Original Article

The Expression Level of a Specific Catalase Isozyme of Maize Mutants Alters Catalase and Superoxide Dismutase during Norflurazon-Induced Oxidative Stress in Scutella

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The effect of catalase (CAT) levels on antioxidant responses to norflurazon (NF)-induced photooxidative stress was examined in 5-day postimbibition (dpi) scutella of a standard maize (*Zea mays*) CAT line (W64A) and various CAT mutants. In W64A and CAT-3 null (WI10D), CAT activities decreased at 1 μ M NF and increased at 10 μ M NF. The CAT activities of CAT-2 null (WA10C) and CAT-2/CAT-3 double null (WDN10) were very low throughout all treatments. The high CAT-2 activity mutant (R6-67) had decreased CAT activity but maintained the highest level among the various CAT lines upon NF treatment, and also had the highest level of SOD activity. Total SOD activity and SOD isozyme patterns were relatively consistent at NF concentrations of 1–10 μ M in scutella of all maize CAT lines examined. The mutants lacking the CAT isozyme were capable of maintaining a high level of SOD in response to NF, demonstrating that SOD is the base-level scavenger of NF-mediated photooxidative stress. An increase in *Cat1*, *Cat2*, and *Cat3* transcript levels occurred in scutella of R6-67, but not W64A following treatment with NF. All *Sod* transcript levels except the level of *Sod1* responded differentially between NF-treated scutella of W64A and R6-67. W64A and maize mutant line expressing strong CAT-2 activity exhibited a different response to NF at the protein and mRNA levels of CAT and SOD. These results indicate that the level of a specific CAT isozyme in the maize mutants affects the levels of CAT and SOD.

Key words: catalase, *Zea mays*, norflurazon, oxidative stress, scutella, superoxide dismutase.

INTRODUCTION

In plants, reactive oxygen species (ROS) have been implicated in damaging effects of various environmental stresses including UV light, xenobiotics, ozone, drought and temperature extremes. To minimize the adverse effects of ROS, plants have evolved numerous enzymatic and nonenzymatic antioxidant defenses.¹⁾ The former include ascorbate peroxidase, glutathione reductase, peroxidase, catalase (CAT) and superoxide dismutase (SOD). Plant catalases are thought to play an important role in the antioxidant defenses in response to environmental as well as physiological stresses.²⁾ Tolerance to environmental stresses such as drought, chilling, and anoxia has been correlated to

increased SOD levels.³⁾ Examples of nonenzymatic antioxidants include glutathione, hydroquinones, ascorbate, α -tocopherol, flavonoids and carotenoids.^{4,5)}

The superoxide radical ($\cdot\text{O}_2^-$) and singlet oxygen ($^1\text{O}_2$) are produced in illuminated chloroplasts. Herbicides that block electron transport or act as alternative electron acceptors and increase the flow of electrons to oxygen significantly increase the production of ROS.⁶⁾ The generation of ROS contributes to the toxic effects of these herbicides. Carotenoids, which are essential components of thylakoid membranes, can effectively quench the excited triplet state of chlorophyll and/or $^1\text{O}_2$.⁷⁾ A potent herbicide norflurazon (NF), which blocks carotenoid biosynthesis by non-competitively binding to phytoene desaturase,⁸⁾ eliminates the important quenchers of the triplet chlorophyll and $^1\text{O}_2$. Thus, this perturbation can cause excess ROS and necessitate additional defenses.

In maize, three unlinked structural genes, *Cat1*, *Cat2* and *Cat3*, encode biochemically distinct CAT isozymes.²⁾ Both

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CAT-1 and CAT-2 are localized in cytosol and glyoxysomes while CAT-3 is mitochondrially associated. During the early stages of germination, both CAT-1 and CAT-2 are expressed in the scutellum.²⁾ As development progresses, CAT-2 becomes the predominant isozyme expressed in scutella while CAT-1 isozyme activity gradually decreases. In maize there are nine SOD isozymes: four Cu/Zn cytosolic isozymes (SOD-2, SOD-4, SOD-4A, and SOD-5), four mitochondrial-associated Mn SODs (SOD-3.1, SOD-3.2, SOD-3.3, and SOD-3.4), and one Cu/Zn chloroplast-associated isozyme (SOD-1).³⁾

The effects of a deficiency in the quenching of ROS by NF on CAT and SOD activities were investigated in 5-day postimbibition (dpi) scutella of the standard maize CAT line W64A and its CAT mutants. Low and high CAT expression mutants were used to determine whether antioxidant responses to NF-induced oxidative conditions are more strongly influenced by any one CAT isozyme. The questions of whether a deficiency or elevation in CAT activity influences SOD levels and/or whether the NF-induced oxidative stress responses of *Cat* and *Sod* genes are affected by an elevated level of CAT activity were examined.

MATERIALS AND METHODS

1. Plant Material and Growth Conditions

The maize (W64A, standard maize CAT line; WA10C, CAT-2 null; WI10D, CAT-3 null; WDN10, CAT-2/CAT-3 double null; R6-67, high CAT-2 activity mutant) seeds were surface-sterilized in 1% sodium hypochlorite, rinsed, immersed in distilled water for 24 hr and then incubated at 25°C in darkness. The isolated scutella from 5 dpi kernels were incubated on Murashige and Skoog (MS) medium (pH 5.8), containing 4.3 g l⁻¹ MS salts, 3% sucrose and 8 g l⁻¹ agar, with or without norflurazon [NF; 4-chloro-5-(methylamino)-2-(3-trifluoromethyl)phenyl-3(2H)-pyridazinone] (Novartis Inc., USA) at 25°C for 24 hr and placed under 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 10 hr. The treated scutella were used for the experiment.

2. Enzyme Assays

The NF-treated scutella were homogenized in 25 mM glycylglycine buffer, pH 7.4. Extracts were centrifuged for 10 min at 14,000 $\times g$ and 4°C. Total CAT activity from crude extracts was measured spectrophotometrically at 25°C in a 3-ml volume containing 50 mM potassium phosphate buffer (pH 7.0) and 20 mM H₂O₂ and by monitoring H₂O₂ destruction at 240 nm.⁹⁾ Total CAT activity is expressed as the change in absorbance per min (unit) per mg of protein. Protein concentration was determined by the method of Lowry *et al.*¹⁰⁾ using bovine serum albumin as the standard.

Total SOD activity in equal protein samples pooled from each NF treatment was determined using the xanthine oxidase-cytochrome *c* method at pH 10.0.¹¹⁾ The assay was performed at 25°C in a 3-ml volume containing 50 mM sodium carbonate buffer (pH 10.0), 0.1 mM EDTA, 0.015 mM

ferricytochrome *c*, and 0.05 mM xanthine. Enzyme activity (units/mg protein) is proportional to $(V/v - 1)/\text{mg protein}$, where *V* and *v* equal the change in absorbance (550 nm) per minute (unit) in the absence and presence of SOD, respectively.

3. Zymogram Analysis and Activity Staining

Equal amounts of protein were separated on 10% non-denaturing polyacrylamide gels at 4°C for 1.5 hr with a constant current of 30 mA. Gels were stained for SOD isoforms by soaking in 50 mM potassium phosphate (pH 7.8) containing 2.5 mM nitroblue tetrazolium in darkness for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 28 mM tetramethylethylenediamine and 28 μM riboflavin in darkness for 30 min and then exposed to light.¹²⁾

4. RNA Extraction and Analyses

Total RNA was isolated from control and NF-treated samples as described,¹³⁾ separated in denaturing 1.6% agarose gel and transferred onto nylon membranes (Schleicher and Schuell Nytran Plus). The blots were sequentially hybridized with ³²P-labeled gene-specific probes for *Cat1*, *Cat2*, *Cat3*,^{14,15)} *Sod1*, *Sod2*, *Sod3*, *Sod4* and *Sod4A*¹⁶⁻¹⁸⁾ in modified Church buffer.¹⁹⁾ After each analysis, probes were removed from the filters by repeated washes in boiling 0.1% SDS/0.1 $\times$ SSC (0.15 M NaCl, 1.5 mM sodium citrate, pH 7.0) and reprobed with a DNA fragment from clone pH2A containing an 18S ribosomal sequence.²⁰⁾

RESULTS

1. Effects of Norflurazon on Catalase in 5-Day Postimbibition Scutella of W64A and CAT Mutants

Total CAT activity was investigated to provide insights into the specific role of this enzyme in 5 dpi scutella of maize variants treated with NF. In the untreated controls, CAT activity levels of WI10D and R6-67 were higher than those of W64A, whereas those of WA10C and WDN10 were nearly undetectable (Table 1). In response to NF, total CAT activity responded differently in the scutella of various maize CAT lines (Table 1). The 5 dpi scutella of W64A and WI10D showed a decline in CAT activity at 1 μM NF and an increase at 10 μM NF (Table 1). The CAT activity levels of WA10C and WDN10 remained very low at all NF concentrations tested. The NF-treated scutella of R6-67 showed slightly decreased CAT activity at all NF concentrations examined but the activity was still much stronger than for W64A (Table 1).

2. Effects of Norflurazon on Superoxide Dismutase in W64A and CAT Mutants

The changes in total SOD activity and SOD isozyme patterns during NF-induced oxidative stress were examined in CAT-deficient mutants and a high CAT-2 activity mutant. In the untreated controls, the 5 dpi scutella of R6-67 had a

Table 1. Effects of norflurazon on catalase activities of 5-day postimbibition scutella of the standard maize CAT line W64A and CAT mutants

NF concentration (μM)	Catalase (units mg^{-1} protein)				
	W64A	WA10C	WI10D	WDN10	R6-67
0	$1.252 \pm 0.012\text{b}$	$0.013 \pm 0.001\text{a}$	$1.837 \pm 0.057\text{b}$	$0.007 \pm 0.001\text{ab}$	$4.704 \pm 0.038\text{a}$
1	$0.829 \pm 0.014\text{c}$	$0.005 \pm 0.001\text{a}$	$1.209 \pm 0.018\text{c}$	$0.013 \pm 0.005\text{a}$	$4.126 \pm 0.021\text{b}$
5	$1.320 \pm 0.054\text{b}$	$0.004 \pm 0.000\text{a}$	$1.819 \pm 0.057\text{b}$	$0.012 \pm 0.005\text{ab}$	$3.554 \pm 0.021\text{c}$
10	$1.689 \pm 0.011\text{a}$	$0.011 \pm 0.007\text{a}$	$2.102 \pm 0.020\text{a}$	$0.004 \pm 0.001\text{b}$	$3.813 \pm 0.126\text{d}$

The isolated scutella from 5 dpi maize kernels (W64A, standard maize CAT line; WA10C, CAT-2 null; WI10D, CAT-3 null; WDN10, CAT-2/CAT-3 double null; R6-67, high CAT-2 activity) were incubated on MS plates or MS plates containing various concentrations of NF at 25°C for 24 hr and then placed under illumination for 10 hr. Data represent the mean $\pm$ SD of two independent experiments with repetitions. Within each column, means with the same letter are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Table 2. Effects of norflurazon on superoxide dismutase activities of 5-day postimbibition scutella of the standard maize CAT line W64A and CAT mutants

NF concentration (μM)	Superoxide dismutase (units mg^{-1} protein)				
	W64A	WA10C	WI10D	WDN10	R6-67
0	$48.8 \pm 2.4\text{ab}$	$40.0 \pm 10.2\text{a}$	$43.5 \pm 1.5\text{a}$	$45.2 \pm 3.6\text{a}$	$69.9 \pm 1.4\text{a}$
1	$49.2 \pm 0.4\text{ab}$	$41.3 \pm 9.2\text{a}$	$47.4 \pm 0.7\text{a}$	$44.5 \pm 6.6\text{a}$	$63.0 \pm 0.7\text{b}$
5	$48.2 \pm 4.1\text{b}$	$41.6 \pm 8.0\text{a}$	$46.7 \pm 5.4\text{a}$	$45.6 \pm 8.6\text{a}$	$65.5 \pm 4.1\text{b}$
10	$51.9 \pm 1.6\text{a}$	$42.0 \pm 11.5\text{a}$	$48.5 \pm 4.1\text{a}$	$47.3 \pm 6.3\text{a}$	$63.9 \pm 2.5\text{b}$

The isolated scutella from 5 dpi maize kernels (W64A, standard maize CAT line; WA10C, CAT-2 null; WI10D, CAT-3 null; WDN10, CAT-2/CAT-3 double null; R6-67, high CAT-2 activity) were incubated on MS plates or MS plates containing various concentrations of NF at 25°C for 24 hr and then placed under illumination for 10 hr. Data represent the mean $\pm$ SD of two independent experiments with repetitions. Within each column, means with the same letter are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

higher total SOD activity level than W64A and all the CAT-deficient mutants (Table 2). Total SOD activities remained almost constant at different NF concentrations in the 5 dpi scutella of all CAT lines examined, and the levels of SOD activity were similar among various maize CAT lines except for greater SOD activity in R6-67 (Table 2).

Gels stained for SOD isozymes corresponded to the induction profile of SOD-specific activity (Table 2 and Fig. 1). In 5 dpi scutella of WI10D, chloroplastic Cu/Zn SOD-1, cytosolic Cu/Zn SOD-2 and SOD-4 increased slightly in response to NF (Fig. 1). In R6-67, SOD-1 decreased slightly in response to NF, but the levels of other SOD isozymes remained constant (Fig. 1). However, in the other CAT lines, amounts of all SOD isozymes remained constant at various NF concentrations (Fig. 1). SOD-4 was a predominant SOD isoform in the 5 dpi scutella of all maize CAT lines, particularly R6-67 (Fig. 1). Overall, total

staining activities of SOD isozymes in R6-67 were much greater than those of other CAT lines, demonstrating that total SOD activity was also higher in the high CAT-2 activity mutant (Table 2). Mitochondrial Mn SOD-3 levels remained very low in all maize CAT lines (Fig. 1).

3. Effects of Norflurazon on Cat Gene Expression in W64A and R6-67

The influence of norflurazon on transcript levels of *Cat* participating in the scavenging of ROS during oxidative stress was examined using gene-specific probes in 5 dpi scutella of W64A and R6-67, which showed a clearly different response to NF-induced oxidative stress (Tables 1 and 2; Fig. 1). In the untreated controls, all *Cat* genes exhibited higher expression levels in R6-67 than in W64A, especially *Cat2* (Fig. 2). NF increased the expression level of all *Cat* genes in 5 dpi scutella of R6-67 (Fig. 2). In contrast, the

Fig. 1. Effects of norflurazon on superoxide dismutase (SOD) isozymes in scutella of the standard maize CAT line W64A and CAT mutants. The isolated scutella from 5-day postimbibition kernels were incubated on MS plates or MS plates containing various concentrations of NF at 25°C for 24 hr and then placed under illumination for 10 hr. Non-denaturing activity gels were run after NF treatment.

Fig. 2. Response to norflurazon exposure of the *Cat* genes in scutella of the standard maize CAT line W64A and CAT mutants. The isolated scutella from 5-day postimbibition kernels were incubated on MS plates or MS plates containing various concentrations of NF at 25°C for 24 hr and then placed under illumination for 10 hr. Total RNA was extracted, separated on 1.6% agarose gels, at 20 μ g per lane, and transferred to membranes. Probing was done with [α - 32 P]-labeled gene-specific DNA fragments. The 18S rRNA is a loading control.

level of *Cat1* transcript was unchanged and that of *Cat2* transcript decreased or remained constant in response to NF in scutella of W64A (Fig. 2). The *Cat3* transcript was almost undetectable at all NF concentrations in W64A (Fig. 2).

4. Effects of Norflurazon on *Sod* Gene Expression in W64A and R6-67

In the untreated controls, the 5 dpi scutella of R6-67 had higher levels of *Sod3* and *Sod4A* than the scutella of W64A (Fig. 3). The *Sod1* transcript level decreased slightly at 5 μ M NF in 5 dpi scutella of W64A and R6-67 (Fig. 3). In response to NF, the scutella of W64A and R6-67 responded differently in terms of the transcript levels of the other *Sods*. The NF-treated scutella of W64A showed a marked increase in the *Sod2* transcript at 1 μ M and 5 μ M NF

Fig. 3. Response to norflurazon exposure of the *Sod* genes in scutella of the standard maize CAT line W64A and CAT mutants. The isolated scutella from 5-day postimbibition kernels were incubated on MS plates or MS plates containing various concentrations of NF at 25°C for 24 hr and then placed under illumination for 10 hr. Total RNA was extracted, separated on 1.6% agarose gels, at 20 μ g per lane, and transferred to membranes. Probing was done with [α - 32 P]-labeled gene-specific DNA fragments. The 18S rRNA is a loading control.

and then a decrease at 10 μ M NF (Fig. 3). In R6-67, the *Sod2* transcript level was unchanged at 1 μ M and 5 μ M NF but slightly decreased at 10 μ M NF (Fig. 3). *Sod3* and *Sod4* transcript levels in NF-treated scutella of W64A remained constant at all NF concentrations examined (Fig. 3). However, in scutella of R6-67, *Sod3* increased slightly at 10 μ M NF and *Sod4* increased greatly at all NF concentrations examined. *Sod4A* transcript levels in scutella of W64A and R6-67 increased at 5 μ M and 1 μ M NF, respectively (Fig. 3).

DISCUSSION

ROS including $\cdot\text{O}_2^-$ and $^1\text{O}_2$ are produced in illuminated chloroplasts.⁷⁾ Perturbation by NF, which drastically reduces nonenzymatic antioxidant carotenoids, may cause excess ROS and necessitate additional defense, enzymatic antioxidants. Standard maize line, CAT-deficient mutant and high CAT mutant plants showed no discernible physiological effects when grown under normal conditions.²⁾ In 5 dpi scutella of maize, the various maize CAT lines responded differently in total CAT activity upon NF treatment, whereas a noticeable change in total SOD activity and SOD isozymes was not observed at different NF concentrations in any maize CAT lines (Tables 1 and 2; Fig. 1). There were differences in *Cat* and *Sod* transcript levels as well as in CAT activity between W64A and R6-67 in response to NF-induced oxidative stress (Tables 1 and 2; Figs. 1-3).

The expression of the catalase gene is not only influenced by genetic and developmental signals, but also significantly influenced by and responsive to various environmental signals, both physical and chemical.²¹⁾ In 5 dpi scutella of W64A and WI10D, NF caused a decline in total CAT activity at $1\ \mu\text{M}$ NF and an increase at $10\ \mu\text{M}$ NF whereas, in R6-67, NF slightly decreased total CAT activity although the level was still higher than in W64A (Table 1). The change in CAT activity appeared to be triggered by photooxidative stress. The levels of all *Cat* transcripts were higher in R6-67 than in W64A and the *Cat* genes responded strongly to NF in R6-67 at all NF concentrations tested (Fig. 2). The *Cat1* transcript level increased at $1\ \mu\text{M}$ NF in 5 dpi scutella of R6-67, but no change in *Cat1* was observed in NF-treated scutella of W64A (Fig. 2). The expression of the *Cat1* gene is weak in almost all tissues and light-independent throughout development.²²⁾ Upon exposure to light, there is rapid accumulation of CAT-2 in leaves due to both increased transcription²³⁾ and increased translation of the *Cat2* message.²⁴⁾ *Cat2* responded sensitively to NF in the 5 dpi scutella of R6-67 (Fig. 2), suggesting that the *Cat2* gene is critical to plants' adaptive responses to NF-induced oxidative stress. *Cat2* expression is rapidly induced during embryo growth, and by day 5 postpollination, CAT-2 becomes the predominant isozyme.²⁾ *Cat3* exhibits a transcriptionally regulated circadian rhythm showing maximal expression late in the light period.^{23,25,26)} *Cat3* transcript was not detected in NF-treated scutella of W64A 10 hr after the light onset but it increased in NF-treated scutella of R6-67 (Fig. 2). Reduced inactivation of photosynthetic genes due to enhanced antioxidant enzymes might lead to the high level of *Cat3* transcript in R6-67. In *Arabidopsis* and *Nicotiana plumbaginifolia*, the accumulation of catalase transcripts in the early stage of the light period coincides with that of photosynthetic genes.²⁷⁾

Effective antioxidant defenses can scavenge copious amounts of ROS generated in illuminated chloroplasts. SOD activities increased in response to treatment with

herbicides that serve as preferred electron acceptors at the reducing side of photosystem I or that are known to block electron transport.^{28,29)} Thus, SOD has often been correlated with mechanisms of herbicide survival or tolerance. However, the NF-treated 5 dpi scutella of the maize CAT lines did not show a noticeable change in total SOD activity and SOD isozymes, and the SOD activity levels were highest in R6-67 among the CAT lines for all the NF treatments (Table 2 and Fig. 1). A high steady-state level of SOD might be necessary at all times to provide adequate protection in 5 dpi scutella of maize. W64A and R6-67 showed a different response to NF-induced oxidative stress at *Sod* transcript levels, with an increase of *Sod2* and *Sod4A* in W64A and an increase of *Sod3*, *Sod4*, and *Sod4A* in R6-67 (Fig. 3). Taken together, R6-67 having high CAT activity also exhibited stronger SOD activity compared to other maize CAT lines (Tables 1 and 2; Fig. 1), implying that the high level of CAT-2 influences the activity of SOD.

NF caused a slight alteration in CAT at the protein level in W64A, WI10D and R6-67, with the highest level of CAT activity in R6-67. All CAT mutants were capable of maintaining a high level of SOD in response to NF, suggesting that SOD is the base-level scavenger in NF-induced oxidative stress. The level of *Cat1* and *Cat3* as well as *Cat2* increased in response to NF-induced photooxidative stress in the scutella of R6-67, but not in W64A. R6-67 also showed higher levels of *Sod3*, *Sod4*, and *Sod4A* as well as SOD isozymes than W64A. NF did not cause a noticeable change in SOD activities, although *Sod* genes changed significantly in response to NF in both W64A and R6-67, indicating a posttranscriptional regulation of these genes. The antioxidant response using CAT nulls and a high CAT activity mutant provided insights into the localization and function of these antioxidant enzymes.

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