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ORIGINAL PAPER

A Thioredoxin-Dependent Glutathione Peroxidase (OsGPX5) Is Required for Rice Normal Development and Salt Stress Tolerance

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Abstract Crops often suffer from oxidative stress due to changes of the environmental conditions. Glutathione peroxidases (GPXs) are a family of enzymes that protect cells against oxidative damage caused by generation of excessive reactive oxygen species (ROS) in vivo. Compared to animal GPXs, the precise physiological role of plant GPXs remained poorly understood, particularly that of endoplasmic reticulum (ER)-type GPXs. Rice OsGPX5 has been reported to have dual subcellular compartments (ER and chloroplast), suggesting that it might play important roles in vivo. In the present study, transcriptional expression pattern analysis of *OsGPX5* indicated that it displayed differential responses to various abiotic stresses or hormone treatments. Additionally, *OsGPX5* was ubiquitously expressed in most of the tissues and organs analyzed and was found to accumulate particularly high transcript abundance in green tissues. Similar to other plant GPX homologs, the purified recombinant OsGPX5 protein showed activities of thioredoxin-dependent peroxidase, catalyzing the reduction of hydrogen peroxide and organic hydroperoxides. Furthermore, disruption of *OsGPX5* via T-DNA insertion had adverse effects on the normal development of rice. The *OsGPX5* mutant line exhibited lower germination potential, shorter length of 6-day-old seedlings, and a smaller number of filled grains compared to wild-type plants. Moreover, the seedlings of *OsGPX5* mutant plants exhibited enhanced sensitivity to salt stress. Beyond being an efficient ROS and peroxide scavenger, *OsGPX5* has therefore been proposed to participate in the interaction between ER stress and redox homeostasis, which is essential for the normal development of rice under stress conditions.

Keywords Abiotic stress · Glutathione peroxidase · Oxidative stress · Rice · Reactive oxygen species · Thioredoxin

Abbreviations

cDNA	Complementary DNA
h	Hour
kb	Kilobase(s)
kDa	Kilodalton
min	Minute
NADPH	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
S	Second

Introduction

Reactive oxygen species (ROS), such as superoxide radical ($O^{2\bullet-}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), play central roles in cell signal transduction and plant

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development (Apel and Hirt 2004; Baxter et al. 2014). The production of ROS is generally enhanced when plants are exposed to environmental stresses. Excessive ROS in vivo can damage cellular components and macromolecules such as DNA, proteins, and lipids. Thus, to maintain ROS at optimal physiological concentrations, plants have evolved efficient non-enzymatic as well as enzymatic defense systems to protect cells from oxidative injuries. Non-enzymatic antioxidants are mainly composed of ascorbate, glutathione, and tocopherols, while antioxidant enzymes include superoxide dismutases (SODs), catalase (CAT), glutathione peroxidases (GPXs), and other members of the diverse superfamily of peroxidase enzymes (Foyer and Noctor 2009).

Plant GPX family enzymes catalyze the reduction of H_2O_2 or organic hydroperoxides to H_2O and respective alcohols using reduced glutathione (GSH) or thioredoxin (Trx) as electron donors (Herbette et al. 2007; Brigelius-Flohé and Maiorino 2013). Since plant GPXs can reduce peroxides much more efficiently by using Trx than GSH, they are often classified into the fifth subgroup of thioredoxin peroxidases (peroxiredoxins (Prx)) (Iqbal et al. 2006; Navrot et al. 2006; Herbette et al. 2007). During the last decade, many GPX gene family members have been cloned and characterized in higher plants, e.g., eight members in *Arabidopsis thaliana* (Milla et al. 2003; Chang et al. 2009; Gaber et al. 2012) and six in *Lotus japonicas* (Ramos et al. 2009). Within the rice genome, five GPX members (OsGPX1–OsGPX5) were identified and experimentally proved to be distributed in distinct subcellular compartments (Li et al. 2000; Kang et al. 2004; Passaia et al. 2013). OsGPX1 and OsGPX3 were localized within mitochondria, OsGPX2 in the cytosol, and OsGPX4 in chloroplasts. OsGPX5 was confirmed to target both in the endoplasmic reticulum and chloroplasts (Passaia et al. 2013).

As efficient ROS scavengers, the expressions of plant GPX genes and/or GPX enzyme activities were often induced by abiotic stresses such as salinity, heat, and H_2O_2 treatment (Milla et al. 2003; Fischer et al. 2006, 2009; Zhai et al. 2013; Wang et al. 2013; Gao et al. 2014; Kim et al. 2014). Publicly available microarray data and/or RT-qPCR analyses of rice *OsGPX* transcripts revealed that they had different stress-responsive, tissue-specific, and/or developmental expression profiles (Agrawal et al. 2002; Kang et al. 2004; Passaia et al. 2013). For example, *OsGPX1* and *OsGPX3* were significantly upregulated in both shoots and roots in response to most of the tested stresses, while *OsGPX2* and *OsGPX4* were only induced by drought and oxidative stress but downregulated by salinity, heat, and cold in shoot (Islam et al. 2015).

In a mammalian system, GPXs are central components of antioxidant metabolism and play crucial roles in the repair of biomembranes. To date, eight types of GPXs have been identified in mammals (Toppo et al. 2008). Among them, GPX1–4 are selenoproteins with selenocysteine (Sec) in their catalytic center, while the remaining four GPXs (GPX5–8, except for human GPX6) are non-seleno GPXs, containing cysteine (Cys) instead of Sec at their catalytic sites (Tosatto et al. 2008; Mariotti et al. 2012). In addition to their antioxidant activity, diverse physiological roles have been elucidated in mammalian GPXs, such as balancing the H_2O_2 homeostasis in signaling cascades, regulation of apoptosis, and involvement in male fertility (Brigelius-Flohé and Maiorino 2013). Compared to animal GPXs, studies on the functional characterization of plant GPXs are lagging behind (Brigelius-Flohé and Maiorino 2013). Numerous reports showed that the function of these enzymes appears to be detoxification of H_2O_2 and organic hydroperoxides, thus protecting cells from oxidative damage (Chen et al. 2004; Herbette et al. 2007, 2011; Margis et al. 2008). In addition, plant GPXs have been suggested to act as ROS sensors and to play critical roles in the redox signal transduction (Miao et al. 2006; Passaia and Margis-Pinheiro 2015; Rouhier et al. 2015). However, the precise physiological functions of different GPXs remain poorly characterized in higher plants. To date, the biological functions of rice GPX isoenzymes have preliminarily been elucidated via transgenic approaches. Knockdown of mitochondrial *OsGPX1* or *OsGPX3* via RNA interference (RNAi) severely affected the normal growth and development of rice (Passaia et al. 2013, 2014a). Furthermore, chloroplastic *OsGPX4* has been shown to be required for in vitro rice regeneration, as no *OsGPX4*-silencing plants could be obtained from calli (Passaia et al. 2014a). These evidences indicated that GPXs were likely to be implicated in plant growth and development by regulation of cellular redox homeostasis (Faltin et al. 2010; Bela et al. 2015; Passaia and Margis-Pinheiro 2015).

It has been reported that rice OsGPX5 protein was not only confined in ER but also localized within the chloroplast (Passaia et al. 2013). To date, several plant ER-type GPX genes have been cloned (Milla et al. 2003; Navrot et al. 2006; Passaia et al. 2013), including rice *OsGPX5* and *Arabidopsis AtGPX2*; however, their precise function remains poorly understood compared to other types of plant *GPXs*. Thus, in the present study, we investigated the expression patterns and biochemical properties of *OsGPX5*. Furthermore, the effects of *OsGPX5* disruption on the rice development were evaluated under normal or salt stress conditions. These data will help to understand the role of ER-type GPXs as well as the mechanism of antioxidant defense systems in plant ER.

Material and Methods

Plant Material and Stress Treatments

Rice (*Oryza sativa* L. ssp. *indica* cv. 9311) seeds were germinated on wet filter paper. The hydroponically cultured seedlings (at 28 °C for 12 days) were transferred to solutions containing 20% (*w*/*v*) PEG6000 for the drought treatment and 200 mM NaCl for the salt stress treatment. The 12-day-old seedlings were transferred to incubators at 40 °C for the heat stress treatment (Islam et al. 2015). To mimic the submergence treatment, 12-day-old seedlings were submerged in water-filled tanks. The water depth was approximately 10 cm above the tips of seedlings to the water surface. Hormone treatments were accomplished by exposure of the seedlings to solutions supplemented with either 25 μM 6-benzylaminopurine (6BA), 50 μM indole-3-acetic acid (IAA), 100 μM abscisic acid (ABA), 100 μM gibberellin (GA), or 100 μM salicylic acid (SA), respectively (Agrawal et al. 2002; Li et al. 2000). Plants were collected at 0, 1, 3, 6, and 12 h after the various treatments and immediately frozen in liquid nitrogen and stored at −80 °C until further use.

Expression Analysis by RT-qPCR

Total RNA was extracted using the TRIzol reagent according to the manufacturer's instructions (Invitrogen®, Carlsbad, CA, USA), and RNA was treated with DNase I (Promega, Madison, USA) to remove all residual genomic DNA. RNA integrity and quantity were evaluated by electrophoresis on 1% agarose gel. First-strand cDNAs were synthesized by use of MMLV reverse transcriptase (Promega, Madison, USA). The expression patterns of *OsGPX5* were analyzed by RT-qPCR in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) using the iQ SYBR Green Supermix (Bio-Rad, USA) for 40 cycles (95 °C for 15 s, 60 °C for 15 s, 72 °C for 45 s), according to the instruction manual. Three reference genes (actin, EDF, and profilin-2) were selected to normalize the transcript level of *OsGPX5* (Wang et al. 2016). Data were derived from three independent replicates. The specific primer sequences are shown in Supplementary Table 1.

Expression and Purification of Recombinant OsGPX5

The coding sequence (CDS) of *OsGPX5* was generated by reverse transcription-PCR (RT-PCR) using the primer sets OsGPX5-F and OsGPX5-R (see Supplementary Table 1). After cutting with *Nde*I and *Xho*I, the *OsGPX5* PCR fragment was cloned into corresponding sites of pET-21b (Novagen, Darmstadt, Germany) with a His-tag fusion at the C-terminus. The resulting plasmid (pET-21b-*OsGPX5*) was transformed into the *Escherichia coli* BL21 (DE3) strain for expression. *E. coli* cells, carrying pET-*OsGPX5*, were cultured in 200-ml LB medium supplemented with 50 μg/ml kanamycin at 37 °C until the OD600 of the culture ranged between 0.5 and 0.8. The recombinant OsGPX5 expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were further incubated at 16 °C for 16 h. Cells were collected by centrifugation at 6000*g* for 10 min at 4 °C, then resuspended in 50 ml of Tris–HCl buffer (pH 8.0, containing 500 mM NaCl and 10 mM imidazole), and disrupted by sonication on ice. The soluble protein was harvested by centrifugation at 10,000*g* for 20 min at 4 °C, purified by His•Bind Resins (Novagen, Darmstadt, Germany), and desalted by 10-kDa ultracentrifugal filter (Millipore, USA) according to the manufacturer's protocol. The purified OsGPX5 protein was confirmed by electrophoresis on 12% SDS-PAGE. Protein concentrations were measured by use of the Bradford assay.

Enzyme Activity Assay

Glutathione-dependent peroxidase activity was measured by spectrophotometric monitoring of the absorbance decrease at 340 nm due to NADPH oxidation. The assay was performed with a glutathione peroxidase assay kit (Beyotime, Nanjing, China) in a 200 μl reaction mixture, containing 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 μM NADPH, 2 mM GSH, 1.0 U glutathione reductase (GR), and 10–50 μg of recombinant OsGPX5 protein (Gaber et al. 2012; Zhai et al. 2013). The reactions were started by addition of 0.5 mM H_2O_2 or hydroperoxides (either cumene hydroperoxide or t-buthylhydroperoxide). Trx-dependent activity was assayed in the same reaction mixture described above, except that GSH and GR were replaced with *E. coli* Trx (4 μM) and Trx reductase (0.2 U), respectively.

For the kinetic analysis of OsGPX5, 200 μl reaction mixture, which contained 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 μM NADPH, 4 μM Trx and Trx reductase, and 0–0.6 mM H_2O_2 or hydroperoxides (cumene hydroperoxide or t-buthylhydroperoxide), was pre-incubated at 30 °C for 5 min; then, the reaction was initiated by addition of 10 μg purified OsGPX5. Enzyme assays were performed in triplicate for each substrate concentration. The kinetic parameters were determined by hyperbolic regression analysis using the Prism program (GraphPad).

Seed Germination, Plant Development, and Salt Stress Treatments

The KO-*OsGPX5* and wild-type (WT) rice seeds (*O. sativa* L. cv. Dongjin) were germinated in H_2O at 28 °C for 6 days. The germination percentages were calculated every 24 h, and the shoot lengths of seedlings were measured after 6 or 12 days. To evaluate the development of the KO-*OsGPX5* plants, comparative analyses of the agronomic traits between KO-*OsGPX5* and WT rice plants were performed at their late

Table 1 Enzymatic activities of purified recombinant OsGPX5

Protein	Substrate	Enzymatic activity (V_{max}) (μmol min^{-1} mg $protein^{-1}$)		K_m (μM)
		GSH	Trx (*E. coli*)	
OsGPX5	t-BuOOH	0	8.7 ± 1.2	320.7 ± 96.2
	CumOOH	0	6.7 ± 0.4	144.8 ± 26.1
	H_2O_2	0	13.1 ± 1.7	204.6 ± 62.1

The GPX activities for H_2O_2, cumene hydroperoxide (CumOOH), and t-buthylhydroperoxide (t-BuOOH) were assayed using GSH, or *E. coli* Trx, as a reducing agent. The procedures are described in the "Material and Methods" section

reproductive stages. For the salt stress treatment, seeds of KO-*OsGPX5* or WT rice were germinated at 28 °C in different concentrations of NaCl solutions (ranging from 0 to 20 mM), respectively. The root and shoot lengths of the resulting seedlings were measured after 12 days of germination. Three biological replicates were analyzed per experiment.

Results

Expression Profile of OsGPX5

Since several plant GPX-encoding genes can be induced in response to various abiotic stresses (Milla et al. 2003; Navrot et al. 2006; Islam et al. 2015), we employed RT-qPCR to investigate the transcriptional expression profiles of *OsGPX5* under different stress conditions or hormone treatments. Firstly, the integrity and quantity of RNA were evaluated via RNA gel, and the melting curve of *OsGPX5* was obtained to confirm the unique fragment amplification in RT-qPCR analyses (Supplementary Fig. S1). Moreover, as shown in Fig. 1a, the transcript level of *OsGPX5* was markedly increased and peaked at 6 h after submergence stress. The *OsGPX5* mRNA level was only weakly upregulated in response to heat; however, it was not significantly changed when seedlings were subjected to salt stress. In addition, exposure of rice seedlings to PEG caused a continuous decrease of *OsGPX5* within 6 h and recovered to normal level after 12 h. On the other hand, *OsGPX5* was upregulated in response to all hormone treatments but exhibited distinct responsive patterns (Fig. 1b). For instance, the *OsGPX5* transcript was strongly induced by GA or SA following a 3-h treatment and reached maximum levels at 12 and 6 h, respectively. For ABA treatment, *OsGPX5* was activated and peaked within 3 h, approximately fourfold of the original level, and then declined to a normal level after 6 h. However, when rice seedlings were exposed to IAA or 6BA, only a weak induction of the *OsGPX5* transcript was observed within 1-h treatment and its expression sustained at this level for 12 h.

The expression profile of *OsGPX5* was also analyzed in various tissues and organs (Fig. 1c). *OsGPX5* was ubiquitously expressed in most of the analyzed tissues and organs, and had very high transcript abundance in green tissues such as node, internode, shoot, and flag leaf. While

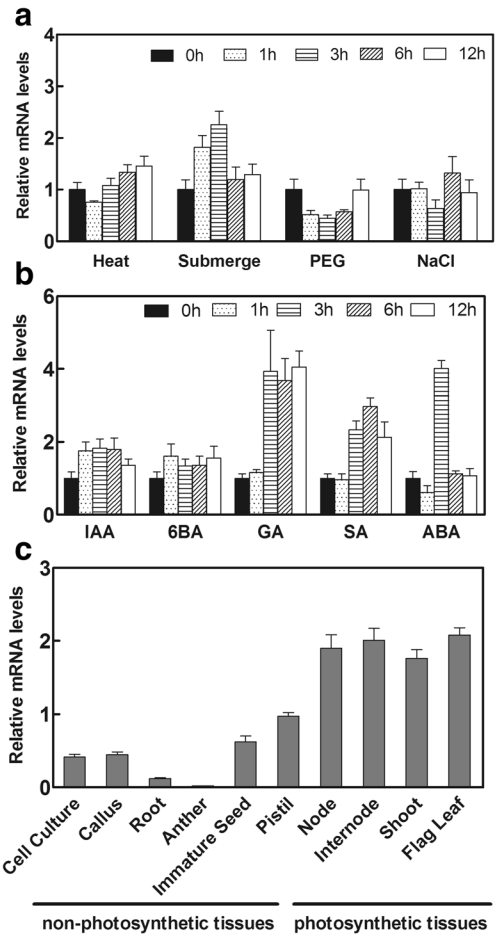

Fig. 1 Quantitative RT-PCR analysis of the relative abundance of rice *OsGPX5* transcript under different abiotic stress conditions (**a**), hormone treatments (**b**), and in various tissues/organs (**c**). Twelve-day-old seedlings were subjected to 20% (*w*/*v*) PEG6000 to simulate drought stress, 200 mM NaCl to simulate salt stress, and 40 °C to simulate heat stress. For hormone treatments, 12-day seedlings were transferred to solutions supplemented with 25 μM 6-benzylaminopurine (6BA), 50 μM indole-3-acetic acid (IAA), 100 μM abscisic acid (ABA), 100 μM gibberellin (GA), or 100 μM salicylic acid (SA), respectively. Data represent the means ± SD (standard deviation) of three independent biological replicates

OsGPX5 exhibited moderate expression levels in cell culture, callus, pistil, immature seeds, and root, it was almost undetectable in anther.

Thioredoxin-Dependent Peroxidase Activity of OsGPX5

To analyze the enzymatic properties of rice GPXs, we produced recombinant OsGPX5 protein fused with a histidine (His)-tag in *E. coli*. SDS-PAGE analysis of the soluble proteins from IPTG-induced *E. coli* cells expressing OsGPX5 showed a strong expression of recombinant protein (Fig. 2). The molecular mass of this fusion protein was approximately 23 kDa, which was in agreement with the theoretically predicted molecular mass of OsGPX5 plus the His-tag.

The peroxidase activity of purified recombinant OsGPX5 was measured using GSH as a reducing agent and cumene hydroperoxide (CumOOH), t-buthylhydroperoxide (t-BOOH), or H_2O_2 as electron acceptor (Table 1). No enzymatic activity was detected under these conditions. However, OsGPX5 was able to rapidly reduce H_2O_2 and organic hydroperoxides with Trx as a reducing agent, indicating it to be a Trx-dependent peroxidase. The V_{max} value of OsGPX5 toward H_2O_2 was 13.13 ± 1.75 μmol min^{-1} mg $protein^{-1}$, which was approximately 1.51–1.96-fold higher than those for CumOOH (V_{max} = 6.7 ± 0.4) and t-BuOOH (V_{max} = 8.7 ± 1.2 μmol min^{-1} mg $protein^{-1}$). The K_m value for CumOOH at a fixed concentration of 4 μM Trx was 144.8 ± 26.1 μM, which was lower than the K_m values for t-BOOH (K_m = 320.7 ± 96.2 μM) and H_2O_2 (K_m = 204.6 ± 62.1 μM).

Disruption of *OsGPX5* Affects Seed Germination and Plant Development

To investigate the physiological role of *OsGPX5*, a T-DNA insertion mutant line (KO-*OsGPX5*, PFG_1B-11,108) was obtained from the Rice Functional Genomic Express Database (http://signal.salk.edu/cgi-bin/RiceGE) (Jeon et al. 2000). For characterization of the T-DNA insertion line, PCR was performed by using a set of three primers (P1, P2, and P3; see Supplementary Table 1) with genomic DNA as template. The results indicated that the KO-*OsGPX5* was a homozygous mutant line (Fig. S2b). Sequencing of the T-DNA insertion sites of KO-*OsGPX5* revealed that the insertion site was located approximately 96 bp upstream of the translation start codon (ATG) of *OsGPX5* (*Os11g0284900*). By RT-PCR analysis, the expression of *OsGPX5* could not be detected in KO-*OsGPX5* (Fig. S2c).

As shown in Fig. 3, knockout of *OsGPX5* by T-DNA insertion had adverse effects on the normal development of rice. The seed germination potential of KO-*OsGPX5* lagged far behind that of WT plants during the first 72 h of incubation (Fig. 3a). For instance, the germination index was only about

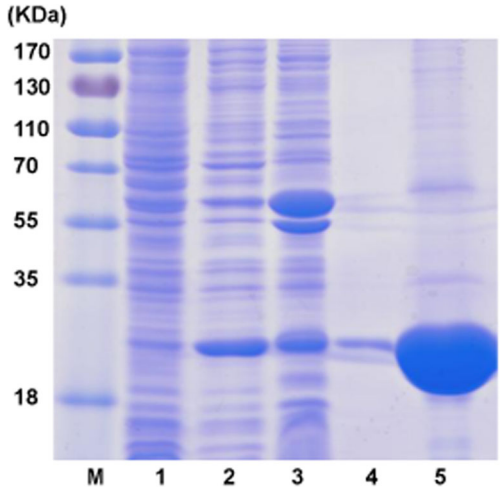

Fig. 2 SDS-PAGE examination of expression and purification of recombinant OsGPX5 protein. Molecular mass protein standard (*M*), total proteins (12 μg) of *E. coli* cells harboring pET-21b-*OsGPX5* prior to induction (*1*), total proteins (10 μg) of *E. coli* cells induced by IPTG for 16 h (*2*), soluble proteins (10 μg) of the induced cells (*3*), recombinant OsGPX5 protein (1 μg) purified with His-Bind Purification Kit (*4*), and purified OsGPX5 protein (10 μg) desalted by ultracentrifugal filter (Millipore) (*5*)

24% after 48 h of incubation in KO-*OsGPX5* plants, whereas it reached 92% in WT plants. However, its germination rate reached up to approximately 94% after 144 h of incubation, which has no significant difference with WT plants. Furthermore, the growth of KO-*OsGPX5* seedlings was significantly inhibited compared to that of WT plants within 6 days (144 h) as judged by their shoot length, while it appeared not to be affected after 12 days of germination (Fig. 3b). The impaired development of KO-*OsGPX5* at the early seedling stage was likely attributed to its lower germination potential during the early stage of germination process.

For further functional characterization of the *OsGPX5*, the agronomic traits of the KO-*OsGPX5* rice plants were assessed during two growing seasons (2013–2014) at different locations (Wuhan and Haikou) (Table 2). KO-*OsGPX5* had a significantly reduced shoot length compared to WT plants (Fig. 3c). Additionally, panicle length, number of branches, and thousand-seed weight in KO-OsGPX5 plants were also shorter or lower compared to WT plants. Although the total seed number displayed no significant difference between KO-*OsGPX5* and WT plants, the filled grains and seed-setting rates of KO-*OsGPX5* plants were much lower in comparison to WT plants (Table 2 and Fig. 3d).

OsGPX5-Disruption Plants Exhibit Enhanced Sensitivity to Salt Stress

GPXs genes are important for the detoxification of ROS that form during environmental stresses, and they often conferred enhanced stress tolerance when plants encounter adverse

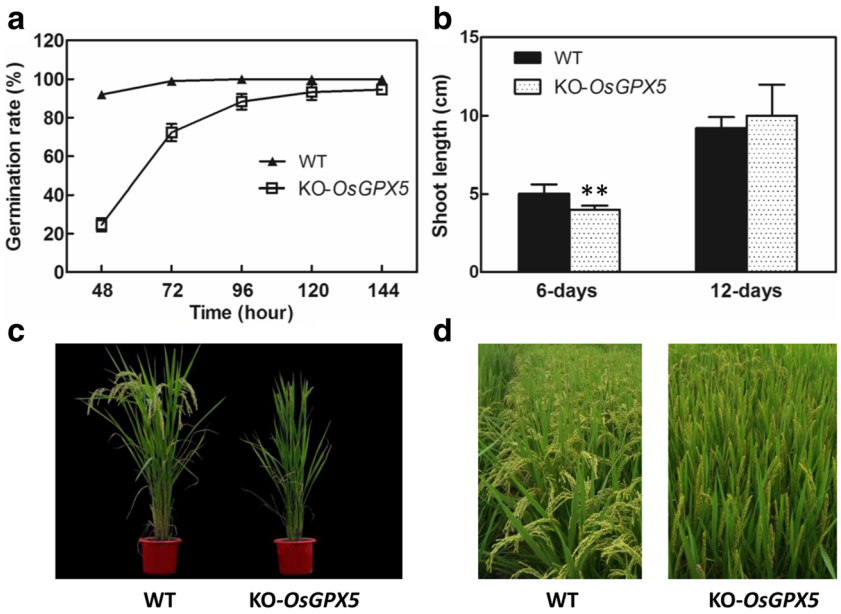

Fig. 3 Disruption of *OsGPX5* impairs the normal development of rice. Seed germination potential (**a**); shoot length of 6- or 12-day-old seedlings (**b**); and the phenotypes of KO-*OsGPX5* and wild-type (WT) rice at their late reproductive stages, which were cultivated either in greenhouse (**c**) or field (**d**) conditions. *Values* represent the means ± SD of three independent biological replicates. *Asterisks* indicate the significant difference (**$P < 0.01$) relative to WT

conditions, such as salinity, drought, and heat (Yoshimura et al. 2004; Gaber et al. 2006; Navrot et al. 2006; Ramos et al. 2009; Zhai et al. 2013). To determine the role of *OsGPX5* under stressful conditions, KO-*OsGPX5* seeds were hydroponically grown at different concentrations of NaCl solution (0–20 mM) for 12 days. When subjected to NaCl solutions with low concentration (0–5 mM), *OsGPX5*-disrupted seedlings had similar phenotypes to WT plants after 12 days of germination (Fig. 4). However, the growth of both KO-*OsGPX5* and WT seedlings was inhibited in higher concentrated NaCl solutions (10–20 mM), and the insertion line was affected to a larger extent by the salt stress. For example, KO-*OsGPX5* seedlings had shorter shoot and root lengths than WT plants under 10 mM NaCl. When KO-*OsGPX5* seedlings were exposed to 15 and 20 mM NaCl solutions, mutant growth was significantly inhibited and their shoots were much shorter compared to WT. Moreover, the elongation of primary roots of KO-*OsGPX5* seedlings was more severely inhibited than in WT under these conditions (15–20 mM). The root length of mutants was only about half of that of WT plants, and almost no lateral roots were observed on the primary roots (Fig. 4c). These results indicate that disruption of *OsGPX5* resulted in an enhanced sensitivity to salt stress during early seedling growth of rice.

Discussion

Rice, one of the most important staple foods, often encounters oxidative stress due to changes of environmental conditions during the entire growth stage. Plant GPXs are considered as

Table 2 Main agronomic traits of *OsGPX5*-disruption and wild-type (WT) rice plants

	WT[a]	KO-*OsGPX5*[a]	WT[b]	KO-*OsGPX5*[b]
Shoot length (cm)	86.0 ± 0.8	73.3 ± 2.4**	71.5 ± 3.3	64.0 ± 2.1*
Tiller number per hill	12.3 ± 1.7	10.1 ± 2.1	14.0 ± 2.0	15.0 ± 2.1
Panicle length (cm)	17.6 ± 0.4	16.0 ± 0.4**	13.1 ± 0.8	11.0 ± 0.5*
Primary branches	9.8 ± 0.4	10.0 ± 0.7	6.4 ± 0.6	5.3 ± 0.3*
Total seeds per hill	1041.0 ± 191.1	783.3 ± 200.9	756.5 ± 196.0	590.0 ± 110.5
The filled grains	594.3 ± 144.7	303.2 ± 161.5*	338.2 ± 89.6	61.25 ± 28.8*
Seed-setting rate (%)	57.5 ± 13.9	37.8 ± 13.7*	45.22 ± 6.3	10.12 ± 3.4**
Thousand-seed weight	24.9 ± 0.1	22.7 ± 1.4*	24.5 ± 3.4	19.69 ± 0.84*

Crops were planted either in Wuhan or Haikou and harvested at the late reproductive stage to evaluate their agronomic traits. Data are means of four replicates ± SD. Asterisks represent the significant differences (*$P < 0.05$, **$P < 0.001$) between WT plants and mutant lines at the same location according to the *t* test

[a] Crops were planted in Wuhan in July 2013 and harvested in October 2013

[b] Crops were planted in Haikou in December 2013 and harvested in March 2014

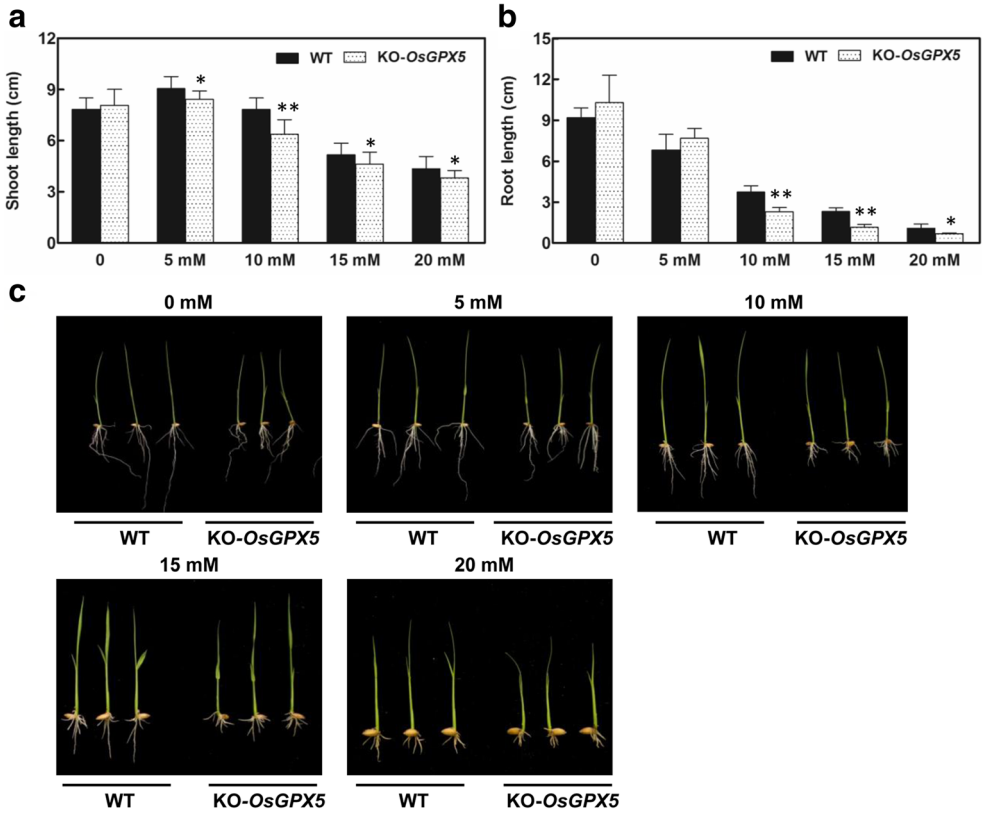

Fig. 4 Responses of wild-type and KO-*OsGPX5* mutant plants to salt treatment. Shoot lengths (**a**) and root lengths (**b**) of WT and KO-*OsGPX5* seedlings grown on H_2O containing different concentrations of NaCl 12 days after germination. **c** Phenotypes of wild-type and KO-*OsGPX5* seedlings grown on H_2O with the addition of 0–20 mM NaCl 12 days after germination. *Values* represent the means ± SD of three independent biological replicates. *Asterisks* indicate a significant difference (*$P < 0.05$, **$P < 0.01$) relative to WT

the main defense strategy to prevent cell membranes from oxidative injuries by scavenging organic hydroperoxides and H_2O_2. Several reports demonstrated that their expressions are regulated by various environmental stresses and/or different plant hormones (e.g. auxin, GA, ABA, SA, and JA) (Li et al. 2013; Zhai et al. 2013). In this study, *OsGPX5* transcript was also observed to be involved in the response to various abiotic stresses or hormone treatments (Fig. 1). Promoter analysis of the region approximately 1.5 kb upstream of *OsGPX5* (using PlantCARE) identified several important *cis*-regulatory elements and stress-responsive motifs (Islam et al. 2015). The presence of multiple stress-related motifs in the promoter region of GPX genes provides a clue to understand their transcriptional regulatory mechanism under different stresses. For instance, the ABA and drought-responsive elements (ABREs) and other ABA-related elements found in the *OsGPX5* promoter might be responsible for the gene responses to ABA or drought stress; CRT/DRE was likely related to the response to salt stress, while antioxidant response element (ARE) was involved in the oxidative stress response. Furthermore, the process of photosynthesis takes place in the chloroplasts, which are the highest production sites of ROS in vivo. Thus, it was suggested that the high level of tissue/organ-specific expression of *OsGPX5* in green tissues, such as shoots, and flag leaves, might be related to its chloroplastic localization, as OsGPX5 can efficiently detoxify excess ROS in situ.

In multiple alignments, the deduced amino acid sequence of rice OsGPX5 showed a high degree of similarity to mammalian phospholipid hydroperoxide GPX (PHGPX) but contained a Cys residue instead of a SeCys in their catalytic triad (Toppo et al. 2008). These non-selenium GPX-like proteins (NS-GPX) were reported to prefer Trx as a reductant to detoxify H_2O_2 and various organic hydroperoxides in vitro (Herbette et al. 2007). In the present study, OsGPX5 was also shown to be a Trx-dependent peroxidase by biochemical assay, and its enzyme kinetic parameters were comparable to those of other plant GPXs (Zhao et al. 2014; Matamoros et al. 2015).

Phylogenetic analysis of rice OsGPX5 with other plant GPXs revealed that OsGPX5 formed a separate clade with plant ER-type GPXs (Margis et al. 2008), including AtGPX2 and AtGPX3. Functional characterization of *Arabidopsis* GPXs via a T-DNA insertion approach showed a lower lateral root density (LRD) in the *Atgpx2* mutant line compared to WT (Col-0), whereas *Atgpx3* plants displayed a shorter primary root length as well as a lower LRD compared to Col-0 (Passaia et al. 2014b). In this study, knockdown of *OsGPX5* expression also had adverse effects on several important processes of rice development, including a lower seed germination potential, shorter shoot lengths of 6-day-old

seedlings, and a smaller number of filled grains at the late reproductive stages in comparison to WT. Plant cells have a complex network of antioxidant systems. In addition to ROS scavengers, GPXs can also act as redox sense proteins in the signal transduction, which are essential for the normal development of plants (Passaia and Margis-Pinheiro 2015). It has been reported that downregulation of rice mitochondrial *OsGPX1* via RNAi led to reduced shoot length and smaller number of seeds (Passaia et al. 2013). Furthermore, OsGPX3-knockdown plants displayed shorter root and shoot lengths and higher accumulation of H_2O_2 in mitochondria compared to WT (Passaia et al. 2014a). Thus, it is likely that a disruption of *OsGPX5* might disturb the in vivo redox homeostasis, thus causing impaired development of plants from germination to grain production in the insertion line.

Salt stress is one of the major limiting factors that affect yield and geographical distribution of numerous important crops. It has been reported that the *Arabidopsis Atgpx8* mutant exhibited an increased sensitivity to oxidative injuries induced by paraquat (Gaber et al. 2012), while overexpression of two wheat chloroplastic GPX genes in *Arabidopsis* conferred high tolerance to salt, H_2O_2, or ABA (Zhai et al. 2013). In this study, exposure of rice seedlings to NaCl did not significantly alter the expression level of *OsGPX5*, whereas reduction of its expression level via T-DNA insertion led to an enhanced sensitivity to salt stress for root elongation compared to WT plants. It seems that the expression of *OsGPX5* does not correlate with the phenotypic data for NaCl tolerance, which can be attributed to the complication of the antioxidant system and signal transduction cross talk in higher plants, particularly under stressful conditions (Zhu 2016). Several salt-responsive antioxidant enzymes can be induced by salinity stress, including other OsGPX members (Ozyigit et al. 2016). For example, it has been reported that the expression levels of *OsGPX1*, *OsGPX2*, and *OsGPX4* were all upregulated by NaCl in the roots of rice seedlings (Islam et al. 2015). In addition, knockdown of mitochondrial *OsGPX1* caused an intensive reduction of growth and seed yield under salt stress, which might be due to a photosynthetic impairment and involved a cross talk mechanism between mitochondria and chloroplast originated from redox changes owing to *OsGPX1* deficiency (Lima-Melo et al. 2016). Therefore, *OsGPX1* and *OsGPX5* are suggested to work coordinately, thus enabling rice to cope with salinity conditions.

In eukaryotic cells, the ER is responsible for proper folding and modification of proteins before they are transferred to their final destinations. Under environmental stresses (such as high salinity), many proteins could not be folded or misfolded because the demand on the cell for folding largely exceeds its folding capacity (Liu et al. 2007; Liu et al. 2011). Accumulated unfolded proteins cause ER stress, which in turn led to excessive ROS production and induced a series of antioxidant defense systems (Ozgur et al. 2014). Therefore, we propose that the ER-type OsGPX5 might participate in the interaction between ER stress and redox homeostasis, which is required for a normal development under stress conditions.

In conclusion, rice *OsGPX5* encodes a thioredoxin-dependent glutathione peroxidase, and its expression could be induced by various environmental stresses as well as hormone treatments. More than an efficient ROS and peroxide scavenger, *OsGPX5* is essential for rice development under both normal and stress conditions.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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