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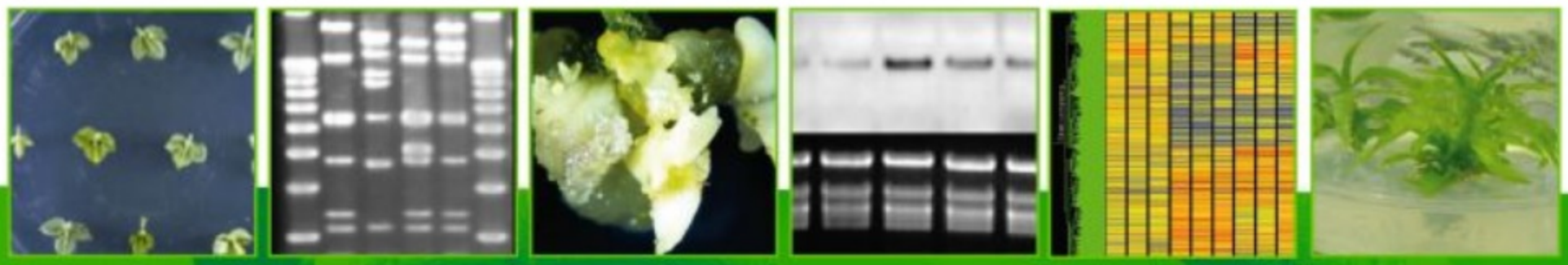
# PCTOC

PLANT CELL, TISSUE AND ORGAN CULTURE

# JOURNAL OF PLANT BIOTECHNOLOGY



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ORIGINAL ARTICLE

# Overexpression of a cytosolic ascorbate peroxidase from *Panax ginseng* enhanced salt tolerance in *Arabidopsis thaliana*

**Johan Sukweenadhi[1] · Yu-Jin Kim[2] · Shadi Rahimi[1] · Jeniffer Silva[1] · Davaajargal Myagmarjav[1] · Woo Saeng Kwon[1] · Deok-Chun Yang[1,2]**

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**Abstract** Ascorbate peroxidase (APX) plays an essential role in the antioxidant defense mechanism in the plant, serving in the ascorbate–glutathione cycle for the cellular $H_2O_2$ metabolism process. As the perennial plant, *Panax ginseng* Meyer encounters a lot of unfavorable growth conditions, and among them soil salinity significantly decreases the yield. Two APX genes from *Panax ginseng* were isolated and designated as *PgAPX1* and *PgAPX2*, which are most similar with previously characterized cytosolic APX of *Daucus carota* and *Spuriopimpinella brachycarpa*, as revealed by sequence analysis of their deduced amino acid sequences. *PgAPXs* transcripts are most abundant in leaf tissue, whereas *PgAPX1* expression level was higher compared to *PgAPX2*. Consistent with higher *PgAPX1* expression during salt stress in ginseng, *PgAPX1*-overexpressing *Arabidopsis* lines ($PgAPX1_{ox}$) increased the germination rate and root length compared with wild-type (WT) under 200 mM NaCl stress treatment. Furthermore, higher chlorophyll content, relative water content, total APX activity, proline content, and lower $H_2O_2$ accumulation were shown in $PgAPX1_{ox}$ plants compared to WT under 200 mM NaCl stress condition. Transcription analysis showed that $PgAPX1_{ox}$*Arabidopsis* lines were more salt-tolerant by upregulating the ion homeostasis mechanism.

**Keywords** Oxidative stress · Ascorbate peroxidase · Salt stress · $H_2O_2$ · *Panax ginseng*

## Introduction

Environmental stresses periodically affect plants during their cultivation. These conditions can disrupt the balance of cells metabolism, resulting in elevated reactive oxygen species (ROS) production, for instance, hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{\cdot}$), singlet oxygen ($^1O_2$) and superoxide ($O_2^{\cdot-}$). Recent study discovered a role of singlet oxygen as initiator of ROS systemic signals (Carmody et al. 2016). At the condition with no stresses, plants produce ROS which act in the oxidative signaling pathway and triggering a lot of cellular functions such as cell apoptosis, stress responses, and developmental processes (Apel and Hirt 2004; Ishikawa et al. 2013; Dietz et al. 2016). However, due to excessive stress exposure, plants can produce higher amount of ROS which induce oxidative damage and reduce plant productivity (Shigeoka et al. 2002).

An efficient antioxidant mechanism in plant integrates antioxidants network and ROS-scavenging enzymes in diverse locations in organelles, cells, and tissues (Ara et al. 2013). Antioxidant enzymes consist of ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxiredoxin (PrxR), glutathione peroxidase (GPX), and catalase (CAT). APX (EC 1.11.1.1) enzymes have an essential function in regulation of ROS levels and $H_2O_2$ scavenging networks from cytosol and chloroplast of plant cells. APX detoxify $H_2O_2$ utilizing ascorbate (AsA) as a substrate. Through

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✉ Yu-Jin Kim
yujinkim@khu.ac.kr

✉ Deok-Chun Yang
dcyang@khu.ac.kr

[1] Graduate School of Biotechnology and Ginseng Bank, College of Life Sciences, Kyung Hee University, Yongin, South Korea

[2] Department of Oriental Medicinal Biotechnology, College of Life Science, Kyung Hee University, Yongin, South Korea

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glutathione (GSH) utilization, electron transfer from ascorbate to a peroxide is catalyzed by dehydroascorbate (DAsA) reductase, yielding DAsA and water as the products. Thus, a combination of APX and AsA–GSH cycle serves to prevent the $H_2O_2$ toxicity in the plant in response to multiple stress reactions (Shigeoka et al. 2002; Blokhina et al. 2003).

APX has been discovered in various cellular compartments: stroma (Asada 1992) and thylakoid membrane (Miyake and Asada 1992) in chloroplasts, cytosol (Mittler and Zilinskas 1991), microbodies (Yamaguchi et al. 1995), and mitochondrial membrane (Leonardis et al. 2000). In *Arabidopsis*, chloroplastic APX; stroma APX (*At4g08390*) and thylakoid APX (*At1g77490*) were found to contribute of photoprotection by regulation of $H_2O_2$-responsive genes during photooxidative stress (Maruta et al. 2010), while cytosolic APX1 (*At1g07890*) was shown to participate in a various biological processes (Davletova et al. 2005). The cytosolic APX1 was constitutively expressed in leaves, stems, and roots (Zimmermann et al. 2004) and it has a common defense role during abiotic and biotic stresses (Wu et al. 2014).

cDNAs encoding the cytosolic APX isoenzymes were isolated and well-studied from many plants, such as pea (Mittler and Zilinskas 1991), *Arabidopsis* (Kubo et al. 1992), corn (Koshiba 1993), spinach (Webb and Allen 1995), tobacco (Orvar and Ellis 1995), rice (Teixeira et al. 2004), cowpea (D'Arcy-Lameta et al. 2006), rose gum (Teixeira et al. 2005), and tomato (Najami et al. 2008). APX isogenes' expression varies depending on tissue (Teixeira et al. 2006), developmental stage (Agrawal et al. 2003), and various environmental stimuli such as high light (Fryer et al. 2003), salt stress (Menezes-Benavente et al. 2004), $H_2O_2$ stress (Yoshimura et al. 2000), heat stress (Bonifacio et al. 2011), cold stress (Zhang et al. 1997), drought stress (Rosa et al. 2010), and pathogen attack (Agrawal et al. 2003).

*Panax ginseng* Meyer (Korean ginseng) is known to possess various pharmacological properties, such as immunostimulant, anticancer, antiemetic, antiproliferative, and antioxidant properties, along with other health benefits (Chung et al. 2016). As a herbaceous perennial plant, ginseng encounters many unfavorable growth circumstances, such as drought, temperature fluctuations, and salinity (Ashraf and Foolad 2013). Among these stresses, soil salinity is crucial environmental stress which significantly decreases the yield of various crops (Zhu 2001) including ginseng growth (Kim et al. 2015). Despite the importance of ginseng, its antioxidant enzyme system is not yet well-studied, and their role in the salt tolerance mechanism remains vague. In the research presented here, we isolated and cloned *APX* gene from *P. ginseng*, examined the phylogenetic relationship with those in other plants, and overexpressed into *Arabidopsis* to understand the possible role of ginseng *APX* against salinity condition. Overexpression of *PgAPX* enhanced salt stress tolerance in *Arabidopsis* via increase of APX activity and proline content, and decrease in the accumulation of $H_2O_2$. It also suggested its possible application in improving the tolerance abilities in crops by the way of genetic modification.

## Materials and methods

### Sequence analysis and cloning of *PgAPX*

cDNA clones encoding the APX genes were found from our EST library constructed previously (Kim et al. 2006) and sequenced by Genotech (Daejeon, Republic of Korea). After sequencing analysis, we found two full-length cDNAs, designated as *PgAPX1* and *PgAPX2* (*P. ginseng* ascorbate peroxidase). By NCBI BLAST feature, the homologous proteins were searched from deduced amino acid sequences as input. Multiple alignments of APXs isolated from *P. ginseng* and other species APXs were performed using ClustalX. A neighbor-joining based phylogenetic tree was constructed using MEGA6 software. Molecular mass, the number of amino acid residue, and isoelectric point (pI) of PgAPX proteins deducted from the open reading frames (ORF) of mature protein were analyzed by *ExPASy Server* according to Gasteiger et al. (2005). The hydropathy value was determined following Kyte and Doolittle method (1982), while the secondary structure was predicted using SOPMA (Geourjon and Deleage 1995). Conserved motifs on APX were predicted by MEME (Bailey et al. 2009). SWISS-MODEL Workspace in automated mode was utilized to predict a three-dimensional model using APX as a template (Arnold et al. 2006) and then the 3-D structure was visualized using UCSF Chimera package. Furthermore, promoter sequence of *P. ginseng* APX (pPgAPX) and gene structure were identified based on ginseng genome database (http://im-crop.snu.ac.kr/new/index.php). PlantCARE was used to estimate cis-acting regulatory elements comprised of promoter region (Lescot et al. 2002). Amplified fragments of pPgAPX were cloned, sequenced, and fused with 3-glucuronidase (GUS) reporter gene in pCAMBIA 1300, using specific primers embedded with PstI and SalI (Supplementary Table 1), designated as *pPgAPX1::GUS* and *pPgAPX2::GUS*. Histochemical analysis through GUS staining for each *pPgAPX1::GUS* and *pPgAPX2::GUS Arabidopsis* line was conducted by using 5 biological replicates. *PgAPX1* overexpression was carried on using pCAMBIA1390 containing CFP (cyan fluorescent protein) and it was expressed under the cauliflower mosaic virus (CaMV) 35 S promoter. From *P. ginseng* cDNA, *PgAPX1* gene was amplified by PCR using specific primers

embedded with SalI and EcoRI (Supplementary Table 1), designated as *35S:PgAPX1:CFP*.

### Localization of PgAPX1

Subcellular localization analysis of PgAPX1 was done utilizing four-day-old seedlings of $PgAPX1_{ox}$ lines. Leica TCS SP5 laser scanning confocal microscope was used for the analysis. With the excitation wavelength of 488 nm and an emission wavelength of 505–530 nm, CFP fluorescent signals were visualized. Meanwhile, at emission wavelength longer than 650 nm, the red auto-fluorescence of chlorophylls was visualized.

### Plant materials and stress treatment

The leaf, stem, and root of four-year-old *P. ginseng* cultivated on ginseng field (Kyung Hee University, South Korea, provided by the Ginseng Bank of Korea) were used for checking the abundance of *PgAPX* genes. For checking the expression of *PgAPX*s under salt stress, two-year-old ginseng roots were used. The *Arabidopsis thaliana* (Columbia ecotype) was used in this study as the model plant. Surface-sterilized seeds were sown on ½MS medium containing 1% sucrose and 0.8% agar with pH 5.7 adjustment using KOH. Two-day-old cold-treated seeds were germinated under long-day conditions (16 h light/ 8 h dark) at 23 °C. *Arabidopsis* transformation of all gene constructs was done using *Agrobacterium tumifaciens* via floral dip according to Clough and Bent's method (1998). Hygromycin (50 µg/mL)-containing plates were used to get transgenic (T2) lines. Utilizing promoter construct, the histochemical analysis was done based on various developmental stages and under salt stress treatment (200 and 300 mM NaCl). For measuring germination rate, 100 sterilized seeds of *Arabidopsis* $PgAPX1_{ox}$ lines were sown on each ½MS medium with various NaCl concentrations (0, 50, 150, 300 mM). It was measured every 12 h by observing penetration of the radicles in seed coats. For measuring root growth, approximately 500 sterilized seeds of *Arabidopsis* $PgAPX1_{ox}$ lines were sown on ½MS medium and 100 of four-day-old seedlings were transferred onto each ½MS medium with various NaCl concentrations (0, 50, 150 and 300 mM), for further vertical growth for another 12 days. Three independent replicates were conducted for each treatment of germination test and root elongation test. For salt stress treatment, 60 of ten-day-old seedlings were transplanted into the vermiculite: peat moss: perlite (1:1:1) mixture and allowed to grow in the same light/dark conditions. 30 of three-week-old seedlings on pots were watered with 200 mM NaCl solution while remaining seedling were watered normally. At various time points of post-treatment, three biological replicates of leaves samples were collected for RNA isolation. The plant samples were quickly frozen using liquid nitrogen and stored at −70 °C until needed.

### Real-time quantitative RT-PCR

Total RNA was extracted from plant samples using the RNeasy mini kit (Qiagen, Valencia, CA, USA) and used as a template for reverse transcription using oligo(dT)15 primer (0.2 mM) and RevertAid H Minus Reverse Transcriptase (200 U/µL) (ThermoFisher Scientific, South Korea). Using 100 ng of cDNA in a 15-µL total reaction volume of SYBR® Green Sensimix Plus Master Mix (Quantace, Watford, England), Real-time PCR was executed. Amplification, detection, and data analysis were carried out with a CFX 96/ Connect Real-Time PCR system (BIO-RAD, South Korea). Specific primers for *PgAPX1, PgAPX2*, and other genes were used to perform real-time PCR (Supplementary Table 1, Supplementary Fig. 1). The thermal cycler conditions were used as follows: 3 min at 95 °C, followed 40 cycles of 95 °C for 30 s, 56.4–62.4 °C for 50 s, and 72 °C 30 s. The annealing temperature of each gene is available in Supplementary Table 1. At the last step of each cycle, the fluorescent product was detected. The number of cycles at which it has significantly higher fluorescence intensity than the background fluorescence at the initial exponential phase of PCR amplification is designated as the threshold cycle (Ct). The Ct value for *β-actin* was used to normalize Ct value of *PgAPX* before calculated relatively to a calibrator using the formula $2^{-\Delta\Delta Ct}$ to determine the relative fold differences in template abundance for each sample. The primer efficiencies were determined according to the method by Livak and Schmittgen (2001) to validate the ΔΔCt method. The efficiencies of the gene and the internal control *β -actin* were equal when the observed slopes were near to zero. Three independent experiments were performed.

### $H_2O_2$ detection by 3,3′-diaminobenzidine (DAB) staining

Modified from previous methods (Daudi et al. 2012), DAB staining was used to do *in situ* detection of $H_2O_2$. The harvested leaf samples were placed in a 12-well-plate. Three mL of DAB staining solution was applied to the leaves in the well until fully immersed. As the control, 2 mL of 10 mM $Na_2HPO_4$ was applied to replicate leaves. The 12-well plates covered with aluminum foil were placed in a desiccator and gently vacuumed for 5 min, and were shaken for 3 h at 100 rpm. After the incubation, bleaching solution (ethanol: glycerol: acetic acid = 3:1:1) was given replacing DAB staining solution, boiled on a water bath for 15 min, and replaced by fresh bleaching solution. After incubation

at room temperature for 30 min, the photograph was taken under uniform lighting.

### Chlorophyll content assay

According to Lichtenthaler method (1987) with some modification, 60 mg leaves were ground into powder with liquid nitrogen and moved to a 1.5 mL tube. Mix with 1 mL of 80% acetone and stand in the dark for overnight. After centrifugation at 4 °C, 2500 rpm for 15 min, the supernatant was moved to a new tube. The absorbance of chlorophyll was measured using spectrophotometry with 80% acetone as a blank control. The concentrations of chlorophyll are calculated as follows; Chlorophyll a = 12.72*A663 − 2.59*A645, Chlorophyll b = 22.9*A645 − 4.67*A663 and Total chlorophyll = 20.31* A645 + 8.05* A663.

### Relative water content (RWC) measurement

Fully expanded terminal leaflets were sampled and fresh weight (FW) was recorded immediately. Turgid weight (TW) was achieved constant weight after overnight keeping the leaf in distilled water at 4 °C while dry weight (DW) was obtained constant weight after keeping the turgid leaf at 70 °C oven. The formula is following: RWC = (FW – DW)/(TW − DW) × 100% (Tambussi et al. 2005).

### Proline determination

Proline extraction was done using 50 times diluted fresh weight (w/v), in a 70:30 ethanol: water mixture (v/v). Extract was mixed with reaction mix (ninhydrin 1% (w/v) in ethanol 20% (v/v), acetic acid 60% (v/v)) with equal volume under light-protected condition. The reaction mixture was heated at 95 °C for 20 min. After cooling to room temperature, it was spin down immediately then transferred to a microplate well and processed the absorbance read at 520 nm. The amount of proline in the extracts:

$$\text{Proline in nmol/mg FW or in µmol/g FW} = (\text{Abs}_{\text{extract}} - \text{blank})/\text{slope} \times \text{Vol}_{\text{extract}}/\text{Vol}_{\text{aliquot}} \times 1/\text{FW}$$

$\text{Abs}_{\text{extract}}$ is the extract absorbance value. Blank (expressed as absorbance) and slope (expressed as absorbance/nmol) are specified through linear regression, $\text{Vol}_{\text{extract}}$ is the extract total volume, $\text{Vol}_{\text{aliquot}}$ is the volume used in the assay, FW (expressed in mg) is the amount of plant material used at first extraction process. It is presumed that $\text{Abs}_{\text{extract}}$ is within the linear range. As standards, proline solutions ranging from 0.04 to 1 mM were prepared in the same medium as the one that used for the extraction (Carillo et al. 2008).

### APX and SOD enzyme activity assay

APX and SOD enzyme activity were determined following the method described by Venisse et al (2001) and Shafi et al (2015), respectively. Leaves were sampled, immediately weighted and ground. All the ground powder was collected into a 1.5 mL microtube in which 1 mL extraction buffer (50 mM sodium phosphate buffer- pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM polyethylene glycol, 8% (w/v) polyvinylpyrrolidone, 0.01% (v/v) Triton X-100) was added and vortex for a few seconds. The mixture was centrifuged at 4 °C, 13,000 rpm for 20 min. The supernatant was collected in a new microtube and incubated on ice for activity analysis. 1 mL APX reaction mixture (0.2 M Tris/HCl buffer, pH 7.8, 0.5 mM $H_2O_2$, 0.25 mM ascorbic acid) was mixed with 50 µl of the crude extract into a 1.5 mL microtube, and recorded the absorbance at 290 nm at the start point of time. The total SOD activity was measured by adding 25 µl enzyme extract to reaction mixture (1 mL) containing 1.5 µm riboflavin, 50 µm NBT (nitroblue tetrazolium), 10 mM Dl-methionine and 0.025% (v/v) triton-X 100 in 50 mM phosphate buffer. After 5 min, reaction sample was moved to the cuvettes and covered with parafilm to prevent the oxidation in the air. The ELISA reader (Synergy-2, Bio-Tek Instruments, Inc., Winooski, VT, USA) was used to read the absorbance at the specific wavelength Coomassie (Bradford) protein assay kit was used to determine the protein concentration of the samples. Value decline in absorbance at 290 and 560 nm represents decrease amount of ascorbate and NBT, respectively. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 µM of substrate per min at 25 °C (µL/mg protein/min).

Enzyme activity = [(A2 – A1)/(T2 – T1)]/mg protein = change of absorbance/per mg protein per min.

## Results and discussion

### Sequence analysis and cloning of two *PgAPX*s

APXs, especially the cytosolic APX, participate in the plant defense against oxidative stress (Shigeoka et al. 2002). Several previous studies demonstrated that cytosolic *APX* overexpression was significantly improving APX activity that protects plants from oxidative damage caused by a salt stress condition. For example, overexpression of *Arabidopsis* cytosolic *APX* (Badawi et al. 2004), pea cytosolic *APX* (Wang et al. 2005), *Lycium chinense* cytosolic *APX* in tobacco (Wu et al. 2014), rice *OsAPXa* and *OsAPXb* in *Arabidopsis* (Lu et al. 2007), and rice *OsAPX2* in Alfalfa (Zhang et al. 2014), amends salt stress tolerance on transgenic plant at various responsive degree. The

isolation and characterization of novel cDNAs of cytosolic *APX* is an access to further understanding of cytosolic *APX* gene. Thus, overexpressing this gene allows new perspectives about regulation and physiological function of these enzymes. In this study, we isolated a cytosolic *APX* gene from *P. ginseng* and its potential function in transgenic *Arabidopsis* were examined under salt stress condition.

From our *P. ginseng* EST library previously built (Kim et al. 2006), two different isozymes from more than ten cDNA clones encoding APX genes have been identified. The full-length cDNA sequences have been assigned to GenBank under the accession numbers (KX388539 and KX388540), named as *PgAPX1* and *PgAPX2* (*P. ginseng* ascorbate peroxidase). Both of PgAPX sequences have 91% identity to each other. The *PgAPX1* and *PgAPX2* ORF sequences were 753 bp long encoding proteins with 250 amino acids in length, and 27.5 kDa of molecular mass. Theoretical pI for PgAPX1 and PgAPX2 is 5.55 and 5.64, respectively. Amino acid sequences of PgAPX1 and PgAPX2 share higher degrees of identity (86 and 91%, respectively) with cytosolic APX proteins of *Daucus carota* (AKH49594) and *Spuriopimpinella brachycarpa* (AAF22246). The phylogenetic analysis showed that PgAPX1 and PgAPX2 belong to the cytosolic APX subgroup (Fig. 1a). Consistent with phylogenetic analysis, confocal microscope analysis of PgAPX1 tagging with CFP showed its expression was gained on cytosol which can suggest the cytosol localization of PgAPX1 (Fig. 1c). Up to date, *Arabidopsis* has total eight types of APXs, consisting three cytosolic APXs, three cytosol membrane-bound APXs, and two chloroplast APXs (Dąbrowska et al. 2007). Some other plants reported have two isoforms of cytosolic APX, such as *Oryza sativa* L, *Capsicum annum, Pinus pinaster, Pissum sativum, Nicotiana tabacum* and *Spinacia oleraceae* (Teixeira et al. 2004). The exact amount of APX family genes present in *P. ginseng* will be known once whole-genome sequencing of ginseng is completed.

MEME analysis found conserved motifs in all plant APX isozymes (Fig. 1b, Supplementary Fig. 2), corresponding to APX's same function in different organelles. The motifs contain the conserved amino acid regions of the APX (Fig. 2a), such as the active site (His 42, Trp 179, Asp 208) (Çelic et al. 2001) and the binding site (His 163) (Lad et al. 2002), which are influential for maintaining the structure

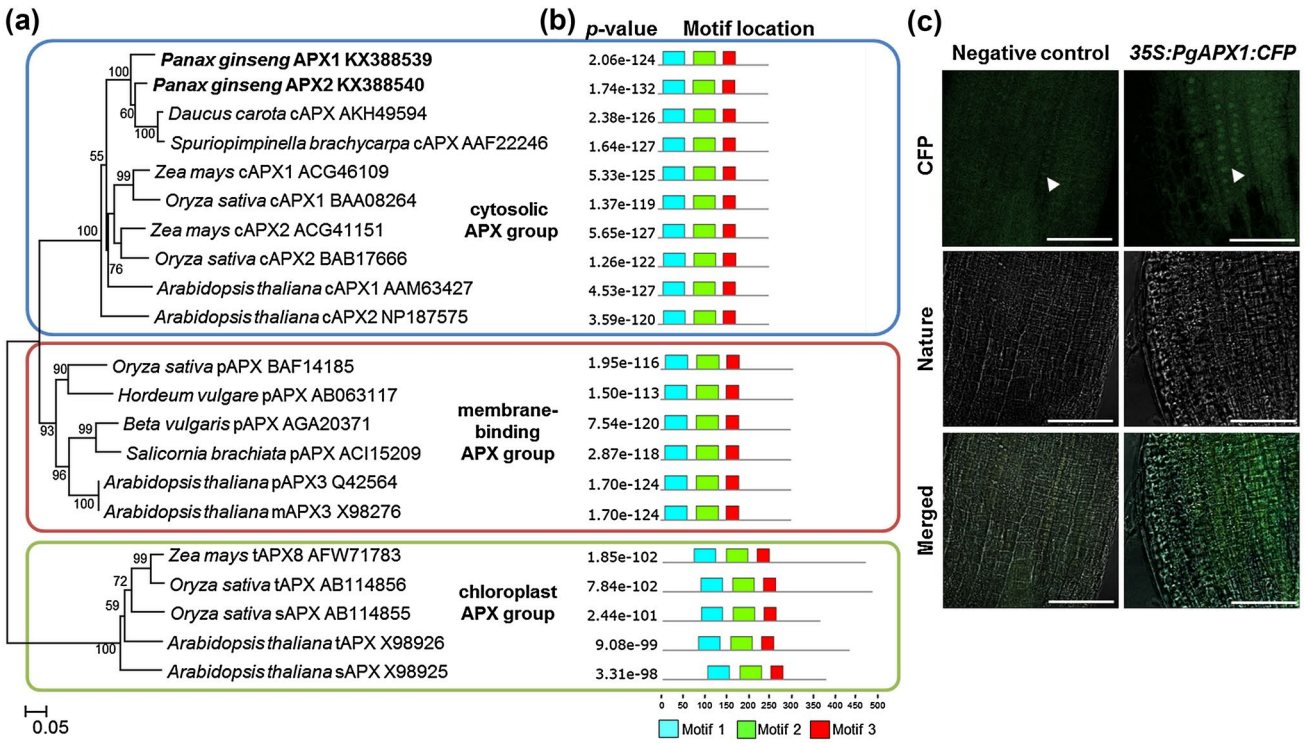


**Fig. 1** **a** Sequence homology analysis of *PgAPXs* with other *APX* genes. The neighbor-joining method was used and the branch lengths are proportional to the divergence, with the scale of 0.05 representing 5% changes. Protein sequences were from the databases indicated in parentheses. **b** Organization of putative motifs in APX identified by MEME. Numbered *color boxes* represent different putative motifs, and the sequences of the motifs are listed in Supplementary Fig. 1. Motifs 1, 2, and 3 are indicated by the *mint, blue*, and *red* boxes, respectively. Motif sizes are indicated at the bottom of the figure. **c** Confocal microscope photo of $PgAPX1_{ox}$ lines (*35S:PgAPX1:CFP*) tissue indicated PgAPX1 tagged with CFP expressed mainly on cytosol compared to negative control which present some basal expression (indicated from *white arrow*). Negative control was CaMV promoter fused with CFP (*35S::CFP*). *Scale bar* indicates 100 μm

and function of APX (Kjaersgard et al. 1997). Furthermore, amino acid sequence of PgAPX1 and PgAPX2 contained the "GFAEA" motif present in C-terminal of cytosolic APXs (Wu et al. 2014). The hydrophobicity profile similarity of the deduced PgAPX and other nearest cytosolic APX group is shown in Fig. 2b. Both active site (His 42, Trp 179, Asp 208) and binding site (His 163) were found on hydrophobic region. The secondary structure of PgAPXs also showed high resemblance compared with the secondary structure of another close-related plant cytosolic APXs (Supplementary Table 2). The tertiary structure of matured protein of PgAPX1 and PgAPX2 was observed by 3-D modeling and it revealed that all important active sites (His 42, Trp 179, Asp 208) located nearby each other which make space for catalytic reaction (Fig. 2c).

Furthermore, construction of full gene sequence of *PgAPX1* and *PgAPX2* revealed two genes (2310 and 2843 bp, respectively) consisting nine exons and eight introns (Supplementary Fig. 3), which is typical characteristic of non-chloroplastic APX isoforms, with the exception only for *AtAPX* (*At1g07890*) with eight exons and seven introns (Teixeira et al. 2004). Numerous regulatory elements contributing on stress responsiveness were predicted within *pPgAPX*s, such as ethylene-responsive element

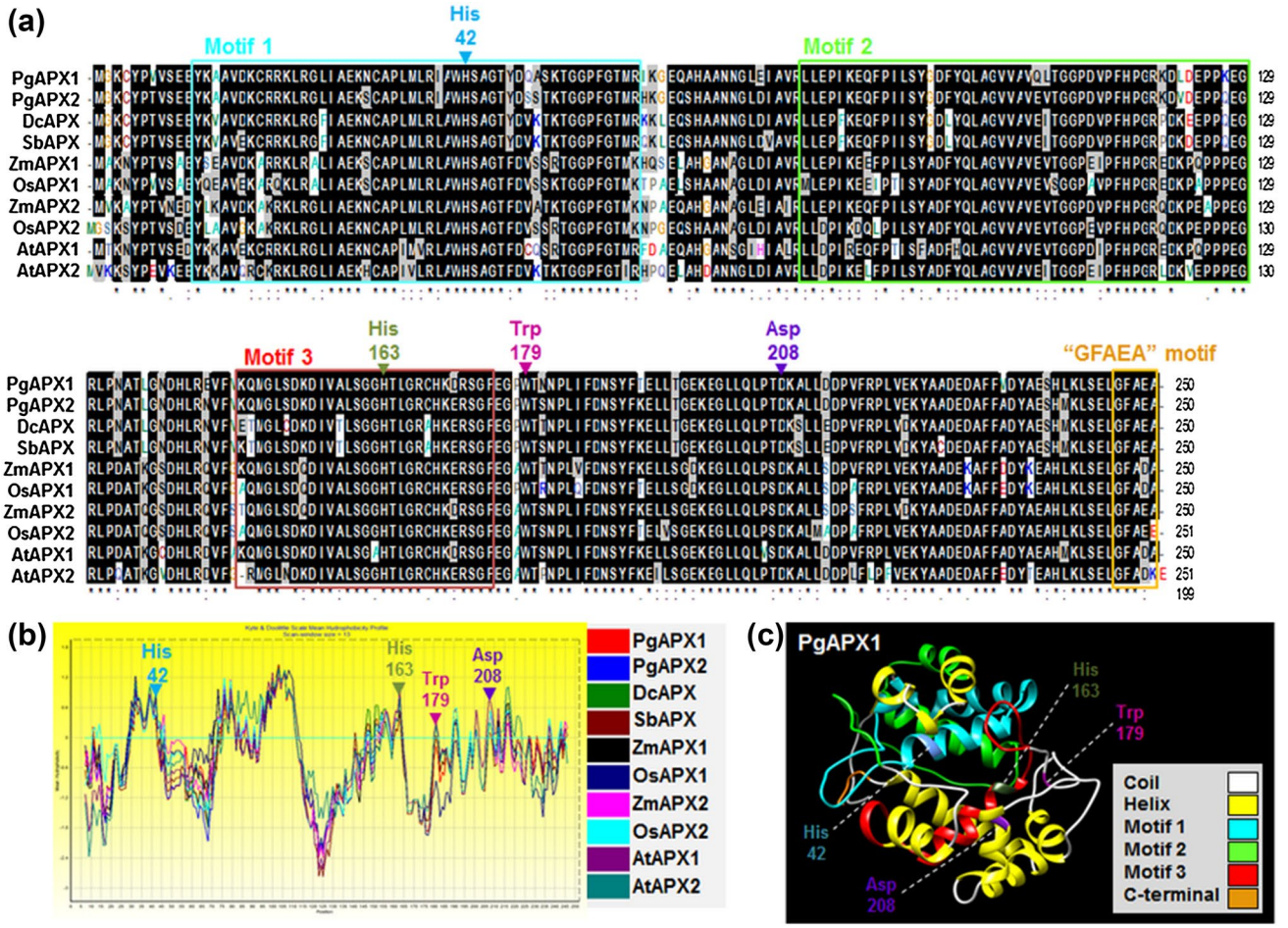


**Fig. 2** **a** Multiple amino acid sequence alignment of PgAPX1 and PgAPX2 with those of proteins encoded *Daucus carota*/ DcAPX (AKH49594), *Spuriopimpinella brachycarpa*/ SbAPX (AAF22246), *Zea mays*/ ZmAPX1 (ACG46109); ZmAPX2 (ACG41151), *Oryza sativa*/ OsAPX1 (BAA08264); OsAPX2 (BAB17666), and *Arabidopsis thaliana*/ AtAPX1 (AAM63427); AtAPX2 (NP187575). *Asterisks* indicate the conserved amino acids residues. The identical and conserved amino acid residues are highlighted with the *black* and *gray* background, respectively. Motifs 1, 2, and 3 are indicated by the *mint, lime green*, and *red boxes*, respectively. The highly conserved motif "GFAEA" found on C-terminal of cytosolic APXs is indicated by *orange color*. **b** Superimposed hydrophobicity profiles and secondary structure predictions for PgAPX group and homologous. Hydrophobic domains are indicated by positive numbers, hydrophilic domains are above the line and hydrophilic domains are below the line. **c** The predicted 3-D structures of PgAPX1. Comparative representation was performed by UCSF Chimera package and helix and coil structures are depicted as *yellow* and *white*, respectively. Motif protein sequences colored the same with the color indicated by MEME. Furthermore, "GFAEA" motif on C-terminal is depicted as an *orange color*. The presence of the conserved amino acid regions of the APX such as the active site (H42, W179, D208) and the binding site (H163) indicated by *blue, magenta, purple* and *dark green color*, respectively

(ERE), ABA-responsive element (ABRE), MYB-binding motif, and TC-rich repeats (Supplementary Table 3). Previous reports on APX of extreme halophyte, *Salicornia brachiata* (Tiwari et al. 2013) and *Beta vulgaris* (Dunajska-Ordak et al. 2014), suggest the possibility that *PgAPX*s can be expressed under broad kind of stresses. Notwithstanding,

### Tissue expression analysis of *PgAPX* genes

*PgAPX* transcription profile was examined in different ginseng tissues using real-time PCR. *PgAPX1* was highly expressed in the stem and leaf, compared to the root, whereas the highest expression level of *PgAPX2* was detected in the leaf (Supplementary Fig. 4a). Overall, most abundant transcript of *PgAPX* was found in leaf tissues although it was detectable in all tested tissues. This result is similar to the recent review in which *APX* gene was expressed in almost all analyzed tissues of *Arabidopsis* with various folds (Ozyigit et al. 2016). Cytosolic *AtAPX1* (*At1g07890*) showed higher expression significantly at roots particularly at its growing zone, while other cytosolic *APX* isoform, *AtAPX6* (*At4g32320*) were expressed mainly in leaf (Ozyigit et al. 2016). In *Solanum tuberosum* L., the cytosolic *APX* transcript was observed at the same level in leaves, shoots, and sprouts (da Costa et al. 2006). Histochemical assay of *pPgAPX1::GUS* and *pPgAPX2::GUS* construct in transgenic *Arabidopsis* showed that both *pPgAPX1* and *pPgAPX2* expression initiated from roots four days after sprout (Figs. 3a, 4 DAS), gradually moved into stems (Fig. 3a, 6 DAS), highly expressed in leaves and barely found in roots of one-week-old seedlings (Fig. 3a, 8 DAS and 10 DAS), and again highly accumulated in stem of two-week-old *Arabidopsis*seedlings (Fig. 3a, 14 DAS). The various change of *pPgAPX*s:*GUS* expression during the seedling growth indicated that the expression of *APX* genes was induced in a tissue-dependent way (Ozyigit et al. 2016).

### Temporal expression of *PgAPX1* and *PgAPX2* gene in response to salt stresses

*PgAPX* expression pattern at several time points was checked in two-year-old *P. ginseng* treated with 200 mM NaCl using real-time PCR (Fig. 3b). Contrary with *PgAPX2, PgAPX1* expression level was up-regulated quickly at 12 h and gained its maximum level at 24 h post-treatment. Meanwhile, *PgAPX2* expression was not significant until 24 h post treatment. Both *PgAPX1* and *PgAPX2* expression levels decreased at 48 h post treatment. Similarly, the expression of APX genes from other species is upregulated during salt stress (Sun et al. 2010; Liu et al. 2014; Singh et al. 2014). On 200–300 mM NaCl treatment, histochemical assay of *pPgAPX1::GUS* and *pPgAPX2::GUS* showed that both *PgAPX1* and *PgAPX2* promoter expression can be driven by salt stress (Supplementary Fig. 4b). However, *pPgAPX1* seems to have

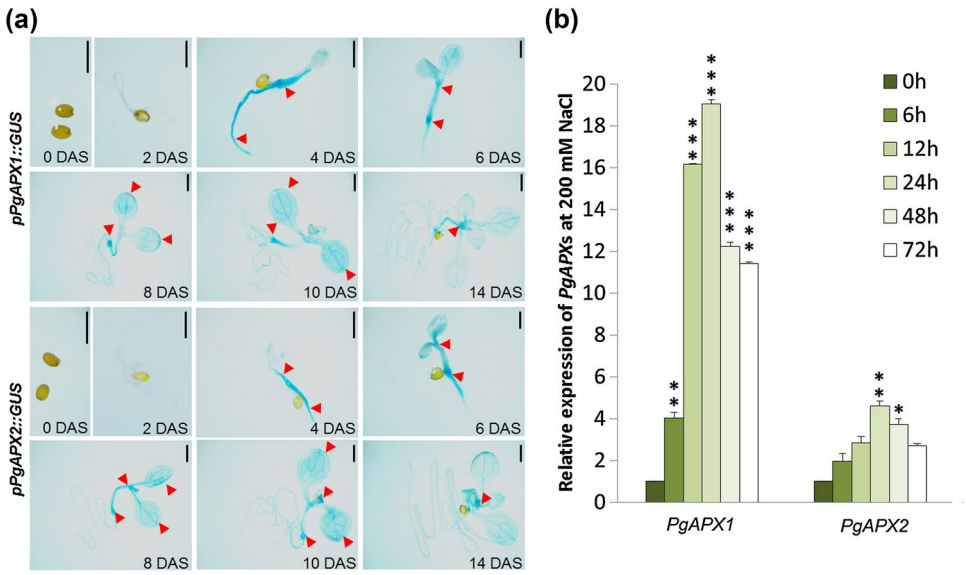


**Fig. 3** **a** Histochemical analysis of transgenic *Arabidopsis* line harboring *pPgAPX1::GUS* and *pPgAPX2::GUS* on various developmental stages. The *red arrows* indicate the concentrated and flow change of GUS expression. For histochemical analysis, 5 biological replicates were used for each treatment. *Scale bar* indicates 5 mm. **b** A relative expression of *PgAPX1* and *PgAPX2* of two-year-old ginseng at various time point of 200 mM NaCl post-treatment. *Bars* indicate the mean value ± SE from three independent experiments. The actin housekeeping gene of ginseng was selected as an internal control. Means of three independent replicates are statistically analyzed and compared with 0 h sample, by using Student's *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

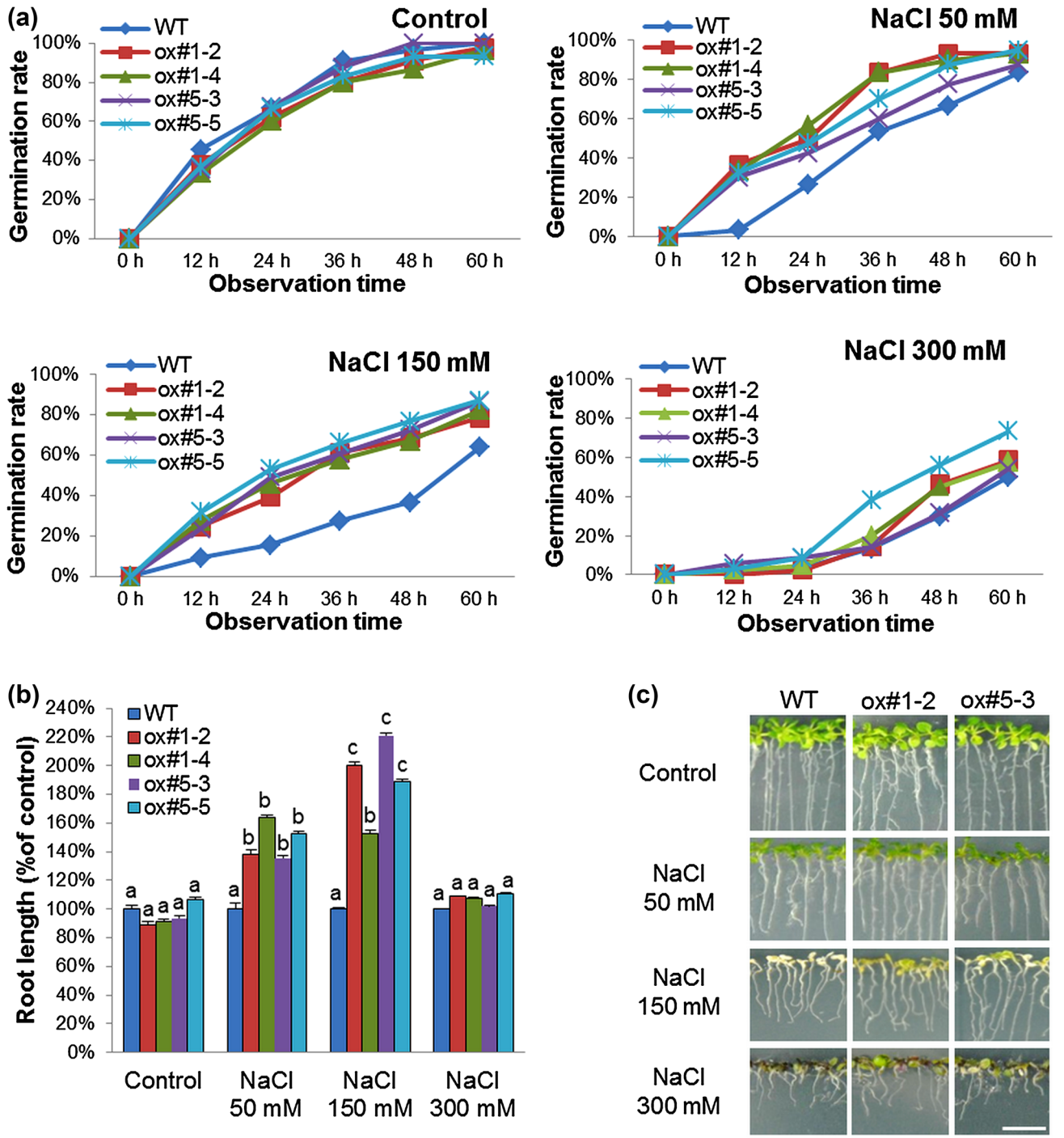


**Fig. 4** **a** Germination rate and **b** root length of *PgAPX1*$_{ox}$ lines on *A. thaliana* under salt stress. *Different letters* on top of the *bars* indicate significant difference at a level of $p < 0.05$, as determined by Duncan's multiple range test (DMRT). The graphical value represents the mean value of 3 independent replicates, consists of 100 seeds/ seedlings for each treatment. **c** Morphological observation of WT and various *PgAPX1*$_{ox}$ lines on ½ MS with various concentration of NaCl. The root length measurement and seedlings morphology documentation were taken at 16-day-old seedlings stage. *Scale bar* indicates 1 cm

stronger expression compared to *pPgAPX2* (Supplementary Fig. 4b). Not only responded to the salt stress, *PgAPX1* promoter was also expressed strongly under peroxide (10 mM $H_2O_2$), heat (37 °C) and cold (4 °C) treatment (Supplementary Fig. 5a), indicating its induction by broad abiotic stresses. Our result corresponds with the previous study that *PgAPX* was up-regulated under abiotic stresses including peroxide, heat, and cold treatment but slight

down-regulation or no significant change of transcript level during pathogen attack (Sathiyaraj et al. 2011) using same *PgAPX1* qRT-PCR primer (Supplementary Fig. 1). Thus, *PgAPX1* was chosen to be constructed with CaMV promoter, transformed into *A. thaliana*, screened with 50 µg/mL hygromycin and finally the successful transgenic plants were detected by PCR. $PgAPX1_{ox}$ #1-2, #1-4, #5-3 and #5-5 transgenic lines were selected for further analysis.

### Stress tolerance in $PgAPX1_{ox}$*Arabidopsis*

It has been reported that the overexpression of APX is essential for the effective protection of plants against oxidative damage caused by salt stress (Diaz-Vivancos et al. 2013a). To examine whether overexpression of *PgAPX* in *Arabidopsis* can improve salt tolerance, germination ratio was counted in ½MS medium containing different levels of NaCl. No clear differences were observed between WT and $PgAPX1_{ox}$ lines for 60 h after sowing at normal media (Fig. 4a). However, $PgAPX1_{ox}$ lines showed higher germination rate compared to WT under 150 mM NaCl (15–23% higher) at 60 h. At 300 mM NaCl condition, only $PgAPX_{ox}$ #5-5 showed higher germination rate compared to WT (23.5% higher). Other treatment results for germination rate of $PgAPX1_{ox}$ lines under heat (37 °C) and cold (4 °C) was either slightly significant or not significant (Supplementary Fig. 5b). In the case of root growth assay, four-day-old germinated seedlings were moved into ½MS with various NaCl concentrations. After 12 days, no visual differences in root growth and leaves color were observed between WT and $PgAPX1_{ox}$ lines under non-salt stress condition (Fig. 4b, c). However, during 50 mM NaCl treatment, the growth of WT plants was slightly inhibited compared with those of $PgAPX1_{ox}$ seedlings after 12 days, as shown shorter than transgenic lines (Fig. 4b, c). Once 150 mM NaCl was given to plants for 12 days, the WT seedlings simply ceased growth and their leaves turned white, whereas the transgenic plants merely grew slowly and their leaves stayed green (Fig. 4b, c). All transgenic lines and WT showed similarly stunted and ceased seedlings under 300 mM NaCl condition at 12 days (Fig. 4c). Overall, growth of all tested lines was suppressed to different degrees under salt stress, although the $PgAPX1_{ox}$ lines showed higher tolerance to NaCl at the seedling stage, which confirmed by higher germination rate and longer roots (Fig. 4). This was concurrent with previous reports of transgenic plants overexpressing the pepper *APX* (Sarowar et al. 2005), rice *APX* (Lu et al. 2007) and *Lycium chinense APX* genes (Wu et al. 2014).

Moreover, to validate the function of the *PgAPX1* gene during salt stress, three-week-old *Arabidopsis* plants (on pot) were watered with or without 200 mM NaCl solution. All plants watered with NaCl solution displayed a common growth inhibition. Compared with more obvious yellow leaves of the WT plants (Fig. 5a), the $PgAPX1_{ox}$ grew as normal, and apparently, a slight number of leaves suffered damage. These symptoms indicated that overexpression of the *PgAPX1* gene conferred a high tolerance to salt stress in transgenic *Arabidopsis* plants. Oxidative damage marked by the accumulation of ROS such as $H_2O_2$ when plants were subjected to salt stress (Hasegawa et al. 2000). Salt stress can trigger $H_2O_2$ accumulation as reported in rice, marine macroalgae, and *Medicago sativa* (Luo and Liu 2011; Hu et al. 2012; Mishra et al. 2013; Guo et al. 2014). The $H_2O_2$ level was high on WT plant under 200 mM NaCl treatment, indicated by intense brown color after DAB staining (Fig. 5b). Meanwhile, all $PgAPX1_{ox}$ lines showed slight or much less brown color after staining. These results indicated that transgenic lines have less level of $H_2O_2$ accumulation in response to salt stress, compared to WT plant.

### Transcription pattern analysis of $PgAPX1_{ox}$ lines under salt stress

Δ1-Pyrroline-5-carboxylate synthetase (*P5CS*) is the rate-limiting enzyme which catalyzing proline biosynthesis (Yoshiba et al. 1999). It was reported that *P5CS1* but not *P5CS2* gene is required for proline accumulation during stress condition in *A. thaliana* (Székely et al. 2008). Higher proline content can be found on $PgAPX1_{ox}$ lines, which was along with higher transcript level of *AtP5CS1* (Fig. 5c). Similar study reported that induction of *AtP5CS1* expression during salt stress can be depend on abscisic acid (ABA) and phospholipase C (Abraham et al. 2003; Parre et al. 2007).

The transcription pattern of several ROS-related genes was analyzed from leaf samples of WT and $PgAPX1_{ox}$*Arabidopsis* at 5 days post treatment of 200 mM NaCl, which showed the distinct result of morphological appearance and DAB staining. *PgAPX1* transcript level was found significantly high in all selected $PgAPX1_{ox}$ lines (Fig. 5c). Transcription level of *AtSOD1* was found to be significantly increased, although expression of *AtAPX1, AtAPX2, AtCAT1, AtGPX1*, and *AtPrxR1* was found as high as WT (Fig. 5c, Supplementary Fig. 6). Gupta et al. (1993) described that overexpression of SOD on pea, not only exhibited SOD transcript and enzyme activity, but also increase level of APX mRNA and enzyme activity. Resembling to this study, overexpression of *SOD* and *APX* in plum (Diaz-Vivancos et al. 2013b) and sweet potato (Yan et al. 2016) increased their salt stress tolerance. In this study, increasing levels of *PgAPX1* and *AtSOD1* can clearly lead to enhanced oxidative protection in plants, as showed by lowering $H_2O_2$ content in $PgAPX1_{ox}$ lines (Fig. 5b) due to higher respective enzyme activity (Fig. 5g–h). Enhanced antioxidant defense system can improve salt tolerance in

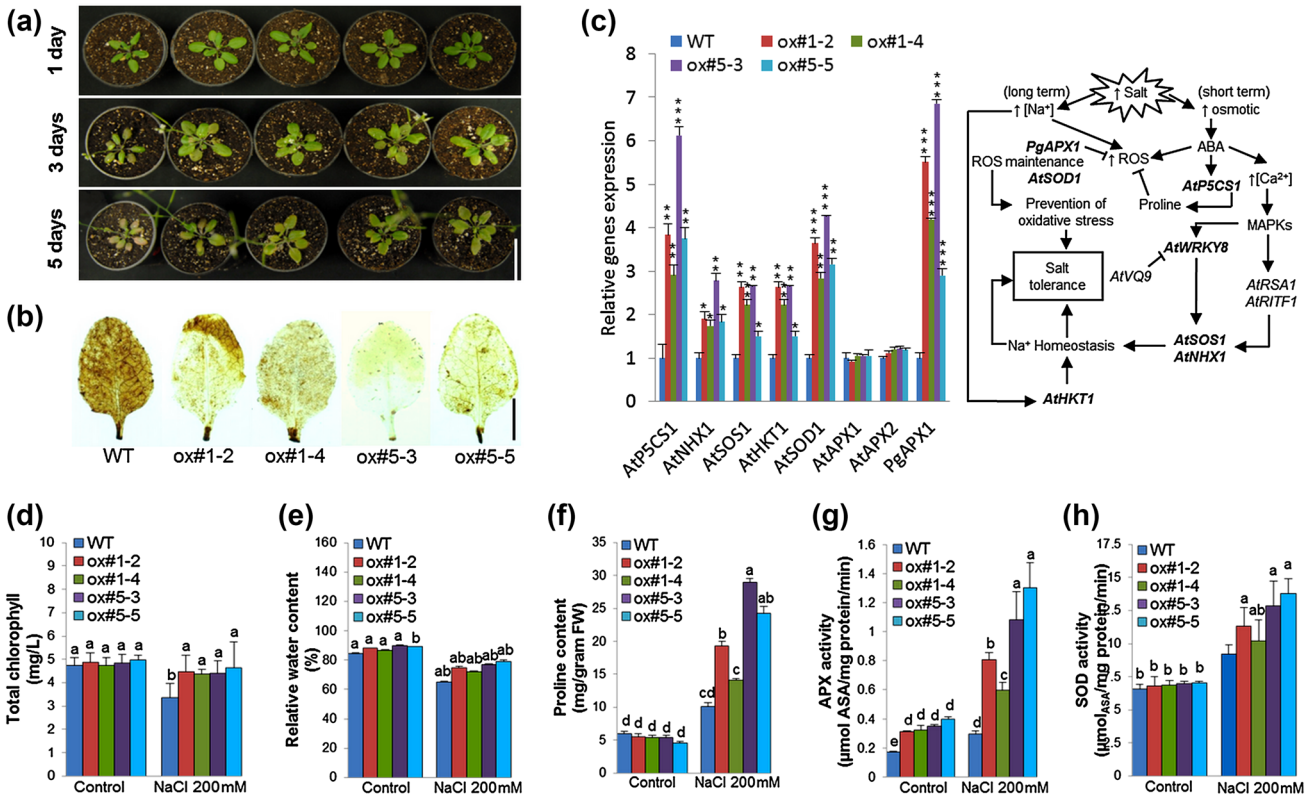


**Fig. 5** **a** Visual symptoms on representative WT plants (WT) transgenic *Arabidopsis* plants overexpressing *PgAPX1* after 200 mM NaCl treatment (*Scale bar* indicated 2 cm) and **b** $H_2O_2$ visualization (DAB staining) of $PgAPX1_{ox}$ lines after 5 days 200 mM NaCl treatment (*Scale bar* indicated 3 mm). For DAB staining analysis, 5 biological replicates were used for each treatment. **c** The transcript levels of *PgAPX1* and other genes in different overexpressed lines were analyzed by qRT-PCR. Total RNA was extracted from leaf samples collected from three-week-old T2 transgenic plants after 5 days 200 mM NaCl treatment. The actin housekeeping gene of *Arabidopsis* was selected as an internal control. Means of three independent replicates are statistically analyzed and compared with control at (*$p<0.05$, **$p<0.01$, ***$p<0.001$) using Student's t-test. A suggested schematic model of overexpressing *PgAPX1* effect on *Arabidopsis* salt stress defense pathway also provided. The *solid line* indicates activating/ increasing while the *broken line* indicates deactivating/ inhibiting. The *bold font* indicates its mRNA expression was found to be upregulated significantly. **d–h** Biochemical analysis of three-week-old $PgAPX1_{ox}$ lines after 5 days salt stress (200 mM): **d** Total chlorophyll, **e** Relative water content, **f** Proline content, **g** APX activity and **h** SOD activity. *Different letters* on *top* of the *bars* indicate significant difference at a level of $p<0.05$, as determined by Duncan's multiple range test

$PgAPX1_{ox}$ lines. Probably, *SOD* and *APX* genes regulation was linked through $H_2O_2$ signaling. ROS generated during salt stress get converted into $H_2O_2$ by SOD and its optimum level was maintained by APX, as reported by Shafi et al. (2015).

*AtWRKY8* (*At5g46350*) expression was higher in $PgAPX1_{ox}$ lines compared to WT, while the expression of *AtVQ9* (*At1g78310*) was not altered (Supplementary Fig. 6). Previous review article stated that *AtWRKY8* regulate signaling cascades in salinity, drought, and oxidative stresses by binding to W-box of downstream genes related to stress response (Banerjee and Roychoudhury 2015). *AtWRKY8* was primarily reacting to high salinity and only specifically interacted with *AtVQ9*, in which its interaction decreased the DNA-binding activity of *AtWRKY8*. In *wrky8* mutants, the expression of Salt Overly Sensitive genes (*SOS1, SOS2*, and *SOS3*) were reduced but increased in *vq9* mutants under salt treatment (Banerjee and Roychoudhury 2015). SOS1 is plasma membrane $Na^+/H^+$ antiporter which acts crucially in $Na^+$ exclusion by the driving force produced by plasma membrane $H^+$-ATPase (Shi et al. 2000). Similarly, the vacuolar $Na^+/H^+$ exchanger (NHX1) helps pushing excess $Na^+$ ions into vacuoles (Ulrich et al. 2014). Three key components of SOS pathway includes SOS1; SOS2, a serine/threonine protein kinase (Liu et al. 2000) and SOS3, a $Ca^{2+}$ sensor (Liu and Zhu 1998). SOS2-SOS3 kinase complex regulates SOS1 activity by phosphorylation-mediated regulation (Sanchez-Barrena et al. 2005). The histidine kinase transporter (HKT) is a low-affinity $Na^+$ ion transporter, which assist the removal of $Na^+$ from xylem sap into surrounding parenchyma cells (Davenport et al. 2007). Meanwhile, nucleus-localized *AtVQ9*

protein mainly expressed in roots and salt stress tolerance was enhanced by its mutation. It was proved that *AtVQ9* acts antagonistically with *AtWRKY8* to mediate salt stress responses by maintaining ion homeostasis, especially at a lower cytosolic $Na^+/K^+$ ratio (Hu et al. 2013). As a transcription factor, expression of *AtWRKY8* is known to be regulated by MAPK (mitogen-activated protein kinases)-mediated phosphorylation, especially calcium-dependent protein kinases (CPDKs), which can promote its DNA binding activity (Tsuda and Somssich 2015). ABA signaling generates ROS and $Ca^{2+}$ signals that activates CPDK. In the present study, similar increasing expression pattern was found for *AtWRKY8, AtHKT1, AtNHX1*, and *AtSOS1* (Fig. 5c, Supplementary Fig. 6); indicating that the cells tried to pump out excess $Na^+$ or distribute it to other cells to prevent the $Na^+$ toxicity (Ulrich et al. 2014).

Furthermore, *AtRSA1* (Short Root in SAlt medium 1; *At3g06590*), the nuclear-localized calcium-binding protein, senses salt-induced changes in nuclear free calcium and interacts with RITF1 (RSA1 Interacting Transcription Factor 1, a bHLH transcription factor), which may be phosphorylated by nuclear-localized MAPKs. The RSA1-RITF1 complex maintains expression of genes involved in detoxification of salt-induced ROS and $Na^+$ homeostasis during salt stress (Guan et al. 2013). However, the expression of *AtRSA1* and *AtRITF1* were similar between WT and $PgAPX1_{ox}$ lines. These results suggested that probably *AtWRKY8* induced *AtSOS1* directly or indirectly in a separate way that *AtRSA1* and *AtRITF1* did. Probably, different MAPKs are responsible for each of these pathways. The high expression level of transcription factor *AtWRKY8* and stable expression of its antagonist, *AtVQ9* in $PgAPX1_{ox}$ lines suggested that probably MAPK triggered WRKY protein and later induced *SOS1* due to lower ROS content. Higher $H_2O_2$ scavenging activity of both PgAPX1 and AtSOD1 were probably involved to lower ROS level.

In summary, salt stress treatment generated ROS production which induced expression of antioxidant genes such as *AtAPX1* and *PgAPX1*. However, the expression of *PgAPX1* was only found higher in $PgAPX1_{ox}$ lines, compared to WT. Overexpression of *PgAPX1* upregulated transcription factor *AtWRKY8* but not its antagonist, *AtVQ9*. These phenomena probably affected *AtSOS1* directly or indirectly which also found to be upregulated in $PgAPX1_{ox}$ lines.

### Biochemical analysis of $PgAPX1_{ox}$ lines under salt stress

Next, some biochemical analysis such as total chlorophyll content, RWC, proline content and APX activity were assayed before (0th day) and after (5th day) the exposure of 200 mM NaCl. Without the NaCl treatment, total chlorophyll content was same in WT and $PgAPX1_{ox}$ lines (Fig. 5d). During salt stress, total chlorophyll content of all tested lines was decreased, but the extent of this decline in $PgAPX1_{ox}$ was not significant and much less than that in the WT plants (Fig. 5d). These results indicated that the $PgAPX1_{ox}$ were less suppressed in growth and they lost less chlorophyll than the WT seedlings. Both WT and $PgAPX1_{ox}$ have similar RWC under control condition. However, WT suffered a significant RWC decline during salt stress as compared to $PgAPX1_{ox}$ lines (Fig. 5e). To relieve the harmful effects of salinity, one of the strategies for plants is to produce more small osmolytes or stress proteins (Zhu 2001). Increased level of proline was observed in transgenic lines under salt stress condition. Proline acts as a stabilizer of the plasma membrane and free radical scavenger, which can protect the plant under stress conditions (Jain et al. 2001). The accumulation of proline was 1.5–3 folds higher in transgenic lines as compared to WT under the salt stress (Fig. 5f). Results in this study suggest that increased proline content under salt stress might have helped plants to avoid the osmotic effect of high salinity. The free proline was used as an osmoprotectant which could help plants to adjust their cellular osmotic potential as well as act as an antioxidant (Verbruggen and Hermans 2008). These results ensure the histochemical assay of $PgAPX1_{ox}$ lines (Fig. 5b) which showed lower $H_2O_2$ accumulation in leaves after 200 mM NaCl treatment. To test whether APX activity affected $H_2O_2$ levels, APX activity was compared between WT and $PgAPX1_{ox}$ plants before and after salt treatment. As shown in Fig. 5g–h, the APX and SOD enzyme activity of all tested transgenic lines were significantly higher than WT plants under normal condition and salt stress conditions, respectively. These outcomes indicated that greater APX and SOD activity by *PgAPX1* overexpression plays an effective role in alleviating the adverse stress effects on *Arabidopsis* growth and development.

In summary, two full-length of *P. ginseng* cDNA encoding APX were identified, and the gene structures were demonstrated. Their expression pattern was analyzed in various ginseng tissues and under salt stresses condition. Phylogenetic comparison with other plants APX group and bioinformatics analysis revealed that both PgAPX1 and PgAPX2 belong to cytosolic APX members in *P. ginseng*. *Arabidopsis* overexpressing *PgAPX1* demonstrated enhanced tolerance against 300 mM NaCl by displaying higher germination rate, longer roots, less leaf chlorosis, less $H_2O_2$ accumulation, and higher chlorophyll content, relative water content, and proline content under salt stress than WT plants, caused by higher *PgAPX1* expression and APX activity. Our results suggest *PgAPX1* can help plant to be more tolerant of salt stress by affecting the ion homeostasis regulation and ROS detoxification regulation. This is the first report describing the isolation of APX genes from *P. ginseng* and it provides significant information for

improving abiotic stress tolerance by genetically engineering of crop/ other plants.

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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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