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Cloning of the *y1* Locus of Maize, a Gene Involved in the Biosynthesis of Carotenoids

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The *y1* gene is one of the genes responsible for the production of β-carotene in the endosperm and leaves of maize. We have cloned a Robertson's *Mutator*-tagged allele of the *y1* gene (*y1-mum*) by using a *Mu3* element as a hybridization probe. We substantiate that the cloned sequence is a portion of the *y1* gene by molecular analyses of a revertant of a putative *Mutator*-induced *y1* allele and the incidence of insertions within the cloned *y1* sequence from several independently derived *Mutator*-induced *y1* mutant stocks. The *y1-mum* sequence was used to isolate the standard *Y1* allele, which conditions the presence of β-carotene in the endosperm of the maize kernel.

INTRODUCTION

Carotenoids are a class of lipid-soluble pigments found in all photosynthetic organisms. These pigments function in part to protect chlorophyll from photooxidation (Anderson and Robertson, 1960, 1961). Carotenoid pigments also are found in some nonphotosynthetic organisms and in various nonphotosynthetic tissues of higher plants, including the root, flower, fruit, and pollen. Maize and sorghum contain carotenoids in the endosperm of their kernels. The function of the carotenoids in the endosperm of these plants is unknown (Goodwin, 1980).

The two major types of carotenoids in the maize endosperm are carotenes and xanthophylls; these pigments are primarily responsible for the yellow color of the grain. The predominant carotene in the maize kernel is β-carotene (Goodwin, 1980).

There are a large number of white endosperm mutants in maize that have been analyzed at the genetic level, many of which give rise to albino plants (e.g., *al*, *w3*, *vp9*, *vp7*; Robertson, 1975). Some of these white endosperm-albino seedling mutants of maize have been shown by biochemical analysis to be devoid of β-carotene, but many of these mutants do accumulate precursors of β-carotene (Robertson, 1975; Fong et al., 1983) in both their leaves and endosperm. These mutants are probably defective in one or more of the enzymes responsible for the biosynthesis of carotenoids. Because the unique accumulation patterns of the precursor of β-carotene for each of these maize mutants is the same in both their leaves and endosperm, it is likely that the biosynthesis of β-carotene is achieved by similar, or identical, pathways in both tissues.

The gene product of the *y1* locus in maize also is involved in the biosynthesis of β-carotene (Robertson and Anderson, 1961). The dominant *Y1* allele conditions β-carotene in the endosperm; the recessive *y1* allele conditions a lack of β-carotene in the endosperm. The endosperm of kernels that are homozygous for the recessive *y1* allele is white to pale yellow depending on the genetic background. In this report, the *y1* phenotype will be referred to as white. An allele of *y1* that conditions a lack of β-carotene in both the leaf and endosperm (i.e., white endosperm-albino seedling) has not been identified, but there are two white endosperm alleles of *y1*, *y1-8549* and *y1-wmut*, that condition a reduced amount of β-carotene in the leaf when grown at 35°C (Robertson and Anderson, 1961). These plants can be grown to maturity and often exhibit a diurnal banding pattern of white and light-green pigmentation on the leaves (Robertson and Anderson, 1961). The tissue-specific expression of the various alleles of the *y1* gene suggests that the *y1* gene product may be a regulator of the carotenoid biosynthetic pathway in maize.

In this report, we describe the cloning of a Robertson's *Mutator*-tagged allele of the *y1* gene from maize. This allele was cloned by using a *Mu3*-specific sequence as a hybridization probe. We substantiate that the cloned sequence is part of the *y1* gene by a molecular analysis of a revertant of a putative *Mutator*-induced *y1* allele and also by a comparison of the cloned *y1* sequence within several independently derived *Mutator*-induced *y1* mutant stocks. We have used this sequence to clone an approximately 11.4-kb fragment of DNA from a plant that was homozygous for a standard *Y1* allele.

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RESULTS

Robertson's *Mutator*-Tagged Alleles of y1

Figure 1A depicts the crossing strategy that was employed to produce a *Mutator*-induced mutation of the *y1* gene. Plants homozygous for the dominant *Y1* allele, which carried copies of *Mu* transposable elements, were crossed to homozygous recessive *y1* plants. The offspring kernels were all expected to have yellow endosperms and to be heterozygous *Y1/y1*, but if a *Mu* transposable element transposed into the *Y1* gene (designated *y1-mum*), some progeny kernels would be expected to have white endosperm and to be heterozygous *y1/y1-mum*. Several hundred independently derived putatively *Mutator*-induced *y1* mutant kernels have been generated using this crossing strategy (Robertson, 1985).

The putative *Mutator*-tagged *y1* alleles were all recessive and resulted in a white to pale-yellow endosperm kernel when homozygous. These *y1-mum* alleles can be separated further into two classes, homozygous *y1-mum* white kernels that germinate into green seedlings and homozygous *y1-mum* white kernels that germinate into green seedlings at 25°C and light-green seedlings (referred to as pastel) at 35°C. Similar temperature-sensitive pastel alleles of *y1* have been described previously and were shown to accumulate reduced levels of β-carotene in their leaves (Robertson and Anderson, 1961). The pastel phenotype is believed to be a consequence of chlorophyll photooxidation. As shown in Figure 1B, mutability of the *y1-mum* pastel phenotype could be observed as small, normal green sectors within the light-green tissues of leaves of homozygous *y1-mum* plants. Mutability could not be detected within the endosperm of plants homozygous for the *y1-mum* alleles. For this attempt to clone the *y1* gene, we have worked exclusively with the *y1-mum* pastel alleles.

Identification and Cloning of a *y1-mum*-Linked *Mutator* Element

Plants that carried a *Mutator*-induced temperature-sensitive pastel allele of *y1* (*Y1/y1-mum2053*) were crossed to homozygous recessive *y1* plants, resulting in yellow (*Y1/y1*) and white (*y1-mum2053/y1*) endosperm kernels in a 1:1 ratio (Figure 1A). DNA was isolated from sibling plants grown from each type of kernel, digested with BamHI (a restriction endonuclease that does not cleave within *Mu3*; K.K. Oishi, personal communication), and analyzed by DNA gel blot hybridization using the HindIII-XbaI internal fragment of the cloned *Mu3* element as a hybridization probe (Oishi and Freeling, 1988). It was expected that the *y1* gene, carrying a *Mu* element insertion, would cosegregate as a single DNA fragment exclusively with the plants derived from white endosperm kernels. As shown in Figure 2, the BamHI hybridization profile contained two DNA fragments, approximately 4.4 kb and 5.1 kb in length, that appeared in the parent mutant plant (*Y1/y1-mum2053*) and in all progeny plants derived from white endosperm

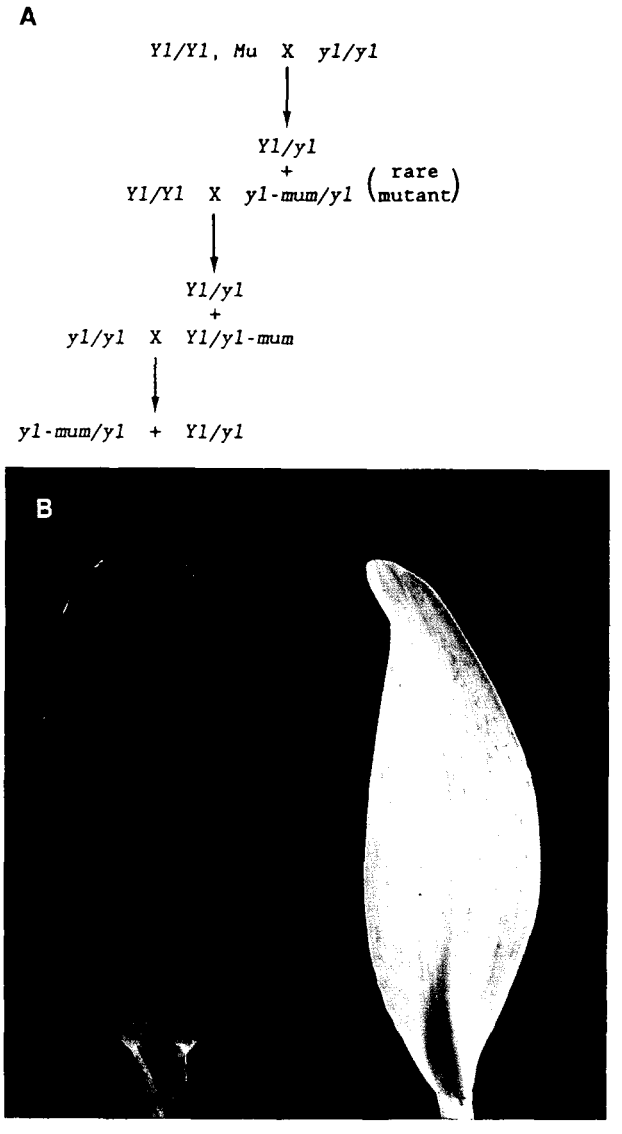

Figure 1. Production, Segregation, and Phenotype of the Robertson's *Mutator*-Induced Mutations of the *y1* Locus.

(A) The crossing strategy used to produce the *Mutator*-induced alleles of *y1* (*y1-mum*) analyzed in Figures 5A and 5B, and the subsequent crosses that resulted in the 1:1 segregation of the *y1-mum* allele analyzed in Figures 2 and 4.

(B) Leaf from a homozygous *y1-mum* plant (right) and from a plant heterozygous for the standard *Y1* and *y1* alleles of *y1* (left). Both plants were grown at 35°C. Transposition of the Robertson's *Mutator* transposable element away from the *y1* locus results in small sectors of normally pigmented tissue.

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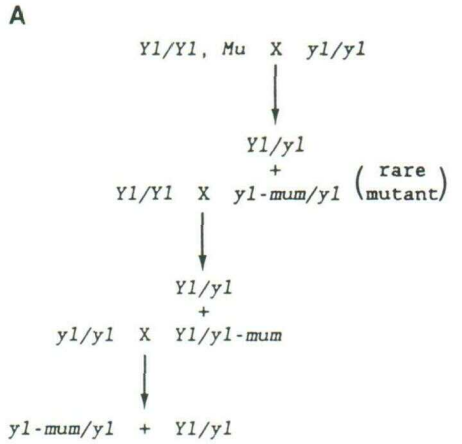

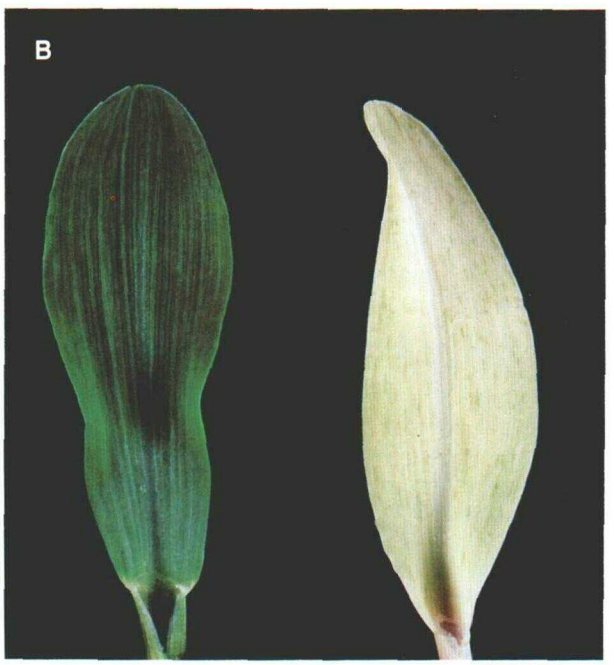

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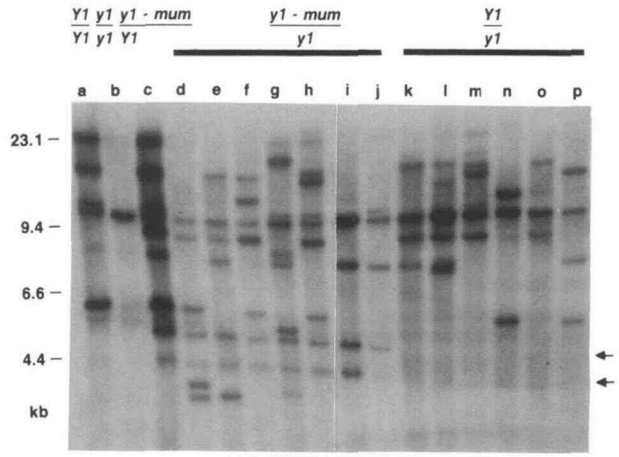

Figure 2. Identification of *Mu3*-Hybridizing BamHI Fragments that Cosegregate with the *y1-mum* Genotype.

Robertson's *Mutator*-induced *y1* mutant plant 2053-6 (*y1-mum/Y1*) was crossed to a homozygous *y1* plant. The resulting ear segregated in a 1:1 ratio for white (*y1-mum/y1*) and yellow (*Y1/y1*) kernels. BamHI-digested DNA from sibling plants derived from white and yellow kernels was analyzed by DNA gel blot hybridization using the internal HindIII-XbaI fragment of *Mu3* as a hybridization probe. The genotype of the plants was as follows: lane a, *Y1/Y1*; lane b, *y1/y1*; lane c, *y1-mum/Y1*; lanes d to j, *y1-mum/y1*; lanes k to p, *Y1/y1*. The arrows indicate the *Mu3*-hybridizing fragments that cosegregate with the *y1-mum* genotype.

kernels (*y1-mum2053/y1*) but were absent in all progeny plants derived from yellow endosperm kernels (*Y1/y1*).

The 4.4-kb *Mu3*-hybridizing BamHI fragment was cloned from a library constructed in λL47 from size-selected BamHI-digested *y1-mum2053/y1* DNA, using the HindIII-XbaI internal fragment of *Mu3* as a hybridization probe. The 4.4-kb DNA insert from this λ clone (designated L47-3) was subcloned into pBR322 and designated pl47-3. The restriction map of this clone showing the approximate location and size of a *Mu3*-hybridizing DNA insertion is shown in Figure 3A. To demonstrate that the cloned DNA is homologous to the 4.4-kb BamHI fragment shown to cosegregate with the white endosperm phenotype, an approximately 150-bp BamHI-PvuII DNA fragment flanking the *Mu3* element from pl47-3 was used as a hybridization probe of BamHI-digested DNA from sibling plants with the genotypes *y1-mum2053/y1* and *Y1/y1*. As shown in Figure 4, this DNA hybridized to both of the *Mu3*-hybridizing DNA fragments that cosegregated with the white endosperm phenotype, as well as 2.4-kb and 3.1-kb fragments of DNA associated with the *Y1* background and a 2.9-kb fragment associated with the *y1* background. This cosegregation indicates that the insert in pl47-3 is homologous to the 4.4-kb *Mu3*-hybridizing fragment that cosegregates with the *y1* phenotype.

One explanation for both the cosegregation of two *Mu3*-hybridizing fragments with the *y1-mum2053* phenotype and the hybridization of the cloned 4.4-kb BamHI fragment to both of these *Mu3*-hybridizing fragments is that a *Mu3* element had transposed into an approximately 2.4-kb BamHI fragment in which one of the BamHI sites is frequently, but not always, methylated. The restriction endonuclease BamHI has been demonstrated to be unable to digest the recognition site 5′-GGATCC when the 3′ cytosine of this sequence is methylated. Therefore, when neither BamHI site is methylated, digestion would result in an approximately 2.4-kb fragment in the progenitor allele and an approximately 4.4-kb fragment in the *Mu3* mutated allele. However, if one of the BamHI sites is frequently, but not always, methylated, digestion would not occur at the methylated site but instead would occur at the next avail-

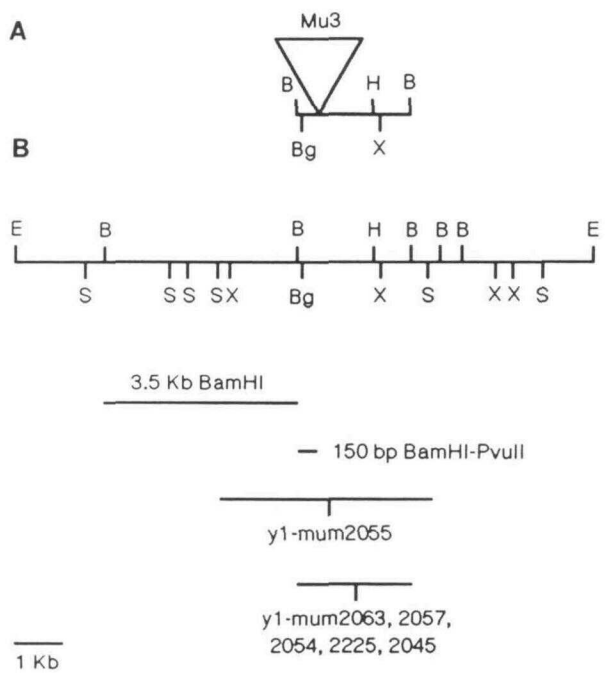

Figure 3. Restriction Endonuclease Map of the *y1-mum* and *Y1* Alleles.

(A) Restriction endonuclease map of 4.4 kb of the cloned *Mu3*-induced allele *y1-mum2053*, showing overlap with the progenitor *Y1* allele and the location of the *Mu3* insert.
(B) Restriction endonuclease map of 11.4 kb of the cloned progenitor *Y1* allele.
The fragments indicated below the transcription unit are the 3.5-kb BamHI and 150-bp BamHI-PvuII hybridization probes described in Results. The *y1-mum* stocks 2055, 2063, 2057, 2054, 2225, and 2045 contain insertions within the region of the *y1* gene indicated by a horizontal line below the restriction map. The restriction endonuclease sites are abbreviated as: E, EcoRI; S, SstI; B, BamHI; X, XbaI; Bg, BglII; H, HindIII.

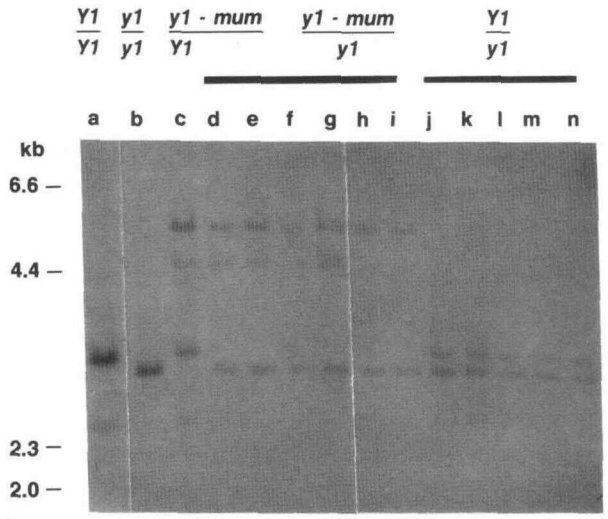

Figure 4. DNA Gel Blot Hybridization Analysis of Sibling Plants Segregating for White and Yellow Endosperm Kernels.

Robertson's *Mutator*-induced *y1* mutant plant 2053-6 (*y1-mum/Y1*) was crossed to a homozygous *y1* plant. The resulting ear segregated in a 1:1 ratio for white (*y1-mum/y1*) and yellow (*Y1/y1*) kernels. BamHI-digested DNA from sibling plants derived from white and yellow kernels was analyzed by DNA gel blot hybridization using as a hybridization probe the 150-bp BamHI-PvuII fragment flanking the *Mu3* element in the cloned *y1-mum* allele. The genotype of the plants was as follows: lane a, *Y1/Y1*; lane b, *y1/y1*; lane c, *y1-mum/Y1*; lanes d to i, *y1-mum/y1*; lanes j to n *Y1/y1*.

able unmethylated BamHI site. This would result in a second, larger BamHI fragment. Both of these fragments are expected to hybridize to the hybridization probe used in this analysis.

Molecular-length determinations indicated that the length difference between the longer and shorter *Y1*-associated fragments (i.e., 3.1 kb and 2.4 kb) is approximately 700 bp, which is the same as the length difference between the *y1-mum2053*-associated fragments (i.e., 4.4 kb and 5.1 kb). The subsequent cloning of the progenitor of the *y1-mum2053* allele (Figure 3 and described below) indicated that an approximately 700-bp BamHI fragment is contiguous on the restriction map with the 2.4-kb BamHI fragment into which the *Mu3* element transposed. These data correlate well with our hypothesis that the *Mu3* element transposed into a 2.4-kb BamHI fragment that contained methylation of one of its BamHI recognition sites.

Analysis of Independently Derived *y1-mum* Alleles

The *y1-mum* alleles used in this study were derived from crosses of *Mu* plants (*Y1/Y1*, *Mu*) as female parents to non-*Mutator* (*y1/y1*) plants as male parents (Figure 1A). Subsequently, all white *y1-mum* kernels found on ears from different plants were derived from independent mutational events. Each of these mutations is likely to be caused by an insertion of a transposable element within the *y1* gene. If the cloned DNA is a portion of the *y1* gene and if it is used as a hybridization probe in a DNA gel blot analysis of various independently derived *y1-mum* plants, it should detect length polymorphisms between the progenitor and *y1-mum* alleles. The cloned sequence would not be expected to detect length polymorphisms if it were not part of the *y1* gene.

DNA from the mutant used to clone the 4.4-kb BamHI fragment and seven independently derived *y1-mum* lines was digested with BamHI and analyzed by DNA gel blot hybridization using as a hybridization probe the 150-bp BamHI-PvuII fragment that flanks the *Mu3* insert in pl47-3. As shown in Figure 5A, in six out of eight *y1-mum* mutant lines analyzed, we detected a length difference between the mutant and progenitor alleles of this sequence. In four *y1-mum* lines, a DNA approximately 1.4 kb longer than the progenitor allele was detected. This correlates well with the length of a *Mu1* element. In *y1-mum* mutant line 2045, a 1-kb BamHI fragment was detected by the hybridization probe. This fragment is shorter than the wild-type allele and, therefore, it is possible that a DNA insertion including a BamHI site may have inserted within the progenitor sequence. The sequences of *Mu1* (Barker et al., 1984), *Mu1.7* (Taylor and Walbot, 1987), *Mu3* (K.K. Oishi, personal communication), *Mu4* (Talbert et al., 1989), and *Mu7* (Schnable et al., 1989) do not contain any BamHI restriction sites; therefore, the DNA insertion in *y1-mum* line 2045 does not appear to be caused by any of these *Mu* elements. We observed two mutant lines, 2207 and 2055, that exhibited no difference between the progenitor and mutant allele when digested with BamHI and hybridized with the 150-bp BamHI-PvuII hybridization probe. It is possible that an insertion outside the BamHI fragment detected by this hybridization probe was responsible for the altered phenotype of these mutants.

To test whether the *y1-mum* mutant lines 2207 and 2055 contain DNA insertions outside the BamHI fragment that has been cloned, we digested DNA from the same mutants as above (excluding *y1-mum2045*) with SstI (a restriction endonuclease that does not cleave within the cloned 4.4-kb BamHI fragment) and analyzed these DNAs by DNA gel blot hybridization using the 150-bp BamHI-PvuII fragment as a hybridization probe. As shown in Figure 5B, this DNA hybridization probe detected a 3.9-kb and a 12.1-kb SstI DNA fragment from *Y1* and *y1* backgrounds, respectively. The DNA hybridization probe detected a 3.9-kb SstI fragment in *y1-mum* line 2207; therefore, this line does not seem to contain a DNA insert in this region of the DNA. In *y1-mum* line 2055, a 2.9-kb SstI fragment was detected by the hybridization probe. This fragment is shorter than the progenitor allele; therefore, it

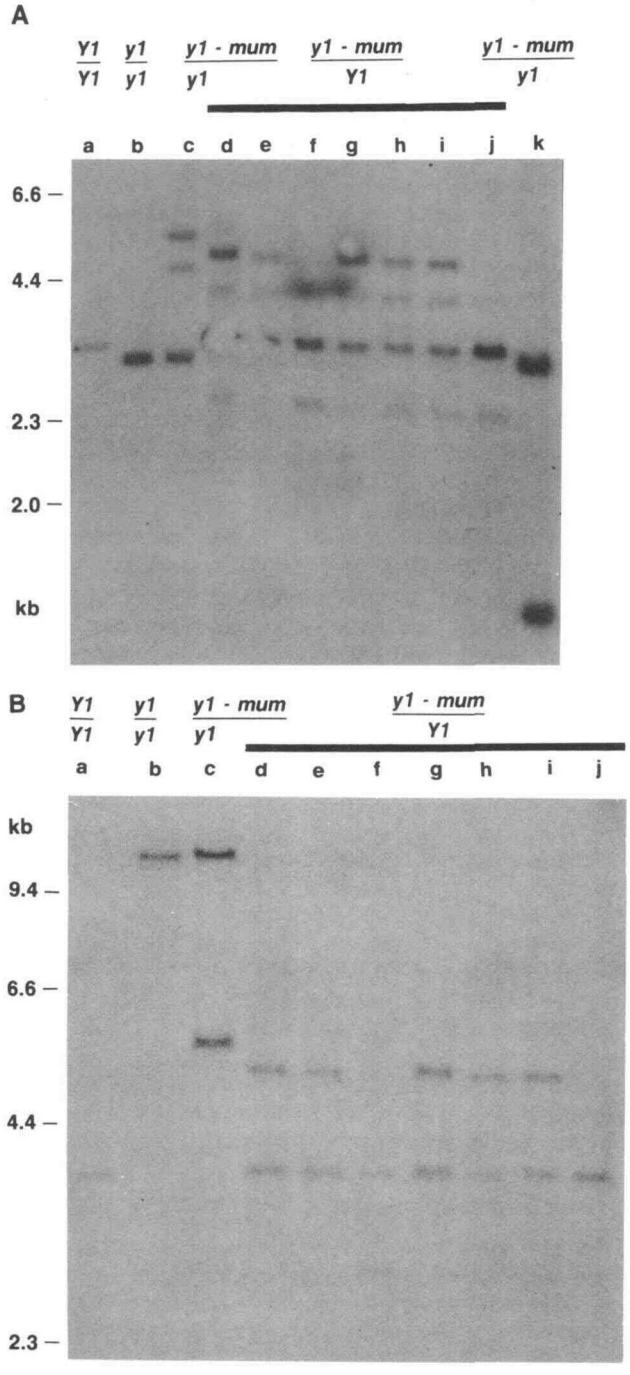

Figure 5. DNA Gel Blot Hybridization Analysis of Independently Derived *y1-mum* Stocks.

DNA was isolated from independently derived *y1-mum* stocks and analyzed by DNA gel blot hybridization using as a hybridization probe the 150-bp BamHI-PvuII fragment flanking the *Mu3* element in the cloned *y1-mum* allele. All *y1-mum* stocks were heterozygous for the *Mutator*-induced *y1* allele and the standard *Y1* or *y1* allele.

(A) BamHI digestion. Lanes a and b are plants with the genotype *Y1/Y1* and *y1/y1*, respectively. Lanes c and k are plants with the genotype *y1-mum/y1*. Lanes d to j are plants with the genotype *y1-mum/Y1*. Lanes c to k are *y1-mum* stocks 2053, 2063, 2057, 2055, 2054, 2225, 2225, 2207, and 2045, respectively.

(B) SstI digestion. Lanes a and b are plants with the genotype *Y1/Y1* and *y1/y1*, respectively. Lane c is a plant with the genotype *y1-mum/y1*. Lanes d to j are plants with the genotype *y1-mum/Y1*. Lanes c to j are *y1-mum* stocks 2053, 2063, 2057, 2055, 2054, 2225, 2225, and 2207, respectively.

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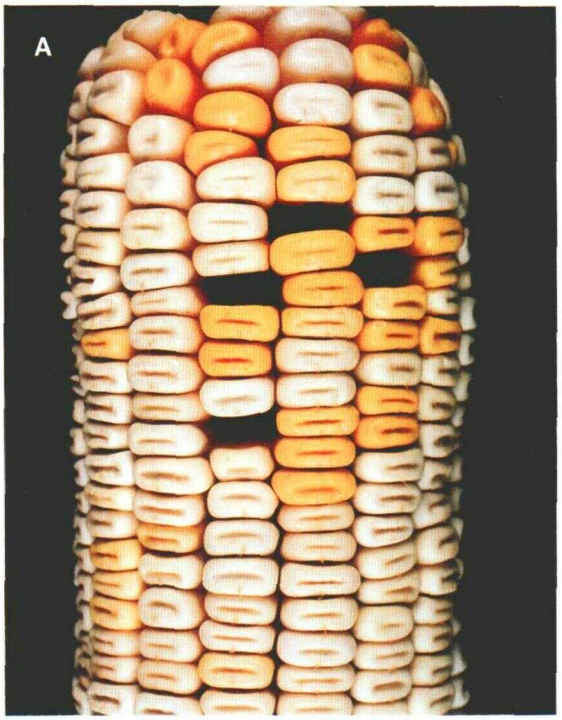
We have shown that six of eight independently derived *y1-mum* lines contain insertions within a 2.4-kb BamHI fragment. Two mutants did not contain an insertion in this BamHI DNA fragment, but one of these two did contain an insert in a 3.9-kb SstI DNA fragment that included the entire 2.4-kb BamHI fragment. In addition, we have demonstrated that the mutations at this sequence have been caused by insertions of DNA similar in size and restriction map to *Mu3* and *Mu1*, as well as other unidentified elements.

Analysis of a Revertant of a *Mutator*-Induced *y1* Mutant

Plants that were homozygous or heterozygous for *waxy* endosperm (*wx*) and also heterozygous for the *y1-mum2045* and standard *y1* alleles were crossed by homozygous recessive *y1/y1*, *wx/wx*, gl1/*gl1* plants. All resulting kernels from this cross would be expected to have a white endosperm. However, if the *Mu* transposable element presumed to be present at the *y1* locus transposes out of the *y1* locus, the *y1-mum* allele may phenotypically revert to a *Y1* phenotype (yellow endosperm kernel). We observed several such putative reversion events, some of which arose as multikernel sectors on the progeny ears, as shown in Figure 6A. As expected, the multikerneled revertant sectors segregated for yellow and white endosperm kernels. The expected phenotype and genotype of these kernels would be yellow kernels from a revertant sector, *Y1-revertant/y1*; white kernels from a revertant sector, *y1/y1*; and white kernels from outside a revertant



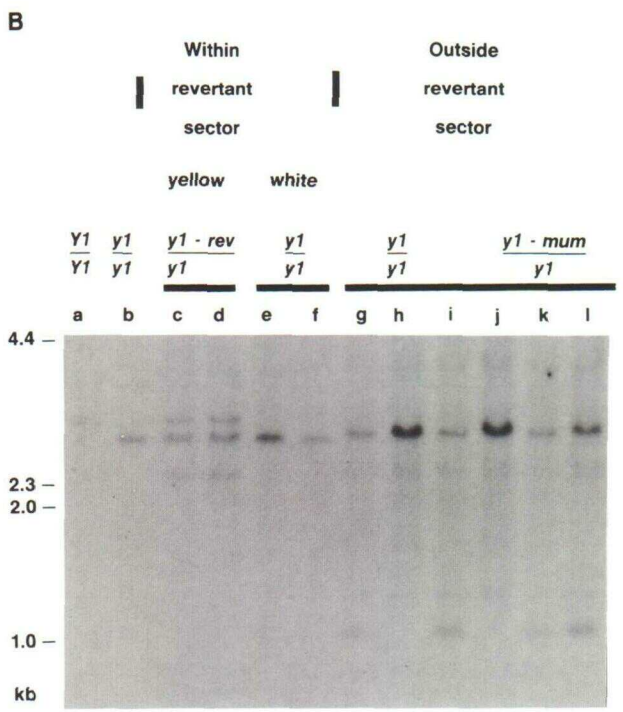


Figure 6. DNA Gel Blot Hybridization Analysis of a Revertant of a Putative *Mutator*-induced *y1* Mutant.

(A) Robertson's *Mutator*-induced *y1* mutant stock 2045 (*y1-mum/y1*) was crossed to a homozygous *y1* plant. The resulting ear contained white kernels with a multiple-kerneled revertant sector that segregated in a 1:1 ratio for white and yellow kernels. The sector, *y1-mum2045/y1* or *y1/y1*. The yellow, putatively revertant, kernels selected for molecular analysis (below) were also *waxy* and gave rise to *glossy* seedlings (*wx/wx*, *gl1/gl1*). Therefore, it is unlikely that the yellow kernels are due to contamination of *Y1* pollen, which would be expected to be *Y1/Y1*, *Wx/Wx*, *Gl1/Gl1*.

Plants were grown from kernels from inside and outside the revertant sector from one of these ears. DNA was isolated from these plants, digested with BamHI, and analyzed by DNA gel blot hybridization using as a hybridization probe the 150-bp BamHI-PvuII fragment that flanks the *Mu3* element in pl47-3. As shown in Figure 6B, the 150-bp BamHI-PvuII fragment hybridized to either a single 2.9-kb fragment or a 1-kb and a 2.9-kb fragment in the plants derived from white endosperm kernels from outside of the revertant sector. Therefore, the plant that produced this ear does carry the 1-kb BamHI length polymorphism of the cloned sequence, as was previously determined (Figure 5A). The 150-bp BamHI-PvuII fragment hybridized to a single 2.9-kb DNA fragment in the plants derived from white kernels from within the revertant sector (suggesting that the genotype of these plants was *y1/y1*) and 2.9-kb, 2.4-kb, and 3.1-kb DNA fragments in the putative *y1* revertant plants. The largest and smallest BamHI DNA fragments were the same length as the DNA associated with the *Y1* allele (the progenitor of the *y1-mum* alleles), suggesting that the genotype of these plants is *Y1-revertant/y1* and that the *y1-mum2045* allele has molecularly reverted to the *Y1* allele. We conclude from these data that pl47-3 contains at least a portion of the *y1* gene from maize.

Cloning the DNA Homologous to the *y1-mum* Allele from a Plant that Was Homozygous for the Standard *Y1* Allele

The 150-bp BamHI-PvuII fragment was used as a hybridization probe to clone an approximately 11.4-kb EcoRI fragment from a plant that was homozygous for the stand-

Figure 6. (continued).

pale-yellow phenotype of some kernels on this ear is not typical of the dominant *Y1* allele but has been observed associated with homozygous *y1-mum* kernels for many of our different mutant lines as described in the text. Missing kernels were removed for genetic and molecular analyses.

(B) BamHI-digested DNA from each type of kernel was analyzed by DNA gel blot hybridization using as a hybridization probe the 150-bp BamHI-PvuII fragment flanking the *Mu3* element in the cloned *y1-mum* allele. Lane a, *Y1/Y1*; lane b, *y1/y1*; lanes c and d, yellow endosperm kernels from within the revertant sector (*y1-revertant/y1*); lanes e and f, white endosperm kernels from within the revertant sector (*y1/y1*); lanes g to l, white endosperm kernels from outside the revertant sector (*y1/y1* or *y1-mum/y1*). Revertant is abbreviated as rev.

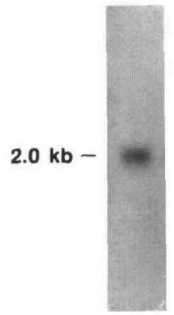


Figure 7. RNA Gel Blot Hybridization Analyses of mRNA from 1-Week-Old Seedlings.

Polyadenylated RNA was isolated from 1-week-old light-grown seedlings (*y1*/*y1*) and analyzed by RNA gel blot hybridization using the 3.5-kb BamHI or 150-bp BamHI-PvuII fragments from the cloned *Y1* allele as hybridization probes. Two separately prepared RNA blots were hybridized, stripped, and rehybridized with each hybridization probe. In each hybridization, a 2.0-kb RNA hybridized to these sequences.

ard *Y1* allele (Figure 3B). Genetic data (not shown) and comparisons of the restriction maps of the *y1-mum* and *Y1* sequences suggest that the cloned *Y1* sequence is the progenitor to the *y1-mum* allele.

Transcription of the *y1* Gene

Young seedlings actively synthesize carotenoids; therefore, the 3.5-kb BamHI and 150-bp BamHI-PvuII fragments from the *Y1* sequence were used separately as hybridization probes of polyadenylated RNA isolated from 1-week-old seedlings that were homozygous for the standard *y1* allele. As shown in Figure 7, a 2.0-kb transcript hybridized to these sequences.

DISCUSSION

A number of maize genes have been isolated by transposon tagging and cloning. These genes include the *a1* (O'Reilly et al., 1985), *bz1* (Fedoroff et al., 1984), *bz2* (McLaughlin and Walbot, 1987; Theres et al., 1987), *C1* (Cone et al., 1986), *c2* (Wienand et al., 1986), *R* (Dellaporta et al., 1988), *vp1* (McCarty et al., 1989), *P* (Lechelt et al., 1989), *o2* (Schmidt et al., 1987; Motto et al., 1988), *hcf106* (Martienssen et al., 1989), and *Kn1* (Hake et al., 1989) loci. The *Mutator* transposable element *Mu1* was used successfully to clone three of these genes: the *bz2*, *hcf106*, and *vp1* loci. We have used a sequence specific to the *Mutator* transposable element *Mu3* as a hybridization probe to identify and clone a *Mu3*-hybridizing *y1*-linked sequence of DNA. DNA gel blot hybridization analysis of a putative *Mutator*-induced *y1* allele, a revertant of this allele, and several other independently derived putative *Mu*-induced *y1* alleles indicated that this cloned sequence is a portion of the *y1* gene. The *y1-mum* sequence was used to clone a standard *Y1* allele.

Mangelsdorf and Fraps (1931) demonstrated a dose dependence between the *Y1* allele and the provitamin A activity (i.e., β-carotene and α-carotene concentration) of the maize endosperm. The level of provitamin A activity was found to be directly proportional to the number of *Y1* allele copies in the endosperm. This relationship has been confirmed more recently by Simcox et al. (1987), who used high-performance liquid chromatography to measure endosperm carotenoid content. Mangelsdorf and Fraps (1931) suggested that the *y1* gene product was not an enzyme involved in the biosynthesis of β-carotene because the rate of an enzymatic reaction, although dependent on concentration, is seldom directly proportional to the concentration of the enzyme. Thus, it is possible that the *y1* gene product regulates the carotenoid biosynthetic pathway. In addition, Robertson (1971) analyzed 532 white endosperm lines of maize and found all of them to be white kerneled as a result of being homozygous for *y1*. These data and the tissue specificity of the various *y1* alleles also suggest that the *y1* gene may be a regulator of the carotenoid biosynthetic pathway in maize. Now that the *y1* gene has been cloned, it should be possible to establish how the *y1* gene product influences carotenoid biosynthesis.

If the *y1* gene product regulates the carotenoid biosynthetic pathway, it could function as a transcriptional activator, interacting directly with the biosynthetic genes (e.g., *al*, *vp7*, *vp9*, *y9*, and *w3*). Alternatively, the *y1* gene product might be essential for the assembly or stability of a carotenoid biosynthetic enzyme complex. Sequence analysis indicates that the products of the maize regulatory genes *C1* (Paz-Ares et al., 1987), *R* (Ludwig et al., 1989), and *o2* (Hartings et al., 1989) have structural similarities to DNA-binding proteins and transcriptional activators. Sequence analysis of a *y1* cDNA should reveal whether the *y1* gene product has similar properties.

Several different alleles of *y1* have been described. Plants that contain a dominant *Y1* allele produce carotenoids in both their endosperm and leaves. Carotenoids are produced in the leaves but not in the endosperm of plants that are homozygous for the recessive *y1* allele. Plants that are homozygous for the temperature-sensitive pastel alleles of *y1* exhibit a phenotype similar to the recessive *y1* allele when grown at moderate temperatures (approximately 25°C). However, at elevated temperatures (approximately 35°C), these plants exhibit reduced amounts of β-carotene in their leaves. The tissue-specific and temperature-sensitive behavior of the *y1* gene could be caused by either a differential transcription of *y1* or by differential activity of the *y1* gene product. Determining the pattern of

expression and the amino acid sequence of the products of different *y1* alleles could distinguish between these two models.

Several hundred *Mutator*-induced *y1* alleles have been generated (Robertson, 1985). Approximately 75% of these are temperature-sensitive pastel alleles. The remainder are similar to the standard *y1* allele. Now that the *y1* gene has been isolated, the genetic defect(s) responsible for these various *y1* alleles can be characterized at the molecular level. The large number of *y1* alleles available for study will allow a detailed mutational map of the *y1* locus to be prepared. Mapping the genetic defects in the different alleles of *y1* might indicate which regions are responsible for the tissue-specific or temperature-sensitive activity.

β-Carotene is the major source of provitamin A in the human diet. Vitamin A is essential for normal vision; prevention of night blindness, xerophthalmia, and blindness; and proper immune function (Sommer et al., 1981; Sommer, 1989). Of the major food grains, only yellow-kerneled maize contains significant levels of β-carotene; therefore, maize can serve as a source of provitamin A. It is apparent that the *Y1* allele of the *y1* gene is necessary for expression of the carotenoid biosynthetic pathway in maize endosperm. An understanding of how the *Y1* allele activates the carotenoid biosynthetic pathway in the endosperm of maize may suggest strategies for activating this pathway in the endosperm of other important grains such as rice or wheat.

METHODS

Genetic Materials and Crosses

The production of the Robertson's *Mutator*-tagged alleles of *y1* has been described previously (Robertson, 1985). The standard *Y1* lines used in this tagging experiment, and all subsequent crosses, are one of two hybrid lines B77/B79 or Q66/Q67. The standard *y1* line used in these experiments was derived from a heterozygous translocation 6-9e stock obtained from the Maize Genetics Cooperative in 1961 (stock no. 57-413-2).

Revertants of the *y1-mum2045* allele were found in an isolation plot in which plants that were homozygous or heterozygous for *waxy* endosperm (*wx*) and also heterozygous for the *y1-mum2045* and standard *y1* alleles were crossed with homozygous recessive *y1/y1*, *wx/wx*, *gl1/gl1* plants. A portion of the *y1-mum2045* plants used in this study were homozygous for *gl1* (*glossy* seedling). In the molecular analysis presented here, putative revertant, *yellow*, *waxy* kernels that gave rise to *glossy* seedlings were analyzed. The *waxy* and *glossy* phenotypes were included as contamination markers because contaminating *Y1* pollen is likely also to be homozygous for *Wx* and *Gl1*.

Isolation of DNA and Blot Hybridization

Maize DNA was isolated by the method of Dellaporta et al. (1983). Maize DNA used in the production of genomic libraries was further purified by ultracentrifugation in CsCl and ethidium bromide by standard methods (Maniatis et al., 1982). DNAs were digested as recommended by the manufacturer (Bethesda Research Laboratories) or in 300-μL digests with 50 units of restriction endonuclease. Digested DNAs were fractionated on agarose gels (Maniatis et al., 1982) and transferred to nylon membranes (Genetran, Plastco Co., Woburn, MA) by the method of Southern (1975). Membranes were hybridized with DNA made radioactive by random primer extension (Boehringer Mannheim Biochemical; according to the manufacturer's instructions) or by Klenow end labeling (Maniatis et al., 1982). Membranes were hybridized, washed, and exposed to x-ray films as previously described (Buckner et al., 1988). DNAs to be made radioactive were isolated by gel electroelution (Maniatis et al., 1982).

Genomic Cloning

To clone the *Mutator*-tagged allele of *y1*, DNA was isolated as described earlier from a plant with the genotype *y1-mum2053/y1* that contained the *Mu3*-hybridizing DNA fragments that cosegregated with the *y1-mum2053* phenotype. This DNA was digested with BamHI, electrophoresed on agarose gels (GT-grade Sea Kem agarose, FMC Corp.), and the 4.0-kb to 5.5-kb DNA fragments were isolated by gel electroelution (Maniatis et al., 1982). This size-fractionated DNA was ligated into BamHI-digested λL47 (Amersham Corp.), packaged into bacteriophage λ (Giga-pack Gold, Stratagene), and transfected into *Escherichia coli*. The library was screened by plaque hybridization (Hames and Higgins, 1987) by using the internal HindIII-XbaI *Mu3* fragment made radioactive as described earlier. A plaque that hybridized to the *Mu3* sequence was purified (designated L47-3), the DNA from this bacteriophage was isolated (Davis et al., 1986), and the maize insert was subcloned into pBR322 and designated pl47-3. The *Y1* allele was cloned from "standard" line Q60 as described above except that 10-kb to 12-kb EcoRI fragments were ligated into λEMBL4, libraries were screened by using the 150-bp BamHI-PvuII fragment from pl47-3, and the maize insert was subcloned into plasmid pGem 7Zf(+) (Promega Biotec).

Isolation of RNA and Blot Hybridization

Total RNA was isolated from 1-week-old seedlings of maize by the method of McCarty (1986). Polyadenylated RNA was purified by affinity chromatography on oligo(dT)-cellulose (Maniatis et al., 1982). The RNA was electrophoresed, blotted to nylon membranes, and hybridized to ^{32}P-labeled DNA as described by Thomas (1980).

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REFERENCES

Anderson, I.C., and Robertson, D.S. (1960). Role of carotenoids in protecting chlorophyll from photo destruction. Plant Physiol. **35,** 531–534.

Anderson, I.C., and Robertson, D.S. (1961). Carotenoid protection of porphyrins from photo destruction. Progress in Photobiology. Third International Congress on Photobiology, Copenhagen, B.C. Christensen and B. Buchman, eds (Amsterdam: Elsevier Publishing), pp. 477–479.

Barker, R.F., Thompson, D.Y., Talbot, D.R., Swanson, J.A., and Bennetzen, J. (1984). Nucleotide sequence of the maize transposable element *Mu1*. Nucl. Acids Res. **12,** 5955–5966.

Buckner, B., Novotny, C.P., and Ullrich, R.C. (1988). Organization of the ribosomal RNA genes of *Schizophyllum commune*. Curr. Genet. **13,** 417–424.

Cone, K.C., Burr, F.A., and Burr, B. (1986). Molecular analysis of the maize anthocyanin regulatory locus *c1*. Proc. Natl. Acad. Sci. USA **83,** 9631–9635.

Davis, L.G., Dibner, M.D., and Battey, J.F. (1986). Basic Methods in Molecular Biology (New York: Elsevier Science Publishers).

Dellaporta, S.L., Woods, J., and Hicks, J.B. (1983). A plant DNA mini preparation: Version II. Plant Mol. Biol. Rep. **1,** 19–21.

Dellaporta, S.L., Greenblatt, I., Kermicle, J.L., Hicks, J.B., and Wessler, S.R. (1988). Molecular cloning of the maize *R-nj* allele by transposon tagging with *Ac*. In Chromosome Structure and Function: Impact of New Concepts, 18th Stadler Genetics Symposium, J.P. Gustafson and R. Appels, eds (New York: Plenum Press), pp. 263–282.

Federoff, N.V., Furtek, D.B., and Nelson, O.E., Jr. (1984). Cloning of the *bronze* locus in maize by a simple and generalizable procedure using the transposable controlling element *Activator* (*Ac*). Proc. Natl. Acad. Sci. USA **81,** 3825–3829.

Fong, F., Smith, J.D., and Koehler, D.E. (1983). Fluridone induction of vivipary during maize seed development. In III International Symposium on Pre-Harvest Sprouting in Cereals, J.E. Krueger and D.E. LaBerge, eds (Boulder, CO: Westview Press), pp. 188–196.

Goodwin, T.W. (1980). The Biochemistry of the Carotenoids, Vol. 1: Plants (New York: Chapman and Hall).

Hake, S., Vollbrecht, E., and Freeling, M. (1989). Cloning *Knotted*, the dominant morphological mutant in maize using *Ds2* as a transposon tag. EMBO J. **8,** 15–22.

Hames, B.D., and Higgins, S.J. (1987). Nucleic Acid Hybridization: A Practical Approach (Washington, DC: IRL Press).

Hartings, H., Maddaloni, M., Lazzaroni, N., Di Fonzo, N., Motto, M., Salamini, F., and Thompson, R. (1989). The *O2* gene which regulates zein deposition in maize endosperm encodes a protein with structural homologies to transcriptional activators. EMBO J. **8,** 2795–2801.

Lechelt, C., Peterson, T., Laird, A., Chen, J., Dellaporta, S.L., Dennis, E., Peacock, W.J., and Starlinger, P. (1989). Isolation and molecular analysis of the maize *P* locus. Mol. Gen. Genet. **219,** 225–234.

Ludwig, S.R., Habera, L.F., Dellaporta, S.L., and Wessler, S.R. (1989). *Lc*, a member of the maize *R* gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the *myc*-homology region. Proc. Natl. Acad. Sci. USA **86,** 7092–7096.

Mangelsdorf, P.C., and Fraps, G.S. (1931). A direct quantitative relationship between vitamin A in corn and the number of genes for yellow pigmentation. Science **73,** 241–242.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Martienssen, R.A., Barkan, A., Freeling, M., and Taylor, W.C. (1989). Molecular cloning of a gene involved in photosynthetic membrane organization that is regulated by Robertson's *Mutator*. EMBO J. **8,** 1633–1639.

McCarty, D.R. (1986). A simple method for extraction of RNA from maize tissue. Maize Gen. Coop. Newslett. **60,** 61.

McCarty, D.R., Carson, C.B., Stinard, P.S., and Robertson, D.S. (1989). Molecular analysis of the *viviparous-1* locus in maize. Plant Cell **1,** 523–532.

McLaughlin, M., and Walbot, V. (1987). Cloning of a mutable *bz2* allele of maize by transposon tagging and differential hybridization. Genetics **117,** 771–776.

Motto, M., Maddaloni, M., Ponziani, G., Brembilla, M., Martta, R., Di Fonzo, N., Soave, C., Thompson, R., and Salamini, F. (1988). Molecular cloning of the *o2-m5* allele of *Zea mays* using transposon marking. Mol. Gen. Genet. **212,** 488–494.

Oishi, K.K., and Freeling, M. (1988). A new *Mu* element from a Robertson's *Mutator* line. In Plant Transposable Elements, O.E. Nelson, ed (New York: Plenum Press), pp. 289–291.

O'Reilly, C., Shepherd, N.S., Pereira, A., Schwarz-Sommer, Z., Bertram, I., Robertson, D.S., Peterson, P.A., and Saedler, H. (1985). Molecular cloning of the *a1* locus of *Zea mays* using the transposable elements. EMBO J. **41,** 877–882.

Paz-Ares, J., Ghosal, D., Wienand, U., Peterson, P., and Saedler, H. (1987). The regulatory *c1* locus of *Zea mays* encodes a protein with homology to *myb* proto-oncogene products and structural similarities to transcriptional activators. EMBO J. **6,** 3553–3558.

Robertson, D.S. (1971). Investigations of the bipartite structure of the white-albino maize mutants. Mol. Gen. Genet. **112,** 93–103.

Robertson, D.S. (1975). Survey of the albino and white-endosperm mutants of maize. Their phenotype and gene symbols. J. Hered. **66,** 67–74.

Robertson, D.S. (1985). Differential activity of the maize mutator *Mu* at different loci and in different cell lineages. Mol. Gen. Genet. **200,** 9–13.

Robertson, D.S., and Anderson, I.C. (1961). Temperature sensitive alleles of the *y1* locus in maize. J. Hered. **52,** 53–60.

Schnable, P.S., Peterson, P.A., and Saedler, H. (1989). The *bz-rcy* allele of the *Cy* transposable element system of *Zea mays* contains a *Mu*-like element insertion. Mol. Gen. Genet. **217,** 459–463.

Schmidt, R.J., Burr, F.A., and Burr, B. (1987). Transposon tagging and molecular analysis of the maize regulatory locus *opaque-2*. Science **238,** 960–963.

Simcox, K.D., Shadley, J.D., and Weber, D.F. (1987). Detection of the time of occurrence of nondisjunction induced by the *r-X1* deficiency in *Zea mays* L. Genome **29,** 782–785.

Sommer, A. (1989). New imperatives for an old vitamin (A). J. Nutr. **119,** 96–100.

Sommer, A., Tarwotjo, I., and Hussaini, G. (1981). Incidence, prevalence and scale of blinding malnutrition. Lancet **I,** 1407–1408.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98,** 503–517.

Talbert, L.E., Patterson, G.I., and Chandler, V.L. (1989). *Mu* transposable elements are structurally diverse and distributed throughout the genus *Zea*. J. Mol. Evol. **29,** 28–39.

Taylor, L.P., and Walbot, V. (1987). Isolation and characterization of a 1.7 Kb transposable element from a *Mutator* line of maize. Genetics **116,** 469–477.

Theres, N., Scheele, T., and Starlinger, P. (1987). Cloning of the *Bz2* locus of *Zea mays* using the transposable element *Ds* as a gene tag. Mol. Gen. Genet. **209,** 193–197.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments to nitrocellulose. Proc. Natl. Acad. Sci. USA **77,** 5201–5205.

Wienand, U., Weydemann, U., Niesbach-Kloesgen, U., Peterson, P.A., and Saedler, H. (1986). Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol. Gen. Genet. **203,** 202–207.