Journal of Experimental Botany, Vol. 62, No. 1, pp. 235–248, 2011
doi:10.1093/jxb/erq261 Advance Access publication 25 August, 2010
This paper is available online free of all access charges (see http://jxb.oxfordjournals.org/open_access.html for further details)

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Haem oxygenase delays programmed cell death in wheat aleurone layers by modulation of hydrogen peroxide metabolism

Mingzhu Wu*, Jingjing Huang*, Sheng Xu*, Tengfang Ling, Yanjie Xie and Wenbiao Shen†

College of Life Sciences, Co. Laboratory of Nanjing Agricultural University and Carl Zeiss Far East, Jiangsu Province, Nanjing Agricultural University, Nanjing 210095, PR China

* These authors contributed equally to this work.
† To whom correspondence should be addressed: E-mail: wbshenh@njau.edu.cn

Received 30 April 2010; Revised 31 July 2010; Accepted 3 August 2010

Abstract

Haem oxygenase-1 (HO-1) confers protection against a variety of oxidant-induced cell and tissue injury in animals and plants. In this report, it is confirmed that programmed cell death (PCD) in wheat aleurone layers is stimulated by GA and prevented by ABA. Meanwhile, HO activity and HO-1 protein expression exhibited lower levels in GA-treated layers, whereas the hydrogen peroxide (H_2O_2) content was apparently increased. The pharmacology approach illustrated that scavenging or accumulating H_2O_2 either delayed or accelerated GA-induced PCD. Furthermore, pretreatment with the HO-1 specific inhibitor, zinc protoporphyrin IX (ZnPPIX), before exposure to GA, not only decreased HO activity but also accelerated GA-induced PCD significantly. The application of the HO-1 inducer, haematin, and the enzymatic reaction product of HO, carbon monoxide (CO) aqueous solution, both of which brought about a noticeable induction of HO expression, substantially prevented GA-induced PCD. These effects were reversed when ZnPPIX was added, suggesting that HO *in vivo* played a role in delaying PCD. Meanwhile, catalase (CAT) and ascorbate peroxidase (APX) activities or transcripts were enhanced by haematin, CO, or bilirubin (BR), the catalytic by-product of HO. This enhancement resulted in a decrease in H_2O_2 production and a delay in PCD. In addition, the antioxidants butylated hydroxytoluene (BHT), dithiothreitol (DTT), and ascorbic acid (AsA) were able not only to delay PCD but also to mimic the effects of haematin and CO on HO up-regulation. Overall, the above results suggested that up-regulation of HO expression delays PCD through the down-regulation of H_2O_2 production.

Key words: Aleurone layers, haem oxygenase, hydrogen peroxide metabolism, programmed cell death, *Triticum aestivum*.

Introduction

Cereal endosperm consists of two distinct tissues: the starchy endosperm and the surrounding aleurone layer. The death of aleurone cells is described as a form of programmed cell death (PCD) following germination, which is tightly regulated by GA and ABA (Bethke *et al.*, 1999). Evidence further suggests that the second messengers or modulators, such as cytosolic Ca^{2+}, reactive oxygen species (ROS), and nitric oxide (NO), are involved in hormone-induced PCD (Kuo *et al.*, 1996; Bethke and Jones, 2001; Fath *et al.*, 2000, 2001, 2002; Beligni *et al.*, 2002; Lamotte *et al.*, 2005). For example, the reduction of nitrite to NO can be achieved non-enzymatically at acidic pH values (Bethke *et al.*, 2004). This reaction occurs in the apoplast of barley aleurone layer cells in response to GA or ABA, two hormones that acidify the apoplastic medium. It has also been reported that catalase (CAT) and ascorbate peroxidase (APX), pivotal enzymes for ROS removal, decrease significantly, while substrate hydrogen peroxide (H_2O_2) increases remarkably as early events of the PCD process, thereby representing a hallmark of cells that have activated a suicide programme (de Pinto *et al.*, 2006; Locato *et al.*, 2008). Therefore, knowledge of how these second messengers or modulators interact is crucial in gaining a better understanding of fundamental plant growth and developmental processes.

The haem oxygenase (HO; EC 1.14.99.3) system is the rate-limiting step in the conversion of haem into biliverdin (BV), carbon monoxide (CO), and free iron (Fe^{2+}). Biliverdin is subsequently reduced by cytosolic biliverdin reductase to form the potent antioxidant bilirubin (BR; Wilks, 2002). To date, three isoforms of mammalian HO protein have been identified: HO-1, HO-2, and HO-3. Under physiological conditions, HO-1, the sole inducible 32 kDa isoform, is highly expressed in liver and spleen; HO-2 is a constitutive 36 kDa isoform, which is expressed mainly in the brain and testis; and HO-3 is a 33 kDa isoform that closely resembles HO-2. Many putative *HO* genes or corresponding proteins, such as those found in animals, have been identified from different plant species, including *Arabidopsis*, soybean, tomato, potato, *Medicago truncatula*, rice, corn, sorghum, etc. (Shekhawat and Verma, 2010). For example, the first map-based cloning of the *Arabidopsis HO* gene is *HY1*, and the product of the *HY1* gene shows significant similarities to animal HOs and contains a possible transit peptide for transport to plastids (Muramoto *et al.*, 1999). Recent results show that four members of *Arabidopsis* HOs cluster into two subfamilies: HY1 (HO1), HO3, and HO4 belong to the HO-1 subfamily, while HO2 is the only member of the HO-2 subfamily, which is unable to bind or degrade haem; therefore it is not a true HO (Gisk *et al.*, 2010). More recently, the wheat (*Triticum aestivum*) HO-1 gene was cloned from total RNA prepared from seedlings. It has been named *TaHO1* and is currently deposited in GenBank with the accession number HM014348. The *TaHO1* gene encodes a single open reading frame of 288 amino acids, with a predicated molecular mass of 31.5 kDa. This includes a 63 amino acid transit peptide, predicting a mature protein of 26 kDa following cleavage of the transit peptide. Expression analysis further showed that the *TaHO1* gene was expressed in leaves, seeds, roots, and aleurone layers.

In animals and plants, evidence has accumulated showing that the expression of HO-1 is triggered by diverse stress-inducing stimuli, including hypoxia (Motterlini *et al.*, 2000), salinity stress (Xie *et al.*, 2008; Ling *et al.*, 2009), heavy metals (Noriega *et al.*, 2004; Balestrasse *et al.*, 2005, 2006, 2008; Han *et al.*, 2008), UV radiation (Keyse and Tyrrell, 1989; Yannarelli *et al.*, 2006), ROS such as H_2O_2 (Chen *et al.*, 2009), and NO (Noriega *et al.*, 2007; Xuan *et al.*, 2008). For example, compared with the NaCl-free control, salinity stress results in the inhibition of HO activity as well as decreased rice seed germination. By contrast, the combination with the HO-1 inducer haematin could reverse the above responses. Consequently, biological functions of HO-1 protein or corresponding genes are believed to be associated with a fundamental adaptive and defensive response against oxidative stress and cellular stress, both in animals and in plants (Motterlini *et al.*, 2000; Noriega *et al.*, 2004; Shekhawat and Verma, 2010). Normally, the protective effects of HO-1 in plants result from enzyme actions. For example, CO aqueous solution applied exogenously, results in a mitigation of the salinity-induced inhibition of wheat root growth and delays PCD, both of which have been related to a decrease in superoxide anion production via up-regulation of superoxide dismutase (SOD) and down-regulation of NADPH oxidase expression (Ling *et al.*, 2009). However, it still remains to be determined whether endogenous H_2O_2 participates in the HO/CO signal transduction pathway.

Although HO can have many, often disparate, effects on plants (Shekhawat and Verma, 2010), there is so far little information demonstrating that endogenous HO acts as a modulator of plant growth and development. In this investigation, two complementary approaches were simultaneously carried out to address the functional significance of endogenous HO in GA-induced PCD. First, HO activity was inhibited by a specific competitive inhibitor, zinc protoporphyrin (ZnPPIX), which was pretreated 6 h prior to different treatments. In the second approach, the HO-1 inducer haematin or the catalytic product of HO, CO aqueous solution, was applied to induce HO activity or HO-1 expression. Compared with the control sample, it was found that GA-induced PCD, which took place significantly after 36 h of treatment in wheat aleurone layers, was preceded by a substantial decrease after a slight rise of HO activity at 12 h of incubation. On the other hand, significant up-regulation of HO by ABA alone, haematin, or CO aqueous solution plus GA treatment contributed to the delay of PCD, which was reversed by the addition of ZnPPIX, respectively. This suggests that HO *in vivo* plays a role in delaying PCD. The interrelationship between HO up-regulation and endogenous H_2O_2 was also studied. Finally, three PCD-blocking antioxidants, butylated hydroxytoluene (BHT), dithiothreitol (DTT), and ascorbic acid (AsA), could mimic the effect of haematin and CO on HO up-regulation, further confirming that endogenous HO may act as an antioxidant in the aleurone layers.

Materials and methods

Chemicals

All chemicals were obtained from Sigma unless stated otherwise. GA was used at 5 μM and 50 μM. ABA was used at 50 μM. Haematin (Ht) was used at concentrations of 0.1, 1.0, 10.0, and 100.0 μM. ZnPPIX, a specific inhibitor of HO-1, was used at 100 μM. 3-amino-1,2,4-triazole (3-AT) was used at 100 μM as an inhibitor of CAT. A 'suicide' inhibitor of APX *p*-aminophenol (*p*-AP) was used at 1 mM. N,N'-dimethylthiourea (DMTU, Fluka) was chosen as a scavenger of H_2O_2 at concentration of 5 mM (Zhang *et al.*, 2007). The fluorescent probes FDA and FM 4-64 were purchased from ICN Biomedicals Inc. and Invitrogen (Molecular Probes), respectively. Three well-known antioxidants BHT, DTT (Merck), and AsA, were used at 100 μM, 5 mM, and 5 mM, respectively.

CO aqueous solution treatment

The preparation of the CO aqueous solution was carried out according to the method described in our previous reports (Liu *et al.*, 2007; Han *et al.*, 2008; Xuan *et al.*, 2008). In this experiment, CO-saturated aqueous solution was freshly produced by bubbling CO gas gently through a glass tube into 50 ml of 5 mM $CaCl_2$ (Kuo *et al.*, 1996) for at least 20 min, a duration long enough to saturate the solution with CO. The saturated stock solution (100% saturation) was then diluted immediately with 5 mM $CaCl_2$ solution to the concentrations required [0.1, 1, and 10% (v/v)].

Preparation of wheat aleurone layers

Aleurone layers were prepared from de-embryonated wheat (*Triticum aestivum* 'Yangmai 13') grains as previously described (Kuo *et al.*, 1996; Mrva *et al.*, 2006). The de-embryonated half-grains were briefly surface-sterilized in 1% sodium hypochlorite solution for 20 min, and rinsed extensively with sterile water several times. The de-embryonated half-grains were then imbibed in sterile water at 25±1 °C for 48 h. Aleurone layers were isolated from the imbibed grain by removing the starchy endosperm under sterile conditions. Isolated aleurone layers were preincubated with water (–ZnPPIX) or with ZnPPIX (+ZnPPIX) at 100 μM for 6 h, and/or incubated directly in a medium containing 5 mM $CaCl_2$ and 5 or 50 μM GA or 50 μM ABA alone, or in the absence or presence of varying concentrations of haematin (Ht), different saturations of CO aqueous solution, 1 mM H_2O_2, 100 μM BHT, 5 mM DTT, 5 mM AsA, 100 μM 3-AT, 1 mM *p*-AP, 5 mM DMTU, or 10 μM each of $FeSO_4$ (Fe^{2+}) and BR for the indicated time. Layers incubated in 5 mM $CaCl_2$ alone were regarded as the control (Con).

O_2 consumption

O_2 consumption was measured using an O_2-sensitive electrode (oxy-lab, Hansatech, UK). Layers were put into a measuring chamber containing 3 ml of sterile distilled water at least 20 min prior to determining the rate of O_2 consumption. Ten aleurone layers were used for per treatment.

Determination of cell viability and death

The number of live and dead cells was determined by double staining with FDA (2 μg ml^{-1} in 5 mM $CaCl_2$) for 15 min, followed by FM 4-64 (20 μM in 5 mM $CaCl_2$) for 3 min (Fath *et al.*, 2001; Beligni *et al.*, 2002; Bethke *et al.*, 2004). Aleurone layers were observed with a fluorescent microscope (Axio Imger A1; Carl Zeiss, Germany) using a ×20 objective. Images of the fluorescent signal were captured using a digital camera. Randomly selected fields from at least four different aleurone layers per treatment were counted to determine the percentage of live cells.

Determination of enzymatic activity

Haem oxygenase (HO), catalase (CAT), and ascorbate peroxidase (APX) activities were analysed using the methods described in our previous reports (Liu *et al.*, 2007; Han *et al.*, 2008). The supernatant was desalted by Sephadex G-25 gel filtration to remove possible interfering materials, and used as the crude enzyme extract. For the HO activity assay, the concentration of BV was estimated using a molar absorption coefficient at 650 nm of 6.25 mM^{-1} cm^{-1} in 0.1 M HEPES-NaOH buffer (pH 7.2). One unit of activity (U) was calculated by taking the quantity of the enzyme to produce 1 nmol BV per 30 min. CAT activity was spectrophotometrically measured by monitoring the consumption of H_2O_2 (extinction coefficient 39.4 mM^{-1} cm^{-1}) at 240 nm. APX activity was determined by monitoring the decrease at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}). One unit (U) of CAT or APX was defined as the decomposition of 1 μmol H_2O_2 or AsA min^{-1}. α-Amylase activity in the aleurone incubation medium was measured using the previously reported starch–iodine procedure (Jones and Varner, 1967; Kuo *et al.*, 1996; Beligni *et al.*, 2002).

Western blotting analysis for HO-1

Rabbit polyclonal antibody was made against the mature wheat HO-1 with a molecular mass of 26 kDa. Homogenates obtained for the HO activity assays were also analysed by Western blotting. Fifty micrograms of protein from homogenates were subjected to SDS-PAGE using a 12.5% acrylamide resolving gel (Mini Protean II System, Bio-Rad, Hertz, UK). Separated proteins were then transferred to polyvinylidene difluoride (PVDF) membranes, and non-specific binding of antibodies was blocked with 5% non-fat dried milk in phosphate-buffered saline (PBS, pH 7.4) for 2 h at room temperature. Membranes were then incubated overnight at 4 °C, with primary antibodies diluted 1:200 in PBS buffer plus 1% non-fat dried milk. Immune complexes were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G. The colour was developed with a solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) as HRP substrate.

Semi-quantitative RT-PCR analysis

Aleurone layers (10) were harvested for RNA extraction. Total RNA was isolated by grinding with liquid nitrogen using a mortar and pestle until a fine powder appeared and by using Trizol reagent (Invitrogen) according to the instructions of the manufacturer. DNA-free total RNA (5 μg) from different treatments was used for first-strand cDNA synthesis in a 20 μl reaction volume containing 2.5 units of avian myeloblastosis virus reverse transcriptase XL (TaKaRa) and 2.5 μM random primer. PCR was performed using 2 μl of a 2-fold dilution of the cDNA, 10 pmol of each oligonucleotide primer, and 1 unit of *Taq* polymerase (TaKaRa) in a 25 μl reaction volume.

cDNA was amplified by PCR using the following primers: for *HO-1* (accession number HM014348), forward (5′-TCAATGTCCAGCAGTGTCGC-3′) and reverse (5′-GCTAAACGCTTGTGAGGTAG-3′), amplifying a 595 bp fragment; for *CAT* (accession no. X94352), forward (5′-ACCGGGAACGCATACCTGAACG-3′) and reverse (5′-CAACCCACCGCTGGAGGAAACG-3'), amplifying a 1191 bp fragment; for *APX* (accession no. EF555121), forward (5′-ACCGTTGAGTTCATCCCTG-3′) and reverse (5′-CCTTACTTGCTCCTCTTGG-3′), amplifying a 550 bp fragment; for *18S rRNA* (accession no. AJ272181), forward (5′-CAAGCCATCGCTCTGGATACATT-3′) and reverse (5′-CCTGTTATTGCCTCAAACTTCC-3′), amplifying a 658 bp fragment. To standardize the results, the relative abundance of *18S rRNA* was determined and used as an internal standard.

Cycle numbers of PCR reactions were adjusted for each gene to obtain visible bands in agarose gels. Aliquots of the PCR reactions were loaded on 1.2% agarose gels using ethidium bromide. Specific amplification products of the expected size were observed, and their identities were confirmed by sequencing.

H_2O_2 content determination

Aleurone layers (30) were used to determine the content of H_2O_2 according to the method described by De Michele *et al.* (2009) and Bellincampi *et al.* (2000), which was based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (Sigma). Standard curves were obtained by adding variable amounts of H_2O_2.

Statistical analysis

Where indicated, results are expressed as mean values ±SD of at least three independent experiments. Statistical analysis was performed using SPSS 8.0 software. For statistical analysis, *t* test (P <0.05 and P <0.01) or Duncan's multiple test (P <0.05) was chosen as appropriate.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers HM014348, X94352, EF555121, and AJ272181.

Results

GA-induced PCD in wheat aleurone layers was prevented by the HO-1 inducer haematin or CO aqueous solution

In accordance with previously reported results on barley aleurone layers (Bethke *et al.*, 1999; Fath *et al.*, 2001;

Beligni *et al.*, 2002), the time-course treatments for 48 h confirmed that compared with the control sample, PCD in the wheat aleurone layers was stimulated by GA, with a maximal response at 50 μM, which is different from the effective GA concentration (5 μM) reported in wheat layers by Kuo *et al.* (1996), and prevented by 50 μM ABA treatment, respectively (see Supplementary Fig. S1 at *JXB* online). As determined by simultaneously staining living and dead cells with the fluorescent probes fluorescein diacetate (FDA) and *N*-(3-triethylammoniumpropyl)-4-(6-[4-(diethylamino) phenyl] hexatrienyl) pyridinium dibromide (FM 4-64, Beligni *et al.*, 2002), approximately 1% and 7% of the cells died when wheat aleurone layers were incubated in 50 μM ABA and $CaCl_2$ solution alone (Con) after 48 h, compared with about 61% and 87% of the cells that died in 5 μM and 50 μM GA treatments, respectively. Results of oxygen consumption (see Supplementary Fig. S2 at *JXB* online) illustrated that relative O_2 consumption was increased by 10% after 48 h of incubation in the ABA-treated aleurone layer with respect to the control (Con) sample, whereas, 50 μM or 5 μM GA-treated samples decreased up to 81% and 42%, respectively. Therefore, 50 μM each of GA and ABA were chosen throughout the study.

The responses of HO activity to GA and ABA were also investigated. An induction of HO activity peaked significantly at 12 h with ABA treatment, followed by a gradual decrease. However, this maintained a higher level of HO activity compared with the control treatment during the 48 h period (Fig. 1), whereas GA treatment brought about a substantial decrease during 24–48 h of incubation (P <0.05 or 0.01, respectively) after an obvious rise of HO activity at 12 h. Meanwhile, GA-induced PCD took place significantly after 36 h of treatment in wheat aleurone layers (see Supplementary Fig. S1 at *JXB* online), suggesting that HO inhibition preceded PCD.

In subsequent experiments, GA-induced PCD was prevented differentially by the application of CO aqueous solution and the HO-1 inducer haematin in the wheat aleurone layers, with a maximal biological response at 1% saturation of CO aqueous solution and 10 μM haematin (P <0.05; Fig. 2A). Responses of relative oxygen consumption displayed similar tendencies (Fig. 2B).

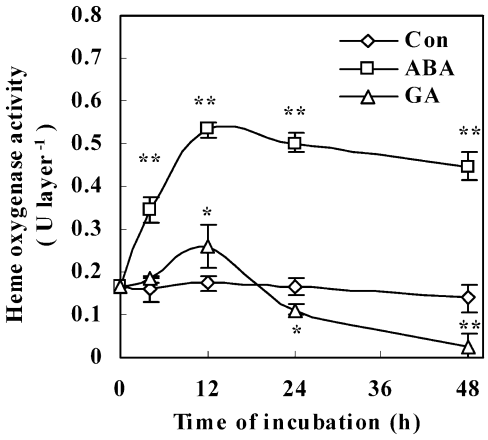

Fig. 1. Changes of haem oxygenase (HO) activity in wheat aleurone layers treated with 50 μM GA or ABA alone. Layers were incubated in a medium containing 5 mM $CaCl_2$ and 50 μM ABA or GA, respectively. Layers incubated in 5 mM $CaCl_2$ alone were regarded as the control (Con). HO activity was determined at the indicated time after various treatments. Data are means ±SD of at least three independent measurements from different experiments. Bars with asterisks are significantly different in comparison with corresponding Con samples at P <0.05 and P <0.01 (t test).

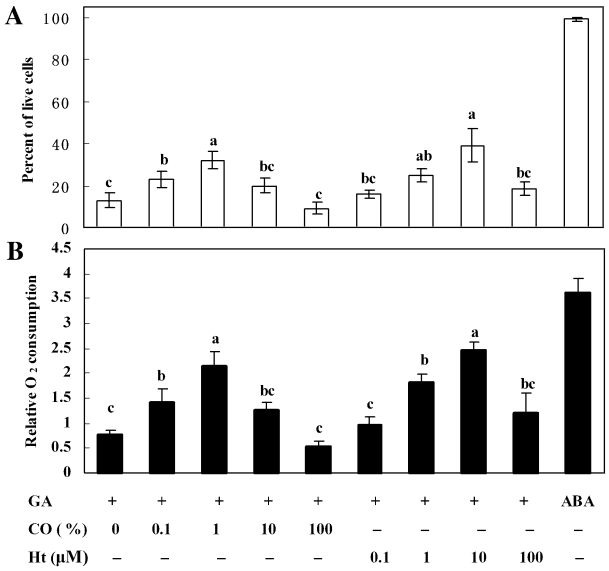

Fig. 2. Effects of CO aqueous solution and the HO-1 inducer haematin on the cell viability of GA-treated wheat aleurone layers. Layers were incubated in a medium containing 5 mM $CaCl_2$ and 50 μM GA alone or in the presence of CO aqueous solution (CO) or haematin (Ht) at the indicated saturations or concentrations. Quantification of viability and death for wheat aleurone layers at 48 h of incubation (A). The data are collected from at least four aleurone layers. Relative rates of O_2 consumption were measured at 48 h of incubation using an O_2 electrode (B). The rate of oxygen consumption was measured for ten layers in 3 ml of sterile water. For comparison, the percentage of live cells and relative O_2 consumption for ABA-treated (50 μM) layers are also shown. Bars denoted by the different letters are different significantly at P <0.05 according to the t test.

Up-regulation of HO caused by haematin and CO aqueous solution

The ameliorating effects of the HO-1 inducer haematin or the CO aqueous solution on GA-induced death of wheat aleurone cells described above prompted us to assess if induction of HO is associated with the above responses. In the following experiments, wheat aleurone layer cells were tested for HO accumulation, through semi-quantitative RT-PCR, enzymatic activity determination, and immunoblot analysis. Based on the results, GA treatment significantly decreased the level of HO-1 mRNA with respect to the ABA-treated sample, by contrast, haematin or CO plus GA reversed the effect of GA (Fig. 3A). HO activity decreased

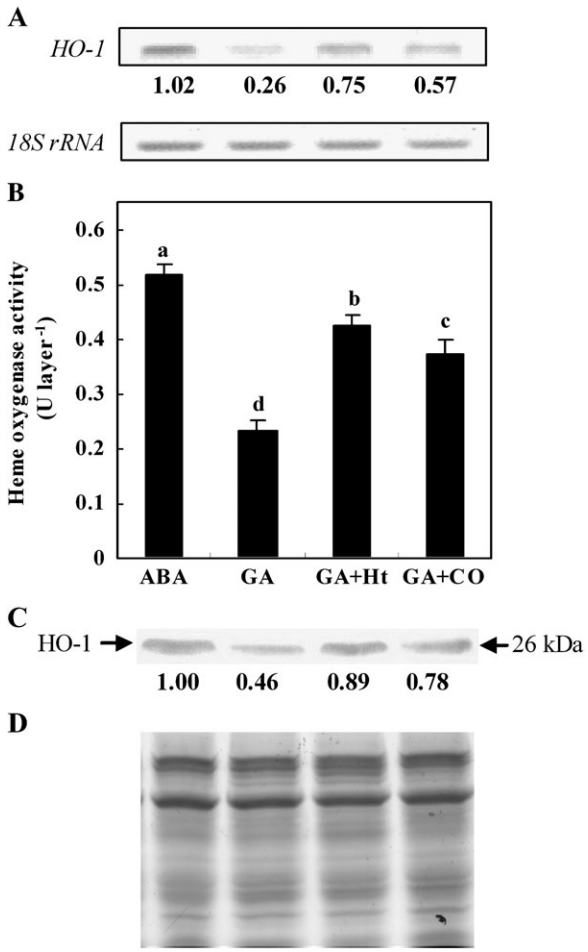

Fig. 3. *HO-1* transcript, HO activity, and HO-1 protein expression in wheat aleurone layers treated with ABA alone or with GA in the absence or presence of the HO-1 inducer haematin or CO aqueous solution. Layers were incubated in a medium containing 5 mM $CaCl_2$ and 50 μM GA or ABA alone, or GA plus 10 μM haematin (Ht) or 1.0% saturation of CO aqueous solution (CO). Wheat *HO-1* transcript was analysed by semi-quantitative RT-PCR after 12 h of various treatments (A), HO activity was also determined (B). Meanwhile, HO-1 protein expression was analysed by Western blotting (C), and Coomassie Brilliant Blue-stained gels are present to show that equal amounts of proteins were loaded (D). The number below the band (A) indicates the relative abundance of the corresponding gene with respect to the loading control 18S rRNA, and the number below the band (C) illustrates the relative abundance of the corresponding HO-1 protein compared with that of the ABA-treated sample. Bars denoted by the different letters (B) are different significantly at $P < 0.05$, according to Duncan's multiple test.

as much as 44.6% after 12 h of incubation in GA-treated aleurone layers compared with ABA-treated samples. An increase close to 83.9% was assessed in incubations carried out in the presence of GA plus haematin. Application of GA plus the CO aqueous solution brought about 62.1% higher HO activity compared with the sample that was treated with GA alone (Fig. 3B). Western blot analysis for HO-1 (Fig. 3C) showed only a single band with a molecular mass of 26 kDa as determined using molecular mass markers (data not shown). This mass is approximately consistent to a previously reported result for mature HO1 of pea and *Arabidopsis* plants (Muramoto *et al.*, 1999; Linley *et al.*, 2006). This assay also demonstrated a positive correlation among HO-1 transcript, enzyme activity, and HO-1 protein expression.

Decrease of HO activity contributes to PCD in wheat aleurone layers

ZnPPIX, the potent HO-1 inhibitor, has been found to inhibit HO activity in both animals and plants (Lamar *et al.*, 1996; Lang *et al.*, 2005; Xuan *et al.*, 2007, 2008). To investigate the physiological function of HO in plant response to PCD, the effects of ZnPPIX on the responses of ABA, GA with or without haematin, or CO aqueous solution were assessed. In this study, it was confirmed that ZnPPIX inhibited HO activity (Fig. 4A, B). Comparatively, pretreatment with 100 μM ZnPPIX brought about fewer inhibitory effects on HO activity in ABA-treated samples compared with GA-treated aleurone layers, which led to the complete inhibition of HO activity. In the following experiments, the addition of 100 μM ZnPPIX notably prevented any further inducible action of 10 μM haematin, or 1.0% saturation of CO aqueous solution plus GA treatment on increased HO activity after a 12 h treatment ($P < 0.05$; Fig. 4A). Meanwhile, incubation of GA-treated aleurone layers in haematin (GA+Ht) or CO (GA+CO) dramatically reduced the rate of PCD as measured with fluorescence microscopy, and the above aleurone cell death was aggravated by the application of ZnPPIX, respectively (Fig. 4C, D). For example, no cells died 42 h after incubation with ABA, compared with only 27% of cells that remained alive in aleurone layers incubated in GA alone. Meanwhile, 46% of cells in GA+Ht and 43% in GA+CO remained alive. After preincubation with ZnPPIX, about 7% and 90% of the cells died in ABA- or GA-treated samples, respectively, suggesting that endogenous HO plays a role in delaying PCD in wheat aleurone layers. Furthermore, cell death in the pretreatment of ZnPPIX brought about 63% and 67% death in GA+Ht and GA+CO samples, respectively. These values are higher than those found for corresponding samples without the addition of ZnPPIX, further confirming that the effect of haematin or CO on PCD is mediated specifically by the up-regulation of HO activity. The changes of PCD after 18 h of incubation exhibited a similar tendency (Fig. 4C).

Three PCD-blocking antioxidants mimic the effect of haematin and CO on HO up-regulation

BHT, DTT, and AsA are efficient antioxidants and reactive oxygen species scavengers. Previous work shows that BHT could slow down GA-induced PCD in barley aleurone layers (Beligni *et al.*, 2002). This observation has been extended in this report. When wheat aleurone layers were stained with fluorescent probes, the cytoprotective effects of BHT, DTT, and AsA became apparent (Fig. 5A, B; some

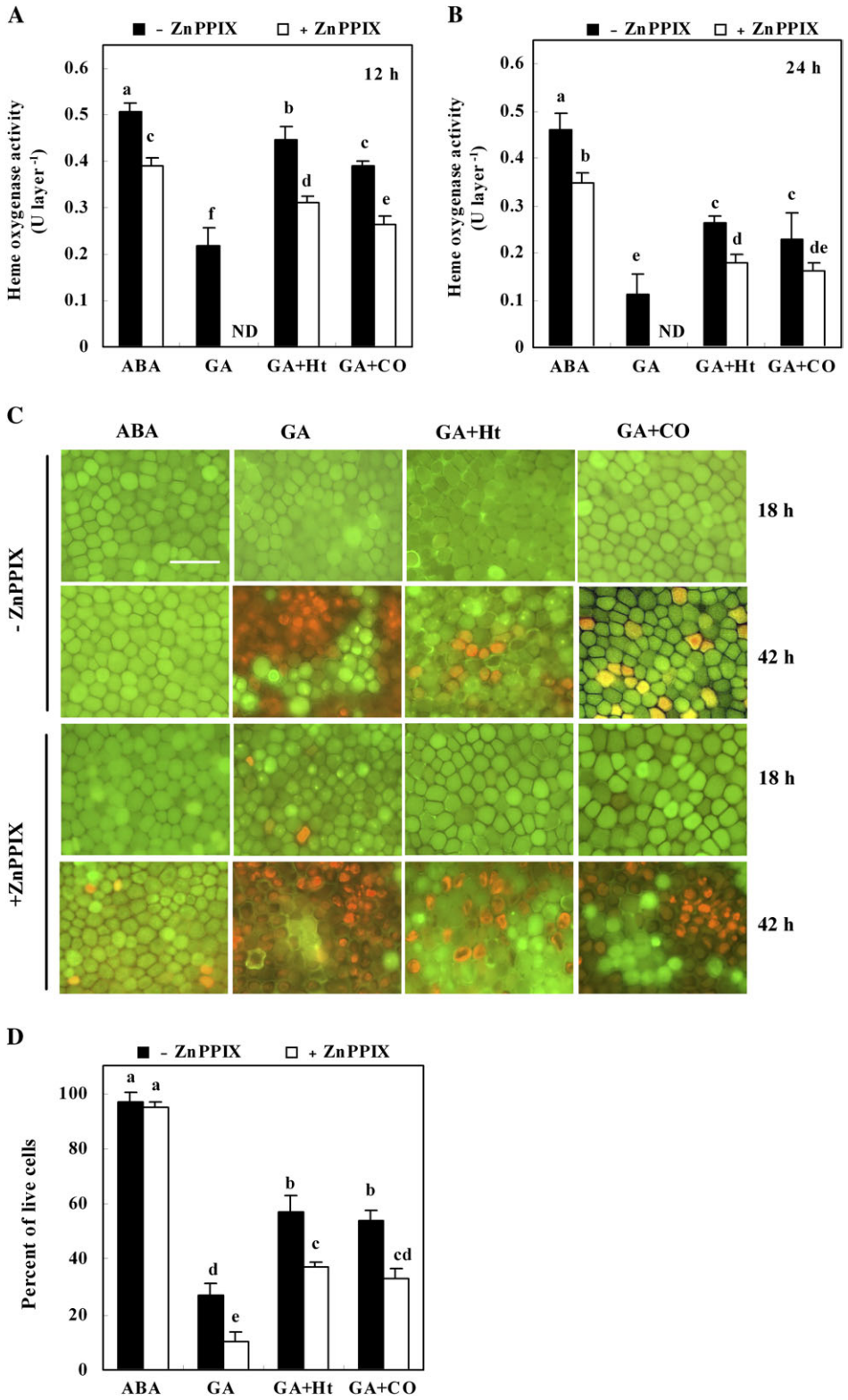

Fig. 4. PCD in wheat aleurone layers is related to the decrease in HO activity. Layers pretreated with water (–ZnPPIX) or 100 μM ZnPPIX (+ZnPPIX) for 6 h, were further incubated in a medium containing 5 mM $CaCl_2$ and 50 μM ABA or GA alone, or GA plus 10 μM haematin (Ht) or 1.0% saturation of CO aqueous solution for another 18 h or 42 h. HO activity was determined at 12 h (A), or 24 h (B) after various treatments. Meanwhile, digital images of fluorescently labelled wheat aleurone cells were further obtained (C). Death for wheat aleurone layers was also quantified from at least four aleurone layers (D). Scale bar, 200 μm. Data are means ±SD of at least four independent samples. Bars denoted by the different letters are significantly different at P <0.05, according to Duncan's multiple test. ND, none detected.

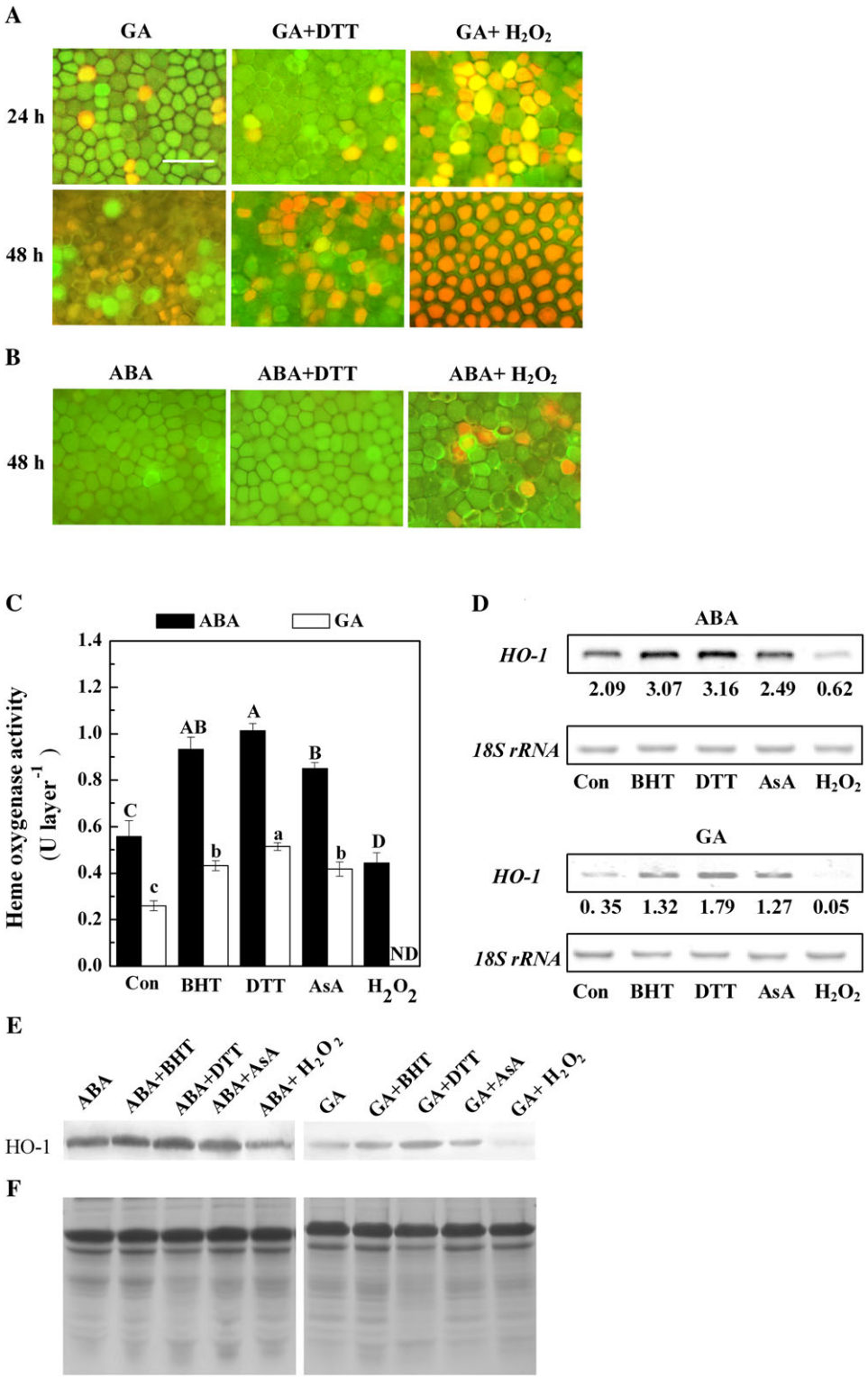

Fig. 5. Effects of DTT, BHT, AsA, and H_2O_2 on PCD, HO activity, HO-1 transcript, and HO-1 protein expression in aleurone layers. Layers were incubated in a medium containing 5 mM $CaCl_2$ and 50 μM GA, or 50 μM ABA alone or in the presence of 5 mM DTT, 100 μM BHT, 5 mM AsA, or 1 mM H_2O_2. Digital images of fluorescently labelled wheat aleurone cells were obtained at the indicated time after various treatments (A, B). HO activity was determined after 12 h of different treatments (C). Meanwhile, *HO-1* transcript was analysed by semi-quantitative RT-PCR (D). The number below the band indicates the relative abundance of the corresponding gene with respect to the loading control 18S rRNA. HO-1 protein expression was analysed by Western blotting (E), and Coomassie Brilliant Blue-stained gels are present to show that equal amounts of proteins were loaded (F). Scale bar=200 μm. Data are means ±SD of at least four independent samples. Bars denoted by the different letters are different significantly at P <0.05, according to Duncan's multiple test.

data not shown). For instance, after 48 h in GA treatment, 87% of cells died, and more than 47%, 41%, and 33% remained alive in aleurone layers incubated with GA plus DTT, BHT, and AsA, respectively. However, no significant difference of cell death was discovered in the ABA-treated samples with or without DTT.

To investigate whether HO is associated with the response of these antioxidants leading to a delay of PCD, detailed studies were conducted on HO activity (Fig. 5C), *HO-1* transcript (Fig. 5D), and the expression of HO-1 protein (Fig. 5E, F). Results showed that the level of HO activity was increased by 81.8% after 12 h of GA plus DTT treatment with respect to control values (GA-treated alone sample, Con). When BHT or AsA was added to GA, these resulted in 67.2% and 58.1% enhancement, respectively. Meanwhile, ABA in the presence of the above antioxidants respectively led to similar inducible tendencies toward the changes of HO enzyme activity. *HO-1* transcript and its protein expression (Fig. 5D–F) were approximately modulated in parallel with the levels of HO activity and PCD. Together, these findings provide preliminary evidence suggesting that the antioxidants BHT, DTT, and AsA can mimic the effects of haematin and CO aqueous solution on the alleviation of PCD and up-regulation of HO expression.

Regulation of endogenous H_2O_2 modulates PCD and HO expression in wheat aleurone layers

ROS are linked to the promotion of PCD in barley aleurone cells (Bethke and Jones, 2001; Fath *et al.*, 2001). The increasing sensitivity of GA-treated barley aleurone cells to H_2O_2 (Bethke *et al.*, 2002; Palma and Kermode, 2003) suggests the existence of a possible interrelationship between H_2O_2 and HO expression during GA-induced PCD process. To test this hypothesis, ABA- and GA-treated aleurone cells were exposed to 1 mM H_2O_2, after which the cell viability and HO expression was measured (Fig. 5). In our test, GA or ABA treatment led to the sensitization of wheat aleurone layers to 1 mM H_2O_2 treatment for 48 h with 100% and 13% dead cells, respectively (Fig. 5A). In comparison, about 87% and 1% of the cells died in samples that were treated with just GA or ABA, respectively. Biochemical tests also showed that the above-mentioned H_2O_2 treatment brought about significantly decreased HO activity, *HO-1* transcript, and HO-1 protein expression in both GA or ABA treatments (P <0.05).

The potential roles of H_2O_2 in GA-induced PCD were investigated using CAT and APX inhibitors. Treatment of aleurone layers with the CAT inhibitor 3-amino-1,2,4-triazole (3-AT) at 100 μM, a concentration deemed effective (Fig. 6B; Havir, 1992), had a significant inducible effect on GA-induced PCD (Fig. 6). A 'suicide' inhibitor of APX, *p*-aminophenol (*p*-AP, 1 mM; Chen and Asada, 1990), displayed slightly pronounced effects. GA-induced PCD and H_2O_2 overproduction were substantially reversed by the scavenger of H_2O_2 N,N'-dimethylthiourea (DMTU, 5 mM). Further, analogous effects were observed when the above-mentioned chemicals were combined with ABA. These

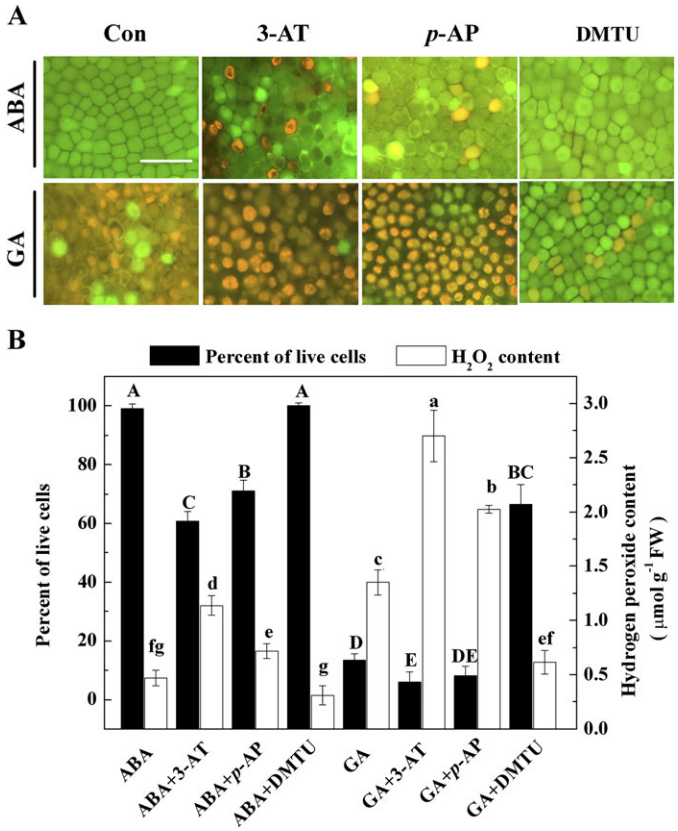

Fig. 6. Effects of an inhibitor of CAT 3-amino-1,2,4-triazole (3-AT), a 'suicide' inhibitor of APX *p*-aminophenol (*p*-AP), and the scavenger of hydrogen peroxide (H_2O_2) N,N'-dimethylthiourea (DMTU) on GA-induced PCD in aleurone layers and H_2O_2 production. Layers were incubated in a medium containing 5 mM $CaCl_2$ and 50 μM ABA or GA alone, or in the presence of 100 μM 3-AT, 1 mM *p*-AP, or 5 mM DMTU. Digital images of fluorescently labelled wheat aleurone cells were obtained (A). Live and dead cells were also quantified (B) at 48 h after various treatments. Meanwhile, H_2O_2 content (B) was determined. Scale bar=200 μm. Data are means ±SD of at least four independent samples. Bars denoted by the different letters are significantly different at P <0.05, according to Duncan's multiple test.

results also confirmed that changes of endogenous H_2O_2 are likely to be involved in GA-induced PCD in wheat aleurone layers, and that both CAT- and APX-mediated H_2O_2 decomposition play important roles in this process.

GA-induced down-regulation of APX and CAT expression, and H_2O_2 overproduction were reversed by haematin and CO

A previous study has reported that GA-treated aleurone cells lose their ability to scavenge ROS, and that this loss ultimately results in oxidative damage and PCD (Fath *et al.*, 2001). The following experiment investigated the response of APX and CAT enzyme activities, corresponding transcripts, and H_2O_2 production. GA treatment led to a declining trend in the activity or mRNA expression of APX and CAT during the 48 h incubation period; by

comparison, ABA brought about an increased tendency (Fig. 7A, B). Comparatively, preincubation with ZnPPIX produced a decrease in the two enzyme activities compared with the values obtained when only ABA or GA was applied (Table 1; Fig. 7A, B). Further results showed that the haematin or CO aqueous solution blocked the decrease of APX and CAT activities induced by GA, resulting in the decrease of H_2O_2 production, all of which were reversed differentially by pretreatment with ZnPPIX (P <0.05; Table 1; Figs 7A, B, 8D).

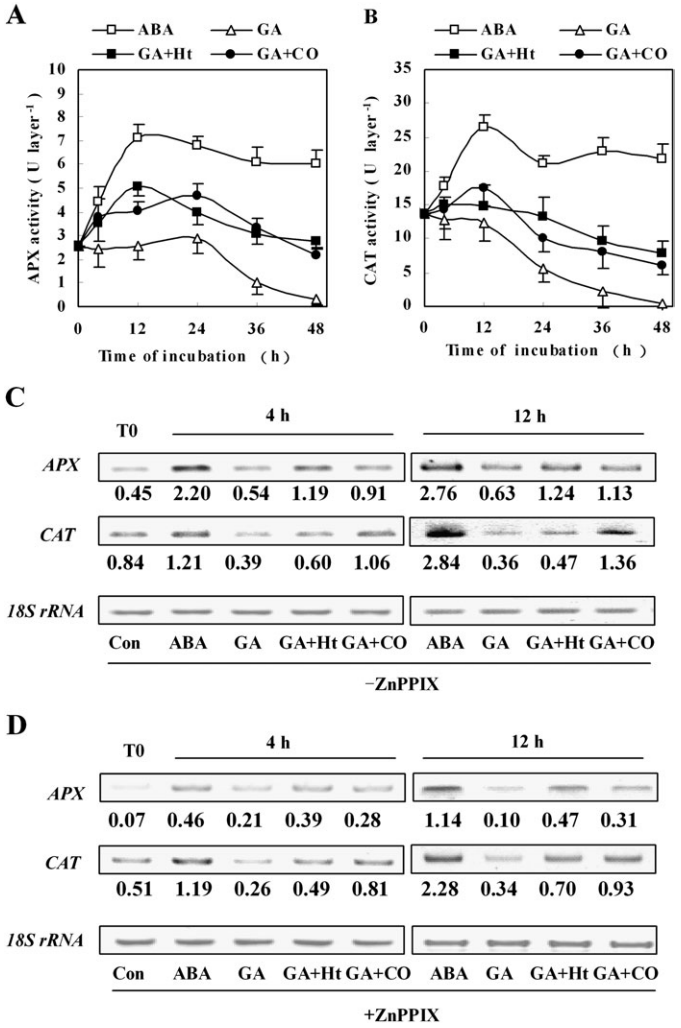

Fig. 7. Effects of ABA, GA, haematin, and CO aqueous solution on the activities or expression of ascorbate peroxidase (APX) and catalase (CAT) in wheat aleurone layers. Layers pretreated with water (–ZnPPIX) or with 100 μM ZnPPIX (+ZnPPIX, only D) for 6 h, were further incubated in a medium containing 5 mM $CaCl_2$ and 50 μM ABA or GA alone, or GA plus 10 μM haematin (Ht), or 1.0% saturation of CO aqueous solution for another 48 h. Layers incubated in 5 mM $CaCl_2$ alone were regarded as the control (Con). APX (A) and CAT activities (B) were determined at the indicated time. The corresponding mRNA expression was analysed by semi-quantitative RT-PCR (C, D). The number below the band indicates the relative abundance of the corresponding gene with respect to the loading control 18S rRNA. Data are means ±SD of at least four independent samples.

Results from semi-quantitative RT-PCR (Fig. 7C, D) supported the observation mentioned above. For example, when haematin or CO aqueous solution was added, the amount of the *APX* and *CAT* transcripts in GA-treated samples remained at a higher level through 12 h of incubation, which could be blocked differentially by pretreatment of ZnPPIX. by contrast, ABA resulted in an increase in *APX* and *CAT* abundance (Fig. 7C; Fath *et al.*, 2001), and prevented cell death significantly (Fig. 4). These results confirm the positive correlation between enzyme activity and gene expression (Table 1; Fig. 7A, B).

Effects of BR and Fe^{2+} on APX and CAT activities, aleurone cell viability, and H_2O_2 production

A previous result has shown that oxidative stress provoked by cadmium (Cd) could be partially prevented by the addition of BV (Noriega *et al.*, 2004). In this test, the behaviour of the CO aqueous solution as an inducer for the up-regulation of antioxidant enzymes prompted the investigation of whether BR and Fe^{2+}, the other end-products of HO activity, could perform similar effects. To explore this possibility, Fe^{2+} or BR was added together with GA, after which it was found that the two chemicals displayed different effects on APX and CAT activities, aleurone cell viability, and H_2O_2 production induced by GA (Fig. 8). For example, activities of APX in aleurone cells differentially increased after 12 h and 48 h of GA plus 10 μM BR treatment, being 55.4% and 822.1% higher than those of BR-free control samples (GA-treated alone). CAT activities were also increased by 31.3% and 681.4%, respectively. Meanwhile, the rate of PCD significantly decreased (P <0.05; Fig. 8C), and the H_2O_2 level declined because H_2O_2 content in wheat aleurone layers were reduced by 49.1% and 36.0% with respect to corresponding samples that were treated with GA alone (Fig. 8D). Reversed responses of APX and CAT activities, and H_2O_2 production were observed when ZnPPIX was pretreated (Table 1). By contrast, the samples treated with Fe^{2+} exhibited weaker or negative effects.

Changes of α-amylase activity

To evaluate if delayed or aggravated PCD could result from a global inhibition or induction of GA-induced responses, the time-course of α-amylase secretion was investigated in wheat aleurone layers upon various treatments. As expected, GA stimulated the secretion of α-amylase, while ABA inhibited it (see Supplementary Fig. S3 at *JXB* online). When haematin, CO aqueous solution, Fe^{2+}, and DMTU were incubated with GA during the 48 h period, the total α-amylase activities were not less than that from layers treated with GA alone, while BR, 3-AT, and *p*-AP, respectively, brought about equal amounts or a slight decrease of α-amylase activity. Therefore, these results suggest that corresponding pharmacological effects of the above-mentioned chemicals (Figs 2, 4, 6, 8) appear to be primarily on PCD.

Table 1. Changes of ascorbate peroxidase (APX), catalase (CAT) activities, and hydrogen peroxide (H_2O_2) content

Layers pretreated with 100 μM ZnPPIX for 6 h were further incubated in a medium containing 5 mM $CaCl_2$ and 50 μM ABA or GA alone, or GA plus 10 μM haematin (Ht), 1.0% saturation of CO aqueous solution, 10 μM each of $FeSO_4$ (Fe^{2+}) or BR for another 12 h and 48 h. Data are means ±SD of at least four independent samples. Different letters within columns are significantly different at P <0.05, according to Duncan's multiple test.

Treatment	APX (U layer^{-1})		CAT (U layer^{-1})		H_2O_2 (μmol g^{-1} FW)	
ZnPPIX→	12 h	48 h	12 h	48 h	12 h	48 h
ABA	5.60±0.61 a	4.80±0.91 a	19.1±1.50 a	7.88±0.83 a	0.54±0.16 d	0.68±0.04 d
GA	2.02±0.18 c	0.16±0.02 c	10.2±0.85 c	0.60±0.13 d	0.93±0.13 a	1.57±0.15 a
GA+Ht	4.17±0.32 b	1.69±0.44 b	12.6±1.54 b	3.95±0.34 b	0.59±0.24 cd	0.94±0.06 c
GA+CO	3.18±1.43 bc	1.44±0.24 b	13.9±1.07 b	2.67±0.78 c	0.75±0.05 bc	1.23±0.07 b
GA+Fe^{2+}	3.35±0.57 bc	1.11±0.33 bc	2.56±0.44d	0.49±0.12 d	0.84±0.24 ab	1.43±0.06 a
GA+BR	4.01±0.46 b	1.46±0.28 b	13.1±0.57 b	3.44±0.45 bc	0.66±0.05 cd	0.97±0.07 c

Discussion

Up-regulation of HO contributes to the delay of PCD in aleurone layers

The present study provides evidence, for the first time in plants, that up-regulation of HO contributes to the delay of PCD in wheat aleurone layers. First, HO inhibition preceded PCD in GA-treated aleurone layers (Fig. 1; see Supplementary Fig. S1 at *JXB* online), and there was a strong correlation between the modulation of the amounts of HO-1 protein, *HO-1* transcript, or HO activity, and the corresponding responses of PCD. For example, the activities of HO, *HO-1* transcript, and HO-1 protein expression significantly decreased in GA-treated aleurone layers compared with those of ABA-treated samples (Fig. 3), indicating a possible interrelationship between HO expression and PCD in aleurone layers. When ZnPPIX, a specific inhibitor of HO-1, proven in wheat aleurone layers (Fig. 4A, B), was preincubated with cells that were later incubated in GA, PCD became accelerated (Fig. 4C). These results suggest that endogenous HO might play a role in delaying PCD.

In human monocytes, HO-1 mRNA is induced by hemin in a dose- and time-dependent manner, as measured by semi-quantitative RT-PCR and flow cytometry (Lang *et al.*, 2005). Treatment with exogenous CO stimulates the expression of manganese SOD and HO in endothelial cells (Thom *et al.*, 2000; Nakao *et al.*, 2008). In our experiments, the exogenous HO-1 inducer, haematin, or the CO aqueous solution, both of which brought about a noticeable induction of HO expression (Fig. 3), ameliorated GA-induced PCD, with a maximal biological response at 10 μM haematin and 1% saturation of CO aqueous solution during the 48 h period of incubation (Fig. 2). In addition, the effects of haematin and CO on cell viability and HO expression can be mimicked by the antioxidants BHT, DTT, and AsA (Fig. 5), further confirming that up-regulation of HO can act as a potent antioxidative enzyme responsible for the protection of plant cells from oxidative stress. A similar finding has previously been reported by Beligni *et al.* (2002), in which they have observed that BHT could imitate the effect of NO donors and slow down GA-induced PCD in barley aleurone layers. The results mentioned above also indicate that maintenance of higher level HO expression in aleurone layers is critical in the delay

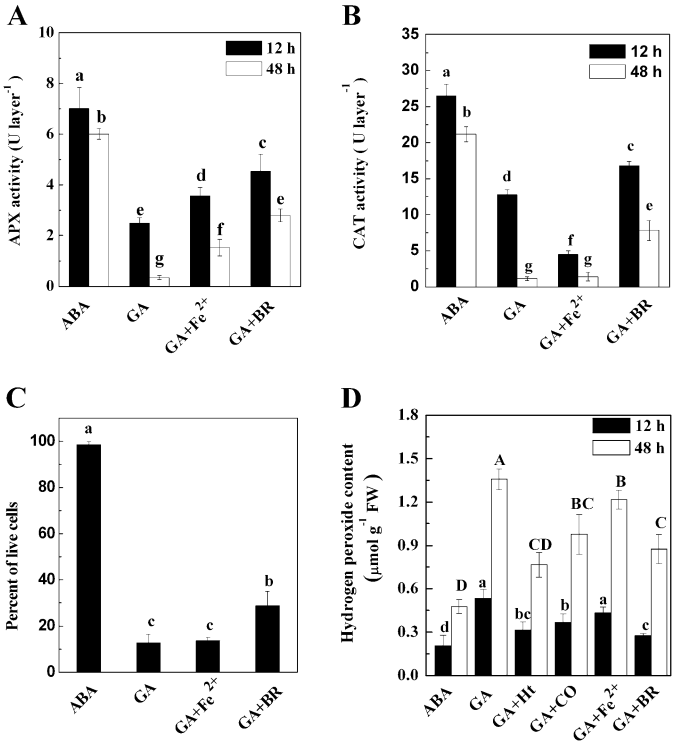

Fig. 8. Effects of ABA, GA, haematin, CO aqueous solution, Fe^{2+}, and bilirubin (BR) on the activities of ascorbate peroxidase (APX), catalase (CAT), PCD, or hydrogen peroxide (H_2O_2) content in wheat aleurone layers. Layers were incubated in a medium containing 5 mM $CaCl_2$ and 50 μM ABA or GA alone, or GA plus 10 μM haematin (Ht) or 1.0% saturation of CO aqueous solution, 10 μM each of $FeSO_4$ (Fe^{2+}) or BR for 48 h. APX (A), CAT activities (B), and H_2O_2 content (D) were determined at the indicated time. Epifluorescence images of live and dead cells at 48 h were quantified as shown in C. Data are means ±SD of at least four independent samples. Bars denoted by the different letters are significantly different at P <0.05 according to Duncan's multiple test.

of PCD, and that HO down-regulation is closely associated with the subsequent PCD. The observations that treatment with GA and 100 μM ZnPPIX can fully inhibit HO activity, speed up PCD in aleurone layer cells, and that these effects were reversed by haematin or CO aqueous solution (Fig. 4), are in line with this proposition.

Cytoprotective roles of HO in animals and plants have previously been reported (Dulak and Józkowicz, 2003; Lang *et al.*, 2005; Han *et al.*, 2008). Our data illustrate that up-regulation of HO can delay GA-mediated PCD in wheat aleurone layers. This could be ascribed to the capability of HO to act as a novel antioxidative enzyme, which has previously been proven in plants (Noriega *et al.*, 2004; Yannarelli *et al.*, 2006; Han *et al.*, 2008). The above protective effects of HO might also be derived from the products of its enzyme catalytic actions. For example, the generation of CO has been found to display antiapoptotic and anti-inflammatory effects (Otterbein *et al.*, 1999; Zhang *et al.*, 2003), and CO applied exogenously mitigates salinity-induced PCD in wheat primary roots (Ling *et al.*, 2009). In addition, BV and BR have antioxidant and cytoprotective effects that may enhance the effects of HO-1 in animals and plants (Stocker *et al.*, 1987; Noriega *et al.*, 2004; Matsumoto *et al.*, 2006). A similar phenomenon was also observed when BR was added with GA in wheat aleurone layers, whereas Fe^{2+} did not induce such effects (Fig. 8). It has been shown that, although BV is converted to BR by biliverdin reductase, BR can be recycled to BV, providing a powerful redox cycle in animals (Baranano *et al.*, 2002). Thus, whether BV is also involved in the above cytoprotective effects, requires further elucidation.

In animals, the cellular content of haem, derived either from the delivery of filtered haem proteins such as haemoglobin and myoglobin, or from the breakdown of ubiquitous intracellular haem proteins, is regulated via the HO enzyme system, thereby exhibiting various physiological roles. In a previous study, induction of endogenous HO-1 protein with the infusion of haemoglobin increased survival in a rat model of lipopolysaccharide-induced inflammatory lung injury (Otterbein *et al.*, 1995). Among haem proteins, haemoglobin exists widely in organisms ranging from prokaryotes to eukaryotes, and the presence of haemoglobin in aleurone layers has also been discovered. Previous results have shown that CO can strongly enhance haemoglobin gene expression in barley aleurone layers (Nie and Hill, 1997), and that Ca^{2+} plays a major role in the signal transduction pathway leading to haemoglobin synthesis (Nie *et al.*, 2006). Non-symbiotic haemoglobin AHb1 in *Arabidopsis thaliana* has been confirmed to scavenge NO and reduce NO emission under hypoxic stress, indicating its role in NO detoxification in plants (Dordas *et al.*, 2003; Perazzolli *et al.*, 2004). Given that NO may be an endogenous modulator of the delay of PCD in barley aleurone cells (Beligni *et al.*, 2002), it is speculated that the up-regulation of HO expression might not only compensate for the lower level of NO due to the induction of haemoglobin expression, but also contribute to the delay of PCD in wheat aleureone layers.

The decreased H_2O_2 production by haematin or CO is involved in delay of GA-induced PCD

ROS have been linked to the death of cells in many mammals (Jabs, 1999), and have also been implicated in the death of plant cells in response to a variety of stimuli, including pathogen invasion and some environmental stresses. Fath *et al.* (2001) showed that ABA maintains high CAT, APX, and SOD expressions to scavenge ROS, and then prevents PCD in barley aleurone layers, whereas GA brings about rapid reduction in these antioxidant enzyme activities and promotes cell death. Therefore, it was investigated whether up-regulation of HO leading to a delay of PCD in aleurone layers is related to the modulation of ROS production or metabolism. Compared with that of the ABA treatment in wheat aleurone layers, the overproduction of H_2O_2 synthesis in response to GA was demonstrated (Table 1; Fig. 6B). These processes are correlated to the biological response of GA-stimulated PCD or ABA-prevented PCD. The pharmacology approach also illustrated that scavenging or the accumulation of H_2O_2 delayed or accelerated GA-induced PCD (Fig. 6). Meanwhile, the incubation of wheat aleurone layers in H_2O_2-containing media (1 mM) resulted in the total death of GA-treated but not ABA-treated aleurone cells (Fig. 5A, B). This outcome is consistent with the results in barley aleurone layers reported by Bethke and Jones (2001), in which GA-treated aleurone protoplasts are less able than ABA-treated protoplasts to tolerate internally generated or exogenously applied H_2O_2. On the other hand, as previously reported (Yannarelli *et al.*, 2006), after the addition of H_2O_2, the decreased HO expression became more noticeable in GA-treated aleurone layers than in ABA-treated samples (Fig. 5C, D). These results are consistent with the responses of cell death, suggesting that interaction between H_2O_2 and HO is implicated in GA-induced PCD (Fig. 5C; Bethke and Jones, 2001; Fath *et al.*, 2001).

Using the HO-1 knockout mice and inhibitor test, Matsumoto *et al.* (2006) have confirmed that CO and BV produced from HO-1 suppresses ROS production generated from NADPH oxidase. Meanwhile, CO can suppress the catalytic activity of cytochrome P450 monooxygenases that are responsible for the endogenous generation of ROS (Kyokane *et al.*, 2001). In our subsequent experiment, results revealed that, in wheat aleurone layers, application of exogenous haematin and CO aqueous solution not only induced HO expression (Figs 3, 4), but also slowed down the GA-induced decrease of CAT and APX activities or their transcripts in a time-dependent manner (Table 1; Fig. 7), thus leading to the significant decrease in H_2O_2 production (Fig. 8D). These findings are consistent with those reported in plants (Xie *et al.*, 2008) and animals by Turkseven *et al.* (2005), in which they found that the vascular cytoprotective mechanism of HO-1 against oxidative stress requires an increase in extracellular SOD and CAT expression, thus leading to an improvement in vascular relaxation in diabetic rats. Meanwhile, the delay of PCD was also observed (Fig. 4C). Overall, an increased

ability to scavenge H_2O_2 may contribute to the delay of GA-induced PCD in wheat aleurone layer cells treated with haematin or CO aqueous solution by reducing the degree of oxidative stress, because ROS, such as H_2O_2 and superoxide anion, play a central role in promoting PCD (Bethke and Jones, 2001; Ling *et al.*, 2009), and ROS are likely targets of BHT, NO, haematin, CO, and BR in plant and animal cells (Fig. 8D; Beligni *et al.*, 2002). Our results also suggested that BR rather than Fe^{2+}, can significantly suppress the increased H_2O_2 generation in GA-treated wheat aleurone layers by enhancing APX and CAT activities at both 12 h and 48 h of incubation (Fig. 8).

In conclusion, this work expanded our previous investigations concerning the physiological roles of the HO/CO system in plants, and illustrated that the delay of PCD was induced by the up-regulation of HO expression. This result also strongly indicates that the antioxidative role of HO involves an alteration of H_2O_2 metabolism. However, further genetic evidence is required unequivocally to elucidate corresponding mechanisms or signal transduction pathways.

Supplementary data

The following supplementary data are available at *JXB* online.

Supplementary Fig. S1. Time-course of PCD in wheat aleurone layers determined by staining with FDA and FM 4-64.

Supplementary Fig. S2. The rate of oxygen consumption by wheat aleurone layers treated with ABA and GA.

Supplementary Fig. S3. Effects of ABA, GA, haematin, CO aqueous solution, BR, Fe^{2+}, DMTU, 3-AT, and *p*-AP on the activities of α-amylase in wheat aleurone layers.

Acknowledgements

This work was supported by the Program for New Century Excellent Talents in University (grant no. NCET-07-0441), and the Fundamental Research Funds for the Central Universities (grant nos KYZ200905 and KYJ200912). We also thank Dr Evan Evans from the University of Tasmania, Australia, for his kind help in writing the manuscript.

References

Balestrasse KB, Noriega GO, Batlle A, Tomaro ML. 2005. Involvement of heme oxygenase as antioxidant defense in soybean nodules. *Free Radical Research* **39,** 145–151.

Balestrasse KB, Noriega GO, Batlle A, Tomaro ML. 2006. Haem oxygenase activity and oxidative stress signaling in soybean leaves. *Plant Science* **170,** 339–346.

Balestrasse KB, Yannarelli GG, Noriega GO, Batlle A, Tomaro ML. 2008. Heme oxygenase and catalase gene expression in nodules and roots of soybean plants subjected to cadmium stress. *Biometals* **21,** 433–441.

Baranano DE, Rao M, Ferris CD, Snyder SH. 2002. From the cover: biliverdin reductase: a major physiologic cytoprotectant. *Proceedings of the National Academy of Sciences, USA* **99,** 16093–16098.

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL. 2002. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiology* **129,** 1642–1650.

Bellincampi D, Dipierro N, Salvi G, Cervone F, De Lorenzo G. 2000. Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxin-regulated rolB gene expression in tobacco leaf explants. *Plant Physiology* **122,** 1379–1385.

Bethke PC, Badger MR, Jones RL. 2004. Apoplastic synthesis of nitric oxide by plant tissues. *The Plant Cell* **16,** 332–341.

Bethke PC, Fath A, Spiegel YN, Hwang Y, Jones RL. 2002. Abscisic acid, gibberellin and cell viability in cereal aleurone. *Euphytica* **126,** 3–11.

Bethke PC, Jones RL. 2001. Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. *The Plant Journal* **25,** 19–29.

Bethke PC, Lonsdale JE, Fath A, Jones RL. 1999. Hormonally regulated programmed cell death in barley aleurone cells. *The Plant Cell* **11,** 1033–1045.

Chen GX, Asada K. 1990. Hydroxyurea and *p*-aminophenol are the suicide inhibitors of ascorbate peroxidase. *Journal of Biological Chemistry* **265,** 2775–2781.

Chen XY, Ding X, Xu S, Wang R, Xuan W, Cao ZY, Chen J, Wu HH, Ye MB, Shen WB. 2009. Endogenous hydrogen peroxide plays a positive role in the upregulation of heme oxygenase and acclimation to oxidative stress in wheat seedling leaves. *Journal of Integrative Plant Biology* **51,** 951–960.

De Michele R, Vurro E, Rigo C, Costa A, Elviri L, Di Valentin M, Careri M, Zottini M, Sanità di Toppi L, Lo Schiavo F. 2009. Nitric oxide is involved in cadmium-induced programmed cell death in Arabidopsis suspension cultures. *Plant Physiology* **150,** 217–228.

de Pinto MC, Paradiso A, Leonetti P, De Gara L. 2006. Hydrogen peroxide, nitric oxide and cytosolic ascorbate peroxidase at the crossroad between defence and cell death. *The Plant Journal* **48,** 784–795.

Dordas C, Rivoal J, Hill RD. 2003. Plant haemoglobins, nitric oxide and hypoxic stress. *Annals of Botany* **91,** 173–178.

Dulak J, Józkowicz A. 2003. Carbon monoxide: a 'new' gaseous modulator of gene expression. *Acta Biochimica Polonica* **50,** 31–47.

Fath A, Bethke P, Beligni V, Jones R. 2002. Active oxygen and cell death in cereal aleurone cells. *Journal of Experimental Botany* **53,** 1273–1282.

Fath A, Bethke PC, Jones RL. 2001. Enzymes that scavenge reactive oxygen species are down-regulated prior to gibberellic acid-induced programmed cell death in barley aleurone. *Plant Physiology* **126,** 156–166.

Fath A, Bethke P, Lonsdale J, Meza-Romero R, Jones R. 2000. Programmed cell death in cereal aleurone. *Plant Molecular Biology* **44,** 255–266.

Gisk B, Yasui Y, Kohchi T, Frankenberg-Dinkel N. 2010. Characterization of the haem oxygenase protein family in *Arabidopsis*

thaliana reveals a diversity of functions. *Biochemical Journal* **425,** 425–434.

Han Y, Zhang J, Chen XY, Gao ZZ, Xuan W, Xu S, Ding X, She WB. 2008. Carbon monoxide alleviates cadmium-induced oxidative damage by modulating glutathione metabolism in the roots of Medicago sativa. *New Phytologist* **177,** 155–166.

Havir EA. 1992. The *in vivo* and *in vitro* inhibition of catalase from leaves of *Nicotiana sylvestris* by 3-amino-1,2,4-triazole. *Plant Physiology* **99,** 533–537.

Jabs T. 1999. Reactive oxygen intermediates as mediators of programmed cell death in plants and animals. *Biochemical Pharmacology* **57,** 231–245.

Jones RL, Varner JE. 1967. The bioassay of gibberellins. *Planta* **72,** 53–59.

Keyse SM, Tyrrell RM. 1989. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proceedings of the National Academy of Sciences, USA* **86,** 99–103.

Kuo A, Cappelluti S, Cervantes-Cervantes M, Rodriguez M, Bush DS. 1996. Okadaic acid, a protein phosphatase inhibitor, blocks calcium changes, gene expression, and cell death induced by gibberellin in wheat aleurone cells. *The Plant Cell* **8,** 259–269.

Kyokane T, Norimizu S, Taniai H, Takeoka S, Tsuchida E, Naito M, Nimura Y, Ishimura Y, Suematsu M. 2001. Carbon monoxide from heme catabolism protects against hepatobiliary dysfunction in endotoxin-treated rat liver. *Gastroenterology* **120,** 1227–1240.

Lamar CA, Mahesh VB, Brann DW. 1996. Regulation of gonadotrophin-releasing hormone (GnRH) secretion by heme molecules: a regulatory role for carbon monoxide? *Endocrinology* **137,** 790–793.

Lamotte O, Courtois C, Barnavon L, Pugin A, Wendehenne D. 2005. Nitric oxide in plants: the biosynthesis and cell signaling properties of a fascinating molecule. *Planta* **221,** 1–4.

Lang D, Reuter S, Buzescu T, August C, Heidenreich S. 2005. Heme-induced heme oxygenase-1 (HO-1) in human monocytes inhibits apoptosis despite caspase-3 up-regulation. *International Immunology* **17,** 155–165.

Ling T, Zhang B, Cui W, Wu M, Lin J, Zhou W, Huang J, Shen WB. 2009. Carbon monoxide mitigates salt-induced inhibition of root growth and suppresses programmed cell death in wheat primary roots by inhibiting superoxide anion overproduction. *Plant Science* **177,** 331–340.

Linley PJ, Landsberger M, Kohchi T, Cooper JB, Terry MJ. 2006. The molecular basis of haem oxygenase deficiency in the *pcd1* mutant of pea. *FEBS Journal* **273,** 2594–2606.

Liu K, Xu S, Xuan W, *et al*. 2007. Carbon monoxide counteracts the inhibition of seed germination and alleviates oxidative damage caused by salt stress in *Oryza sativa*. *Plant Science* **172,** 544–555.

Locato V, Gadaleta C, De Gara L, de Pinto MC. 2008. Production of reactive species and modulation of antioxidant network in response to heat shock: a critical balance for cell fate. *Plant, Cell and Environment* **31,** 1606–1619.

Matsumoto H, Ishikawa K, Itabe H, Maruyama Y. 2006. Carbon monoxide and bilirubin from haem oxygenase-1 suppresses reactive oxygen species generation and plasminogen activator inhibitor-1 induction. *Molecular and Cellular Biochemistry* **291,** 21–28.

Motterlini R, Foresti R, Bassi R, Calabrese V, Clark JE, Green CJ. 2000. Endothelial haem oxygenase-1 induction by hypoxia. Modulation by inducible nitric oxide synthase and S-nitrosothiols. *Journal of Biological Chemistry* **275,** 13613–13620.

Muramoto T, Kohchi T, Yokota A, Hwang I, Goodman HM. 1999. The Arabidopsis photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid haem oxygenase. *The Plant Cell* **11,** 335–347.

Mrva K, Wallwork M, Mares DJ. 2006. α-Amylase and programmed cell death in aleurone of ripening wheat grains. *Journal of Experimental Botany* **57,** 877–885.

Nakao A, Kaczorowski DJ, Zuckerbraun BS, Lei J, Faleo G, Deguchi K, McCurry KR, Billiar TR, Kanno S. 2008. Galantamine and carbon monoxide protect brain microvascular endothelial cells by haem oxygenase-1 induction. *Biochemical and Biophysical Research Communications* **367,** 674–679.

Nie X, Durnin DC, Igamberdiev AU, Hill RD. 2006. Cytosolic calcium is involved in the regulation of barley hemoglobin gene expression. *Planta* **223,** 542–549.

Nie X, Hill RD. 1997. Mitochondrial respiration and hemoglobin gene expression in barley aleurone tissue. *Plant Physiology* **114,** 835–840.

Noriega GO, Balestrasse KB, Batlle A, Tomaro ML. 2004. Heme oxygenase exerts a protective role against oxidative stress in soybean leaves. *Biochemical and Biophysical Research Communications* **323,** 1003–1008.

Noriega GO, Yannarelli GG, Balestrasse KB, Batlle A, Tomaro ML. 2007. The effect of nitric oxide on heme oxygenase gene expression in soybean leaves. *Planta* **226,** 1155–1163.

Otterbein LE, Kolls JK, Mantell LL, Cook JL, Alam J, Choi AMK. 1999. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *The Journal of Clinical Investigation* **103,** 1047–1054.

Otterbein L, Sylvester SL, Choi AM. 1995. Hemoglobin provides protection against lethal endotoxemia in rats: The role of heme oxygenase-1. *American Journal of Respiratory Cell and Molecular Biology* **13,** 595–601.

Palma K, Kermode AR. 2003. Metabolism of hydrogen peroxide during reserve mobilization and programmed cell death of barley (*Hordeum vulgare* L.) aleurone layer cells. *Free Radical Biology and Medicine* **35,** 1261–1270.

Perazzolli M, Dominici P, Romero-Puertas MC, Zago E, Zeier J, Sonoda M, Lamb C, Delledonne M. 2004. Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *The Plant Cell* **16,** 2785–2794.

Shekhawat GS, Verma K. 2010. Haem oxygenase (HO): an overlooked enzyme of plant metabolism and defence. *Journal of Experimental Botany* **61,** 2255–2270.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. 1987. Bilirubin is an antioxidant of possible physiological importance. *Science* **235,** 1043–1046.

Thom SR, Fisher D, Xu YA, Notarfrancesco K, Ishiropoulos H. 2000. Adaptive responses and apoptosis in endothelial cells exposed

to carbon monoxide. *Proceedings of the National Academy of Sciences, USA* **97,** 1305–1310.

Turkseven S, Kruger A, Mingone CJ, Kaminski P, Inaba M, Rodella LF, Ikehara S, Wolin MS, Abraham NG. 2005. Antioxidant mechanism of heme oxygenase-1 involves an increase in superoxide dismutase and catalase in experimental diabetes. *American Journal of Physiology-Heart and Circulatory Physiology* **289,** H701–H707.

Wilks A. 2002. Heme oxygenase: evolution, structure, and mechanism. *Antioxidants and Redox Signaling* **4,** 603–614.

Xie YJ, Ling TF, Han Y, *et al*. 2008. Carbon monoxide enhances salt tolerance by nitric oxide-mediated maintenance of ion homeostasis and up-regulation of antioxidant defense in wheat seedling roots. *Plant, Cell and Environment* **31,** 1864–1881.

Xuan W, Huang L, Li M, Huang B, Xu S, Liu H, Gao Y, Shen W. 2007. Induction of growth elongation in wheat root segments by haem molecules: a regulatory role of carbon monoxide in plants? *Plant Growth Regulation* **52,** 41–51.

Xuan W, Zhu FY, Xu S, Huang BK, Ling TF, Qi JY, Ye MB, Shen WB. 2008. The heme oxygenase/carbon monoxide system is involved in the auxin-induced cucumber adventitious rooting process. *Plant Physiology* **148,** 881–893.

Yannarelli GG, Noriega GO, Batlle A, Tomaro ML. 2006. Heme oxygenase up-regulation in ultraviolet-B irradiated soybean plants involves reactive oxygen species. *Planta* **224,** 1154–1162.

Zhang A, Jiang M, Zhang J, Ding H, Xu S, Hu X, Tan M. 2007. Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of the mitogen-activated protein kinase cascade involved in antioxidant defense in maize leaves. *New Phytologist* **175,** 36–50.

Zhang X, Shan P, Otterbein LE, Alam J, Flavell RA, Davis RJ, Choi AM, Lee PJ. 2003. Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. *Journal of Biological Chemistry* **278,** 1248–1258.