A Tandem Duplication Causes the *Kn1-O* Allele of *Knotted*, a Dominant Morphological Mutant of Maize

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ABSTRACT

Molecular and genetic techniques are used to define *Kn1-O*, a mutation which interferes with the normal differentiation of vascular tissue in leaves. Sequences associated with a previously cloned allele, *Kn1-2F11*, were used as hybridization probes in a Southern analysis of *Kn1-O*. By this analysis, *Kn1-O* lacks the *Ds2* transposable element that causes *Kn1-2F11* but instead is associated with a sequence duplication. Sequence and restriction analysis of genomic clones show that the duplication consists of a tandem array of two 17-kb repeats. Analysis of *Kn1-O* derivatives indicates that the duplication itself conditions the mutant phenotype; a severely knotted line, *Kn1-Ox*, has gained a repeat unit to form a triplication, whereas normal derivatives have either lost a repeat unit or sustained insertions that disrupt the tandem duplication. These insertions map near the central junction of the tandem duplication, suggesting that the mutant phenotype results from the novel juxtaposition of sequences. We discuss models that relate the tandem duplication of sequences to altered gene expression.

PHOTOSYNTHETIC leaves are complex organs whose function depends on the orderly differentiation of many specialized cell types. In maize, this process has been analyzed on several levels. Anatomical studies (SHARMAN 1942; ESAU 1943; RUSSELL and EVERT 1985; DALE 1988) have provided a relatively detailed description of the organization of tissues in the mature leaf. Maize leaves exhibit a Kranz type anatomy typical of C4 plants in which veins are surrounded by the bundle sheath (BROWN 1975). Physiological studies indicate this specialized organization is instrumental in the efficient fixation of carbon by C4 metabolism (EDWARDS and HUBER 1981). The developmental basis for this organization is less clear. Although a number of studies (reviewed by NELSON and LANGDALE 1989; also see POETHIG 1984; DENGLER, DENGLER and HATTERSLEY 1985; LANGDALE, ROTHERMEL and NELSON 1988; LANGDALE *et al.* 1989) have examined the lineage relationships between different tissues, thus providing a more refined description of leaf development, relatively little is known of how this process is regulated.

We have approached this question with the analysis of a group of dominant mutations, collectively referred to as *Knotted* (hereafter, *Kn1*), that interfere with the differentiation of specific tissues within the leaf. These mutations define the *Kn1* locus based on their close linkage to *Adh1* on chromosome *1* and their similar mutant phenotypes in which sporadic outpocketings or knots develop on the growing leaf (BRYAN and SASS 1941; GELINAS, POSTLETHWAIT and NELSON 1969; FREELING and HAKE 1985). Although these knots can lend affected plants a disorganized, even grotesque appearance (Figure 1), two aspects of the phenotype suggest that *Kn1* mutants perturb normal leaf development in a specific fashion. First, knots are almost always restricted to the blade portion of the leaf, only rarely forming in the more proximal sheath. Second, knots appear to initiate within a small subset of cells associated with the lateral veins. Specialized cell types associated with the bundle sheath of these veins fail to differentiate properly, resulting in vein clearing and sporadic knot formation (FREELING and HAKE 1985).

Characteristic differences between the mutant phenotypes of *Kn1* alleles suggest further specificity in their action. For example, *Kn1-O* most frequently causes prominent knots on leaves initiated late in development (GELINAS, POSTLETHWAIT and NELSON 1969). By contrast, knots caused by *Kn1-2F11* (CHEN, FREELING and MERKELBACH 1986) are restricted to the first 3 embryonic leaves. The mutant phenotypes are further distinguished by the displacement of ligule, a flap of tissue that normally forms perpendicular to the long axis of the leaf at the junction of the blade and sheath. While this displacement toward the blade tip is common on knotted leaves of *Kn1-O*, it rarely occurs in *Kn1-2F11*.

Toward understanding these characteristic expression patterns, we have begun to compare the organization of *Kn1* alleles where the basis for this specificity must reside. In an earlier study (HAKE, VOLLBRECHT and FREELING 1989), *Kn1-2F11* was cloned and shown to result from the presence of the nonautonomous transposable element, *Ds2* (MCCLINTOCK 1947, 1948). In the present study, we use the *Kn1-2F11* clone to isolate homologous sequences from the *Kn1-O* allele. Using a combination of mutational and molecular

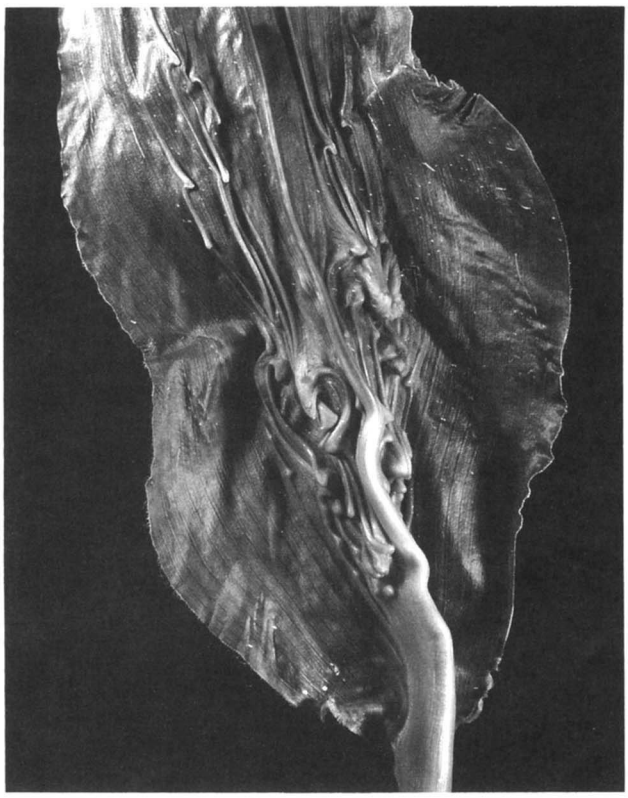

FIGURE 1.—Leaf of a plant homozygous for *Kn1-O*. The plate shows the abaxial (lower) surface of a severely knotted leaf blade.

analysis, we show that a 17-kb direct tandem duplication causes the *Kn1-O* mutation. We discuss models for the origin of this duplication and how it might condition aberrant gene expression.

MATERIALS AND METHODS

Maize stocks and plasmids: *Kn1-O* was obtained from the Maize Genetics Cooperative, Department of Agronomy, University of Illinois. The *Adh1-F6* allele was obtained from the laboratory of D. SCHWARTZ, University of Indiana. Lines carrying Mutator were a gift from D. ROBERTSON, Iowa State University. The *Mu1* plasmid, pAB5, the *Mu* end-specific plasmid, pDTE (CHANDLER, RIVIN and WALBOT 1986), and the *Mu6* plasmid were gifts from V. CHANDLER, University of Oregon.

Southern analysis: All Southern analysis was performed on nylon filters (Nytran; Schleicher and Schuell) as previously described (HAKE, VOLLBRECHT and FREELING 1989; STROMMER *et al.* 1982).

Characterization of restriction fragment length polymorphisms (RFLPs): Because *Kn1* is less than 1 map unit from *Adh1*, easily scored electrophoretic variants of *Adh1* (FREELING and SCHWARTZ 1973) can be used to follow restriction site polymorphisms linked with *Kn1*. For example, 1s2p, a line homozygous for *Adh1-S*, *kn*, and a 2.0-kb *Hin*dIII fragment visualized with the H2 probe, can be crossed with another isogenic line homozygous for *Adh1-F*, *Kn1-O*, and a 2.2-kb *Hin*dIII H2 hybridizing fragment. In a backcross of the resulting hybrid to the *kn* line, the close linkage of the 2.2-kb RFLP with both *Kn1* and *Adh1-F* is indicated by its cosegregation with these markers. In practice, the linkage of RFLPs with particular chromosomes, especially those carrying the recessive *kn1* alleles, was most easily established by showing cosegregation with a particular *Adh1* isozyme marker.

Genomic cloning: Except where noted, recombinant DNA methods were adapted from protocols described by MANIATIS, FRITSCH and SAMBROOK (1982). The cloning of *Kn1-2F11* has been described previously (HAKE, VOLLBRECHT and FREELING 1989). A 10-kb *Bcl*I/*Sal*I fragment, which contains most of the clone, was subcloned into pUC18 to create pEV100. This clone was subcloned further into pSK (Stratagene) as fragments corresponding to the individual hybridization probes. H2 is a 2.2-kb *Hin*dIII fragment. H3 is a 1-kb *Hin*dIII/*Eco*RI fragment. SP is a 200-bp *Sac*I/*Pvu*II fragment. B3 is a 300-bp *Bam*HI/*Sal*I fragment.

Sequences corresponding to the tandem duplication of *Kn1-O* were cloned from two separate lambda genomic libraries prepared with size selected DNA from a 2-month-old *Kn1-O* homozygote. DNA was purified by the method of MURRAY and THOMPSON (1980). In the first library, 5 EMBL3 phage clones containing a 13-kb *Bam*HI fragment were selected from 300,000 by hybridization to the H2 probe. One of these was subcloned into pSK+ to make pBV200. In the second library, *Hin*dIII-digested genomic DNA was cloned into *lambda* 2001 and screened by hybridization to probe H3. Seven clones containing a 15-kb fragment and 1 containing a 19-kb fragment were purified from a total of 100,000 phage. One 15-kb clone and one 19-kb clone were subcloned into pSK+ to make pBV201 and pBV202, respectively. Sequences that include the transposon insertion site in the three Mutator derivatives were cloned from separate EMBL3 libraries as *Bam*HI fragments using the H2 probe. The cloned *Bam*HI fragments were then subsequently subcloned into pSK+. The sequences that include the insertion site in the non-Mutator derivative, *Kn1-O204*, were cloned as a *Hin*dIII fragment into *lambda* 2001. In each case, DNA was prepared from one individual.

Polymerase chain reaction (PCR) amplification: The right boundary of the tandem duplication array was cloned on a 1-kb fragment by PCR amplification (Perkin Elmer-Cetus). A primer within the repeated sequence was selected from sequence determined for the right end of the left repeat unit (see Figure 4a). An opposing primer that was assumed to derive from sequence outside the repeated array was selected from sequence determined from *Kn1-2F11* subclones (Figure 2a). Reaction conditions were those recommended by the manufacturer.

DNA sequencing: Sequences of single stranded templates were determined with the Sequenase system of U.S. Biochemicals. Templates were generated from pSK+ "phagemids" (Stratagene) into which 1–2-kb *Kn1-O* fragments had been subcloned as discrete restriction fragments. The sequence presented for *Kn1-O* derives from both strands of the subcloned sequences. Sequencing of the insertion site of the derivatives utilized *Kn1*-specific primers and a combination of double and single stranded protocols.

Mutational analysis: Postmeiotic tassels of plants homozygous for *Kn1-O* were X-irradiated as described previously (HAKE, VOLLBRECHT and FREELING 1989).

Mutator elements were introduced into *Kn1-O* lines as follows: *Kn1-O Adh1-F6* plants were crossed as males to plants carrying Mutator elements, then backcrossed as females to homozygous *Kn1-O Adh1-F6*. Homozygous *Kn1-O Adh1-F6* plants were then crossed as males to detasseled females carrying a different *Adh1* isoelectric allele, *Adh1-F*. Approximately 10,000 plants were grown to maturity and examined for the presence of ligule displacement or knots. Only individuals that were normal and still carried *Adh1-F6* were followed.

RESULTS

A sequence duplication is associated with *Kn1-O*: The structure of *Kn1-O* was initially characterized by Southern analysis. This analysis indicates that *Kn1-O* lacks any large insertion comparable to the *Ds2* that causes the *Kn1-2F11* mutation, but is instead closely associated with a sequence duplication. Genomic DNAs from knotted and normal individuals were hybridized to probes derived from nonrepetitive sequences of the *Kn1-2F11* clone (Figure 2a). In some individuals, these probes visualize two distinct fragments as a result of RFLPs between the two chromosome homologs (HELENTJARIS *et al.* 1986). Where such RFLPs were encountered, it was relatively simple to demonstrate their association with a particular chromosome by their cosegregation with either the dominant *Kn1* gene, or closely linked (< 1 map unit) *Adh1* isozyme variants (see MATERIALS AND METHODS).

Figure 2b shows the restriction patterns obtained when three probes spanning the cloned region of *Kn1-2F11* were successively hybridized to a blot of genomic DNAs digested with *Hin*dIII. The pattern obtained with each of these probes suggests the duplication of sequences in *Kn1-O*. For example, H2, a 2.2-kb probe derived from sequences to the left of the *Ds2* insertion site, visualizes two fragments whose size difference reflects an RFLP. The 2.0-kb fragment segregates with chromosomes bearing *kn1 Adh1-S* (lanes 1, 2 and 3) while the 2.2-kb fragment segregates with chromosomes bearing *kn1 Adh1-F* (lane 1), *Kn1-O Adh1-F6* (lane 2) and *Kn1-Ox Adh1-F6* (lane 3). Although both fragments are common to all lanes, the dosage of the larger 2.2-kb fragment is elevated in DNA from knotted individuals. In a *Kn1-O* heterozygote, the 2.2-kb band is approximately twice as intense as in the normal, suggesting a duplication of sequences. *Kn1-Ox*, a severely knotted heterozygous derivative of *Kn1-O*, (see subsequent results), gives a 2.2-kb fragment which is approximately three times as intense as the normal, suggesting a triplication of sequences. In contrast, the smaller 2.0-kb band is equally intense in both knotted and normal individuals. The signal from hybridization to this fragment, present in one dose in each of the three individuals, serves as a convenient internal standard to substantiate relative copy number differences seen for the larger 2.2-kb fragment. Density ratios between the 2.2-kb and the 2.0-kb bands (see Figure 2 legend) are consistent with estimates presented above that presumed equal loadings of DNA. Hybridizations with other probes provide additional evidence for the duplication of sequences in *Kn1-O*. When the blot was stripped and rehybridized with H3, a probe derived from sequences to the left of H2, three bands are seen for *Kn1-O* and *Kn1-Ox* compared to only one for the normal plant (Figure 2b). The 10-kb and 19-kb bands, equally intense in the knotted plants, reflect heterozygosity for a *Hin*dIII site polymorphism to the left of H3, each band deriving from a different homolog. The normal individual, lacking polymorphism in the H3 region, shows a single 10-kb band that is roughly twice as intense as its knotted counterpart because of the superimposed signals from both homologues. The 19-kb band, though characteristic of both knotted individuals in this experiment, appears unrelated to the mutant phenotype since it is present in certain normal lines (as will be shown in Figure 5). The 15-kb band, in contrast, is specifically associated with *Kn1-O*. Moreover, its intensity appears proportional to the severity of the mutant phenotype. In *Kn1-O*, the 15- and 19-kb bands have comparable intensities, while in the more knotted *Kn1-Ox*, the 15-kb band is significantly more intense.

Hybridization with probe SP, derived from sequence to the right of the *Ds2* insertion site, also visualizes a 15-kb *Kn1-O* specific band. Like H3, the fragment is twice as intense in *Kn1-Ox* as in *Kn1-O*. A larger 17-kb fragment is present in both knotted and normal individuals. Its intensity in the normal (lane 1) reflects the superimposition of fragments from both the *Adh1-S* and *Adh1-F* bearing homologs. Given the comparable intensity of the 17 kb-band in all the lanes, it appears that the knotted individuals are also homozygous for this length restriction fragment and that the 15-kb band derives from additional copies of SP containing sequences.

Two models would account for the distinct size of the duplication fragments visualized by both the H3 and SP probes. The first (Figure 3a) supposes that the

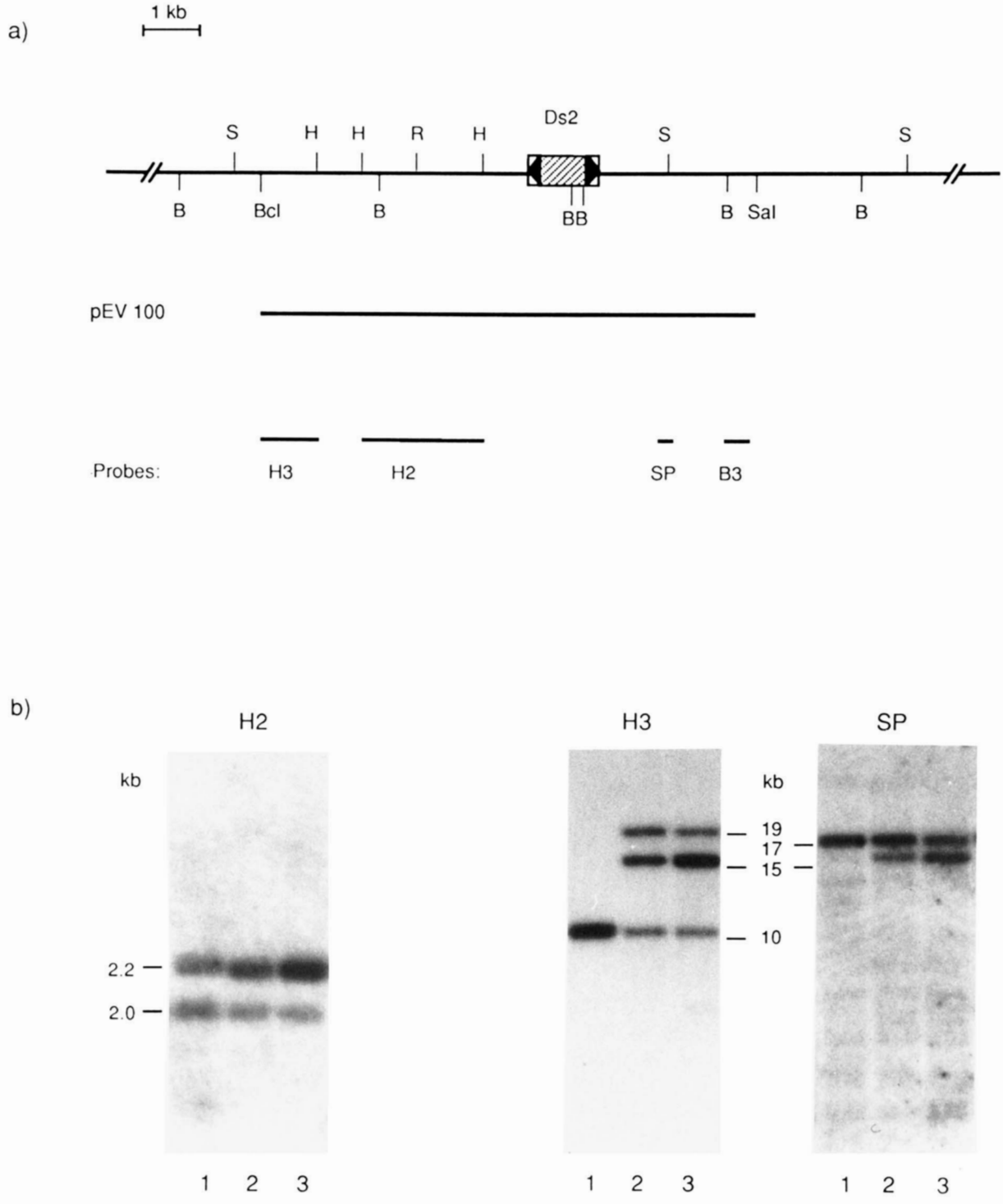

FIGURE 2.—(a) Restriction map of *Kn1-2F11*. Top line indicates the restriction map of genomic sequences as determined by Southern analysis of genomic and subcloned DNAs. B = *Bam*HI, H = *Hin*dIII, P = *Pst*I, S = *Sac*I, R = *Eco*RI, V = *Eco*RV. Hybridization probes H3, H2, SP and B3 are indicated below the map. The middle line, aligned with the genomic restriction map above, shows the extent of a plasmid subclone, pEV100, from which the hybridization probes were derived. (b) The three panels show Southern analysis of 1) normal (*kn Adh1-F/kn Adh1-S*) 2) *Kn1-O*/normal (*Kn1-O Adh1-F6/kn Adh1-S*) and 3) *Kn1-Ox*/normal (*Kn1-Ox Adh1-F6/kn Adh1-S*) genomic DNAs digested with *Hin*dIII, then successively probed with H2, H3 and SP. Ratios of the densities of the 2.2-kb vs 2.0-kb fragments (2.2/2.0) in the H2 panel are: lane 1, 1.1; lane 2, 2.2; lane 3, 3.5. The greater than integral values for these ratios probably reflects slightly more efficient hybridization of the H2 probe to the larger 2.2-kb fragment.

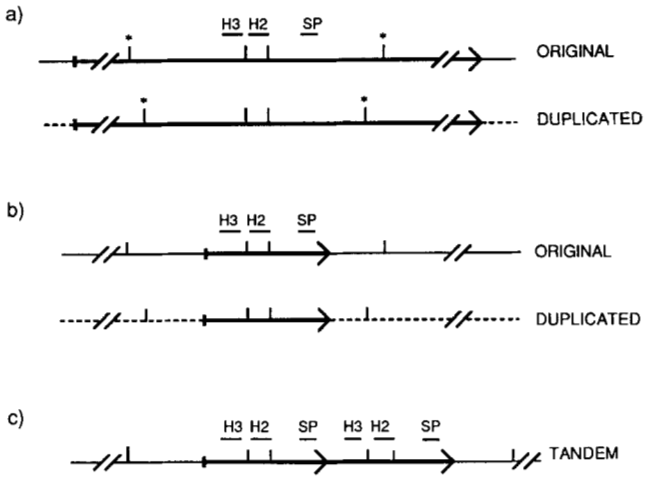

FIGURE 3.—Models for duplication suggested by Southern analysis. The diagrams compare the relationship between repeated sequence (heavy lines) and flanking unique sequence for both the original and duplicated copies. Probes used in the Southern analysis are aligned above the map. Hatch marks denote *Hin*dIII restriction sites. (a) Duplication containing *Hin*dIII RFLPs. Polymorphic sites are denoted with an asterisk. (b) a perfect duplication with novel *Hin*dIII junction fragments. (c) a perfect tandem duplication with a single novel *Hin*dIII junction fragment.

fragments are contained entirely within the sequence duplication, their distinct size reflecting RFLPs between the original and duplicated sequences. An alternative model (Figure 3b) notes that a novel duplication fragment would likely result if it extended across a junction separating the duplication from flanking sequence. The exact size of such a fragment would depend on the position of restriction sites in sequences flanking the duplication. By this model, the novel fragments detected by H3 and SP would define the intervals that contain the left and right boundaries of the duplicated sequence, with the unaltered 2.2-kb H2 fragment contained entirely within the duplication. A refinement of the latter model (Figure 3c) accounts for the common 15-kb size of fragments visualized by the H3 and SP probes by supposing a tandem duplication. Such a rearrangement contains a single novel junction that would juxtapose H3 and SP sequences.

Duplicated sequences occur in a tandem array: A more detailed analysis of genomic clones substantiates the tandem organization suggested by the previous analysis. Figure 4a gives a restriction map of the duplication inferred by Southern analyses of genomic DNAs and analysis of genomic clones that contain duplicated sequences (aligned below the restriction map). pBV201, cloned as a *Kn1-O* specific 15-kb *Hin*dIII fragment that hybridizes with H3, extends leftward from the right repeat across the single novel junction of the duplication into the adjacent repeat. The clone accounts for the common 15-kb *Hin*dIII band visualized by the H3 and SP probes (Figure 2b) since it contains sequences corresponding to both probes. pBV200, cloned as a *Kn1-O*-specific 13-kb *Bam*HI fragment, also spans the central junction and shows extensive overlap with pBV201 by restriction mapping.

A comparison of pBV201 with pBV202, a 19-kb *Hin*dIII fragment that hybridizes with H3 (Figures 2b and 4a) suggests the position of a repeat boundary. The clones overlap from their right ends but diverge to the left of common *Eco*RV sites. The right boundary of the tandem array was suggested by hybridization experiments. B3, a probe derived from the *Kn1-2F11* clone (Figures 2a and 4a) does not detect duplication fragments (data not shown), suggesting it lies to the right of the array. The boundaries of the repeat units were defined more precisely by sequencing portions of the genomic clones. A fragment containing the middle junction was subcloned from pBV200, while the left boundary of the duplication was subcloned from pBV202. The right boundary was cloned by PCR amplification (MATERIALS AND METHODS). Earlier attempts to isolate the right boundary as a larger fragment in lambda vectors were complicated by rearrangements of cloned sequences, a problem that was peculiar to this region.

A comparison of sequences of the left and right boundary fragments with that of the middle junction reveals the extent of the duplication. Since sequences of the left and right boundary are found juxtaposed in the middle junction fragment, the duplication appears complete (Figure 4b). It is not possible to precisely assign the positions of the repeat boundaries because of their association with a 5-bp repeat, TATGT, which occurs at both the right and left boundaries and the middle junction. The duplication as characterized so far appears to be exact with no differences in the restriction maps or the approximately 1 kb of sequence determined for each of the repeats.

The *Kn1-O* phenotype is tightly associated with the duplication: The duplication characterized in the foregoing analysis is tightly linked to the *Kn1-O* mutation. In over 100 *Kn1-O* individuals which have been analyzed by Southern hybridization (data not shown), all contained duplication-associated fragments, whereas in a comparable number of normals, these fragments were consistently lacking. To address whether there is causal relationship between the duplication and *Kn1-O* mutation, we undertook a mutational analysis in which phenotypically distinct derivatives of *Kn1-O* were characterized on a molecular level.

In the first study, derivatives were produced by crossing normal plants with pollen from *Kn1-O* homozygotes that had been X-irradiated. Both of the *Kn1-O* bearing chromosome *1* homologs were marked with a closely linked *Adh1-F6* allele to reduce the possibility of confusing normal individuals derived from contaminating pollen with true revertants. Four normal

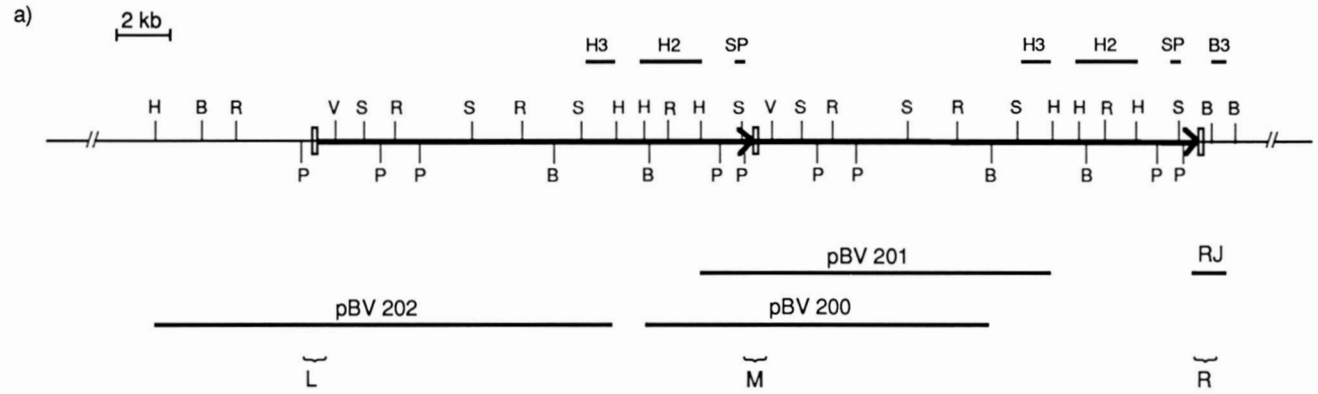

FIGURE 4.—(a) Restriction map of *Kn1-O*. Sites were determined by a combination of genomic Southern analysis and restriction mapping of genomic subclones. The restriction sites are as for Figure 2. Probes B3, H2, H3, and SP used in the Southern analysis are aligned over the restriction map. Genomic clones pBV200, pBV201 and pBV202 are indicated below the map. Brackets L, M, and R indicate positions of sequences presented in b). RJ corresponds to the PCR product described in methods.

plants and one severely knotted plant were found in a screen of 10,000 resulting progeny, with the remainder of the plants showing knots typical of *Kn1-O* heterozygotes.

Southern analysis of the exceptional plants shows a direct correlation between the number of repeats and the severity of the knotted phenotype. Three of the normals give a restriction pattern which lacks the *Kn1-O* specific 15-kb duplication fragment. The 19-kb fragment characteristic of the *Adh1-F6* bearing homolog is retained, suggesting that the reversion to a normal phenotype is the result of the relatively precise deletion of a duplication repeat. A similar frequency of reversion by loss of a repeat was observed in an unirradiated population (1 in 3000), suggesting these loss events may be unrelated to irradiation. The remaining normal, *Kn1-Od*, lacks both the 15-kb and 19-kb fragments, suggesting that both copies of the repeat and some flanking sequence have been deleted (HAKE, VOLLBRECHT and FREELING 1989). It is unlikely that the alterations described in these analyses reflect pollen contamination artifacts since the derivatives retain linkage with the *Adh1-F6* allele and RFLPs characteristic of the *Kn1-O* progenitor line.

The severely knotted plant, *Kn1-Ox*, gives the same restriction pattern as *Kn1-O* (Figure 2b); however, densitometric analysis indicates that fragments resulting from the duplication in *Kn1-O* (Figure 2b) are present in an additional copy in *Kn1-Ox*.

Insertions that revert the knotted phenotype map to either side of the central junction of the tandem duplication: In a second experiment, we defined sequences that influence the expression of *Kn1-O* by correlating the positions of transposon insertions with

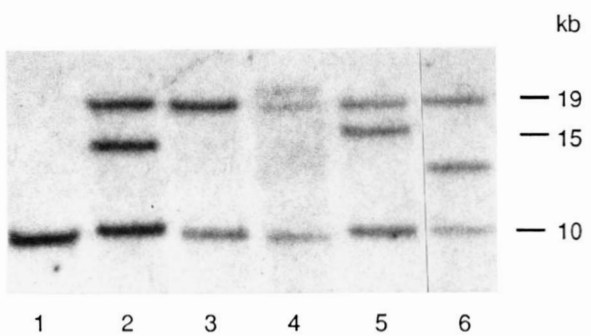

FIGURE 5.—Southern analysis comparing normal derivatives of *Kn1-O* with the *Kn1-O* progenitor. All the individuals are heterozygous with a normal allele. Genomic DNAs were digested with *Hin*dIII and probed with H3. The 15-kb *Hin*dIII fragment of *Kn1-O* is either altered or missing in the derivatives. lane 1, normal; lane 2, *Kn1-O*; lane 3, derivative that does not carry an insertion but has lost the duplication; 4, *204*; lane 5, *174*; lane 6, *167*.

loss or alteration of the mutant phenotype. Ten thousand plants were screened that were progeny of normal females crossed by *Kn1-O* homozygous males from an active Mutator stock (ROBERTSON 1978). Mutator lines typically show a 50-fold increase in the rate of spontaneous mutation, presumably the result of transposon insertions. The vast majority of plants screened displayed the knotted phenotype expected of a *Kn1-O* heterozygote; however, in nine plants, knotting was absent or much reduced.

Southern analysis of these exceptional individuals strengthens the correlation between the duplicated sequences and the *Kn1-O* phenotype. In five normal plants, one of the duplication repeats had been lost (Figure 5, lane 3). We have been unable to determine which of the two repeats was lost since we have not found any distinguishing polymorphisms. Four other

normal or near normal individuals show alterations to the 15-kb *Hin*dIII fragment that spans the central duplication junction (Figure 5). Derivative *Kn1-O204* (*204*) contains a *Hin*dIII fragment that is approximately 4 kb larger while derivatives *Kn1-O174* (*174*) and *Kn1-O169* (*169*) (not shown) both contain fragments that are 1.3 kb larger. A fourth derivative *Kn1-O167* (*167*) contains a fragment that is smaller than the corresponding fragment in *Kn1-O*. Genomic libraries were prepared for each derivative and the isolated *lambda* clones were subcloned for analysis (see MATERIALS AND METHODS). Preliminary restriction mapping of the derivatives indicated that all four contained insertions in the 2.3-kb *Sac*I fragment that spans the central junction of the tandem duplication. Sequence analysis places the insertions to the left and right of the breakpoint (Figure 6). The insertions that result in derivatives *169* and *174* are in the exact same location, 310 bp to the left of the central junction. The *167* derivative has an insertion 6 bp to the right of the duplication junction, and the *204* derivative carries an insertion 1105 bp to the right.

The identity of the insertions was determined by hybridizing the cloned insertions to different *Mu* elements. All *Mu* elements contain similar 200-bp inverted repeats, but often differ completely in the region between the repeats (BARKER *et al.* 1984; OISHI and FREELING 1988; TALBERT, PATTERSON and CHANDLER 1989). We first determined that the insertions in derivatives *174*, *167* and *169* belong to the Mutator family by hybridizing each insertion to a *Mu* end specific fragment (CHANDLER, RIVIN and WALBOT 1986). Restriction mapping and hybridization analysis of the element in *174*, suggest it is *Mu1* (STROMMER *et al.* 1982). The insertion in derivative *169* shares sequence homology with *Mu8* (S. WESSLER, personal communication). The *167* insertion hybridizes to *Mu6*, a partial *Mu* element isolated by V. CHANDLER (personal communication), but also shares considerable sequence homology with *rcy* (SCHNABLE, PETERSON and SAEDLER 1989) (data to be presented elsewhere). The insertion in *204* is much larger than known *Mu* elements and did not hybridize to any *Mu* sequences. We have not yet determined the identity of this element. Thus, four different elements have inserted at, or very close to the central junction of the tandem duplication, strongly implicating this region in the *Kn1-O* mutation.

DISCUSSION

In the current study, we describe a sequence duplication that is associated with the *Kn1-O* allele and present evidence that the duplication itself conditions the mutant phenotype. The mutation contrasts with that which causes *Kn1-2F11*, an insertion of the transposable element, *Ds2* (HAKE, VOLLBRECHT and FREELING 1989). By comparing *Kn1* mutants on a molecular level, we hope to identify common attributes that would account for their effect on leaf development. Such comparisons might also explain the characteristic phenotypes of different *Kn1* alleles.

By Southern analysis and cloning of genomic DNA fragments, we find the duplication consists of a tandem repeat of 17 kb of normal sequence. It is unclear how this duplication arose, though unequal crossing over between misaligned homologs affords one obvious explanation. Although such an event would by definition be accompanied by the exchange of markers that flank the locus, it is impossible to substantiate without progenitors of the individual in which the mutation arose. We have examined DNA flanking the repeat unit for repeated sequences that might promote the mispairing of homologous chromosomes as have been described for other duplications (TARTOF 1988), but thus far we have found only a 5-base motif that flanks each repeat unit. Short repeats such as these have been implicated in several examples of illegitimate recombination in both prokaryotes and eukaryotes, though the exact role they play in this process is not yet understood (reviewed by ANDERSON 1987).

Several lines of evidence argue that the duplication itself causes the *Kn1-O* mutation. *Kn1-O* cannot be separated from the duplication by recombination, a result consistent with their supposed identity. Moreover, those sequences duplicated in *Kn1-O* include the site at which the insertion of *Ds2* produces the *Kn1-2F11* mutation. Finally, mutational studies show that loss of the mutant phenotype coincides with loss or disruption of a duplication repeat unit, while the gain of a third repeat unit exacerbates the knotted phenotype.

The reversion of *Kn1-O* associated with the loss of a repeat unit can be likened to that seen with the duplicated *Bar* locus (STURTEVANT 1925; BRIDGES 1936), the frequency of such events in both systems approaching 10^{-3} (this study; ZELENY 1921). The majority of *Bar* reversion events appear to result from unequal crossing over since loss events are accompanied by exchange of flanking markers (STURTEVANT and MORGAN 1923; STURTEVANT 1925). We have recently begun a similar analysis of *Kn1-O* reversion, exploiting RFLPs that flank the duplicated sequences as markers of recombination.

Loss events mediated by unequal crossing over should be balanced by a reciprocal class of events in which a repeat unit is gained. *Ultrabar*, a severe derivative of the duplicated *Bar* mutation, conforms to expectations of the model, with the gain of a repeat unit to create a triplication accompanied by the exchange of flanking markers (STURTEVANT 1925). *Kn1-Ox*, a triplication derivative of *Kn1-O*, might be an analogous case, but, since the mutation occurred in a homozygous line, we can not determine if it was

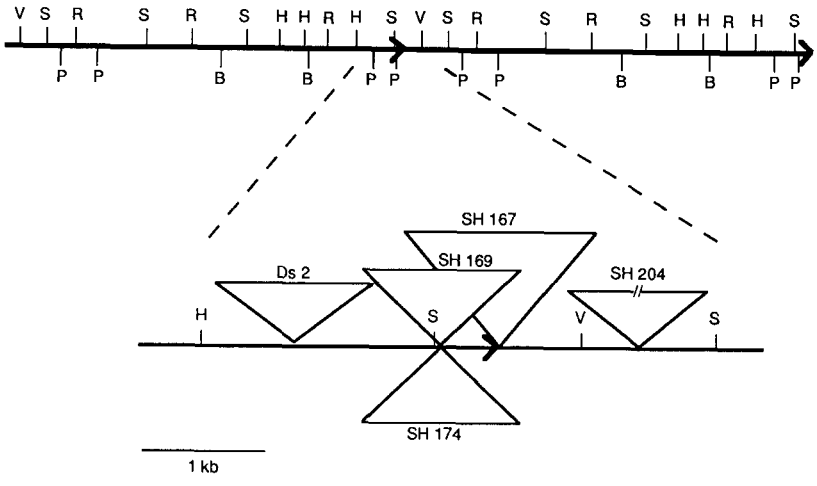

FIGURE 6.—Map designating the location of the insertions that revert the *Kn1-O* phenotype in relation to Ds2, an insertion that produces the *Kn1-2F11* knotted phenotype. Restriction sites are as indicated in Figure 2.

associated with unequal crossing over. If so, repeat loss events should be balanced by an equal number of repeat gains. Screens for derivatives of *Kn1-O* fail to show this parity, with 9 repeat loss events (normal revertants) isolated compared to only 1 gain (*Kn1-Ox*). However, as was the case with *Ultrabar*, it is possible that *Kn1-Ox* individuals are underrepresented due to the reduced vigor associated with the mutant phenotype.

One model for how the duplication might lead to the formation of knots supposes that they form as the result of the overexpression of a normal gene contained entirely within the repeat unit with the expression proportional to the copy number of repeat unit. Thus, a *Kn1-O* heterozygote, which carries three repeat units compared to the normal two, would express the gene at 150% the normal value. This model supposes that a 50% increase in the expression of the *Kn1* gene is sufficient to induce the formation of knots. While this model is consistent with the semidominant character of *Kn1-O* and the increased severity of knotting in *Kn1-Ox*, it is not supported by genetic data. Increasing the dosage of the repeat unit and flanking sequences from two to four in an 18 map unit segmental tetrasome does not produce a knotted phenotype (FREELING and HAKE 1985). It is possible however, that increases in the dosage of other factors in this larger duplication effects dosage compensation (BIRCHLER 1979, 1981) at the *Kn1* locus.

In the same study, *Kn1* was suppressed in a line monosomic for the long arm of chromosome *1* (1L) as would be expected by the dosage model; the extra repeat dose contributed by *Kn1-O* is balanced by the absence of a repeat on the deficient homolog to give a normal dose of two. However, in more recent studies, in which *Kn1-O* is placed opposite a smaller deficiency, *Kn1-Od*, knots are not suppressed (our unpublished results). We have not yet accounted for the suppression conferred by the larger deficiency. Suppression may be effected by reducing the dosage of factors encoded on 1L that are required specifically for *Kn1* expression. Alternatively, the suppression might be an indirect consequence of altered growth generally associated with hypoploidy. Experiments are in progress to distinguish between these explanations.

We favor an alternative to the dosage model that supposes aberrant gene expression results from the juxtaposition of sequences at the novel central junction of the duplication. *Kn1-O* contains one such junction, while the more severely knotted derivative, *Kn1-Ox*, contains two. The involvement of these sequences is supported by results of our mutational analysis in which a loss or reduction of knotting is associated with insertions of transposable elements to either side of the junction. These data strongly suggest that the repeat junction itself is essential for expression of the *Kn1-O* phenotype. These sequences might exert their effect through the production of a novel transcript that spans the central junction. Such a transcript could encode a chimeric protein, or behave anomalously by way of an altered stability. Alternatively, the mutant phenotype might result from the misexpression of a normal gene product. Such altered expression might result if transcribed sequences within one repeat unit were juxtaposed by the rearrangement with sequences in the adjacent repeat that have novel regulatory activity.

The latter alternative is simpler to reconcile with the organization of other *Kn1* mutations that are not associated with duplications, but that instead result from the insertion of transposable elements. Four of these have been characterized to date, *Kn1-2F11*, caused by a *Ds2* element, and three recently isolated *Kn1* mutations that contain insertions of Mutator related elements (WALKO and HAKE 1989; R. WALKO, unpublished data). In all four cases, the insertions map approximately 2 kb to the left of sequences that form the central junction of the tandem duplication in *Kn1-O*. It is possible that these insertions give rise to an altered protein that acts like one produced by *Kn1-O*. However, given the distinct sequences affected by these mutations, it seems more plausible that the

mutations exert their effect by altering the spatial or temporal expression pattern of a normal or near normal transcript. Experiments are in progress to more clearly define the structure and expression pattern of the *Kn1* transcription unit in both knotted and normal plants.

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