Endogenous Hydrogen Peroxide Plays a Positive Role in the Upregulation of Heme Oxygenase and Acclimation to Oxidative Stress in Wheat Seedling Leaves

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Abstract

Pretreatment of lower H₂O₂ doses (0.05, 0.5 and 5 mM) for 24 h was able to dose-dependently attenuate lipid peroxidation in wheat seedling leaves mediated by further oxidative damage elicited by higher dose of H₂O₂ (150 mM) for 6 h, with 0.5 mM H₂O₂ being the most effective concentrations. Further results illustrated that 0.5 mM H₂O₂ pretreatment triggered the biphasic production of H₂O₂ during a 24 h period. We also noticed that only peak I (0.25 h) rather than peak II (4 h) was approximately consistent with the enhancement of heme oxygenase (HO) activity, HO-1 gene expression. Meanwhile, enhanced superoxide dismutase (SOD) activity, Mn-SOD and Cu,Zn-SOD transcripts might be a potential source of peak I of endogenous H₂O₂. Further results confirmed that 0.5 mM H₂O₂ treatment for 0.5 h was able to upregulate HO gene expression, which was detected by enzyme activity determination, semi-quantitative reverse transcription-polymerase chain reaction and western blotting. Meanwhile, the application of N,N’-dimethylthiourea, a trap for endogenous H₂O₂, not only blocked the upregulation of HO, but also reversed the corresponding oxidation attenuation. Together, the above results suggest that endogenous H₂O₂ production (peak I) plays a positive role in the induction of HO by enhancing its mRNA level and protein expression, thus leading to the acclimation to oxidative stress.

Key words: acclimation to oxidative stress; endogenous hydrogen peroxide; heme oxygenase; signal transduction; Triticum.


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Hydrogen peroxide (H₂O₂), as well as the other reactive oxygen species (ROS), generated in a variety of biotic and abiotic stimuli, was viewed as a toxic molecule to plant cellular structures and some macromolecules such as lipids, proteins and nucleic acids (Halliwell 1981). However, much evidence has recently emerged that H₂O₂ also functions as an important signal molecule mediating the phenomena of acclimation and cross-tolerance, in which previous exposure to one stress can induce tolerance of subsequent exposure to the same or different stresses (Neill et al. 2002a, 2002b). However, the mechanism(s) by which H₂O₂ mediated acclimation and cross-tolerance is currently not fully understood in plants.

Heme oxygenase (HO, EC 1.14.99.3), the rate-limiting step in heme degradation to carbon monoxide (CO) and biliverdin (BV), exists in inducible (HO-1) and constitutive (HO-2 and HO-3) isofoms, the synthesis and activation of which are differentially regulated in mammalian tissues (Maines et al. 1986; Maines 1997). Disparate conditions and a number of pathological states including hypoxia, endotoxic shock, atherosclerosis, and inflammation have been found to promote overexpression of
the HO-1 gene and increased HO activity. Furthermore, ample results illustrated that the mechanism by which HO-1 acts as antioxidant machinery most likely involves its enzymatic reaction products, including CO and BV (Maines et al. 1986; Maines 1997; Dulak and Józkwicz 2003). In animals, it has also been suggested that various conditions associated with increased production of ROS and reactive nitrogen species (RNS) favor the activation of the HO-1/CO signal pathway, which is now regarded as an important cellular stratagem to counteract and resist different stress insults (Maines 1997; Foresti and Motterlini 1999). In plants, however, the role of HOs was initially focused on its association with the pathway leading to phytochrome chromophores metabolism, and functioning in light signaling (Muramoto et al. 1999; Davis et al. 2001). In Arabidopsis, for example, four HO genes (HY1, HO2–4) have been found to be responsible for the biosynthesis of the phytochrome chromophore by using pharmacological, biochemical and molecular genetic approaches, and HY1 has been shown to be chloroplast-localized (Muramoto et al. 1999; Davis et al. 2001; Emborg et al. 2006). More recently, we provided pharmacological, physiological and molecular evidence that HO/CO also represent a new signal system with significant impact on auxin-induced adventitious root development (Xuan et al. 2008).

A similar role of HO as antioxidant machinery has been reported for the first time by an Argentine research group (Noriega et al. 2004). In their reports, they demonstrated a cytoprotective role of HO against cadmium (Cd)- or ultraviolet-B (UV-B) irradiation-induced oxidative stress in soybean leaves and nodules, in which HO enzyme activities, HO-1 transcripts and its protein expression were enhanced (Noriega et al. 2004; Balestrasse et al. 2005, 2006; Yannarelli et al. 2006; Noriega et al. 2007). Administration of BV, another product of HO-1, and which can act as an efficient ROS scavenger, partially prevented the oxidative effects caused by Cd in soybean leaves (Noriega et al. 2004). Additionally, upregulation of HO gene expression in the above events was preliminarily thought to be a result of excessive exogenous ROS or nitric oxide (NO) (Yannarelli et al. 2006; Noriega et al. 2007), an intercellular and intracellular signaling molecule recently proven in plants (Neill et al. 2008). However, the role of endogenous H2O2 as a signal molecule in HO induction, rather than a cellular toxic component, is not well elucidated in plants.

In the present study, an effort was made to elucidate a possible interrelationship among endogenous H2O2, acclimation to oxidative stress and HO. First of all, we discovered that 0.5 mM H2O2 pretreatment for 24 h triggered wheat seedlings to survive a further higher concentration of 150 mM of H2O2 for 6 h, in which the thiobarbituric acid reactive substances (TBARS) content, an indicator of lipid peroxidation in the tissues (Yannarelli et al. 2006), was inhibited in seedling leaves. Furthermore, the time-course of endogenous H2O2 accumulation, HO activity, HO-1 transcripts, were monitored during 24-h pretreatment period, as well as the modulation of two H2O2 producing enzymes such as NADPH oxidase (NOX) and superoxide dismutase (SOD) in the leaves of wheat plants when exposed to 0.5 mM H2O2 pretreatment for 0.25 h and 4 h. The effects of pretreatment with a trap of endogenous H2O2 N,N’-dimethylthiourea (DMTU) (Levine et al. 1994; Zhang et al. 2007) or one product of HO CO used as its aqueous solution (Cao et al. 2007), on the levels of TBARS, HO enzyme activity, HO-1 protein expression and its gene expression were also investigated. All together, the above experiments were an attempt to discern the contribution of endogenous H2O2 in the upregulation of HO and further cellular adaptation to oxidative stress.

**Results**

**Effects of exogenous H2O2 pretreatment on lipid peroxidation**

Reactive oxygen species are regarded as initiators of peroxidative cell damage. TBARS formation in plants exposed to detrimental environmental conditions is a reliable indicator of free radical generation. To examine whether exogenous H2O2 could alleviate oxidative damage, 2-week-old wheat seedlings were pre-treated with 0, 0.05, 0.5, 5 and 50 mM H2O2 for 24 h, and grown for a further 6 h in the solution with 150 mM H2O2. The above wide range of H2O2 doses enabled us to mimic the physiological responses *in vivo* elicited by various abiotic and biotic stresses (Neill et al. 2002b; Uchida et al. 2002).

Figure 1 shows that 150 mM H2O2 treatment alone (without H2O2 pretreatment) significantly increased TBARS content in comparison with H2O2-free control (Con). Meanwhile, pretreatment of lower H2O2 doses (0.05, 0.5 and 5 mM) was able to dose-dependently attenuate lipid peroxidation mediated by further oxidative damage elicited by higher dose of H2O2 (150 mM), also with 0.5 mM H2O2 being the most effective concentrations (*P < 0.05*), which was then used to investigate the role of H2O2 in acclimation to oxidative stress throughout the study. For example, the production of TBARS in 0.5 mM H2O2 pretreated seedling leaves was inhibited by 45% compared with a non-pretreated sample, whereas, 50 mM H2O2 pretreatment did not show any protective effect. Seedling leaves also exhibited wilting to some extent, in comparison with the control samples. The above results clearly confirmed that H2O2 pretreatment caused oxidative stress at high concentrations, while low levels of H2O2 conferred a cytoprotecitve role (Uchida et al. 2002).

**Exogenous H2O2 caused biphasic changes of endogenous H2O2 production**

It seemed possible that the acclimation to higher doses of H2O2 caused by a low dose of H2O2 pretreatment might involve,
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Figure 1. Effects of pretreatment of H2O2 on thiobarbituric acid reactive substance (TBARS) concentration in wheat seedling leaves under further H2O2 treatment.

Two-week-old seedlings were pretreated with different H2O2 concentrations (0, 0.05, 0.5, 5, 50 mM) for 24 h and then exposed to 150 mM H2O2 for 6 h. Meanwhile, sample treated with distilled water alone was regarded as H2O2-free control (Con). Values are means ± SE of three different experiments with at least three replicated measurements. Bars with different letters are significantly different at \( P < 0.05 \) according to Duncan’s multiple range test.

Figure 2. Time course of endogenous levels of H2O2 in wheat seedling leaves under exogenous H2O2 treatment.

Two-week-old seedlings were treated in the solution with or without 0.5 mM H2O2. Endogenous H2O2 levels were measured over 24 h. The time course test showed a biphasic reaction (Figure 2); namely, a fast but weak burst of endogenous H2O2 production at 0.25 h with 0.5 mM H2O2 (peak I), then followed by a sharp decrease, and another stronger peak at 4 h (peak II). Thus, there is a potentially significant, biphasic increase in endogenous H2O2, which could be part of the mechanism for acclimation caused by 0.5 mM H2O2 exogenously applied.

Effects of exogenous H2O2 treatment on HO activity and HO-1 gene expression

To evaluate whether HO-1 is involved in the exogenous H2O2-mediated acclimation to oxidative stress, we also investigated the effects of 0.5 mM H2O2 treatment on HO activity and HO-1 gene expression. The time course experiments (Figure 3) showed that, in comparison with H2O2-free control (Con), both HO activity and HO-1 transcripts abruptly increased during the 24 h treatment period with H2O2 treatment. The increase peaked between 0.25 and 0.5 h, followed by a rapid decrease to the basal levels of control plants until 24 h.
Figure 3. Time-course analysis of heme oxygenase activity (A) and HO-1 transcripts (B) in wheat seedling leaves under H$_2$O$_2$ treatment.

Two-week-old seedlings were treated in the solution with or without 0.5 mM H$_2$O$_2$. HO activity and HO-1 transcripts were measured over 24 h. HO-1 transcripts were analyzed by semi-quantitative reverse transcription-polymerase chain reaction. The relative abundance of 18S rRNA was used as the internal standard. Values are means ± SE of three different experiments with at least three replicated measurements.

Con, H$_2$O$_2$-free control.

Effects of pretreatment with DMTU and CO on lipid peroxidation

In order to determine whether the decrease of lipid peroxidation induced by low dose of exogenous H$_2$O$_2$ (0.5 mM) pretreatment for 24 h results from the increase in the production of endogenous H$_2$O$_2$ (peak I or II), 2-week-old wheat seedlings were further pretreated with DMTU, the trap or scavenger of H$_2$O$_2$, or 10% CO-saturated aqueous solution, another product of H$_2$O$_2$, both in animals and plants, for 0.5 h (only peak I appeared) and then exposed to 150 mM H$_2$O$_2$ for 6 h. Experimental results showed that the pretreatment with DMTU plus H$_2$O$_2$ fully blocked the decrease of TBARS conferred by 0.5 mM H$_2$O$_2$ pretreatment alone. However, the addition of CO aqueous solution weakened the above effect, and CO pretreatment produced a similar response to that of the H$_2$O$_2$ pretreatment alone (Figure 4). Interestingly, the application of 0.5 mM H$_2$O$_2$ for either 0.5 h or 24 h exhibited the similar attenuation effects of lipid peroxidation elicited by further 150 mM H$_2$O$_2$ treatment for 6 h (data not shown). Together, the above results suggest that H$_2$O$_2$ production of peak I elicited by exogenously applied H$_2$O$_2$, at least in our experimental condition, contributed a major role in the acclimation to oxidative stress.

Effect of DMTU on endogenous H$_2$O$_2$-induced upregulation of HO activity, HO-1 gene expression and its protein expression

To further examine the mechanism of HO induction in the H$_2$O$_2$ signal pathway, we applied DMTU to investigate whether the peak I of endogenous H$_2$O$_2$ was able to induce HO from transcript and protein levels. As expected, simultaneous treatment with H$_2$O$_2$ and DMTU for 0.5 h produced a decrease in the HO enzyme activity in comparison with H$_2$O$_2$ treatment alone (Figure 5A). Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) revealed that DMTU brought about the full block of the enhancement of HO-1 transcripts, and the level of 18S was unaffected throughout all experiments (Figure 5B). Additionally, western blotting analysis for HO-1 (Figure 5C) showed only a single band with a molecular mass of 32 kDa, determined by using molecular mass markers (data not shown), a similar value to that reported for soybean leaves (Yannarelli et al. 2006). This assay also demonstrated a positive correlation among HO enzyme activity, HO-1 gene expression
and its protein level (Figure 5). All of these results suggested that endogenous peak I of \( \text{H}_2\text{O}_2 \) production might upregulate HO activity by enhancing its mRNA levels and protein expression.

**Figure 5.** Effects of treatment of \( \text{H}_2\text{O}_2 \) and \( \text{N,N'-dimethylthiourea (DMTU)} \) on heme oxygenase (HO) activity (A), HO-1 transcripts (B) and its protein expression (C) in wheat seedling leaves.

Two-week-old seedlings were treated in solutions containing either 0.5 mM \( \text{H}_2\text{O}_2 \) and 5 mM DMTU or containing two of the above chemicals for 0.5 h. HO-1 transcripts were analyzed by semi-quantitative reverse transcription-polymerase chain reaction. The relative abundance of \( 18S \) rRNA was used as the internal standard. HO-1 protein expression was analyzed by western blotting. The Coomassie Bright Blue-stained gels (D) are present to show that equal amounts of proteins were loaded. Values are means ± SE of three different experiments with at least three replicated measurements. Bars with different letters are significantly different at \( P < 0.05 \) according to Duncan’s multiple range test. Con, \( \text{H}_2\text{O}_2 \)-free control.

### Table 1. Effects of \( \text{H}_2\text{O}_2 \) treatment on NADPH oxidase (NOX) and superoxide dismutase (SOD) activities in wheat seedling leaves

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NOX (( \text{O}_2^- )) nmol/min per mg protein</th>
<th>SOD (U/g FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>time (h)</td>
<td>Con ( \text{H}_2\text{O}_2 )</td>
<td>Con ( \text{H}_2\text{O}_2 )</td>
</tr>
<tr>
<td>0</td>
<td>42.0 ± 6.8(^b)</td>
<td>42.1 ± 6.8(^b)</td>
</tr>
<tr>
<td>0.25</td>
<td>41.3 ± 0.5(^a)</td>
<td>43.8 ± 1.4(^\text{ab})</td>
</tr>
<tr>
<td>4</td>
<td>43.5 ± 0.3(^a)</td>
<td>69.4 ± 2.6(^a)</td>
</tr>
</tbody>
</table>

Two-week-old seedlings were treated in solutions with or without 0.5 mM \( \text{H}_2\text{O}_2 \) for 0, 0.25 and 4 h. Con, \( \text{H}_2\text{O}_2 \)-free control; FW, fresh weight. Values are means ± SE of three different experiments with at least three replicated measurements. Different letters within columns indicate significant differences (\( P < 0.05 \)) according to Duncan’s multiple range test.

**Discussion**

Various stresses, such as heavy metals, UV-B irradiation, salt stress and pathogen infection, are detrimental to plant cells by generating excessive ROS. As a result, upregulation of plant redox systems will result in resistance in a wide range of biotic or abiotic stresses. For instance, \( \text{H}_2\text{O}_2 \), a famous reactive and toxic molecule, has been reported to be a multifunctional trigger to induce acclimatory stress tolerance by mediating redox balance in plant cells (Foyer et al. 1997). To date, recent evidence has validated \( \text{H}_2\text{O}_2 \) roles in acclimation tolerance. For example, pretreatment of \( \text{H}_2\text{O}_2 \) could induce salinity tolerance in maize and wheat plants (de Azevedo Neto et al. 2005;
Figure 6. Effect of treatment of H$_2$O$_2$ on the expression of superoxide dismutase (SOD) in wheat seedling leaves.

Two-week-old seedlings were treated in solutions with or without 0.5 mM H$_2$O$_2$ for 0, 0.25 and 4 h. Mn-SOD and Cu,Zn-SOD transcripts were analyzed by semi-quantitative reverse transcription-polymerase chain reaction. The relative abundance of 18S rRNA was used as the internal standard. Con, H$_2$O$_2$-free control.

Wahid et al. (2007), and heat tolerance in rice (Uchida et al. 2002). In the present paper, we confirmed that lower doses of H$_2$O$_2$ pretreatment led to the acquired-acclimation to oxidative stress induced by higher doses of H$_2$O$_2$ (Figure 1), and first time discerned the contribution of endogenous H$_2$O$_2$ (peak I), produced by H$_2$O$_2$ applied exogenously, in the upregulation of HO and further cellular adaptation to oxidative stress.

It is well known that, unlike the other small molecules of ROS such as $\cdot$O$_2^-$ and OH$, H$_2$O$_2$ is fusible to cross the plasma membrane (Foyer et al. 1997; Uchida et al. 2002; de Azevedo Neto et al. 2005; Wahid et al. 2007), and at the same time its comparatively stable quality also add to the points for H$_2$O$_2$ to campaign for the role of a signal molecule (Neill et al. 2002a, 2002b). In the present study, the time course change in H$_2$O$_2$ content was measured during a 24 h pretreatment period. Interestingly, we discovered a biphasic burst of endogenous H$_2$O$_2$, including peaks I and II (Figure 2), a phenomena very similar to that after pathogen infection, which has a primary peak 1–2 h after infection followed by a greater secondary peak 3–6 h after infection (Lamb and Dixon 1997). A similar biphasic oxidative burst is also observed in ozone exposure (Sandermann et al. 1998), considered as an abiotic trigger of the pathogen defense response.

In plants, H$_2$O$_2$ is continually generated in organelles such as chloroplasts, mitochondria, microbodies and peroxisomes during normal metabolism, and the enzymatic sources and cellular localization can be especially important for H$_2$O$_2$ signaling (Neill et al. 2002a, 2002b). Additionally, H$_2$O$_2$ production has been proved to increase under various abiotic stresses and to enhance gene expression of ROS scavenging enzymes. The results presented in Table 1 and Figure 6 illustrate the enhancement of total SOD activity and Mn-SOD and Cu/Zn-SOD gene expression 0.25 h after H$_2$O$_2$ exposure, both of which are consistent with the time of the first weak peak of H$_2$O$_2$ (peak I, Figure 2). Additionally, plasma membrane-bound NADPH oxidase (NOX) has become an intense focus in H$_2$O$_2$ signaling (Neill et al. 2002a, 2002b). A significant augment in NOX activity has been found at 4 h after treatment (Table 1), indicating that it might be another major source of the secondary H$_2$O$_2$ peak (peak II, Figure 3) besides SOD enzyme (Table 1 and Figure 6).

Expression of stress proteins is an important adaptive strategy of environmental stress tolerance. Recent results indicated that 0.08 mM H$_2$O$_2$ treatment was able to induce the expression of two heat-stable proteins with apparent molecular masses of 32 and 52 kDa in wheat plants (Wahid et al. 2007). Meanwhile, earlier studies also demonstrated the expression of small heat shock proteins (HSPs), including mitochondrial HSP22, as an acclimation response to H$_2$O$_2$ application in tomato (Banzet et al. 1998) and Arabidopsis (Pnueli et al. 2003). In animal tissues, HO-1, one type of HSPs proven in rats (Ewing and Maines 1991), is induced by a great deal of factors such as heme, several heme-proteins, heavy metals, hyperthermia, UV-B irradiations, hypoxia, hyperoxia, and H$_2$O$_2$ (Dulak and Józkowicz 2003). Recent results (Yannarelli et al. 2006) also revealed that in the presence of different exogenous H$_2$O$_2$ concentrations, an induction of HO-1 mRNA in the absence of UV-B occurred in soybean leaves. In our experimental conditions, the Figures 2 and 3 give confirmation that the changes of HO activity and HO-1 transcripts after H$_2$O$_2$ exposure during 1 h coincided with the behavior of H$_2$O$_2$ (including peak I). Furthermore, semi-quantitative RT-PCR and western-blotting were carried out to investigate the mechanism of HO induction by H$_2$O$_2$. Our results also demonstrated that, in response to exogenous 0.5 mM H$_2$O$_2$ pretreatment for 0.5 h (only peak I appeared), HO was upregulated in enzymatic, transcriptional and protein levels. TBARS content was decreased, and these effects were blocked by the application of an H$_2$O$_2$ trap or scavenger, DMTU (Figures 4 and 5). Interestingly, the application of 0.5 mM H$_2$O$_2$ for either 0.5 h or 24 h exhibited similar attenuation effects of lipid peroxidation elicited by a further 150 mM H$_2$O$_2$ treatment for 6 h (data not shown). As a result, we deduced that in our experimental conditions, a fast but weak peak of H$_2$O$_2$ (peak I) is associated with a complicated gene transduction network related to the induction of the acclimation or tolerance against oxidative stress. However, whether HO-1 induced by H$_2$O$_2$
pretreatment in wheat seedling leaves belongs to HSPs should be elucidated in the near future.

On the other hand, the application of 10% CO-saturated aqueous solution was able to mimic the effect of 0.5 mM H2O2 pretreatment on the alleviation of lipid peroxidation (Figure 4), further suggesting that HO could act as antioxidative enzyme in plants, and the product of HO, CO was involved in this cytoprotective effect (Xie et al. 2008). Similar results were also reported by our research groups (Liu et al. 2007; Han et al. 2008). In plants, SOD is localized in chloroplasts, mitochondria, cytosol and peroxisomes (McKersie et al. 1993). In view of the fact that HO is mainly distributed in chloroplasts (Muramoto et al. 1999; Davis et al. 2001; Emborg et al. 2006), combined with the results shown in this paper (Figures 2–5), it is reasonable to confirm the idea that the early peak of H2O2 (peak I) might be responsible for the upregulation of HO expression.

To sum up, the above results suggested that endogenous H2O2 production (peak I) resulting from the increase of SOD activity elicited by exogenous H2O2, might mediate a complex of the signal transduction network by the induction of HO through enhancing its mRNA level and protein expression, thus inducing acclimation or tolerance against oxidative stress.

Materials and Methods

Plant materials, growth condition and treatments

Selected seeds of wheat (Triticum aestivum L., Yangmai 158) were sterilized in 2% NaClO for 5 min, washed in distilled water and germinated at 25 °C in the dark for 1 d. The identical buds were selected and moved into the rectangular boxes in a growth chamber (12-h light period, 25 °C, humidity 50% ± 4%; 12-h dark period, 18 °C, humidity 56% ± 5%, MGC-300B, Shanghai Yiheng Technology Co., Ltd., China) with modified Hoagland solution containing 3 mM KNO3, 1 mM NH4H2PO4, 0.5 mM MgSO4, 5.5 mM Ca(NO3)2, 50 mg of FeEDTA per liter (10% iron), 25 μM KCl, 12.5 μM H3BO3, 1 μM MnSO4, 1 μM ZnSO4, 0.25 μM CuSO4, and 2 μM H2MoO4. The irradiance was approximately 300 μmol/m2 per s provided by fluorescent lamps. The culture solution was renewed every other day until two fully expanded leaves appeared (about 2 weeks). Afterwards, 20 uniform wheat seedlings were transferred to 500 mL of distilled water with or without different H2O2 concentrations (0, 0.05, 0.5, 5, 50 mM), 5 mM DMTU and 10% CO-saturated aqueous solution or containing two or three of the above chemicals for 24 h or indicated time, then exposed to distilled water or 150 mM H2O2 for 6 h at 25 °C with a continuous light intensity of 300 μmol/m2 per s. The plants treated with distilled water under the same conditions during the whole period served as controls for the above (H2O2-free control). After various treatments, the second seedling leaves were sampled, washed thoroughly, and immediately frozen under liquid nitrogen, and then stored at −80 °C until further analysis, except for those needed for isolation of plasma membranes and determination of endogenous H2O2 content, both of which were conducted immediately after sampling and washing.

Chemicals

NADPH, xylene orange, sodium, 3′-(1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzensulfonic acid hydrate (XTT), 2-thiobarbituric acid (TBA) and superoxide dismutase, were from Sigma (St Louis, MO, USA). N,N′-dimethylthiourea (DMTU), chosen as the trap or scavenger of H2O2 (Levine et al. 1994; Zhang et al. 2007), was from Fluka. Additionally, the remaining chemicals were of analytical grade from Chinese companies.

CO-saturated aqueous solution preparation

Carbon monoxide aqueous solution was prepared according to the method described in our previous report (Han et al. 2008). In our experiments, CO-saturated aqueous solution was freshly obtained by bubbling CO gas gently through a glass tube into 500 mL of distilled water in a glass beaker for at least 30 min. Then the saturated stock solution (100% of saturation) was immediately diluted with distilled water to the required concentration for 10% of saturation. Preliminary studies using various concentrations (1%, 10%, 50% and 100% of saturation) of CO aqueous solution have shown that 10% CO-saturated aqueous solution was the suitable treatment because of its better positive results against 150 mM H2O2-induced oxidative stress determined by TBARS content compared with other treatments (data not shown).

TBARS content determination

Lipid peroxidation was estimated by the amount of TBARS as described by Liu et al. (2007). About 500 mg fresh tissue was ground in 0.25% TBA in 10% trichloroacetic acid (TCA) using a mortar and pestle. After heating at 95 °C for 30 min, the mixture was quickly cooled in an ice bath and centrifuged at 10 000 g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The blank was 0.25% TBA in 10% TCA. The concentration of lipid peroxides together with oxidatively modified proteins of plants were thus quantified in terms of TBARS level using an extinction coefficient of 155/mM per cm and expressed as nmol/g fresh weight (FW).
H$_2$O$_2$ content determination

The content of H$_2$O$_2$ was measured according to Bellincampi et al. (2000) in the extracellular phase. Briefly, an aliquot of supernatant (500 μL) was added to 500 μL assay reagent (500 μM ferrous ammonium sulfate, 50 mM H$_2$SO$_4$, 200 μM xylene orange, 200 mM sorbitol). After 45 min incubation, the peroxide-mediated oxidation of Fe$^{2+}$ to Fe$^{3+}$ was determined by measuring the absorbance at 560 nm of the Fe$^{3+}$-xylene orange complex.

Enzyme activities assays

Heme oxygenase and SOD activities were analyzed following the method by Han et al. (2008). For the HO activity test, the concentration of biliverdin IX was estimated using a molar absorption coefficient at 650 nm of 6.25/mM per cm 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. Total SOD activity was measured on the basis of its ability to reduce nitroblue tetrazolium (NBT) by the superoxide anion generated by the riboflavin system under illumination. One unit of SOD (U) was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50%. Isolation of plasma membrane and determination of NADPH oxidase (NOX) activity of isolated plasma membrane vesicles were carried out by the method described by Jiang and Zhang (2003). The assay mixture of 1 mL contained 50 mM Tris-HCl buffer (pH 7.5), 0.5 mM XTT, 100 μM NADPH and 15–20 μg of membrane proteins. The reaction was initiated with the addition of NADPH, and XTT reduction was determined at 470 nm. Corrections were made for background production in the presence of 50 units SOD. Rates of O$_2$•− generation were calculated using an extinction coefficient of 2.16 x 10$^4$/M per cm. Protein was evaluated by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Western-blotting analysis for HO-1

Homogenates obtained for HO activity assays were also analyzed by western-blotting. Sixty micrograms of protein from homogenates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide resolving gel (Mini Protean II System, BioRad, 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 raised against Oryza sativa HO-1 (OsHO1) diluted 1: 200 in PBS buffer plus 1% non-fat milk. Immune complexes were detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G. The color was developed with a solution containing DAB (3,3′-diaminobenzidine tetrahydrochloride) as the HRP substrate.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from 100 mg of fresh-weight wheat seedling leaves ground with mortar and pestle in liquid nitrogen until a fine powder appeared and used Trizol reagent (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer’s instructions. 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 U of AMV reverse transcriptase XL (Takara) and 1 μM of oligo-dT primer. PCR reactions were carried out using 2 μL of a twofold dilution of the cDNA, 10 pmol of each oligonucleotide primer and 1 U of Taq polymerase (Takara) in a 25 μL reaction volume.

HO-1 (according to the method described by Baudouin et al. (2004), the CK162568, CK217201 and CJ706509 sequences were assembled to construct the HO-1 cluster sequence), forward 5′-AGTTCAGACCTGACGGACAT-3′ and reverse 5′-CCCAGCAGTATTTATTTCCATTTT-3′ (amplifying a 363 bp fragment); Mn-SOD (accession number AF092524), forward 5′-ACCGAAAGCACCCGCCATCTC-3′ and reverse 5′-GCTCCAGACATCAATTTACAAACAAA-3′ (amplifying a 417 bp fragment); Cu,Zn-SOD (accession number U69632), forward 5′-CCTCTTCTCCAGGTCTCCTGG-3′ and reverse 5′-ATGAAACACACAGCCTCCCC-3′ (amplifying a 465 bp fragment); 18S rRNA (accession number AJ272181), forward 5′-CAAGCCATCCTGCTCTGATT-3′ and reverse 5′-CCTGTTATGGCCTCAAACCTCC-3′ (amplifying a 658 bp fragment). To standardize the results, the relative abundance of 18S rRNA was determined and used as the internal standard.

The cycle numbers of the 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 with the use of ethidium bromide (EB). Specific amplification products of the expected size were observed and their identities were confirmed by sequencing.

Statistical analysis

Values are means ± SE of three different experiments with at least three replicated measurements. Differences among treatments were analyzed by one-way ANOVA, taking P < 0.05 as significant according to Duncan’s multiple range test.

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References


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