Proline metabolism regulation in *Spartina alterniflora* and *SaP5CS2* gene positively regulates salt stress tolerance in transgenic *Arabidopsis thaliana*

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**ABSTRACT**

It has been discovered that many plants accumulate proline in response to osmotic stress. However, the interaction between salinity stress and proline metabolism-related genes has not been unequivocally identified in *Spartina alterniflora*. In our research, we successfully cloned key genes: *SaP5CS1* and *SaP5CS2*, *SaOAT*, *SaProT* and correlated with expression profiles including *SaPDH* were also investigated. As the key enzyme genes for proline metabolism, the up-regulated expression of *SaP5CS2* played a leading part under salt stress. Chlorophyll contents decreased gradually degree in leaves with prolonged salt stress time. Additionally, transgenic Arabidopsis overexpressing *SaP5CS2* showed high tolerance to salt stress and accumulated more proline and H$_2$O$_2$ content was also reduced in salt stress condition. This study provided a foundation for deciphering the molecular osmoregulation underlying proline metabolism in *S. alterniflora* and *SaP5CS2* may play an important role in conferred salinity tolerance by the regulation of proline.

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1. Introduction

*Spartina alterniflora* (also called *Sporobolus alterniflorus* or smooth cordgrass), which grows in salt marshes along the coast, is a perennial deciduous species belonging to Poaceae family (Yu et al., 2020). It was initially introduced to China in 1979 from the coast of North America for coastal protection purposes and is widespread in Southeast of China (Chung, 2006). It can complete growth and develop normally in soil with 1 to 3% sodium salt (mainly NaCl) and is considered a useful model to explore salt stress tolerance genes (Baisakh et al., 2006; Baisakh et al., 2008). Extensive investigations described effects on physical and chemical properties and biological communities of soil (Wan et al., 2009; Zuo et al., 2017), environmental protection effects in the absorption of metal substances (Alvarez et al., 2018; Robertson et al., 2017), inhibition of algal blooms (Xu et al., 2019), antioxidant system (Mesa-Marín et al., 2018), ion balance (Baisakh et al., 2012) under salt stress, and so on, which mainly focus on physiologic properties and economic values (Xie and Han, 2018). To date, no exhaustive investigations have been focused on proline metabolism-related genes in *S. alterniflora* seedlings in response to salt stress and its transgenic work.

Salinity is a devastating abiotic factor restricting plant’s development and growth (van Zelm et al., 2020). Due to ebb and flow, *S. alterniflora* often encounters severe salinity fluctuations during the whole growth period, which requires the ability to regulate osmosis. The response of plants to osmotic stress is complex and employs several physiological and sophisticated mechanism changes withholding undesirable environmental conditions (Xie and Han, 2018). Under environmental stresses, plants can increase the osmotic potential of their cells by synthesizing and accumulating compatible osmolytes, such as polyols, sugars, betaine, glycine, proline, and so on (Ashraf and Foolad, 2007; Pérez-Llano et al., 2020). Proline accumulation is one of the most common responses that plays a role in counteracting osmotic stress in a wide variety of species including bacteria, fungi, algae, invertebrates, and plants (Delauney and Verma, 1993). The biosynthesis of proline was firstly elucidated in *Escherichia coli* and then people found that it was slightly different in plants (Caonka et al., 1988; Hu et al., 1992).

In higher plants, the metabolic pathway of glutamate (Glu) or ornithine (Orn) plays a leading role in proline synthesis and is often closely related to the plant species, stress conditions, developmental stage, and nitrogen level (Moukhtari et al., 2020). In the Glu pathway, glutamate is first catalyzed by delta 1-pyrroline-5-carboxylate synthetase (*P5CS*) to generate γ-glutamyl semialdehyde (GSA), and spontaneously converted to delta 1-pyrroline-5-carboxylate (P5C). Proline is produced from the P5C intermediate when it is reduced by delta 1-pyrroline-5-carboxylate reductase (*P5CR*) using NADPH and H$^+$ (Szabados and Savouré, 2010). The P5CS is generally considered to be the rate-limiting enzyme in the Glu pathway (Funk et al., 2020). In the Orn pathway, the precursor ornithine is transaminated to P5C by ornithine-δ-aminotransferase (OAT), and then P5C is transported to the cytosol and converted to proline by *P5CR*. The OAT is considered to be the key enzyme in this pathway (Mansour and Ali, 2017). Proline catalysis is mainly through proline dehydrogenase or proline oxidase (PDH or POX) producing P5C from proline and P5C dehydrogenase (*PSCDH*) converts P5C to glutamate (Schertl et al., 2014). Besides, proline content under stress conditions is connected with transport processes, suggesting...
that proline transporters (ProT) are also vital for the protective role of proline (Mansour and Ali 2017). Generally, the Glu pathway plays a dominant role under osmotic stress and nitrogen limitation, whereas the Orn pathway displays more prominent under non-stress, nitrogen excess, and seedling development (Hayat et al. 2012).

Previous studies elicited that high salt or drought conditions increased proline accumulation in Arabidopsis, moth bean (Vigna aconitifolia), and rice (Oryza sativa), accompanied by an increase in the P5CS message levels (Siripornadulsil et al. 2002; Fabro et al. 2004). However, P5CS expression patterns were not correlated with proline concentration in tomatoes (Fujita et al. 1998) and also proline content was not consistent with salt tolerance in barley (Widodo et al. 2009). So, some researchers believed that there were positive correlations between the ability of proline accumulation and salinity tolerance (Hussain et al. 2000), others have challenged the importance of proline as a positive indicator of salt-resistance (Blum et al. 1996). Therefore, if regulation expression of P5CS is important for the control of proline synthesis under salt stress, as reported in Arabidopsis and rice, then S. alterniflora, being a moderately salt-tolerant, is expected to accumulate much more proline by overexpression in Arabidopsis, which would be highlighted as a success halophyte (van Zelm et al. 2020).

Until now, a plethora of genes related to salt tolerance and transgenic work attempts have been well established and carried out (Sawahel and Hassan 2002; Jin and Wu 2004). It is well known that during the long-term evolution of plants, there are various ways to respond adapt to salt stress involved in many related genes and these genes have been exploited previously in different plants (Zhu 2002). However, the mechanism-related genes of proline biosynthesis, catabolism, and transport have not been fully understood in S. alterniflora. In particular, the relationship between proline accumulation and salt tolerance has not yet been investigated. In this study, the key pathway and enzymes of proline were identified and the gene expression under salinity stress was revealed. The chlorophyl contents during prolonged salt stress period were also measured. Moreover, the salt tolerance function of SaP5CS2 gene was preliminarily verified by overexpression experiment in Arabidopsis thaliana with salt stress. This study will provide new insights into the molecular responses mechanism of S. alterniflora to salt stress and help us to cultivate salt-tolerant crops by transgenic engineering technology.

2. Results

2.1. Isolation and sequences characterization of proline metabolism-related genes

S. alterniflora cDNA libraries under normal condition obtained from salt mash in Qingdao were established using Illumina Hiseq™ RNA at SanGon Biotech company (Shanghai, China). And the transcriptome information was stored in our laboratory. Based on the transcriptome information, the cDNA sequences of the proline metabolic pathway including SaP5CS1, SaP5CS2, SaOAT, SaPDH, SaProT were identified and extracted from the transcriptome database of S. alterniflora. To ensure the correctness of the sequences, appropriate fragments were selected blasting on National Center for Biotechnology Information (NCBI). Blast analysis showed these fragments shared significant homologies with genes in the databases of NCBI. Then, by means of RT-PCR and RACE, the full-length nucleotide sequences of P5CS1, P5CS2, OAT, and ProT genes were obtained from S. alterniflora and named as SaP5CS1, SaP5CS2, SaOAT, and SaProT, respectively. And their basic information was shown in Table 1.

2.2. Bioinformatic analysis of four proline metabolism-related gene

Multiple sequence alignments revealed that proline metabolism-related genes had high-level identities with known genes in GenBank. SaP5CS (SaP5CS1, SaP5CS2) genes encode 701 and 728 amino acids and SaP5CS1 showed 74.21% homology to the SaP5CS2 at the level of amino acid sequence. Multiple amino acid sequence analysis showed a high homology between SaP5CS and other plant P5CS proteins (AtP5CS1, AtP5CS2, DpP5CS1, DpP5CS2). The duplicate SaP5CS showed conserved domains as other species, such as ATP binding sites, NAD(P)H-binding sites, Glu-5-kinase, GSA-DH domains, and Leu-rich regions (Figure 1). A conserved residue was also found in the sequences of SaP5CS (Phe, at position 128 in SaP5CS1, at position 142 in SaP5CS2) (Figure 1), which functions as proline feedback inhibition (Strizhov et al. 1997). The SaOAT protein also shared high identities with other plants (Fig. S1) and the putative SaProT protein had lower level of identity compared with SaP5CS and SaOAT with their homologues (Fig. S2). The phylogenetic tree analyzed the evolutionary relationship of proline metabolism-related genes and other species involved in P5CS, OAT, ProT from amino acid sequence using a Neighbor-Joining method in the MEGA 7.0 program (Figure 2). The phylogenetic tree of P5CS proteins was divided into two major groups from monocots to dicots except PtP5CS. The duplicate SaP5CS were clustered into the monocot group (Figure 2(a)). SaP5CS1 was closely related to SbP5CS1, whereas SaP5CS2 had significant similarity to ZmP5CS. The SaOAT was related closely to ZmOAT and SiOAT in the phylogenetic tree (Figure 2(b)). And the SaProT was closely related to SiProT2 and SvProT2 (Figure 2(c)).

Ga: Gossypium arboreum; Nt: Nicotiana tabacum; Lc: Lycium chinense; Sl: Solanum lycopersicum; Vu: Vigna unguiculata; Ha: Helianthus annuus; Sb: Sorghum bicolor; Os: Oryza sativa Japonica Group; Ta: Triticum aestivum; Zm: Zea mays; Sv: Setaria viridis; Si: Setaria italica; Pt: Phaedactylus tricornutum; Ph: Panicum hallii; Bd: Brachypodium distachyon; Td: Triticum dicoccoides; Pd: Phoenix dactylifera; Kp: Kosteletzkya pentacarpos; Bn: Brassica napus; Te: Theobroma cacao; Cs: Citrus sinensis (Figure 2).

2.3. Expression levels of five proline metabolism-related genes in leaves

qRT-PCR analysis was performed on RNA extracted preliminarily determining the level of SaP5CS, SaOAT, SaPDH,
SaProT from leaves. Underexposed to different concentrations of salinity seawater for 12 h, the expression of SaP5CS1 was slightly increased by 10‰ salt stress, and then apparently increased in response to 24‰ salt stress which was approximately 40-fold higher than control, but fell back at 32‰ seawater. Similarly, the SaP5CS2 displayed a similar trend compared with SaP5CS1. But it is apparent that SaP5CS2 is expressed much higher treated with 24‰ and fell back less slightly at 32‰ than SaP5CS1. The level was approximately 60 times higher in plants treated 24‰ salinity compared with the control.

The expression of SaOAT showed a slight increase with the increasing salt concentrations and play a key role under low salinity stress and its increasing trend is consistent with SaP5CS. Expression pattern of SaProT showed a similar trend with SaP5CS1. In the expression of SaPDH under salt treatment no significant difference was observed compared to the control. Quantitative analysis of the results presented in Figure 3 showed that proline accumulation is at least partially due to increased SaP5CS expression, especially SaP5CS2 plays a dominant role under higher salinity stress conditions. Our preliminary analysis of the result demonstrated that the SaP5CS enzymes perform non-redundant functions, and that SaP5CS2 may be more essential for high salinity stress.

2.4. Salt stress induced proline accumulation

To determine whether proline accumulation is correlated with organ distribution under salt tolerance, we first examined the proline content of S. alterniflora in roots, stems, leaves under non-stress treatment and treated the plants with 24‰ salinity for 12 h. Under non-stress condition, the proline content was the lowest in roots, while in leaves, it was the highest. Under 24‰ salt stress with seawater, the proline content in stems and leaves increased more remarkably than that in roots. It was about 2.07- and 2.30-fold higher, respectively, than the corresponding controls.
Furthermore, there were significant differences between the three organs. Obviously, the highest proline content appeared in leaves under salt stress.

2.5. Expression levels of five proline metabolism-related genes in different tissues

Given that SaP5CS2 has the obvious response to salt treatment, we further studied proline metabolism genes expression patterns in roots, stems, and leaves of S. alterniflora treated with 24% concentration of seawater for 12 h. Figure 5(a) shows that the expression of SaP5CS1 in root is lower than SaProT and SaOAT, and that SaP5CS2 is pronounced. This explanation proline accumulation in root appears to be attributed to the Glu and the Orn pathway and proline transportation. And in stems (Figure 5(b)), proline metabolism-related genes have a similar trend to roots and the expression level was slightly increased. The proline degradation pathway involved in SaPDH has almost no effect on proline accumulation and even repressed. As shown in Figure 5(c), SaP5CS2 had striking expression level in leaves and was found to be approximately 150 times higher exposed to 24‰ salinity stress than in the control. However, the expression level of SaP5CS1, SaOAT, SaPDH, SaProT were not obvious compared with SaP5CS2. Proline transportation may transport proline from roots, stems to leaves and result in proline overaccumulation in leaves. These results revealed that the expression of SaP5CS2 was largely accumulated in leaves, at a relatively low level in root and stem.

2.6. Chlorophyll contents decrease under salt stress

The results showed, that with the salt stress duration lengthened time, chlorophyll contents in leaves gradually decreased. Upon salinity 24‰ treatment at 3 h, the chlorophyll contents slightly decreased but not obviously (Figure 6(a)). As shown in Figure 5, the S. alterniflora plants had a sharp reduction for 6 h. The total chlorophyll content was reduced by 28.17% at 6 h (Figure 6(a)). Consistently, chlorophyll a content was reduced by 28.40%, and chlorophyll b content showed 27.2% reduction treated with 24‰ seawater under the same condition (Figure 6(b,c)). Subjected to 24‰ for 48 h, the chlorophyll content was about the lowest, the total chlorophyll content decreased by 58.25%, and chlorophyll a and b showed a reduction of 57.63% and 59.89%, respectively (Figure 6(b,c)), indicating that salinity stress caused chlorophyll degradation and affected the physiological condition of plants to a certain extent.

2.7. Inducible expression of SaP5CS2 by salinity in transgenic Arabidopsis plants

Under 24‰ seawater, the target gene SaP5CS2 of S5 and S9 was significantly upregulated, while the expression SaP5CS2 in wild-type was inhibited at 3 h (Figure 7). With the extension of salt stress time, the relative expression of SaP5CS2 in A. thaliana of lines S5, and S9 increased and then slightly decreased, while the expression of SaProT in wild-type A. thaliana was inhibited due to salt stress. The preliminary results showed that the increased expression of SaP5CS2 in Arabidopsis seedlings transfected with SaP5CS2 could promote proline synthesis.

2.8. Physiological and molecular evaluation of transgenic Arabidopsis plants under salt stress

After 24‰ salt stress for 7 days, two leaves of wild-type and SaP5CS2-transfected A. thaliana were taken, respectively.
Wild-type *A. thaliana* showed wilting phenomenon and yellow, while transgenic lines S5 and S9 transected with SaP5CS2 displayed slightly vigorous and healthy growth (Figure 8). To better understand physiological and biochemical mechanism of salt stress response in overexpressed-SaP5CS2 transgenic lines, we measured the amount of free proline (Figure 9). In our study, the proline content between wild-type and transgenic SaP5CS2 Arabidopsis S5 and S9 was not significant without salinity stress. After 7 days of 24% seawater stress, the content of proline increased significantly in both wild-type and transferred SaP5CS2 target genes. Wild type was more 26.53 times proline than unstressed, and S9 proline accumulated more 30.79 times than unstressed and significantly higher than wild type. In another study carried out to evaluate the functional roles of SaP5CS2 in transgenic lines under salt stress, we determined the content of H₂O₂ in salt stress condition applied to transgenic plants and wild type before and after (Figure 9). The content of H₂O₂ in the transgenic plants was not significantly different from the wild-type plants without salt stress. The content of H₂O₂ was increased in both wild Arabidopsis and transgenic SaP5CS2 plants, but both transgenic lines had significantly lower content H₂O₂ than the wild-type.

3. Discussion

Proline overaccumulation has been considered to play a cardinal role as an osmotic protector exposed to hyperosmotic stresses throughout evolution in plants as well as in bacteria (Sharma and Dietz 2006; Szabados and Savouré 2010). Stress-induced accumulation of proline achieved by biosynthesis from glutamate in combination with proline transport between different tissues or cell types as well as suppression of proline degradation in mitochondria have been elucidated in *A. thaliana* (Kiyosue et al. 1996), *Kosteletzky Virgínica* (Wang et al. 2015), rice (*Oryza sativa*) (Lutts et al. 2010), *Helianthus tuberosus* (Huang et al. 2013), and other plants (Kishor et al. 2004), but the regulatory proline metabolism-related genes during osmotic stress have been unexplored in *S. alterniflora*. In this study, we favorably cloned ORF of proline synthetase (SaP5CS1, SaP5CS2, and SaOAT) and proline transporter (SaProT) and the partial sequence of SaPDH was also cloned and analyzed their molecular characterization, biochemical properties, expression profiles, chlorophyl contents under salt stress and functional SaP5CS2 gene from *S. alterniflora* overexpression in *Arabidopsis*. Bioinformatics analysis demonstrated that deduced amino acid sequences of these genes had high similarities with other plants. As characterized by other plant species (Szabados and Savouré 2010), there were isolated two P5CS genes in *S. alterniflora*, which encoded 701 and 728 amino acid polypeptides. The SaP5CS1 presents 74.21% amino acid identity with the protein sequence coded by SaP5CS2 gene. Molecular phylogenetic analysis indicated that P5CS genes were classified into monocot and dicot clades with exception of PtP5CS and two SaP5CS proteins were clustered with those other monocots. Moreover, SaOAT and SaProT are closely related to SiOAT, SvOAT, and SiProT, SvProT, respectively, belonging to monocots clades. It is pity that SaPDH obtained its partial sequence used for qRT-PCR analysis in this research under its normal condition in leaves because the sequence itself has a high 73% GC content (Fig. S3). After a great deal of work, we have been cloned to the 3′ end by RACE, the 5′ end is still dropping for conditions. Next, we’re going to change the growing conditions according to qRT-PCR result or try to sample different organs, such as roots or stems, making the gene express up-regulated or a little bit higher. It is also worth noting that many plants have more than one ProT genes, which may have a different function in proline accumulation according to available research (Zhang 2014). For instance, two proline transport proteins were isolated in *Arabidopsis*, while ProT2 was strongly induced by NaCl stress than ProT1 (Rentsch 1996). In the next work, we would focus on exploring whether exists another ProT gene from *S. alterniflora*. In addition, further studying its pathway of transportation and respective function during osmoregulation should be a deal of work.

To uncover whether proline metabolism-related genes have different spatial expression patterns in *S. alterniflora*, we sampled different organs treated in 24% salinity. In roots, stems, and leaves, we found that salt stress induced a significant proline metabolism-related genes in roots, stems, and leaves. To date, it has been reported that tomato (Fujita et al. 1998), green gram (Misra and Gupta 2005), mulberry (Surabhi et al. 2008), canola (Xue et al. 2009), Sweet Christmas (Surabhi et al. 2008), Jerusalem arishoke (Huang et al. 2013), and *Kosteletzky Virgínica* (Wang et al. 2015) showed proline accumulation with temporal and spatial patterns. Many key enzymes in plant metabolic pathways are encoded by duplicated genes, which are thought to perform redundant functions, thus avoiding the possibility of single gene mutations leading to lethal auxotrophy (Briggs et al. 2006; Szabados and Savouré 2010). However, in some species like *Medicago truncatula*, P5CS is possibly encoded by three genes (Kim and Nam 2013). In *Arabidopsis*, *AtP5CS1* is induced by salt, drought, glucose, sucrose, light, and abscisic acid (ABA) treatments, whereas AtP5CS2 was expressed to support development at shoot and root meristems. The essential roles of *AtP5CS1* and *AtP5CS2* were verified by overexpression and loss-of-function mutants in stress tolerance and development. Overexpression *AtP5CS1* increased proline accumulation, while *AtP5CS1* T-DNA insertion mutants resulted in reducing proline accumulation and hypersensitivity to salt stress. In contrast, *AtP5CS2* knockout mutants displayed embryo abortion (Székely et al. 2008). In rice, *OsP5CS1* and *OsP5CS2* are both induced exposed to salt stress which differs in *Arabidopsis*. *OsP5CS2* had high

**Figure 4.** The proline content in roots, stems, and leaves under non-stress and 24‰ seawater for 12 h. Values are expressed as mean ± SD; n = 3, ns represents no significance, *represents P < 0.05, **represents P < 0.01, ***represents P < 0.001.
expression levels in mature plants and knocked OsP5CS2 mutants showed more sensitivity to salt conditions; but under normal conditions, OsP5CS2 mutants can grow well and produce seeds with no difference in quality. OsP5CS1 was highly expressed in vegetative and reproductive organs (Hur et al. 2004). These data demonstrated that OsP5CS genes are non-redundant and OsP5CS2 is essential for salt treatment. And in sweet sorghum, the result was similar to the rice and two close related P5CS genes were stressed induction, while SbP5CS1 was at higher expression level under salt. SbP5CS1 had low expression in normal growth condition. On the contrary, SbP5CS2 was ubiquitously expressed. Researchers considered that SbP5CS1 played an important role in response to stress and SbP5CS2 was housekeeping gene that mainly functions in basic proline metabolism (Man et al. 2011). MitP5CS3, a novel gene isolated from Medicago truncatula, plays a critical role in stress-induced proline accumulation during symbiotic nitrogen fixation. MitP5CS1 transcript levels were correlated with proline levels in the different plant organs but transcript abundance was unaffected by osmotic stresses. MitP5CS2 transcripts were poorly detected in all organs but were strongly accumulated under salt-stressed in shoots. As above described, these results generalized that the duplicated P5CS genes had distinct functions and were non-redundant. Unlike prior study, Tomomichi Fujita concluded that the high-level accumulation of proline in pollen was not correlated with a detectable induction in the levels of the tompRO1 and tompRO2, specifying P5CS, the first enzyme of proline biosynthesis, transcripts. There was >60-fold increase in proline levels in roots and leaves treated with 200 mM NaCl. However, a <3-fold increase in the accumulation of the tompRO2 transcript and no detectable induction was observed in the level of the tompRO1 transcript in response to NaCl stress, indicating that P5CS is probably not important for the osmotic regulation of proline synthesis in tomato (Fujita et al. 1998). In the present study, we also found the rate-limiting step in proline synthesis is controlled by a bifunctional P5CS enzyme (Zhang et al. 1995), which is encoded by two highly homologous genes in S. alterniflora. SaP5CS genes have the same expression patterns as rice genes and also have different expression patterns in roots, stems, and roots. The result showed that both SaP5CS genes were stress-inducible, but SaP5CS2 expression level remained higher than SaP5CS1 throughout salt stress, indicating that the proline synthesis is mainly regulated by SaP5CS2. On the one hand, SaPDH could play a role in the inhibition of proline degradation whether in different organs during salt stress or treated in different salinity seawater, which made contributions to proline accumulation. On the other hand, SaOAT expression, as another key enzyme gene for proline synthesis through Orn pathway, showed a slight increase in stems or different salinity stress. It is consistent with prior research that proline biosynthesis from Glu is considered to be the predominant pathway under stress conditions (Zhang et al. 1995). Notable is, SaProT in stems, had a significantly higher level than in roots and leaves, which demonstrated that proline transporter made an exert to transporting proline from roots to leaves during salt stress. These data demonstrate that proline accumulation response to salt stress was linearly correlated with P5CS2 enzyme expression level. Furthermore, we have preliminary evidence that SaP5CS2 plays positive role in plant salt stress response by overexpression SaP5CS2 in Arabidopsis. The above results indicated that overexpression SaP5CS2 gene promoted proline synthesis in transgenic Arabidopsis, increased the concentration of cell fluid, and protected against salinity stress. It is preliminarily shown that transgenic Arabidopsis has strong antioxidase vitality and can effectively reduce oxidative. This study provides a wealth of reference data to further research the mechanism of SaP5CS2 osmoregulation in S. alterniflora conferred salt stress response, but we would like to emphasize that at this stage we do not have sufficient data to verify P5CS2 as an ideal candidate to target in the effort to improve salt tolerance in S. alterniflora. So far, considerable studies have been focused on transgenic plants, we are no exception, and we will perform overexpression SaP5CS2 in Arabidopsis to examine whether expression of SaP5CS genes would

Figure 5. The expression analysis of SaP5CS, SaOAT, SaPDH, SaProT in roots (a), stems (b), leaves (c) from S. alterniflora under 24‰ seawater for 12 h. Values are expressed as mean ± SD; n = 3, ns represents no significance, *represents P < 0.05, **represents P < 0.01, ***represents P < 0.001.
increase salt tolerance in MDA accumulation, chlorophyll content, and related genes up-regulation or down-regulation. Additional research is needed to explore its function conferred on salt stress.

4. Materials and methods

4.1. Plant materials and growth conditions

*S. alterniflora* seedlings (3–5 leaf stages) in this study were obtained from coastal salt marsh in Qingdao, China (36° N1548′; 120° E19′0″). Then they were transferred into plastic pots (with drain holes in the bottom) filled with silt and sand mixture (3:1) for two weeks acclimated the situation before the experiment treatment and cultivated in a greenhouse 16 h light/8 h dark at 26°C day/24°C night with relative air humidity of 60%, respectively. Each group contains three uniform seedlings and were on regular basis irrigated with tap water every 3 days.

4.2. Stress treatment and sampling

Salt stress was applied after growing in a greenhouse for two weeks. To explore the expression proline metabolism-related genes, roots, stems, and leaves were sampled in the unstressed condition and 24‰ seawater salt stress at 12 h, respectively. The plant seedlings were divided into four groups treated with different concentrations of seawater (0, 10, 24, 32‰) for salt stress at 12 h. Leaves samples were collected respectively after the initiation of the treatment. Each treatment had three independent replications and the sample from each pot was mixed together as a replication. All samples were rapidly dipped in liquid nitrogen, and stored at −80°C for RNA isolation and quantitative real-time PCR (qRT-PCR) analysis.

4.3. RNA isolation and first-strand cDNA synthesis

Total RNA from each sample was extracted following the manufacturer’s instructions of TransZol (TransGen Biotech, Beijing, China) with minor modifications. RNA degradation and quality were checked by 1% agarose gel electrophoresis and NanoVue ultra-micro spectrophotometer (GE Healthcare, America), respectively. Furthermore, the samples of A260/280 ranging from 1.9 to 2.0 was performed in the next step. The first-strand cDNA was synthesized with TransScript All-in-One First-Strand cDNA Synthesis SuperMix for PCR and qRT-PCR (TransGen Biotech) according to the manufacturer’s instructions.

4.4. Cloning of proline metabolism-related genes and sequence analysis

For amplification of the proline metabolism-related genes from *S. alterniflora*, the specific primers of *P5CS1, P5CS2, OAT, PDH, ProT* were designed and amplified based on the conserved region of each gene annotated transcriptome analysis (Table S1, S2). After that, the amplified fragments were ligated into the pEASY®-Blunt Zero Cloning Vector (TransGen Biotech) and transformed *E. coli* Trans1-T1 Phage Resistant Chemically Competent Cell (TransGen Biotech). Five clones were picked randomly and sequenced, and the results were aligned by BLAST to confirm sequences correctly. Subsequently, the 5′ end and 3′ end of the full-length cDNA were further amplified according to the instruction of Single Cell Full Length mRNA-Amplification Kit N712 (Vazyme Biotech, Nanjing, China). The above PCR products were isolated from 1% agarose gel and the expected bands...
were recycled and purified, then transferred into the pEASY®-Blunt Zero Cloning Vector and sequenced. At length, the above-obtained sequences of each gene were assembled into the full-length cDNA using DNAMAN software. Amino acid homology analysis of each gene was performed using DNAMAN software. Moreover, the phylogenetic tree was constructed using the neighbour-joining method with MEGA7.0.

4.5. Expression analysis of isolated genes
Total RNA isolated and first-strand cDNA synthesized as described previously were used for qRT-PCR. The reaction system was carried out in accordance with TransStart® Green qPCR SuperMix instructions. To investigate the effects of salinity stress on proline metabolism-related genes, we conducted salinity 0, 10, 24, 32‰ at different salinity ranges. To explore the expression changes in organ-specific distribution of proline, roots, stems, and leaves were sampled under salt stress (24‰) for 12 h. One group was cultured under 0‰ as control. Three replications of each experiment were tested. The other conditions were the same. The *S. alterniflora* alpha-tubulin gene was selected as an internal control and specific primers for qRT-PCR analysis (Table S3). Experiments were repeated three times and the CT values of the triplicate PCR were averaged. The quantification of the relative expression levels was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2002).

4.6. Proline determination
Proline content was determined using the Proline Assay Kit (Nanjing JianCheng Bioengineering Institute, Nanjing, China). The sample consisting of three pots from each treatment was mixed together as a replicate. The proline content in roots, stems, and leaves was determined at 520 nm by a UV-VIS spectrophotometer UV-2550 (SHIMADZU, Japan).

4.7. Chlorophyll quantification
Chlorophyll contents of leaves subjected to 24‰ seawater were monitored at 0, 3, 6, 12, 24, and 48 h by using Chlorophyll Assay Kit (Nanjing JianCheng) with minor modifications. Accordingly, approximately 0.1 g (fresh weight) of leaves was washed out and cut into pieces, and added grinding powder, then incubated in 9 mL ethanol and acetone mixture (1:2, V/V) in the dark until the bottom residues turned white. The absorbance of the supernatant was determined using a UV-VIS spectrophotometer UV-2550 (SHIMADZU, Japan), scanning at 663 and 645 nm, respectively.

Figure 8. Seedling conditions of T3-generation transgenic *A. thaliana* under salt stress for 0 (a), 7 days (b).
Zero was set with ethanol and acetone mixture (1:2, V/V) as described above. Each sample was run as three replications.

4.8. Generation of SaP5CS2 overexpressing A. thaliana

The PCR product of SaP5CS2 ORF sequence with BsmBI/Esp3I was inserted into the pBWA(V)HS vector transformed from the vector PCAMBIA1300 (Fig. S4). The 35S:SaP5CS2 plasmid was induced into the Agrobacterium tumefaciens EHA105 strain. The transformation of A. thaliana was performed via floral dip as previously described (Clough and Bent 1998). The seeds of positive transgenic lines were collected individually. The regenerating resistant plants were detected by PCR using the primer pair Hyg-F/R (Table S1). T3 homozygous Arabidopsis transgenic lines were selected for further investigation. The qRT-PCR using PerfectStart Green qPCR SuperMix (+Dye I) was employed to analyze the expression of SaP5CS2 with the primer pair qP5CS2-F/R (Table S1). Transcription data with three biological replicates were calculated using the 2^-ΔΔCt method as described above. Positive lines S5 and S9 with highest expression levels of SaP5CS2 were subjected to conferred salinity (Fig. S5).

4.9. Quantification of salinity SaP5CS2 in overexpressing A. thaliana

To analyze the expression levels of SaP5CS2 responsive to salinity stress, the overexpressing plants S5, S9 and wild-type A. thaliana at six to eight leaf stage were irrigated with 24‰ salinity. And they were sampled at 0, 3, 6, 12, 24 h. The experiment included three biological replicates. Actin-F/R was used as the reference gene (Table S3).

4.10. Physiological characterization of transgenic lines

For the salinity tolerance assay, the SaP5CS2 overexpressing plants S5, S9, and wild-type A. thaliana at six to eight leaf stage were irrigated with 24‰ seawater concentration for 7 days and observed the physiological growth condition of transgenic and wild-type Arabidopsis for 0 day and 7 days. To evaluate the potential roles of SaP5CS2 gene in transgenic Arabidopsis under salt stress condition, the amount of proline and ROS production of H2O2 were determined using leaf tissues following the introduction described by Jian-Cheng NanJing Kit.

4.11. Primer

Primers for gene cloning, RACE, and qPCR are listed in Supplementary Table S1, Table S2, and Table S3.

5. Conclusions

In general, our work represents the first isolation of the complete ORF proline metabolism-related genes including SaP5CS1, SaP5CS2, SaOAT, and SaProT in S. alterniflora and explore its expression profiles in temporal expression patterns including SaPDH based on the partial of the sequence that has been amplified under salt stress. Results provide valuable information on salinity adaptation proline mechanism and uncover proline synthesis pathway serving as an important role in salt tolerance in S. alterniflora. Furthermore, based on genes expression, SaP5CS enzymes perform non-redundant functions, and that SaP5CS2 plays a leading role in proline accumulation during hyperosmoregulation. Chlorophyll contents decreased with prolonged time under salt stress, demonstrating that the seeding was damaged to a certain extent. Here, overexpression SaP5CS2 gene in Arabidopsis played an intermediate role in proline accumulation and oxidative stress.

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Disclosure statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure 9. Changes of proline (a) content and H2O2 (b) content in transgenic A. thaliana under 24‰ salt stress for 7 days. Values are expressed as mean ± SD; n = 3, ns represents no significance, *represents P < 0.05, **represents P < 0.01, ***represents P < 0.001.
Author contributions
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Data availability statement
All the data used in this study have been provided in the main text and Supplementary Materials.

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