Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5930–5934, June 1995
Developmental Biology

Developmentally related responses of maize catalase genes to salicylic acid

(gene expression/*Zea mays*/hydrogen peroxide)

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Communicated by Irwin Fridovich, Duke University Medical Center, Durham, NC, March 13, 1995 (received for review February 17, 1995)

ABSTRACT **The response of the maize catalase genes (*Cat1*, *Cat2*, and *Cat3*) to salicylic acid (SA) was examined at two distinct developmental stages: embryogenesis and germination. A unique, germination-related differential response of each maize catalase gene to various doses of SA was observed. During late embryogenesis, total catalase activity in scutella increased dramatically with 1 mM SA treatment. The accumulation of *Cat2* transcript and CAT-2 isozyme protein provided the major contribution to the observed increase in total catalase activity. This increase was paralleled by the enhanced growth of germinated embryos at that stage. In a CAT-2 null mutant line, a full compensation of total catalase activity by the CAT-1 isozyme was observed in the presence of SA. This suggests that catalase is important for maintenance of normal cellular processes under stress conditions. SA at 1 mM, which enhances growth of precociously germinated embryos, appeared to inhibit seed germination at 1 day after imbibition. Furthermore, *Cat2* transcript accumulation was inhibited at this stage. SA is probably not a direct signal for the induction of the catalase genes. Other signals, possibly germination-related regulator(s), might be responsible for the induction of the catalase genes. The effect of SA on the activity of purified catalase protein was also examined.**

Activated oxygen—in the form of free radicals, such as superoxide and the hydroxyl radical, and hydrogen peroxide—is toxic (1). To minimize the damaging effects of these activated oxygen species, aerobic organisms evolved enzymatic as well as nonenzymatic antioxidant defense systems. Enzymatic defenses include the catalases and superoxide dismutases, which detoxify hydrogen peroxide and the superoxide radical (2). Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6) is a tetrameric heme-containing enzyme found in all aerobic organisms and participates in converting hydrogen peroxide to water and molecular oxygen.

In maize, three unlinked structural genes, *Cat1*, *Cat2*, and *Cat3*, encode three biochemically distinct isozymes of catalase, CAT-1, CAT-2, and CAT-3 (3–5). The CAT-1 and CAT-2 isozymes are found in the cytosol and/or in glyoxysomes/peroxisomes, whereas CAT-3 is coisolated with the mitochondrial fraction of cells (4, 6). Expression of the catalase genes in maize is highly regulated temporally and spatially (7–9). In addition to developmental regulation, catalase activity is affected by many environmental signals, including light (10, 11) and temperature (12). The catalase genes also respond differently to exogenously applied abscisic acid (13) and the fungal toxin cercosporin (14). Thus, the catalases of maize provide an excellent model system to study the differential regulation of plant genes during embryogenesis and to examine the functional roles of their products in normal plant development and under environmental stress.

Salicylic acid (SA) can be found in the leaves and reproductive structures of many plant species (15) and is believed to modulate many biological processes in plant cells (16). Recent evidence indicated that SA may serve as an endogenous signal molecule in the induction of systemic acquired resistance in tobacco (17). Additionally, a cellular SA-binding protein (SABP) which shares high sequence identity with plant catalase was identified in tobacco leaves (18). It has been proposed that SA can bind to catalase and inhibit its activity, leading to an increase in cellular hydrogen peroxide. It was hypothesized that hydrogen peroxide may transduce the signal that mediates a response to pathogen attack triggering plant defense mechanisms such as systemic acquired resistance (18). We are interested in understanding the mechanisms of antioxidant gene responses to environmental signals, particularly of the maize catalase genes. Here we report on the response of the individual catalase genes of maize to SA in different tissues and developmental stages.

MATERIALS AND METHODS

Plant Materials and Treatment Conditions. The standard catalase inbred maize line W64A and the CAT-2 null line A338F, maintained by our laboratory, were used in these studies. Embryos used were dissected from kernels of field- and greenhouse-grown plants at 25 and 28 days postpollination (dpp) and from germinating seeds at 1 day postimbibition (dpi). Plant ears were harvested in the morning and whole embryos were excised on the same day. Excised embryos were placed on Murashige–Skoog (MS) basic medium (19) supplemented with SA at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mM. Plates were incubated at 25°C for 24 hr in the dark. At the end of each treatment, scutella were isolated and half the samples were used for catalase activity assays and gel electrophoresis. The remaining samples were frozen in liquid nitrogen and stored at −70°C. Young (10-day-old) maize leaves were also used for SA treatments. Leaves were excised and dissected into small pieces. Leaf disks were treated in Petri dishes by flotation in 10 ml of water containing various concentrations of SA. The treatments were performed in the dark for 24 hr.

Catalase Enzyme Assay, Zymogram Analysis, and Protein Determination. Total catalase activity in equal protein samples pooled from each SA dose treatment was determined (20). Catalase activity is expressed as the change in absorbance per min per mg of protein. Protein concentration was determined according to Lowry *et al.* (21) with bovine serum albumin as standard. Gel electrophoresis was performed with equal protein samples and the Tris/citrate buffer system for catalase analyses (22). Western blot analysis was performed (23) with monospecific maize catalase polyclonal antibodies.

***In Vitro* Assay for Catalase Activity After SA Treatment.** Scutella (3 dpi) and 10-day-old leaves were collected and finely

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Abbreviations: SA, salicylic acid; SABP, SA-binding protein; dpp, day(s) postpollination; dpi, day(s) postimbibition.

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ground in protein extraction buffer containing 25 mM glycyglycine (scutella) or 50 mM Hepes buffer (pH 7.5) with 10 mM dithiothreitol (leaves). SA solution was added (1 mM) to the protein extract. The mixed solution was held on ice for 5, 10, 20, 30, or 60 min and total catalase activity was determined at each time point.

Effect of SA on Purified CAT-2 Protein *in Vitro*. CAT-2 protein was purified from 10-dpi scutella as described (24). About 15 μg of purified CAT-2 protein was added to 1 ml of SA binding solution containing 20 mM citrate (pH 6.5) and 5 mM $MgSO_4$ (25). SA was added (1 or 5 mM) to the protein solution. The mixed solution was held at 4°C for 10, 20, 30, or 60 min, and CAT-2 protein (150 ng) was used to determine the catalase specific activity at each time point.

RNA Analyses. Total RNA was isolated from control and SA-treated samples by a modification of the cold phenol extraction method (26). For Northern analysis, total RNA (20 μg) from each sample was separated in denaturing 1.2% agarose gels and transferred onto nitrocellulose (27). The blot was hybridized with ^{32}P-labeled gene-specific probes for *Cat1, Cat2*, and *Cat3* (28, 29). After each analysis was performed, old probes were removed from the filters by repeated washes in boiling 0.1× SSC/0.1% SDS (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

RESULTS

Effects of Increasing Concentrations of SA on Germination and Catalase Expression in Immature Developing Embryos.

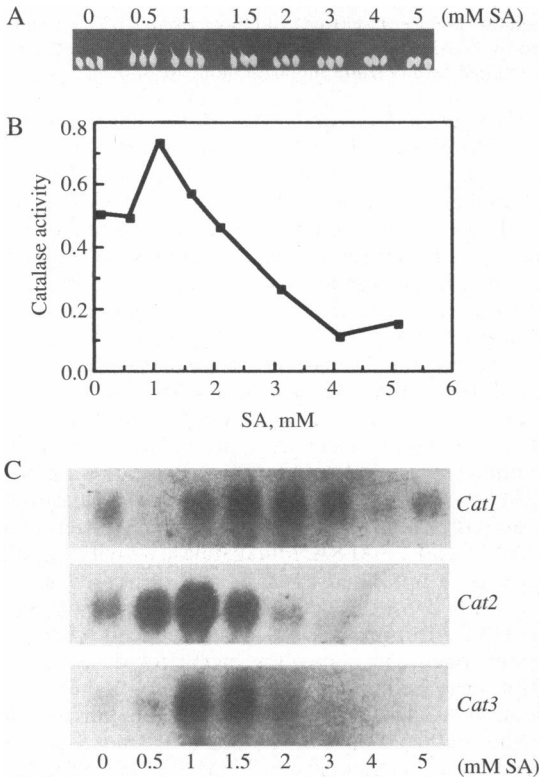

FIG. 1. Responses of maize catalase genes to SA at 25 dpp. Immature embryos were isolated from 25-dpp kernels of field-grown W64A plants and treated with various doses of SA as described. Total catalase activity is expressed as the change in absorbance per min per mg of protein. Total RNA was isolated from each sample for Northern blot hybridization with *Cat1, Cat2*, and *Cat3* gene-specific probes. (*A*) Morphological changes of W64A embryos in response to exogenously applied SA. Three embryos shown here are representative of the total of 25 embryos from each treatment. (*B*) Changes in catalase activity in the presence of SA. Equal amounts of protein from pooled samples for each SA treatment were examined for total catalase activity. (*C*) Changes in catalase transcript accumulation in the presence of various doses of SA.

The effect of SA on developing embryos was examined at several times during seed maturation. Embryos were excised from seeds at 25 and 28 dpp and placed on MS culture medium supplemented with various amounts of SA. After SA treatment for 24 hr, morphological changes in embryo size were observed. In the absence of applied SA in the culture medium, the control 25-dpp embryos started to germinate within 24 hr, as expected. Further, with low concentrations of SA (0.5–1.5 mM) the growth rate was increased significantly; the average embryo axis length was almost twice as long in comparison to the control. However, higher doses of SA (3–5 mM SA) completely inhibited germination (Fig. 1*A*). Total protein in isolated scutella from each SA treatment, as well as untreated controls, was analyzed for changes in total catalase activity as well as individual catalase isozyme activity. In 25-dpp scutella, total catalase activity increased about 50% at 1 mM SA, but decreased at ≥3 mM SA (Fig. 1*B*). The zymogram patterns showed an increase in total catalase activity due to accumulation of CAT-1 and CAT-2 isozymes. There was an increase in *Cat1* transcript at 1–2 mM SA with a maximum at 1.5 mM SA. *Cat1* transcript levels decreased at high concentrations of SA. There was a significant increase in *Cat2* and *Cat3* transcripts at 1 mM SA; the *Cat2* and *Cat3* transcript levels decreased with ≥2 mM SA (Fig. 1*C*). At 28 dpp, dramatic changes in germination and catalase activity were observed. Enhanced growth of germinated embryos was observed over a wide range of SA doses from 0.5 to 3 mM. Total catalase activity increased upon 0.5–3 mM SA treatment, with the maximum increase (4-fold) observed at 1 mM SA. The major contributor to the increased catalase activity was the CAT-2 isozyme, although CAT-1 also increased, but to a lesser extent, after SA treatment (Fig. 2*A*). Western blot analysis using CAT-2 antibody indicated that CAT-2 protein also increased at 0.5–3 mM SA (Fig. 2*B*). In 28-dpp scutella, *Cat1* transcript was low at 0–1 mM SA and increased at ≥1.5 mM SA. In

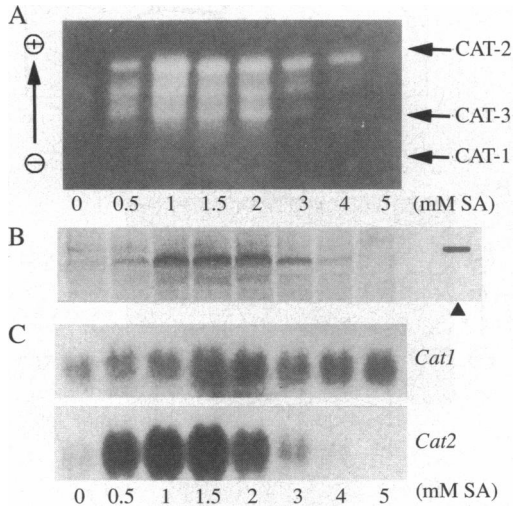

FIG. 2. Changes in catalase activity and isozyme protein of developing embryos in the presence of SA. Embryos were isolated from 28-dpp kernels of field-grown W64A plants and incubated on MS medium supplemented with 0–5 mM SA. Scutella were isolated from treated embryos and equal amounts of protein were examined by zymograms and Western blot analysis using CAT-2 antibody. (*A*) Zymogram pattern of 28-dpp scutella treated with SA. The three catalase homotetramers are indicated. The rest of the bands are CAT-1/CAT-2 heterotetramers, which occur when *Cat1* and *Cat2* are coexpressed. (*B*) Changes in CAT-2 protein after SA treatment as shown by Western blot analysis. Arrowhead indicates lane with purified CAT-2 protein. (*C*) Changes in catalase transcripts shown by Northern blot analysis of 28-dpp W64A scutella treated with various doses of SA. Total RNA was hybridized with the *Cat1* and *Cat2* gene-specific probes.

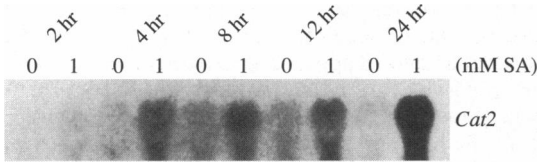

FIG. 3. Kinetics of accumulation of the *Cat2* transcript in the presence of exogenous SA. Embryos were dissected from developing kernels of maize inbred line W64A at 28 dpp. Excised embryos were incubated in the dark on MS medium supplemented with 0 and 1 mM SA. Embryos were harvested at 2, 4, 8, 12, and 24 hr after the beginning of incubation. Total RNA was extracted for Northern blot analysis with the *Cat2* probe.

contrast, the *Cat2* transcript increased dramatically at SA concentrations from 0.5 mM to 3 mM, with the maximum transcript accumulation found at 1 and 1.5 mM SA. *Cat2* RNA levels decreased at the higher doses of SA (4 and 5 mM) (Fig. 2*C*). In a time-course experiment, 28-dpp W64A embryos were isolated and placed on MS medium supplemented with 0 and 1 mM SA for 2, 4, 8, 12, and 24 hr in the dark. Total RNA was isolated from each time point for Northern analysis with the *Cat2*-specific probe. *Cat2* transcript started to accumulate after 4 hr of SA treatment and reached a maximum at 24 hr (Fig. 3). The level of catalase activity was also examined in the CAT-2 null line A338F at 28 dpp. Similar changes were observed, with the total catalase activity increasing upon

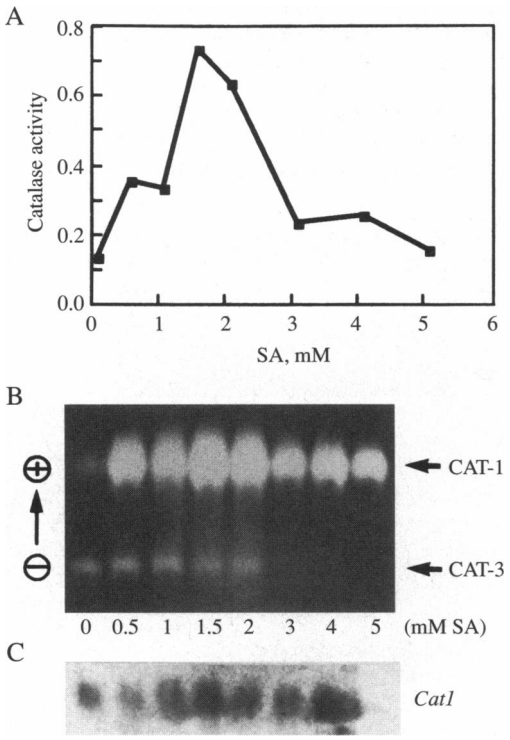

FIG. 4. Changes in catalase activity, isozymes, and transcripts in a CAT-2 null mutant in the presence of SA. Embryos were excised from 28-dpp kernels of the CAT-2 null mutant maize line A338F and treated with 0–5 mM SA as described. (*A* and *B*) Scutella were isolated from treated embryos and equal amounts of protein for each dose treatment were used for catalase activity assays (*A*) and for zymogram analysis. The CAT-1 and CAT-3 homotetramers are indicated. Note that A338F contains the CAT-1 F allele, which migrates faster than the CAT-1 V allele in W64A. Since CAT-3 is compartmentalized in different cell types and organelles than CAT-1 and CAT-2, no hybrid heterotetramers are formed when CAT-3 is coexpressed with the other catalase isozymes. (*C*) Northern blot analysis of 28-dpp A338F scutella treated with increasing doses of SA. Total RNA (20 μg per lane) was hybridized with the *Cat1* probe.

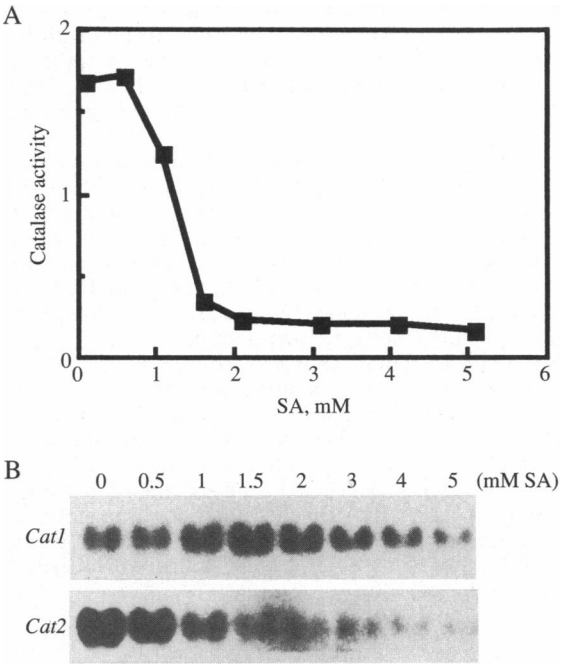

FIG. 5. Response of catalase genes to SA in mature maize embryos. W64A embryos were excised from 1-dpi seeds and treated with 0–5 mM SA as described. Scutella were isolated from treated embryos and examined for catalase activity (*A*) and transcripts (*B*). Transcripts were detected by Northern blot hybridization with *Cat1* and *Cat2* probes. Representative results from duplicate blots are shown.

treatment with up to 4 mM SA; the maximum increase in catalase activity was at 1.5 mM SA (6-fold increase) (Fig. 4*A*). CAT-1 was the only isozyme that contributed to the increase in total catalase activity in the CAT-2 null line. CAT-3 activity remained unchanged at low SA concentrations and decreased at higher SA concentrations (Fig. 4*B*). Thus, CAT-1 appears to compensate for the absence of CAT-2. The *Cat1* transcript in A338F showed an accumulation pattern similar to that observed in the standard line W64A (Fig. 4*C*).

CAT Isozyme and Transcript Accumulation in Scutella of Mature Maize Embryos in Response to SA. The effects of SA on the various catalases in 1-dpi embryos were examined. The rate of germination was slightly inhibited after 0.5 and 1 mM SA treatment and was totally inhibited after treatment with ≥1.5 mM SA. Changes were observed in total catalase activity as well as in individual isozymes in response to different doses of SA. At 0.5 and 1 mM SA, total catalase activity was almost the same as in untreated controls and decreased at higher doses of SA (1.5–5 mM) (Fig. 5*A*). CAT zymograms showed that the CAT-2 isozyme was drastically reduced, while CAT-1 activity remained constant at the higher SA doses. The *Cat1* transcript increased in the presence of 1–2 mM SA and decreased at ≥3 mM SA. Conversely, *Cat2* transcript levels were higher in the absence of SA, decreased at 0.5–1 mM SA, and failed to accumulate at 1.5–5 mM SA (Fig. 5*B*). As with

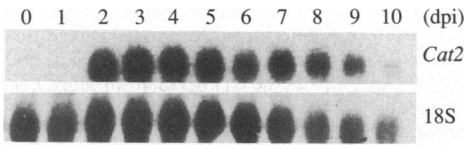

FIG. 6. Accumulation of *Cat2* transcript in scutella of germinated maize embryos. W64A embryos were isolated from germinated seeds at 0–10 dpi. Total RNA was isolated, and the *Cat2* transcript was determined by Northern blot analysis. The blot was reprobed with a DNA fragment containing a sequence from 18S rRNA, to ensure similar loading.

Table 1. Effect of SA on CAT-2 specific activity *in vitro*

SA, mM	Activity, absorbance change per min per mg of protein				
	0 min	5 min	10 min	20 min	60 min
0	830	827	830	793	790
1	825	763	735	750	703
5	830	606	531	515	490

Cat2, the highest *Cat3* transcript accumulation was observed in control samples and decreased with increasing doses of SA; however, the levels of *Cat3* RNA remained low in comparison to *Cat2* RNA. We also examined *Cat2* RNA accumulation in the scutella of mature embryos at 0–10 dpi. At 0 and 1 dpi, no *Cat2* transcript was detected. High levels of *Cat2* transcript were observed at 2 dpi, when the germination process begins, reaching peak levels at 3–5 dpi. *Cat2* RNA decreased to low levels between 8 and 10 dpi (Fig. 6).

Changes in Catalase Activity in Maize Leaves in the Presence of SA. Leaves were excised from 10-day-old light-grown W64A seedlings. The leaves were dissected into small pieces and floated on 10 ml of water supplemented with 0, 1, or 5 mM SA for 24 hr at 25°C in the dark. Leaf samples were collected and assayed for catalase specific activity. Activity increased in the leaves treated with 1 mM and 5 mM SA (data not shown).

Effect of SA on Purified CAT-2 Protein *in Vitro*. Active CAT-2 protein was isolated from 10-dpi scutella and diluted in SA binding buffer (25). Catalase activity was decreased slightly (9%) by mixing with 1 mM SA for 60 min at 4°C. A 35% decrease in activity was found after mixing CAT-2 protein with 5 mM SA for 60 min (Table 1). Similar results were obtained with catalase in crude protein extracts of maize leaves and scutella with 1 mM SA *in vitro* for up to 60 min.

DISCUSSION

Utilizing excised developing embryos and mature embryos, we have demonstrated that each of the three maize catalases responds differently to exogenously applied SA at different developmental stages. As embryogenesis progresses, CAT-1 responded less to SA, while CAT-2 became the predominant isozyme that responded positively to a wide range of SA doses. The drastic increase in scutellar *Cat2* transcript levels with 1 mM SA at 28 dpp is consistent with the observation that both the CAT-2 isozyme protein and CAT-2 activity increased following the same treatment. The *Cat1* transcript level did not change with 1 mM SA; however, an obvious increase in CAT-1 activity was observed on zymograms. A 5-fold increase in total catalase activity was observed in the standard maize line W64A at 28 dpp with 1 mM SA. Time-course experiments with RNA from W64A showed that the *Cat2* transcript started to accumulate after 4 hr of 1 mM SA treatment and did not reach the maximum until 24 hr. This implies that SA may not be the direct signal responsible for the induction of *Cat2* at that stage. In the CAT-2 null line A338F, total catalase activity increased 6-fold with 1 mM SA treatment, and the CAT-1 isozyme was solely responsible for the increase. RNA blot analysis indicated that the increase in CAT-1 activity in A338F was not exclusively due to transcriptional activation. *Cat1* transcript did not accumulate with either 0.5 or 1.0 mM SA, which increased CAT-1 activity in A338F. Thus, in the line missing one catalase isozyme (i.e., CAT-2 null) another catalase isozyme (CAT-1) can be increased to compensate for the loss of catalase activity in order to maintain normal cellular functions. A different response pattern was observed in the scutella of postimbibition maize seeds. The *Cat1* transcript was increased with 1.5 mM SA treatment; however, the *Cat2* transcript failed to accumulate with the same treatment. These results indicated that the response of maize catalases to SA is different at two separate developmental stages—i.e., embryogenesis and germination. Similar responses of catalases to the fungal toxin cercosporin, which generates free radicals, was also reported (14).

The effects of SA on plant growth and development had been examined; but the data reported were ambiguous. SA was classified as a germination and growth inhibitor (30, 31). It was also reported that SA stimulates germination and growth (32). Our studies on maize revealed two distinct and converse effects of SA on embryo germination and CAT expression at two developmental stages. We have found that SA can stimulate growth of precociously germinated embryos at 25–28 dpp. However, SA was found to stunt growth of germinated mature seed. We observed a direct correlation between the effect of SA on germination and CAT-2 activation. Growth of precociously germinated 25-dpp embryos was enhanced by a narrow range of SA (0.5–1.5 mM), while at 28 dpp, the same effect was observed over a wider range (0.5–3 mM). *Cat2* transcript and CAT-2 protein accumulation followed the same pattern as the growth rate of precociously germinated embryos at the two developmental stages. At 1 dpi, *Cat2* transcript failed to accumulate and paralleled the lack of effect of SA on the germination process. These observations suggest that the *Cat2* gene might be induced by a signal, possibly a hormone, during germination. The time-course experiments also support this notion, since a massive accumulation of *Cat2* transcript occurred after 12 hr of 1 mM SA treatment, when radicle elongation actually started. It is likely that the observed effects on germination by SA mimic the natural germination process, where CAT-2 is the predominant isozyme at that stage. During normal germination, *Cat2* transcript accumulation starts at 2 dpi (Fig. 6), when radicle elongation has just begun. This observation also supports the assumption that *Cat2* is induced by a germination-related signal. In contrast to *Cat2*, *Cat1* transcript accumulation occurred at SA doses not favoring germination, being in line with the observation that CAT-1 is the major isozyme during embryogenesis. During the germination process, much energy is required for the massive increase in cell division and radicle elongation. An embryo can germinate only if its respiratory activity and ATP production are sufficient. High levels of activated oxygen, such as superoxide radical and hydrogen peroxide, can be generated with elevated levels of activities in the mitochondria and other biochemical processes within the cell during germination. It is possible that increases in *Cat2* transcript and CAT-2 protein are necessary to deal with oxidative stress caused by SA-stimulated metabolic activities in the cell. The mechanisms by which SA stimulates germination and growth are not clear. It has been proposed that SA possesses chelating properties (33, 34) and the chelation of some important elements of cellular and organellar membranes leads to increased permeabilities by SA, allowing the free access of some metabolites involved in the induction of germination.

A cDNA encoding a SABP with significant identity to catalase has been isolated from tobacco leaves (18). It was suggested that SA, which is believed to be the inducer of specific plant defenses, acts by inhibiting catalase activity. Increased hydrogen peroxide can be detected in SA-treated tobacco leaves, resulting from catalase inhibition, and may serve as a second messenger in the activation of pathogen-related genes. We found that the tobacco SABP has a sequence identity of 76% to our maize CAT-1, 72% to CAT-2, and 69% to CAT-3. In maize, therefore, CAT-1 and CAT-2 are the best candidates to serve as SABPs. We performed the on-plate assay to study the effects of SA on maize catalases and were surprised to find that both CAT-1 and CAT-2 activities were increased in scutella after 24 hr treatment with 1 mM SA. In maize leaves, catalase activity also increased slightly after 24 hr of treatment with 1 mM or 5 mM SA. CAT-2 is the major catalase isozyme in green leaves and was the major contributor to the changes in total catalase activity. We conducted SA

binding experiments with total protein extracted from leaves and scutella, as well as with purified CAT-2 protein. Our results show only slight inhibition of catalase activity with 1 mM SA for 1 hr. Because the sequence identity between each of the three maize catalases and the reported tobacco SABP is not very high, the maize catalases may not serve as SABPs and an alternative mechanism might be involved in maize.

We observed that each maize catalase responded differently to exogenously applied SA. We also observed a significant increase in *Cat2* transcript and CAT-2 isozyme activity with 1 mM SA treatment. This event was paralleled by an enhancement in growth of germinated embryos during late embryogenesis. In the CAT-2 null mutant line, a full compensation of catalase activity by the CAT-1 isozyme was observed. These data support the idea that catalase is important in dealing with stresses caused by elevated metabolic activities and helps to maintain normal cellular functions. Our data also imply that the maize catalases, unlike the tobacco catalase, may not serve as SABPs and that the observed differential—and, most likely, indirect—responses to SA may be indicative of unique metabolic roles for each of the three maize catalases, in addition to their role in scavenging hydrogen peroxide. Our results and those from tobacco (18) suggest that the mechanism of SA action, with respect to catalases and hydrogen peroxide, may be different between monocots and dicots.

We thank Stephanie Ruzsa and Sheri Kernodle for expert technical assistance. This research was supported in part by Grant 94-37-100-0690 from the National Research Initiative Competitive Grants Program, U.S. Department of Agriculture, to J.G.S.

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