was cloned and sequenced. Using the SH1 primer in a 5′ to 3′ direction, colinearity with *Kn1-O* was found up to the position where the *Mu1* element had resided (Figure 3). At this point, colinearity was found with sequences outside the distal repeat (Figures 3 and 4).

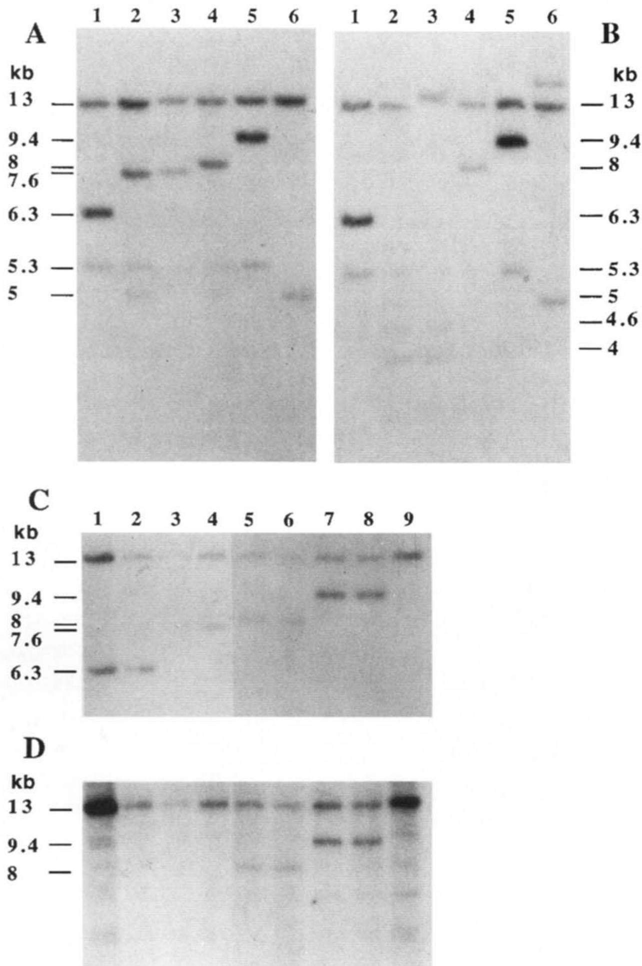

FIGURE 2.—DNA blots of *Kn1-O174* derivatives. (A) DNA samples from (1) *Kn1-O/Kn1-O,* (2) *174/kn1+,* (3) *174/kn1+,* (4) *174c/kn1+,* (5) *174a/174a,* (6) *kn1+/kn1+* were digested with *Eco*RI and the DNA blot was hybridized to the A probe. (B) *Eco*RI and *Bst*EII digested DNA hybridized with the A probe. The individuals are the same as in A. (C) *Eco*RI digested DNA from (1 and 2) *Kn1-O/Kn1-O,* (3 and 4) *174/kn1+,* (5 and 6) *174c/kn1+,* (7 and 8) *174a/174a,* (9) *kn1+/kn1+*. The DNA blot was hybridized with the A probe. (D) The same blot in C was hybridized with the B probe (see Figure 1).

To verify the cloning and PCR results with DNA gel blots, we reasoned that the novel 9.4- and 8-kb *Eco*RI bands of *174a* and *174c,* respectively, would hybridize not only to the A probe, but should also hybridize to the B probe outside the tandem repeat (Figures 1 and 4). Figure 2D shows that only the 13-kb band of *Kn1-O, 174,* and *kn1+* hybridizes to B (Figure 2D, lanes 1–4, 9), whereas the 8-kb band of *174c* and the 9.4-kb band of *174a* also hybridize to the B probe (Figure 2D, lanes 5–8). Faint hybridizing bands result from repetitive sequence within the B probe. The blot was stripped and rehybridized to probe A for comparison (Figure 2C). These results confirm the insertion of sequences that flank the tandem repeat into the central junction region.

The recombination events that produced *174c* and *174a* could have occurred from the sister chromatid or the homologous chromosome. Because the parent of *174a* and *174c* was heterozygous (*174 adh1-F6/kn1+ adh1-F*), we compared p174a with sequences 5′ of *kn1* in *174* and the *kn1+ adh1-F* allele, to distinguish between these possibilities. We used the primer combination J6B and RKNL1 to amplify a 3-kb fragment (Figure 4) that was reamplified using nested primers and the PCR reactions were sequenced. No genotype-specific differences were detected (data not shown), and thus we were not able to identify the chromosome from which *174a* was derived.

Phenotype of derivatives: Leaf morphology of 10 or more *174, 174a, 174c,* and *Kn1-O* plants was observed throughout their development. Comparisons were made to the inbred Berkeley slow, since *174, 174a,* and *174c* had been crossed into this line at least once. We found that the first and second leaves were particularly indicative of the overall phenotype (Figure 5). DNA was prepared from each *174* and *174c* individual to monitor any additional changes at the *kn1* locus.

All 16 *174a* plants were identical to wild type at each leaf. In field experiments, we have grown ~500 *174a* individuals and never found any indication of the knotted phenotype (data not shown).

Two families carrying *174c* were examined. In one family, 3479, the knotted phenotype was mostly absent and when it did appear, it was very mild. Three plants had a slightly ragged ligule on the first or second leaf (Figure 5). All the plants were normal from leaf 3 to leaf 11. The knotted phenotype on the adult leaves was confined to a single bump on the sheath immediately below the ligule. These sheath knots differ from blade knots in that a number of veins participate in a sheath knot, whereas the knots in the blade are focused over a single vein. The ligule was never displaced and ectopic ligule and knots were rare.

The second *174c* family examined (3474) showed a stronger knotted phenotype throughout leaf development, although most of the plants were normal from leaf 3 to leaf 6. By leaf 11, all plants had one to two knots or a patch of ectopic ligule along the veins near the midrib. The ligule was not displaced. All adult leaves on the plants in this family had sheath knots. Previous generations of *174c* produced phenotypes more similar to 3479 than 3474.

Two of the 10 *174* individuals were normal throughout development. DNA gel blot analysis with probe A on an *Eco*RI digest showed only the 13- and 5.3-kb band (data not shown), suggesting that one copy of the repeat and the *Mu* element were lost, similar to recombination events documented before (LOWE *et al.* 1992). Another individual was very knotted throughout development; each leaf showed displaced or missing ligule and the knots extended beyond the midrib to the adjacent three to six lateral veins. DNA gel blot analysis using *Mu1* as a hybridization probe to *Hin*fI digested

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                                  ▼                                              Pvu II
Kn1-0     AAGAAGCCCC CACAAACACC ACTCCACACT AGTGCACTGT CTCTCTCCAA AGGCAGCTGC ATTGGCCTCC AGCCTTTTCC CTACTGTGCC

174a      AAGAAGCCCC CACAAACACC ACTCCACACT AGTGCACTGT CTCTCTCCAA AGGCAGCTCG AGTCAAGTCT TTTCCATTTG TTTTTTCTCT
                                                                  |||  |||
distal    GCCCGGACAC TGTACCAGAC AGTCCGGTGC ACCCAGACTG AGCAGAGTCT TGGCTGCTCG AGCCAAGTCT TTTCCATTTG TTTTTTCTCT
5' region

174c      AAGAAGCCCC CACAAACACC AAGTGGTGGC CATCCCTAAA TATGGTGTTT TGTCGTATTT GTTTTTTAAC TCGTGCACAC TTTGTTTTCG
                             || |
distal    GGTCAAAGTT CTAACTATCC AAGTGGTGGC CATCCCTAAA TATGGTGTTT TGTCGTATTT GTTTTTTAAC TCGTGCACAC TTTGTTTTCG
5' region
```

FIGURE 3.—Sequence analysis at border of insertions. The sequence of p174a and the PCR product obtained from *174c* using JM1 and SH1 were compared to the junction region of *Kn1-O* and the distal 5′ region. Sequence repeats that may have facilitated the resolution of the putative gene conversion event are indicated with vertical lines. ▼ represents the position of the *Mu* excision site. A *Pvu*II site in *Kn1-O* (underlined sequence) is altered in p174a; instead an *Xho*I site appears, which is also found in the distal flanking sequence of wild type (underlined).

DNA of this individual revealed that the *Mu1* elements were methylated (data not shown). Methylated *Mu* elements are correlated with inactive *Mu* elements (CHANDLER and WALBOT 1986; BENNETZEN 1987) and our previous work has shown that *174* individuals with methylated *Mu* elements resemble *Kn1-O* plants (LOWE *et al.* 1992). The remaining seven *174* plants showed displaced ligules on the first leaf. Half of the plants had a period of normal leaf development until leaf 10, at which point, they all showed slight aspects of the knotted phenotype. Similar to the *174c* family, #3474, the disturbances were restricted to veins near the midrib, such as small patches of ectopic ligule or thickened veins.

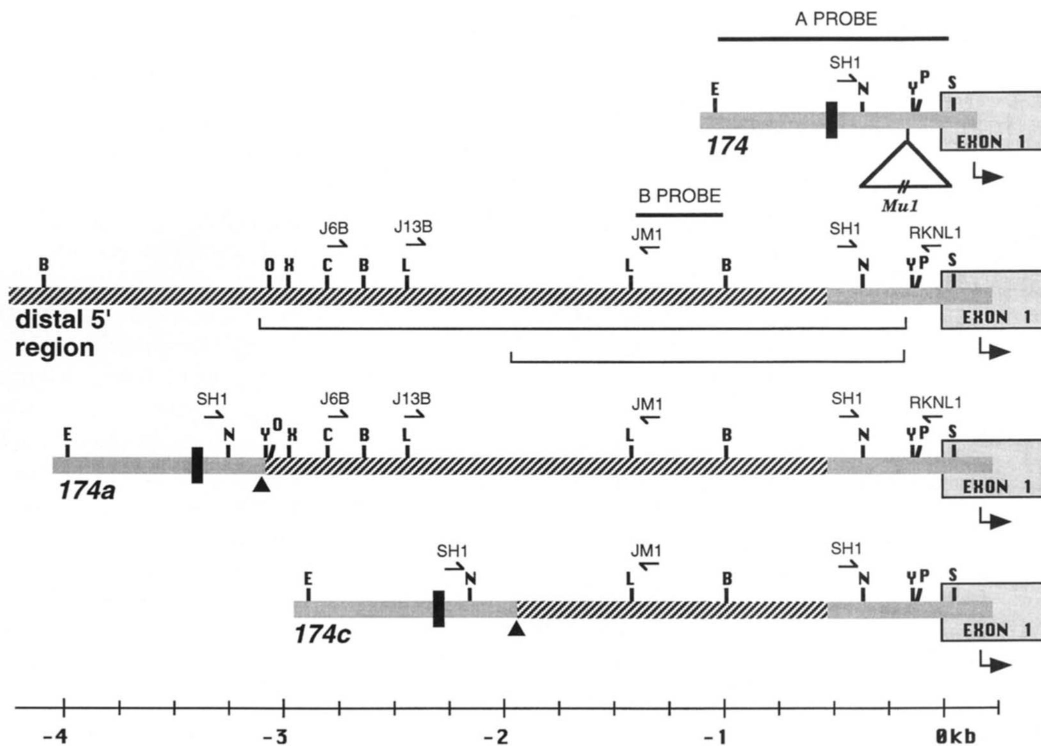

FIGURE 4.—Map of *174a* and *174c* rearrangements. The junction of the tandem repeats in *174* is compared with the junction regions of *174a* and *174c* and with the distal 5′ region, the sequences flanking the distal repeat. Sequences 5′ of the distal repeat are equivalent to 5′ sequences in *kn1+*. The position of *Mu1* in *174* is indicated as a large triangle and the position where *Mu1* is excised from *174a* and *174c* is indicated as a small filled triangle. In *174a, Mu1* is removed and 3 kb of sequences extending from the position of *Mu1* into the DNA flanking the distal repeat are inserted 30 bp 3′ of the *Mu1* excision site. In *174c, Mu1* is removed and 1.7 kb of sequences extending into the flanking DNA are inserted precisely at the position of the *Mu1* excision site. Lines underneath the distal region denote the extent of the DNA copied into *174a* and *174c*. The vertical black rectangle denotes the junction between the tandem repeats. Sequences distal to the tandem repeat are shown as hatched lines. Primers used for PCR and sequencing are indicated. Probes A and B used in DNA gel blot analysis are indicated by horizontal lines. A few restriction sites are indicated for orientation: B, *Bam*HI; C, *Bcl*I; E, *Eco*RV; L, *Sal*I; N, *Nhe*I; O, *Xho*I; P, *Pvu*II; S, *Sac*I; X, *Xba*I; Y, *Spe*I.

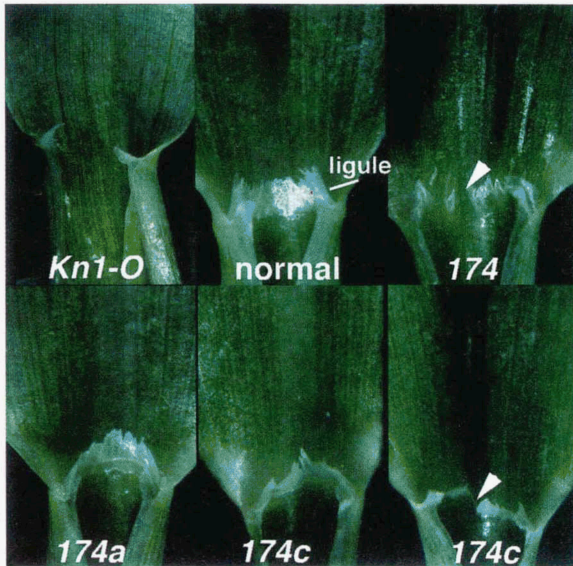

FIGURE 5.—Leaf phenotypes. The ligule region from the second leaf of *Kn1-O,* a normal plant (Berkeley slow), *174, 174a* and *174c* is shown. Note the absence of ligule on *Kn1-O* and gaps in the ligule in *174* and *174c* (arrowhead).

Kn1-O consistently showed ligule alterations and frequently had knots, similar to the methylated *174* individual. No normal ligules were detected on any leaf of any of these *Kn1-O* plants (Figure 5).

RNA analysis: *kn1* is strongly expressed in the meristem and stem of wild-type plants but is restricted from expression in leaves. Previous analysis has shown that the dominant *Kn1* alleles resulting from transposon insertion are correlated with ectopic *kn1* expression in leaf primordia (SMITH *et al.* 1992; GREENE *et al.* 1994; JACKSON *et al.* 1994; E. VOLLBRECHT and S. HAKE, unpublished data). We isolated RNA from *Kn1-O* and derivatives to determine if the appearance of knots and ligule displacement was also correlated with *kn1* expression in the leaf. Figure 6 shows detectable *kn1* expression in *Kn1-O, 174* and *174c* leaf RNA. The level of RNA detected is approximately the same as seen in leaf primordia from other dominant *Kn1* alleles (Figure 6). No *kn1* expression was detected in *174a* or *kn1+* leaf primordia. RNA was also isolated from meristem-enriched tissue where *kn1* is highly expressed. Only the 1.6-kb message is present in each sample. The RNA blot was rehybridized to the maize ubiquitin gene to assess the quantity and quality of the RNA loaded. The amount of *kn1*-hybridizing message in meristem-enriched tissue appears to be roughly the same, whether the sample is from Berkeley slow, which has one copy of the *kn1* coding region, or from *174a,* which has two. Thus, the copy number of the coding region does not appear to determine *kn1* expression levels.

DISCUSSION

The ability of transposons to create additional alleles has been well documented in many plants (SCHWARZ-SOMMER *et al.* 1985). New alleles often result from the footprint that is left upon transposon excision. New alleles also result from rearrangements that occur during transposition (COEN 1989). *Mu* element excisions have resulted in deletions (ROBERTSON and STINARD 1987) and complex rearrangements (KLOECKENER-GRUISSEM and FREELING 1995). We have characterized two new *Kn1* alleles that resulted from *Mu1* excision. The *Mu* element was deleted and additional sequences normally present 17 kb away were inserted near the *Mu* excision site. The rearrangements appear to be mediated by gene conversion and affect the dominant leaf phenotype. We previously showed that a high frequency of recombination occurs at derivatives of *Kn1-O* that contain insertions of active *Mu* elements (LOWE *et al.* 1992). Due to the lack of flanking marker exchange, we hypothesized that either sister chromatid exchange, intrachromatid exchange, or gene conversion occurred. Since the frequency of recombination events was much higher when *Mu* was active, the results suggested that gene conversion was the prevailing mechanism (LOWE *et al.* 1992).

Gap repair model: The Drosophila transposable element, *P,* is thought to excise and transpose duplicatively via gene conversion (ENGELS *et al.* 1990). Gene conversion, or gap repair, occurs by a process in which the broken ends that result from transposon excision are digested by exonuclease activity to create a gap. The 3′ ends then invade a homologous duplex and serve as primers for DNA synthesis, thereby replacing both strands of the gap. The template for repair may be the sister chromatid, the homologous chromosome or homologous sequences at ectopic sites (ENGELS *et al.* 1990; GLOOR *et al.* 1991; NASSIF *et al.* 1994). Not only *P* elements, but also *Mu* elements in maize are thought to employ the mechanism of gap repair. The model explains how duplicative transposition of *Mu* elements could occur upon excision and successful reinsertion somewhere else in the genome (LISCH *et al.* 1995). If the sister chromatid is used as a template for repair, two elements result, one at the original location and one at a new location.

The generation of truncated *Mu* elements is thought to occur when gene conversion is interrupted (LISCH *et al.* 1995; HSIA and SCHNABLE 1996). Interruption of gene conversion occurs when the growing 3′ strands contain direct repeats that hybridize to one another. Resolution of the gap through the repeat leads to the deletion of one of the repeats and all intervening DNA. HSIA and SCHNABLE (1996) analyzed the endpoints of six deletions that occurred in *MuDR,* the autonomous *Mu* element, and compared them with the DNA that was deleted. In five of the six examples, repeats of two to five bases in length could be found at the deletion endpoints. We also found short repeats at the ends of our insertions, *i.e.,* at the position where *174a* and *174c* switch from sequence identity with *Kn1-O* to identity

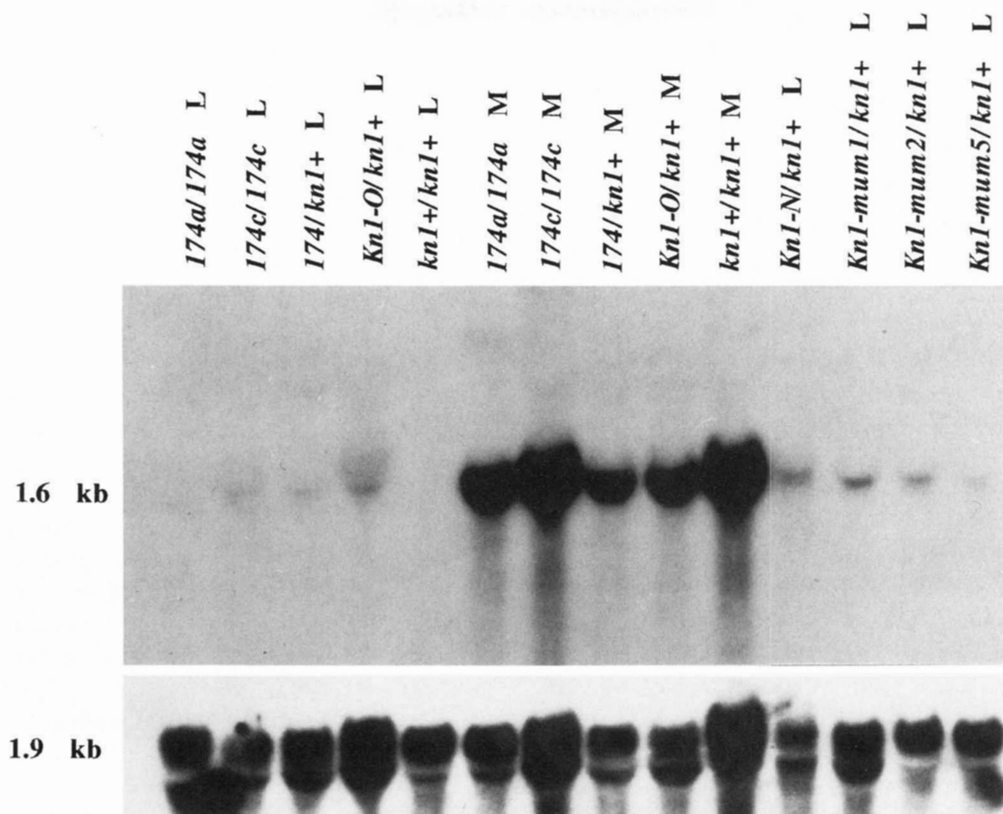

FIGURE 6.—RNA blot. RNA prepared from 2-week-old seedlings was hybridized to the *kn1* cDNA (VOLLBRECHT *et al.* 1991) and to the ubiquitin cDNA for a loading control (CHRISTENSEN *et al.* 1992). L is young leaf tissue and M is meristem-enriched (see MATERIALS AND METHODS). *kn1+* RNA was isolated from the inbred Berkeley slow. RNA from similar leaf samples of other *Kn1* mutants (GREENE *et al.* 1994) was included on the blot for comparison.

with the distal flanking sequence. Figure 3 shows that six of seven bases are identical in *Kn1-O* and the distal region, adjacent to the insertion of *174a,* and three bases are identical in *Kn1-O* and the distal sequences adjacent to the insertion of *174c.* It is likely that these repeats contributed to the resolution of the gene conversion.

The rearrangements of *174a* and *174c* are most easily understood using a modified gene conversion model (NASSIF *et al.* 1994). This model differs from previous models by acknowledging the fact that the two free 3′ ends may conduct independent searches for homology and use different templates for repair. The invading DNA displaces the duplex DNA in a localized region, producing two bubbles that migrate. The two single strands anneal at a position of homology and any unpaired DNA is removed by exonuclease (KURKULOS *et al.* 1994; NASSIF *et al.* 1994). We propose that the excision of *Mu1* caused a double strand break in the proximal repeat of *174* thereby producing two free 3′ ends (Figure 7). Three possible templates could have been used for gap repair: the proximal repeat on the sister chromatid, the distal repeat on the sister chromatid, or the allele on the homologous chromosome, which was *kn1+* and not a duplication. Based on the sequences in *174a* and *174c,* we propose that the proximal 3′ end invaded sequences near the 5′ region of the 17-kb repeat and primed DNA synthesis into the distal flanking sequences. The template could have been the distal repeat of the sister chromatid or the homologous chromosome. The template for the distal 3′ end would depend on the size of the gap. Because neither proximal flanking sequence nor the *Mu* element itself are part of *174a* and *174c,* we propose that the gap was small (JOHNSON-SCHLITZ and ENGELS 1993) and that repair took place from the 5′ region of the distal repeat unit.

Our sequencing efforts were not able to identify polymorphisms that would allow us to determine whether gene conversion occurred using the distal repeat of the sister chromatid or the homologous chromosome as a template. Previously, we observed a decrease in frequency of recombination when the *Kn1-O167* allele was hemizygous (LOWE *et al.* 1992). A similar result was documented for the frequency of *P*-element excision (ENGELS *et al.* 1990). Hemizygosity, however, did not suppress the frequency of precise excision of *Mu1* from *bz1-mum1* in somatic tissue (DOSEFF *et al.* 1991; LISCH *et al.* 1995), suggesting that the sister chromatid was used as a template. As discussed by LISCH and colleagues, the recombination events observed at *Kn1-O* are germinal, whereas the events observed at *bz1-mum1* are somatic. It may be that gap repair occurs from the homologue

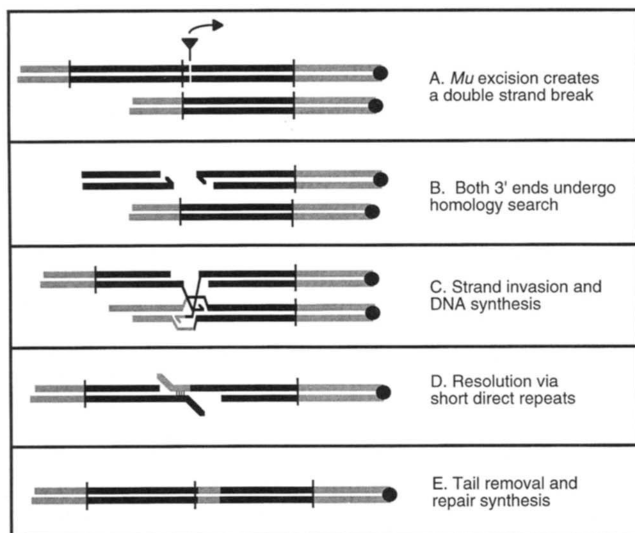

FIGURE 7.—Model for gene conversion at *174* (adapted from NASSIF *et al.* 1994). The tandem duplication of *Kn1-O* is shown as black lines. The region outside the tandem duplication is represented by gray lines. The centromere is represented by a black circle. The *Mu1* element is a black triangle. The vertical lines represent the ends of the 17-kb repeats. Gene conversion may have occurred from the homologous chromosome that carried a single 17-kb region or from the distal repeat of the sister chromatid (not shown). (A) *Mu* excision results in a double strand break. (B) Both free 3′ ends, indicated by arrowheads, undergo homology search and (C) invade homologous DNA. (D) After a period of DNA synthesis, the two single strands anneal at a short region of homology, indicated as vertical thin lines. (E) The 3′ overhangs are cleaved and DNA synthesis fills the gaps.

during meiosis but occurs from the sister chromatid during mitosis (LISCH *et al.* 1995). An alternative explanation is that the 3′ ends produced following *Mu* excision in *167* differ from those in *174* since the *Mu* element in *167* lies within the distal repeat. Over 100 *167* individuals were analyzed by DNA gel blot analysis, yet loss of one repeat was the only recombination event we observed (LOWE *et al.* 1992). Thus, it is possible that the results for *167* would not reflect other gene conversion events at *Kn1-O*. To determine whether the gene conversion events are occurring from the sister chromatid or homologous chromosome, *kn1+* alleles with polymorphic sequences in the 5′ region are required.

Regulation of *kn1* gene expression: *Kn1-O* is a highly expressive mutation; almost every leaf has alterations to the ligule and the appearance of knots. Although it is theoretically possible that the mutant phenotype results from a simple increase in transcript levels due to the tandem duplication, the results shown here support the hypothesis that regulatory sequences are altered by the duplication thus permitting ectopic expression in leaves (VEIT *et al.* 1990; LOWE *et al.* 1992).

The *kn1* coding region contained in the distal repeat of *Kn1-O* is assumed to be in a wild-type context; the restriction site maps of 5′ sequences extending at least 3 kb in a distal direction are identical to those of *kn1+* alleles in inbred lines (data not shown). We refer to the sequences that flank the distal repeat of the tandem duplication as the distal 5′ region (Figure 1). Since expression of *kn1* is not detected in leaves, we hypothesize that regulatory elements in this distal region keep *kn1* expression off in leaves. The coding region in the proximal repeat, on the other hand, has 5′ sequences that diverge 420 bp from the start of transcription. We hypothesize that the divergent 5′ sequences, referred to as the junction 5′ region (Figure 1), permit expression of *kn1* ectopically, thereby causing the knotted phenotype. Thus, in the tandem duplication of *Kn1-O,* one copy of the coding region is in a wild-type context and the other copy of the coding region is in a mutant context.

174a and *174c* have retained the tandem duplication but have the distal 5′ region inserted between the proximal coding region and the junction 5′ region. *174c,* with an insertion of 1.7 kb of the distal 5′ region, has a mild knotted phenotype, and *174a,* with an insertion of 3 kb of the distal 5′ region appears normal. The *174a* and *174c* rearrangements argue strongly against a simple dosage model for the *Kn1-O* mutant phenotype. The rearrangements lend support to the idea that the close proximity of the junction region and the proximal coding region results in the mutant phenotype. One possibility is that silencer sequences in the distal 5′ region block expression of the distal transcription unit, but are ineffective in repressing the proximal coding region either due to size of the intervening DNA (17 kb), or due to specific sequences contained within. A second nonexclusive possibility is that enhancer sequences in the junction 5′ region activate *kn1* transcription during leaf development. Future experiments using the junction region as an enhancer could distinguish between these possibilities.

Leaf phenotypes: Examination of the knotted phenotype has revealed five different attributes that change with severity: (1) timing during development, (2) distance of knots from the midvein, (3) number of knots or ectopic ligule patches, (4) position along the length of the leaf, and (5) alterations of the normal ligule. *Kn1-O* and the methylated *174* individual showed alterations at each leaf (data not shown), whereas *174* and *174c* were affected in seedling leaves and adult leaves, but often had normal juvenile leaves. When disturbances occurred to *174* and *174c,* only the veins at the midrib were affected in contrast to *Kn1-O* in which up to eight veins from the midrib outward were affected. Many of the *174c* leaves had a very subtle defect that consisted of a single patch of ectopic ligule. In *Kn1-O* and the methylated *174* individual, the number of knots or patches of ectopic ligule was much greater than *174* or *174c.* In the mildest *174c* individuals, the only adult knotted phenotype was a knot on the sheath, whereas the severely affected *Kn1-O* individuals had knots extending almost to the tip of the blade. Finally, we no-

ticed that *Kn1-O* always had a displaced ligule, but *174c* derivatives rarely did.

It is interesting to consider the timing of events that led to the knotted phenotype. From examining RNA expression data for *Kn1-N*, SMITH *et al.* (1992) showed that *kn1* was ectopically expressed in cells surrounding the vasculature during plastochron 5, *i.e.*, the fifth leaf from the meristem. The ligule of *Kn1-N* is rarely affected, and there are many mild knots along the length of most veins. The phenotype is clearly not restricted to the midrib (SMITH *et al.* 1992; SINHA and HAKE 1994). Since the ligule is initiated in plastochron 2 (SYLVESTER *et al.* 1990), the perturbations that affect *Kn1-O* must happen earlier than plastochron 5. The midrib is also considered an early marker of leaf development; a median provascular strand is just visible in plastochron 2 (SHARMAN 1942). One might hypothesize that ectopic expression in *Kn1-O* occurs much earlier than the ectopic expression documented for *Kn1-N*. The *174* derivatives are somewhat of an enigma, since they still affect cells along the midrib, but the phenotype is restricted to the midrib. We might expect that ectopic expression of *kn1* occurs early in leaf development in *174* derivatives, but unlike *Kn1-O*, expression does not stay on continually during leaf development. FOWLER *et al.* (1996) have also noted changes in phenotypic defects suggesting differences in the timing of ectopic gene expression during leaf development.

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