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Expression of the catalase and superoxide dismutase genes in mature pollen in maize

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Summary. The expression of the *Cat* and *Sod* genes encoding the multiple catalases and superoxide dismutases in maize has been studied in maize pollen at anther dehiscence. Pollen from three catalase activity variant inbred lines was examined. Zymorgram analysis, immunoassays, and RNA blots show that, in the lines used, only the CAT-1 catalase isozyme is expressed in mature pollen; the CAT-2 and CAT-3 isozymes are not. The data presented further demonstrate that the *Cat1* gene is transcribed and translated after tetrad formation. The relative protein levels of the various superoxide dismutase (SOD) isozymes appear to be similar in pollen and scutella, and correspond to the relatively low levels observed for the different *Sod* transcripts in these tissues. The presence of "double" transcripts for *Sod3* and *Sod4A* is discussed.

Key words: *Zea mays* – Catalase – Gene expression – Pollen – superoxide dismutase

Introduction

The development of spores and pollen occurs during a critical phase in the lifecycle of land plants, spanning the transition between the diploid sporophytic and the haploid gametophytic generations (Blackmore and Crane 1988). In maize (*Zea mays* L.) the tricellular, mature pollen is an arrested developmental stage. At anther dehiscence (anthesis), pollen grains are released into the environment, ready to germinate as soon as they are deposited on the hairs of the style. Upon germination, they transfer to a new cell the haploid genetic complement they carry.

Previous studies of gene expression during the developmend of the haploid, male gametophyte in graminaceous species have employed a variety of approaches at the enzyme activity, transcriptional, and translational levels (Singh et al. 1985; Stinson and Mascarenhas 1985; Stinson et al. 1987; Vergne and Dumas 1988; Raghavan 1989). Under heat-shock conditions pollen synthesizes a set of new proteins, some of which are not induced in sporophytic tissues and, therefore, are specifically associated with the gametophytic phase (Frova et al. 1989). Thus, two gene expression programs, the haploid and the diploid, overlap within the plant lifecycle. The meiotic division of the mother cells marks the beginning of the development of male and female gametophytes. The gametophytic stage (haploid program) ends with the double fertilization in the embryo sac, and the sporophytic stage (diploid program) starts with the fertilization of the egg cell and extends throughout the remainder of the life-cycle. Both catalase (E.C., 1.11.1.6; $H_2O_2:H_2O_2$ oxidoreductase; CAT) and superoxide dismutase (E.C., 1.15.1.1. $\cdot O_2^- : \cdot O_2^-$ oxidoreductase; SOD) have been studied primarily during the sporophytic developmental phase of maize (Baum and Scandalios 1979, 1982; Scandalios 1979, 1987). Relatively little information is available from rigorously examining the expression of these genes during the gametophytic phase of maize development (Scandalios 1964; Frova et al. 1987).

In maize, the three catalase isozymes (CAT-1, CAT-2, CAT-3) are encoded by the three unlinked genes, *Cat1*, *Cat2*, and *Cat3*, respectively (Scandalios et al. 1980). The catalase genes are highly regulated temporally and spatially, and respond to various environmental signals (Scandalios 1983). Six superoxide dismutases have been identified in maize: SOD-1 (chloroplastic), SOD-3 (mito-

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chondrial), and SOD-2, SOD-4, SOD-4A, and SOD-5 (cytosolic). These are encoded by the corresponding genes *Sod1, Sod2, Sod3, Sod4, Sod4A,* and *Sod5* (Baum and Scandalios 1979, 1982; Cannon and Scandalios 1989). The possibility exists that *Sod4A* and *Sod5* may be allelic.

Both catalase and superoxide dismutase are believed to play a central role in the defense of the maize plant, and all aerobic eukaryotes, against oxidative stress. Consequently, a study of how these genes are regulated and expressed at a critical stage like anthesis is deemed important. This is so, particularly for plants, because of their oxidative metabolism during photosynthesis, respiration, and photorespiration, times at which various active oxygen intermediates are generated that can lead to damage or cell death. In addition, plants in their natural field environments are constantly bombarded by damaging free radicals from various environmental pollutants. Herein, we report on the expression of these two important antioxidant gene families in the mature pollen of maize.

Materials and methods

Genetic stocks

Three maize (*Zea mays* L.) lines – R6-67 (high CAT activity), W64A (normal CAT activity), and A338 (null for CAT-2; low activity) – were the source of all tissues analyzed in this report. All three lines are maintained by our laboratory.

Inbred R6-67, W64A, and A338 plants were grown in the field, while F_1 crosses between A338 and W64A were grown in the greenhouse. W64A staminate spikelets, dissected from tassels harvested 1 week before anthesis, were frozen in liquid nitrogen and stored at −70 °C until processed for RNA extraction. Mature pollen was collected at anther dehiscence, between 8:00 a.m. and 10:00 a.m., 24 h after tassels were bagged. Pollen was vacuum dessicated over CaCl pellets at 4 °C for 24 h and stored at −20 °C until use.

W64A, A338, and F_1 (A338 × W64A) kernels were surface-sterilized for 10 min in 1% sodium hypochlorite and soaked in distilled water for 24 h. Kernels were then placed on moistened germination paper in sterilized germination boxes. Germination was accomplished at 25–26 °C in a dark growth chamber. W64A, A338, and F_1 (A338 × W64A) scutella, as well as W64A epicotyls dissected from dark-grown seedlings 1 and 4 days postimbibition, were immediately processed for zymogram analysis as catalase and superoxide dismutase isozyme markers. Tissue for RNA isolation was frozen in liquid nitrogen and stored at −70 °C.

Enzyme assays

Catalase and superoxide dismutase were assayed spectrophotometrically as previously described (Beers and Sizer 1952; Beauchamp and Fridovich 1971). An extraction concentration of 1 g fresh weight tissue for 5 ml of 25 m*M* glycylglycine NaOH buffer, pH 7.4 (1 g.f.w./5 ml) was used. Catalase-specific activity and total superoxide dismutase activity are expressed as the change in absorbance/minute/gram fresh weight (ΔA/min/g.f.w.). Protein concentrations were determined as described (Lowry et al. 1951) using bovine serum albumin as standard. Each activity measurement represents the average of two independent experiments with repetitions.

Zymogram analysis

For catalase, starch gel (12%) electrophoresis was performed using a TRIS-citrate buffer system (Scandalios 1968). For superoxide dismutase, electrophoresis was conducted using a lithium hydroxide-boric acid:TRIS-citrate buffer system (Baum and Scandalios 1979). Histochemical staining of the zymograms was a previously described (Scandalios 1968; Baum and Scandalios 1979).

Immunoelectrophoresis analysis

Rocket immunoelectrophoresis (Laurell 1966) was performed as modified by Tsaftaris and Scandalios (1981).

Total RNA isolation and analysis

Total RNA was isolated as previously described (Wadsworth et al. 1988). Total cellular RNA, which will henceforth be referred to as RNA, was separated on denaturing agarose gels (1.2%), transferred to nitrocellulose paper, prehybridized and hybridized at 42 °C for 24 h using 10^6 cpm/ml of radiolabelled (α-^{32}P)dCTP (800 Ci/mmol), gene-specific sequences for *Cat2* (Bethards et al. 1987), *Cat1* and *Cat3* (Redinbaugh et al. 1988), *Sod2* (Cannon et al. 1987), *Sod4* and *Sod4A* (Cannon and Scandalios 1989), and a full-length sequence for *Sod3* (White and Scandalios 1988). Following hybridization, the filters were sequentially washed with 0.1% SDS in 5 × SSC at room temperature for 10 min, 0.1% SDS in 2 × SSC at 42 °C for 1 h, and 0.1% SDS in 0.1 × SSC at 60 °C for 1 h. Autoradiograms were produced by 6-day exposure of Kodak XAR-5 films to the filters between two Kronex intensifying screens.

Results

Isozyme distribution

In the lines examined, zymogram analysis of mature pollen at anthesis showed that CAT-1 is the only detectable catalase isozyme expressed in pollen (Fig. 1). This was deduced from the fact that the observed catalase band comigrates with the CAT-1V (product of the *Cat1V* allele) isozyme, which is the only form known to be expressed in the milky endosperm stage, 18 days postpollination, of W64A and R6-67 kernels. Likewise, pollen of the inbred A338 expresses only the CAT-1F isozyme; this line is homozygous for and expresses only the *Cat1F* allele at the milky endosperm stage (18 days postpollination) of kernel development. Neither CAT-2 nor CAT-3 were detected in pollen extracts by zymogram analysis. The absence of CAT-2 and CAT-3 protein was further verified by rocket immunoelectrophoresis (Fig. 2).

In order to determine whether the expression of CAT-1 in the pollen was of gametophytic (haploid tissue) or sporophytic (diploid tissue) origin, the zymogram patterns of CAT-1 from pollen and scutellum from CAT-1 heterozygous plants were compared. F_1 plants from the cross A338 (*Cat1F/Cat1F*) × W64A (*Cat1V/Cat1V*) would be expected to produce pollen grains one-half of

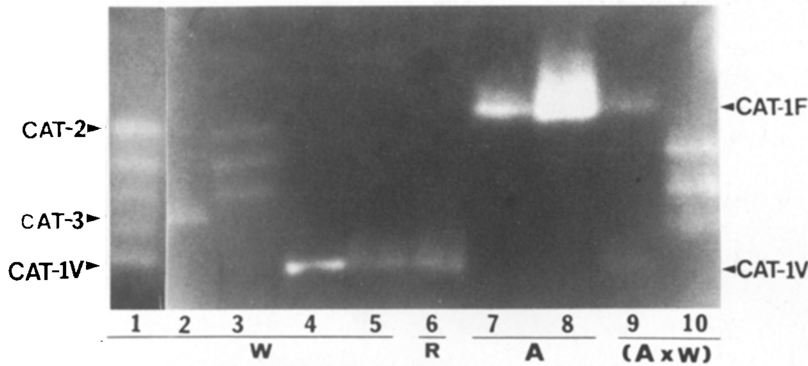

Fig. 1. Zymogram showing the expression of the CAT-1 catalase isozyme in pollen of maize lines W64A (W), R6-67 (R), A338 (A), and the F_1 hybrid A338 × W64A (A × W). *1* – W64A scutellum at 5 DPI showing the characteristic expression of the CAT-1 and CAT-2 isozymes and the hybrid, tetrameric catalases due to intergenic interactions. *2* – Epicotyl at 4 DPI characteristically expressing only the CAT-3 isozyme. *3* – Scutellum at 8 DPI showing shift to CAT-2, which is the only catalase expressed in the scutellum by 10 DPI. *4* – Milky endosperm at 18 DPP, typically expressing only the CAT-1V isozyme. *5* – Pollen of W64A expressing only the CAT-1V isozyme. *6* – Pollen of R6-67 expressing only the CAT-1V isozyme (both W and R are homozygous for the *Cat1V* allele). *7* – Milky endosperm at 18 DPP expressing only the CAT-1F isozyme. *8* – Pollen of A338 expressing only the CAT-1F isozyme (A338 is homozygous for the *Cat1F* allele). *9* – Pollen form the F_1 (A × W) expressing both the CAT-1F and CAT-1V homotetrameric catalases but not hybrid catalases. *10* – Endosperm of the F_1 (A × W) at 30 DPP, showing the CAT-1F and CAT-1V homotetramers and the three intermediate heterotetramers. Mobility is anodal. DPI, days postimbibition; DPP, days postpollination

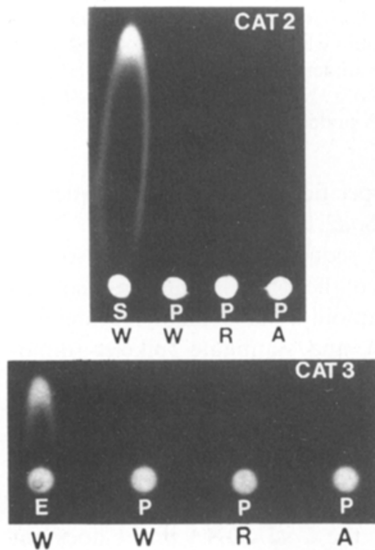

Fig. 2. Rocket immunoelectrophoresis shows the absence of CAT-2 and CAT-3 proteins in W64A (W), R6-67 (R), and A338 (A) pollen. Monospecific, polyclonal Ab for CAT-2 and CAT-3 was used. *S* – W64A scutellum (4 DPI), which normally expresses CAT-2 (except in line A, which is null for CAT-2), was used as CAT-2 positive control. *E* – W64A epicotyl (4 DPI), which normally expresses CAT-3, was used as a control. DPI, days postimbibition. Neither CAT-2 nor CAT-3 protein is detectable in pollen (P)

which carry the *Cat1V* allele, while the other half carries the *Cat1F* allele. Since catalase is a tetrameric enzyme (Scandalios 1965), the resulting zymogram would show a five-banded pattern, the two homotetramers plus the three hybrid heterotetramers, if the CAT-1 in pollen was of sporophytic origin (i.e., translation of CAT-1 occurs premeiotically). Thus, the zymogram pattern from pollen and scutellum would be identical. Alternatively, if *Cat1* mRNA is translated postmeiotically, then a two-banded pattern (only the two homotetramers, CAT-1F and CAT-1V) would be observed in the pool of pollen produced in the F_1 plants. That the latter is, in fact, the case was demonstrated (Fig. 1). Thus, CAT-1 synthesis occurs during the gametophytic phase.

Unlike catalase, superoxide dismutases do not exhibit any obvious tissue or stage specificity during the maize lifecycle. All SOD isozymes characteristic of the inbred lines examined were expressed in the pollen (Fig. 3).

Catalase and superoxide dismutase activities in mature pollen grains

The activities of catalase and superoxide dismutase were measured spectrophotometrically in mature pollen extracts from the inbred maize lines R6-67, W64A, and A338. Since only the CAT-1 isozyme is expressed in pollen, the activity measured is specifically that of CAT-1. Unlike catalase, the superoxide dismutase activity assay measured the activity of all five isozymes expressed in the pollen. The total superoxide dismutase and catalase activities did not vary significantly in the pollen extracts from the three inbred lines (data not shown).

RNA blot analysis of Cat and Sod transcripts

RNA blot analyses were performed to determine whether the steady-state RNA levels corresponded with the levels of maize catalase and superoxide dismutase isozymes in the tricellular, mature pollen grains.

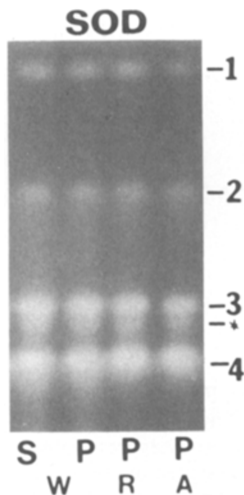

Fig. 3. Zymogram of superoxide dismutases (SOD) in mature pollen (P) and scutellum (S) of lines W64A (W), R6-67 (R), and A338 (A). The SOD isozymes are expressed in all maize tissues examined. The mitochondrial isozyme (SOD-3) and the slower minor band (indicated by *asterisk*) are both resistant to millimolar concentration of cyanide, whereas SOD-1, -2, -4 are sensitive (not shown). Migration is anodal

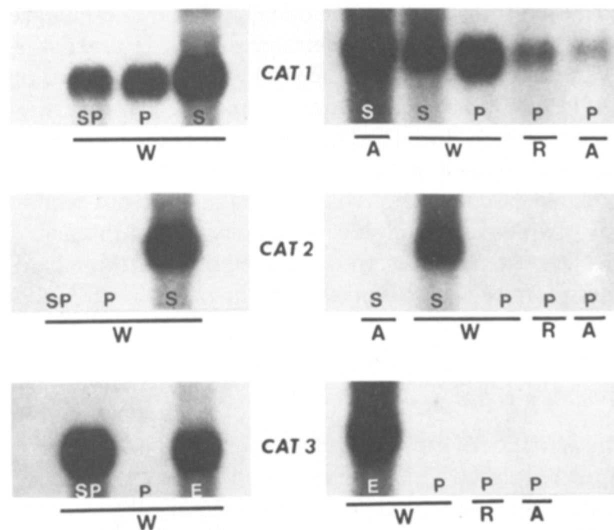

Fig. 4. RNA-blot analyses of *Cat1*, *Cat2*, and *Cat3* RNA levels in mature pollen (P), scutellum (S), epicotyl (E), and staminate spikelet (SP). Left panel shows the accumulation of the catalase transcripts in diplo-haploid (SP), haploid (P), and diploid (S) tissues of W64A (W). In both right and left panels, triplicate RNA samples, isolated from pollen of lines W64A (W), R6-67 (R), and A338 (A), were electrophoresed on a 1.2% formaldehyde, denaturing agarose gel, transferred to nitrocellulose filters, and sequentially probed with a *Cat1*, *Cat2* and *Cat3* gene-specific probe as indicated. Each lane contained 10 µg of total RNA from the tissue indicated. RNA isolated from staminate spikelet (SP) and 4 DPI scutellum (S) were used as controls in *Cat1* and *Cat2* RNA blots; 4 DPI epicotyl RNA (E) and staminate spikelet RNA were used as controls for *Cat3* RNA-blots. Note that pollen expresses only the *Cat1* transcript. DPI, days postimbibition

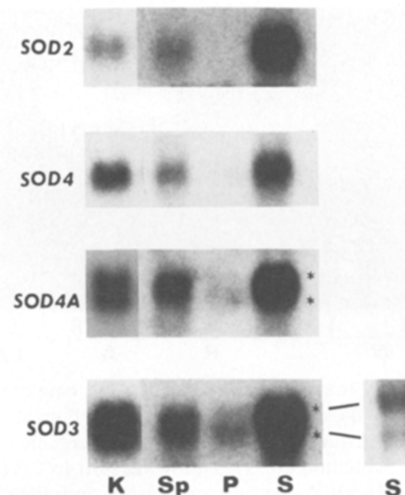

Fig. 5. RNA blots probed with *Sod2*, *Sod4*, and *Sod4A* gene-specific sequences, and with *Sod3* full-length sequence. (*Sod3* does not cross-hybridize to the other *Sods*.) Quadruplicate samples were electrophoresed on a 1.2% formaldehyde, denaturing agarose gel, transferred to nitrocellulose filters, and probed as indicated. Each lane contains 10 µg of total RNA isolated from W64A whole kernel 30 DPP (K), staminate spikelet (SP), pollen (P), and scutellum 4 DPI (S). The *asterisks* indicate double transcripts observed for *Sod4A* and *Sod3*. The double transcripts become more apparent when a 2% (instead of a 1.2%) gel is used for electrophoresis under otherwise identical conditions (right, offset panel for *Sod3*). Note that *Sod2* and *Sod4* transcripts are undetectable in pollen

^{32}P-labeled, gene-specific, 3′-end cDNA sequences for *Cat1*, *Cat2*, *Cat3*, *Sod2*, *Sod4*, *Sod4A*, and a ^{32}P-labeled full-length cDNA sequence for *Sod3* were isolated and used to probe blots of the total cellular RNA isolated from mature pollen (haploid tissue), epicotyl, scutellum, kernel (diploid tissues), and staminate spikelet (diplo-haploid tissue).

Our results demonstrated that the *Cat1* transcript is present in W64A, R6-67, and A338 mature pollen, in W64A and A338 scutellum, and in W64A staminate spikelet (Fig. 4). *Cat2* transcripts are detected only in W64A scutellum. Since the *Cat2* cDNA probe does not crosshybridize with the truncated *Cat2* RNA (Bethards and Scandalios 1988) in the CAT-2 null line A338, no *Cat2* transcripts are seen in A338 (Fig. 4). *Cat3* transcripts accumulated only in W64A epicotyls and staminate spikelets, indicating that *Cat3* is not transcribed in mature pollen (Fig. 4).

W64A staminate spikelets harvested 1 week before anthesis histologically combine sporophytic (glume, lemma, palea, anther) and gametophytic (pollen grain) tissues. As indicated (Fig. 4), only *Cat1* and *Cat3* transcripts are found in staminate spikelets. Since no tissue dissection was achieved in the staminate spikelets, it was not possible to associate *Cat1* and *Cat3* transcripts with the sporophytic tissues and *Cat1* alone with immature pollen in these tissues.

Superoxide dismutase RNA levels are low in mature pollen (Fig. 5). RNA blot analysis on W64A pollen RNA resolves two *Sod3* (Mn-SOD) transcripts. Using this assay, *Sod* transcripts coding for cytosolic forms are barely detectable in the case of *Sod4A* or undetectable in the case of *Sod2* and *Sod4*. Similar results were obtained for R6-67 and A338 pollen (data not shown). This is in contrast to the higher levels of accumulation of *Sod* transcripts in the scutellum, staminate spikelet, and 30-day, post pollination kernel. As in RNA from scutella and 30-day, postpollination, whole kernels, pollen also expresses double transcripts for *Sod3* and *Sod4A*, while single transcripts are observed for *Sod2* and *Sod4* in the other tissues.

Discussion

A single catalase gene is expressed in pollen and its activation and translation is postmeiotic

Based upon zymogram, immunological, and RNA analyses, we have demonstrated that the mature pollen of the maize lines examined express solely the *Cat1* catalase gene. This is in agreement with an earlier report (Scandalios 1964) that a single catalase protein is observed in maize pollen. All catalase activity measurable at this stage of pollen development is specifically due to the CAT-1 isozyme.

RNA blot analysis shows that the steady-state levels of mRNA encoding each isozyme correspond to the tissue-specific expression of each catalase isozyme (Fig. 4). The *Cat1* transcripts have been found in all maize tissues screened to date (Redinbaugh et al. 1990; Wadsworth and Scandalios 1989). The fact that pollen RNA hybridizes only with the *Cat1* gene-specific cDNA sequence is, therefore, consistent with the "constitutive" nature of *Cat1* expression. To date, nulls have been found and characterized for CAT-2 (Tsaftaris and Scandalios 1981; Bethards and Scandalios 1988) and for CAT-3 (Wadsworth and Scandalios, 1990). The apparent lack of a CAT-1 null phenotype among the many lines we screened, in addition to its apparent "constitutive" expression, suggest that CAT-1 expression may be crucial to the viability of maize at some stage in its lifecycle. Loss of functional catalases in *Drosophilia melanogaster* (Mackay and Bewley 1989), *Escherichia coli* (Loewen et al. 1985), and *Saccharomyces cerevisiae* (Cohen et al. 1985) leads to H_2O_2 hypersensitivity and reduced viability. These data suggest that catalases provide protection against oxygen-free radical induced cellular damage by scavenging H_2O_2 and, hence, limiting the formation of the highly reactive, hydroxyl radical

Expression of the CAT-1 protein is postmeiotic. Thus, the CAT-1 isozyme in mature pollen is of gametophytic origin, and protein translation coincides with a period of time in which pollen is exposed to numerous types of environmental stresses. Because transmission of the sperm cells' genetic complement and fertilization depend on the successful germination of the pollen grain under stress, *Cat1* expression may be critical for maize reproduction. If so, one might expect male germ cells carrying a *Cat1* null mutation to exhibit reduced viability. This may coincidentally explain why *Cat1* nulls have not been recovered by normal breeding techniques. In addition, the fact that a single, constitutive catalase gene is expressed in a tricellular tissue makes the mature pollen of maize an exciting system for studying the response to oxidative stress and cellular damage.

Superoxide dismutase transcripts are present at low levels in pollen

Zymograms of pollen extracts show the same SOD isozyme pattern as the scutellum (sporophytic tissue) from the line used (Fig. 3). Although zymograms are not quantitative, the relative levels of the various SODs appear to be the same in pollen and scutellum. This repeatable pattern of SOD isozymes has been reported for other maize tissues such as milky endosperm, scutellum, pericarp, root, primary leaf, coleoptile, sheath, and mesocotyl. (Baum and Scandalios 1979). SOD isozymes, therefore, do not appear to be tissue specific in their expression.

These data correspond to the relative levels of the various SOD transcripts seen in RNA blot analyses (Fig. 5). While the levels of SOD RNAs are much lower in pollen, the relative levels of the various transcripts are similar to those seen in other tissues. Mature pollen represents a terminal stage in pollen development. Therefore, if superoxide dismutase activation, transcription, and translation occur at a premeiotic stage, it may be possible to observe significant levels of SOD proteins due to their higher stability, and low or undetectable SOD steady-state RNA levels due to RNA degradation. It has been demonstrated that the steady-state RNA levels for the *Sod* genes are similar in every maize tissue examined (Cannon and Scandalios 1989). It is also interesting to note that two distinct *Sod3* transcripts are detectable in both pollen and 30-day, postpollination kernel. Also, the accumulation of two *Sod4A* transcripts is distinguishable in kernel RNA. These double transcripts for *Sod3* and *Sod4A* may be explained by (a) transcription initiation-site heterogeneity, (b) transcriptional termination or 3′ processing-site heterogeneity, (c) cross reaction in the *Sod4A*, gene-specific sequence, (d) allelic variation, or (e) multiple genes. Preliminary results from this laboratory using primer extension assays have revealed multiple

transcription initiation sites for *Sod4A* (R. E. Cannon and J. G. Scandalios, unpublished results). Northern analysis often shows multiple-sized transcripts for individual plant mitochondrial genes. The complexity of these transcriptional patterns is largely accounted for by heterogeneity in the 5′ RNA termini. It appears that multiple 5′ termini observed for other maize protein coding transcripts are due to multiple sites of transcriptional initiation rather than multiple RNA processing sites (Levings and Brown 1989). A similar explanation might account for the multiple *Sod3* RNAs observed. Conversely, the *Sod3* mRNA may terminate at different polyadenylation signals (White and Scandalios 1988). The 3′ noncoding regions of *Sod4* and *Sod4A* cDNAs both contain multiple polyadenylation consensus signals (AATAAA), with *Sod4A* having two as direct repeats (Cannon and Scandalios 1989). However, these may or may not be significant, since multiple polyadenylation consensus sequences have been reported for *Cat1* and *Cat3* (Redinbaugh et al. 1988) and for *Sod2* (Cannon et al. 1987), all of which produce a single transcript.

If the length of the transcripts is, indeed, the reason for finding multiple-sized transcripts for *Sod3* and *Sod4A* on northern blots, then these transcripts could be due to allelic variation of these genes. If so, a segregation of different-sized transcripts would be expected to occur. Since only double transcripts for *Sod3* and *Sod4A* have been found, allelic variation might not be the case. This is in agreement with the fact that the lines examined are highly inbred. Therefore, heterozygous loci are not expected to be present unless a mechanism of permanent or fixed heterozygosity (i.e., balanced lethals) is preserving heterozygosity at a given locus.

Finally, data reported by Cannon and Scandalios (1989) indicate that the *Sod4A* gene-specific sequence does not cross-react either with *Sod2* or with *Sod4* sequences. Although there are still no conclusive data indicating that a second isozyme for SOD-3 exists in W64A, the two *Sod3* transcripts detected with RNA blot analysis, together with the existence of two cyanide-resistant electrophoretic forms observed on zymograms, and the slight disparity reported by White and Scandalios (1988) between the N-terminal protein sequence data and their deduced amino acid sequence for SOD-3, suggest that this might be the case.

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