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Cloning *Knotted*, the dominant morphological mutant in maize using *Ds2* as a transposon tag

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The *Kn1-2F11* mutation causes protrusions or knots along the lateral veins of the first few leaves of the maize plant. The phenotype is visible when an unlinked gene, presumably *Ac*, is present in the genome. The mutation is closely linked to a genetically unstable *Adh1* mutation that resulted from the insertion of a *Ds2* element (Döring *et al.*, 1984; Chen *et al.*, 1986). Using a unique sequence from the *Ds2* element as a hybridization probe, a genomic restriction fragment that cosegregated with the knotted phenotype was cloned. It carries the *Kn1-2F11* locus by the following criteria. (i) Cosegregation of the fragment is tightly linked to the phenotype. (ii) Somatic and germinal excision produce a fragment which is the expected size of a revertant fragment; progeny containing the revertant size fragment are normal. (iii) The sequences that hybridize to this fragment are significantly altered in the chromosome containing the original knotted mutation, *Kn1-O*. (iv) The cloned fragment does not hybridize to a chromosome that contains a deletion of *Kn1-O*.

Key words: dominant mutant/*Activator* element/*Zea mays*/morphological mutant/*Knotted*

Introduction

Dominant mutants of the *Knotted* locus (*Kn1*) in maize profoundly alter leaf morphology. Most significantly, protrusions or knots are formed along the lateral veins as a result of new cell divisions and a change in the plane of growth. In addition, the veins are associated with non-green cells, making the veins clear and more prominent than normal veins. The severity of the *Kn1* phenotype is variable in different stocks. In a mildly expressing plant, clear veins may be the only sign of the mutation, whereas severely affected plants are grotesquely distorted. The ligule is also affected in some of the *Kn1* mutations. It is displaced into the leaf blade or is produced *de novo* (Freeling and Hake, 1985).

The original knotted mutation of maize, *Kn1-O*, was recovered as a spontaneous mutation by Bryan and Sass (1941). It is very expressive, causing ligule displacement at most leaves, midrib distortion and occasional knots. Other knotted mutations have since been identified which map to the same region on chromosome 1 (~1 map unit from *Adh1*). All the knotted mutations are more severe in the homozygote than in the heterozygote. Dosage analyses using B-A translocations and reciprocal translocations were carried out with three of the *Kn1* mutants. The data suggest that *Kn1* is a neomorphic mutation, causing either a vast overproduction of a normal product, an altered non-competing product or an entirely new product for the leaf (Freeling and Hake, 1985).

The *Kn1-2F11* mutation was first recognized in seedlings that carried an *Adh1* mutation, *Adh1-2F11*. This mutation was recovered following allyl alcohol selection for *Adh1* nulls from plants carrying the transposable elements *Ac* and *Ds* (Döring *et al.*, 1984). *Ds* elements are defined as non-autonomous elements that transpose or cause chromosomal breaks only in the presence of the autonomous element, *Ac* (McClintock, 1947, 1949, 1951). *Adh1-2F11* resulted from insertion of a *Ds* element into the fourth exon of *Adh1* (Merckelbach *et al.*, 1986). *Adh1-2F11* is closely linked to the knotted mutation but is not its cause; *Adh1* revertants, in which *Ds* has excised from the *Adh1* gene, remain knotted (Chen *et al.*, 1986). The phenotype of the *Kn1-2F11* mutation is very mild in comparison to other *Kn1* mutations. In typical *Kn1-2F11* plants, only the first few leaves have knots and clearing of the veins (Figure 1), although an occasional leaf near the tassel may have a knot. Furthermore, penetrance is low in some lines with only 50% of the seedlings that carry the mutation actually showing knots.

In this paper, we show that the expression of *Kn1-2F11* requires the presence of an unlinked *Ac* element, or a gene closely linked to *Ac*, and that a *Ds2* element segregates with the *Kn1-2F11* mutation. We have cloned a DNA fragment containing a *Ds2* element, and show genetic and physical linkage of the *Ds2* insertion and the knotted phenotype.

Fig. 1. The first leaf of a plant homozygous for *Ac* and for *Kn1-2F11*.

Results

The expression of Kn1-2F11 depends on Ac

Mutations caused by *Ds* elements are destabilized by the presence of an *Ac* element *in trans* (McClintock, 1951). For example, in kernels containing the *bz2-m* mutation, in which a *Ds* element has inserted at the *Bz2* gene (Neuffer, 1954), the aleurone is bronze in the absence of *Ac*. When *Ac* is present in the genome, revertant purple sectors appear against the bronze background. The maize stock carrying *Kn1-2F11* also contains *bz2-m* on 1L and an *Ac* element located on 9S (K.Dawe, personal communication). We used the *bz2-m* marker as an indicator of *Ac* activity to test whether *Ac* is required for *Kn1-2F11* expression. Because the *bz2-m* mutation often becomes stable due to imprecise excision of the *Ds2* element, a hypomethylation assay for *Ac* activity (Chomet *et al.*, 1987) was used for those kernels which appeared to lack *Ac* (see Materials and methods).

In general, we found that the *Ac* element on 9S was required for the plants to exhibit the knotted phenotype. When knotted plants were crossed to normal plants and then self-pollinated (see the cross described in Materials and methods), the only progeny that were knotted carried *Ac* and at least one dose of the *Kn1-2F11* allele. *Adh1* alleles were used to mark the *Kn1-2F11* and normal ('*kn*') chromosomes, allowing us to score unambiguously for the presence or absence of the mutation. We examined 33 families for dependence on *Ac* for *Kn1-2F11* expression (Table I). Depending on the family, the expression of knots varied from 50% to 100%. In some families, the penetrance was particularly weak when the mutation was heterozygous. The poor penetrance may reflect a number of factors such as modifying background effects, changes in *Ac* activity (McClintock, 1964), or a high rate of germinal excision.

We found two exceptions to the requirement of *Ac* for knotted expression. A family was found that had *bz2* kernels, indicative of inactive *Ac* elements, but five of 17 individuals were knotted (Table I: family 6812-3). One knotted individual was self-pollinated and 22 of 41 progeny were knotted (family 110-1). These individuals did not contain active *Ac* elements by the molecular assay (see Materials and methods) and by genetic crosses to the *Ac* tester, *bz2-m*. In a second family, 473-7, 80 kernels were scored for the presence of *bz2-m* sectors and planted. DNA was prepared from all of them and crosses made to the *bz2-m* tester. Forty five individuals carried at least one allele of *Kn1-2F11*; 37 of these were knotted. 34 of the *Kn1-2F11* individuals contained *Ac* and were knotted, but three individuals, all heterozygous for *Kn1-2F11*, were found that had knots but no *Ac*. The progeny of the outcross do not carry *Ac* and are still quite knotted (one-half to one-third of the progeny show knots). Possible explanations are discussed later.

We examined the effect of varying the dosage of *Ac* on the *Kn1-2F11* phenotype by comparing individuals that segregated for *Ac* in homozygous *Kn1-2F11* families. The *Ac* dosage was determined by the *bz2-m* marker of both the seed before germination and the resultant progeny of a test cross. An increase in *Ac* dosage results in less excision of *Ds*, as indicated by fewer and less frequent *bz2-m* sectors (McClintock, 1948, 1949). In general, we observed that (i) the timing of *Kn1* expression was not altered by a change of *Ac* dose and, (ii) with increasing copies of *Ac* there were more knots on the first leaf, and the second and third leaves were often knotted as well.

Table I. The dependence on *Ac* for the knotted phenotype of *Kn1-2F11*

	+*Ac*		−*Ac*	
	knots	none	knots	none
Homozygous *Kn1-2F11*				
5573-8 ⊗	–	–	–	8
5574-19 ⊗	29	–	–	–
5574-13 ⊗	–	–	1[a]	43
5574-4 ⊗	3	3	–	–
30-1 ⊗	–	–	–	9
32-2 ⊗	10	–	–	–
32-3 × sib 4	17	–	–	–
101-10 ⊗	–	–	–	25
101-6 ⊗	20	4	–	14
101-13 ⊗	11	6	–	6
6812-3 ⊗[b]	–	–	5	17
110-1 ⊗			22	19
474-13 ⊗	8	4	–	–
474-11 ⊗	10	12	–	–
474-10 ⊗	13	6	–	6
551-5 ⊗	3	13	–	2
Segregating *Kn1-2F11*				
33-7 ⊗	6	6	–	2
33-2 × sib-1	3	11	–	7
98-6 ⊗	36	9	–	–
101-8 ⊗	15	10	–	–
473-7 ⊗[c]	34	5	3	3
101-3 ⊗	8	4	–	8
Kn1-2F11 outcross[d]				
475-15 × 474-3	8	46	–	–
474-2 × 475-17	10	46	–	–
475-17 × 474-2	11	42	–	–
483-6 × 474-2[e]	2	98	–	–
566-6 × 565-1	6	14	–	–
565-6 × 566-2	4	16	–	–
565-3 × 566-3	6	14	–	–
565-2 × KD494-1[f]	2	40	–	–
568-4 × 565-1	–	–	–	20
476-3 × 482-15[g]	1	–	–	19
476-9 × 482-15	43	2[h]	–	20

Seedlings were examined for knots after determining if there were *bz2-m* sectors on the aleurone of the kernel (indicating an active *Ac* is present). In certain families, the *Pvu*II test for an active *Ac* was performed (see Materials and methods).

[a]One plant had a single mild knot. No further tests were performed.

[b]6812-3 ⊗ (110) was *bz2* and did not have *Ac* by the *Pvu*II test. One plant was self-pollinated (110-1 ⊗). Its progeny were also knotted. One knotted individual was crossed to *bz2-m* (no *Ac*); the resulting kernels were *bz2*.

[c]473-7 ⊗ analysis was done on selected seedlings that carried an allele of *Kn1-2F11*. One of the three individuals that was knotted without *Ac* had a *bz2-m* aleurone but did not contain *Ac* in the plant, possibly due to nonconcordance between endosperm and embryo. The other two had *bz2* aleurones.

[d]A number of these crosses were designed to reactivate the knotted phenotype by crossing in *Ac*. 474 and 565 are homozygous *Kn1-2F11*, no *Ac*, no knots. 475 and 566 are no *Kn1-2F11*, homozygous *Ac*, no knots.

[e] 483 was homozygous for the *Ac* at *P*, *P-vv*. The knots were mild.

[f]KD 494-1 was heterozygous for the *Ac* at *waxy*, *wx-m7*. This *Ac* is known to cycle. The knots were mild.

[g]568 and 482 are the *bz2-m* tester; no *Ac*, no *Kn1-2F11*.

[h]The two normals with *Ac* were revertants.

In order to test further the *Ac* dependence of *Kn1-2F11*, we reactivated the knotted phenotype in plants without *Ac* but homozygous for *Kn1-2F11*, as determined by the closely

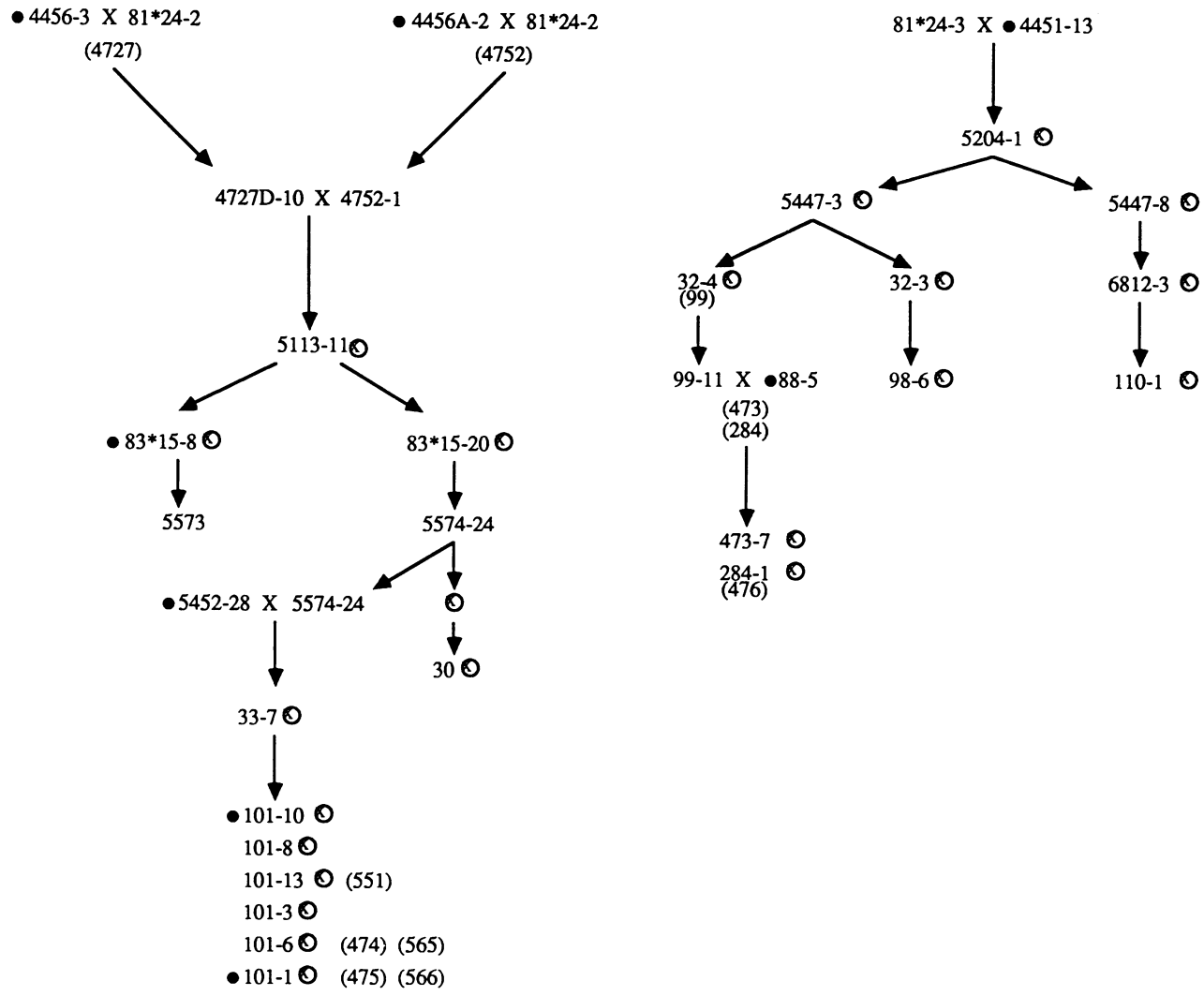

Fig. 2. Genetic crosses used in generating material segregating for *Kn1-2F11* and *Ac*. The original family was 81*24. All individuals crossed were knotted except those with dots. 88 and 5452 are *bz2-m* testers, without *Ac* and without *Kn1-2F11*.

linked *Adh1-2F11* allele. These plants were crossed to *Ac*-containing normal ('*kn*') plants marked by the *Adh1-F* allele. As a control, we crossed each of these parents to the *bz2-m* tester which contains neither *Ac* nor the *Kn1-2F11* allele. As expected, none of the parents were knotted. Only progeny of the *Ac* containing line crossed to the *Kn1-2F11* line were knotted. Approximately 25% of the progeny had knots at the seedling stage and 90% showed sporadic clearing of the veins. The clear vein phenotype appears to be a mild form of knotted expression since heterozygous parents with clear veins and no knots sometimes give rise to progeny with knots. In further tests similar to the one described above, the *Ac* at the *P* locus, *P-vv*, and the *Ac* at *waxy*, *wx-m7* were evaluated for reactivation of knotted expression. Crosses to *P-vv* produced only two knotted individuals out of 100 progeny. The knotted individuals were selfed and produced two knotted progeny of 40. The outcross of *wx-m7* to *Kn1-2F11* produced only two very mildly knotted individuals of 40 examined. We have initiated experiments to test whether the poor ability of other *Ac* elements to activate *Kn1-2F11* is due to background effects, or whether the *Ac* present on 9S specifically interacts with *Kn1-2F11*, or if this effect is actually a second gene closely linked to the *Ac* on 9S and not *Ac* at all.

Cosegregation of the Kn1-2F11 phenotype with a Ds2 element

The *Adh1-2F11* mutation is caused by a *Ds2* insertion (Döring *et al.*, 1984). Because transposable elements frequently transpose to closely linked sites (Greenblatt, 1984), it seemed plausible that similar *Ds2* elements could be responsible for both mutations. Therefore we used *Ds2* in a cosegregation analysis that employed outside markers (*Adh1* and *lw*) to score unambiguously the non-knotted individuals. The crosses for generating seed to follow segregation are described in Materials and methods. Southern blots of genomic DNA isolated from normal and *Kn1-2F11* plants were hybridized to an internal 100 bp probe, 'pAM', subcloned from and unique to the *Ds2* element (Merckelbach *et al.*, 1986). Using the restriction enzyme *Bcl*I, two fragments, 12 kb and 19 kb, hybridized to the pAM probe and were unique to DNA extracted from pooled seedlings scored for the knotted phenotype (Figure 3). The 12 kb and 19 kb fragments did not correspond to *Adh1-2F11* or *bz2-m* [which is 20 map units away and also contains a *Ds2* element (Theres *et al.*, 1987)] since DNA was prepared from lines in which *Ds2* had excised from both *Adh1* and *Bz2*. Further hybridizations with the pAM probe were carried out to DNA from individual seedlings. The smaller 12 kb frag-

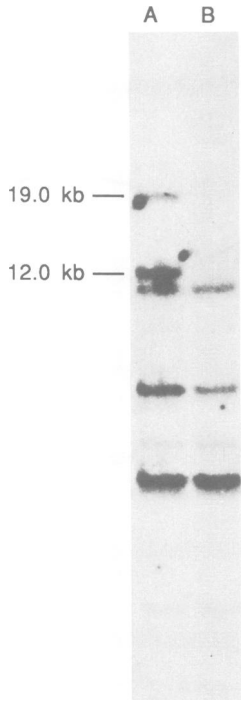

Fig. 3. Southern blot of DNA from *Kn1-2F11* and *lw* seedlings probed with the subclone from *Ds2*, pAM. DNA was made from 25 pooled seedlings that were knotted and green (**lane A**), and 20 sibling seedlings that were albino (**lane B**). The DNA was digested with *Bcl*I, electrophoresed on an 0.8% gel, transferred to nitrocellulose and hybridized to the pAM probe. The DNA preparation from the knotted seedlings was used to construct the EMBL3 library.

ment was always found to segregate with *Kn1-2F11* and away from *lw* or *Adh1-F*, whereas the 19 kb fragment was not always present in *Kn1-2F11* individuals.

Cloning the Ds2 insertion and demonstration of linkage between the insertion and the Kn1 phenotype

A genomic DNA library was constructed by cloning *Bcl*I restricted *Kn1-2F11* DNA into EMBL3. Lambda clones hybridizing to the pAM probe were isolated and one was found to contain the 12 kb fragment observed in genomic Southern analysis (Figure 3). Figure 4 diagrams the map of the *Ds2*-containing subclone, Ds10B, and the maps inferred from genomic Southern analysis of DNA from the three maize lines which have been used for segregation analysis. The number and placement of restriction sites in the region of pAM hybridization is identical in our clone and in the *Ds2* element at *Adh1-2F11* (Merckelbach *et al.*, 1986). Ds10b was analyzed by hybridizing total genomic DNA to a Southern of the clone digested with various enzymes in order to find single copy sequences to use as probes for further segregation analysis. The 2 kb *Hin*dIII fragment (H2) was chosen as a probe, although most of the 12 kb is represented by unique sequences (Figure 4).

We found tight linkage of the *Ds2* insertion to the *Kn1* phenotype by hybridizing the H2 probe to DNA from 80 knotted or normal seedlings (using *lw* or *Adh1* markers to score them for *Kn1-2F11*). Fragments generated by the enzyme *Bcl*I showed complete agreement (i) between the 12 kb fragment and *Kn1-2F11* individuals; (ii) between an 11 kb fragment and individuals carrying the *Adh1-F* marker; and (iii) between a 10.5 kb band and *lw* individuals. Two individuals were recovered that reflected recombination events; they were both *lw* and knotted. These individuals were found to carry the 12 kb fragment characteristic of *Kn1-2F11* when DNA was digested with *Bcl*I.

The knotted phenotype reverts to normal upon Ac dependent Ds2 excision

The true progenitor of *Kn1-2F11* and *Adh1-2F11* is not known (Döring *et al.*, 1984). We can infer the structure of the wild-type progenitor locus, and therefore the expected structure of the revertant locus, by comparing restriction site patterns between alleles. The *Sac*I restriction sites and other sites that are clustered in the region of the H2 probe are conserved in all lines we have examined (Figure 4 and our unpublished data). From comparison of other restriction sites, it appears that the *lw* and *Kn1-2F11* line are related. The predicted restriction map of *Kn1-2F11* following excision of *Ds2* is identical to the map of the *lw* line when the DNA is cleaved with *Sac*I, *Bam*HI or *Bcl*I (Figure 4).

The predicted size of the resulting restriction enzyme fragment corresponding to the revertant *Kn1-2F11* allele is revealed by reintroducing *Ac* into a *Kn1-2F11* line. Figure 5 shows a 5.2 kb *Bam*HI fragment of the *Adh1-F* parent (lane A) and the 3.5 kb *Bam*HI fragment of *Kn1-2F11* parent (lane B). *Ac* is present in the *Adh1-F* line. When individuals of these genotypes are crossed, some of the progeny have an additional 5.0 kb fragment that has a weaker intensity of hybridization (lanes I, J and L). A 5.0 kb *Bam*HI fragment is predicted if the *Ds2* element excises, suggesting that somatic excision of this element occurred frequently during development of the plant. It should be noted that some seedlings were knotted without any indication of excision (lane K) while other seedlings appeared normal but still carried evidence of excision (lane I). We have never seen evidence of *Ds2* excision in the absence of *Ac*.

In order to find germinal revertants we crossed a homozygote from a family that expressed knots well (~100%) to our *bz2-m Adh1-F* tester. Fifty kernels carrying *Ac*, as determined by the *bz2-m* phenotype, were grown to the seedling stage and scored for the presence of knots. Forty three seedlings showed knots. Of the seven that appeared normal at the seedling stage, five later showed knots on upper leaves. The two that remained normal no longer carried the *Kn1-2F11* (3.5 kb) fragment, but only the revertant (5.0 kb) and *Adh1-F* tester (5.2 kb) fragments (Figure 6, lanes D and F). In other similar experiments in which we have examined progeny of knotted individuals, seedlings carrying the 5.0 kb fragment, but not the 3.5 kb fragment, are always normal. Thus, germinal revertants have been selected in which *Ds2* has excised from the *Kn1-2F11* gene rendering the plants normal. This demonstrates that the insertion of the *Ds2* element is responsible for the knotted phenotype of *Kn1-2F11*.

Analysis of another knotted mutant and its deletion derivative

We hybridized a fragment derived from our *Ds2* clone to DNA from *Kn1-O* plants in order to determine if there was polymorphism specific to *Kn1-O* near the site of the *Ds2* insertion. *Kn1-O* and *Kn1-2F11* both map near *Adh1* and affect development of the lateral veins, although *Kn1-O* is much more severe in phenotype. A 5.0 kb fragment cosegregates with *Adh1-F* and a 4.8 kb fragment

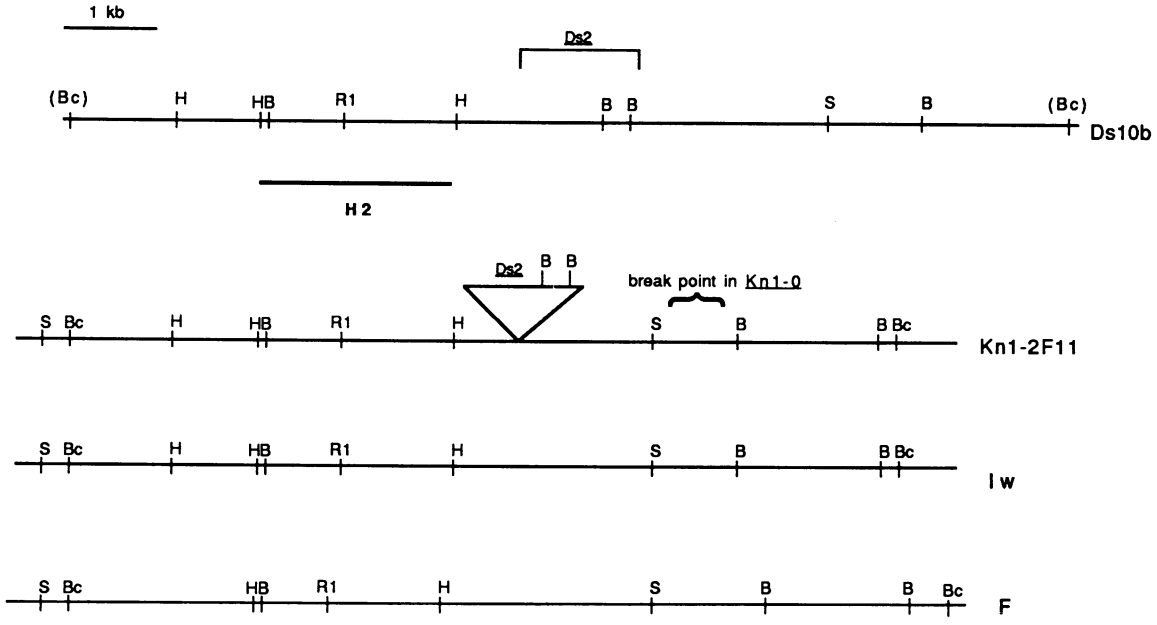

Fig. 4. Restriction map of the *Kn1-2F11* clone and Southern mapping of genomic DNA. The top line, Ds10B, represents the *Bcl*I fragment cloned into lambda and the position of *Ds2* within the clone is indicated. (The *Bcl*I sites were destroyed in the cloning process). The lines below are genomic maps for three maize lines (*Kn1-2F11*, *lw*, *bz2-m Adh1-F*). S, *Sac*I; Bc, *Bcl*I; H, *Hin*dIII; B, *Bam*HI, R1, *Eco*RI; H2, a 2.2 kb *Hin*dIII fragment that was used as a hybridization probe.

cosegregates with *Adh1-S* when H2 is used as a probe (see map in Figure 4) and the DNA is cleaved with *Bam*HI (Figure 7, lanes A and E). Hybridization to *Kn1-O* DNA, which is linked to *Adh1-F* in this experiment, detects not only a 5.0 kb fragment, but also a novel 15 kb fragment (Figure 7, lanes C and D). The 15 kb and 5 kb fragments cosegregate with the *Kn1-O* phenotype in all lines we have looked at. Genomic Southern blots of *Kn1-O* DNA cleaved with *Bcl*I also reveal an additional fragment, but not when the DNA is cleaved with *Sac*I (unpublished data). The data suggest that the novel 15 kb fragment in *Kn1-O* is a duplication, the breakpoint of which lies between the *Sac*I site and the *Bam*HI site on the map in Figure 4.

X-ray mutagenesis of *Kn1-O* plants was initiated in order to create a *Kn1* deletion stock. Tassels of plants homozygous for *Kn1-O* and *Adh1-F6* were irradiated with 1000 rads prior to pollinating normal plants carrying *Adh1-S*. Approximately 10 000 heterozygous plants were screened for normal phenotype. Of the 10 normal plants found, one seemed to carry a deletion as indicated by transmission of *Adh1-F6* marker through the female gametophyte only (McClintock, 1942). The deletion does not affect the *Adh1* or *lw* locus (data not shown).

To determine if the deletion overlaps the Ds10b clone, we isolated DNA from seedlings that were heterozygous for the deletion. DNA from plants that were heterozygous for the *Adh1-S* and *Adh1-F* markers and either *Kn1-O* or normal, served as controls. (*Kn1-O* is linked to *Adh1-F* in this control). DNA was cleaved with *Bam*HI and Southern blots were hybridized to the H2 probe. A 4.8 kb fragment segregates with the *Adh1-S* marker (Figure 7, lane A). Both the 5.0 kb and the 15 kb fragments unique to *Kn1-O* are missing in the DNA of the plant heterozygous for the deletion (lane B). Only the fragment of the normal homolog, linked to *Adh1-S*, is present. This suggests that the deletion removes the part of the *Kn1* locus that is included in the Ds10b clone.

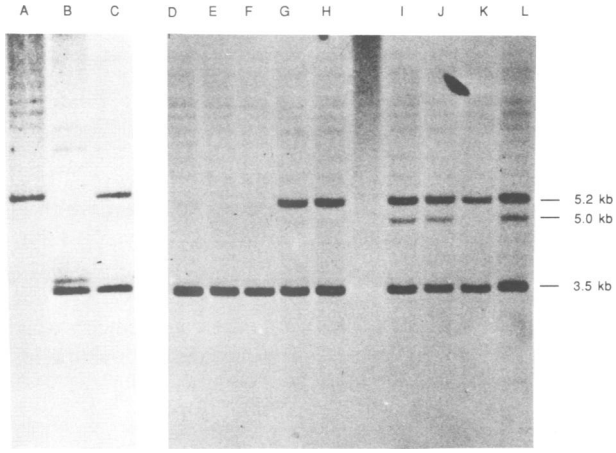

Fig. 5. Evidence for somatic excision of *Ds2* after reintroducing *Ac* into the *Kn1-2F11* line. A plant without *Ac* and homozygous for *Kn1-2F11* as determined by the *Adh1-2F11* allele (**lane B**) was crossed to a plant with *Ac* and carrying *Adh1-F* (**lane A**). These plants were not knotted but some of their progeny were (**lanes J, K** and **L**). The genotypes and culture numbers of these plants are as follows: homozygous *Ac kn Adh1-F*, 475-17 (**lane A**); homozygous *Kn1-2F11 Adh1-2F11* (no *Ac*), 474-2 (**lane B**); *kn Adh1-F/Kn1-2F11 Adh1-2F11 Ac*, 475-15×474-3 (**lane C**); homozygous *Kn1-2F11 Adh1-2F11* (no *Ac*), 474-3, 474-4, 474-2 (**Lanes D, E** and **F**); *kn Adh1-F/Kn1-2F11 Adh1-2F11 Ac*, 475-15×474-3 (**lanes G** and **H**); *kn Adh1-F/Kn1-2F11 Adh1-2F11 Ac*, 474-2×475-17 (**lanes I** and **J**); *kn* Adh1-F/*Kn1-2F11 Adh1-2F11 Ac*, 475-17×474-2 (**lanes K** and **L**). Seedlings with knots (**lanes C, G, H, J, K** and **L**). DNA was digested with *Bam*HI, electrophoresed and transferred to Nytran; the hybridization probe was H2 (**Figure 4**).

Discussion

Most *Ds* elements have not been considered suitable transposon tags due to their high copy number (Geiser *et al.*, 1982; Döring and Starlinger, 1986). Using the *Ds* element isolated from *sh-m5933* (Courage-Tebbe *et al.*, 1983), we

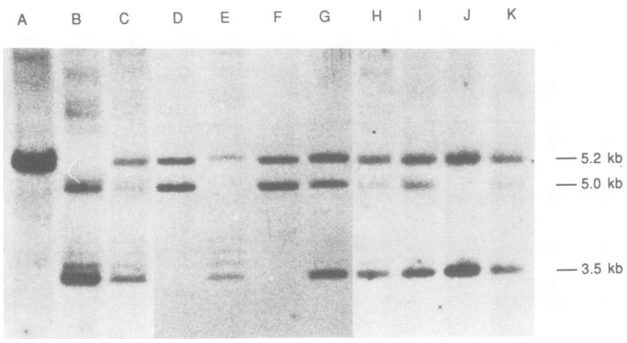

Fig. 6. Evidence for germinal excision of *Ds2* resulting in progeny with normal phenotype. A knotted plant homozygous for *Kn1-2F11* and heterozygous for *Ac* (**lane B**) was outcrossed to the *bz2-m* tester (**lane A**). 43 of the progeny were knotted as seedlings; one is shown (**Lane K**). Five of the progeny had sporadic knots on upper leaves (**lanes C, E, G, H** and **I**). Two remained normal and are revertants (**lanes D** and **F**). For a control, one individual is shown that is not carrying *Ac* as determined by the kernel marker, *bz2-m* (**lane J**). Genomic DNA isolated from seedlings was restricted with *Bam*HI, electrophoresed and blotted onto Nytran.

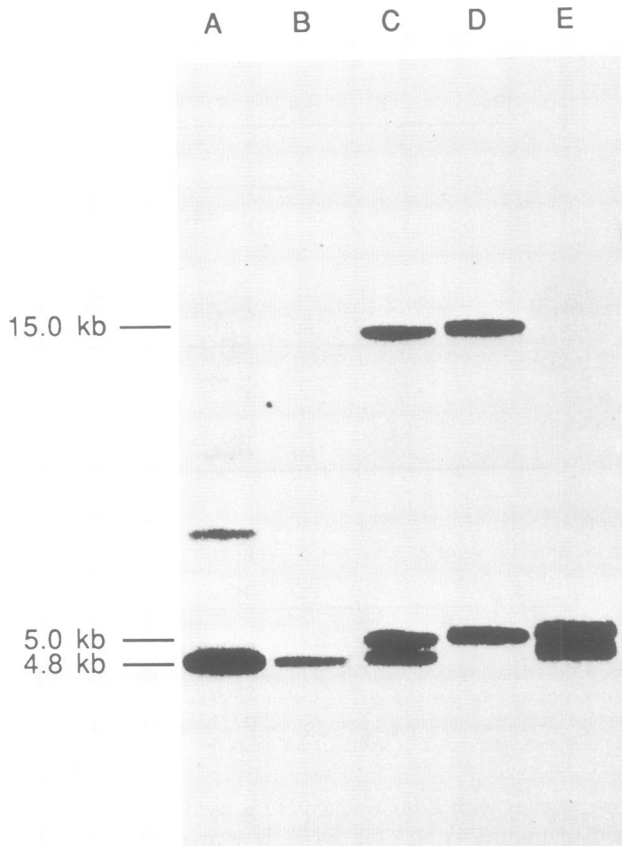

Fig. 7. Hybridization to DNA from *Kn1-O* and a deletion derivative of *Kn1-O*. The genotypes are as follows: homozygous *kn Adh1-S* (**lane A**). (The larger fragment results from partial homology to unlinked DNA and is not seen in repeated experiments.) *Kn1-O-deletion Adh1-F6/kn Adh1-S* (**lane B**). *Kn1-O Adh1-F/kn Adh1-S* (**lane C**). Homozygous *Kn1-O Adh1-F* (**lane D**). *kn Adh1-S/kn Adh1-F* (**lane E**). The DNA was digested with *Bam*HI, electrophoresed and blotted onto nitrocellulose. The hybridization probe was H2. *Bam*HI detects a polymorphism between *Adh1-S* and *Adh1-F*, and between knotted and normal. The 4.8 kb fragment is linked to *Adh1-S* and the 5.0 kb fragment is linked to *Adh1-F* or *Adh1-F6*. The 15 kb and 5.0 kb fragments always cosegregate together in *Kn1-O* lines. Complete enzyme digestion was assured by hybridization of the DNA to other probes, excluding the possibility that the novel fragment resulted from partial digestion.

were unable to detect any segregating fragments in our *Kn1-2F11* lines (our unpublished results), although Theres and coworkers (Theres *et al.*, 1987) were able to clone the *Bz2* locus using that *Ds* as a probe. *Ds2*, however, has an internal region that is not homologous to other *Ac* or *Ds* elements (Merckelbach *et al.*, 1986) and is present in only 10−15 copies in our lines. In this paper we describe the cloning of the *Kn1-2F11* mutation with *Ds2*.

Our data show that the *Kn1-2F11* mutation results from the insertion of a *Ds2* element. Germinal excision of *Ds2* returns the phenotype to normal. But unlike other mutations caused by *Ds* insertions, *Kn1-2F11* appears to require an active *Ac* for the mutant phenotype and an increase in *Ac* dosage results in a more severe mutant phenotype. In other *Ds* mutations, such as *bz2-m* (which we have used to score *Ac* dosage in our experiments), the mutant phenotype does not require *Ac* activity and excision of the *Ds* element, which produces wild-type sectors, is delayed and occurs less frequently with an increase in *Ac* dosage (McClintock, 1948, 1949).

The initial *Kn1-2F11* mutation was described as being 30% penetrant (Freeling and Hake, 1985). One explanation for the poor penetrance is the mutation's apparent *Ac* dependence. If *Ac* is unlinked and heterozygous, then 1/4 (no *Ac*) of the seedlings would not be knotted and only 1/4 (homozygous *Ac*) would show the phenotype strongly following self-pollination. Also, the high rate of germinal excision may add to an apparent lack of penetrance. We found two plants out of 50 to have lost the *Ds2* element at *Kn1-2F11*.

Before discussing possible models to explain the dependence of *Kn1-2F11* expression on *Ac*, we must consider the possibility that the *Ac* effect is due to a *trans*-acting gene closely linked to *Ac* and not *Ac* at all. If there was a modifier closely linked to the *Ac* on 9S that was required for knotted expression, one might find occasional recombination events that separate the two genes. The resulting progeny following recombination would contain *Ac* but not show knots, or would show knots in the absence of *Ac*. We find plants that carry *Ac* and *Kn1-2F11* but do not express knots quite frequently. We have attributed this low expression to a problem in penetrance and a high rate of reversion. We have also found two exceptions that are knotted in the absence of *Ac*. In both exceptions the *Ds2* element still resides at the *Kn1-2F11* locus by molecular analysis (our unpublished results). These exceptions could be due to a change in the *Kn1-2F11* locus or could result from recombination between a modifying gene and the *Ac* on 9S. In order to explore the possibility that a modifying gene could be separated from *Ac* by recombination, we have crossed a different *Ac*, *P-vv*, with *Kn1-2F11*. We find that this *Ac* is not able to activate *Kn1-2F11* to the same extent as the *Ac* on 9S. This result supports the possibility that the *trans*-acting gene is not *Ac* but a linked modifier, but it could also reflect differential activity of *Ac* elements on different *Ds* elements. For example, *Ac2* only causes transposition of *Ds2* elements (Rhoades and Dempsey, 1982). Definitive proof will result from the ability of another *Ac*, or this *Ac* transposed to a new location, to activate *Kn1-2F11* to the same extent as this *Ac* on 9S. Alternatively, if the modifier that activates

Kn1-2F11 is now unlinked from *Ac* in the two exceptions, then individuals from those families should be capable of activating the phenotype.

Our first model for the dependence of *Kn1-2F11* on *Ac* is based on the excision of *Ds2*, of which we have molecular evidence. If *Ds* excises imperfectly quite frequently in the developing leaf, it could cause small deletions in the '*kn*' gene in some cells. The new product that results from the deletion could interfere with normal leaf development and cause knot formation. This model does not explain the dosage effect of *Ac*, assuming the temporal regulation of *Ds* excision is the same at *Kn1-2F11* as it is at *bz2-m*. Furthermore, we do not detect germinal stable revertants that remain knotted without *Ds* and without *Ac*, although this may result from the fact that the cells which contribute to the first few leaves do not necessarily contribute to the rest of the plant (Poethig *et al.*, 1986). Therefore, the knotted leaves of *Kn1-2F11* are not necessarily of the same lineage as the 'germline' of the plant. The excision model is also weakened by the fact that occasionally DNA samples from knotted seedlings do not contain any detectable sign of *Ds2* excision (Figure 5, lanes G, H and K) and likewise there is molecular indication of *Ds2* excision from seedlings that are normal in phenotype (Figure 5, lane I).

The other model proposes that the insertion of *Ds2* results in the novel *Kn1-2F11* phenotype when a *trans*-acting gene, which we believe is *Ac*, is present, and that excision results in a normal phenotype. A higher rate of somatic excision occurs with one dose of *Ac* than with two, and therefore there is less chance for the plant to exhibit the knotted phenotype with one *Ac*. By this model, we might also expect to find patches of revertant normal tissue which coincide with perfect excision of *Ds*. As seen in Figure 1, the *Kn1* phenotype is only evident on the lateral veins. The knots and wide veins are sporadic and sectors of normal tissue are present, but whether they represent revertant tissue or just the erratic nature of the mutation is difficult to establish.

Interactions such as proposed between *Ac* and *Kn1-2F11*, have not been described for *Ac* before, but have been described for another maize transposon, *Spm* (*En*) (Peterson, 1953; McClintock, 1954; reviewed by Fedoroff, 1983). Masson and coworkers (Masson *et al.*, 1987) have designated McClintock's defective *Spm* alleles, such as *a-m2-7995, Spm-dependent* alleles. They contain a non-autonomous, defective *Spm* at the *a* locus. These kernels are colorless in the absence of a *trans*-acting, non-defective *Spm*. In its presence, there are two types of color expression: a mildly pigmented background resulting from partial activation of the *a* gene by the *trans*-acting *Spm*, and deeply pigmented spots resulting from excision of the defective *Spm*. In these alleles, the *a* locus appears to have come under control of the *trans*-acting *Spm* element. Molecular analysis of the defective *Spm* elements suggested that it is not the element itself that predicts the nature of the interaction, but the insertion site (Masson *et al.*, 1987; Schwarz-Sommer *et al.*, 1987). If such an interaction were taking place between *Ac* and *Kn1-2F11*, the mild knotted phenotype would be analogous to the pigmented background present only when a *trans*-acting transposon is present and would explain why no knots are seen without *Ac*. Even if excision of *Ds2* at *Kn1-2F11* decreases with *Ac* dose, the positive interaction described above might increase. In either case, the knotted expression would increase with *Ac* dose. The interaction might involve binding of an *Ac* product to *Ds* thereby altering transcription or splicing and making a new *kn* product. Splicing of *Ds* elements from transcribed genes has been described for *Adh1* (Simon and Starlinger, 1987) and *waxy* (Wessler *et al.*, 1987).

One of the questions that will be interesting to pursue is how the different *Kn1* mutants are related. They all affect the lateral veins, either by inhibiting greening and proper differentiation of the surrounding mesophyll and bundle sheath cells, by making knots along the veins, by displacing the ligule up into the leaf blade or by inducing *de novo* ligule formation parallel to the veins. The mutants differ in their severity and timing. Our data suggest that *Kn1-2F11* arose from the insertion of a transposable element. Although insertions normally do not cause dominant mutants, the inhibitor of color expression in maize, *C1-I*, is a dominant insertion mutant (Cone *et al.*, 1986). The inhibitor allele produces a transcript which is more abundant than the normal *C1* allele and of a slightly different size. Perhaps the gene product of *C1-I* competes with the wild-type product (Cone *et al.*, 1986). There are more examples of dominant mutations caused by insertions in *Drosophila*, such as *Hairywing* (Campuzano *et al.*, 1986), the *glued* locus (Swaroop *et al.*, 1985), one of the alleles at the *white* locus, w^{DZL} (Zachar and Bingham, 1982), and some of the dominant *Antennapedia* alleles (Scott *et al.*, 1983). Our data from hybridization to *Kn1-O* DNA suggest there may be a duplication of genomic DNA rather than an insertion. This is reminiscent of the *Hairywing* mutation in *Drosophila*, where both the insertion of an element and position-effect rearrangements can produce the same excess function phenotype (Campuzano *et al.*, 1985; Garcia-Bellido, 1979). The aneuploid studies that were carried out with three of the *Kn1* mutants, but not *Kn1-2F11*, suggested that *Kn1* is a neomorphic mutation, either making a product new to the leaf or vastly overproducing a normal product (Freeling and Hake, 1985). Perhaps a new product has been made at the *kn* locus, albeit by different mechanisms. Finally, we must also consider the possibility that only the timing of expression is altered in the *Kn1* mutants and that the 'new product' is actually a wild-type product seen by new cells or at a new time.

Materials and methods

Maize stocks and recombinant clones

The albino *lw* marker, the *Adh1-F6* and the *bz2-m* marker were obtained from the laboratory of D.Schwartz. The *P-vv* stock was a gift from I.Greenblatt. The *Adh1-2F11* mutation came from P.Starlinger (Döring *et al.*, 1984). Our *Kn1-O* stock came from the Maize Genetics Cooperative, Department of Agronomy, IL, USA.

The *Ds2* clone was a gift of A.Merckelbach of the Starlinger laboratory in Cologne, FRG. We subcloned a 100 bp *Bam*HI–*Eco*R1 fragment from their Ds 8/3 deletion subclone into pUC9. The pAc9 clone was kindly made available to us by N.Fedoroff.

Genetic crosses for segregation analysis

The albino mutation, *lw*, is 1–2 map units from *Kn1* (Chen *et al.*, 1986) *Kn1-2F11* plants were crossed to plants heterozygous for the albino mutation and not knotted (*kn Lw/kn lw*), then self-pollinated for two generations to generate material that was segregating for *lw*. Albino seedlings were *kn/kn*.

The *Adh1-2F11* mutation is also closely linked to *Kn1-2F11*. Because the *Ds2* element at *Adh1-2F11* has excised improperly in our lines, the *Adh1-2F11* allele remained null. The *Adh1* genotypes were determined by pollen genotyping (Freeling, 1976) or starch gel electrophoresis (Freeling

and Schwartz, 1973) in the following cross:

Ac bz2-m Adh1-2F11 × *Adh1-F bz2-m*

↓

Adh1-2F11/Adh1-F Ac/+ bz2-m/bz2-m

⊗

↓

Adh1-2F11-/Adh1-2F11 *Adh1-2F11/Adh1-F* *Adh1-F/Adh1-F*

Ac segregated independently of *Adh1* in the resulting progeny. Seeds carrying an active *Ac* as determined by the *bz2-m* marker were knotted if they also carried *Adh1-2F11*.

Tests for active Ac

Knotted plants were crossed to a *bz2-m* line without *Ac* to determine if there was an active *Ac* present, indicated by sectored kernels. When kernels were without sectors, DNA was isolated from seedling leaves, digested with *Pvu*II, electrophoresed and hybridized to the 1.6 kb *Hin*dIII fragment of the pAc9 plasmid (Fedoroff *et al.*, 1984). The *Pvu*II sites in *Ac* are only cleaved when the *Ac* is active, whereas the *Pvu*II sites in the cryptic, inactive, *Ac* elements are not sensitive to digestion (Chomet *et al.*, 1987). Thus, the inactive elements are separated from the active elements by gel electrophoresis.

Genomic cloning

DNA was extracted by the method of Murray and Thompson (1980) from 2 week old, green, knotted seedlings from a population that was segregating for *lw* and *Kn1-2F11*. The DNA was digested with *Bcl*I and fractionated through a 10–40% sucrose gradient. Fragments in the 10–20 kb range were recovered and cloned into the *Bam*HI site of EMBL3 (arms prepared by Stratagene). The library of 500 000 recombinants was screened with plasmid subclone, pAM. Six phage plaques were purified, DNA was prepared and digested with *Sal*I. One recombinant had a 12 kb insert and was subcloned into pUC18. This clone, Ds10B, was further analyzed by restriction enzyme digestion and blot hybridization. The cloning and subsequent techniques were done according to Maniatis *et al.* (1982).

Southern blot hybridization

Restriction digests of plant genomic, phage and plasmid DNAs were fractionated on 0.8% agarose gels and transferred to either nitrocellulose or Nytran filters (Schleicher and Shuell). The filters were hybridized and washed as described (Strommer *et al.*, 1982). DNA preparations (except the DNA used in cloning) were done by modifications to the unpublished method of R.Martienssen. 1–2 g of leaf tissue were pulverized in a mortar and pestle with liquid nitrogen, resuspended in 5 ml of extraction buffer [0.1 M Tris–HCl pH 8.5, 0.1 M NaCl, 50 mM EDTA, 2% SDS, 0.4% diethyldithiocarbamic acid and 100 μg/ml Proteinase K (Sigma)], and ground gently before thawing. The slurry was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol precipitated, followed by one additional extraction and ethanol precipitation.

X-irradiation of post-meiotic tassels

Irradiation was done to the tassels of mature *Kn1-O Adh1-F6* plants in pots at Lawrence Berkeley Labs at 80 rad/min for a total of 1000 rads 10 days prior to pollinating. The pollen was collected and used to pollinate detasseled females of *kn Adh1-S* genotype.

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