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Tissue and Induction Expression Profiles of Rice Phospholipid Hydroperoxide Glutathione Peroxidase at Protein Level *

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Abstract Proteins are the major molecules performing life activities, and their spatial and temporal expression profiles in organisms are very important for understanding their accurate functions. Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a unique antioxidant enzyme that directly reduces lipid hydroperoxides in biomembranes and plays a vital role in defense against membrane peroxidation damage. The protein expression profiles of rice PHGPx (OsPHGPx) were investigated in different rice tissues and under various stress treatments by using Western blot analysis. The results showed that in mature rice plants, OsPHGPx was mainly distributed in leaves, especially flag leaves, and in rice seedlings, OsPHGPx was detected in shoots and leaves. Moreover, OsPHGPx expression in rice seedlings could be markedly induced by H_2O_2 and NaCl, but weakly influenced by several plant hormones. Time- and dose-dependent effects were observed in both H_2O_2 and NaCl treatments, and the strongest induction was observed when rice seedlings were treated with 0.5 mmol/L H_2O_2 for 12 h or 500 mmol/L NaCl for 24 h. Additionally, dimethylthiourea, a H_2O_2 trap, inhibited H_2O_2-enhanced expression of OsPHGPx, but did not impair the enhanced effect of NaCl, implying that NaCl-induced OsPHGPx expression was not mediated by H_2O_2. These results will contribute greatly to further study the exact physiological function of OsPHGPx in rice.

Key words rice, PHGPx, protein expression profile, oxidative stress, salt stress
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In the post-genomic era, a number of powerful techniques including differential display[1], DNA microarray[2] and serial analysis of gene expression[3] can conveniently establish global and quantitative mRNA expression profiles of cells and tissues in species for which the sequence of all the genes is known. However, mRNA expression patterns are necessary but are by themselves insufficient for understanding the expression of protein products, as additional post-transcriptional mechanisms may influence the level of a protein present in a given cell or tissue[4, 5]. Proteins are the main undertakers of life activities, and protein expression profiles are crucial for uncovering their biological functions. Thus, recent studies have focused on characterization of protein expression using proteome analysis for proteins at large scale and global level or Western blot analysis for an individual protein[6].

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is a unique antioxidant enzyme that can directly reduce lipid hydroperoxides and is generally considered the main line of enzymatic defense against oxidative biomembrane destruction in animals[7]. Recently, PHGPx has also been found existing in plants and PHGPx genes have been cloned and characterized from a number of plant species[8~10], but the function of plant PHGPx has not been fully understood yet. Expression pattern analysis in transcript level showed that plant PHGPx genes were involved in plant responses to a broad range of biotic

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and abiotic stresses[11]. Due to possible discrepancies between responses at RNA and protein levels, the expression profiles of PHGPx protein remain to be elucidated.

Rice PHGPx gene (*OsPHGPx*) was first cloned by Li *et al*.[9], and it is a single copy gene, which encodes a protein of 169 amino acids with a calculated molecular mass of 19 234 u. Biochemical characterization of recombinant OsPHGPx demonstrated its broad specificity for hydroperoxide substrates including H_2O_2, organic hydroperoxides and lipid peroxides, providing significant insight into the physiological function of OsPHGPx[12]. Taking into consideration the potential importance of OsPHGPx in oxidative stress and the lack of any study on its responses to environmental stresses at protein level, we conducted this study to investigate the tissue and induction expression profiles of OsPHGPx in detail.

1 Materials and methods

1.1 Plant materials

Rice (*Oryza sativa* L. cv. Zhonghua 10) was grown in the greenhouse at 25℃ and 70% relative humidity with a 16 h light and 8 h dark cycle. Rice leaves of 2-week-old seedlings were used for all treatments as reported previously[13]. In briefly, leaves were cut into 2 cm long segments and floated on solutions of the test compounds in sterile Petri dishes. The control (labeled as CON in the figures) was treated with distilled water. Because the accumulation of *PHGPx* induced by some treatment is influenced by light[13], all the incubations were done under continuous light (wavelength 390～500 nm, 150 μmol·m^{-2}·s^{-1}) at 25℃ and 70% relative humidity. Leaves were harvested at the times indicated, frozen immediately in liquid nitrogen and stored at –80℃ till use.

1.2 Chemicals

Jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA), gibberellin (GA_3) and dimethylthiourea (DMTU) were purchased from Sigma (St. Louis, MO, USA). JA, ABA and GA_3 were dissolved in methanol as stock solutions of 100 mmol/L, and used at concentrations described in the figure legends. All the other chemicals used in this study were at least of analytical grade.

1.3 Protein extraction

Protein extraction was performed according to Cui *et al*.[14] with some modifications. About 2 g plant materials were ground to a fine powder in liquid nitrogen and homogenized in 4 ml ice-cold extraction buffer containing 50 mmol/L Tris-HCl, pH 7.8, 10% glycerol, 2% β-mercaptoethanol, 1 mmol/L PMSF, and 1 mmol/L EDTA. After incubation on ice for 30 min, the homogenate was centrifuged at 16 000 *g* for 30 min at 4℃. The resulting supernatant was transferred to another clean tube and suspended in 5 volumes of acetone at –20℃ overnight. Precipitated proteins were recovered by centrifugation at 16 000 *g* for 30 min at 4℃. The protein pellet was collected and lyophilized. After lyophilization, 5～10 mg protein precipitants were gained for each extraction and dissolved correspondingly in 0.5～1 ml SDS sample buffer without β-mercaptoethanol. Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, USA).

1.4 Western blot analysis

Western blot was carried out as described previously[15, 16]. The protein extracts (50 μg/lane) were separated on 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The immunodetection with rabbit anti-OsPHGPx serum was performed at a dilution of 1/4000 and goat anti-rabbit antibodies conjugated to horseradish peroxidase (Jackson, Pennsylvania, USA) were used as secondary antibodies. Binding of antibody was detected using the enhanced chemiluminescence reagents (ECL) (Pierce, Rockford, USA). Equal protein loading and transferring were confirmed by SDS-PAGE stained with Coomassie Brilliant Blue (CBB) or by staining membranes with Ponceau's solution. The resulting bands of OsPHGPx were quantified using TotalLab software.

2 Results and discussion

2.1 Tissue expression patterns of OsPHGPx

To investigate the expression patterns of OsPHGPx in various rice tissues and at different developmental stages, total protein from rice organs of mature plants and 2-week-old seedlings were subjected to Western blot analysis. As shown in Figure 1, in the mature plants, OsPHGPx was mostly distributed in the leaves, especially in the third and fourth leaves, while in the other tissues including stems, roots, panicles and seeds, the expression levels were much lower. Similarly, in the 2-week-old seedlings, OsPHGPx was distributed in the leaves and shoots, and it was nearly absent in the roots. Previous work has demonstrated that *OsPHGPx* transcripts exhibited a tissue-specific

expression manner, and the highest level of *OsPHGPx* mRNA was found in flag leaves, which was 2, 44 and 32 times higher than that in stems, roots and seeds respectively[9]. Our results at protein level were almost consistent with those at mRNA level. In plants, a lot of reactive oxygen species (ROS) are generated during the process of photosynthesis and the highest photosynthesis efficiency is found in green leaves. Therefore, the tissue distribution of OsPHGPx corresponding to photosynthetic organs suggested that OsPHGPx might play a vital role in protection against oxidative damage of ROS generated by photosynthesis.

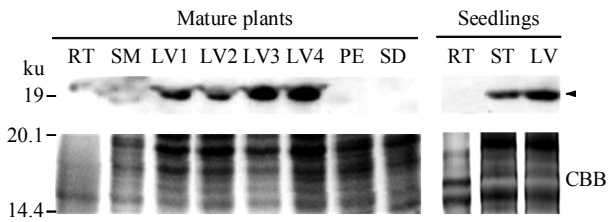

Fig. 1 Expression of OsPHGPx in different rice tissues and at different development stages

Total protein (50 μg/lane) extracted from roots (RT), stems (SM), the first leaves (LV1), the second leaves (LV2), the third leaves (LV3), the fourth leaves (LV4), panicles (PE), seeds (SD) of mature plants, and roots (RT), shoots (ST), leaves (LV) of 2-week-old seedlings. The arrowhead indicated the OsPHGPx protein (approximately 19 ku). CBB stained proteins (14.4～20.1 ku) were shown to verify equal loading.

2.2 OsPHGPx expression in response to various stresses and plant hormones

Evidence connecting OsPHGPx protein to stress responses came from observed changes in *OsPHGPx* gene expression under stress conditions. For example, exposure of rice seedlings to H_2O_2 and aluminum resulted in increased *OsPHGPx* mRNA levels [9]. Agrawal *et al*.[13] also reported that signaling molecules including JA, SA and ABA could potently up-regulate the *OsPHGPx* expression. To confirm these results at the protein level, we examined OsPHGPx protein expression in response to salinity, oxidative stress and plant hormones by Western blot analysis. The results showed that salt and oxidative stress caused a relatively remarkable increase at the level of OsPHGPx protein (Figure 2). OsPHGPx exhibited approximately 2-fold increase in NaCl treatment and 1.5-fold increase in H_2O_2 treatment compared with the control (CON). Furthermore, plant hormones had different effects on the OsPHGPx expression. In hormone treatments, only ABA was slightly effective in up-regulating the OsPHGPx expression, whereas JA or SA had a negligible effect and GA_3 had a negative effect. Interestingly, OsPHGPx was also responsive to H_2O and/or cut, for its level of CON was higher than that of untreated samples (UT). These results implied a complexity in regulation of OsPHGPx expression and a significant role of OsPHGPx in scavenging ROS produced by salt and oxidative stresses.

It should be noted that these stress treatments were able to bring evident increase of *OsPHGPx* mRNA in the previous research. As mentioned above, phytohormones, particularly ABA, can potently induce the *OsPHGPx* mRNA accumulation. However, our results showed that ABA could only have a subtle effect on the OsPHGPx expression. The likely explanation for the apparent inconsistency between the high ABA-induced *OsPHGPx* mRNA level and the very low ABA-induced OsPHGPx protein level, is that ABA might affect the regulation of posttranscriptional mechanisms controlling OsPHGPx protein translation rate or degradation rate, impairing the induced expression of OsPHGPx protein. Additionally, the possibility that these plant hormones may influence the OsPHGPx expression in a time- or dose-dependent manner can not be ruled out. On the other hand, our results could be supported by evidence of *PHGPx* promoter analysis, which revealed that ABA failed to activate the *PHGPx* promoter[17]. Therefore, compared with H_2O_2 and NaCl, ABA may be a rather weak inducer of OsPHGPx protein expression.

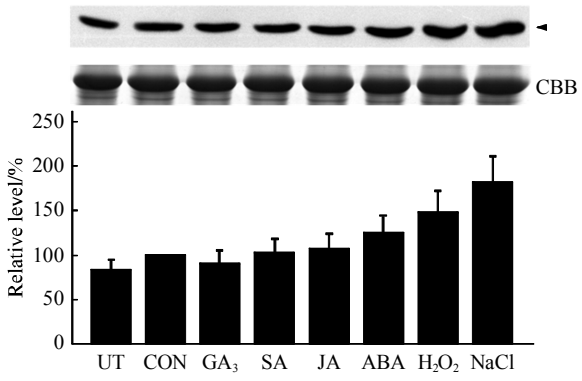

Fig. 2 Expression of OsPHGPx exposed to various stress and hormone treatments

Western blot analysis of total protein (50 μg/lane) isolated from leaves untreated(UT), or treated with H_2O(CON), 100 μmol/L SA, 100 μmol/L ABA, 100 μmol/L JA, 100 μmol/L H_2O_2, 100 mmol/L NaCl for 24 h, respectively. The arrowhead indicated the OsPHGPx protein. CBB stained proteins (RuBisCO bands) were shown to verify equal loading. CON was used as normalized reference and its relative OsPHGPx level was set at 100%. The values represented the $\bar{x} \pm s$ of relative OsPHGPx level in three independent experiments.

2.3 Induction of OsPHGPx expression by H_2O_2 and NaCl

Since H_2O_2 and NaCl treatments could increase protein expression levels of OsPHGPx effectively, we first studied the effects of their concentrations on OsPHGPx expression. For H_2O_2 treatment, rice leaves of 2-week-old seedlings were exposed to 0～5 mmol/L H_2O_2 for 24 h under continuous light. As shown in Figure 3a, elevated H_2O_2 concentration caused obvious increase in abundance of OsPHGPx in 0～0.5 mmol/L range, and 0.5 mmol/L H_2O_2 treatment gave the highest level of OsPHGPx expression. However, higher H_2O_2 treatment concentrations (0.5～5 mmol/L) seemed to impair the effect on up-regulating OsPHGPx expression, and 5 mmol/L H_2O_2 even inhibited its expression. This phenomenon probably results from the balance between antioxidant enzymes activities in plant cells. It can be inferred that OsPHGPx plays a central role in responding to low concentration of H_2O_2 (μmol/L range), while at higher concentration of H_2O_2 (mmol/L range), other antioxidant enzymes such as catalase (CAT) have a main effect on ROS scavenging[18].

In the NaCl treatment, the induced level of OsPHGPx was found to be dependent on the dose of NaCl (Figure 3a). OsPHGPx expression increased gradually with NaCl concentrations elevating from 0 to 500 mmol/L, and reached the highest level at 500 mmol/L NaCl. As is well known to us, salt stress triggers an increased production and accumulation of ROS, affecting cell redox homestasis and causing oxidative stress[19]. Response patterns of OsPHGPx in salt stress indicated that OsPHGPx participated in the antioxidant defense system to ensure proper protection against ROS generated after salt treatment.

In order to further investigate the responses of the OsPHGPx in oxidative stress and salt stress in detail, a time course experiment was carried out using 2-week-old rice seedlings treated with H_2O_2 (0.5 mmol/L), NaCl (500 mmol/L) and in parallel with H_2O. As shown in Figure 3b, H_2O_2 could up-regulate the OsPHGPx protein expression within 6 h, which increased in abundance with time till 12 h followed by a stepwise decline thereafter. On the other hand, a marked increase in the level of OsPHGPx protein was observed following NaCl stimulation at 24 h, but then a rapid decrease was found after 36 h. Samples under H_2O treatment were used as control, and density scanning revealed that OsPHGPx amount remained at low levels during the duration of the experiment, except for a slight increase at 60 h. Because ROS is produced directly by H_2O_2 and indirectly by NaCl, OsPHGPx expression responds to H_2O_2 more rapidly than NaCl, providing further evidence for an involvement of OsPHGPx in oxidative stress defense. These results convincingly demonstrate that OsPHGPx is a key member of ROS-scavenging enzymes in oxidative stress and salt stress.

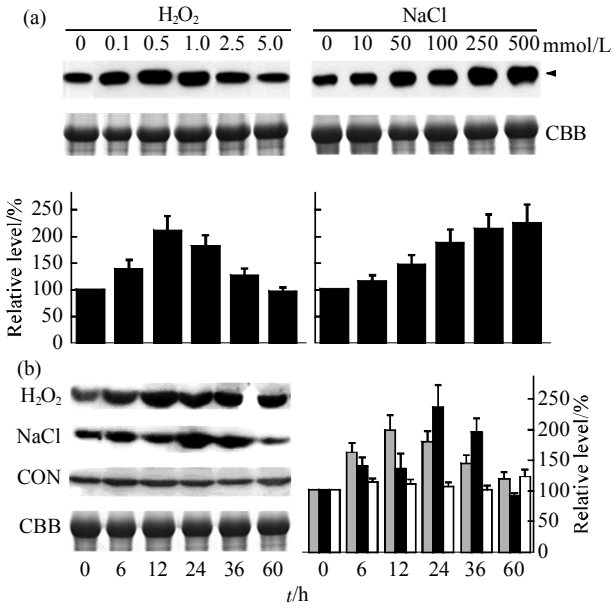

Fig. 3 Expression profiles of OsPHGPx induced by H_2O_2 and NaCl

(a) Effects of H_2O_2 and NaCl treatment concentrations on OsPHGPx expression. Rice leaves were exposed to 0 (CON), 0.1, 0.5, 1, 2.5 and 5 mmol/L H_2O_2 or 0 (CON), 10, 50, 100, 250 and 500 mmol/L NaCl for 24 h, respectively. (b) Effects of H_2O_2 and NaCl treatment time on OsPHGPx expression. Rice leaves ware treated with 0.5 mmol/L H_2O_2, 500 mmol/L NaCl or H_2O (CON), respectively. Total protein (50 μg/lane) was extracted at the times indicated in the figure (0, 6, 12, 24, 36 and 60 h, respectively). The arrowhead indicated the OsPHGPx protein. CBB stained proteins (RuBisCO bands) were shown to verify equal loading. The relative values of expression intensity were indicated at the bottom for (a) and at the right for (b). All the values represented the $\bar{x} \pm s$ of relative OsPHGPx level in three independent experiments. ▨: H_2O_2; ■: NaCl; □: CON.

2.4 Effect of DMTU on OsPHGPx expression under H_2O_2 and NaCl stresses

As reported previously, NaCl induced the accumulation of H_2O_2 in rice[20, 21]. Although we have shown that NaCl and H_2O_2 both effectively up-regulated the OsPHGPx expression, it was not clear whether NaCl-induced OsPHGPx expression was specific to NaCl or NaCl-enhanced H_2O_2. To separate the effect of NaCl from that of NaCl-enhanced H_2O_2,

we pretreated leaves with DMTU, a chemical trap for H_2O_2 [22]. For each treatment, leaves of rice seedlings were pretreated with 5 mmol/L DMTU or in parallel with H_2O for 12 h. As indicated in Figure 4, when rice leaves were pretreated with DMTU, H_2O_2-induced OsPHGPx expression was almost abolished completely and the abundance of OsPHGPx protein went down to normal level, whereas NaCl-induced OsPHGPx expression remained unchanged. Therefore, DMTU was effective in inhibiting OsPHGPx expression induced by H_2O_2, but not by NaCl.

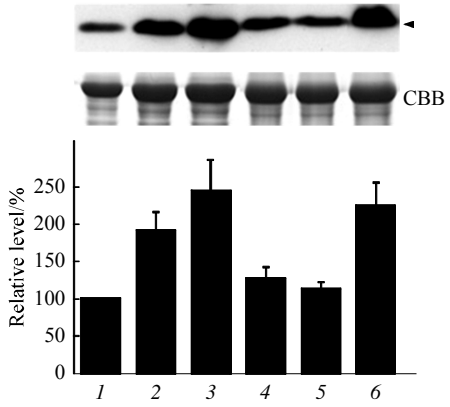

Fig. 4 Effect of DMTU on OsPHGPx expression in H_2O_2 and NaCl treatments

Rice leaves were pretreated with H_2O or 5 mmol/L DMTU for 12 h, and then transferred to H_2O, 0.5 mmol/L H_2O_2 or 500 mmol/L NaCl for 24 h, respectively. The arrowhead indicated the OsPHGPx protein. CBB stained proteins (RuBisCO bands) were shown to verify equal loading. H_2O treatment (CON) was used as normalized reference and its relative OsPHGPx level was set at 100%. The values represented the $\bar{x} \pm s$ of relative OsPHGPx level in three independent experiments. *1*: $H_2O \rightarrow H_2O$; *2*: $H_2O \rightarrow H_2O_2$; *3*: $H_2O \rightarrow$NaCl; *4*: DMTU $\rightarrow H_2O$; *5*: DMTU $\rightarrow H_2O_2$; *6*: DMTU→NaCl.

Despite the fact that salt stress produces H_2O_2, there are differences in the responses of H_2O_2- and NaCl-induced expression of OsPHGPx to DMTU. The failure of DMTU to reduce NaCl-induced expression suggests that other forms of ROS, rather than H_2O_2, or other signaling molecules probably mediate this signaling pathway activated by NaCl. There are also other evidences showing that NaCl-enhanced activities and gene expression of antioxidant enzymes are unaffected by H_2O_2 scavengers[17, 21]. However, allowing for different subcellular localizations of antioxidant enzymes and resulting local differences of H_2O_2 levels in diverse cellular compartments, we need further evidence to clarify whether H_2O_2 is involved in NaCl-induced expression of OsPHGPx or not.

In conclusion, our analysis revealed that OsPHGPx distribution was highly relevant to photosynthetic organs and its expression in leaves of rice seedlings was responsive to H_2O_2 and NaCl treatment in a time- and dose-dependent manner. Moreover, the different effects of DMTU on the OsPHGPx expression induced by H_2O_2 and NaCl suggested that H_2O_2 produced by salt stress might not be involved in the signaling pathway regulating OsPHGPx expression in salt stress. This work focused on protein expression profiles of OsPHGPx and provided more direct insight into its role in defense against oxidative damage in rice, which was advantageous to uncover the exact physiological function of this important enzyme in plant cells.

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水稻磷脂氢谷胱甘肽过氧化物酶在蛋白质水平上的组织和诱导表达特征 *

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摘要 蛋白质是生命活动的主要承担分子，了解蛋白质在有机体中的时空分布对于正确解析蛋白质的功能十分重要．磷脂氢谷胱甘肽过氧化物酶 (PHGPx) 是目前发现的唯一能够直接还原膜上脂类过氧化物的抗氧化酶，在保护生物膜免受过氧化损伤方面有着重要作用．采用 Western blot 技术，分析了水稻 PHGPx (OsPHGPx) 在水稻不同组织以及多种胁迫条件下的蛋白质表达特征．结果表明，OsPHGPx 在成熟水稻植株内主要分布于叶组织中，以旗叶中含量最高，而在水稻幼苗中则在茎及叶组织中均检测到较强的杂交信号．OsPHGPx 在幼苗中的表达受到 H_2O_2 和 NaCl 的强烈诱导，但植物激素对其表达的影响较弱．H_2O_2 和 NaCl 的诱导效果呈现出时间及剂量的相关性，当用 0.5 mmol/L H_2O_2 处理 12 h 或用 500 mmol/L NaCl 处理 24 h，此时 OsPHGPx 表达量达到最大值．对 H_2O_2 清除剂二甲基硫脲处理的水稻幼苗，外源 H_2O_2 的再处理并不能诱导 OsPHGPx 的表达，而 NaCl 的诱导效果并不受影响，说明 H_2O_2 可能并不介导 NaCl 诱导 OsPHGPx 的表达．这些结果为进一步研究 OsPHGPx 在水稻中生物学功能奠定了基础.

关键词 水稻，磷脂氢谷胱甘肽过氧化物酶，蛋白质表达谱，氧化胁迫，盐胁迫
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