Two Wheat Glutathione Peroxidase Genes Whose Products Are Located in Chloroplasts Improve Salt and H$_2$O$_2$ Tolerances in *Arabidopsis*

Chao-Zeng Zhai$^{1,2}$*, Lei Zhao$^2$, Li-Juan Yin$^1$, Ming Chen$^1$, Qing-Yu Wang$^2$, Lian-Cheng Li$^1$, Zhao-Shi Xu$^{1,*}$, You-Zhi Ma$^{1,*}$

$^1$ Institute of Crop Science, Chinese Academy of Agricultural Sciences/National Key Facility for Crop Gene Resources and Genetic Improvement, Key Laboratory of Biology and Genetic Improvement of Triticeae Crops, Ministry of Agriculture, Beijing, China, $^2$ College of Plant Science, Jilin University, Changchun, China

**Abstract**

Oxidative stress caused by accumulation of reactive oxygen species (ROS) is capable of damaging effects on numerous cellular components. Glutathione peroxidases (GPXs, EC 1.11.1.9) are key enzymes of the antioxidant network in plants. In this study, *W69* and *W106*, two putative GPX genes, were obtained by *de novo* transcriptome sequencing of salt-treated wheat (*Triticum aestivum*) seedlings. The purified His-tag fusion proteins of *W69* and *W106* reduced H$_2$O$_2$ and t-butyl hydroperoxide (t-BHP) using glutathione (GSH) or thioredoxin (Trx) as an electron donor *in vitro*, showing their peroxidase activity toward H$_2$O$_2$ and toxic organic hydroperoxide. GFP fluorescence assays revealed that *W69* and *W106* are localized in chloroplasts. Quantitative real-time PCR (Q-RT-PCR) analysis showed that two GPXs were differentially responsive to salt, drought, H$_2$O$_2$, or ABA. Isolation of the *W69* and *W106* promoters revealed some cis-acting elements responding to abiotic stresses. Overexpression of *W69* and *W106* conferred strong tolerance to salt, H$_2$O$_2$, and ABA treatment in *Arabidopsis*. Moreover, the expression level of key regulatory genes (SOS1, RbohD and ABI1/ABI2) involved in salt, H$_2$O$_2$ and ABA signaling were altered in the transgenic plants. These findings suggest that *W69* and *W106* not only act as scavengers of H$_2$O$_2$ in controlling abiotic stress responses, but also play important roles in salt and ABA signaling.

**Introduction**

In higher plant cells, several metabolic processes (e.g. photosynthesis and respiration) and adverse environmental conditions (e.g. high-salt, drought, mechanical stimulation, chemical toxicity, pathogen infections and extreme temperatures) promote generation of reactive oxygen species (ROS), including hydroxyl radicals (OH$^-$), superoxide radicals (O$_2^-$), and hydrogen peroxide (H$_2$O$_2$) [1–5]. ROS are capable of causing damage to membrane lipids, proteins and nucleic acids and these highly reactive molecules are believed to be the major contributing factors in causing rapid cell damage [6]. Exogenous H$_2$O$_2$ induces the expression of defense genes as well as initiating programmed cell death in plants [7,8]. The steady-state level of ROS is mainly determined by the activity of antioxidant system in plant cells [9]. Several endogenous antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) play important roles in protecting plants cell from oxidative injury [10]. GPX is a key enzyme for scavenging H$_2$O$_2$ in plant cells [11].

As an antioxidant enzyme, GPX in mammals reduces H$_2$O$_2$ and organic hydroperoxides to water and correspondingly alcohols using glutathione (GSH) to protect cells from oxidative damage [12,13]. Based on their amino acid sequences, substrate specificity, and tissue localization, the GPX family in mammals can be divided into five classes, cytosolic GPX (GPX1), gastro-intestinal GPX (GPX2), plasma GPX (GPX3), phospholipid hydroperoxide GPX (PHGPX, also called GPX4), and seleno-independent epididymis GPX (GPX5) [12,14]. Among GPX isoforms, vertebrate GPX5 proteins lack the selenocysteine residue replaced by a cysteine residue in the catalytic site [15,16]. The selenocysteine residue in its presumed catalytic site is important for the catalytic activity of GPXs, but the replacement of selenocysteine by cysteine greatly reduces the activity of enzymes in animals [17]. PHGPX is an antioxidant selenoenzyme present in a variety of adult and embryonic tissues. It is the principal basis of a defense system that intimately participates in the repair of disrupted biomembranes by interacting directly with peroxidized phospholipids in biomembranes [18–21].

The plant GPX family shares highest sequence homology to animal PHGPXs, rather than to any of the other members of the animal GPX family [22–26]. These proteins from plants possess a Cys residue, rather than selenocys, in their presumed catalytic site, suggesting that GPX in plant is not a Se-dependent protein [27]. However, Amino acid replacement of the catalytic selenocys by Cys results in a relatively low activity in plants compared to...
homologous animal GPXs [28]. GPXs are ubiquitously occurring enzymes in subcellular organelles, including the cytosol, nucleus, chloroplast, mitochondria, and peroxisome, where GPXs use GSH or thioredoxin (Trx) as a reducing agent to reduce H$_2$O$_2$, organic hydroperoxide, and lipid hydroperoxides [2,12,29–32]. Plant GPX isoenzymes display an obvious preference to Trx [24,33].

Besides GPXs another group of enzymes capable of scavenging peroxides in plants have been studied extensively. It is known that certain plant glutathione-S-transferase (GST) displayed also strong GPX activity (GST/GPX) toward organic hydroperoxides [2,12,29–32]. Plant GPX isoenzymes display an obvious preference to Trx [24,33].

Plant GPXs are involved in responses to many abiotic stresses and hormones [27,35–37]. A rice GPX gene was strongly expressed in normal tissues of high photosynthetic efficiency as well as in tissues stimulated by oxidative stresses [38]. Overexpression of the tobacco GST/GPX gene confers salt and chilling tolerance due to enhanced ROS scavenging and reduces membrane damage in transgenic tobacco plants [10]. It was demonstrated that Arabidopsis GPX3 plays dual roles in H$_2$O$_2$ scavenging and ABA signal transduction [39]. Furthermore, chloroplastic GPXs in Arabidopsis play a role in cross talk between photooxidative stress and immune responses [5,40]. As has been reported, expressing a tomato PHGPx in tobacco could prevent Bax induced cell death, indicating the role of GPX in PCD in plant [41,42].

Wheat (Triticum aestivum L.) is one of the most important cereal crops in the world; however, ROS accumulation in cells induced

---

**Figure 1. Alignment of W69 and W106 with other GPXs from plants and their exon-intron structures.** (A) Alignment of GPX protein sequences with other species: Oryza sativa (OsGPX01, accession number: NC008397), Zea mays (ZmGPX01, AY542310) and Homo sapiens (HsGPX04, P36969). Gray background represents strictly conserved amino acids. Boxed sequences represent highly conserved domains (G1,G2,and G3). The SeCys residues of the mammalian PHGPXs (HsGPX04) are denoted by “U”, the three conserved Cys of these isoenzymes are marked by inverted triangles. (B) Exon-intron structure of the GPX homologs. The chromosomal structures of the GPXs were constructed by comparing mRNA sequences with their respective genomic sequences. The length of each exon (square) and intron (line) is given. The W69 gene starts with ATG at position 75 bp in exon 1 and ends with TAG at position 81 bp in exon 6; the W106 gene starts with ATG at position 56 bp in exon 1 and ends by TGA at position 29 bp in exon 6.

doi:10.1371/journal.pone.0073989.g001
by environmental stresses can cause severe oxidative damage to wheat growth and grain yield [43]. At present, there is no report on the role of GPXs in protecting wheat plants from oxidative damage. In this study, we isolated two wheat cDNAs encoding GPX proteins for the first time by de novo transcriptome assembly technology. The isoenzymatic characteristics, subcellular localization, and transcript accumulation of the recombinant protein when transgenic plants were subjected to different stresses were investigated.

Results

Isolation and characterization of W69 and W106

We generated an extensive expressed gene catalog for salt-treated wheat using Illumina mRNA Sequencing technology and de novo assembly (unpublished data). Two up-regulated putative GPX cDNA fragments, designated as W69 (GenBank Accession No. KF031945) and W106 (GenBank Accession No. KF031946), were selected from the total transcript-derived assembled unigenes. Full-length cDNA of W69 and W106 were cloned successfully from wheat cDNA by reverse transcription PCR (RT-PCR) using special primers sets. The predicted W69 protein consists of 214 amino acids with a calculated molecular mass of 21.3 kDa, and W106 protein consists of 211 amino acids and calculated molecular mass of 19.2 kDa. Multiple sequence alignment of W69 and W106 with other reported GPXs from animals and mammalian GPX sequences. Cysteine residues coded by UGU signature 2), and WNF(S/T)KF) existed within most plant and different species (Fig. 1A). Three completely conserved motifs GKVLLIVNASRCG (GPX signature 1), LAFPCNQ (GPX signature 2), and WNF/S/TKF) existed within most plant and mammalian GPX sequences. Cysteine residues coded by UGU located in the “GPX signature 1” are the specific structure of selenium-independent plant GPXs, whereas selenium-dependent HsGPXs from mammals possess a seleno-cysteine residue coded by UGA (Fig. 1A). A phylogenetic tree of GPXs from plants revealed that GST family can be classified into five classes, W69 and W106 belong to one group located to chloroplasts (Fig. 2B).

Isolation of the genomic sequences of the wheat GPXs revealed both W69 and W106 comprised six exons and five introns (Fig. 1B). All of the exon/intron splice junctions in W69 DNA sequence conform to the canonical GT/AG boundary [44], intron 3 of W106 gene has a GT/GC sequence.

To gain insight into the mechanism responsible for transcriptional regulation, we isolated a 1,862 bp promoter region upstream of the W69 ATG start codon, and 1,600 bp of W106 from genomic DNA using a PCR strategy. We searched for putative cis-acting elements in the promoter regions using the database Plant cis-acting Elements, PLACE (http://www.dna.affrc.go.jp/PLACE/) (Table 1). A number of regulatory elements responsive to drought, salt, low-temperature, and ABA were recognized, including ABRE, DRE, DRE/CRT, and DBP binding sequences. In addition, a gibberellin responsive element (GARE), and an ethylene responsive element (ERE) were identified. More importantly, the W69 and W106 promoter regions have antioxidant-responsive elements, ARE, which was found in plant and human promoters (Table 1).

Expression patterns of W69 and W106 under stress

The W69 and W106 promoter regions have multiple abiotic stress-responsive elements (Table 1). To assess responses to abiotic stresses, we quantitatively surveyed the transcript expressions of W69 and W106 using quantitative real-time PCR (Q-RT-PCR). As shown in Fig. 3, both transcripts began to respond to environmental stress at early stages, but exhibited different expression patterns.

Both W69 and W106 were rapidly activated to peak within 0.5 h after salt treatment, then declined to normal levels after 1 h, and were barely detectable within 24 h. Both were also rapidly induced by drought and ABA, reached a maximum level at 4 h and 1 h, respectively, and then declined. W69 was strongly induced by drought, but only weakly by ABA. In contrast, W106 was highly activated by ABA, but weakly by drought. In addition, W106 was immediately activated by H2O2 and absent after 6 h; however, the transcript of W69 was barely detectable over a 24 h treatment with H2O2. These results implied that the GPX subfamily may function mainly in oxidative responses induced by salt, H2O2, and ABA stresses, but with some divergence in roles between W69 and W106.

GPX activities of W69 and W106 in vitro

Overexpression of recombinant GPXs in E. coli BL21 cells was observed after IPTG induction. Samples of total proteins and purified soluble protein fractions were separated on SDS-PAGE. W69 (21 kDa) and W106 (19 kDa) were successfully expressed (Fig. 4A).

The purified protein was prepared for enzymatic analysis. Results showed that the recombinant isoforms could catalyze H2O2 and t-BHP using E. coli Trx or GSH as electron donors. However, the isoforms reduced hydroperoxides with Trx under a higher rate than with GSH, indicating that Trx was likely the favored electron donor (Fig. 4B). In contrast to t-BHP, the isoforms exhibited a higher affinity for H2O2 and GPX activities of W69 were detected at a higher level than W106 utilizing Trx, which is the main electron donor during redox reactions. As expected, the eET-28A-transformed control had no obvious GPX activity (Fig. 4B).

Chloroplast localization of W69 and W106

GPX proteins in plants were found in the cytosol, chloroplast, and mitochondria. The PSORT program for predicting subcellular localization, indicated that both W69 and W106 with a chloroplastic N-terminal transit peptide would most probably localize in chloroplasts. To investigate the biological activity of the W69 and W106 proteins, the full-length cDNAs of W69 and W106 were fused in frame with the green fluorescent protein (GFP) gene under the control of the CaMV 35S promoter, and transferred into wheat mesophyll protoplasts. Fluorescence of both W69-GFP and W106-GFP was specifically detected in chloroplasts, whereas the control expressing hGFP alone showed fluorescence distributed throughout the protoplasts (Fig. 5). Given their localization in chloroplasts, possible roles for W69 and W106 would be in protection of chloroplasts against oxidative damage induced by photosynthesis or external stresses.

Improved salt tolerance in transgenic Arabidopsis

GPX genes are important in detoxification of many cellular degradation products formed during oxidative stress. Additionally, GPXs could impart stress tolerance through their peroxidase activities [45]. To further investigate the biological functions of the two GPXs, two T3 generation transgenic Arabidopsis lines were chosen to examine effects on germination and growth under salt stress. Transgenic and wild-type Arabidopsis seeds were grown on MS medium for 3 d at 22 °C, and then transferred on MS medium containing different NaCl concentrations (50–200 mM). Transgenic W69 and W106 seedlings had similar phenotypes to wild-type seedlings under normal conditions. As expected, salt...
stress reduced the growth of both transgenic seedlings and wild-type seedlings to some extent (Fig. 6). At 150 mM NaCl, transgenic plants remained green, whereas wild-type plants displayed chlorosis and growth inhibition after 15 days. The root lengths of W69-7 lines were almost 4-fold those of wild-type plants (Fig. 6A). Furthermore, transgenic Arabidopsis plants showed higher germination rates than wild-type under high salt stress. Transgenic seeds germinated in up to 200 mM NaCl, whereas wild-type seeds failed to germinate under the same conditions (data not shown). Therefore, overexpression of W69 and W106 resulted in enhanced early tolerance to high salt stress.

Figure 2. Phylogenetic tree of W69 and W106 with other GPXs from plants. All GPXs proteins were clustered using ClustalX, and the phylogenetic tree was generated by MEGAS using the neighbor-joining algorithm. In addition to the sequences of W69 and W106, the tree was constructed using sequences from Arabidopsis thaliana: AtGPX01, At2g25080; AtGPX02, At2g31570; AtGPX03, At2g43350; AtGPX04, At2g48150; AtGPX05, At3g63080; AtGPX06, At4g11600; AtGPX07, At4g31870; AtGPX08, At1g63460. Brassica napus: BnGPX01, AD158654; BnGPX02-1, AC189433; BnGPX04, ES268656; BnGPX08, TC10360. Citrus sinensis: CsGPX06, X66377. Gossypium hirsutum: GhGPX01, A1729829; GhGPX04, DV849230; GhGPX05, DW496848; GhGPX08, DW518187. Helianthus annuus: HaGPX02, CX945161; HaGPX04, CX947851. Hevea brasiliensis: HbGPX02, EC609359. Hordeum vulgare, HvGPX02, AK357226. Lycopersicon esculentum: LeGPX01, B1934604; LeGPX02, AY301280; LeGPX08, DB689713. Medicago truncatula: MtGPX01, AC143339; MchGPX02, AF346906. Nicotiana tabacum: NtGPX01a, DV999781. Oriza sativa ssp japonica: OsGPX01, NC008397; OsGPX02, NC008396; OsGPX03, NC008395; OsGPX04, NC008399; OsGPX05, NC008404. Pisum sativum: PsGPX01, AJ000508. Populus trichocarpa: PtGPX08, EEE84340. Selaginella moellendorffii: SmGPX01a, DN838361. Sorghum bicolor: SbGPX01, AAT42166; Spinacia oleracea: SoGPX06, D63425. Triticum aestivum: TaGPX01, AJ010455; TaGPX03, JP2114946. Vitis vinifera: VvGPX08, EE064395. Zea mays: ZmGPX01, AY542310; ZmGPX04, EU971245.

doi:10.1371/journal.pone.0073989.g002

Two Wheat GPXs Improve Salt and H₂O₂ Tolerances

PLOS ONE | www.plosone.org 4 October 2013 | Volume 8 | Issue 10 | e73989
Table 1. Putative cis-acting elements in the wheat *W69* and *W106* promoters.

| Element                  | Promoter | Sequence     | Function                              | Reference |
|--------------------------|----------|--------------|---------------------------------------|-----------|
| ARE                      | W69, W106 | AGTGACNNNGC  | Antioxidant-responsive elements       | [76]      |
| ABRE                     | W69, W106 | ACGTG(G/T)C  | ABA and drought responsive elements   | [77]      |
| CBF                      | W69      | RYCGAC       | Dehydration responsive element        | [78]      |
| DRE                      | W69      | ACCGAC       | ABA and drought responsive elements   | [79]      |
| CRT/DRE                  | W69      | CCGAC        | Drought, high-salt and cold responsive elements | [79] |
| DPBF binding site        | W69, W106 | ACACNG      | ABA responsive and embryo specification elements | [80] |
| GT1GMSCAM4               | W69, W106 | GAAAAA      | Pathogen and salt responsive elements | [81]      |
| MYB recognition site     | W69, W106 | C/TAACNG/G  | ABA and drought responsive elements   | [82]      |
| MYC recognition site     | W69, W106 | CATGTG       | ABA and drought responsive elements   | [83]      |
| HSE                      | W69, W106 | AGAAAAATCG   | Heat shock responsive element         | [84]      |
| TC-rich repeats          | W69, W106 | ATTTCTTCA    | Element involved in defense and stress | [85]      |
| W-box                    | W69, W106 | TTGAC        | Wound and pathogen responsive elements | [86]      |
| ERE element              | W106     | ATTTCAA      | Ethylene responsive elements          | [85]      |
| GARE-motif               | W106     | AAACAGA      | Gibberellins responsive element       | [87]      |
| TGACG-motif              | W69, W106 | TGACG        | MeJA responsive elements              | [88]      |
| AuxRE                    | W69      | GGTCAT       | Auxin responsive elements             | [89]      |

doi:10.1371/journal.pone.0073989.t001

Figure 3. Expression patterns of *W69* and *W106* under various stress conditions, including salinity (A), drought (B), H$_2$O$_2$ (C), and ABA (D). Total RNA was isolated from leaves of wheat seedlings. The actin gene was used as an internal reference. Relative expression of *W69* and *W106* were normalized to the transcript abundances in untreated controls (normalized as 1). Error bars represent standard deviation (SD) among three biological replicates. Asterisks indicate a significant difference (*P*<0.05; Student’s t-test) relative to untreated control (at 0 hours). doi:10.1371/journal.pone.0073989.g003
Germination of W69 and W106-overexpressed seeds is insensitive to ABA

To investigate the effect of ABA on germination of W69- and W106-overexpressing seeds, we planted seeds on MS medium containing different ABA concentrations. As shown in Fig. 8A, there was no difference in seed germination between the wild-type and transgenic plants under normal conditions. At 0.5 μM ABA there was some inhibition of germination of both wild-type and transgenic seeds, but the latter were affected to a lesser extent. Approximately 42% of W69-7 overexpressed seeds and 39% of W106-2 overexpressed seeds developed cotyledons and became green compared with only 10% wild-type germination after 6 days (Fig. 8B). Addition of 1 μM ABA entirely arrested the emergence of cotyledons of both transgenic and wild-type seeds (Fig. 8B). These results indicated that GPX overexpression relieves the ABA-mediated inhibition during seed germination and early seedling growth.

W69 and W106 displayed changed stress-responsive gene expressions

SOS1, RbohD and ABI1/ABI2 are key regulators in response to salt, oxidative stress and ABA, respectively [39,46–48]. To determine whether stress response pathways were affected by the GPX overexpression, we used Q-RT-PCR to analyse these genes in wild-type and transgenic Arabidopsis. Overexpression of W69 and W106 indeed changed the transcript levels of these marker genes. For example, W69 activated SOS1 and RbohD expression to levels about 1.8- and 1.5-fold higher, respectively, than those in wild-type. In contrast, the expression levels of SOS1 and RbohD were only slightly higher in W106 seedlings than in wild-type. Furthermore, ABI1 and ABI2 transcript levels in both transgenic lines appeared to be slightly lower than in wild-type (Fig. 9). These findings suggest that these GPXs may be signaling components in the ROS, ABA and salt-response pathways.

Discussion

Animal GPXs are 20–22 kD monomer proteins, that can directly reduce phospholipid hydroperoxides, fatty acid hydroperoxides, and cholesterol hydroperoxides produced in peroxidized membranes [18]. Animal GPXs are considered crucial for protecting membranes from oxidative stress. Though GPX-like cDNAs had been found in a diversity of plants [22,23,38,49–51], their physiological functions in plants had not been clearly resolved. Plant GPXs belong to a small GPX multigenic subfamily involved in response to various environmental stresses and had been considered to be the main defense strategy against oxidative membrane damage by detoxifying organic hydroperoxides as well as H_{2}O_{2} [31,52–55].

In the present study, we describe the identification and function of two wheat GPXs, designated W69 and W106, that share relatively high levels of sequence similarity with other plant GPXs and human GPX04 (Fig. 1). Genomic sequences analysis of the two GPXs revealed that both comprise six exons and five introns. The nucleotide sequences and sizes of exons 2–5 of the isoenzymes genes were very similar, although the nucleotide sequences and sizes of the corresponding introns were highly divergent. These results provided evidence supporting tightly conserved exon-intron structures of GPX homologs across the plant kingdom [13]. In particular, there was a high degree of conservation among plant species in the lengths of exons 2–5, whereas the lengths of exons 1 and 6 were quite divergent [56]. The homologous human HgGPX04 gene contains seven exons. Plant and mammalian GPXs have very divergent genomic organization patterns and analogous

We also examined tolerance to drought stress in the transgenic plants, but observed no detectable increases in tolerance (data not shown).

Transgenic Arabidopsis has increased tolerance to H_{2}O_{2}

Three-day-old seedlings of transgenic and wild-type Arabidopsis were transferred to MS medium plates supplemented with 1 mM H_{2}O_{2}. At 15 d, the seedlings of W69 and W106 grew vigorously and were larger than wild-type seedlings. At 1 mM H_{2}O_{2}, the transgenics had longer primary roots than wild-type plants. There were also significant increases in the number and total lengths of the lateral roots compared to wild-type (Fig. 7A). As shown in Fig. 7B, H_{2}O_{2} treatment significantly decreased the fresh and dry weights of transgenic and wild-type plants, but the transgenics were less affected by H_{2}O_{2} treatment than wild-type plants. In addition, leaves of wild-type plants tended to become yellow during growth under H_{2}O_{2} treatment whereas those of transgenic plants remained green (Fig. 7A). Thus the transgenic seedlings apparently had increased peroxide-scavenging capacity and enhanced tolerance to H_{2}O_{2}.
functions, reinforcing the hypothesis of an independent evolutionary scenario accompanying the structural diversification but functional similarity [13].

Salinity stress, mainly caused by NaCl and a serious threat to crop productivity, causes osmotic and ionic imbalances in cells [57]. On the other hand, salinity increases production and accumulation of ROS, which affects cell redox homeostasis and causes oxidative damage [58]. In the present work, we showed that overexpression of \( W69 \) and \( W106 \) increased the seed germination rate and boosted seedling growth in transgenic \( \textit{Arabidopsis} \) plants during exposure to salt stress conditions (Fig. 6). In addition, exposure of wheat seedlings to NaCl or \( \text{H}_2\text{O}_2 \) induced rapid accumulation of \( W69 \) and \( W106 \) transcripts (Fig. 3). Therefore, it was likely that increased mRNA accumulation in \( W69 \) and \( W106 \) was an early response in ROS reduction or a manifestation of adjustment to environmental stress [59]. \( \text{H}_2\text{O}_2 \) is a central signaling molecule in stress and wounding responses, pathogen defense, and regulation of the cell cycle and cell death. Exogenous applications of \( \text{H}_2\text{O}_2 \) promote the formation and development of adventitious roots in seedlings, but a relatively higher concentrations \( \text{H}_2\text{O}_2 \) and longer times of exposure lead to inhibitory effects on rooting [60,61]. Measurement of GPX activities showed that the two wheat GPXs can catalyze \( \text{H}_2\text{O}_2 \) or t-BHP \textit{in vitro}. High level of GPX involved in the scavenging of \( \text{H}_2\text{O}_2 \) in plants helps to protect against \( \text{H}_2\text{O}_2 \)-induced tissue injury and increases tolerance to oxidative damage induced by salt or ROS [62,63]. Under exogenous \( \text{H}_2\text{O}_2 \) treatment, we found that transgenic plants became stronger than wild-type. In addition, the transgenic seedlings had greater total lateral root lengths than wild-type and improved tolerance to ROS stress (Fig. 7). \( W69 \) and \( W106 \) were located in the chloroplasts (Fig. 5), cell components with the highest production sites of ROS [64,65]. These results indicated that overexpression of \( W69 \) and \( W106 \) conferred enhanced antioxidant capacity and protected plants against oxidative damage.

ABA is a phytohormone controlling seed dormancy and preventing seed germination during early embryogenesis. It also plays an essential role in stress response to drought, cold, and oxidative and osmotic stresses in the vegetative growth phase [66,67]. Some reports indicate that ABA can trigger the production of \( \text{H}_2\text{O}_2 \) [68–70]. \( \textit{Arabidopsis} \) seeds overexpressing \( W69 \) and \( W106 \) displayed higher germination rates than wild-type after ABA treatment, suggesting that transgenic seedlings have weakened ABA control due to the scavenging effects of \( \text{H}_2\text{O}_2 \) (Fig. 8). Therefore, \( W69 \) and \( W106 \) might play an important role in the ABA signal transduction. It was reported that mutation of \( \textit{AtGPX3} \) affected the expression of ABA-responsive genes and that the \( \textit{AtGPX3} \) gene might act as an oxidative signal transducer in ABA and drought stress signaling [71]. Q-RT-PCR indicated that overexpression of \( W69 \) and \( W106 \) also affected the transcriptional levels of key regulator genes involved in ABA, \( \text{H}_2\text{O}_2 \) and salt signaling. This was in agreement with the important role of the GPX gene family in responding to environment stresses.

Figure 5. Subcellular localization of \( W69 \) and \( W106 \) proteins. The wheat \( W69 \) and \( W106 \) GFP fusion proteins localize to the chloroplasts in transiently transformed wheat leaf cells. After 12 h incubation, GFP fluorescence was detected. (A–D) control (16318hGFP) fluorescence detection; (E–H) \( W69 \)-GFP fusion protein fluorescence detection; (I–L) \( W106 \)-GFP fusion protein fluorescence detection. (A, E and I) bright field; (B, F and J) confocal fluorescence; (C, G and K) chloroplast auto fluorescence; (D, H and L) overlay images. Scale bars = 10 \( \mu\text{m} \). Subcellular localization analysis was carried out four times with similar results.

doi:10.1371/journal.pone.0073989.g005
Analysis of upstream cis-regulating elements of W69 and W106 provided a way for us to understand how GPX expression was controlled. For instance, ABRE and other ABA-related elements (such as the DRE and DPBF binding sites) found in the promoters of W69 and W106 might be responsible for the responses of genes to ABA. The CRT/DRE and GT1GMS/CM4 elements present in the promoters of the two isoenzymes might be responsible for genetic responses to NaCl. More importantly, ARE, an antioxidant-responsive element in rat is also present in the promoters of the two isoenzymes, and might be related to genetic response to oxidative stress (Table 1). Collectively, W69 and W106 were not only participated directly in ROS signaling, but were also involved in ABA and salt signaling cascades.

In summary, overexpression of W69 and W106 in Arabidopsis seedlings provided protection from salinity stress during germination and seedling growth. This protective effect appeared to allow transgenic plants to retain high levels of metabolic activity and growth due to increased GPX expression and reduced levels of oxidative damage compared to wild-type seedlings. Our study should be beneficial for obtaining detailed insights into the complex function of plant GPXs, and for analyzing specialized physiological characteristics of this antioxidant system in other organisms [19]. The GPXs might be excellent candidates for genetic engineering of crop plants with improved salt tolerance.

Materials and Methods

Plant materials and stress treatments
Wheat (T. aestivum cv. Xiaobaimai) seedlings grown hydroponically at 25 °C for 10 d were subjected to various abiotic stresses. Seedlings were exposed to air on filter paper for rapid induction of water stress. To mimic salinity and ABA treatments, roots were submerged in 100 mM NaCl or 100 μM ABA, respectively. For hydrogen peroxide treatment, seedlings were transferred to solutions containing 0.1 mM H2O2. Materials were collected at 0, 1, 2, 4, 16, 12, and 24 h after various treatments. Harvested plants were dropped immediately into liquid nitrogen and stored at −80 °C for RNA extraction.

General bioinformation
Putative amino acid sequences used in this study were from GenBank databases. Conserved motifs were investigated by multiple alignments using DNAMAN version 6.0. Phylogenetic
trees were constructed with ClustalX using the neighbor-joining (NJ) method.

Quantitative real-time PCR

Expression patterns for the two GPX genes were measured by Quantitative real-time PCR (Q-RT-PCR), using the ABI Prism 7300 system (Applied Biosystems, USA). Total RNA was extracted from young leaves of 2-week-old wheat plants using Trizol reagent according to the manufacturer’s protocol (TianGen, Beijing, China) and DNase I digestions were applied (TaKaRa, Japan). First-strand cDNA was synthesized with AMV Reverse Transcriptase (TaKaRa, Japan). Each PCR was repeated three times in total volumes of 20 μl containing primer (4 μM), cDNA (40 ng) and 9 μl RealMasterMix (TianGen, Beijing, China). Quantification of the target gene expression under each stress was carried out by the relative 2−ΔΔCT method [72]. The actin gene was used as an internal control for normalization of template cDNA. The gene-specific primers were as follows: W69, 5′-ACAGAGGATTTCCGGAGGACT-3′ and 5′-AGGAGGACCTCCCCTTGTA-3′; W106, 5′-ACAAGGGGAAAGTCCTGCTT-3′ and W106-RTR 5′-CTGGTTCCTGTCCAGCAAAT-3′; wheat actin, 5′-CTCCCTTACACAAACCGC-3′ and 5′-TACCCAGGACCTCCCATTACAC-3′. Q-RT-PCR was also performed to analysis the expression of stress- and ABA-responsive genes, using 2-week-old transgenic and wild-type Arabidopsis seedlings. The actin gene was used as an internal control. The specific primers were as follows: RbohD (At5g47910), 5′-CCCTCAACACACACCCCTCCT-3′ and 5′-GGAAGGGGCTTGAGATG-3′; ABI1 (At4g26080), 5′-TAAAGGAGGGTGGATGA-3′ and 5′-CTGTATCCGGACGTTGACA-3′; ABI2 (At3g7050), 5′-TCCGGGCGTAAAGGAGAT-3′ and 5′-TTCCCTTTTTGCGAAAGCATT-3′; SOS1 (At2g01980): 5′-ACCGGCAGACTC-TAATGACG-3′ and 5′-CTCCGCTACTGTCAGTCA-3′;

| H₂O₂ treatment | Wild-type | Ox-W69-1 | Ox-W69-7 | Ox-W106-2 | Ox-W106-8 |
|----------------|-----------|----------|----------|-----------|----------|
| Primary root length (cm) | | | | | |
| 0 mM | 2.9 ± 0.3 | 3.0 ± 0.4 | 3.2 ± 0.2 | 3.2 ± 0.3 | 3.3 ± 0.2 |
| 1 mM | 1.5 ± 0.2 | 2.2 ± 0.1 | 2.4 ± 0.4 | 2.1 ± 0.3 | 1.7 ± 0.5 |
| Number of lateral roots | | | | | |
| 0 mM | 30 ± 2 | 32 ± 2 | 29 ± 2 | 29 ± 2 | 32 ± 3 |
| 1 mM | 24 ± 2 | 26 ± 3 | 24 ± 2 | 24 ± 4 | |
| Total lateral length (cm) | | | | | |
| 0 mM | 18.9 ± 2.4 | 22.4 ± 2.1 | 23.5 ± 1.2 | 17.6 ± 1.4 | 18.3 ± 2.4 |
| 1 mM | 1.5 ± 0.1 | 13.2 ± 0.2* | 15.4 ± 0.3* | 16.5 ± 0.2* | 14.3 ± 0.2* |
| Fresh weight (mg) | | | | | |
| 0 mM | 16.5 ± 2.6 | 17.0 ± 3.6 | 17.5 ± 1.9 | 16.8 ± 1.3 | 16.7 ± 1.1 |
| 1 mM | 8.2 ± 1.6 | 13.4 ± 2.1* | 14.1 ± 1.7* | 14.8 ± 1.3* | 14.6 ± 1.6* |
| Dry weight (mg) | | | | | |
| 0 mM | 1.5 ± 0.3 | 1.7 ± 0.4 | 1.7 ± 0.2 | 1.7 ± 0.3 | 1.6 ± 0.3 |
| 1 mM | 0.8 ± 0.3 | 1.5 ± 0.2* | 1.6 ± 0.1* | 1.6 ± 0.2* | 1.5 ± 0.1* |

Figure 7. Responses of the wild-type and transgenic plants to H₂O₂ treatment. (A) Phenotypes of wild-type, W69 and W106 seedlings under H₂O₂ treatment. Three-day-old seedlings of four transgenic lines and wild-type Arabidopsis were planted on MS media with added H₂O₂ (1 mM) for 15 d after germination. (B) Effect of exogenous H₂O₂ on biomass production and root development of wild-type, W69 and W106 seedlings. After growing in MS media with added H₂O₂ (1 mM) for 15 d after germination, seedlings were collected and analysed. Data are given for the length of the primary root, lateral root number, total lateral root length, seedling fresh weight and dry weight. Data are means ± SD of three independent experiments. Asterisks indicate a significant difference (*P < 0.05; Student’s t-test) relative to wild-type control. doi:10.1371/journal.pone.0073989.g007
Actin2 in Arabidopsis, 5'‐GGTAACATTGTGCTCAGTGGTG‐3' and 5'‐C GACCTTAATCTTCATGCTGC‐3'.

Detection of enzyme activities of the GPX isoenzymes

The two GPX cDNAs were amplified by reverse transcription PCR (RT-PCR) using the primer sets W69‐PET (5’‐TACGGATCCATGGGGGCGTCCGAATCT‐3' and 5’‐TAGCTCGAGAACCTCCAACAGCTTCTTGATG‐3') and W106‐PET (5’‐TACGGATCCATGGGTGCGGCAGAGT‐3' and 5’‐TAGCTCGAGAACCTCCAACAGCTTCTTGATG‐3'). The PCR product was cut by BamHI and XhoI and then subcloned into corresponding sites of pET‐28a (Novagen) with a Histidine‐tag at the N‐terminus. The fusion plasmids and empty vector (PET‐28a) were transformed into E. coli BL21 (DE3) strain.

E. coli cells carrying pET‐W69, pET‐W106, and PET‐28a were grown in 40 ml LB medium containing 50 mg/ml kanamycin at 37°C and induced with 0.8 mM isopropyl‐1‐thio‐bD‐galactopyranoside (IPTG) at 22°C. Cells were extracted at 4 h and were collected by centrifugation at 3000 g for 10 min, resuspended in pre‐chilled sodium phosphate buffer (14 mM NaCl, 0.27 mM KCl, mM Na₂HPO₄, 0.18 mM KH₂PO₄, 1 mM EDTA-2Na and 1% polyvinylpyrrolidone, pH 7.0), and disrupted by sonication. His‐tag fusion pET‐W69/pET‐W106/and/PET‐28a proteins were purified using Ni‐NTA Agarose (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

Figure 8. Responses of the wild‐type and transgenic plants to ABA treatment. (A) Phenotypes of wild‐type, W69 and W106 seedlings under ABA treatment. Seedlings of mixed transgenic lines and wild‐type Arabidopsis were planted on MS media for 15 d. (B) Statistical analysis of seed germination on MS plates with added ABA (0.8 µM). 60 transgenic and wild‐type seeds were plated on MS plates containing 0.8 µM ABA. Asterisks indicate a significant difference (P < 0.05; Student's t‐test) relative to wild‐type control.

doi:10.1371/journal.pone.0073989.g008

Figure 9. Expression effects of ABA‐ and stress‐responsive genes in W69 and W106 Arabidopsis using Q‐RT‐PCR. Total RNA was isolated from leaves of 2‐week‐old Arabidopsis seedlings. The actin2 gene was used as an internal reference. Relative expression of W69 and W106 were normalized to the transcript abundances in wild‐type controls (normalized as 1). Error bars represent standard deviation among three biological replicates. Asterisks indicate a significant difference (P < 0.05; Student's t‐test) relative to wild‐type control.

doi:10.1371/journal.pone.0073989.g009
Protein concentrations were quantified using the Bradford method. Purified proteins from each sample were loaded for sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Enzyme activity was measured spectrophotometrically using a Glutathione Peroxidase Assay Kit (Biotium systems, USA) with minor modifications. E. coli Trx and t-BHP were purchased from Sigma Aldrich (St. Louis, MO, USA). The reaction was started by addition of H$_2$O$_2$ or t-butyl hydroperoxide. The absorbance was immediately read at OD$_{340}$ nm and again at 4 min using a Perkin-Elmer Lambda 35 UV/VIS spectrometer (Perkin-Elmer Instruments, USA). The unit activity (U/L) is the amount of GPX that produces 1 mM of oxidized glutathione (GSSG) per min at pH 7.6 and room temperature.

Transient expression of green fluorescent protein-fused GPXs

Green fluorescent protein (GFP)-fused GPXs, coexpression plasmids were constructed for the expression studies. The full open reading frames of the W69 and W106 genes were respectively cloned into 16318hGFP vectors, fused with the GFP reporter gene under the control of the 35S promoter [73]. The recombination W69-hGFP and W106-hGFP fusion plasmids were transformed to common wheat mesophyll protoplasts by the PEG-mediated method [74]. The expression of fusion proteins was monitored after 12 h of incubation in a dark room, and images were captured under a laser scanning confocal microscope (Zeiss LSM700, Germany). GFP fluorescence was collected in the 500–570 nm wavelength range. For chloroplast auto fluorescence, the wavelength range was 630–700 nm.

Generation of transgenic Arabidopsis lines with GPX genes

Fragments of W69 and W106 were separately ligated into the modified pB121 vector under control of the CaMV 35S promoter [75]. Kanamycin-resistant Arabidopsis transformants carrying W69 or W106 were generated using the vacuum infiltration method. Transformed plants were cultured on Murashige and Skoog (MS) medium containing 0.8% agar and 50 μM Kanamycin in a day/night regime of 16/8 h under white light (50 photons m$^{-2}$s$^{-1}$) at 22°C for 2 weeks and were then transferred to soil. The T3 generation seeds obtained were used for further analysis.

Stress treatments

Seeds of transgenic (T3 generation) and wild-type Arabidopsis plants were grown on 10×10 cm MS agar plates. They were routinely kept for 3 days in the dark at 4°C to break dormancy and transferred to a tissue culture room under a day/night of 16/8 h under white light (50 photons m$^{-2}$s$^{-1}$) at 22°C for 3 d.

For salt treatment, 7-day-old seedlings were transferred to MS agar plates containing 150 mM NaCl for 7 days. For H$_2$O$_2$ treatment, the plants were placed on MS agar plates supplemented with H$_2$O$_2$ (1–5 mM) for 7 days. For ABA treatment, the seeds of transgenic and wild-type Arabidopsis were grown on MS agar plates supplemented with 0.5–1 μM ABA for 7–14 days. Seeds were considered germinated when radicles had completely emerged from the seed coat.

The root lengths of Arabidopsis were measured with a ruler, and lateral roots were counted and measured with a dissection microscope. The fresh weight was measured on an analytical balance immediately after plant harvest. The samples used for dry weight determinations were measured after 48 h at 70°C.

Acknowledgments

We are grateful to Dr RA McIntosh (Plant Breeding Institute, University of Sydney) for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: ZSX YZM. Performed the experiments: CZZ LZ LJY. Analyzed the data: ZSX. Contributed reagents/materials/analysis tools: MC QYW LCL. Wrote the paper: ZSX CZZ.

References

1. Bartoli CG, Simontacchi M, Tambussi E, Beltrano J, Montaldi E, et al. (1999) Drought and water-deficient oxidative stress. Effect on antioxidant content in Triticum aestivum L. leaves. J Exp Bot 50: 301–303.

2. Rodriguez MM, Maurer A, Rodriguez HA, Gustafson JP (2003) Glutathione peroxidases in Arabidopsis are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. Plant J 36: 602–615.

3. Mitler R, Vanderwoude S, Golemy M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9: 490–498.

4. Victor VM, Rocha M, De la Fuente M (2004) Immune cells: free radicals and antioxidants in sepsis. Int Immunopharmacol 4: 327–347.

5. Chang CC, Sliski J, Jordi L, Sorokav A, Melzer M, et al. (2009) Arabidopsis chloroplastic glutathione peroxidases play a role in cross talk between photosynthetic stress and immune responses. Plant Physiol 150: 670–683.

6. McHardy JM (2000) The evolution of free radicals and oxidative stress. Am J Med 109: 652–659.

7. Levine A, Trehahken R, Dixon R, Lamb C (1994) H$_2$O$_2$ from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79: 583–593.

8. Desikan R, Reynolds A, Hancock JT, Nell SJ (1998) Harpin and hydrogen peroxide both initiate programmed cell death but have different effects on defence gene expression in Arabidopsis suspension cultures.

9. Depege N, Varenne M, Boyer N (2000) Induction of oxidative stress and GPX-like protein activation in tomato plants after mechanical stimulation. Physiol Plantarum: 209–214.

10. Ruxa VP, Lodhi SA, Garrett DK, Mahan JR, Allen RD (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. Plant Cell Physiol 41: 1229–1234.

11. Aitken RJ (2009) Glutathione peroxidases. Methods Enzymol 252: 38–53.

12. Ursini F, Maiorino M, Brigelius-Flohe R, Aumann KD, Roveri A, et al. (1995) Diversity of glutathione peroxidases. Methods Enzymol 252: 38–53.

13. Margis R, Dunand C, Teixeira FK, Margis-Pinheiro M (2008) Glutathione peroxidase family-an evolutionary overview. PloS Biol 6: 3959–3976.

14. Kühn H, Borchert A (2002) Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. Free Radic Biol Med 33: 154–172.

15. Vernet P, Aitken RJ, Drevet JR (2004) Antioxidant strategies in the epiphyllosis. Mol Cell Endocrinol 216: 31–39.

16. Aitken RJ (2009) Gpx2 protects the family jewels. J Clin Invest 119: 1849–1851.

17. Maiorino M, Aumann KD, Brigelius-Flohe R, Doria V, van den Heuvel J, et al. (1995) Probing the presumed catalytic triad of selenium-containing peroxidases by mutational analysis of phospholipid hydroperoxide glutathione peroxidase (PHGPx). Biol Chem Hoppe Seyler 376: 651–660.

18. Imai H, Narashima K, Arai M, Sakamoto H, Chiba N, et al. (1998) Suppression of leukotriene formation in RBL-2H3 cells that overexpressed phospholipid hydroperoxide glutathione peroxidase. J Biol Chem 273: 1990–1997.

19. Bai YX, Cai GB, Kim SH, Zo YG, Kong Y (2009) Modular evolution of glutathione peroxidase genes in association with different biochemical properties of their encoded proteins in invertebrate animals. BMC Evol Biol 9: 72.

20. Schneider M, Vogt WD, Seiler A, Bornkamm GW, Brudehofer M, et al. (2006) Embryonic expression profile of phospholipid hydroperoxide glutathione peroxidase. Gene Expr Patterns 6: 409–494.

21. Back H, Jung K, Jung Y, Lee SR, Lee BJ, et al. (2011) Phospholipid hydroperoxide glutathione peroxidase gene is regulated via an estrogen and estrogen receptor signaling in cultured mouse fetuses. In Vitro Cell Dev Biol Anim 47: 533–540.

22. Cirujo MC, Jamet E, Parmentier Y, Marbach J, Durr A, et al. (1992) Isolation and characterization of a plant cDNA showing homology to animal glutathione peroxidase. Plant Mol Biol 12: 623–627.

23. Depege N, Drevet J, Boyer N (1996) Molecular cloning and characterization of tomato cDNAs encoding glutathione peroxidase-like proteins. Eur J Biochem 235: 445–451.
24. Herbst S, Meun AL, Rousselle P, Ameglio T, Falin Z, et al. (2005) Modification of photosynthetic rhythm in tomato overexpressing glutathione peroxidase. Biochim Biophys Acta 1724: 108–118.

25. Herbette S, Roeckel-Drevet P, Drevet JR (2007) Seleno-independent glutathione peroxidase homologues in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol 142: 1364–1379.

26. Ahmad P, Jaleel CA, Salem MA, Nabi G, Sharma S (2010) Roles of enzymic and nonenzymic antioxidants in plants during abiotic stress. Crit Rev Biotechnol 30: 161–175.

27. Avsian-Kretchmer O, Gueta-Dahan Y, Lev-Yadun S, Gollop R, Ben-Hayyim G (1998) Molecular characterization, organ distribution and stress-mediated induction of two glutathione peroxidase-encoding mRNAs in sunflower mesophyll cells. Plant Physiol 113: 1685–1696.

28. Eshed Y, Holland D, Fahn Z, Ben-Hayyim G (1997) Plant glutathione peroxidases. Physiol. Plant. 100: 234–240.

29. Maierino M, Gregolin C, Ursini F (1990) Phospholipid hydroperoxide glutathione peroxidase. Methods Enzymol 169: 449–457.

30. Haluskova L, Valentovicova K, Huttova J, Mistrik I, Tamas L (2009) Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiol Biochem 47: 1069–1074.

31. Navrot N, Collin V, Gualberto J, Gelhaye E, Hirasawa M, et al. (2006) Plant glutathione peroxidases are functional peroxidases distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol 142: 1364–1379.

32. Li S, Xue L, Xu S, Feng H, An L (2009) Hydrogen peroxide acts as a signal molecule in the adventitious root formation of mung bean seedlings. Environ Exp Bot 65: 63–71.

33. Iqbal A, Yabuta Y, Takeda T, Nakano Y, Shigeoka S (2006) Hydroperoxide peroxidase homologues in Arabidopsis thaliana. Plant Cell Physiol 47: 1484–1495.

34. Cummins I, Cole DJ, Edwards R (1999) A role for glutathione transferases functioning as glutathione peroxidases in resistance to multiple herbicides in blackgrass. Plant J 18: 295–302.

35. Rouhier N, Gelhaye E, Gualberto JM, Jordy MN, De Fay E, et al. (2004) Molecular characterization of salt-stress-associated protein in citrus: cDNA sequence homology to mammalian glutathione peroxidases. Plant Mol Biol 51: 923–927.

36. Liu W, Feng H, Fan JH, Zhang RQ, Zhao NM, et al. (2000) Molecular cloning and expression of a phospholipid hydroperoxide glutathione peroxidase homolog in Oryza sativa. Biochim Biophys Acta 1493: 225–230.

37. Xue Z, Xue L, Zhong Q, Liu JY (2006) A chloroplast-localized and auxin-inducible Arabidopsis gene encoding a CRT/DRE-binding factor responsive to drought, low-temperature, or high-salt stress. FEBS Lett 587: 443–447.

38. Miao Y, Lu D, Wang P, Wang XC, Chen J, et al. (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Physiol 142: 2749–2766.

39. Liang L, Xue L, Xu S, Liu W, Xu J, Tang S, et al. (2006) The Arabidopsis thaliana gene encoding a CRT/DRE-binding factor responsive to drought, low-temperature, or high-salt stress. FEBS Lett 587: 443–447.

40. Liss C, Van Moerbeke K, Huttova J, Mistrik I, Tamas L (2009) Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiol Biochem 47: 1069–1074.

41. Chen S, Vaghchhipawala Z, Li W, Asard H, Dickman MB (2004) Tomato glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Physiol 130: 639–648.

42. Qi YC, Liu WQ, Qiu LY, Zhang SM, Ma L, et al. (2010) Overexpression of glutathione S-transferase gene increases salt tolerance of Arabidopsis. Russ J of Plant Physiol 57: 253–258.

43. El-Shabrawi H, Kumar B, Kaul T, Reddy MK, Single-Airakel SL, et al. (2010) Redox homeostasis, antioxidant defense, and methylglyoxal detoxification as markers for salt tolerance in Pokkali rice. Proteoplasma 245: 85–96.

44. Rouhier N, Gelhaye E, Gualberto JM, Jordy MN, De Fay E, et al. (2004) Poplar peroxidin Q. A thioredoxin-linked chloroplast antioxidant functional in pathogen defense. Plant Physiol 134: 1027–1038.

45. Li SW, XG, Lu X, Xu J, Feng H, An L (2007) Hydrogen peroxide involvement in formation and development of adventitious roots in cucumber. Plant Growth Regul 52: 173–180.

46. Li S, Xue L, Xu S, Feng H, An L (2009) Hydrogen peroxide acts as a signal molecule in the adventitious root formation of mung bean seedlings. Environ Exp Bot 65: 63–71.

47. Xue Z, Xue L, Zhong Q, Liu JY (2006) A chloroplast-localized and auxin-inducible Arabidopsis gene encoding a CRT/DRE-binding factor responsive to drought, low-temperature, or high-salt stress. FEBS Lett 587: 443–447.
elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant J 11: 1237–1251.

81. Park HC, Kim MI, Kang YH, Jeon JM, Yoo JH, et al. (2004) Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. Plant Physiol 135: 2150–2161.

82. Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5: 1529–1539.

83. Abe H, Yamaguchi-Shinozaki K, Urao T, Iwasaki T, Hosokawa D, et al. (1997) Role of Arabidopsis MYC and MYB homologs in drought- and abscisic acid-regulated gene expression. Plant Cell 9: 1859–1868.

84. Gurley WB, Key JL (1991) Transcriptional regulation of the heat-shock response: a plant perspective. Biochemistry 30: 1–12.

85. Ishaki H, Woodson WR (1993) Characterization of an ethylene-responsive glutathione S-transferase gene cluster in carnation. Plant Mol Biol 22: 43–58.

86. Yu D, Chen C, Chen Z (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell 13: 1527–1540.

87. Suttif TD, Lanahan MB, Ho TH (1993) Gibberellin treatment stimulates nuclear factor binding to the gibberellin response complex in a barley alpha-amylase promoter. Plant Cell 5: 1681–1692.

88. Rouster J, Leah R, Mundy J, Cameron-Mills V (1997) Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. Plant J 11: 513–523.

89. Sakai T, Takahashi Y, Nagata T (1996) Analysis of the promoter of the auxin-inducible gene, parC, of tobacco. Plant Cell Physiol 37: 906–913.