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Differential antioxidant responses in catalase-deficient maize mutants exposed to norflurazon

S. JUNG*, S.U. CHON and Y.I. KUK

School of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul, 151-921, Korea

Abstract

Maize catalase (CAT) mutants lacking in CAT isozymes were used to investigate the response of CAT and superoxide dismutase (SOD) to norflurazon (NF) caused oxidative stress in 18-d post-pollination scutella. NF treatment caused an increase in malonyldialdehyde content, which is an indicator of oxidative stress, and a decrease in a content of photoprotectant carotenoids in the scutella. CAT activity decreased greatly with increasing NF concentration in standard maize line (W64A) and CAT-2 null mutant (WA10C), however, it remained relatively constant in CAT-2/CAT-3 double null mutant (WDN10). *Cat1* transcript increased in the NF-treated scutella of W64A and WDN10, with a greater *Cat1* transcript in WDN10, whereas *Cat2* transcript decreased in the NF-treated scutella of all lines examined. In all lines, SOD activities were not changed noticeably at various NF concentrations. In the scutella of W64A, *Sod4A* decreased in response to NF but the other *Sod* transcript contents were unchanged. The CAT-deficient mutants reacted with raised transcripts of *Sod1*, *Sod3*, *Sod4* or *Sod4A*.

Additional key words: carotenoids, catalase, oxidative stress, superoxide dismutase, *Zea mays*.

Introduction

Various environmental factors including high irradiance, drought, temperature extremes, and herbicides produce excess of reactive oxygen species (ROS), which are eliminated efficiently by an integrated system of enzymatic and non-enzymatic antioxidants (Asada 1994). Among these, the combined action of catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) is critical in mitigating the effects of oxidative stress. Alterations in CAT and SOD activities against oxidative processes have been observed in many plants, underscoring the critical role of these enzymes (Slooten *et al*. 1995, Van Camp *et al*. 1996, Goel and Sheoran 2003, Sairam *et al*. 2003/4). The non-enzymatic reductants consist of ascorbate, glutathione, α-tocopherol, carotenoids, and phenolic compounds (Alscher and Hess 1993, Foyer *et al*. 1994, Jung 2004). Especially, carotenoid-dependent energy dissipation in the light harvesting antennae is thought to play an important protective role by mitigating oxidative damage.

The transgenic maize plants provided an excellent model for determining the role of CAT and SOD in response to NF. In maize, three CAT isozymes are encoded by three genes *Cat1, Cat2* and *Cat3*. CAT-1 and CAT-2 are found in peroxisomes and glyoxysomes as well as in cytosol (Scandalios *et al*. 1997). CAT-3 is associated with mitochondria. The expression of each maize catalase gene is tissue- and developmental stage-dependent (Scandalios *et al*. 1997). *Cat1* is expressed in the scutellum during seed maturation, reaching high levels in the late stages of embryogenesis. *Cat2* transcript starts to accumulate during the late stages of seed maturation and drops to undetectable levels in dry seeds. *Cat3* is detected during very early post-pollination kernel development, but declines rapidly shortly after pollination. Maize SOD consists of nine distinct isozymes encoded by *Sod1*, *Sod2*, *Sod3.1*, *Sod3.2*, *Sod3.3*, *Sod3.4*, *Sod4*, *Sod4A*, and *Sod5* genes (Scandalios 1997). SOD-1 is a Cu,Zn enzyme associated with chloroplasts. SOD-2,

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Abbreviations: CAT - catalase; dpi - days post-imbibition; dpp - days post-pollination; MDA - malonyldialdehyde; MS - Murashige and Skoog; NF - norflurazon; SOD - superoxide dismutase.
* Fax: (+82) 2 8735260, e-mail: sjung@snu.ac.kr

SOD-4, SOD-4A and SOD-5, also Cu,Zn enzymes, are cytosolic. MnSOD-3, encoded by the multigene *Sod3* family, is localized in mitochondria.

Photooxidative damage is exacerbated by herbicides, which generate ROS either by direct involvement in radical production or by inhibition of biosynthetic pathways (Elstner *et al*. 1988, Ezhova *et al*. 1997). A potent herbicide norflurazon (NF) blocks carotenoid biosynthesis by non-competitive binding to phytoene desaturase (Bramley 1993). It eliminates important quenchers of the triplet chlorophyll and $^1O_2$, thus inducing photooxidative processes. The NF-caused oxidative damage to cucumber leaves resulted in a reduction in contents of chlorophyll and carotenoids, and CAT activity, with a concomitant increase in SOD activity (Jung *et al*. 2000). A substantial increase in *Cat1*, *Cat2* and *Sod1* transcripts occurred in the NF-treated leaves of maize plants (Jung *et al*. 2001).

In the present study, 18-d post-pollination (dpp) scutella of maize were used to investigate the NF-induced responses of the tissue at this developmental stage. We examined whether NF-induced oxidative stress influences an H_2O_2-producing enzyme, SOD, and an H_2O_2-scavenging enzyme, CAT, at mRNA and protein levels in 18 dpp scutella. In addition, transcript levels of *Cat*s and *Sod*s and activities of CAT and SOD were compared in scutella of the standard maize line W64A and its CAT variant lines, WA10C and WDN10.

Materials and methods

The maize (*Zea mays* L.) lines (W64A, standard maize line; WA10C, CAT-2 null mutant; WDN10, CAT-2/CAT-3 double null mutant) were used for this study. The shorter *Cat2* transcript of *Cat2* null line was about 1400 nucleotides, compared to the 1850-nucleotide *Cat2* transcript in standard maize line (Alber and Scandalios 1991). The missing portion of the transcript is present in the genomes of line null for the CAT-2 isozyme. A rearrangement of the *Cat2* gene had occurred in the CAT-2 null line, suggesting that this CAT-2 null mutation in maize is due to a DNA insertion into the *Cat2* gene. Significant structural alterations in the *Cat3* gene of CAT-3 nulls suggest that the molecular basis for the CAT-3 null phenotype is due to a deletion in the 5′ end of the *Cat3* gene (Wadsworth and Scandalios 1990). For immature embryos, maize ears were harvested from field plants at 18 d post-pollination and whole embryos were extracted from kernels on the same day. The isolated scutella from 18 dpp kernels were incubated on Murashige and Skoog (1962; MS) plates or MS plates containing various concentrations of norflurazon [NF; 4-chloro-5-(methylamino)-2-(3-trifluoromethyl)phenyl-3(2*H*)-pyridazinone] (*Novartis*, Seoul, Korea) at 25 ºC for 12 h in the dark and then exposed to 250 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) for 12 h. The treated scutella were used for experiments.

Carotenoids were determined in 100 % acetone extract with a *Shimadzu UV-2401PC* spectrophotometer (*Shimadzu*, Tokyo, Japan) according to the method of Lichtenthaler (1987). The absorbance of the solution was measured at 470, 645 and 662 nm.

For the assessment of oxidative stress in scutella, the thiobarbituric acid (TBA) test, which determines content of malonyldialdehyde (MDA) as an end product of lipid peroxidation (Heath and Packer 1968), was used. Scutella (0.1 g) of control and NF treatment were homogenized in 5 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 12 000 *g* for 15 min and 0.5 cm^3 of the supernatant was added to 1 cm^3 of 0.5 % (m/v) TBA in 20 % TCA. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. Then the samples were centrifuged at 10 000 *g* for 5 min, and the absorbance of the supernatant at 532 nm for each sample was recorded and corrected for nonspecific turbidity at 600 nm. MDA concentration was calculated using a coefficient of absorbance of 156 $mmol^{-1}$ cm^{-1}.

Scutella from each treatment were homogenized in a mortar and pestle in 25 mM glycylglycine buffer, pH 7.4. Extracts were centrifuged for 10 min at 14 000 *g* and 4 ºC. The crude supernatant was used for enzyme assay. Total catalase activity in equal protein samples was determined spectrophotometrically at 25 ºC in a 3-cm^3 volume containing 50 mM potassium phosphate buffer (pH 7.0) and 20 mM H_2O_2 and by monitoring H_2O_2 destruction at 240 nm (Beers and Sizer 1952). Total catalase activity is expressed as the change in absorbance per min (unit) per mg of protein. Total SOD activity was determined using the xanthine oxidase-cytochrome *c* method (Spychalla and Desborough 1990). The assay was performed at 25 ºC in a 3-cm^3 volume containing 50 mM sodium carbonate buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome *c*, and 0.05 mM xanthine by adding xanthine oxidase. Enzyme activity [U mg^{-1}(protein)] is proportional to (V/v - 1)/mg(protein), where V and v equal the change in absorbance (550 nm) per min in the absence and presence of SOD, respectively. Protein concentration was determined according to Lowry *et al*. (1951). Bovine serum albumin (BSA) was used as standard.

All samples were frozen in liquid nitrogen and stored at -80 ºC for analysis. For studying the activation of specific *Cat* and *Sod* genes, total RNA was isolated from control and NF-treated samples as according to Thompson *et al*. (1983). For northern analysis, total RNA (20 μg) from each sample was separated in denaturing 1.6 % agarose gel and transferred onto nylon membranes (*Schleicher* and *Schuell Nytran Plus*). The blots were

sequentially hybridized with ^{32}P-labeled gene-specific probes for *Cat1*, *Cat2*, *Cat3*, *Sod1*, *Sod2*, *Sod3*, *Sod4* and *Sod4A* in modified Church buffer (Church and Gilbert 1984), containing 7 % SDS, 1 mM EDTA, 0.25 M $NaHPO_4$ and 1 % BSA. Full-length *Cat2* probe was also used to identify the aberrant shortened *Cat2* transcript in the CAT-2 nulls. After 2-h prehybridization at 65 °C, the prehybridization solution was substituted with hybridization solution. Hybridization proceeded at 65 °C overnight, and washes were performed once for 20 min with 2 × SSC (0.15 M NaCl, 1.5 mM sodium citrate, pH 7.0) and twice for 30 min with 0.1 × SSC at 65 °C. Blots were finally probed with a DNA fragment from clone pHA2, containing an 18S ribosomal sequence (Jorgensen *et al*. 1987).

Data were analyzed by Duncan's Multiple Range Test at $P < 0.05$.

Results

In untreated control, carotenoid contents were similar in 18 dpp scutella of maize line W64A and CAT-null mutants, WA10C and WDN10 (Fig. 1*A*). Carotenoid contents significantly decreased at 1 μM NF concentration and further decreased with increasing NF concentrations in all maize lines examined (Fig. 1*A*). The magnitude of decrease in carotenoids was less in WDN10 at 5 μM NF, compared to the other lines. In NF-treated scutella, the decline in carotenoids occurred concomitantly with an increase in MDA content, which indicates a lipid peroxidation (Fig. 1*A*,*B*). The MDA content of W64A and WA10C was greater than that of WDN10 at 5 μM NF (Fig. 1*B*).

The 18 dpp maize scutella were used to assess the NF-caused response of catalase. In untreated control, W64A and WDN10 showed a greater CAT activity than WA10C (Fig. 1*C*). In the NF-treated scutella of W64A, CAT activities decreased considerably at 5 and 10 μM NF. The scutella of WA10C and WDN10 showed less pronounced decrease in CAT activities with NF treatment in comparison to W64A; WDN10 had a greater CAT activity than WA10C in all NF treatments (Fig. 1*C*). In untreated control, SOD activities were almost the same in all CAT lines examined (Fig. 1*D*). The SOD activities were not changed noticeably in response to NF treatment and they were not different between W64A and CAT-null mutants (Fig. 1*D*).

In untreated control of 18 dpp scutella, *Cat1* transcript levels were higher in WDN10 than in W64A and WA10C (Fig. 2). They increased in the NF-treated scutella of W64A and WDN10, but remained almost constant in WA10C (Fig. 2). In WDN10, they increased gradually with increasing NF concentrations. *Cat2* transcript levels were similar among various lines in untreated scutella (Fig. 2). In the NF-treated scutella of W64A and WA10C, *Cat2* transcripts decreased at 5 and 10 μM NF, and in WDN10 they decreased at 10 μM NF (Fig. 2). *Cat3* transcript was not detected in all lines examined (data not shown).

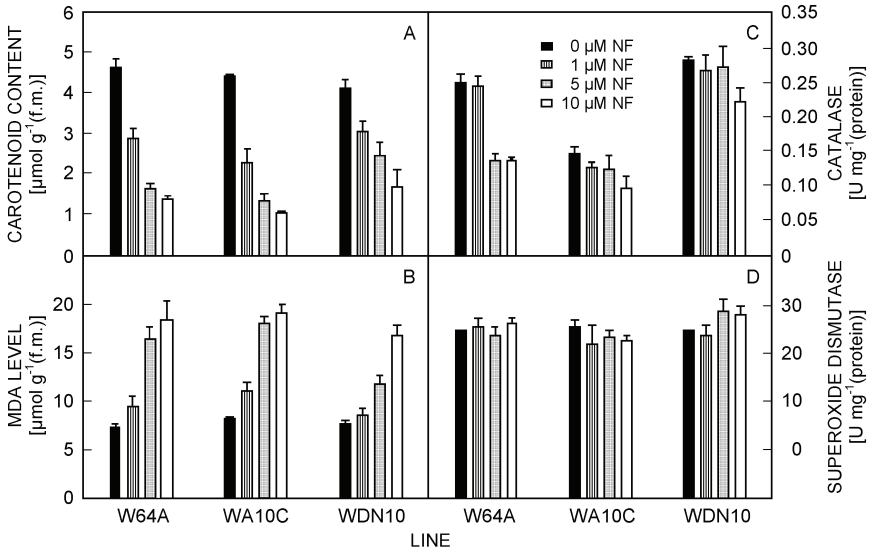

Fig. 1. Effects of norflurazon on carotenoid content (*A*), malonyldialdehyde content (*B*), catalase activities (*C*), and superoxide dismutase activities (*D*) of 18-d post-pollination scutella of the maize catalase variants. The isolated scutella from 18-d post pollination kernels of the standard maize line W64A and CAT-null mutants, WA10C and WDN10, were incubated on MS plates or MS plates containing various concentrations of NF at 25 ºC for 12 h in the dark and then exposed to 250 μmol m^{-2} s^{-1} of PAR for 12 h. Data represent the mean ± SE of two independent experiments with three repetitions.

Both *Sod1* and *Sod2* transcript levels remained quite low in untreated control and in various NF treatments of all maize lines, except a significant increase of *Sod1* transcript level in WDN10 treated with 10 μM NF (Fig. 3). In all lines, *Sod3* transcript levels were unchanged in response to NF (Fig. 3). In untreated control, *Sod4* transcript levels were higher in WDN10 compared to those of W64A and WA10C. *Sod4* transcripts increased slightly in the NF-treated scutella of W64A and WA10C, but they decreased in WDN10 (Fig. 3). *Sod4A* transcripts decreased in W64A in response to NF treatment, whereas they increased up to 5 μM NF and then decreased in both WA10C and WDN10 (Fig. 3).

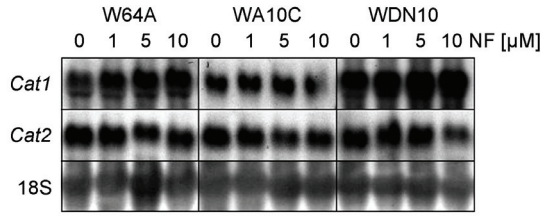

Fig. 2. Response to norflurazon exposure of the *Cat* gene expression in 18-d postpollination scutella of the maize catalase variants. The scutella were subjected to the same treatments as in Fig. 1. Total RNA was extracted, separated on 1.6 % agarose gels, at 20 μg per lane, and transferred to membranes. Probing was done with [α-^{32}P]-labeled gene-specific DNA fragments. *Cat2* probe used was full-length which can also identify the aberrant shortened *Cat2* transcript in the CAT-2 nulls. The 18S rRNA is a loading control.

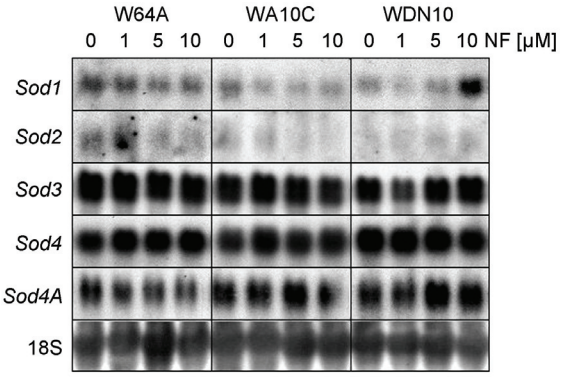

Fig. 3. Response to norflurazon exposure of the *Sod* gene expression in 18-d postpollination scutella of the maize catalase variants. The scutella were subjected to the same treatments as in Fig. 1. Total RNA was extracted, separated on 1.6 % agarose gels, at 20 μg per lane, and transferred to membranes. Probing was done with [α-^{32}P]-labeled gene-specific DNA fragments. The 18S rRNA is a loading control.

Discussion

Carotenoids can effectively quench the excited triplet state of chlorophyll and $^1O_2$ (Knox and Dodge 1985). The treatment with NF drastically reduces concentration of carotenoids in the 18 dpp maize scutella. Lowered amount of carotenoids would lead to increased formation of $^1O_2$. Unavoidably, this directly leads to increased peroxidation reaction, which is indicated by an increased content of MDA (Fig. 1*A*,*B*). The various maize CAT lines responded differently in CAT activities, whereas SOD activities remained relatively constant at different NF concentrations in all maize lines examined (Fig. 1*C*,*D*). In addition, the 18 dpp scutella of maize CAT-deficient mutants demonstrated differences in the *Cat* and *Sod* transcript levels from W64A in their responses to NF (Figs. 2,3).

Unlike a great decline of CAT activity in the 18 dpp scutella of W64A in consequence of NF treatment, a decrease in CAT activity in the presence of NF was not so marked in WA10C and WDN10; overall CAT activities were much higher in WDN10 than in WA10C (Fig. 1*C*). Despite of decreased CAT activity in response to NF, *Cat1* transcript levels increased with NF treatment in W64A and WDN10, indicating a post-transcriptional regulation of this gene (Figs. 1*C*,2). CAT appears to degrade in the scavenging process of H_2O_2 in 18 dpp maize scutella during NF-induced oxidative stress. The decline in CAT activity has also been observed in the leaves of NF-treated cucumber and maize (Jung *et al*. 2000, 2001). The increase of *Cat1* transcript in the leaves of NF-treated 12 dpi maize was dependent on photoinhibition caused by a lack of carotenoids (Jung *et al*. 2001). *Cat2*, rapidly induced with greening of the leaf tissue in the light (Skadsen and Scandalios 1987, Scandalios 1994), responded sensitively to the NF-caused oxidative stress in the 12 dpi leaves of maize plants (Jung *et al*. 2001). However, *Cat2* decreased at high NF concentrations in the 18 dpp scutella of all lines (Fig. 2), showing that the expression of *Cat2* is developmental and tissue-specific. A considerable level of *Cat2* transcripts in WA10C and WDN10 (Fig. 2) implies that the *Cat2* gene products are regulated post-transcriptionally. No detection of *Cat3* transcript indicates that WA10C and WDN10 should be quite the same at this developmental stage, however, both mutants exhibited different NF-induced responses. Further study on CAT isozyme pattern along with transcript change might explain this phenomenon.

The NF-treated leaves of W64A plants were capable of increasing SOD activity (Jung *et al*. 2001). However, in the 18 dpp scutella of all maize lines examined, SOD activities remained almost unchanged in response to NF (Fig. 1*D*). The high steady-state level of SOD activity

might be necessary at all times to provide adequate protection in the 18 dpp scutella of maize and the NF-caused response is likely to be developmental and tissue-specific. In the NF-treated 18 dpp scutella of all maize lines, *Sod1* and *Sod2* transcript levels maintained low in all treatments, whereas the only increase in *Sod1* occurred in WDN10 treated with 10 μM NF (Fig. 3). The two closely related Cu,Zn *Sod4* and *Sod4A* transcripts responded differently to NF, with the *Sod4* accumulation in W64A and the *Sod4A* increase in WA10C and WDN10 (Fig. 3), demonstrating that the scutella of W64A and CAT mutants respond differently in *Sod* transcripts in response to NF-caused oxidative stress.

Maize mutants lacking in CAT isozymes resulted in differential responses in CAT activities and in *Cat* and *Sod* transcript levels from the standard maize line W64A in their response to NF. However, the deficiency in CAT isozymes did not significantly influence the oxidative stress response in 18 dpp scutella, which is induced by the inhibition of carotenoid biosynthesis. This might result from the sustained SOD activity throughout NF treatments in all maize lines.

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