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Transposon Tagging and Molecular Analysis of the Maize Regulatory Locus *opaque-2*

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Genetic analyses suggested that the *opaque-2* (*o2*) locus in maize acts as a positive, trans-acting, transcriptional activator of the zein seed storage-protein genes. Because isolation of the gene is requisite to understanding the molecular details of this regulation, transposon mutagenesis with the transposable element *suppressor-mutator* (*Spm*) was carried out, and three mutable *o2* alleles were obtained. One of these alleles contained an 8.3-kilobase autonomous *Spm*, another a 6.8-kilobase nonautonomous *Spm*, and the third an unidentified transposon that is unrelated to *Spm*. A DNA sequence flanking the autonomous *Spm* insertion was verified to be *o2*-specific and provided a probe to clone a wild-type allele. Northern blots indicated that the gene is expressed in wild-type endosperm but not in leaf tissues or in endosperms homozygous for a mutant allele of the *O2* gene. A transcript was detected in endosperms homozygous for mutations at *opaque-7* and *floury-2*, an indication that *O2* expression is independent of these two other putative regulators of zein synthesis.

THE ZEIN STORAGE PROTEINS OF maize constitute a family of approximately 15 to 22 polypeptides. By SDS–polyacrylamide gel electrophoresis they can be separated into two major size classes of approximately 22 and 19 kD (*1*). Synthesis of all zein polypeptides in the endosperm is coordinately regulated, beginning at 12 days after pollination (DAP) and peaking at about 22 to 25 DAP. Zein proteins are sequestered in protein bodies derived from the endoplasmic reticulum, and translation of zein messenger RNA (mRNA) is accomplished by polysomes located directly on the surface of these protein bodies (*2*). At seed maturity zeins may represent 60% or more of the total protein (*3*).

Several mutations that decrease the amount of zein in the seed have been described (*1*). In contrast to wild-type kernels that have hard, translucent endosperms, mutant kernels have endosperms that are soft and opaque. One of these mutations, *opaque-2* (*o2*), can result in a 50 to 70% reduction in zein content (*3*). In some inbred strains—for example, Oh43, W22, or W64A—the 22-kD class of zeins is affected substantially more than the 19-kD class. In plants homozygous for *o2*, this selective decrease cannot be attributed to defects in synthesis, transport, or protein processing but is rather the result of a deficiency in the zein mRNA's for the 22-kD subgroup (*4*). Genetic linkage analysis has placed the *o2* locus on the short arm of chromosome 7, whereas several of the genes for the 22-kD zeins have been mapped to chromosomes 4 and 10 (*1*). Although a few zein structural genes have been mapped to the same chromosome arm as *o2*, they are not the ones affected by *o2* mutations. These results suggest that the *O2* gene is a trans-acting regulator of zein expression. At the molecular level, the nature and complexity of the role that *O2* plays in zein expression can be addressed only after the gene is cloned.

Since the product of the *O2* gene has not been identified and is not likely to be abundant, transposon tagging appears to be the best approach to clone this regulatory locus. Two mutable alleles of *o2* have been described by Salamini and his colleagues (*5*): one contains a *Ds* element (nonautonomous *Ac*), and the other a novel, nonautonomous transposon that they named *Bergamo* (*Bg*). Unfortunately, neither transposon has thus far proved useful as a molecular tag; molecular probes for the *Bg* element do not exist, and molecular analysis of *Ds* insertions is complicated because DNA sequences of *Ds* elements can be very dissimilar from each other as well as from *Ac* (*6, 7*). Therefore, we attempted to introduce a different transposable element into the *O2* gene (Fig. 1A),

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namely the *suppressor-mutator* (*Spm*) or *enhancer* (*En*) element (*8*). This decision was based on the fact that molecular probes for *Spm* have recently proved successful in cloning several maize genes containing both autonomous and nonautonomous *Spm* insertions (*9, 10*). An F_1 population of approximately 530,000 seeds was screened for mutable *o2* (*o2-m*) kernels. Three kernels bred true for the mutable phenotype and have been designated *o2-m20, o2-m21*, and *o2-m22* (*11*).

Genetic crosses with the *o2-m*'s were initiated to test for the presence of *Spm* insertions. Simultaneously, we attempted to identify an *Spm* at the *o2* locus by determining whether a specific restriction fragment could be correlated with the opaque-mutable phenotype. Plants were grown from F_2 crosses segregating for *o2* and *o2-m* seeds and also from seeds of both parental types. DNA's prepared from leaf samples of individual plants were digested with each of ten methyl-sensitive enzymes (*12*). The decision to use methyl-sensitive enzymes was based on the observation that maize DNA is highly methylated, but sequences in and around active transposable elements tend to be undermethylated (*6, 13*). Our laboratory recently used this approach to clone the *C1* gene via the active *Spm* of *c1-m5* (*10*). Comparisons of Southern blots probed with an *Spm*-specific probe (*14*) showed that a unique restriction fragment could be correlated with the mutable phenotype for *o2-m20* and *o2-m21*, but not for *o2-m22*. An example from a Cla I digest for *o2-m20* is shown in Fig. 1B. The *o2-m20* plants contained a novel 8.4-kb Cla I band absent in both parents and missing from at least some of the individuals that had been classified as opaques (Fig. 1B). Those kernels that had been classified as opaque but possessed the 8.4-kb Cla I fragment were evidently not mutable because of failure of the *Spm* to transpose, or else transposition occurred so late in endosperm development that revertant sectors were undetectable, giving the kernels an opaque rather than an obvious opaque-mutable phenotype. The Cla I fragment was cloned into λgtWES · λB (*15*). Phage plaques were screened by hybridization with an *Spm* probe. Restriction enzyme mapping of the clones from *o2-m20* revealed the presence of an apparently full-length, autonomous 8.3-kb *Spm* and an adjacent sequence of about 150 bp. This non-*Spm* region was sequenced, and an Rsa I site was found just 19 bp from the junction with *Spm*. A 0.1-kb Rsa I–Eco RI subclone was generated from this region. To test whether this fragment was *o2*-specific, we adopted a combined genetic and molecular approach.

Maize inbreds exhibit such a high degree of restriction fragment length polymorphism (RFLP) that one inbred can readily be distinguished from another with any given probe by the use of just a few restriction enzymes (*16*). One can therefore use Southern blots to examine individuals in a backcross population segregating for the allele of choice and affirm that the presumptive probe detects parallel changes predicted from the segregation analysis. For this reason, Oh43 *o2/o2* plants were pollinated by W22 *O2/O2* inbreds. The F_1 heterozygotes were then backcrossed to the Oh43 *o2/o2* parent, producing opaque (*o2/o2*) and normal (*o2/O2*)

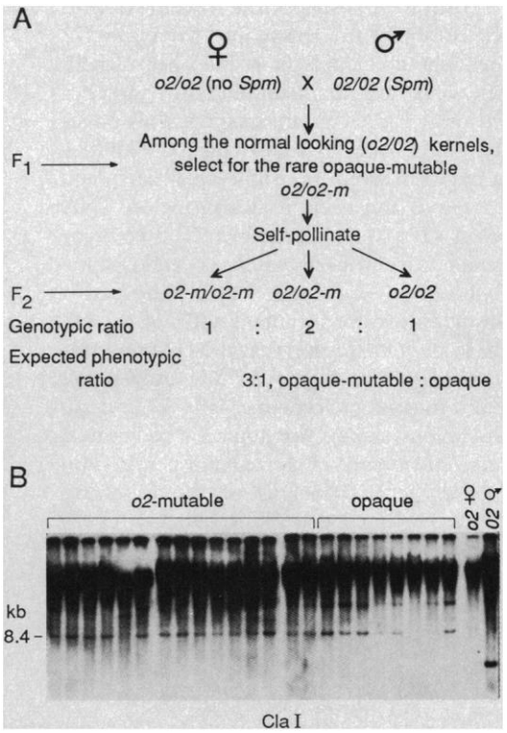

Fig. 1. (**A**) Diagram of the transposon tagging scheme used to generate and subsequently identify *Spm* insertions into the *O2* gene (*23*). (**B**) Autoradiogram of a Southern blot showing cosegregation of the *o2*-mutable phenotype with an 8.4-kb Cla I band that hybridizes with a probe derived from the *dSpm* at *wx-m8* (*14*). DNA was prepared from young leaves of individual plants from F_2 seed that were segregating for opaque-mutable or opaque kernel phenotypes, as well as from both parents. Neither the opaque female parent (*o2* ♀) nor the normal male parent (*O2*♂) has the 8.4-kb band. This is consistent with the idea that this band represents a new transposition of *Spm* into the *O2* gene. Individuals from the opaque class that had the 8.4-kb band produced opaque-mutable progeny when self-pollinated. Thus, these plants must have been carrying at least one copy of the *o2-m20* allele but, because of very late transpositions, were classified as opaque rather than opaque-mutable. One individual in the *o2*-mutable class did not have the 8.4-kb band. However, when the mature plant was self-pollinated, wild-type and opaque kernels were produced in a 3:1 ratio. This indicates that it was genetically *o2/O2*, yet it was mistakenly classified as an opaque-mutable. Self-pollination of all the other individuals produced the kernel phenotype expected on the basis of their original phenotypic classification. Isolation of DNA was performed as described (*16*) except that banding of DNA in CsCl was omitted. For each individual, approximately 1 μg of DNA was digested overnight with 3 units of Cla I (*12*). Restriction fragments were separated by 0.5% agarose gel electrophoresis, blotted to nitrocellulose, and hybridized with either of two *Spm*-specific probes (*14*) that were nick-translated. All conditions were as described (*16*) except that hybridizations were performed at 50°C.

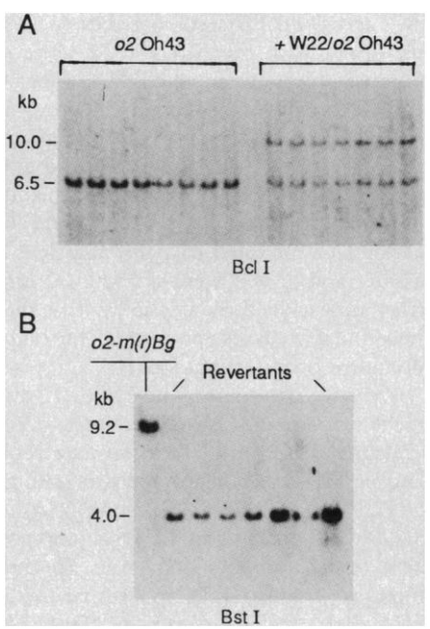

Fig. 2. (**A**) Southern blot hybridized with a putative clone of *o2* showing cosegregation of the RFLP's with the *o2* phenotype. The probe was a 0.1-kb Rsa I–Eco RI subclone of DNA flanking the *Spm* element cloned from *o2-m20* (see text). Plants represented a backcross population segregating 1:1 for kernels that had either opaque or normal phenotypes. For each sample, approximately 1 μg of DNA was digested with Bcl I under conditions specified in (*14*). Other details of the method are described in the legend to Fig. 1B, except that the probe was labeled with ^{32}P by random priming (*26*) and the hybridization was performed at 42°C. (**B**) Southern hybridization analysis of *o2-m(r)Bg* and revertants (*20*). DNA samples were digested with Bst I and probed with the 0.1-kb Rsa I–Eco RI subclone derived from *o2-m20*. A 9.2-kb restriction fragment hybridized in the plant homozygous for the *o2-m(r)Bg* allele. In all seven revertants, which were independently derived, a fragment that is 5.2 kb smaller was recognized, and presumably represented a fragment from which the *Bg* element had been excised. Preparation of DNA for digestion with the restriction enzyme Bst I, agarose gel electrophoresis, preparation of the Southern blot, and hybridization conditions were performed as for (A). The sizes of the restriction fragments hybridizing to the probe are indicated.

kernels segregating in a 1:1 ratio. Figure 2A shows the results when DNA's from individual plants of the backcross population, which had been classified as either opaque or normal on the basis of kernel phenotypes, were hybridized with the putative *o2* probe. All the plants derived from opaque (*o2/o2*) seeds showed a single 6.5-kb fragment, whereas plants from seeds with a normal phenotype (*o2/O2*) had a 10-kb fragment in addition to the 6.5-kb fragment. This demonstrates that the 0.1-kb subclone derived from *o2-m20* represents a single-copy sequence that cosegregates with alleles of the *o2* locus.

As a further demonstration that the Rsa I–Eco RI subclone from *o2-m20* was *o2*-specific, DNA from plants carrying the mutable allele *o2-m(r)Bg* was examined along with DNA from several homozygous stable revertants (*17*), provided by F. Salamini (Fig. 2B). The consistent diminution in

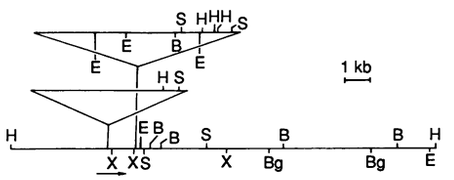

Fig. 3. Restriction map of a 17-kb Hind III genomic clone of the *O2* gene showing the insertion sites of the *Spm* elements in *o2-m20* and *o2-m21*. The labels B, Bg, E, H, S, and X represent restriction sites for Bam HI, Bgl II, Eco RI, Hind III, Sal I, and Xho I, respectively. The position of the 6.8-kb *dSpm* in *o2-m21* was deduced from a 14-kb Hind III genomic clone extending from the Hind III site in the *dSpm* to the Hind III site on the right side of the restriction map. The orientation of the 8.3-kb *Spm* in *o2-m20*, which is inserted within a Cla I fragment of about 150 bp (sites not indicated), was determined by Southern blots of genomic DNA using restriction enzymes that cut within the element and probes that hybridized to either side of the insertion. DNA for genomic cloning was prepared as described (*16*). Hind III fragments were cloned into the Hind III site of λ2001 (*27*). Methods for packaging and screening recombinant phage were as described (*28*). Direction of transcription (arrow) was determined by hybridizing RNA blots of polyadenylated [poly(A)$^+$] enriched endosperm RNA (legend to Fig. 4) with an end-labeled plasmid clone. The 0.9-kb Xho I fragment that lies between the two *Spm* insertions was subcloned into pUC9 (*29*), digested with restriction enzymes that cut on either side of the insert, and end-labeled with α-^{32}P-ATP (adenosine triphosphate) using T4 polymerase as described (*28*). Labeled plasmids were digested with a second enzyme cutting on the opposite side of the insert, and the end-labeled fragment was purified from 1% low melting point agarose gels. The orientation of the 0.9-kb Xho I fragment in pUC9 relative to the restriction map was determined by the presence of an asymmetrically located Pst I restriction site that is not indicated on the map. These results were confirmed with the use of single-stranded probes generated from the same Xho I fragment cloned into M13mp10 and M13mp11 (Bethesda Research Laboratories).

fragment size associated with genetic reversion provides additional evidence that the subclone represents a portion of the *o2* locus. This subclone was used as a probe to clone a 17-kb Hind III fragment of the *O2* allele from the *c1-m5* parent. Figure 3 shows a restriction map of the cloned fragment and the *Spm* insertion in *o2-m20*.

If *o2* is a transcriptional activator of zein expression, then transcripts from the *O2* gene should be present in endosperm where the zeins are abundant, but not in other tissues where zeins are missing. This possibility was tested by subcloning a 0.9-kb Xho I fragment from the wild-type allele shown in Fig. 3 and using it as a probe on RNA blots (Fig. 4). A prominent 2.0-kb transcript is present in wild-type endosperms but not in leaf tissue or in endosperms homozygous for a mutant allele of the *O2* gene. It is interesting that the *O2* transcript is present in both *opaque-7* (*o7*) and *floury-2* (*fl2*) mutant endosperms. The *o7* and *fl2* mutations, like *o2*, are nonlethal and reduce the zein content in maize endosperms (*1*).

Our genetic analysis of the following cross confirmed that the 8.3-kb *Spm* in *o2-m20* was autonomous:

$$\frac{\textit{o2-m20(Spm), wx}}{\textit{o2, wx}} \times$$

$$\frac{\textit{o2, wx-m8 (dSpm), no Spm}}{\textit{o2, wx-m8 (dSpm), no Spm}}$$

Activation of the defective *Spm* (*dSpm*) in *wx-m8* required the presence of the *o2-m20* allele as evidenced by the cosegregation of the *o2-m* phenotype with the *wx-m* phenotype in 61 of 127 individuals.

In addition to *o2-m20*, our tagging experiment generated two other *o2-m* alleles, *o2-m21* and *o2-m22*. We compared these mutable alleles with the parental *O2* allele on Southern blots of genomic DNA, using the Rsa I–Eco RI *o2* probe derived from *o2-m20*. The sizes of the restriction fragments that hybridized with the probe indicated that both *o2-m21* and *o2-m22* contained the insertion of DNA not present in the *O2* allele. This indicated that the insertions in *o2-m21* and *o2-m22* were affecting *O2* function and excluded the possibility that these two mutable alleles represented unrelated, dominant opaque mutations.

We have cloned a 14-kb Hind III fragment from *o2-m21* that contains a 0.9-kb sequence that hybridizes to an *Spm* probe and shares restriction enzyme sites with the 5′ end of *Spm* (Fig. 3). In contrast to *o2-m20*, which contains an 8.3-kb autonomous *Spm*, restriction mapping from Southern blots of genomic DNA shows that the *o2-m21* allele contains a 6.8-kb *dSpm*. This observation is consistent with our having selected stable, opaque kernels from *o2-m21* that still have the 6.8-kb *dSpm* insert. Mutability at the locus was restored in these lines when they were crossed onto homozygous

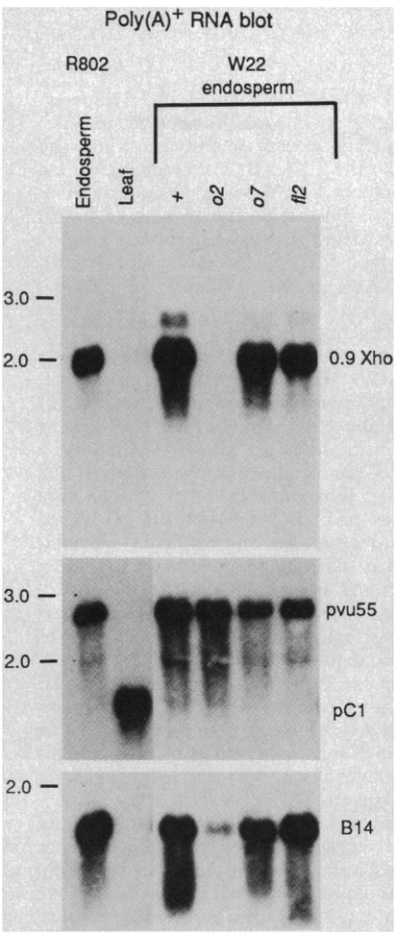

Fig. 4. RNA blot hybridization analyses. Poly(A)$^+$ RNA was obtained from endosperm tissue at 22 DAP or from young leaf tissue. Extraction and purification were as described (*10*) except that the poly(A)$^+$ RNA was purified by passage over Hybond-mAP paper (Amersham) as described by the manufacturer. The RNA was extracted from the leaves or endosperms of the standard maize inbred R802, or from the endosperm of the maize inbred W22 that were normal (+), or homozygous for mutations at *o2*, *o7*, or *fl2*. Approximately 2.5 μg of poly(A)$^+$ enriched RNA was loaded in each lane, except for the lane containing the leaf RNA where 5 μg was loaded. Electrophoresis and blotting conditions are described in (*10*). The nick-translated probe was a 0.9-kb Xho I subclone of the *O2* genomic clone shown in Fig. 3. The subjacent panels show the same blot reprobed with three nick-translated probes (*30*): pvu55, a subclone of sucrose synthetase; pC1, a probe for the small subunit of ribulose bisphosphate carboxylase; and B14, a zein cDNA of the 22-kD zein class. Hybridization with B14 was performed at 50°C. Positions and sizes of maize ribosomal RNAs present in the gel are indicated. Hybridization with pvu55 and pC1 served as a control to demonstrate that RNA was present in all lanes. Hybridization with B14 demonstrated that zein message is not detected in leaves and is severely reduced in the *o2* mutant that is devoid of the *O2* transcript.

o2 plants carrying an active *Spm*.

Molecular and genetic analyses indicated that the insertion in the third unstable opaque mutation, *o2-m22*, is unrelated to the *Spm* family of transposons. On Southern blots in which DNA's were digested with methyl-sensitive enzymes, the mutable phenotype in *o2-m22* could not be correlated with a restriction fragment hybridizing with the *Spm* probes. When plants homozygous for *o2-m22* and the *c1* allele were self-pollinated, opaque-mutable kernels were obtained. When these same individuals were crossed to plants homozygous for a 1.1-kb *dSpm* in the *C1* gene (*10*), no colored sectors were produced in the aleurone, an indication that the mutable phenotype of *o2-m22* is not due to an active *Spm*. We have not yet succeeded in cloning this allele.

Several maize transposons have now been cloned and have provided molecular probes for isolating other maize genes with alleles containing related transposon insertions (*6, 7, 9, 10*). Since such alleles of *o2* were nonexistent, we initiated the genetic crosses necessary to obtain transposon-induced mutations of the locus using the transposon *Spm*. Over a period of 4 years we had conducted three other experiments to tag the *o2* locus with *Mu* (*mutator*) (see *6, 7*), *Ac*, and *Spm*. None of these proved successful. In the tagging experiment reported here, the frequency of *Spm* insertions from an unlinked site into *o2* was about 3.7×10^{-6}. The frequency we detected is within the range of 1.5×10^{-6} to 9.4×10^{-6} reported by Peterson (*18*), who used an *En* (*Spm*) at an unlinked position in the genome to select for mutable *A2* (chromosome 5S) and *C1* (chromosome 9S) alleles. Both these values are low compared to the frequency of *Spm* insertions into linked sites (*19, 20*) for which values as high as 4.4×10^{-4} have been reported.

Morot-Gaudry and Farineau (*21*) reported that the recessive *o2* allele had a detrimental effect on the photosynthetic capacity of the plant. As the *O2* gene does not appear to be expressed in leaves (Fig. 4), the reason for the decrease in photosynthesis of *o2* plants remains obscure but apparently is not directly related to the *o2* allele.

The mutations *o2, o7*, and *fl2* all cause profound reductions in the accumulations of zeins in maize endosperms. Their effects differ, however, in that *o2* reduces the expression of the 22-kD class of zeins to a greater extent than the 19-kD class, whereas *o7* affects primarily the 19-kD class, and *fl2* reduces the accumulation of both the 19- and 22-kD classes. The interaction between these mutant alleles was investigated earlier by examining the zeins in double mutants with all possible pairwise combinations (*22*). The results suggested that the effects of *o2* and *o7* were epistatic to *fl2*, whereas the effect of *o2* and *o7* on zein expression was additive. Our observation that *o7* and *fl2* endosperms have apparently normal levels of an *O2* transcript (Fig. 4) was predicted from these genetic and biochemical analyses. Whether the products of these three loci act independently or in concert is not yet clear. However, if there is a hierarchy in the regulation of zein expression, our results suggest that the product of the *o2* locus is not regulated by *o7* or *fl2*.

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12. Ava I, Cla I, Eco RII, Hae II, Nru I, Pst I, Pvu I, Pvu II, Sal I, and Sst I were all supplied by Bethesda Research Laboratories and used as specified by the manufacturer except that all digests were performed in the presence of 4 m*M* spermidine.
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14. Probes for *Spm* were a 450-bp Ban II–Xba I fragment from the *dSpm* at *wx-m8* and a 2.3-kb Xba I–Sal I fragment derived from *c1-m5* (*10*). These and all of the cloned inserts used as probes were isolated from the plasmid vector by digestion with the appropriate restriction enzymes and separated on 0.6% low melting point agarose gels. Fragments were labeled directly from the low melting point agarose by the methods indicated in the figure legends.
15. Genomic DNA (40 μg), prepared from young leaves as described (*16*), was digested overnight with 120 units of Cla I (*12*). The digested DNA was fractionated on a 0.6% low melting point agarose gel, and the 8.4-kb fragment was excised and purified by Elutips under conditions specified by the manufacturer (Schleicher & Schuell). Blunt ends were generated and Eco RI linkers were attached as described (*28*). The fragment was then cloned into the Eco RI site of λgtWES · λB [P. Leder, D. Tiemeier, L. Enquist, *Science* **196**, 175 (1977)]. Packaging and screening for recombinant phage were performed as described by Maniatis *et al.* (*28*).
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23. The *Spm* material used for the crosses was a McClintock strain bearing *c1-m5* (*24*), a mutable allele of *C1* containing an autonomous *Spm*. *C1* is a regulatory gene in anthocyanin biosynthesis. Also present was *wx-m8*, which contains a 2.2-kb nonautonomous, defective derivative of *Spm* (d*Spm*) (*25*). [The wild-type *waxy* allele (*Wx*) encodes the starch granule–bound uridine 5′-diphosphate (UDP)-glucose glucosyl transferase.] Plants from mutable *c1-m5, wx-m8* kernels were used as pollen donors for homozygous recessive *o2* plants in either the Oh43 or W22 inbred background. Kernels having an opaque-mutable phenotype were selected from the F_1, grown to maturity, and self-pollinated. Those producing opaque-mutable kernels in the F_2 were saved for subsequent molecular and genetic analyses. Among the three ears producing opaque-mutable kernels, we expected to see a phenotypic segregation of 3:1 opaque-mutable to opaque. In fact, all three ears showed a greater than 25% representation of opaque kernels. The excess of opaque kernels was presumed to be due to *o2*-mutable kernels in which excisions of the *Spm* element from *O2* occurred so late in endosperm development that revertant sectors were unrecognizable. Alternatively, if a *dSpm* had inserted into *O2* and an autonomous *Spm* that controlled the activity of the *dSpm* was segregating independently, then again, the ear could show a large proportion of phenotypically opaque kernels. Regardless of the explanation, it is a given fact that some of the phenotypically opaque kernels will be homozygous for the *o2* allele from the female parent.
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