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The CAT-2 null phenotype in maize is likely due to a DNA insertion into the *Cat2* gene

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Summary. The molecular basis for a shorter *Cat2* transcript in maize lines null for the CAT-2 catalase isozyme was investigated using cDNA libraries and genomic DNA blots. Sequence comparison of partial *Cat2* cDNAs obtained from two CAT-2 null lines and a wild-type CAT-2 encoding cDNA showed that the *Cat2* null transcripts diverged from the wild-type transcript but remained homologous to each other. Genomic DNA blots indicated that the missing portion of the transcript is present in the genomes of lines null for the CAT-2 isozyme. Differences in the hybridization patterns of normal and null lines were revealed when genomic DNA blots were probed with the full-length *Cat2* cDNA, a *Cat2* gene-specific probe, and a "null sequence" probe. Together, the DNA blotting results suggest that a rearrangement of the *Cat2* gene has occurred in the CAT-2 null lines. The available data suggest that the CAT-2 null mutation in maize is due to a DNA insertion into the *Cat2* gene.

Key words: Catalase null – *Zea mays* – Catalase transcript – Transposable element

Introduction

Aerobic organisms have evolved many compounds and enzymes to protect themselves against the damage caused by such active oxygen species as hydrogen peroxide (H_2O_2) and the superoxide ($\cdot O_2$-) and hydroxyl ($\cdot OH$) radicals. These oxygen derivatives can quickly react with membranes (Mead 1976), proteins (Fucci et al. 1983), nucleic acids (Brawn and Fridovich 1981), and other cellular components, impairing cellular metabolism or causing cell death.

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is a tetrameric, heme-containing enzyme that converts H_2O_2 to water and molecular oxygen. Thus, catalase prevents oxidative damage to the cell by eliminating H_2O_2 accumulation and preventing H_2O_2 from reacting with $\cdot O_2$- to generate the most potent oxidant, $\cdot OH$.

In maize (*Zea mays* L.), there are three catalase isozymes (CAT-1, CAT-2, and CAT-3) encoded by three unlinked structural genes (*Cat1*, *Cat2*, and *Cat3*) (Scandalios et al. 1980; Chandlee et al. 1983). A16, A338, and A340 are maize lines that are null for the CAT-2 isozyme (Tsaftaris and Scandalios 1981; Chandlee and Scandalios 1984; Bethards and Scandalios 1988). These three lines express a shorter 1.4-kb transcript that hybridizes with the normal 1.8-kb *Cat2* transcript from R6-67 (a high CAT-2 maize line) on an RNA blot (Bethards et al. 1987; Bethards and Scandalios 1988). Further RNA blot analyses showed that the null transcripts do not hybridize to the 3′ end of the R6-67 cDNA (Bethards and Scandalios 1988). S_1 nuclease analyses indicated that the null transcripts hybridize to the first 1,080 nucleotides of the R6-67 cDNA (Bethards and Scandalios 1988).

This investigation further characterizes the molecular basis for the CAT-2 null phenotype. We show that homology between the R6-67 cDNA and the A16 and A340 cDNAs ends at nucleotide 1,093 of the R6-67 cDNA, whereas the two null transcripts remain homologous to one another. Results from genomic DNA blots indicate that the CAT-2 null mutation is likely due to a DNA insertion into the *Cat2* gene.

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Materials and methods

Materials

Maize (*Zea mays* L.) lines W64A (normal CAT-2 expression, normal transcript), R6-67 (high CAT-2 expression, normal transcript), and A16, A338, and A340 (null for CAT-2, short transcript) were used in these studies. All of these lines are maintained by our laboratory.

cDNA libraries

Total RNA was extracted from scutella of 5-day-old, dark-grown, germinating kernels by a guanidinium thiocyanate extraction method (Chirgwin et al. 1979) with modifications (Kopczynski and Scandalios 1986). Poly $(A)^+$ RNA was selected on an oligo(dT)-cellulose column (Aviv and Leder 1972). Double-stranded cDNAs were synthesized by the method of Gübler and Hoffman (1983), ligated into the EcoRI site of the Lambda Zap vector (Stratagene), in vitro packaged (Gigapack, Stratagene), and the resultant phage (2×10^4 recombinants) were plated on a lawn of XL-1 cells (Stratagene). A ^{32}P-labeled (Feinberg and Vogelstein 1983) *Cat2* cDNA probe ($\approx 5 \times 10^7$ cpm/ml) from maize line R6-67 (Bethards et al. 1987) was used to screen the library.

Nucleic acid hybridizations were performed for 16–20 h at 42 °C using 10^6 cpm/ml ^{32}P-labeled cDNA probes (Maniatis et al. 1982). Filters were washed for 15 min at room temperature in 5 × SSC with 0.1% SDS, for 25 min at 42 °C in 2 × SSC with 0.1% SDS (twice) and, finally, for 1 h at 60–65 °C in 0.1 × SSC with 0.1% SDS. Autoradiography was performed as described (Bethards et al. 1987).

Bacteriophage and plasmid DNA isolations were performed as described (Birnboim and Doly 1979; Maniatis et al. 1982). Positive bacteriophage clones were subcloned into bacteriophage vector M13 mp18 or mp19 (Norrander et al. 1983) and sequenced by the dideoxy chain-termination method (Sanger et al. 1977).

Genomic DNA blots

Genomic DNA was isolated from 12-day-old maize seedling leaves as described (Redinbaugh et al. 1988), cut with appropriate restriction enzymes, and run on a 0.7% agarose gel for 18 h in circulating TAE buffer. The DNA was then blotted onto Nytran filters (Schleicher and Schuell Inc., 1987), hybridized, and washed as above. ^{32}P-labeled cDNAs (full-length R6-67 *Cat2* cDNA, the HincII/EcoRI fragment at the 3′ end of the R6-67 *Cat2* cDNA, and the PstI/EcoRI fragment at the 3′ end of the A340 *Cat2* cDNA) were used to probe the filters.

Results

Previous studies showed that the CAT-2 null lines (A16, A338, and A340) produce a 1,400-nucleotide, polyadenylated *Cat2* transcript (Bethards and Scandalios 1988). Partial *Cat2* cDNAs were obtained from two of the three null lines upon screening the cDNA libraries with radiolabeled *Cat2* cDNA from line R6-67. The restriction maps of the *Cat2* cDNAs from lines A16 and A340 are aligned with the *Cat2* cDNA restriction map of the high CAT-2 line (R6-67) in Fig. 1. Both *Cat2* null cDNAs overlap the region of the R6-67 *Cat2* cDNA where the null and normal *Cat2* transcripts diverge. The truncated *Cat2* cDNAs obtained from A16 and A340 were not polyadenylated, possibly due to poor second-strand synthesis.

Dideoxynucleotide sequencing (Sanger et al. 1977) of the A16 and A340 *Cat2* cDNAs showed that the null transcripts diverge from the normal transcript at nucleotide 1,094 (Fig. 2). The null and normal *Cat2* cDNAs are nearly 100% homologous until nucleotide 1,094, whereupon all homology between the null and normal *Cat2* cDNAs ends. Beyond nucleotide 1,094 the null *Cat2* cDNAs remain identical to each other. A search of the GENBANK/EMBL database revealed no significant homology between the unique *Cat2* null sequence (nucleotides 1,094–1,147 of the A340 cDNA, Fig. 2) and any other published DNA sequence.

Genomic DNA from maize lines containing normal *Cat2* genes (W64A and R6-67) and lines null for CAT-2 (A16, A338, and A340) was blotted onto Nytran filters. The blots were probed with full-length R6-67 *Cat2* cDNA, a *Cat2* gene-specific probe (GSP), and a 75-bp *Cat2* "null sequence" probe (NSP) (Fig. 1). The NSP extends from the PstI restriction site (nucleotide 1,073 in Fig. 2) to the 3′ end of the A340 *Cat2* cDNA. Figure 3 shows the different hybridization patterns obtained with the different probes.

The CAT-2 null lines have similar hybridization patterns when probed with the full-length *Cat2* cDNA,

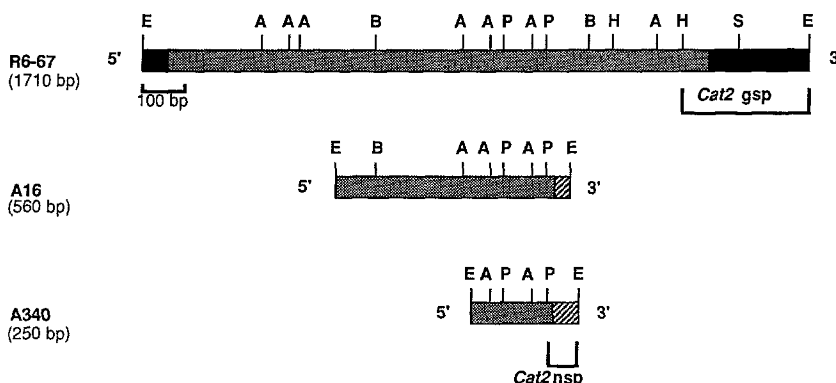

Fig. 1. Restriction maps of the R6-67, A16, and A340 *Cat2* cDNAs. A16 and A340 are CAT-2 null lines and R6-67 is a high CAT-2 line. *Black areas* represent the noncoding regions of the *Cat2* gene, *gray areas* represent the coding region, and the *hatched areas* represent the *Cat2* null sequences that are not homologous to R6-67. E = EcoRI (vector), A = AvaI, B = BamHI, P = PstI, H = HincII, S = SacI. The *Cat2* gene-specific probe (*Cat2* gsp) and the *Cat2* "null sequence" probe (*Cat2* nsp) are indicated beneath the cDNAs

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         994  1000          1010          1020          1030          1040          1050
R6-67    TTC GCG GAG AAC GAG CAG CTG GCC TTC TGC CCG GGC CTC ATC GTC CCT GGT ATC TAC TAC
A16      *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** **C **G *** *** ***
A340     *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** **C **G *** *** ***

         1054 1060          1070          1080          1090          1100          1110
R6-67    TCC GAC GAC AAG CTG CTG CAG ACC AGG ATC TTC TCC TAC TCC GAC ACG CAG CGC CAC CGC
A16      *** *** *** *** *** *** *** *** *** *** *** *** *** *TG TTA G*A ACA *CT GGG AA*
A340     *** *** *** *** *** *** *** *** *** *** *** *** *** *TG TTA G*A ACA *CT GGG AA*
                                                             ▲
                                           Homology ends here

         1114 1120          1130          1140          1150          1160
R6-67    CTC GGC CCC AAC TAC CTG CTG CTA CCG GCC AAC GCG CCC AAG TGC GCA CAC CAC .....
A16      GGG *AT *GG
A340     GGG *AT *GG *GA GGA AGA GCT *AG AG* AGA *TG *
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Fig. 2. Partial sequence comparison of the R6-67, A16, and A340 *Cat2* cDNAs. Numbers indicate nucleotide position in the R6-67 *Cat2* cDNA. Homologous bases between the R6-67 *Cat2* cDNA and the *Cat2* null cDNAs (A16 and A340) are denoted by an *asterisk* (*). Homology ends at base 1,093 of the R6-67 *Cat2* cDNA. Note that the A16 and A340 sequences are completely identical through the end of the A16 *Cat2* cDNA

whereas the normal *Cat2* lines have different hybridization patterns (Fig. 3A). A338 and A340 have six fragments in common, whereas A16 has four fragments in common with A340 and five fragments in common with A338. The fragments common to all three null lines are the 2.9-kb BamHI fragment, the 2.5-kb BamHI fragment, the 23.4-kb HindIII fragment, and the 3.0-kb HindIII fragment. W64A and R6-67 have no hybridization fragments in common when probed with the R6-67 *Cat2* cDNA. The 2.9-kb BamHI fragment hybridizes to the full-length cDNA probe in R6-67, A16, A338, and A340. Thus, W64A and R6-67 show *Cat2* hybridization patterns that differ from each other, as well as the CAT-2 null lines, when probed with the full-length R6-67 *Cat2* cDNA.

Very simple hybridization patterns occur in both the normal *Cat2* lines and the CAT-2 null lines when genomic DNA blots are probed with a gene-specific *Cat2* fragment (Fig. 3B). W64A has a single hybridization fragment in each lane. None of the hybridization fragments in W64A appear in the CAT-2 null lines. A 2.9-kb BamHI fragment hybridizes to the *Cat2* gene-specific probe in R6-67, A16, A338, and A340, the only common fragment between a normal *Cat2* line and the CAT-2 null lines. A16, A338, and A340 show nearly identical hybridization patterns in Fig. 3B. The only differences between the CAT-2 null lines probed with *Cat2* gene-specific probe are the different sized EcoRI fragments that hybridize in each line.

Probing identical genomic DNA blots with the 75-bp *Cat2* "null sequence" probe yields the complex hybridization patterns shown in Fig. 3C. The normal *Cat2* lines show very similar hybridization patterns, with 11 of the 14 hybridization fragments conserved between W64A and R6-67. CAT-2 null lines A338 and A340 have 18 fragments in common, whereas A16 shares nine fragments with A338 and seven fragments with A340. Four fragments are found in both the normal CAT-2 lines and the CAT-2 null lines. The four fragments common to all five lines are the 5.9-kb EcoRI fragment, the 6.7-kb BamHI fragment, the 11.7-kb HindIII fragment, and the 3.3-kb HindIII fragment.

Discussion

We have examined the molecular basis for the CAT-2 null phenotype in three maize lines using truncated *Cat2* cDNA clones and genomic DNA blots. Previous results (Bethards and Scandalios 1988) indicated that lines null for the CAT-2 protein possessed a 1,400-nucleotide *Cat2* transcript whose sequence diverged from the R6-67 *Cat2* cDNA around nucleotide 1,080. The *Cat2* cDNAs obtained from the A16 and A340 scutellar libraries confirm these results (Figs. 1 and 2). Homology between the normal *Cat2* transcript and the null *Cat2* transcripts ends at nucleotide 1,093 of the R6-67 cDNA (Fig. 2). The null *Cat2* cDNAs remain identical to each other throughout their length (Fig. 2). These results suggest a common cause for the sequence divergence from the normal *Cat2* transcript.

The genomic DNA blots in Fig. 3 reveal much about the molecular basis of the CAT-2 null mutation. These fragment hybridization patterns indicate that there are some conserved chromosomal regions in all five maize lines associated with the NSP; however, the normal *Cat2* lines show greater homology to each other than to the CAT-2 null lines and vice versa. The large number of fragments hybridizing to the NSP indicates that the probe sequence occurs many times in the genomes of the maize lines examined and that it is not specific to the CAT-2 null phenotype. The fact that more fragments hybridize to the NSP in the null lines than in the normal lines implies that there are more copies of the "null se-

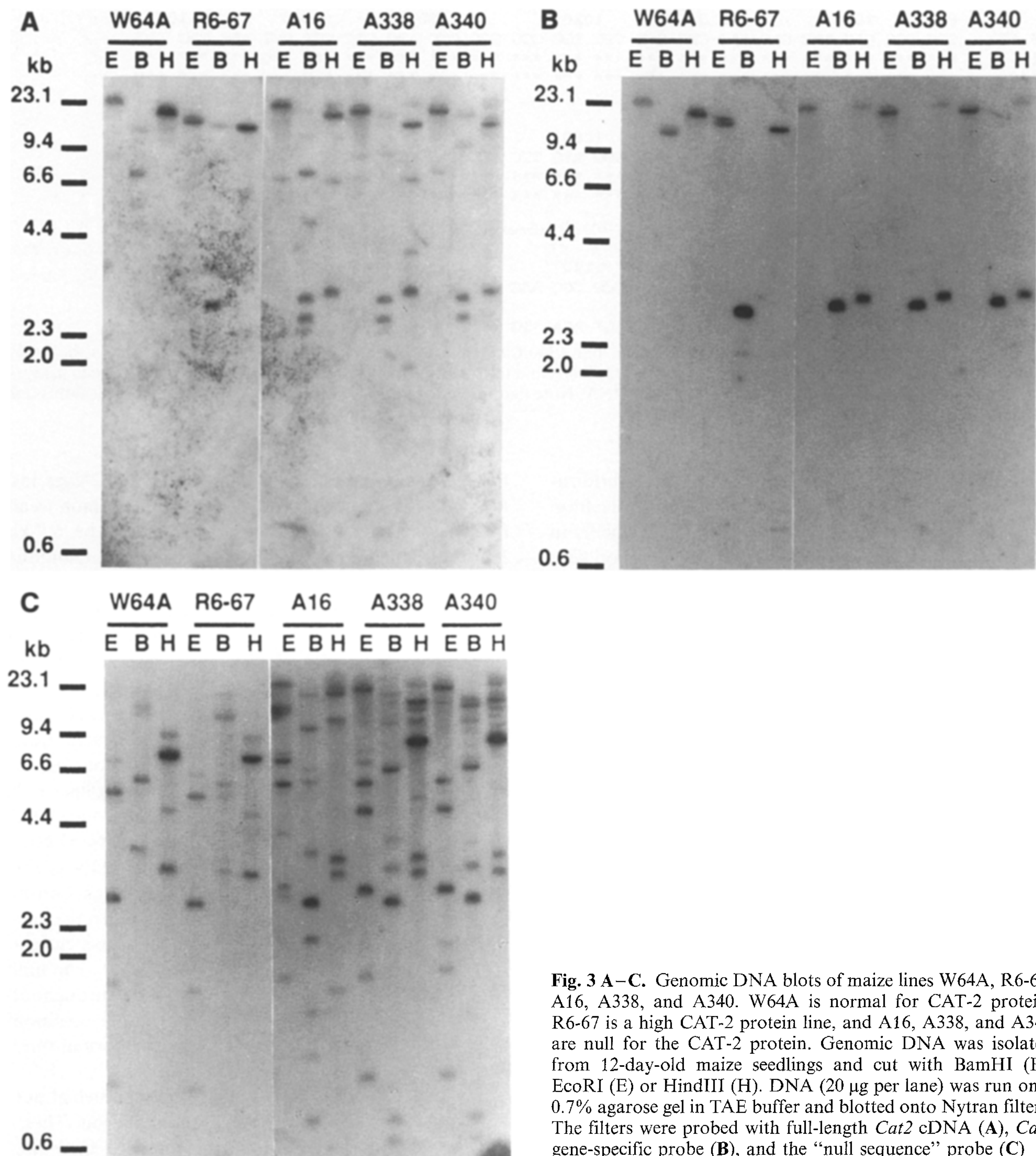

Fig. 3 A–C. Genomic DNA blots of maize lines W64A, R6-67, A16, A338, and A340. W64A is normal for CAT-2 protein, R6-67 is a high CAT-2 protein line, and A16, A338, and A340 are null for the CAT-2 protein. Genomic DNA was isolated from 12-day-old maize seedlings and cut with BamHI (B), EcoRI (E) or HindIII (H). DNA (20 μg per lane) was run on a 0.7% agarose gel in TAE buffer and blotted onto Nytran filters. The filters were probed with full-length *Cat2* cDNA (**A**), *Cat2* gene-specific probe (**B**), and the "null sequence" probe (**C**)

quence" (nucleotides 1,094–1,147 of the A340 cDNA, Fig. 2) in the CAT-2 null lines than there are in the normal *Cat2* lines.

Fragments that hybridize to the NSP and either the full-length cDNA probe or the *Cat2* gene-specific probe (or both) indicate an association of the "null sequence" with the *Cat2* gene. Nine fragments in the CAT-2 null lines hybridize to all three probes and six other fragments hybridize to both the full-length cDNA probe and the NSP. For every restriction (EcoRI, BamHI, and HindIII) in every CAT-2 null line there is at least one fragment that hybridizes to the NSP and either the GSP or the full-length R6-67 *Cat2* cDNA probe. Therefore, the "null sequence" is associated with the *Cat2* gene in the genome of all three CAT-2 null lines. R6-67 has no fragments that hybridize to the NSP and either of the

other probes. Thus, the "null sequence" is not associated with *Cat2*, although it is present in the R6-67 genome. Neither is the "null sequence" associated with *Cat2* in W64A, although a 13-kb BamHI fragment appears to hybridize to all three probes. The "null sequence" probe should also hybridize to the 25-kb EcoRI fragment and the 18.4-kb HindIII fragment of W64A if it is associated with the *Cat2* gene. Because the "null sequence" did not hybridize to either of the aforementioned fragments, we conclude that the "null sequence" is not associated with the *Cat2* gene in W64A, although the "null sequence" is present in W64A's genome.

Several possible models to explain the molecular basis of the CAT-2 null mutation in maize exist, including an unspliced or misspliced *Cat2* transcript, a deletion of the 3′ end of the *Cat2* gene, a translocation or inversion involving the *Cat2* gene, and a DNA insertion into the *Cat2* gene.

The NSP does not hybridize to any of the fragments that hybridize to the full-length *Cat2* cDNA or the GSP in genomic DNA blots of W64A or R6-67. If the "null sequence" was part of an intron, the NSP would hybridize to fragments that also hybridize to the GSP or the full-length *Cat2* cDNA probe in lines W64A and R6-67. Thus, the CAT-2 null mutation is not the result of an unspliced or misspliced transcript.

Neither is the mutation the result of a simple deletion of the 3′ end of the *Cat2* gene. The *Cat2* GSP hybridizes to restriction fragments in the CAT-2 null lines (Fig. 3 B), indicating that the 3′ end of the *Cat2* gene is still in the genomes of the CAT-2 null lines. Although the 3′ end of the *Cat2* gene appears in the genomes of the CAT-2 null lines, it is not transcribed (Bethards and Scandalios 1988).

Similarly, neither a translocation nor an inversion is a good model for the cause of the CAT-2 null phenotype in maize. A translocation with a breakpoint in the middle of the *Cat2* gene would separate the 5′ end of the *Cat2* gene from the 3′ end. The large (>20 kb) EcoRI fragment of all three null lines hybridizes to both the 5′ (data not shown) and 3′ (Fig. 3 B) ends of the *Cat2* cDNA as well as the NSP (Fig. 3 C). Therefore, it is unlikely that a translocation caused the CAT-2 null mutation, because the 5′ and 3′ ends of the *Cat2* gene are still linked in the null lines. An inversion would fit the hybridization patterns of the null lines if the "null sequence" was near the *Cat2* gene. The evidence against this model is that none of the large GSP or full-length *Cat2* cDNA probe hybridization fragments hybridize to the NSP in W64A or R6-67 (compare Fig. 3 B to Fig. 3 C). Thus, an inversion is unlikely to be the cause of the CAT-2 null mutation in maize.

A DNA insertion into the *Cat2* gene is presently the best model for the cause of the CAT-2 null mutation in maize. Several lines of evidence support the DNA insertion model. First, the large (>20 kb) EcoRI fragments of all three null lines hybridize to both the 5′ and 3′ ends of the *Cat2* cDNA and to the NSP as well. Second, the NSP hybridizes to many fragments in all five lines. W64A and R6-67 appear to have three to five copies of the "null sequence" in their genomes, whereas the CAT-2 null lines have five to ten copies. Third, a precedent for such a mutation in maize exists. A *Mu*I transposon inserted into the first intron of a maize *Adh* gene interferes with transcription downstream of the insertion (Vayda and Freeling 1986). Thus, the CAT-2 null phenotype in maize is likely the result of a DNA insertion.

The nature of such an insertion is not yet clear. The insertion of a transposable element into the *Cat2* gene is consistent with the blotting data. However, a database search of the GENBANK/EMBL sequence repositories for the *Cat2* "null sequence" yielded no significant homology to any known mobile genetic elements. New classes of maize transposons are still being identified, so it is possible that the "null sequence" may represent a previously uncharacterized transposon. However, many conserved hybridization fragments appear in the normal *Cat2* lines, W64A and R6-67, when they are probed with the NSP (Fig. 3 C). The same is true of the null lines (Fig. 3 C). A transposable element would probably not generate restriction fragments that are conserved among lines as it moved around the host's genome. Further characterization of the *Cat2* gene in the null maize line is necessary to determine the nature of a possible DNA insertion.

The similarity of the hybridization patterns of the CAT-2 null lines in Fig. 3, coupled with the sequence data from Fig. 2, indicate that the same aberration in the *Cat2* gene is responsible for the CAT-2 null phenotype in all three CAT-2 null lines. It is not known whether the mutations arose independently or if all three CAT-2 null lines are distant descendants of the same mutation. A338 and A340 are much more similar to one another than to A16 (Fig. 3). These relationships are not surprising because A16 is an Eastern European line and A338 and A340 are individual lines derived from ten generations of reciprocal recurrent selection in North Carolina (R. H. Moll, personal communication). Although the present data suggest that the molecular basis for the CAT-2 null mutation in maize is a DNA insertion into the *Cat2* gene, further studies will help to elucidate the cause(s) unequivocally.

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References

Aviv H, Leder P (1972) Purification of biologically active globin mRNA by chromatography on oligothymidylic acid-cellulose. Proc Natl Acad Sci USA 69:1408–1412

Bethards LA, Scandalios JG (1988) Molecular basis for the CAT-2 null phenotype in maize. Genetics 118:149–153

Bethards LA, Skadsen RW, Scandalios JG (1987) Isolation and characterization of a cDNA clone for the *Cat2* gene in maize and its homology with other catalases. Proc Natl Acad Sci USA 84:6830–6834

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Brawn K, Fridovich I (1981) DNA strand scission by enzymically generated oxygen radicals. Arch Biochem Biophys 206:414–419

Chandlee JM, Scandalios JG (1984) Analysis of variants affecting the catalase developmental program in maize scutellum. Theor Appl Genet 69:71–77

Chandlee JM, Tsaftaris AS, Scandalios JG (1983) Purification and partial characterization of three genetically defined catalases of maize. Plant Sci Lett 29:117–131

Chirgwin JM, Przybyla AE, McDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry 24:5294–5299

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction enzyme fragments to high specific activity. Anal Biochem 132:6–13

Fucci L, Oliver CN, Coon MJ, Stadtman ER (1983) Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implications in protein turnover and aging. Proc Natl Acad Sci USA 80:1521–1525

Gübler U, Hoffman BJ (1983) A simple and very efficient method for generating cDNA libraries. Gene 25:263–269

Kopczynski CC, Scandalios JG (1986) *Cat2* gene expression: developmental control of translatable CAT-2 mRNA levels in maize scutellum. Mol Gen Genet 203:185–188

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor/NY

Mead JF (1976) Free radical mechanisms of lipid damage and consequences for cellular membranes. In: Pryor WA (ed) Free radicals in biology, vol 1. Academic Press, New York, pp 51–68

Norrander J, Kempe T, Messing J (1983) Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. Gene 26:101–106

Redinbaugh MG, Wadsworth GJ, Scandalios JG (1988) Characterization of catalase transcripts and their differential expression in maize. Biochim Biophys Acta 951:104–116

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Scandalios JG; Tong WF, Roupakias DG (1980) *Cat3*, a third gene locus coding for a tissue-specific catalase in maize: genetics, intracellular location, and some biochemical properties. Mol Gen Genet 179:33–41

Schleicher and Schuell, Inc. (1987) Transfer and immobilization of nucleic acids to S&S solid supports. Schleicher and Schuell, Inc.

Tsaftaris AS, Scandalios JG (1981) Genetic and biochemical characterization of a *Cat2* catalase null mutant. Mol Gen Genet 181:158–163

Vayda ME, freeling M (1986) Insertion of the *Mu*I transposable element into the first intron of maize *Adh1* interferes with transcript elongation but does not disrupt chromatin structure. Plant Mol Biol 6:441–454