

Genome-wide analysis of ascorbate peroxidase and functional characterization of SpAPX249b and SpAPX285c for salt tolerance in *Sesuvium portulacastrum*

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1 **Title: Genome-wide analysis of ascorbate peroxidase and functional characterization of**
2 ***SpAPX249b* and *SpAPX285c* for salt tolerance in *Sesuvium portulacastrum***

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Abstract

Key message We identified 33 *SpAPXs* from the genome of *Sesuvium portulacastrum* and found *SpAPX249b* and *SpAPX285c* are important for salt tolerance in true halophyte.

Abstract Ascorbate peroxidase (APX) is a vital antioxidant enzyme, involving in plant development and stress response by scavenging excessive reactive oxygen species (ROS). The *APX* genes have been characterized in many organisms, however, its role in *Sesuvium portulacastrum* has not yet to be fully investigated. Here, we identified 33 *SpAPXs* from the the genome of *S. portulacastrum*, and found them to be divided into five subgroups across the 16 chromosomes. *Cis*-element analysis of their promoters indicated that all the detected *SpAPXs* show potential roles in response to biotic and abiotic stresses as well as phytohormone effects on the plant growth and development. Transcriptomic data of the different tissues revealed that the 9 *SpAPX* genes were specifically expressed in root and 13 *SpAPX* genes were specifically expressed in leaves, with *SpAPX249b* prominently expressed in root and *SpAPX285c* prominently expressed in leaves. Moreover, quantitative real-time PCR analysis revealed that both *SpAPX249b* and *SpAPX285c* genes expressed only after NaCl application and were sharply induced in the high concentration of NaCl treatments. Our findings suggest that *SpAPX249b* and *SpAPX285c* may be associated with palnt salt tolerance and can serve as valuable genes for enhancing salt tolerance in other plants.

Keywords: Ascorbate peroxidase; Genome-wide analysis; Salt stress; *Sesuvium portulacastrum*; Transcriptomic study; True halophyte.

Introduction

Plants are subjected to various abiotic environmental stresses, such as drought, cold, and salinity during growth and development (Fancy et al. 2017; Gong et al. 2020; Zhu 2016). Salt stress is a particular stress factor, which can cause many adverse effects on plants, including ionic toxicity, osmotic stress and oxidative stress, and seriously impede plant yield and growth (Yang and Guo 2018; Ma et al. 2022). In addition, salt stress triggers the production of reactive oxygen species (ROS), the form of hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^\cdot$), and superoxide anion (O^{2-}) (Mahajan and Tuteja 2005; Huang et al. 2019). Adverse conditions lead to excessive ROS and damage to the plant cell membranes and organelles through lipid peroxidation and protein oxidation (Mittler et al. 2022). In response to the balance between normal and excessive production of ROS, plants use various scavenging mechanisms, including enzymatic and non-enzymatic scavenging systems, and have evolved a complete antioxidant system (Mittler 2017; Shigeoka et al. 2002).

Sesuvium portulacastrum is a true halophyte, adapted to grow naturally on beaches (Kulkarni et al. 2023). Previously reported demonstrated that it contains a variety of genes related to salt tolerance, and these genes are beneficial to improvement of salt tolerance in cash crops and promote the more effective utilization of saline plants for the restoration of saline ecosystems (Cao et al. 2023). Among them, *SpSOS1* and *SpAHA* can alleviate salinity toxicity by enhancing Na^+ transport efficiency and preserving K^+ homeostasis in *S. portulacastrum* (Fan et al. 2019). *P152*, which putatively encoding late embryogenesis abundant in *Arabidopsis thaliana*, enriched under salty and drought conditions in *S. portulacastrum* (Zeng et al. 2006). This true halophyte displays tolerance to the combined heavy metal pollution of copper, zinc, and cadmium (Cd), and is capable of accumulating a certain amount of heavy metals, and salt stress enhances Cd^{2+} biosorption (Kulkarni et al. 2021). It is potential to be utilized as

a phytoremediation plant for the remediation of coastal heavy metal pollution (Feng et al. 2018).

Ascorbate peroxidase (APX) is a key antioxidant enzyme that plays an important role in the metabolism of ROS in plants. APX catalyzes the formation of O_2^{2-} and H_2O from the substrate H_2O_2 to protect plants from oxidative damage caused by the excessive accumulation of H_2O_2 (Li 2023; Sousa et al. 2015). In addition, APX is critical in plant response to high salinity in many plants (Maruta et al. 2016). *A. thaliana* *AtAPX1* plays a pivotal role to adapt to drought and high temperature stress (Koussevitzky et al. 2008). *AtAPX3* and *AtAPX5* in *A. thaliana* responded significantly to salt, drought, and heat stresses (Li et al. 2019). *Actinidia chinensis* *APX1* and *APX2* can improve salt resistance by overexpression in *A. thaliana* (Guo et al. 2022). The protection ability of transgenic tomato against cold and salt stress has been significantly increased by overexpression of *APX* gene (Wang et al. 2005). Similarly, overexpression of the *Populus* peroxisome *PpAPX* gene significantly improved both drought and salt tolerance in tobacco (Li et al. 2009). Overexpression of *Solanum lycopersicum* *APX* gene improves tobacco osmotic stress (Sun et al. 2010). Overexpression of *Camellia azalea* *CaAPX* gene in tobacco results in the increased cold and heat resistance (Wang et al. 2017). Additionally, overexpression of *Apium graveolens* *AgAPX1* in *A. thaliana* increases ascorbic acid content and improves drought tolerance (Liu et al. 2019).

Here, we conducted a comprehensive analysis of the phylogenetic relationship, chromosome distribution, gene structure, and expression patterns of *SpAPXs* in *S. portulacastrum*. Furthermore, transcriptomic analysis and quantitative real-time PCR were used to determine the expression patterns for two *SpAPX* genes involved in NaCl stress response in different tissues of this halophyte. These results provided basic and comprehensive information for further functional studies of *APX* genes in plants under different abiotic stresses.

Material and Methods

Plant Materials and Treatments

S. portulacastrum plants were obtained from the beach of Qinglan Harbor, Wenchang City, Hainan Province, and 3 cm long stem segments with nodes and two opposite leaves were intercepted from the top of the mother plant and cultivated in tap water for 30 days; then, they were switched to 1/2 Hoagland nutrient solution for cultivation. We treated *S. portulacastrum* plants with 1/2 Hoagland's solution (Ding et al. 2022) containing 0.8 M NaCl for stress treatment. *Nicotiana benthamiana* (Tobacco) plants were grown in a greenhouse under natural light in soil mixed with rich nutrients and perlite (2:1).

Identification of the APX gene family in *S. portulacastrum*

APX family members in the *S. portulacastrum* genome were characterized based on the similarity of homologous sequences of the APX family in the *A. thaliana* genome. The amino acid sequences of eight AtAPXs were obtained from TAIR (<https://www.arabidopsis.org/>). The local database of the protein sequences was created using the blast-2.9.0+ software to find SpAPX candidates. First, eight AtAPX amino acid sequences were used as a query with the e-value set to 1e-20, and the redundant sequences were removed. Next, we used the Pfam website (<http://pfam.janelia.org/>) to obtain the Pfam ID (PF00141) for the APX gene and further manually identified SpAPX gene family members.

Characterization of APX gene family in *S. portulacastrum*

ExPASy (<http://web.expasy.org/protparam/>) was used to identify the physicochemical characteristics of APX proteins. Cell-PLoc 2.0 online (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2>) was used to project APX protein subcellular localization. Gene Structure Display Server (GSDS) (<http://gsds.cbi.pku.edu.cn>) was used to analyze the structural feature by comparing CDSs and genomes of each APX gene. MEME (<http://meme-suite.org/tools/meme>) was used to identify conserved motif

analysis.

Multiple sequence alignment and phylogenetic analysis

Using MUSCLE version 3.8.1, multiple sequence alignments were performed for the protein sequences of *S. portulacastrum*, *A. thaliana*, and *Oryza sativa*. Using MUSCLE version 3.8.1, multiple sequence alignments were performed for the protein sequences of *S. portulacastrum*, *A. thaliana*, and *O. sativa*. Phylogenetic trees were created using the iQ-Tree online software (<https://itol.embl.de/>) and the Maximum Likelihood (ML) method with bootstrap option n = 1000.

Chromosomal Location and Collinearity

The chromosomal localization of APX family members was obtained from the genome-annotated GFF files of *S. portulacastrum*, and then the organized gene localization information was visualized using TBtools software. The Multiple Collinearity Scan toolkit (MCScanX) program was used to detect gene duplication event types of APX family genes in *S. portulacastrum*, which were visualized by Circos software. Finally, the Dual Systemy Plotter program of TBtools software was used to analyze collinearity between *S. portulacastrum* and *A. thaliana*, *O. sativa*, *Populus trichocarpa*, *Glycine max*, *S. lycopersicum* and *Zea mays*.

Cis-element analysis in the SpAPX gene promoters

To predict cis-elements, 2000 bp sequences were extracted from the *SpAPX* genes using TBtools software and submitted to PlantCARE (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Expression Profile of SpAPX gene in different tissues and response to salt stress by RNA-seq

Previously reported expression data were used to analyze *SpAPX* gene expression profiles in various tissues and salt stresses (Ding et al. 2022), and the heatmaps were created with TBtools software to display gene expression profiles.

qRT-PCR

The leaves of *S. portulacastrum* grown in 0.8 M NaCl at 0, 3, 6, and 12 hours after identification to verify gene expression. Total RNA of *S. portulacastrum* was extracted using RNA simple Total RNA Kit (TianGen, Beijing, China), cDNA first-strand synthesis was conducted by reverse transcription using a NovoScript Plus All-in-one SuperMix kit (Novoprotein, Shanghai, China). Subsequently, Real-time PCR was performed using ChamQTM Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). StepOne Plus Real-Time PCR system was used with the following thermal cycling conditions: 95°C for 30 s, 40 cycles at 95°C for 10 s, 60°C for 30s and 72°C for 30 s. *SpGAPDH* was used as an endogenous control to normalize the samples (Zhou et al. 2015). Primer 3 (<http://primer3.ut.ee/>) was used to design PCR primers based on the cDNA sequences of *SpAPX* genes. Three technical replicates were performed for each sample. Relative transcript levels in the samples were calculated using the comparative $2^{-\Delta\Delta CT}$ method (Rao et al. 2013). Primers for the *SpAPX* gene used in the qRT-PCR assay are listed in Supplementary Table 1.

Subcellular Localization

SpAPX249b and *SpAPX285c* CDS were amplified using the gene-special primers (Supplementary Table 1), and fusion to pCAMBIA1300-GFP vector to 35S::SpAPX249bGFP, and 35S::SpAPX2285cGFP, respectively, with the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). Using *Agrobacterium*-mediated methods, pCAMBIA1300-SpAPX249b: GFP and pCAMBIA1300-SpAPX285c: GFP plasmids were transformed into leaves of one-month-old tobacco. Confocal laser scanning microscopy (Carl Zeiss SAS, Jena, Germany) was used to detect fluorescence signals after two days of incubation.

Results

Identification and Characterization of APX Genes in *S. portulacastrum*

A total of 33 SpAPXs proteins were identified as peroxidase and then named based on their number of amino acids (Table 1). Next, we examined their physical and chemical properties and fundamental characteristics. These proteins contain amino acid residues ranging from 56 (SpAPX56) to 461 (SpAPX461), with estimated molecular weights ranged from 5.95 kDa to 50.24 kDa. Their theoretical isoelectric points varied from 4.81 (SpAPX56) to 9.42 (SpAPX243), and the instability indices from 28.66 (SpAPX245) to 47.89 (SpAPX428). Protein indexes of the 23 SpAPXs are all less than 40, indicating that 69% SpAPXs are stable. Moreover, all SpAPXs had negative grand average of hydropathicity scores, suggesting that these proteins are hydrophilic. In addition, subcellular localization prediction showed that there are 16 SpAPX proteins in the cytoplasm, with ten SpAPX proteins located in peroxisome, three in chloroplast, three in chloroplast and mitochondrion, and one (SpAPX56) has non-specific location.

Phylogenetic Analysis of *SpAPX*

In order to evaluate the phylogenetic relationships of the detected APXs in *S. portulacastrum*, a phylogenetic tree was constructed using the 33 members from *S. portulacastrum*, 8 from *A. thaliana*, and 8 from *O. sativa* (Fig. 1). The result showed that the 33 SpAPX proteins could be divided into five subgroups. Subgroups II and V are the most extensive, containing 25 proteins. Three proteins were classified into subgroup I and III, and two proteins in IV, respectively.

Gene Structures and Conserved Motifs of *SpAPX* Genes

To gain further insight into the structural and functional divergence of these *SpAPXs*, we conducted a comprehensive analysis of their phylogenetic tree, exon-intron structure, and conserved motifs. We identified nine conserved motifs using the MEME server, named motifs 1 to 9 (Fig. 2B). Motifs 7 and 8 were existent in all SpAPX proteins, and most SpAPX proteins carried motifs 2, 3, 6, and 8, indicating that the functions of these motifs are conserved during evolution. In addition, members within the same

group displayed similar motif compositions and locations. However, distinct differences were observed between different groups, implying that SpAPX proteins may perform similar biological functions within the group and differences between groups. Analysis of exon-intron showed that the members in subgroup III are longer and contain more than 11 exons, whereas subgroup II members are shorter with only two to three exons (Fig. 2C). Typically, introns, UTR, and exons exhibited more similarity between subgroups from the same evolutionary branch of the tree.

Chromosomal Location and Duplications of *SpAPX* Genes

To ascertain the chromosomal distribution of *SpAPX* genes on chromosomes, we applied TBtools software. The *SpAPXs* were distributed across 16 chromosomes in *S. portulacastrum* genome, with one in chromosomes 2, 4, 8, 9, 11, 15, 21, and 23, two in chromosomes 3, 16, 22, and 24, three in chromosome 17, four in chromosomes 10 and 13, and six in chromosome 7, respectively (Fig. 3). Furthermore, duplication events were analyzed using MCScanX software to explore the amplification mechanism of the *SpAPX* genes. We found that six tandem duplication events and 32 pairs segmentally duplicated genes were distributed on the 16 chromosomes (Fig. 4).

Collinearity Analysis of *SpAPX* Genes in *S. portulacastrum*

We subsequently examined the covariance of SpAPX with APX genes in other species and their evolutionary origins, and performed a comparative collinearity analysis within the other six representative plants, including four dicotyledonous plants (*A. thaliana*, *G. max*, *P. trichocarpa*, and *S. lycopersicum*) and two monocotyledons plants (*O. sativa* and *Z. mays*). Our results revealed that 19, 32, 53, and 23 collinear gene pairs among *A. thaliana*, *G. max*, *P. trichocarpa*, *S. lycopersicum*, and *S. portulacastrum*, whereas only ten and four gene pairs were found in *O. sativa*, *Z. mays*, and *S. portulacastrum* (Fig. 5). Additionally, we noticed that 15 *SpAPXs* showed collinear relationship with four dicotyledonous plants, and only *SpAPX215* had collinear relationship with four dicotyledonous plants and two monocotyledons plants (Supplementary Table 2).

Promoter *cis*-Element Analysis

The Plant CARE website was employed to conduct a further investigation of the function of the APX gene family in *S. portulacastrum* by means of an analysis of the promoter region located 2000 bp upstream of the transcriptional start site (Supplementary Table 3). Our results revealed that these *cis*-elements could be primarily categorized into four main categories involved in stress, hormone, light responsiveness, and plant development (Fig. 6). Interestingly, we observed that, except for *SpAPX243*, *SpAPX285a* and *SpAPX428*, the remaining 30 *SpAPX* genes contain *cis*-elements related to stress-related elements, such as drought, low-temperature, and defense elements. Except for *SpAPX335a*, the other *SpAPXs* have hormone-responsive elements, especially for abscisic acid, methyl-jasmonate, and gibberellins.

Expression Profiles of *SpAPX* Genes in Various Tissues and Response to Salinity

The expression profiles of *SpAPX* genes at different growth stages, including young root, mature root, young stem, mature stem, leaf margin, and mature leaf, were analyzed to explore the potential function of *SpAPX* genes for plant growth and development. The results showed that each *SpAPX* expressed differently in different tissues (Fig. 7A). Specifically, *SpAPX243*, *SpAPX215*, and *SpAPX326* exhibited higher expression levels in young root, while *SpAPX335a*, *SpAPX335b*, *SpAPX245*, *SpAPX250*, *SpAPX249a*, and *SpAPX249b* were highly expressed in mature root. *SpAPX325a* and *SpAPX325b* were highly expressed in young stem, whereas *SpAPX318a* and *SpAPX317* showed significantly higher transcript levels in mature stem. Additionally, *SpAPX325c*, *SpAPX317*, and *SpAPX319* exhibited significantly higher expression in leaf margins. Notably, ten genes, including *SpAPX461*, *SpAPX285c*, *SpAPX428*, *SpAPX340*, *SpAPX56*, *SpAPX285a*, *SpAPX285b*, *SpAPX318b*, *SpAPX225a*, and *SpAPX352* displayed markedly higher expression in leaf margin and mature leaf.

As *cis*-element analysis of *SpAPXs* indicated their potential responsiveness to environmental stresses, we further measured the expression profiles of *SpAPXs* under treatment of different ions and different concentrations of NaCl. Our results revealed that *SpAPXs* were induced in response to both K⁺ and Na⁺ (Fig. 7B). Additionally, the expression levels of most *SpAPXs* were significantly increased after treating with 0.2, 0.4 and 0.6 M NaCl. We also observed that the expression of seven genes, named *SpAPX225a*, *SpAPX352*, *SpAPX245*, *SpAPX322*, *SpAPX340*, *SpAPX56* and *SpAPX319*, increased at low NaCl concentration, but decreased significantly with the application of increased NaCl concentration (Fig. 7C). Interestingly, we noted that *SpAPX285a*, *SpAPX285b*, *SpAPX285c*, and *SpAPX249b* showed a continuously increasing trend in leaves, and their expression was significantly higher in high concentration NaCl treatment. *SpAPX249b* and *SpAPX285c*, which exhibited high expression levels in roots and leaves and demonstrated a robust response to elevated NaCl concentrations were selected for future investigation.

Expression Profiles and Subcellular Localization of *SpAPX249b* and *SpAPX285c* proteins

Based on the detected expression profiles, we conducted quantitative reverse transcription polymerase chain reaction (qRT-PCR) and subcellular localization analysis of the *SpAPX* genes (*SpAPX249b* and *SpAPX285c*). *SpAPX249b* was upregulated, while *SpAPX285c* was suppressed compared with control by treatment with NaCl (Fig. 8A). Moreover, we further found that *SpAPX249b* expressed in both the cell membrane and the nucleus and *SpAPX285c* protein was expressed in the cell membrane and the cytoplasm in tobacco leaves (Fig. 8B).

Discussion

The APX enzyme is important for the clearance of H₂O₂ from plant cells involved in various growth and development processes, as well as responses to various stresses (Gilroy et al. 2016; Tao et al. 2018).

Previous reports identified *APXs* in various plant genomes, 8 in *A. thaliana*, 10 in *Oryza sativa*, 24 in *Brassica napus*, 21 in wheat, 118 in the *Brassica rapa*, 65 in pitaya, 11 in *P. trichocarpa*, 9 in pepper (Aleem et al. 2022; Jardim-Messeder et al. 2023; Leng et al. 2021; Ma et al. 2021; Panchuk et al. 2002; Pan et al. 2022; Pang et al. 2023; Teixeira et al. 2004; Tyagi et al. 2020; Zaman et al. 2023). A total of 33 *SpAPX* genes were identified in the *S. portulacastrum* genome with the conserved acid peroxidase domain. More number of *SpAPXs* are found in *S. portulacastrum* than in *A. thaliana* or rice, suggesting that *SpAPX* genes may perform more complex and diversity functions in this halophyte. Importantly, all *SpAPXs* are hydrophilic and predicted to be located in the cytoplasm, peroxisome, chloroplast, and mitochondrion, which is consistent with the fact that *APX* enzymes regulate the detoxification of ROS in subcellular compartments to maintain redox homeostasis (Pandey et al. 2017).

During evolution, tandem duplications and segmental replications are thought to provide the genetic material that generated new genes and promoted the creation of gene families (Leister 2004). In this work, 6 tandem duplication events and 32 pairs of segmentally duplicated genes were identified. Location of *APX* genes on chromosomes shows that most *SpAPX* genes were located on the ends or beginnings site, which were the chromosomes dynamic zones and tended to occur as segmental or tandem duplicate events. Therefore, we hypothesized that the higher number of *APX* genes compared to *O. sativa* and *A. thaliana* may be attributed to the duplication of *SpAPX* genes. These findings indicate that *SpAPXs* have originated from gene duplication and that segmental duplication event is a major driver of evolution in the *SpAPX* gene family. Collinearity analysis showed that *SpAPX* genes had more collinear gene pairs with dicotyledonous plants than monocotyledonous, indicating a gradual loss trend of the *APX* gene family in the path of plant evolution from dicotyledonous to monocotyledonous. We also observed that 15 *SpAPX* genes showed collinear relationship with 4 dicotyledonous plants and one *SpAPX* gene with 4

dicotyledonous plants and two monocotyledonous plants, which may reflect that these *APX* genes are conserved and play an indispensable role in the evolution of dicotyledonous and monocotyledonous plants.

Promoter *cis*-element is crucial for regulating plant adaptation to stress, growth, and development by regulating target gene expression (Ding et al. 2022; Su et al. 2023; Wang et al. 2024). Furthermore, some phytohormones (abscisic acid, salicylic acid, jasmonic acid, and ethylene) are associated with the regulation of plant defenses (Santner and Estelle 2009). Here, we identified numerous *cis*-elements in the promoter region of *SpAPX* genes, including stress-responsive, developmental regulation, and hormone-related response elements, suggesting that *SpAPX* genes have diverse functions in plant hormone and abiotic stress responses. Moreover, heat map analysis of gene expression revealed variable spatial expression profiles of *SpAPX* genes, with high expression levels in specific tissues, suggesting their involvement in regulating tissue development in *S. portulacastrum*. Similarly, the high tissue expression levels of *SpAPX* genes were consistent with previous reports on plants, such as *A. thaliana*, maize, and pepper (Miller et al. 2010; Pnueli et al. 2003; Suzuki et al. 2013). We also identified seven *SpAPX* genes that were not expressed in the selected samples, suggesting that these genes may be expressed explicitly in other tissues or periods. Besides, numerous studies have shown that APX family members may exhibit different expression profiles in various stress environments, such as ROS, high temperature, and drought (Chen et al. 2014; Pang et al. 2023; Qu et al. 2020). Therefore, we analyzed the expression patterns of *SpAPX* genes in leaves under different salt stress conditions. A significantly up-regulated expression trend of *SpAPX* genes under salt stress was found in different salt ions in the leaves, implying that they might be broadly related to the regulation of *S. portulacastrum* in response to salt stress. Previous studies have shown that *S. portulacastrum* can maintain vigorous growth under 0.1-

0.4 M NaCl, with optimal growth at 0.2 M NaCl and significant inhibition above 0.4 M NaCl (Fan et al. 2009). This suggests that the expression profiles of *SpAPX* genes may vary under different salt concentrations, with genes up-regulated at low salinity being involved in growth and development, while those up-regulated at high salinity are involved in salt stress responses. Because *SpAPX249b* and *SpAPX285c* showed continuously up-regulated expression under different NaCl concentrations treatment, qRT-PCR was used to evaluate the expression profiles of *SpAPX249b* and *SpAPX285c* under 0.8 M NaCl treatment. The expression of *SpAPX249b* in the leaves showed a fluctuating trend, while the expression level of *SpAPX285c* showed a continuous decrease. We hypothesized that this may be due to the expression of *SpAPX* genes were various in response to salt stress, ensuring a dynamic balance of ROS.

Several studies have shown that APX has five isoforms, cytosolic (*cAPX*), peroxisomal (*pAPX*), mitochondrial (*mitAPX*), stromal (*sAPX*), and thylakoidal (*tAPX*) (Shigeoka et al. 2002; Liao et al. 2020). Fourteen *SpAPXs* were classified in sungroup two along with *AtAPX6*, which located in cytosolic, and six *SpAPXs* close relationship to *AtAPX5* in subgroup five (Chen et al. 2014). Previous studies have shown that *cAPX* is central to the ROS scavenging network and plays a key role in regulating H₂O₂ levels and signaling (Davletova et al. 2005; Ribeiro et al. 2017). We observed significant changes in the expression levels of *SpAPX249b* and *SpAPX285c* induced under high NaCl concentrations, with both mainly expressed in the cell membrane. These findings suggest potential key roles for *SpAPX249b* and *SpAPX285c* in salt tolerance. However, the function of *SpAPX249b* and *SpAPX285c* in abiotic stress needs to be further investigated.

Conclusion

This study identified and comprehensively analyzed the 33 *SpAPX* genes in *S. portulacastrum* genome, and described their physio-chemical properties, phylogenetic tree, intron-exon structure,

conserved motif, *cis*-elements and gene expression patterns. In addition, we have identified two candidate genes (*SpAPX249b* and *SpAPX285c*) that may play important roles for salt response in *S. portulacastrum*. Overall, this work provides valuable information for further functional studies of *SpAPX* genes, it offers a opportunity for the cultivation of superior salt-tolerant plants.

Declarations

Conflicts of interest: The authors declared that they do not have any commercial or associative interest that represents a conflict of interest in connection with this work.

Data availability Data will be made available on request.

Author contributions XC W designed the study; BX Y, LX H and YF W provided the experimental materials. HL Z and YX L conceived the experiments and wrote the manuscript. JY W and SG H revised the manuscript. ZZ X, QQ N and ZC Y performed the experiments. All authors approved the manuscript.

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Supporting information available

Supplementary table 1 The primers used in this study.

Supplementary table 2 The inferred *SpAPXs* orthologs in *A. thaliana*, *P. trichocarpa*, *G. max*, *S. lycopersicum*, *O. sativa*, and *Zea mays*.

Supplementary table 3 Predicted *cis*-elements results.

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Figures and legends

Fig. 1 Phylogenetic tree of APX family genes in *A. thaliana*, *S. portulacastrum*, and *Oryza sativa*.

APX protein sequences was drawn by neighbor-joining (NJ) method in MEGA7.0. Three species are marked by purple, blue and green circles, respectively. All APXs are classified into five branches indicated by specific colors. Numbers at each node in the phylogenetic tree represent bootstrap values

Fig. 2 Conserved motifs and gene structures of *SpAPX* genes. (A) Phylogenetic tree of APX family in *S. portulacastrum*. (B) Distribution of conserved motifs. Colored rectangles distinguish the specific motifs. (C) Gene structural structures. UTRs, CDS and introns are denoted in blue boxes, yellow boxes and black lines, respectively

Fig. 3 Distribution on chromosomes and duplication events analysis.

Blue vertical bars represent the *S. portulacastrum* chromosomes, chromosome number indicates to the left of each chromosome (blue), and gene number indicates to the right of each chromosome (black).

Fig. 4 The duplication events of APX genes in *S. portulacastrum*.

Red lines represent collinear blocks and syntenic APX gene pairs, respectively

Fig. 5 Synteny analysis of APX genes between *S. portulacastrum* with *A. thaliana* (A), *Glycine max* (B), *Populus trichocarpa* (C), *Solanum lycopersicum* (D), *Oryza sativa* (E), and *Zea mays* (F).

Red highlight lines indicated the syntenic APX gene pairs, and gray lines in the background indicated the collinear blocks within *S. portulacastrum* and other plant genomes

Fig. 6 Analysis of *cis*-elements in *SpAPX* promoters.

(A) *Cis*-elements in the promoters of *SpAPXs* are presented. The number of different promoter elements is indicated by the different colors and numbers of grids. (B) Sum of the *cis*-elements in each category is further highlighted by the histograms with different colors

Fig. 7 Heatmap of expression patterns for *SpAPX* genes in different samples.

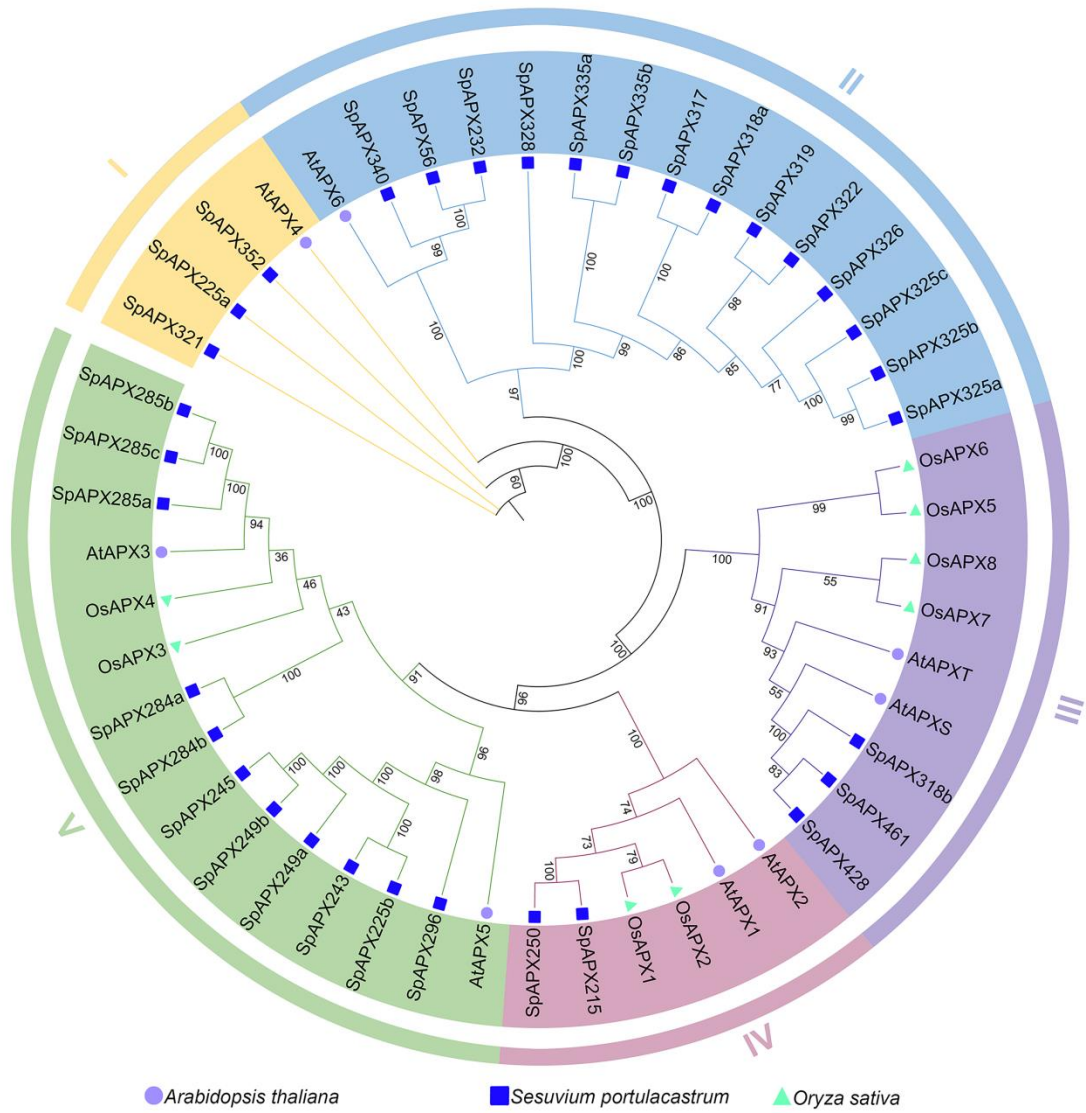
The genes expressed in the six detected tissues (A), leaves under different salt ions (B) and different concentrations of NaCl treatments are highlighted (C). The bars at the top of the heatmap are represented with the scale of $\log_2 (1 + \text{FPKM})$ signal values of each gene

Fig. 8 Expression profile and subcellular localization analysis of *SpAPX249b* and *SpAPX285*.

(A) Changed patterns of gene expression in leaves for *SpAPX249b* and *SpAPX285c* in response to 800 mM NaCl treatment at different time. Error bars represent significant differences among the three replicates. * represent significant difference between 0h (ck) and other time points at $P < 0.05$. (B) Subcellular localization of *SpAPX249b* and *SpAPX285c* in tobacco leaves was also determined. Scale bar = 20 μm .

541 Figures

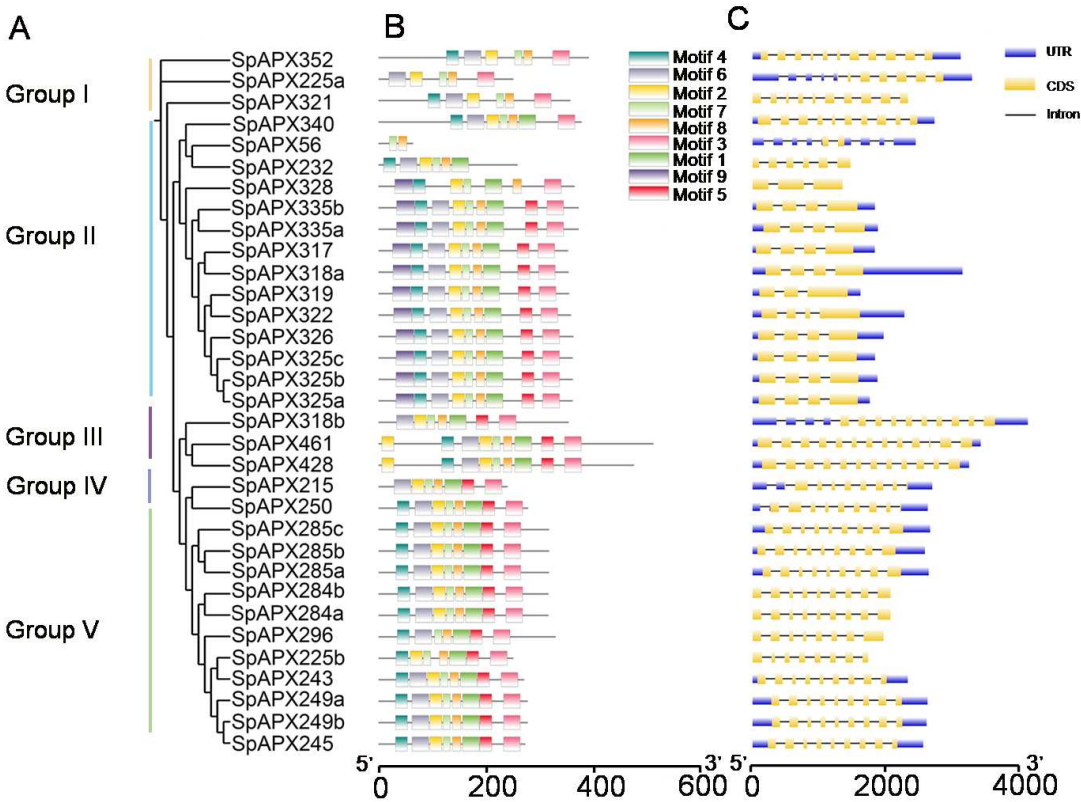
542 **Fig. 1**



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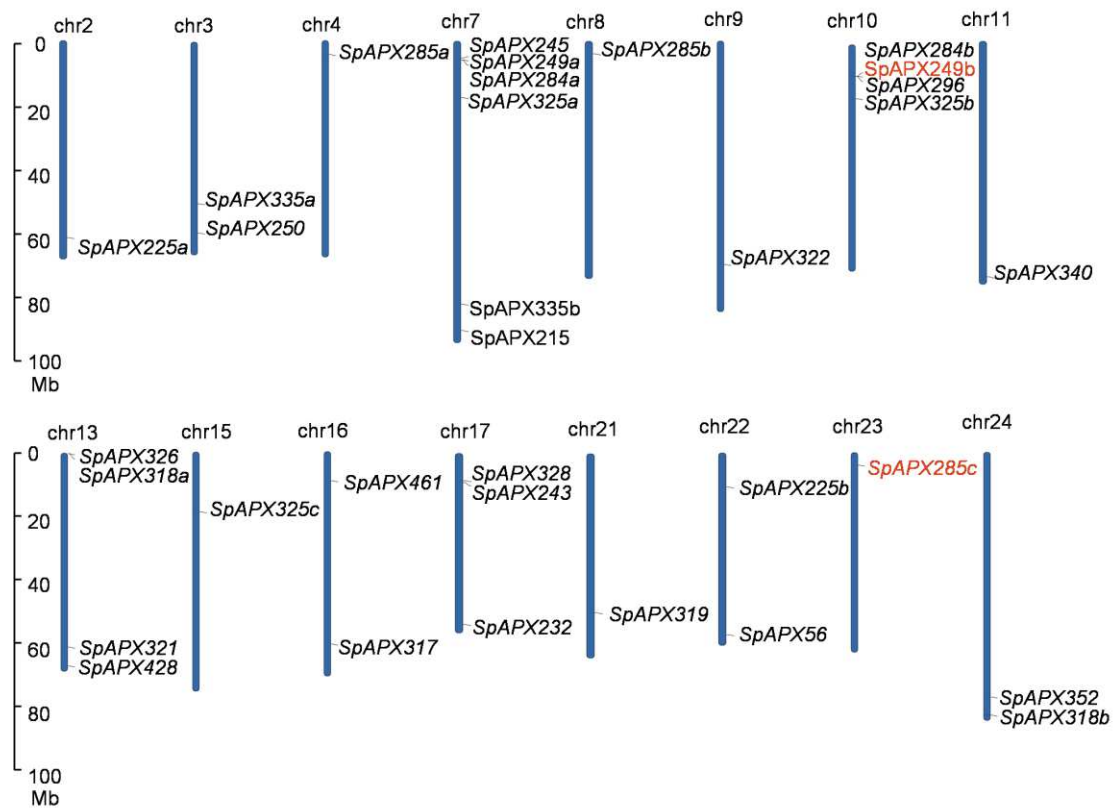


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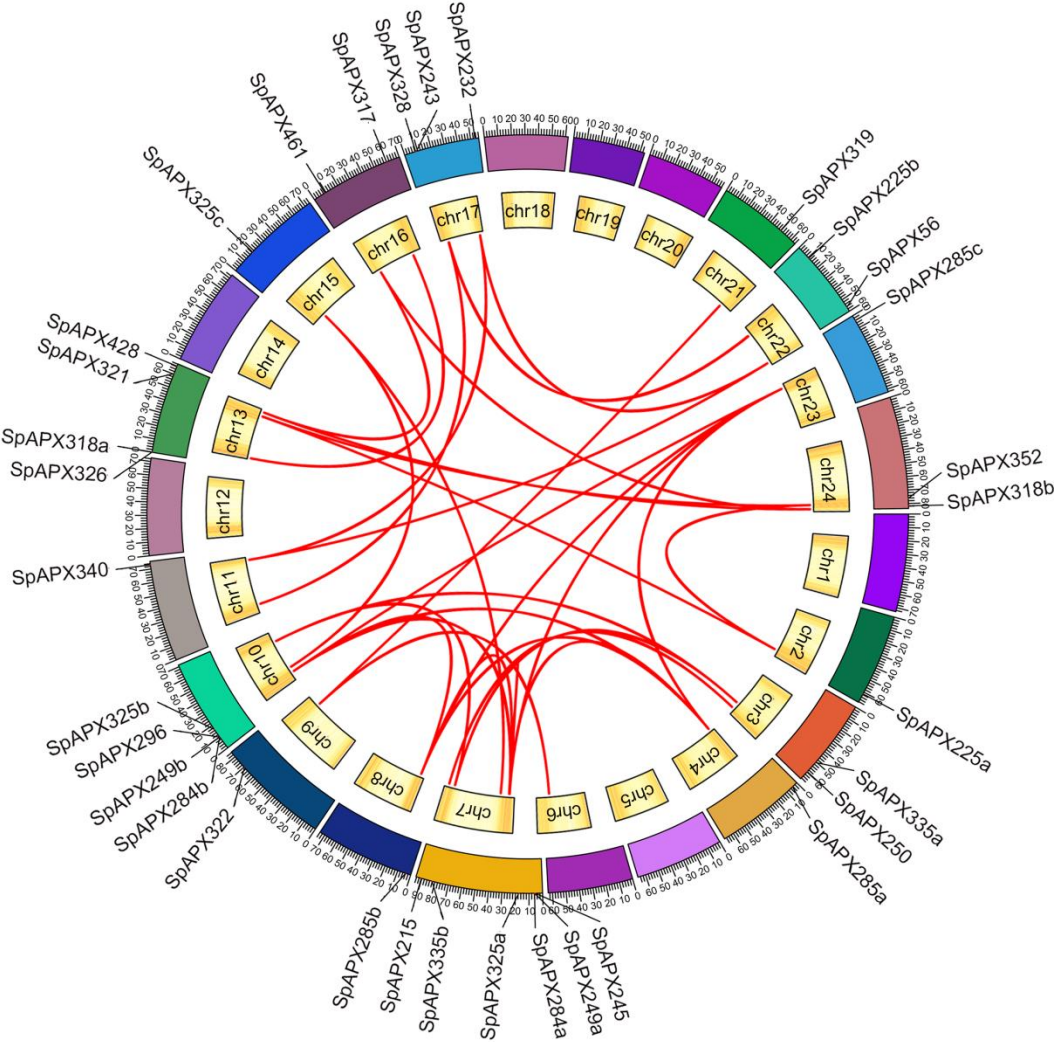
550 **Fig. 3**



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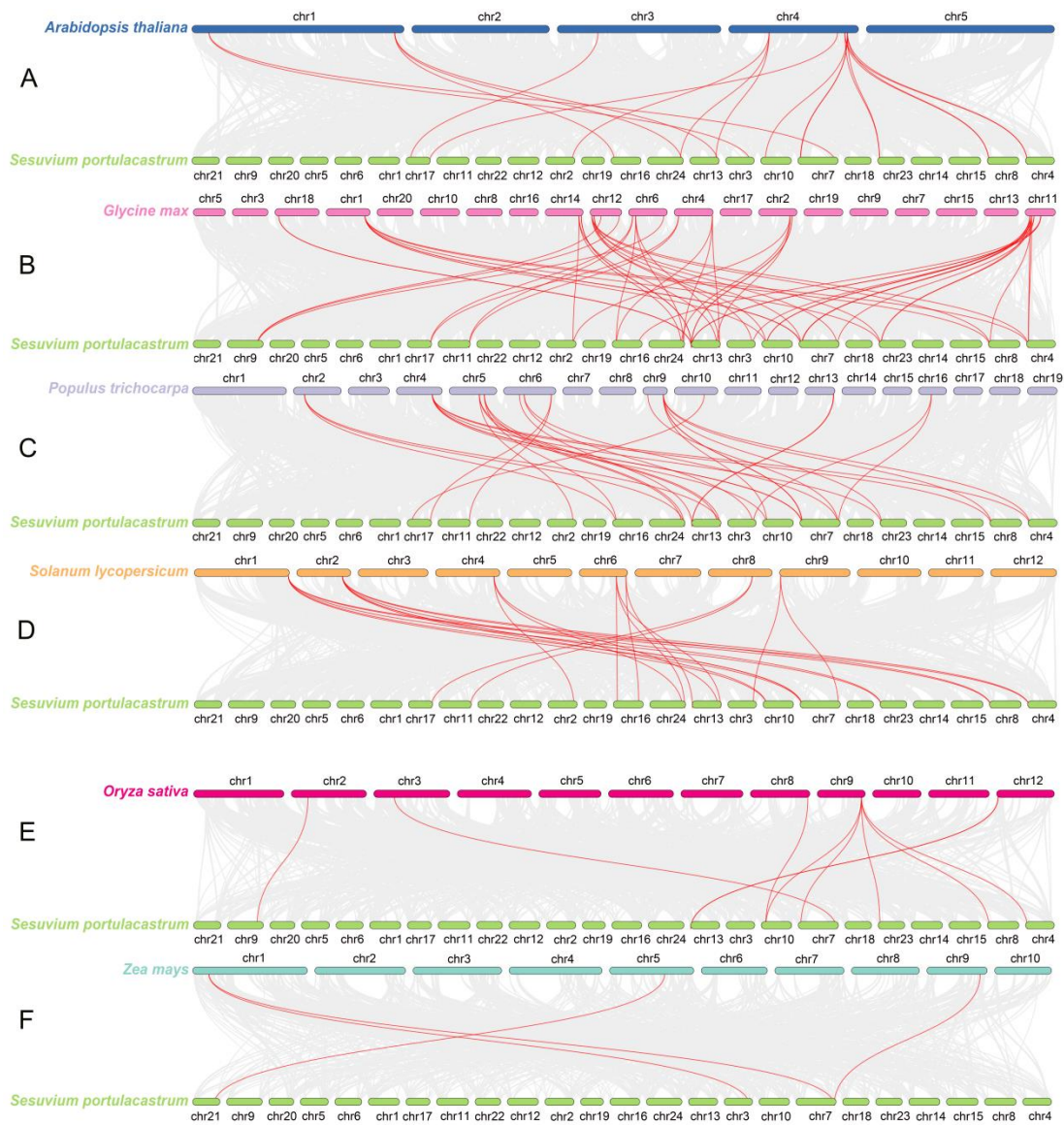
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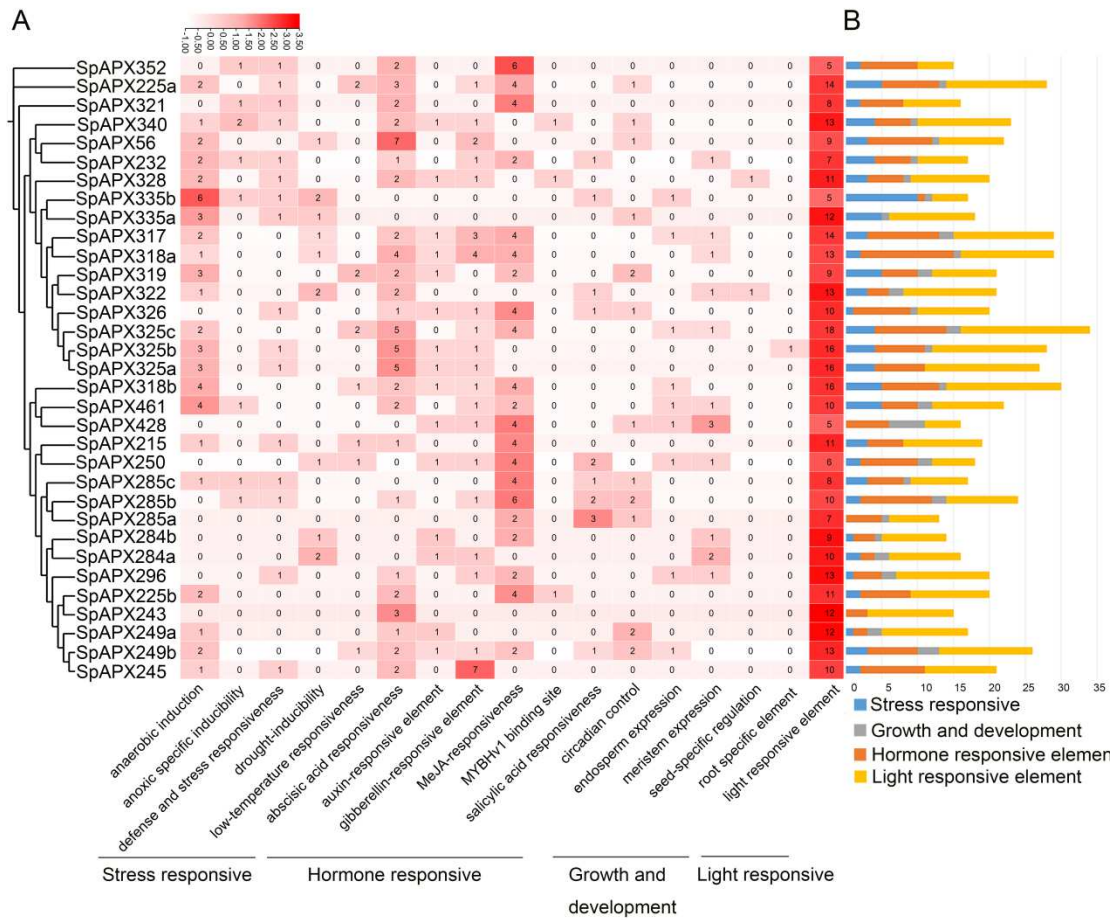
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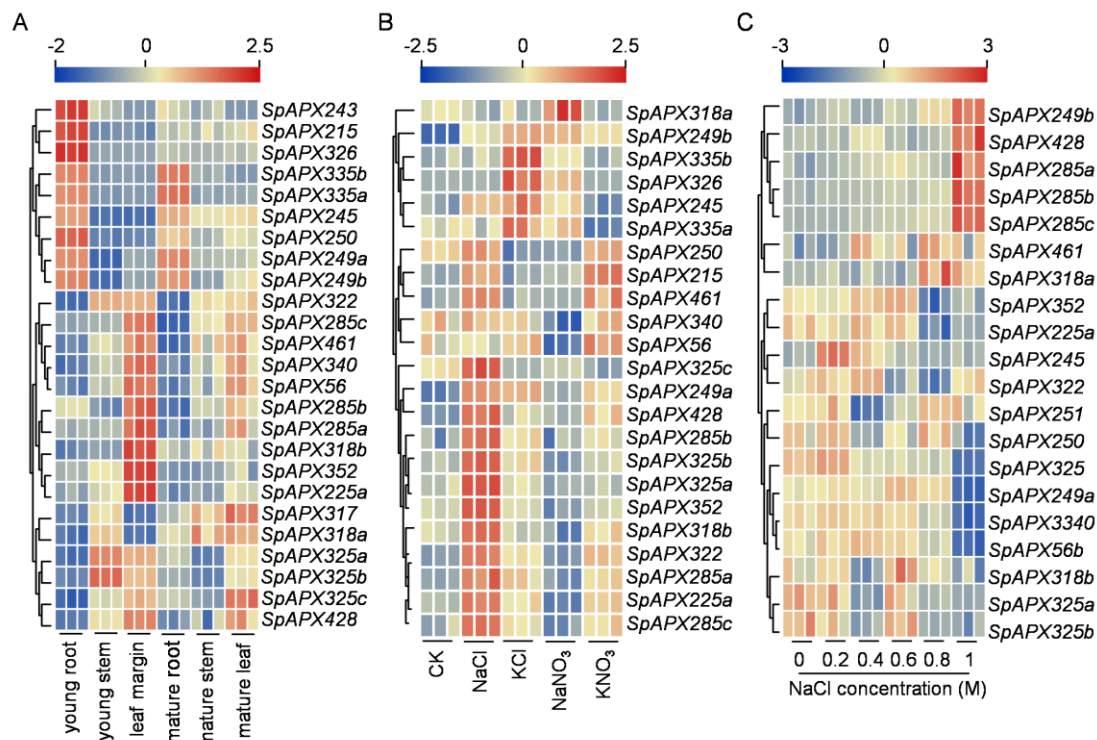


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566 **Fig. 7**

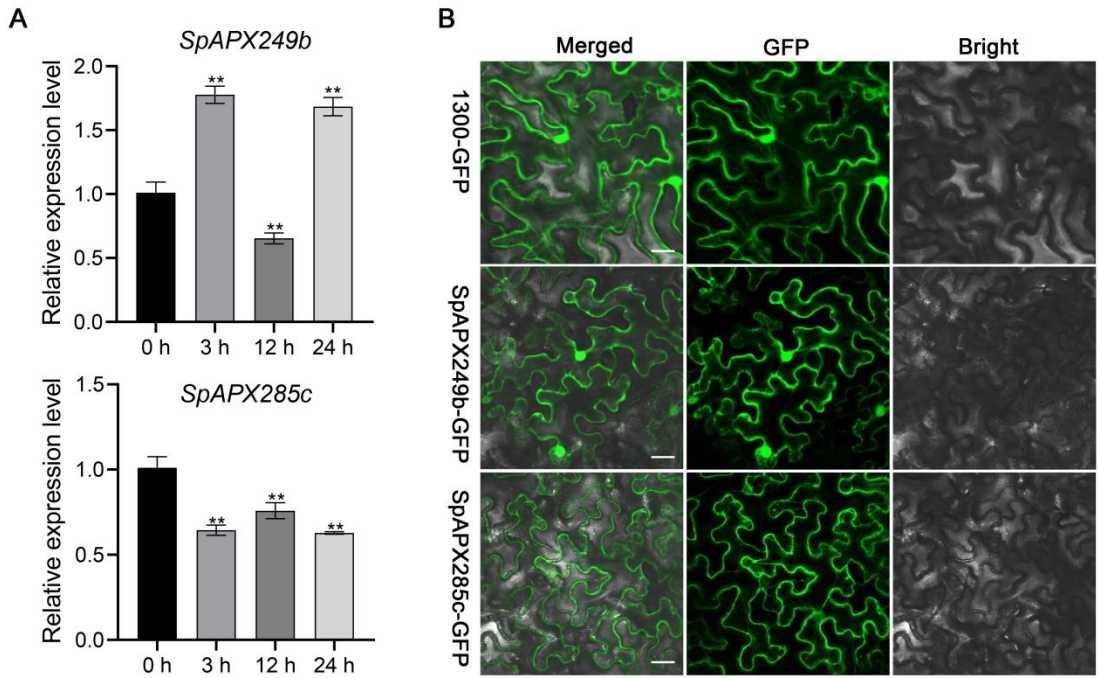


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Fig. 8



574 **Table 1 Identification of 33 APX genes in the genomes of *S. portulacastrum***

Name	Gene ID	Protein (aa)	MW (kDa)	Theoretical pI	Instability Index	Aliphatic Index	GRAVY	Subcellular Localization
SpAPX56	Spor0536800.1	56	5.95	4.81	36.96	80.18	-0.18	Chloroplast/Cytoplasm Nucleus/Peroxisome
SpAPX215	Spor0027930.1	215	23.66	4.97	37.66	78.09	-0.399	Peroxisome
SpAPX225a	Spor0309170.1	225	24.78	5.46	36.22	76	-0.477	Chloroplast
SpAPX225b	Spor0523000.1	225	24.79	7.76	32.17	80.27	-0.314	Cytoplasm
SpAPX232	Spor0581470.1	232	24.84	5.78	41.32	84.05	-0.034	Cytoplasm
SpAPX243	Spor0568330.1	243	26.49	9.42	28.66	79.55	-0.356	Cytoplasm
SpAPX245	Spor0004110.1	245	26.91	6.24	38.65	78.86	-0.323	Peroxisome
SpAPX249a	Spor0004610.1	249	27.32	6.05	39.01	77.99	-0.327	Peroxisome
SpAPX249b	Spor0237300.1	249	27.32	6.05	38.24	78.39	-0.323	Peroxisome
SpAPX250	Spor0391590.1	250	27.56	5.41	40.09	76.16	-0.468	Cytoplasm
SpAPX284a	Spor0004620.1	284	31.63	6.16	33.27	82.43	-0.351	Peroxisome
SpAPX284b	Spor0237290.1	284	31.78	6.72	28.74	84.82	-0.334	Peroxisome
SpAPX285a	Spor0350160.1	285	31.33	7.02	36.19	83.54	-0.373	Peroxisome
SpAPX285b	Spor0139730.1	285	31.25	7.67	34.28	83.58	-0.351	Peroxisome
SpAPX285c	Spor0470830.1	285	31.23	7.02	36.67	83.93	-0.357	Peroxisome
SpAPX296	Spor0237310.1	296	32.06	7.05	39.43	98.18	-0.139	Cytoplasm
SpAPX317	Spor0279260.1	317	33.69	6.05	38.42	81.55	-0.068	Cytoplasm
SpAPX318a	Spor0314980.1	318	33.62	6.58	37.93	81.32	-0.042	Cytoplasm
SpAPX318b	Spor0082650.1	318	34.99	5.55	42.88	69.78	-0.543	Chloroplast/ Mitochondrion
SpAPX319	Spor0434720.1	319	34.41	8.96	41.69	80.25	-0.151	Cytoplasm
SpAPX321	Spor0340980.1	321	35.17	5.89	38.84	79.44	-0.393	Chloroplast
SpAPX322	Spor0042890.1	322	35.02	8.55	36.72	85.84	-0.093	Cytoplasm
SpAPX325a	Spor0013610.1	325	35.02	9.08	32.04	85.88	-0.106	Cytoplasm
SpAPX325b	Spor0242210.1	325	35.02	8.97	32.57	85.88	-0.106	Cytoplasm
SpAPX325c	Spor0119770.1	325	35.06	8.98	32.02	86.8	-0.118	Cytoplasm
SpAPX326	Spor0314960.1	326	35.04	9.05	40.34	79.91	-0.149	Cytoplasm
SpAPX328	Spor0567940.1	328	35.86	5.15	43.39	91.31	0.081	Cytoplasm
SpAPX335a	Spor0387020.1	335	36.93	5.08	38.95	87.04	-0.067	Cytoplasm
SpAPX335b	Spor0021600.1	335	36.87	5.17	38.11	86.48	-0.036	Cytoplasm
SpAPX340	Spor0104780.1	340	36.81	8.49	40.41	84.91	-0.1	Cytoplasm
SpAPX352	Spor0078150.1	352	38.35	6.65	40.81	82.39	-0.295	Chloroplast

SpAPX428	Spor0345660.1	428	46.69	8.63	47.89	72.17	-0.426	Chloroplast/ Mitochondrion
SpAPX461	Spor0265090.1	461	50.24	6.69	44.1	77.79	-0.327	Chloroplast/ Mitochondrion

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