PHYSIOLOGIA PLANTARUM 106: 112–120. 1999
Printed in Ireland—all rights reserved

ISSN 0031-9317

Role of hydrogen peroxide and different classes of antioxidants in the regulation of catalase and glutathione S-transferase gene expression in maize (*Zea mays* L.)

Alexios N. Polidoros and John G. Scandalios*

Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614, USA
**Corresponding author, e-mail: jgs@unity.ncsu.edu*

Received 3 February 1999

The role of hydrogen peroxide (H_2O_2) and various antioxidants in the regulation of expression of the three *Cat* and *Gst1* genes of maize (*Zea mays* L.) has been investigated. Low concentrations of H_2O_2 appeared to inhibit *Cat1*, *Cat3*, and *Gst1* gene expression, while higher doses strongly induced these genes. Time course experiments indicated that high concentrations of H_2O_2 induced *Cat1*, *Cat2*, and *Gst1* gene expression to higher levels, and in less time, than lower H_2O_2 concentrations. Induction of *Cat3* was superimposed on the circadian regulation of the gene. These results demonstrate a direct signaling action of H_2O_2 in the regulation of antioxidant gene responses in maize.

The effects of the antioxidant compounds N-acetylcysteine, pyrrolidine dithiocarbamate, hydroquinone, and the electrophile antioxidant responsive element (ARE)-inducer β-naphthoflavone were quite different and specific for each gene/compound/concentration combination examined. The response of each gene to each antioxidant compound tested was unique, suggesting that the ability of these compounds to affect expression of the maize *Cat* and *Gst1* genes may not be the result of a common (antioxidant) mode of action. A putative regulatory ARE motif involved in the regulation of antioxidant and oxidative stress gene responses in mammalian systems is present in the promoter of all three maize catalase genes and we tested its ability to interact with nuclear extracts prepared from 10 days post-imbibition senescing scutella. Protein-DNA interactions in the ARE motif and the U2 snRNA homologous regions of the *Cat1* promoter were observed, suggesting that ARE may play a role in the high induction of *Cat1* in a tissue which, due to senescence, is under oxidative stress.

Introduction

Aerobic organisms depend on oxygen (O_2) as the electron acceptor in controlled electron transfer reactions. However, during the stepwise one-electron reduction of O_2, cells continuously produce the stable O_2 intermediates superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), commonly known as reactive oxygen species (ROS), and can cause damage to the cell (Scandalios 1993). To minimize the adverse effects of ROS, aerobic organisms evolved numerous antioxidant defenses, including catalases (CAT) and glutathione S-transferases (GST). Antioxidant defenses are activated in response to unbalanced increases of ROS that cause oxidative stress. The mechanisms by which cells sense H_2O_2 and $O_2^{\bullet -}$ are not very well understood, but a number of transcriptional factors that regulate the expression of antioxidant genes, or genes whose activities are modulated by oxidation and reduction, are known. In *Escherichia coli* and other prokaryotes the transcription factor OxyR activates a battery of genes inducible by H_2O_2, while the transcription factors SoxR/SoxS mediate responses to $O_2^{\bullet -}$. Similarly, in the yeast *Saccharomyces cerevisiae*, a lower eukaryote, there are two distinct adaptive stress responses, one towards H_2O_2 and one towards $O_2^{\bullet -}$ (Jamieson and Storz 1997).

In higher eukaryotes, oxidative stress responses are more complex and modulated by several different regulators. There is increasing evidence suggesting that eukaryotic re-

Abbreviations – AP-1: activator protein-1; ARE: antioxidant responsive element; β-NF: β-naphthoflavone; Cat/CAT: catalase gene/enzyme; Gst/GST: glutathione S-transferase gene/enzyme; HQ: hydroquinone; NAC: N-aceltylcysteine; NF-κB: nuclear factor-κB; PDTC: pyrrolidine dithiocarbamate; ROS: reactive oxygen species.

sponses to different kinds of stress, including starvation, heat shock, osmotic stress, and heavy metal intoxication, overlap oxidative stress responses. In mammalian systems, many studies point to the significance of two classes of transcription factors, nuclear factor κB (NF-κb) and activator protein-1 (AP-1), in the regulation of oxidative stress response (Angel and Karin 1991, Schreck et al. 1991, Meyer et al. 1993). However, AP-1 responds strongly to the antioxidants N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC), and only weakly to H_2O_2. This led to the suggestion that AP-1 is a secondary antioxidant-responsive factor (Meyer et al. 1993). Antioxidant-specific gene induction has also been reported for a number of enzymes involved in phase II xenobiotic metabolism, which is mediated by a regulatory motif common in the promoter region of these genes. This motif, named 'antioxidant responsive element' (ARE), 'electrophile responsive element' (EpRE), or GPE1, is present in the promoter of mammalian GST, NADPH-quinone oxidoreductase (NQOR), γ-glutamylcysteine synthetase, metalothioneine-I (MT-1), and manganese superoxide dismutase (MnSOD) genes (Kahl 1997). The signal transduction pathway leading to gene induction through the ARE has not yet been elucidated. The significance of the antioxidant properties of many of the inducers has been questioned, as compounds with no antioxidant activity are also potent inducers. In contrast, many of the inducers are Michael reaction acceptors (electrophilic), and their potency parallels their efficiency in Michael reactions (Talalay et al. 1988). ROS can also activate the ARE, as H_2O_2 itself is a capable inducer (Rushmore et al. 1991). An ARE motif has not yet been identified in any plant *Gst* gene (Marrs 1996). However, ARE-like motifs are present in the promoter region of the three maize *Cat* genes (Scandalios et al. 1997), but the effects of antioxidants on the expression of these plant genes are unknown.

In plants, ROS have been implicated in damaging effects of various environmental stress conditions including UV light and other forms of radiation, xenobiotics (herbicides), ozone, temperature extremes, especially in relation with high light, drought, and pathogen attack (Scandalios 1993). Many plant defense genes are activated in response to these conditions. H_2O_2 appears to be a signaling intermediate in plant defense, activating gene expression (Bi et al. 1995, Levine et al. 1994, Neuenschwander et al. 1995), hypersensitive cell death (Levine et al. 1994), and promoting programs leading to immunity (Wu et al. 1995). H_2O_2 scavengers like catalase can modulate H_2O_2 homeostasis and consequently its signaling capacity. However, information on the effects of H_2O_2 on the expression of catalase genes is limited. Similarly, there are a few reports on H_2O_2-induced *Gst* gene expression (Levine et al. 1994, Tenhaken et al. 1995). The maize *Cat* and *Gst* antioxidant defense genes provide an excellent system for investigation of antioxidant gene responses to antioxidants and oxidants. Catalases catalyze the dismutation of H_2O_2 into O_2 and H_2O, preventing the damaging effects of H_2O_2 accumulation and protecting cells from oxidative stress. Presence of ARE-like motifs in the maize *Cat* genes renders them suitable tools for characterization of their potential regulatory roles in plants (Scandalios et al. 1997). Glutathione S-transferases are a family of enzymes that catalyze the conjugation of glutathione via the sulfhydryl group to a variety of electrophilic centers of hydrophobic compounds. This reaction renders the compound more soluble to H_2O and facilitates its transport to vacuole or apoplast. Among the substrates of GSTs are many plant secondary metabolites like phenolic antioxidants and anthocyanins (Marrs et al. 1995), and many xenobiotics (Kreuz et al. 1996). Importantly, GSTs are also responsible for detoxification of the strongly electrophilic and highly cytotoxic lipid peroxidation products such as 4-hydroxyalkenals, which are generated when lipid membranes are damaged by oxidative stress (Marrs 1996).

In the present study, we examined the responses of the *Cat* and *Gst1* genes of maize to different classes of antioxidants and to H_2O_2-induced oxidative stress. We also examined potential involvement of promoter regions of *Cat1*, including a sequence harboring an ARE-like motif, in the regulation of *Cat1* expression in scutella at 10 days post-imbibition (dpi), when the tissue is under senescence-induced oxidative stress.

Materials and methods

Plant material

The maize inbred line W64A was used in this study. Seeds were surface sterilized by soaking in 1% hypochlorite for 10 min and then washed 5–6 times with deionized water. Seeds were then soaked in distilled water for 24 h and planted in soil mix. Germination occurred in controlled environment with 12 h/12 h D/L photoperiod and constant 25°C temperature.

Treatments

After 9 days dpi, seedlings were removed from the soil with the root, the root was rinsed in tap water and placed in open 50-ml tubes (four plants per tube) containing 20 ml of each compound's solution. The compounds NAC, PDTC, and hydroquinone (HQ) were applied to study antioxidant responses, β-naphthoflavone (β-NF) to study response to an electrophile ARE-inducer, and H_2O_2 to study responses under oxidative stress. Control plants were placed in tubes containing water or the appropriate concentration of the compound's solvent in water. The plants were then placed in the growth chamber for 24 h.

Northern analysis

Leaf material was harvested after a 24-h treatment with each compound. All samples were frozen in liquid nitrogen and stored at -80°C for analysis. Total RNA from leaf material was extracted (Thompson et al. 1983), separated on denaturing 1.2 or 1.6% agarose gels, and stained with ethidium bromide to ensure equal loading. It was then transferred onto nylon membranes and hybridized with ^{32}P-labeled *Cat* (Bethards et al. 1990, Redinbaugh et al. 1988) or *Gst1* gene-specific probes. The *Gst1* gene-specific

probe was prepared with polymerase chain reaction (PCR) using the phi080 set of primers (Senior et al. 1996). Hybridization buffers and conditions were as follows: the pre-hybridization solution contained 6 × SSPE, 5 × Denhardt's, 7% SDS, 200 μg ml^{-1} salmon sperm DNA, and 1% dextran sulfate. After 4 h pre-hybridization at 65°C, the pre-hybridization solution was substituted with hybridization solution that was the same as the pre-hybridization solution without 5 × Denhardt's, and probe was added. Hybridization proceeded at 65°C for 24–36 h, and washes were twice for 30 min with 0.1 × SSC/0.1% SDS at 65°C.

Isolation of nuclei and preparation of nuclear extracts

Nuclei were isolated from post-germination scutella (Hall and Spiker 1994). Scutella were ground to a fine powder with mortar and pestle using liquid nitrogen, and transferred to a beaker sitting on ice. All subsequent manipulations were carried out at 4°C. Nuclei isolation buffer 1 (NIB1: 0.5 *M* hexylene glycol, 0.5% Triton X-100, 20 m*M* HEPES pH 7.4, 20 m*M* Thiodiglycol, 1 m*M* PMSF, 0.5 m*M* EDTA pH 7.4, 2 μg ml^{-1} aprotinin, 50 m*M* spermine, 125 m*M* spermidine) was added in a volume of 10 ml g^{-1} of scutella. The slurry was homogenized with a Polytron homogenizer at low setting for 30 s. Nuclei were filtered away from cell debris through a tiered setup of 300, 200, 100, 50, and 30 μm nylon mesh screens. Aliquots of 25-ml filtrate were layered above a 30–50% Percoll/NIB1 gradient and centrifuged at 900 *g* for 20 min. The supernatant above the Percoll cushion was removed by aspiration. The nuclei were extracted from the 30–50% Percoll interface using a glass pipette and pelleted by centrifugation at 3000 *g* for 15 min. The pellet was resuspended and washed twice with 20 ml cold NIB2 (NIB2: as NIB1 without Triton X-100). The final pellet was resuspended in 1 ml nuclei suspension buffer (NSB: 50% glycerol, 50% 2 × NIB2) and stored at −80°C until use.

Nuclear protein preparation was essentially as described in Holdsworth and Laties (1989), with minor modifications. Nuclei were recovered from NSB with a brief centrifugation, and resuspended in nuclear protein isolation buffer (NPIB: 40 m*M* KCl, 20 m*M* HEPES pH 7.7, 0.5 m*M* EDTA pH 7.7, 5 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 1m*M* PMSF, and 2 μg ml^{-1} aprotinin). Nuclei were lysed with the addition of 1/10 vol saturated ammonium sulfate pH 7.6 on ice for 30 min. Debris was removed by centrifugation at 15000 *g* for 15 min. Nuclear proteins were precipitated from the supernatant with the addition of solid powdered ammonium sulfate to a 65% saturation (337 mg ml^{-1} in the 10% saturated solution), and collected by centrifugation at 15000 *g* for 15 min. Pelleted nuclear proteins were resuspended in dialysis buffer (NPDB: 40 m*M* KCl, 20 m*M* HEPES pH 7.7, 0.5 m*M* EDTA pH 7.7, 2 m*M* dithiothreitol, 1 m*M* PMSF, 20% glycerol) containing 2 μg ml^{-1} aprotinin and dialyzed against two changes of 1000 ml dialysis buffer for 2 h, using Spectapore membrane tubing (Amicon, Beverly, MA, USA) with 3500 MW cutoff. The dialysate was aliquoted, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined using the Bio-Rad protein assay kit and BSA as a standard.

Electrophoretic mobility shift assays

Probes for electrophoretic mobilibty shift assay probes (EMSAs) (Fig. 5) were prepared as follows: a 0.8 kb *Xba*I fragment from the *Cat1* promoter (Guan and Scandalios 1993) was digested with *Sau*3A, generating a 90 bp *Xba*I-*Sau*3A fragment containing the U2 snRNA homologous region, which was subcloned into an *Xba*I-*Bam*HI digested pBluescript vector, and a 158 bp *Sau*3A fragment containing the ARE motif, which was subcloned into the *Bam*HI site of the pBluescript. The two probes were labeled by fill-in 5′ overhangs with α-^{32}P-dCTP using Klenow polymerase. Binding reactions were performed using the Bandshift kit (Pharmacia, Piscataway, NJ) according to instructions. Briefly, the reaction mixture contained 10000–40000 cpm-labeled target DNA (0.1–1 ng DNA) in 10 m*M* Tris-HCl pH 7.5, 50 m*M* NaCl, 0.5 m*M* dithiothreitol, 10% glycerol, 0.05% NP-40, and 0.25 μg poly(dI-dC). Nuclear proteins in a range of 0.1–0.25 μg were added and the mixture incubated at room temperature for 20 min. DNA-protein complexes were separated from unbound DNA probe by electrophoresis on native 4% polyacrylamide gels in Tris-acetate-EDTA buffer (7 m*M* Tris-HCl pH 7.5, 3 m*M* sodium acetate, 1 m*M* EDTA). Gels were vacuum dried and autoradiographed at −70°C for 0.5–2 h.

Results

Responses of the maize *Cat* and *Gst1* genes to antioxidants, electrophilic compounds, and H_2O_2

Specific responses of the maize *Cat* and *Gst1* genes were observed upon treatment with antioxidants, electrophilic compounds, and H_2O_2. The responses were not uniform with the different compounds used. Thus, each gene-compound combination resulted in a different response.

Responses of Cat1

In young maize leaves, *Cat1* is normally expressed at a constant low level and is not affected by light (Scandalios et al. 1997). *Cat1* expression was decreased in response to low concentrations of NAC up to 5 m*M* (Fig. 1A), and increased at 10 m*M*. Treatment with PDTC did not affect *Cat1* expression, while treatment with HQ resulted in gradually increasing *Cat1* expression with increasing (1–10 m*M*) HQ concentrations (Fig. 1A). *Cat1* expression was not affected by ethanol, which was used as solvent for β-NF (Fig. 1B). β-NF resulted in a slight decrease at 0.25 m*M*, followed by an increase at 1 m*M*, and the expression was similar to the control at higher concentrations (Fig. 1B). Treatment with low (0.5 m*M*) concentration of H_2O_2 decreased transcript level. At 1 m*M* H_2O_2, *Cat1* expression was slightly lower than the control, while it was comparable with the control at higher concentrations (Fig. 1C). It is known that in other tissues like scutellum, *Cat1* is induced when the scutellum is excised from the kernel and incubated in control agar plates for 24 h (L. Guan and J. G. Scandalios, unpublished results). This induction was explained as a result of wounding and/or hormone imbalance. In this experiment, *Cat1* expression in leaves is significantly induced

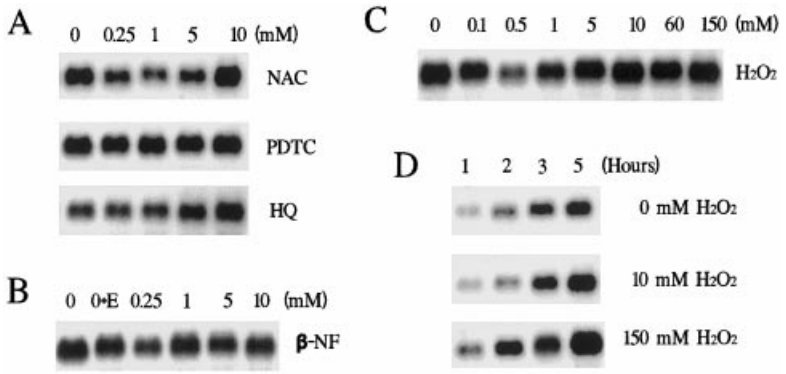

Fig. 1. Response of the maize *Cat1* gene to antioxidants, electrophiles, and H_2O_2. Seedlings (10 dpi) were treated hydroponically with increasing concentrations of the indicated compounds, and *Cat1* transcript in leaves was detected after hybridization with ^{32}P-labeled *Cat1* gene-specific probe. Equal loading of the gels was confirmed by ethidium bromide staining prior to transfer. A. Responses to 24-h treatments with the antioxidants NAC, PDTC, and HQ. B. Responses to 24-h treatments with the electrophile ARE-inducer β-NF. The 0 + E lane is control with the β-NF solvent ethanol. C. Responses to 24-h treatments with H_2O_2. D. Time course responses to 10 and 150 m*M* H_2O_2. Abbreviations are: NAC, N-acetylcysteine; PDTC, pyrrolidine dithiocarbamate; HQ, hydroquinone; β-NF, beta-naphthoflavone; E, ethanol; H_2O_2, hydrogen peroxide.

within 1 h after submersion of the roots in water, and gradually increases during the next 5 h (Fig. 1D). Induction continues for 24 h, as is evident comparing the 5-h control (0 m*M*) in Fig. 1D with the 24-h control (0 m*M*) in Fig. 1C. The observed induction is enhanced by H_2O_2. Addition of 10 m*M* H_2O_2 in the solution results in enhanced induction after 3–5 h. Addition of 150 m*M* H_2O_2 enhances *Cat1* induction after 1 h of treatment and results in higher induction after 5 h. However, enhancement of the induction by H_2O_2 is not observed after 24 h of treatment, indicating that elevated *Cat1* expression due to submersion of the roots in water is reaching a maximum that is not affected by addition of H_2O_2.

Responses of Cat2

Expression of *Cat2* in young maize leaves is constant at low levels (Scandalios et al. 1997). Thus, we investigated *Cat2* responses examining a sample of antioxidant and H_2O_2 treatments. There was no significant induction upon submersion of the roots in water as is evident by comparison of the 24-h control in Fig. 2A with the 0-h control in Fig. 2B. NAC caused a decrease of *Cat2* transcript at 0.25–2.5 m*M*, followed by an increase at 5 m*M* (Fig. 2A). A similar response was observed for HQ, but the decrease was at 0.1–0.25 m*M* and the level of *Cat2* transcript was similar to the control at 0.5–1 m*M*. A high induction was evident at 2.5–5 m*M* HQ (Fig. 2A). *Cat2* was induced by H_2O_2 (60–150 m*M*) within 1 h after submersion of the roots in H_2O_2 solution, and was even stronger 24 h later (Fig. 2B). It was also evident that 150 m*M* H_2O_2 induced *Cat2* to higher expression levels than 60 m*M* H_2O_2 at both 1 and 24 h.

Responses of Cat3

Expression of *Cat3* in young maize leaves is regulated by a circadian rhythm (Scandalios et al. 1997). Thus, analyses regarding *Cat3* responses should be in the context of the circadian expression of the gene. In the present experiments a normal circadian *Cat3* expression was observed in seedlings with roots submerged in water for 24 h, as long as the light regime was maintained at 12 h/12 h L/D photoperiod. NAC increased the *Cat3* transcript in all the concentrations applied (Fig. 3A). PDTC caused a strong induction at 0.25 m*M* that declined at higher concentrations, and expression was inhibited at 10 m*M* (Fig. 3A). HQ caused a high induction between 0.25–5 m*M*, while at 10 m*M* induction was lower (Fig. 3A). With β-NF, a high induction was observed at 0.25–1 m*M* and complete inhibition at higher concentrations (Fig. 3B). Ethanol also inhibited *Cat3* gene expression (Fig. 3B). The effects of H_2O_2 on *Cat3* transcription were examined at two different time points: circadian time 10 (CT-10: 10 h after the onset of light), which represents the time point of the highest *Cat3* expression, and CT-4, which represents a time point with low and increasing *Cat3* transcription. Results were similar and those obtained at CT-4 are shown in Fig. 3C. Low doses (0.1–0.5 m*M*) of H_2O_2 caused a significant decrease on *Cat3* transcript levels. Transcription was similar with the control at 1 m*M* H_2O_2 and significantly increased at 5–60 m*M*. The transcript at 150 m*M* was lower than that of 60 m*M*, but still higher than that of the control.

Responses of Gst1

Expression of *Gst1* in maize is constitutive in young seedling roots, but can be enhanced by herbicide safeners (Jepson et

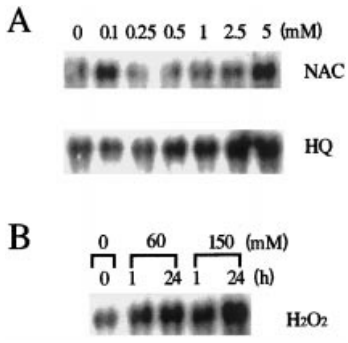

Fig. 2. Response of the maize *Cat2* gene to antioxidants and H_2O_2. Transcripts in leaves of 10 dpi seedlings treated hydroponicaly were detected after hybridization with ^{32}P-labeled *Cat2* gene-specific probe. Equal loading of the gels was confirmed by ethidium bromide staining prior to transfer. A. Responses to 24-h treatments with NAC and HQ. B. Responses to treatments with 60 and 150 m*M* H_2O_2 for 1 and 24 h. Abbreviations are as in Fig. 1.

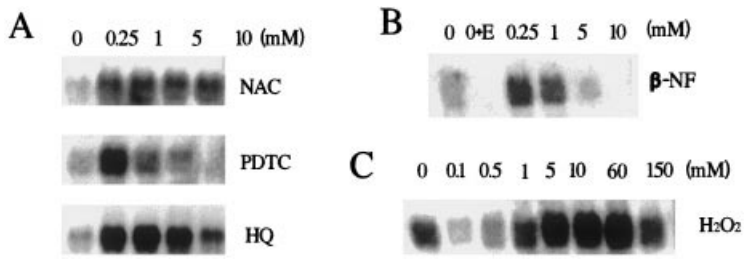

Fig. 3. Response of the maize *Cat3* gene to antioxidants, electrophiles, and H_2O_2. Seedlings (10 dpi) were treated hydroponically for 24 h with increasing concentrations of the indicated compounds, and *Cat3* transcript in leaves was detected after hybridization with ^{32}P-labeled *Cat3* gene-specific probe. Equal loading of the gels was confirmed by ethidium bromide staining prior to transfer. Treatment began 4 h after the onset (CT-4) of light and the plants were maintained at 12 h/12 h L/D photoperiod in order to examine the effects of the compounds in the context of the *Cat3* circadian rhythm. A. Responses to the antioxidants NAC, PDTC, and HQ. B. Responses to the electrophile ARE-inducer β-NF. The 0 + E lane is control with the β-NF solvent ethanol. C. Responses to H_2O_2. Abbreviations are as in Fig. 1.

al. 1994). *Gst1* responses (Fig. 4) were very similar with those of *Cat3* (Fig. 3). NAC increased the *Gst1* transcript, and the highest induction was observed at 10 m*M* (Fig. 4A). PDTC caused a strong *Gst1* induction at 0.25 m*M* that declined at higher concentrations (Fig. 4A). HQ caused a stronger induction at 0.25, 5, and 10 m*M*, while at 1 m*M* the response was weaker (Fig. 4A). β-NF caused a high induction between 0.25 and 1 m*M* and expression was at the levels of the control at 10 m*M* (Fig. 4B). Ethanol also repressed *Gst1* gene expression (Fig. 4B). With H_2O_2 treatments, the *Gst1* transcript decreased at low (0.1 m*M*) concentrations and was comparable with that of the control at 0.5–1 m*M* (Fig. 4C). Significantly higher transcription was evident at higher (5–150 m*M*) H_2O_2 concentrations (Fig. 4C). *Gst1* expression is not affected by submersion of the roots in water, as no difference in transcript was observed between the 24 h control in Fig. 4C and the controls at different time points in Fig. 4D. However, H_2O_2 treatments induced *Gst1* expression within 3–5 h at 10 m*M* and as early as 1 h at 150 m*M* (Fig. 4D).

Protein-DNA interactions in specific *Cat1* promoter regions

Interactions of nuclear proteins, isolated from 10-day-old germinating scutella, with promoter regions of *Cat1* have been examined by electrophoretic mobility shift assays. Results indicated that a 158-bp fragment containing the ARE core sequence as well as a 90-bp fragment highly homologous with the U2 snRNA gene of maize (Guan and Scandalios 1993), located 5′ and in continuous sequence with the 158-bp fragment (Fig. 5), were strongly interacting with nuclear proteins. A very strong interaction was observed for the ARE-harboring fragment as almost the total amount of the probe was shifted on the gel (Fig. 6). Specificity of the observed binding was further analyzed with the addition of 30 × cold probe in a binding reaction. Results (Fig. 6) suggest that the observed binding is highly specific, as with the addition of cold probe the shifted band disappears. A similar strong and specific interaction was observed with the U2 snRNA homologous fragment (Fig. 7). In both experiments, specificity was also examined with the addition of salmon sperm DNA as non-specific competitor in the binding reaction and the binding was maintained (data not shown).

Discussion

Regulatory role of antioxidants in the expression of antioxidant genes

The effects of antioxidants on *Cat* and *Gst1* gene expression were different and specific for each compound and concentration examined. Even compounds whose antioxidant activity is the result of similar chemical properties, like the –SH-containing compounds NAC and PDTC, affected the same genes in different ways. Thus, the responses do not seem to be due solely to the antioxidant properties of the compounds.

There are studies indicating that compounds that exhibit antioxidant activity in vitro can be metabolically transformed, or act as oxidants in vivo (Pinkus et al. 1996). The possibility exists that the observed responses might, in part, result from such conversions. Consistent with this hypothesis, NAC can produce thiyl and other sulfur-containing radicals (Aruoma et al. 1989), which can oxidize glutathione and weaken the antioxidant defenses of the cell. In our experiments, 10 m*M* NAC induced all the genes examined. This concentration is much lower than the 30 m*M* reported to be effective in inhibition of NF-κB in mammalian cell cultures (Meyer et al. 1993). However, 100 m*M* NAC in hydroponic treatments resulted in severe stress of the plants from which they could not recover, high induction of *Cat1*, and complete inhibition of *Cat3* (data not shown). These results suggest that a mechanism of NAC-induced stress could be responsible for the observed responses. PDTC, due to its metal-chelating properties, can inhibit Cu/Zn superoxide dismutase activity, potentiate oxygen toxicity in animal tissues, and cause a decrease in glutathione peroxidase activity and thiol levels (Goldstein et al. 1979). In the present study, PDTC was a very potent inducer of *Cat3* and *Gst1* at low concentrations (0.25 m*M*), while it exhibited inhibitory effects at higher concentrations. However, it did not affect *Cat1* expression. In this study, HQ, which can undergo reversible oxidation-reduction generating ROS, was a potent inducer of all genes examined. *Cat3* exhibited a strong induction at low concentrations, which was gradually decreased as the concentration increased. The other genes examined (*Cat1*, *Cat2*, and *Gst1*) exhibited weaker but persistent induction at the higher concentrations.

The data presented suggest that diverse antioxidants are potent inducers of antioxidant gene expression in maize.

This apparently paradoxical situation is not unique, as both antioxidants and H_2O_2 have been reported to serve as signals inducing oxidative stress responsive factors in animal cells (Bergelson et al. 1994, Meyer et al. 1993). However, the possibility exists that oxidative mechanisms are responsible for the apparent effects of antioxidants in *Cat* and *Gst1* gene expression in maize.

Regulatory role of H_2O_2 in the expression of antioxidant genes

Plants, like other living organisms, sense increased levels of ROS and react with antioxidant mechanisms, finely coordinated and expressed, to effectively protect the organism from oxidative stress. In the progress of these events, H_2O_2, a well-established second messenger in animal systems (Ramasarma 1982), appears to be a significant signaling intermediate in plants as well (Alvarez and Lamb 1997). In the present study, we investigated the role of H_2O_2 in the expression of the *Cat* and *Gst1* genes of maize. Catalases are among the primary H_2O_2 scavenger enzymes and could play a central role in the control of H_2O_2 homeostasis and consequently in the H_2O_2-dependent signaling pathway. This has been indirectly established in several experimental systems by addition of catalase, or the catalase inhibitor aminotriazole, demonstrating differential effects on H_2O_2-induced gene expression (Alvarez and Lamb 1997, Chen et al. 1993, Levine et al. 1994). However, the role of H_2O_2 in the regulation of *Cat* gene expression has not yet been thoroughly investigated. Thus, we examined the effects of exogenously applied H_2O_2 in the regulation of *Cat* and *Gst1* gene expression in maize. We used a wide range of H_2O_2 concentrations (100 μM–150 mM) in hydroponic treatments of whole plants, and examined the transcript level of each gene in leaves 24 h later. With this experimental scheme, the actual concentration of H_2O_2 was changing throughout the treatment period. Developing roots of young maize seedling are known to secrete catalase in the incubation medium as well as in the root apoplast (Salguero and Boettger 1995). As soon as the seedling roots were submerged in the H_2O_2 solution, bubbles appeared on the surface of the roots, indicating very active H_2O_2 decomposition. Depletion of H_2O_2 from the culture medium has also been reported in rat hepatocyte cultures with H_2O_2 half-life between 2.5 and 8 min, depending on the initial H_2O_2 concentration (1–2.5 mM) and the age of the culture (Rohrdanz and Kahl 1998). This rapid decomposition probably caused an early depletion of H_2O_2 at low concentrations, while it decreased the actual concentration of H_2O_2 at the higher concentrations applied.

Low doses of H_2O_2 appeared to reduce *Cat1*, *Cat3*, and *Gst1* steady-state mRNA levels, while higher doses enhanced the amount of the *Cat2*, *Cat3*, and *Gst1* mRNA. Induction of *Cat2* and *Gst1* increased as the concentration of H_2O_2 increased up to 150 mM (the highest concentration used), while the induction of *Cat3* reached a maximum at 60 mM and declined at 150 mM. Time course experiments indicated that a high concentration of H_2O_2 induced *Cat1*, *Cat2*, and *Gst1* gene expression at higher levels and in less time than a lower concentration. *Cat1* expression was affected by the control treatment as it was induced by submersion of the roots into water. However, addition of H_2O_2 enhanced *Cat1* induction within 1 h of treatment, and increased expression over the control level could be observed for the next 5 h. At 24 h, the level of *Cat1* expression in samples treated with high H_2O_2 concentrations (5–150 mM) was similar with the control, indicating that there is a time point where high H_2O_2 concentrations can no longer enhance *Cat1* expression. Taken together, these results demonstrate that responses of the *Cat* and *Gst1* genes are specific for the concentration and the duration of treatment, suggesting a regulatory role for H_2O_2 in the expression of these antioxidant genes.

As mentioned above, very rapid decomposition of H_2O_2 near the roots of the seedlings occurred immediately after submersion in the H_2O_2-containing solution. This could lead to a rapid depletion of the lower concentrations of H_2O_2 used. Thus, treatment with low H_2O_2 concentrations could represent a short transient oxidative burst. The effects of this transient exposure to H_2O_2 could be distinct from those observed for prolonged exposures to higher concentrations. The transient exposure to increased concentrations of H_2O_2 is reminiscent of the oxidative burst in the hypersensitive response of plants to pathogen attack. This localized oxidative burst at the root caused systemic inhibition of *Cat* gene expression observable 24 h later in the leaf. Thus, systemic H_2O_2 accumulation could be accomplished by inhibition of *Cat* gene expression. It has been suggested that H_2O_2 accumulation and initiation of the signaling pathway leading to systemic acquired resistance (SAR) are due to specific inhi-

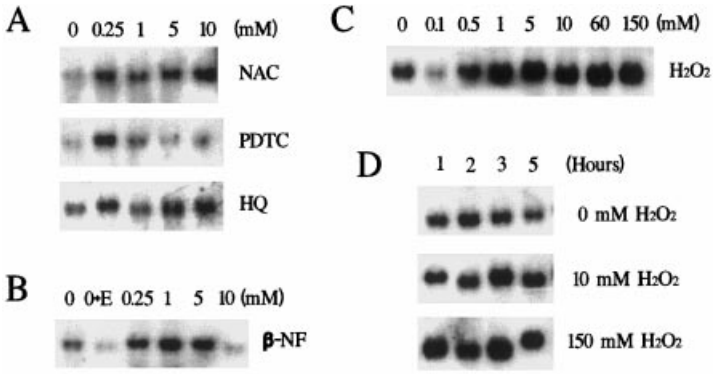

Fig. 4. Response of the maize *Gst1* gene to antioxidants, electrophiles, and H_2O_2. Seedlings (10 dpi) were treated hydroponically with increasing concentrations of the indicated compounds, and *Gst1* transcript in leaves was detected after hybridization with ^{32}P-labeled *Gst1* gene-specific probe. Equal loading of the gels was confirmed by ethidium bromide staining prior to transfer. A. Responses to 24-h treatments with the antioxidants NAC, PDTC, and HQ. B. Responses to 24-h treatments with the electrophile ARE-inducer β-NF. The 0 + E lane is control with the β-NF solvent ethanol. C. Responses to 24-h treatments with H_2O_2. D. Time course responses to 10 and 150 mM H_2O_2. Abbreviations are as in Fig. 1.

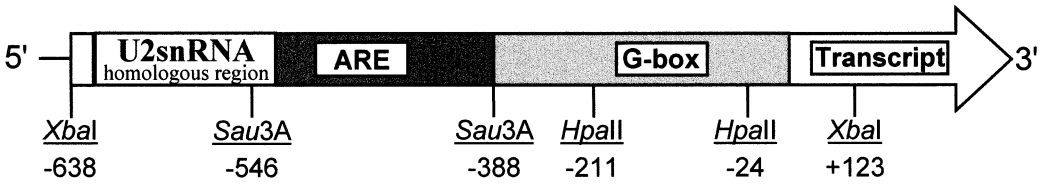

Fig. 5. Schematic of the *Xba*I fragment of the *Cat1* promoter that was used in preparation of electrophoretic mobility shift assay probes. The restriction enzyme sites and their distance from the initiation of transcription + 1 are shown. Transcript is represented as an arrow. The putative regulatory motifs are boxed. ARE is the antioxidant responsive element, and G-box is the ABA responsive element. The U2 snRNA homologous region is also shown.

bition of catalase activity by salicylic acid (SA) (Chen et al. 1993), which allows accumulation of systemic H_2O_2. However, there is also evidence that the H_2O_2 signal does not act downstream of SA in the development of SAR, as *NahG* transgenic plants that cannot accumulate SA do not induce PR-1 expression upon treatment with H_2O_2 and do not develop SAR (Bi et al. 1995, Neuenschwander et al. 1995). Involvement of H_2O_2 in disease resistance has been demonstrated in transgenic plants expressing the H_2O_2-generating glucose oxidase enzyme, and increased resistance of the transgenics could be counteracted with addition of catalase (Wu et al. 1995). Our results, in addition to the reported ability of H_2O_2 to induce SA accumulation (Bi et al. 1995, Neuenschwander et al. 1995), could provide a mechanism for H_2O_2 signaling in the induction of SAR, in which catalase inhibition and H_2O_2 act before SA accumulation (upstream of SA).

High concentrations of H_2O_2 rapidly induced *Cat* and *Gst1* gene expression, indicating that oxidative stress directly induces antioxidant responses. Particularly for *Cat3*, which is regulated by a circadian rhythm (Redinbaugh et al. 1990), its induction overrode the default circadian expression. Our data demonstrate that at CT-4, when *Cat3* transcript is very low, H_2O_2 treatment overrides the circadian regulation and induces high expression with high H_2O_2 concentration (Fig. 3C). Induction over the normal levels was also observed at CT-10, when the *Cat3* transcript reaches maximal expression (data not shown). This suggests that H_2O_2 can induce *Cat3* expression at different time points of the circadian cycle. However, the two time points we examined represent times in the circadian cycle where *Cat3* expression is increasing (CT-4) or is at its maximum (CT-10). Whether induction can also be observed at time points of declining expression, and whether the induced expression is still circadian, remains to be investigated.

Regulatory sequences in the *Cat1* promoter

The maize *Cat* genes harbor the ARE regulatory motif, which has a well-characterized role in induction of mammalian gene expression in response to oxidative stress. In order to investigate the role of ARE in maize *Cat* gene expression, we examined protein-DNA interactions in the ARE region of the *Cat1* promoter. The *Cat1* promoter has been sequenced revealing several regulatory elements, and has been used for the construction of promoter-reporter gene fusions that can drive the expression of the reporter gene GUS in transgenic tobacco plants (Guan and Scandalios 1993). A minimal 800 bp promoter fragment is sufficient for the regulation of GUS expression with a pattern similar to the *Cat1* expression observed in maize (Guan and Scandalios 1993). An ABA response element, located − 110 relative to the transcription start site, displays differential binding of nuclear proteins isolated from germinated and developing embryos, as well as from embryos grown in the presence and absence of absisic acid; this indicates that ABA up-regulates *Cat1* transcript accumulation in the scutellum during embryogenesis, but not during germination (Williamson and Scandalios 1994). However, *Cat1* transcript accumulates to high levels in the scutellum of germinating maize seeds. The promoter regions responsible for the control of *Cat1* expression during germination were not identified. The increase of *Cat1* transcript in the scutellum during germination could be an antioxidant response to oxidative stress. Active oxidative metabolism is well characterized in germinating seeds. During germination of oilseeds,

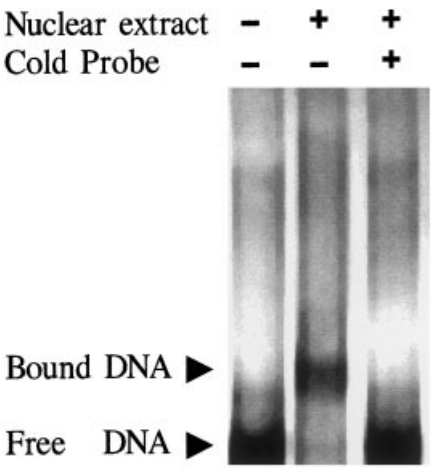

Fig. 6. Protein-DNA interactions in the *Cat1* promoter region harboring the ARE motif. Electrophoretic mobility shift assay was probed with a 158 bp *Sau3*A *Cat1* promoter fragment containing the ARE motif. The probe (0.1 ng ^{32}P-labeled DNA) in binding buffer containing 100 ng poly(dI-dC) non-specific competitor was incubated with 0.1 μg nuclear extract prepared from 10 dpi germinating scutella of maize. The free and bound probes were separated on 4% native acrylamide gel, vacuum dried, and autoradiographed. Specificity of the interaction was determined by addition of 30 × cold probe as specific competitor in the assay. Lanes are: Probe alone control (−, −), probe with nuclear extract and no specific competitor (+, −), and probe with nuclear extract and 30 × cold probe as specific competitor (+, +). Arrowheads indicate the bands of free and bound probe. Note the very strong interaction (+, −) revealed by the retardation of the whole amount of probe used in the assay.

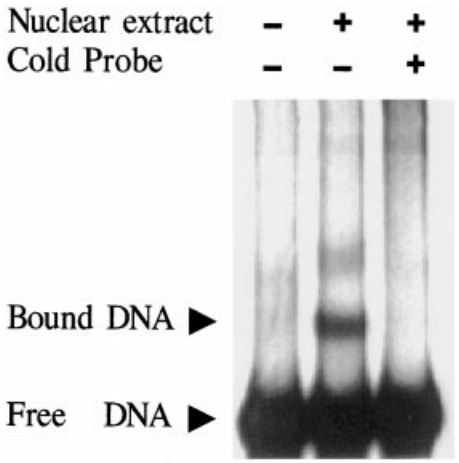

Fig. 7. Protein-DNA interactions in the U2 snRNA homologous region of the *Cat1* promoter. Electrophoretic mobility shift assay was probed with a 90 bp *Xba*I-*Sau3*A *Cat1* promoter fragment containing the U2 snRNA homologous region. The probe (1 ng ^{32}P-labeled DNA) in binding buffer containing 100 ng poly(dI-dC) non-specific competitor was incubated with 0.1 μg nuclear extract prepared from 10 dpi germinating scutella of maize. The free and bound probes were separated on 4% native acrylamide gel, vacuum dried, and autoradiographed. Specificity of the interaction was determined by addition of 30 × cold probe as specific competitor in the assay. Lanes are: probe alone control (−, −), probe with nuclear extract and no specific competitor (+, −), and probe with nuclear extract and 30 × cold probe as specific competitor (+, +). Arrowheads indicate the bands of free and bound probe.

fatty acids are converted into carbohydrates by hydrolysis of stored triglycerides, followed by the β-oxidation of fatty acids in specialized peroxisomes called glyoxysomes (Olsen and Harada 1995). Glyoxysomal catalase plays a vital role decomposing the H_2O_2 generated during β-oxidation. In addition, at the late stages of germination in maize, the scutellum, a terminally differentiated tissue is senescing. Senescence of plant organs and tissues is associated with formation of free radicals and oxidative stress (Thompson 1988). Thus, we examined the regulatory role of nuclear proteins isolated from 10 dpi scutella, when the tissue is under oxidative stress due to senescence. We assume that these nuclear factors may play a role in activation of the gene under oxidative stress conditions.

The 158 bp fragment that harbors the putative regulatory element ARE displayed a strong specific interaction with nuclear extracts prepared from 10 dpi germinating scutella. Specificity of the interaction was confirmed by the addition of 30 × cold probe, which resulted in elimination of the interacting band. These results indicate that regulatory element(s) are present on the 158 bp *Cat1* promoter fragment, and are strongly interacting with transcription factor(s) present in the 10 dpi germinating scutellum. In this region of the *Cat1* promoter, the only putative regulatory element recognized after computer searches for similarities with published regulatory sequences was the ARE motif. Numerous studies in animal systems demonstrate that the ARE motif is necessary and sufficient for regulation of gene responses to H_2O_2 via specific protein-DNA interactions in the region of ARE (Kahl 1997). Our data suggest a similar role of ARE in plants, and further experiments are designed to demonstrate its role in oxidative stress-induced gene expression.

Interestingly, the 90 bp *Cat1* promoter fragment upstream of the ARE motif also interacts with nuclear proteins prepared from 10 dpi scutella in a specific manner. Sequence comparisons of this fragment with published regulatory motifs show that no known regulatory element is present. However, most of the fragment contains a region 80% homologous to the first 100 bp of the coding region of the U2 snRNA gene of maize and other organisms. The major function reported for U2 products is in the formation of a ribonucleoprotein complex for intron splicing (Steitz et al. 1988). Thus, interaction of U2 snRNA homologous fragments with nuclear extracts could be expected. Our results raise the possibility that this fragment could also have a regulatory function in *Cat1* expression.

Acknowledgements – This work was supported, in part, by Research Grants from the US EPA (No. R819360 and No. R822546) and the NRI/CGP/USDA (No. 94371) to J.G.S. We thank Stephanie Ruzsa and Sheri Kernodle for expert technical assistance.

References

Alvarez A, Lamb C (1997) The oxidative burst of plant-pathogen interactions. In: Scandalios JG (ed.) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, New York, pp 567–568. ISBN 0-87969-502-1

Angel P, Karin M (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072: 129–157

Aruoma OI, Halliwell B, Hoey BM, Butler J (1989) The antioxidant action of N-acetylcysteine: Its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med 6: 593–598

Bergelson S, Pinkus R, Daniel V (1994) Intracellular glutathione levels regulate Fos/Jun induction and activation of glutathione-S-transferase gene expression. Cancer Res 54: 36–40

Bethards LA, Skadsen RW, Scandalios JG (1990) Isolation and characterization of a cDNA clone for the *Cat2* gene in maize and its homology with other catalases. Proc Natl Acad Sci USA 87: 6927

Bi YM, Kenton P, Mur L, Darby R, Draper J (1995) Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. Plant J 8: 235–245

Chen Z, Silva H, Klessig DF (1993) Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262: 1883–1886

Goldstein BD, Rozen MG, Quintavalla JC, Amoruso MA (1979) Decrease in mouse lung and liver glutathion peroxidase activity and potentiation of the lethal effects of ozone and paraquat by the superoxide dismutase inhibitor diethyldithiocarbamate. Biochem Pharmacol 28: 27–30

Guan L, Scandalios JG (1993) Characterization of the catalase antioxidant defense gene *Cat1* of maize, and its developmentally regulated expression in transgenic tobacco. Plant J 3: 527–536

Hall GEJ, Spiker S (1994) Isolation and characterization of nuclear scaffolds. In: Gelvin SB Schilperoort RA (eds) Plant Molecular Biology Manual. Kluwer Academic Publisher, Dordrecht, Boston, pp 1–12. ISBN 0-79232-858-2

Holdsworth MJ, Laties GG (1989) Site-specific binding of a nuclear factor to the carrot extensin gene is influenced by both ethylene and wounding. Planta 179: 17–23

Jamieson DJ, Storz G (1997) Transcriptional regulators of oxidative stress responses. In: Scandalios JG (ed.) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, New York, pp 91–115. ISBN 0-87969-502-1

Jepson I, Lay VJ, Holt DC, Bright SWJ, Greenland AJ (1994) Cloning and characterization of maize herbicide safener-induced cDNAs encoding subunits of glutathione S-transferase isoforms I, II and IV. Plant Mol Biol 26: 1855–1866

Kahl R (1997) Phenolic antioxidants: Physiological and toxicological aspects. In: Packer L, Cadenas E (eds) Handbook of Synthetic Antioxidants. Marcel Dekker, Inc., New York, pp 177–224. ISBN 0-82479-810-4

Kreuz K, Tommasini R, Martinoia E (1996) Old enzymes for a new job: Herbicide detoxification in plants. Plant Physiol 111: 349–353

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583–593

Marrs KA (1996) The functions and regulation of glutathione S-transferases in plants. Annu Rev Plant Physiol Plant Mol Biol 47: 127–158

Marrs KA, Alfenito MR, Lloyd AM, Walbot V (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. Nature 375: 397–400

Meyer M, Schreck R, Baeuerle PA (1993) Hydrogen peroxide and antioxidants have opposite effects on activation of NF-kappa-B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. EMBO J 12: 2005–2015

Neuenschwander U, Vernooij B, Friedrich L, Uknes S, Kessmann H, Ryals J (1995) Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? Plant J 8: 227–233

Olsen LJ, Harada JJ (1995) Peroxisomes and their assembly in higher plants. Annu Rev Plant Physiol Plant Mol Biol 46: 123–146

Pinkus R, Weiner LM, Daniel V (1996) Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. J Biol Chem 271: 13422–13429

Ramasarma T (1982) Generation of H_2O in biomembranes. Biochim Biophys Acta 694: 69–93

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

Redinbaugh MG, Sabre M, Scandalios JG (1990) Expression of the maize *Cat3* catalase gene is under the influence of a circadian rhythm. Proc Natl Acad Sci USA 87: 6853–6857

Rohrdanz E, Kahl R (1998) Alterations of antioxidant enzyme expression in response to hydrogen peroxide. Free Rad Biol Med 24: 27–38

Rushmore TH, Morton MR, Pickett CB (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 266: 11632–11639

Salguero J, Boettger (1995) Secreted catalase activity from rrots of developing maize (*Zea mays* L.) seedlings. Protoplasma 184: 72–78

Scandalios JG (1993) Oxygen stress and superoxide dismutases. Plant Physiol 101: 7–12

Scandalios JG, Guan L, Polidoros AN, (1997) Catalases in plants: gene structure, properties, regulation, and expression. In: Scandalios JG (ed.) Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, New York, pp 343–406. ISBN 0-87969-502-1

Schreck R, Rieber P, Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. EMBO J 10: 2247–2258

Senior ML, Chin ECL, Lee M, Smith JSC, Stuber CW (1996) Simple sequence repeat markers developed from maize sequences found in the GENBANK database: map construction. Crop Sci 36: 1676–1683

Steitz PA, Black DL, Gerke V, Parke KA, Kramer A, Frendeway D, Keller W (1988) Small nuclear ribonucleoprotein particles. In: Birnstiel ML (ed.) Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. Springer-Verlag, New York, pp 115–154. ISBN 0-38718-410-4

Talalay P, DeLong MJ, Prochaska HJ (1988) Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. Proc Natl Acad Sci USA 85: 8261–8265

Tenhaken R, Levine A, Brisson LF, Dixon RA, Lamb C (1995) Function of the oxidative burst in hypersensitive disease resistance. Proc Natl Acad Sci USA 92: 4158–4163

Thompson JE (1988) The molecular basis for membrane deterioration during senescence. In: Nooden LD, Leopold AC (eds) Senescence and Aging in Plants. Academic Press, New York, pp 51–83. ISBN 0-12520-920-7

Thompson WF, Everett M, Polans NO, Jorgensen RA, Palmer JD (1983) Phytochrome control of RNA levels in developing pea and mung bean leaves (*Pisum sativum*, *Vigna radiata*). Planta 158: 487–500

Williamson JD, Scandalios JG (1994) The maize (*Zea mays* L.) *Cat1* catalase promoter displays differential binding of nuclear proteins isolated from germinated and developing embryos and from embryos grown in the presence and absence of abscisic acid. Plant Physiol 106: 1373–1380

Wu GS, Shortt BJ, Lawrence EB, Levine EB, Fitzsimmons KC, Shah DM (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7: 1357–1368

Edited by L. McIntosh