Journal of Experimental Botany, Vol. 58, No. 6, pp. 1301–1312, 2007
doi:10.1093/jxb/erl292 Advance Access publication 20 February, 2007

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella of maize

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Received 11 August 2006; Revised 27 November 2006; Accepted 30 November 2006

Abstract

There is circumstantial evidence implicating reactive oxygen species (ROS) in the highly ordered temporal and spatial regulation of expression of the *Cat* and *Sod* antioxidant genes during seed development and germination in maize. In order to understand and provide experimental data for the regulatory role of ROS, the expression patterns of the *Cat1*, *Cat2*, *Cat3*, *GstI*, *Sod3*, *Sod4*, and *Sod4A* genes, as well as catalase (CAT) and superoxide dismutase (SOD) activity responses, were examined after treatments with ROS-generating xenobiotics in developing and germinated maize scutella. CAT and SOD activities increased at both stages in response to each xenobiotic examined in a dose-dependent and stage-specific manner. Individual *Cat* gene expression patterns were co-ordinated with isozyme patterns of enzymatic activity in scutella of developing seeds. This was not observed in germinated seeds where, although *Cat1* expression was highly induced by ROS, there was not a similar increase of enzymatic CAT1 activity, suggesting the involvement of post-transcriptional regulation. Enhanced enzyme activities were synchronous with increases in steady-state transcript levels of specific *Sod* genes. The steady-state transcript level of *GstI* was elevated in all samples examined. Gene expression responses derived from this study along with similar results documented in previous reports were subjected to cluster analysis, revealing that ROS-generating compounds provoke similar effects in the expression patterns of the tested antioxidant genes. This could be attributable to common stress-related motifs present in the promoters of these genes.

Key words: Antioxidants, benzyl viologen, catalase, glutathione *S*-transferase, juglone, methyl viologen, paraquat, reactive oxygen species, ROS, superoxide dismutase.

Introduction

Seed development, germination, and post-germination seedling growth are well-regulated processes that involve high metabolic activity and generation of reactive oxygen species (ROS) in the cell (Bailly, 2004). In the maturation of orthodox seeds such as maize, ROS have mostly been involved in the final stage of seed desiccation in relation to the acquisition of desiccation tolerance, but are also related to high metabolic activity and mitochondrial respiration during embryo development. After germination of oil seeds, ROS production has been mainly associated with β-oxidation of fatty acids and reserve mobilization, while many metabolic cellular and molecular events related to radicle extension are also involved. Increased production of ROS may lead to oxidative stress and cellular damage resulting in seed deterioration (Bailly, 2004; Kranner *et al*., 2006).

To cope with oxidative stress, organisms have evolved several enzymatic and non-enzymatic systems, among which superoxide dismutase (SOD; EC 1.15.1.1) reduces superoxide radical ($O_2^{\cdot-}$) to H_2O_2, and catalase (CAT; EC 1.11.1.6) reduces H_2O_2 to water and dioxygen, thus

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Abbreviations: ABA, abscisic acid; ABRE, ABA-responsive element; ANOVA, analysis of variance; BV, benzyl viologen; CAT, catalase; 2,4-D, 2,4-dichlorophenoxy acetic acid; dpi, days post-imbibition; dpp, days post-pollination; GSH, glutathione; GST, glutathione S-transferase; IAA, indole-3-acetic acid; JA, jasmonic acid; JU, juglone; LSD, least significant difference; MV, methyl viologen; NF, norflurazon; PCD, programmed cell death; ROS, reactive oxygen species; SA, salicylic acid; SOD, superoxide dismutase.

preventing the formation of the highly reactive hydroxyl radical (·OH) that can cause lipid peroxidation, protein denaturation, and DNA mutations. To date, the antioxidant gene/enzyme systems for CAT and SOD have been well characterized in maize as well as other plants (Willekens *et al*., 1995; Scandalios, 1997; Scandalios *et al*., 1997). CAT in maize exists as three distinct isozymes, CAT1, CAT2, and CAT3, encoded by unlinked nuclear genes. SOD exists as nine distinct isozymes encoded by nuclear genes. SOD2, SOD4, SOD4A, and SOD5 are localized in the cytosol; SOD1 is localized in chloroplasts; and SOD3 is a mitochondria-associated enzyme encoded by a multigene family composed of the *Sod3.1*, *Sod3.2*, *Sod3.3*, and *Sod3.4* genes. Each *Cat* and *Sod* gene exhibits temporal, spatial, cell, and organelle specificity in its expression, and each responds variably to different environmental and chemical signals (Scandalios, 1997; Scandalios *et al*., 1997). These are common characteristics found in antioxidant genes of many plant species (Pastori *et al*., 2000; Luna *et al*., 2005), and their importance in stress tolerance and plant survival under adverse conditions has been well documented, especially through transgenic knockouts and overexpressing plants (Willekens *et al*., 1997; Kingston-Smith and Foyer, 2000; Dat *et al*., 2001; Polidoros *et al*., 2001; Rizhsky *et al*., 2002; Vandenabeele *et al*., 2004).

The regulation of *Cat* gene expression has been studied intensively throughout seed development, germination, and post-germination seedling growth in maize, and displays a complex pattern of shift from *Cat1* to *Cat2* with succession of developmental stages (Scandalios *et al*., 1997). This prompted studies to examine the role of plant phytohormones, such as abscisic acid (ABA), in seed development and auxin in germination, regarding the regulation of *Cat* gene expression in maize (Guan and Scandalios, 1998*a*, 2002). Results suggested that differential *Cat* gene expression might in part be regulated by phytohormones, although there was evidence for the involvement of oxidative stress. It was shown that ABA could cause increased generation of H_2O_2 which plays an important role in the ABA signal transduction pathway leading to the induction of the *Cat1* gene (Guan *et al*., 2000). The kinetics of *Cat1* transcript accumulation in response to auxin were similar to those in response to ABA. *Cat* gene responses to auxin were evident at high hormone concentration which may act as a ROS-generating xenobiotic (Guan and Scandalios, 2002). Indirect evidence for the involvement of ROS in differential regulation of *Cat* and *Sod* gene expression in seed maturation and germination was also provided by examining the effects of arsenic which may result in ROS generation (Hartley-Whitaker *et al*., 2001), in developing seeds and seedlings and in young maize leaves (Mylona *et al*., 1998).

Although these studies indicated that ROS generation might be involved in the regulation of antioxidant gene expression in seed development and germination, data providing direct evidence for the effects of exogenously applied ROS-generating compounds are largely missing. To elucidate further the role of ROS, the effects of ROS-generating redox-cycling xenobiotics in the regulation of the *Cat*, *Sod*, and *Gst* genes were examined in developing and germinated maize scutella. Redox-cyling xenobiotics may block organelle activities and promote ROS generation and oxidative stress. Methyl viologen (MV) and benzyl viologen (BV) belong to the bipyridylium herbicide family and, like other amphiphilic viologens, can bind to thylakoid or mitochondrial membranes by hydrophobic interactions and undergo redox cycling, intercepting their electron transport chains, thus promoting ROS generation. Juglone (JU), a natural naphthoquinone produced by walnut trees, once in the cell undergoes redox cycling to generate the naphthosemiquinone radical which reduces oxygen to superoxide radicals and reforms the quinone.

In order to survive when exposed to such chemicals, plants must simultaneously inactivate xenobiotics and defend themselves from the damage of ROS-induced oxidative stress. Consequently, induction and co-ordination of both detoxification and antioxidant responses is of great importance for efficient defence. Detoxification of xenobiotics typically involves activation through hydrolysis or oxidation catalysed by cytochrome P_{450} mono-oxygenases followed by covalent linkage to an endogenous hydrophilic molecule, such as glutathione (GSH), glucose, malonate, or an amino acid to form a water-soluble, and often a less toxic conjugate. When GSH is the ligand, its thiolate anion conjugates to an electrophilic site of the activated metabolite. Glutathione *S*-transferases (GSTs, EC 2.5.1.18), enzymes located in the cytosol, catalyse the reaction. In maize, GSTs comprise a multigene family with at least 42 members that display specific patterns of developmentally regulated expression and induction upon exposure to xenobiotics (Sari-Gorla *et al*., 1993; McGonigle *et al*., 2000). The type I/class Phi *GstI* (GenBank accession no. M16901) is constitutively expressed at low levels in roots and leaves (McGonigle *et al*., 2000). However, *GstI* follows the expression pattern of *Cat1* in post-pollination scutella, accumulating at higher levels between 17 d and 21 d after pollination and at lower levels thereafter (Guan *et al*., 2000). The expression pattern of *GstI* in germinating seeds is not well defined, but its presence has been detected in previous studies as well as in the present study at 5 d after imbibition. *GstI* responds readily to H_2O_2, KCN, and salicylic acid (SA), and has been used as an indicator of oxidative stress in various systems (Polidoros *et al*., 2005). Nevertheless, knowledge is rather limited regarding *GstI* co-ordination with other antioxidant genes in the defence responses during oxidative stress.

In this study, the redox-cycling xenobiotics MV, BV, and JU were used to induce oxidative stress and to examine the responses of the *Cat*, *Sod*, and *Gst* genes of

maize in developing and germinated maize scutella. The results of this study may help to understand the role of ROS in the induction of differential antioxidant gene responses in order to protect the seed effectively from oxidative stress.

Materials and methods

Plant materials and treatment conditions

Zea mays L. inbred line W64A was used in these studies. Scutella at 28 d post-pollination (dpp) and 5 d post-imbibition (dpi) were harvested as described (Mylona *et al*., 1998) and placed on Murashige–Skoog (MS) basic medium supplemented with chemicals at 0.01, 0.1, 1, and 10 mM. Plates were incubated at 25 °C for 24 h. At the end of each treatment, scutella were isolated and half the samples were used for CAT and SOD activity assays and gel electrophoresis. The remaining samples were frozen in liquid nitrogen and stored at −70 °C for subsequent RNA analyses.

Chemicals

MV (also known as paraquat; 1, 1′-dimethyl-4, 4′bipyridinium dichloride), BV (1, 1′-dibenzyl-4, 4′ bipyridinium dichloride), and JU (5-hydroxy-1, 4-naphthoquinone) were purchased from Sigma (St Louis, MO, USA). All chemical reagents used in this study were ACS grade reagents.

Enzyme activity assays, protein determination, and zymogram analysis

Five to 10 scutella from each chemical treatment were used to prepare each sample for protein determination, CAT and SOD enzyme activity assays, and zymogram analysis as previously described (Mylona *et al*., 1998). Enzyme activities were measured in three independent experiments, and each measurement was repeated twice. Enzymatic activities were compared by performing analysis of variance (ANOVA) with the XLSTAT (Addinsoft, New York, NY, USA) add-in module for Microsoft Excel. One-way ANOVA was used to test for the effect of each xenobiotic–dose combination on enzyme activities at each developmental stage. The significance of stage-dependent activity changes and the interactions of stage with the xenobiotic–dose combination were examined by two-way ANOVA applying a type III sum of squares analysis. Fisher's least significant difference (LSD) multiple comparison test was used to determine if means of the dependent variables were significantly different at the 0.05 probability level.

Gene expression analysis

Total RNA was isolated from 10 scutella of control and chemical-treated seeds (Thompson *et al*., 1983). For northern analysis, total RNA (20 μg) from each sample was separated in a denaturing 1.6% agarose gel, transferred onto nylon membranes and hybridized with ^{32}P-labelled gene-specific probes for *Cat1*, *Cat2*, *Cat3*, *Sod3*, *Sod4*, *Sod4A*, and *GstI* as previously described (Mylona *et al*., 1998).

Results from this study along with results from other studies on expression of *Cat*, *Sod*, and *GstI* genes in developing and germinated scutella treated with arsenate and arsenite (Mylona *et al*., 1998), ABA (Guan and Scandalios, 1998*a*, *b*), 2,4-dichlorophenoxy acetic acid (2,4-D) and indole-3-acetic acid (IAA; Guan and Scandalios, 2002), jasmonic acid (JA; Guan and Scandalios, 2000), and norflurazon (NF; Jung *et al*., 2001, 2006; Jung and Kuk, 2003) were analysed using the image processing and analysis software ImageJ 1.36b (http://rsb.info.nih.gov/ij/) and transformed to quantitative data by calculating the mean of the pixel values in a standard area surrounding each hybridizing band. These data were then transformed to fold change of gene expression relative to the control, named for simplicity 'relative expression'. Relative expression comparisons were performed by two-way ANOVA applying type III sum of squares analysis, and means were compared by Tukey's HSD (honestly significant difference) test at the 0.05 probability level. Average linkage clustering estimating Pearson's correlation of $\log_2$-transformed relative expression was performed using the Cluster program and visualized using the TreeView program (Eisen *et al*., 1998), http://rana.lbl.gov/EisenSoftware.htm).

Regulatory motifs in the 5′ upstream region of antioxidant genes

Information for the sequence of the 5′ upstream region was available for *Cat1*, *Cat2*, *Cat3*, *Sod4*, and *Sod4A* (Scandalios, 1997; Scandalios *et al*., 1997). To determine if 5′ upstream sequences for *GstI* and *Sod3* were available in the plant genome database (www.plantgdb.org), BLAST searches were performed using as query the mRNA sequences of these genes. Searching with the *GstI* cDNA sequence [M16901 (Shah *et al*., 1986)], the 9053 bp ZmGSStuc11-12-04.2203.1 contig was retrieved, which contained a chorismate mutase gene at the 5′ end and *GstI* at the 3′ end, both in 5′–3′ orientation. Presumably the spacer region is the 5′ upstream sequence of the maize *GstI* gene. The exact 3′ end of the maize chorismate mutase was located based on sequence similarity to a maize expressed sequence tag (EST) (AY103806) at 2240 bp of the contig, and the translation start of *GstI* was located at 4606 bp. The intervening 2366 bp sequence was used in motif searches for the maize *GstI* gene. Searching for *Sod3* 5′ upstream regions was more difficult since four highly homologous *Sod3* mRNAs have been identified (Scandalios, 1997). BLAST searches using each one of the four cDNAs retrieved several contigs, but only ZmGSStuc11-12-04.96701.1 was informative for 5′ upstream sequences. This contig was analysed further and found to correspond to the originally isolated *Sod3* cDNA that was later renamed *Sod3.1* (Scandalios, 1997). However, only a short region 496 bp upstream of the translation start codon was present in the contig and was analysed for occurrence of regulatory motifs.

Results

Effects of redox-cycling xenobiotics on antioxidant enzyme activities

The effects of MV, BV, and JU on CAT and SOD activities were examined in the scutella of developing and germinated maize seeds in three independent experiments and two measurements per sample in each experiment. CAT activity increased in 28 dpp scutella as the concentration of xenobiotics increased from 0.01 mM to 1 mM, with a maximum observed at 0.1 mM for MV and 1 M for BV and JU. At a higher dose (10 mM), total CAT activity dropped below control levels (Fig. 1A). ANOVA revealed statistically significant differences of means (F=16.44, P <0.001), and comparisons using the LSD method defined homogeneous groups denoted with the same letters on top of the bars in Fig. 1A. MV proved to be the most effective compound able to induce significantly higher CAT activity at low (but not at higher) doses, while there were no significant differences between

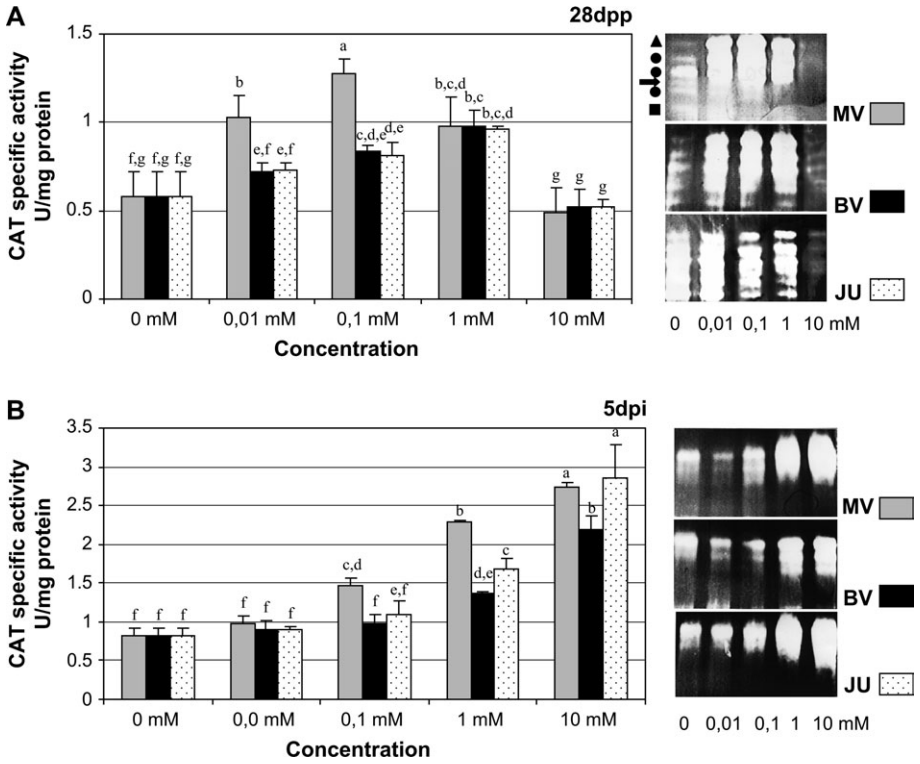


Fig. 1. CAT activity responses to xenobiotics. Histograms of CAT specific activities after treatments with MV, BV, and JU, and gel electrophoretic analysis of the CAT isozymes in 28 dpp (A) and 5 dpi (B) seeds. Scutella were isolated from 28 dpp kernels of greenhouse-grown W64A plants or 5 dpi germinated W64A seeds and incubated on MS medium supplemented with the indicated increasing concentrations of MV, BV, or JU. Scutella were isolated from treated seeds, and equal amounts of protein were used for CAT activity assays. CAT specific activity in response to the chemicals is represented with grey (MV), black (BV), and dotted (JU) bars indicating the mean ±SD of three independent experiments. Different letters on top of the bars indicate statistically significant differences of means (P <0.05) compared with the Fisher's LSD. Gels at the right side of the histograms display the isozyme composition of CAT activity, and the positions of CAT1 (rectangle), CAT2 (triangle), CAT3 (arrow), and CAT1/CAT2 heterotetramers (circle) are shown on the left of the top gel.

BV and JU. The response of each CAT isozyme was assessed with on-gel activity assays after electrophoresis. Untreated 28 dpp scutella revealed the presence of CAT1, CAT2, and CAT3 homotetramers, as well as the three heterotetramers formed between CAT1 and CAT2 subunits (Fig. 1A). The major contributors to the observed increase of CAT activity in MV and BV treatments were CAT1 and CAT2, with CAT2 being higher at high concentrations. In JU treatments, CAT1 and CAT2 seemed to contribute equally to the increase of CAT activity, while CAT3 might also be present (Fig. 1A).

A different picture emerged when the effects of MV, BV, and JU were tested in germinated maize seeds (Fig. 1B). Total CAT activity continuously increased with increasing concentrations of each chemical applied, reaching the highest levels at 10 mM. Statistical ANOVA detected significant differences of means (F=60.19, P <0.001). The three compounds were significantly more effective at the higher dose tested, and homogeneous groups of means are denoted with the same letters on top of the bars in Fig. 1B. The increase of CAT activity was due to a large increase of CAT2 and an increase of CAT1, as judged by the formation of CAT2 homotetramers and CAT1–CAT2 heterotetramers with participation of more CAT2 subunits (Fig. 1B).

The role of the developmental stage on CAT responses after treatment with the tested xenobiotics was estimated, and a significant effect (F=5.246, P <0.05) was recorded. The interaction of developmental stage with the compound–dose combination was highly significant (F=57.91, P <0.0001), with germination correlated with higher CAT activity than seed maturation, especially at higher dose treatments.

SOD activity was also examined, and a trend similar to that observed for CAT activity was recorded. In immature seeds, total SOD activity increased more at low than at high concentrations of the tested compounds, and statistically significant differences were detected (F=26.18, P <0.001). SOD activity reached the highest levels at 0.01 mM JU or 0.1 mM MV and BV, although the differences among these concentrations were not significant as denoted by the mean SOD activity comparison (Fig. 2A). At a higher concentration (1 mM), SOD activity was still higher than that of the control but lower than that

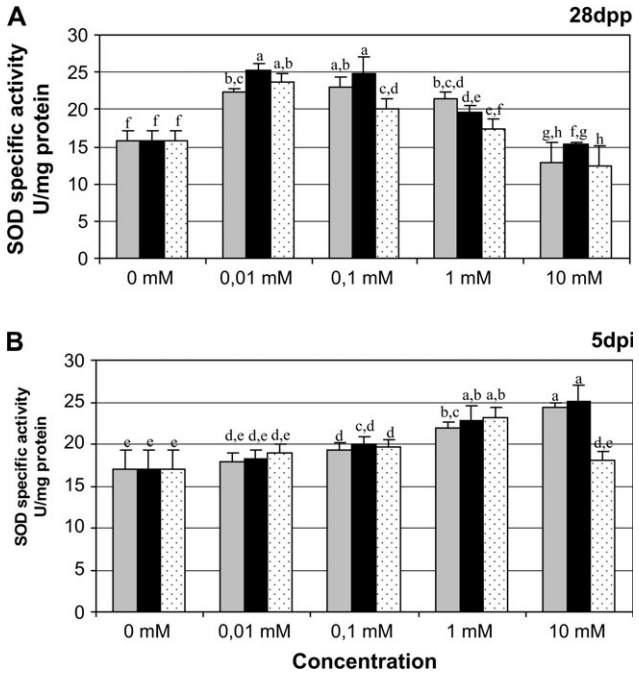


Fig. 2. SOD activity responses to xenobiotics. Histograms of SOD activity in the presence of MV, BV, or JU in developing 28 dpp (A) and germinated 5 dpi (B) maize seeds. Scutella were isolated and treated as described in Fig. 1 and equal amounts of protein were used for SOD activity assays. SOD specific activity in response to the chemicals is represented with grey (MV), black (BV), and dotted (JU) bars indicating the mean ±SD of three independent experiments. Different letters on top of the bars indicate statistically significant differences of means (P <0.05) compared with the Fisher's LSD.

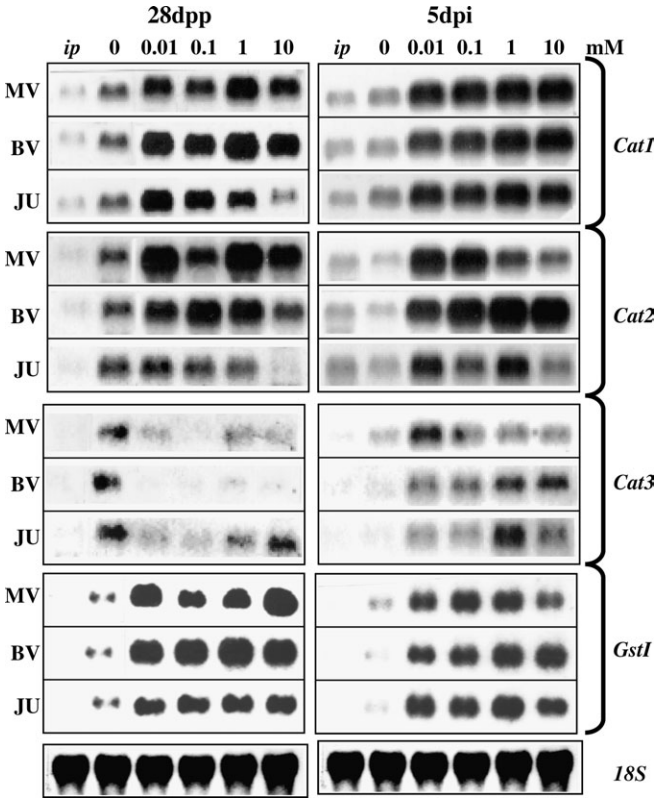


Fig. 3. Accumulation of *Cat* and *GstI* transcripts in 28 dpp and 5 dpi scutella treated with MV, BV, or JU. Total RNA (20 μg) was separated by electrophoresis on denaturing 1.6% agarose gels and transferred onto nylon membranes. Blots were hybridized with *Cat1*, *Cat2*, *Cat3*, and *GstI* gene-specific probes. Hybridization of the same filters with the *pHA2* probe containing an 18S ribosomal sequence was performed to ensure equal loading. Results are representative of two independent experiments.

observed at 0.1 mM. As the dose of the chemicals further increased to 10 mM, SOD activity dropped to levels equal to (for MV) or slightly below (BV and JU) those of the control. At 5 dpi, total SOD activity increased gradually with increasing concentrations of MV or BV, and statistically significant differences were detected (F=10.25, P <0.001). Maximum activity was recorded at the highest concentration of 10 mM for MV and BV. In the JU treatment, SOD activity increased gradually with increasing concentrations, reaching a peak at 1 mM, and was similar to the control at 10 mM (Fig. 2B). Regarding the role of developmental stage on SOD activity, the effect was not significant (F=1.247, P >0.05), but the interaction between stage and the compound–dose combination was highly significant (F=24.55, P <0.0001).

Gene expression in response to redox-cycling xenobiotics

Cat, *GstI*, and *Sod* transcript accumulation was examined by northern hybridization in developing and germinated maize seeds after treatment with the tested xenobiotics (Figs 3, 4). Detailed descriptions of the results in these figures, as well as the statistical analysis of changes in gene expression are given in the Supplementary data at *JXB* online. Gene expression after each treatment was quantified by image analysis, and relative expression (fold change relative to the untreated control) was calculated. Mean relative expression of each gene–stage–dose combination calculated from two independent experiments and two repetitions of image analysis was compared by ANOVA, revealing significant gene responses in both developing and germinated seeds. Statistical analysis (see Supplementary Figs S1–S14 at *JXB* online) revealed significant effects for dose and xenobiotic–dose interaction on gene expression in all the experiments. A significant increase of relative expression comparing treated scutella and untreated control scutella at both 28 dpp and 5 dpi was obtained with all the three compounds and at least one concentration for *Cat1*, *GstI*, *Sod3*, *Sod4*, and *Sod4A*, while for *Cat2* a significant increase was estimated only in MV and BV treatments. In contrast, *Cat3* at 28 dpp displayed a significant decrease in all treatments except with 10 mM JU, while at 5 dpi it displayed a significant increase in at least one concentration with all the three compounds.

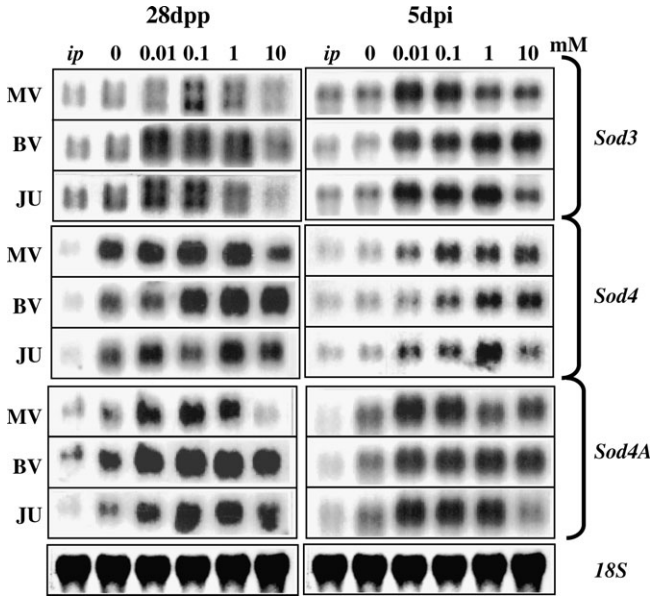


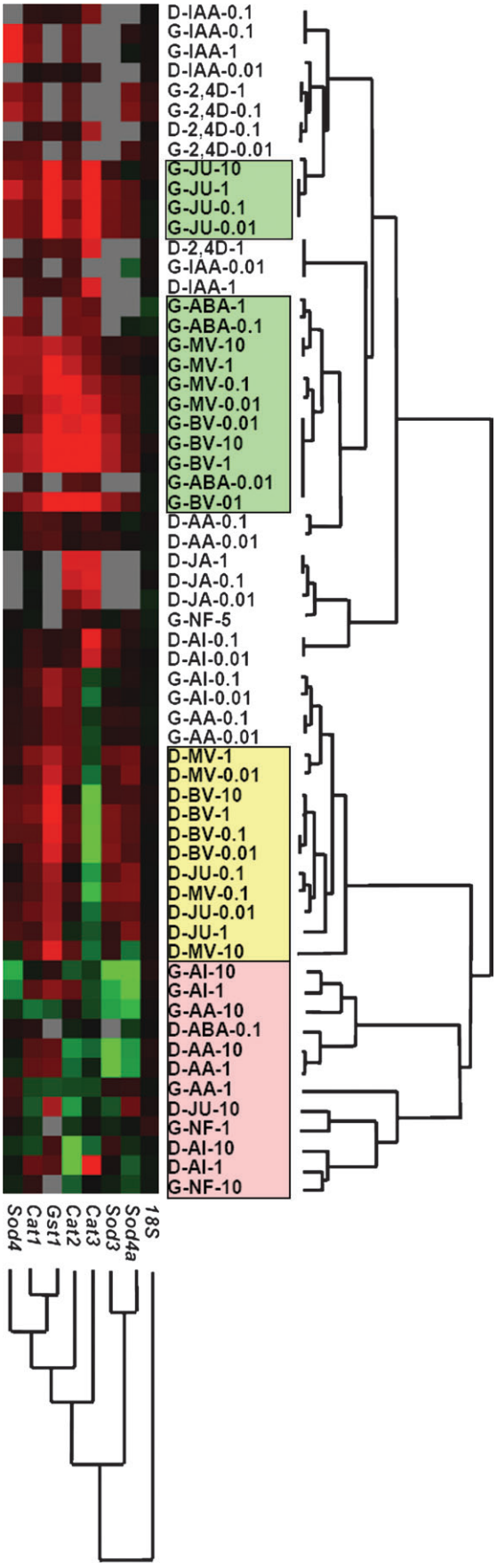
Fig. 4. Accumulation of *Sod* transcripts in 28 dpp and 5 dpi scutella treated with MV, BV, or JU. Total RNA (20 μg) was separated by electrophoresis on a denaturing 1.6% gel and transferred onto a nylon membrane. Transcripts were detected by northern blot hybridization using *Sod3*, *Sod4*, and *Sod4A* gene-specific probes. Hybridization of the same filters with the *pHA2* probe containing an 18S ribosomal sequence was performed to ensure equal loading. Results are representative of two independent experiments.

Cluster analysis of gene expression among different treatments

Previous studies (cited in the Materials and methods) have estimated gene expression profiles of maize antioxidant genes in maturing and germinated seeds under various hormone and xenobiotic treatments. In order to make comparisons of gene expression patterns identified in this study with those published previously, experiments that were conducted in 28 dpp developing and 5 dpi germinated seeds were selected and data were retrieved for a total of seven maize antioxidant genes after treatment with 10 compounds including those in the present study. Expression levels were analysed from published figures and Figs 3 and 4 in the present study using ImageJ, and hybridization signals were transformed to quantitative data. Changes in expression levels of the three *Cat* genes, *GstI*, *Sod3*, *Sod4*, *Sod4A*, and 18S RNA used as normalization control, after treatment with the 10 compounds (described in the Materials and methods) at different doses and two stages, making 61 stage–compound–dose combinations, were recorded. A two-dimensional transcription matrix (genes versus treatments) describing the change in 428 individual mRNAs was constructed from these experiments. To make results comparable across all the experiments, transcript levels of the treated samples were compared with those of the corresponding controls, and fold changes were used for further clustering analysis. The results presented in Fig. 5 display clusters of treatments that provoke similar antioxidant gene responses and clusters of genes with similarities in expression patterns. Groups of treatments that induce distinct expression patterns are indicated in rectangles of different colours. Most of the redox-cycling compounds used in this study were grouped together in each stage. At seed maturation, all the redox-cycling compounds highlighted with a yellow rectangle had similar effects in the expression of the tested genes, with strong up-regulation of *GstI* and to a lesser degree of all other genes except *Cat3*, which was down-regulated. One exception was the treatment with a high concentration of JU due basically to the down-regulation of *Sod3*, *Cat1*, and *Cat2*. In post-germination scutella (green rectangles), the JU treatment formed a distinct group due to lower induction of *Cat2* in comparison with the other compounds. MV and BV treatments clustered together with ABA treatments in a distinct group. Finally, a group of treatments that resulted in down-regulation of the majority of the tested genes is highlighted by a pink rectangle and includes high doses of arsenic at both stages, and NF and ABA at seed development. However, regarding NF and ABA, it should be noted that the effects were of low magnitude. In this group, sporadic up-regulation of specific genes was also detected. This analysis also revealed that low doses of arsenic treatments at 5 dpi produced a similar expression pattern as the redox-cycling compounds at 28 dpp. Hormone treatments with JA, IAA, and 2, 4-D revealed specific patterns although the strength of this assumption is low since several genes had not been examined.

Promoter analysis

Similarities of expression patterns among the maize antioxidant genes raised the possibility that common regulatory circuits might drive expression of certain genes under specific conditions. To examine whether such common expression patterns were related to similarities in promoter architecture, 5′ upstream sequences of the tested genes were searched for the occurrence of 70 different stress-related motifs. It was found that 31 ROS- and stress-related *cis*-elements were present in the 5′ upstream regions of these genes (Table 1). Five stress-related motifs were over-represented in the putative promoter sequences (up to −500 bp from the ATG codon) of more than three of the tested genes. These were the TGACG motif that is a promoter element responsive to JA, auxins, SA, and oxidative stress (Ulmasov *et al*., 1994; Xiang *et al*., 1996; Garreton *et al*., 2002), the ABA-responsive element (ABRE)-like motif that is important for ABA-induced gene transcription (Shinozaki and Yamaguchi-Shinozaki, 2000), the ACGT core sequence that is recognized by transcription factors of the bZIP family (Jakoby *et al*., 2002), the anaerobic response element found in the maize cytosolic glyceraldehyde 3-phosphate dehydrogenase *gpc4* and supposed to play a role either in induction by



anaerobiosis or as a general enhancer element (Manjunath and Sachs, 1997), and the Skn-1 motif that is part of the rice glutelin promoters conferring endosperm-specific expression (Washida *et al*., 1999). Analysis of *Sod3* was not included in Table 1 since only a very short promoter sequence could be retrieved for one of the four different genes. However, within the ~500 bp 5′ upstream region of *Sod3.1*, a W-box and a Skn-1 site were recognized.

Discussion

The role of ROS in the regulation of antioxidant responses in maize seeds at 28 dpp and 5 dpi was assessed using the redox-cycling xenobiotics MV, BV, and JU that are widely used to generate oxidative stress experimentally in living organisms. CAT and SOD activities increased at both stages after treatment in a dose-dependent manner. In developing seeds, the activities of both enzymes increased rapidly at very low concentrations of the compounds, while they seemed to be slightly inhibited at a high concentration. The response was different in germinated seeds since both enzymes displayed a continuous increase in activity with increasing doses (except SOD with JU). These data revealed that ROS can induce enzymatic activities of CAT and SOD in maize seeds, but developmental stage has a critical role in determining how antioxidant defences will be regulated. Increased CAT activity in developing seeds was due to an equal increase of both CAT1 and CAT2, while in germinated seeds the major contributor was CAT2 with minor CAT1 input, somewhat similar to responses of developing and germinated seeds to H_2O_2 (Scandalios *et al*., 1997). These profiles matched previously reported CAT activity patterns after ABA (Guan and Scandalios, 1998*a*) and auxin (Guan and Scandalios, 2002) treatment, pointing to a

Fig. 5. Expression profiles of the maize *Cat1*, *Cat2*, *Cat3*, *Sod1*, *Sod3*, *Sod 4*, *Sod4A*, and *GstI* genes in developing 28 dpp and germinated 5 dpi scutella treated with different xenobiotic/hormone compounds. The fold change values for each sample, relative to untreated control samples, were $\log_2$ transformed and subjected to average linkage hierarchical clustering, as described in the Materials and methods. Expression values higher, equal to, and lower than those of the control are shown in red, black, and green, respectively. The higher the absolute value of a fold difference, the brighter the colour. Missing values are grey. Treatments applied for each experiment are shown on the right with a format that indicates in order stage–chemical–dose. Stage is shown as D, 28 dpp developing scutella; and G, 5 dpi germinated scutella. Chemicals are 2,4D, 2,4-dichlorophenoxy acetic acid; AA, arsenate; AI, arsenite; ABA, abscisic acid; BV, benzyl viologen; IAA, indole-3-acetic acid; JA, jasmonic acid; JU, juglone; MV, methyl viologen; NF, norflurazon. Doses are in mM. The vertical dendrogram (right) indicates the relationship among experiments across all of the genes included in the cluster analysis. The horizontal dendrogram (bottom) indicates the relationship of induction patterns of the tested genes. The rectangles indicate groups of treatments that provoke similar expression patterns at 5 dpi (green) and 28 dpp (yellow). A group of treatments with high doses of different compounds which reduce expression of most genes is also shown (pink rectangle).

Table 1. *Predicted* cis *elements in the 5′ upstream genomic sequence (up to −2500 bp) of maize antioxidant genes*

Sequences were analysed *in silico* for the presence of *cis*-regulatory elements +, or shown in bold when present up to −500 bp, which can be correlated with stress, metabolism, or hormone signaling, regardless of orientation. Analysis was performed by searching the PlantCARE database and using the signal search utility of GeneQuest (DNAStar) with a data set of ROS- and stress-specific elements previously described (Chen *et al*., 2002; Mahalingam *et al*., 2003; Geisler *et al*., 2006).

Element	Gene						Sequence	Function (responsiveness)
	Cat1	*Cat2*	*Cat3*	*GstI*	*Sod4*	*Sod4A*		
ABRE-like	+	+	+	+	+	+	BACGTGKM	ABA
ACGT core	+	+	+	+	+	+	ACGT	bZIP-binding factors
Anaerobic response element	+	+	+	+	+	+	TGGTTT	Anaerobic response
ARE	+	+	+	−	−	+	RTGAYNNNGC	Antioxidants–electrophiles
AtMyb4	+	+	+	+	+	+	AMCWAMC	Defence pathogens
AuxRR core	+	+	−	−	−	−	GGTCCAT	Auxin
CCGTCC motif	+	+	−	−	−	−	CCGTCC	Meristem activation
DRE	+	−	−	+	−	+	RCCGAC	Dehydration, low temperature, salt stress
ERE	−	−	−	−	+	+	ATTTCAAA	Ethylene
GARE motif	−	−	+	−	−	+	TCTGTTG	Gibberellin
G-box	+	−	−	+	+	−	CACGTG	Anaerobiosis, light, hormones
GCN4 motif	−	−	−	+	+	−	TGAGTCA	Endosperm expression
HS-3	−	−	−	+	+	−	TAAAGGG	Heat shock
HS-4	+	−	+	−	+	+	CAANNTTC	Heat shock
HS-2	+	−	−	−	−	−	GTTMTAGA	Heat shock
L-box	+	+	+	−	−	+	GATTGG	Low temperature
LTR	+	+	+	+	+	−	CCGAAA	Low temperature
MBS	−	−	+	−	+	−	CAACTG	Drought
MYC	−	−	+	+	+	+	CACATG	Dehydration
RY motif	−	−	+	−	−	−	CATGCATG	Late embryogenesis regulation
SA-induced	+	+	+	−	−	+	ACGTCA	Salicylic acid
Skn-1	+	+	+	+	+	+	GTCAT	Endosperm-specific expression
SUC/ROS-3	−	+	−	−	−	−	CATGCCTC	ROS, metabolism
SUC/ROS-4	−	−	−	−	+	−	CAGGCATG	ROS, metabolism
TC-rich repeats	−	−	−	−	−	+	ATTTTCTTCA	Stress-defence related
TGACG motif	+	+	+	+	+	+	TGACG	ROS-MeJA, auxin, SA
TGA element	−	+	+	+	+	+	AACGAC	Auxin
UGPe-1	−	+	+	−	+	−	SAKGCRKG	RY-motif-related
W-box	−	−	+	−	+	+	TTGACY	Wounding, infection, senescence
WRKY-like	+	+	+	−	−	−	BBWGACYT	Wounding, infection, senescence
WUN motif	−	−	−	−	+	−	TGTGGWWWG	Wounding

possible involvement of ROS in the signalling cascade affecting CAT responses. Elevation of ROS during seed maturation has been associated with seed desiccation and during germination with metabolic activity. After germination, the seed undergoes senescence and programmed cell death (PCD) that also involves ROS signalling. However, signs of PCD are not visible until 11–12 dpi in maize aleurone (Dominguez *et al*., 2004) and in barley scutella (Lindholm *et al*., 2000). Although comparable results for maize scutella are not available (to the best of our knowledge), based on the above data it can be argued that initiation of PCD in maize scutellum is not probable at 5 dpi. It is also characteristic that, in barley, ROS-scavenging enzymes (CAT and SOD) are down-regulated before PCD (Fath *et al*., 2001) while at 5 dpi maize scutella CAT activity reaches the maximum. Scutellum has to support the initial stages of seedling growth and thus has an important role to play for a longer time before senescence and PCD. Acquisition of desiccation tolerance in developing seeds as well as effective protection of germinating seeds from ROS has been clearly associated with induction of antioxidant defences (Bailly, 2004). It is conceivable that ROS themselves could be one of the signals responsible for induction of antioxidant systems, a capacity of ROS that has been well documented (Mittler, 2002; Foyer and Noctor, 2005).

Increased enzymatic activities of CAT1 and CAT2 could be correlated with specific induction of *Cat1* and *Cat2* at seed maturation. However, at 5 dpi, the high increase of CAT activity was basically due to a large increase of CAT2 and less of CAT1, while both genes were equally induced at least at the low doses of each compound. Thus, at 5 dpi, the isozyme activity profile did not correlate with the expression pattern of the respective genes. Interestingly, in normal seed germination, the *Cat2* mRNA profile increases and decreases in parallel with the CAT2 protein, whereas the accumulation of steady-state *Cat1* mRNA increases as the CAT1 protein decreases during the same scutellar developmental period (Scandalios *et al*., 1997). These data indicate that the differential expression of the two genes in this tissue involves both transcriptional and post-transcriptional regulation which is

superimposed on the responses of the genes to elevated levels of ROS. *Cat3* transcript levels increased in response to MV, BV, and JU in germinated seeds, but the respective enzymatic activity was not detected. *GstI* also displayed a consistent high induction by the three tested compounds in every stage. Specific GSTs are induced during oxidative stress in both plants (Marrs, 1996; Edwards *et al*., 2000) and animals (Raza *et al*., 2002), and might act to detoxify metabolites that arise from oxidative damage (Kilili *et al*., 2004). *GstI* was induced by oxidative stress in maize (Polidoros and Scandalios, 1999), and the observed responses suggest that they were related to ROS generation by the redox-cycling xenobiotics. Differential expression patterns of *Sod3*, *Sod4*, and *Sod4A* genes were induced in response to each xenobiotic examined. Previous studies have shown that *Sod4* and *Sod4A* genes are induced in response to H_2O_2, possibly due to the presence of specific motifs in the promoter region of both genes (Scandalios, 1997).

The observed differences of antioxidant gene responses to different superoxide-generating compounds, although relatively small in comparison with the similarities in expression patterns of each gene to the tested treatments, merit some attention. Differences may be explained by the different redox potentials of the compounds, since the more negative are poorer redox cyclers. In this account, MV has a more negative redox potential ($E_0^{'} = -446$ mV; Wardman, 1989) and is a weaker redox cycler in comparison with BV ($E_0^{'} = -359$ mV; Wardman, 1989) and JU ($E_0^{'} = -95$ mV; Inbaraj and Chignell, 2004) which is the strongest redox cycler of this group. However, MV's effects are much more dramatic in plants, hence its use as a herbicide. The major target of MV in plant cells is the chloroplast, while in non-photosynthetic tissues mitochondria have also been implicated (Vicente *et al*., 2001). Toxicity can be enhanced by metal ions such as copper and iron through a Fenton-type reaction (Sutton and Winterbourn, 1989), and it has been suggested that an increase of catalytic iron in MV-treated plants is a major component of MV toxicity (Iturbe-Ormaetxe *et al*., 1998). BV, although a stronger redox cycler than MV, proved to be ~100 times less inhibitory to photosynthesis than MV (Lewinsohn and Gressel, 1984). On the other hand, superoxide formation was more pronounced in the presence of BV than MV in animal cells (Bonneh-Barkay *et al*., 2005). The phytotoxic effects of JU have been attributed to its ability to disrupt electron transport functions in both isolated chloroplasts and mitochondria (Hejl *et al*., 1993). However, JU can react with the thiol groups in proteins as well as GSH, leading to GSH depletion and increase of H_2O_2 and GSSG (Gant *et al*., 1988). Reaction with GSH generates slower redox-cycling conjugates (Cenas *et al*., 1994), providing an explanation for the attenuated effects of high concentration JU treatments in the present experiments. Other major effects of JU concern inhibition of the enzymatic activity of H-ATPase (Hejl and Koster, 2004) and parvulin peptidyl-propyl isomerase (Hennig *et al*., 1998), and inhibition of transcription by RNA polymerase II (Chao *et al*., 2001). Taken together, these data might explain the diversification of JU effects on gene expression and the formation of a separate branch in cluster analysis (Fig. 5).

Cluster analysis of gene expression under the different treatments revealed that *Cat1* and *GstI* displayed very similar expression patterns, as did *Sod3* and *Sod4A*, indicating that the examined conditions induced coordinated responses in these two pairs of genes. The rest of the genes did not display strong expression pattern similarities. *Cat1* is expressed in all tissues/stages and appears to be induced in response to many different challenges in maize (Scandalios *et al*., 1997; Guan and Scandalios, 1998*a*; Mylona *et al*., 1998; Polidoros and Scandalios, 1999; Guan *et al*., 2000; Guan and Scandalios, 2002), suggesting that the *Cat1* gene may represent the basic mechanism for CAT defence against oxidative stress. Similarly, *GstI* is constitutive in young seedlings, but can be enhanced by the herbicide safener (Jepson *et al*., 1994) and oxidative stress (Polidoros and Scandalios, 1999), suggesting a similar role to that of *Cat1* as a basic mechanism for GST stress responses. On the other hand, the expression patterns of *Cat2* and *Cat3* display developmental and tissue specificity and are quite different in response to radical-generating xenobiotic compounds in the same developmental stage. Similarities observed between *Sod3* and *Sod4A* together with the different compartmentalization of the two gene products provide evidence for similar roles in protection of mitochondria and cytosol, respectively.

An interesting observation from cluster analysis was that ABA treatments at 5 dpi fall into the same branch as redox-cycling compound treatments, although it could be argued that since there are several missing values for expression in ABA treatments, this observation could be circumstantial. However, there is an increasing body of evidence suggesting that ABA action is mediated by oxidative signals in plant cells (Guan *et al*., 2000; Jiang and Zhang, 2001; Kwak *et al*., 2003; Laloi *et al*., 2004). It has been shown that endogenous ABA does not play a major role in *Cat1* expression via ABRE2 in 21 dpp seeds, and the observed ABA effect on *Cat1* is indirectly mediated via oxidative stress (Guan and Scandalios, 1998*a*). Results of cluster analysis also point to a possible role for ROS, but further experiments are needed to clarify the role of ROS in mediating ABA responses in developing and germinated maize scutella.

Several other treatments clustered together and formed separate branches such as, for example, the auxin (Guan and Scandalios, 2002) and the arsenic (Mylona *et al*., 1998) treatments. However, NF treatments did not form a branch and were allocated randomly in the tree,

suggesting that NF's effects were not consistent with a specific mode of action. Although NF can block carotenoid synthesis by non-competitively binding to phytoene desaturase and indirectly causing oxidative stress in the chloroplast in the presence of light (Jung *et al*., 2001), it is not a redox-cycling compound and, unlike MV, it cannot induce oxidative stress in non-photosynthetic tissues. Thus, its effects on antioxidant gene expression were not similar to those of ROS-producing compounds in the scutellum.

How are gene expression patterns of these different genes regulated? The number, order, and type of protein-binding sequences present in promoters are major determinants of the differences in expression patterns of genes (Mahalingam *et al*., 2003). Several well characterized promoter elements related to stress were identified in the 5′ upstream regions of the genes examined (Table 1). However, the promoter architecture of these genes was different and probably combinations of specific elements were important for the detected effects. A stress-related motif present in all the proximal promoters of the antioxidant genes (except *Cat1* that contained it further upstream) is the TGACG motif. This element is part of the activation sequence-1 (*as-1*), characterized by two TGACG motifs that bind basic/leucine zipper transcription factors of the plant TGA family *in vitro* and *in vivo* (Xiang *et al*., 1997; Johnson *et al*., 2001). It is interesting that this promoter element is also responsive to high concentrations of auxins and methyl jasmonate (Ulmasov *et al*., 1994; Xiang *et al*., 1996), is activated by SA via oxidative stress (Garreton *et al*., 2002), and is over-represented in studies that analyse the promoter architecture of stress-responsive genes (Chen *et al*., 2002; Mahalingam *et al*., 2003; Geisler *et al*., 2006). The presence of the TGACG motif in all the promoters of the examined antioxidant genes provides a strong indication for a regulatory role of oxidative stress in antioxidant gene expression in maize seeds.

Among other stress-related promoter elements, the ARE (antioxidant response element) that has been shown to play a role in *Cat1* gene expression during scutellum senescence (Polidoros and Scandalios, 1999) and the *Arabidopsis AtPer1* induction by H_2O_2 and hydroquinone, as well as in embryo- and endosperm-specific expression (Haslekas *et al*., 2003), was present in the promoter region of *Cat1*, *Cat2*, and, further upstream, in the *Cat3* and *Sod4A* genes. An ABRE-like motif that is important for ABA-induced gene transcription could be found in *Cat1*, *Cat2*, and *Sod4A*. However, the ABRE family is similar to the G-box sequence group that is present in many promoters responsive to environmental stimuli such as UV, wounding, and anaerobiosis (Pastori and Foyer, 2002). The presence of ABRE does not guarantee a role for ABA in the induction of antioxidant gene responses as ABA induction has only been observed in 20% of genes with ABRE, which is significantly different from the genome as a whole but still does not explain why the other 80% of ABRE-containing genes are not induced by ABA (Geisler *et al*., 2006). Finally, two elements were present in all the genes examined: the Skn-1 motif that confers endosperm-specific expression in the rice glutelin gene (Washida *et al*., 1999), indicating that it may play a role in seed-specific expression, and the anaerobic response element supposed to play a role either in induction by anaerobiosis or as a general enhancer element (Manjunath and Sachs, 1997).

In conclusion, the results presented in this study confirmed that ROS induced antioxidant gene expression and caused a substantial increase of the respective enzymatic activities of CATs and SODs in developing and germinated maize seeds. Individual *Cat* gene expression patterns at seed maturation were co-ordinated with isozyme patterns of enzymatic activity. This was not evident in germinated seeds where, although *Cat1* expression was highly induced by ROS, there was not a similar increase in enzymatic CAT1 activity, suggesting the involvement of post-transcriptional regulation. Comparison of gene expression patterns between different experiments involving ROS, hormones, and xenobiotics suggested that similarities could be explained by ROS production in treatments that were not intended to examine ROS-dependent effects. Promoter elements that have been recognized as important regulatory components conferring stress-induced and ROS-regulated gene expression were identified in the promoter region of the antioxidant maize genes and could be critical in mediating induction after treatment with ROS-producing xenobiotics.

Supplementary data

The supplementary data, which can be found at *JXB* online, provide a detailed description of Figs 3 and 4, and the statistical analysis of changes in gene expression after each treatment shown in these figures quantified by image analysis.

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