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Chemico-Proteomics Reveal the Enhancement of Salt Tolerance in an Invasive Plant Species via H_2S Signaling

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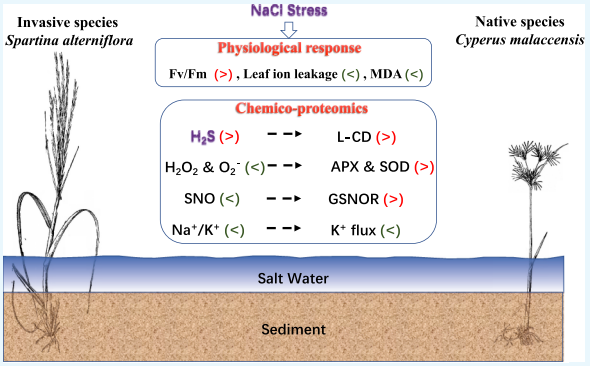
ABSTRACT: H_2S is a small molecule known to have multiple signaling roles in animals. Recently, evidence shows that H_2S also has signaling functions in plants; however, the role of H_2S in invasive plants is unknown. *Spartina alterniflora* is a typical invasive species growing along the beaches of southern China. A physiological comparison proves that *S. alterniflora* is highly tolerant to salinity stress compared with the native species *Cyperus malaccensis*. To decipher the mechanism that enables *S. alterniflora* to withstand salinity stress, a chemico-proteomics analysis was performed to examine the salt stress response of the two species; an inhibitor experiment was additionally designed to investigate H_2S signaling on salinity tolerance in *S. alterniflora*. A total of 86 proteins belonging to nine categories were identified and differentially expressed in *S. alterniflora* exposed to salt stress. Moreover, the expression level of enzymes responsible for the H_2S biosynthesis was markedly upregulated, indicating the potential role of H_2S signaling in the plant's response to salt stress. The results suggested that salt triggered L-CD enzyme activity and induced the production of H_2S, therefore upregulating expression of the antioxidants ascorbate peroxidase, superoxide dismutase, and S-nitrosoglutathione reductase, which mitigates damage from reactive nitrogen species. Additionally, H_2S reduced the potassium efflux, thereby sustaining intracellular sodium/potassium ion homeostasis and enhancing *S. alterniflora* salt tolerance. These findings indicate that H_2S plays an important role in the adaptation of *S. alterniflora* to saline environments, which provides greater insight into the function of H_2S signaling in the adaptation of an invasive plant species.



1. INTRODUCTION

Although the small gas molecule hydrogen sulfide (H_2S) has long since been thought to be toxic, evidence shows that H_2S is the third endogenous gaseous transmitter besides nitric oxide (NO) and carbon monoxide.[1,2] H_2S functions in a majority of physiological processes in animals, including neuronal excitability, vasorelaxation, anti-inflammatory response, smooth muscle relaxation, and blood pressure regulation.[3−5] Recently, the positive effect of H_2S in improving the ability of plants to adapt to multiple environmental stimuli has also been reported.[6,7] In addition, H_2S acts as a critical signal in response to abiotic stress, modulating processes such as stomatal closure, iron availability, leaf senescence, osmotic stress, heat shock, and hypoxia stress.[8−13] Cystathionine β-synthase (CBS, EC4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1) are mainly used by mammalian cells for the synthesis of H_2S. CBS hydrolyses L-cysteine to L-serine and CSE hydrolyses l-serine to H_2S, pyruvate, and ammonia.[14] In plants, H_2S generation is mainly related with the pyridoxal 5′-phosphate (PLP)-dependent D/L-cysteine desulfhydrases (D/L-CDs) enzymes. Meanwhile, another PLP-dependent enzyme (β-cyanoalanine synthase) could convert cysteine and cyanide to H_2S and β-cyanoalanine.[15] However, H_2S is consumed by the cysteine synthesis complex in the synthesis of L-cys from O-acetyl serine which is catalyzed by O-acetylserine-thiol-lyase.[16] *Arabidopsis* DES1 encodes the cytosolic L-CDes, which modulates endogenous H_2S levels in the cytosol.[17] Abscisic acid (ABA), which triggers stomatal closure, induces a *DES1* expression in *Arabidopsis* guard cells. ABA treatment could not close the stomata in isolated epidermal strips of the *des1* mutant, and this defect was restored because of exogenous H_2S addition.[18] These data demonstrate the essential function of H_2S in response to the environmental stress in the plant.

The cross-communication between H_2S and other small molecular signals, such as NO and reactive oxygen species (ROS), has been thoroughly investigated in plants exposed to biotic or abiotic stress. For example, *Arabidopsis* DES1 is involved in ABA-dependent NO production and stomatal closure, and NO acts downstream of H_2S in the ABA-induced stomatal closure.[18] H_2S treatment also improves the tolerance

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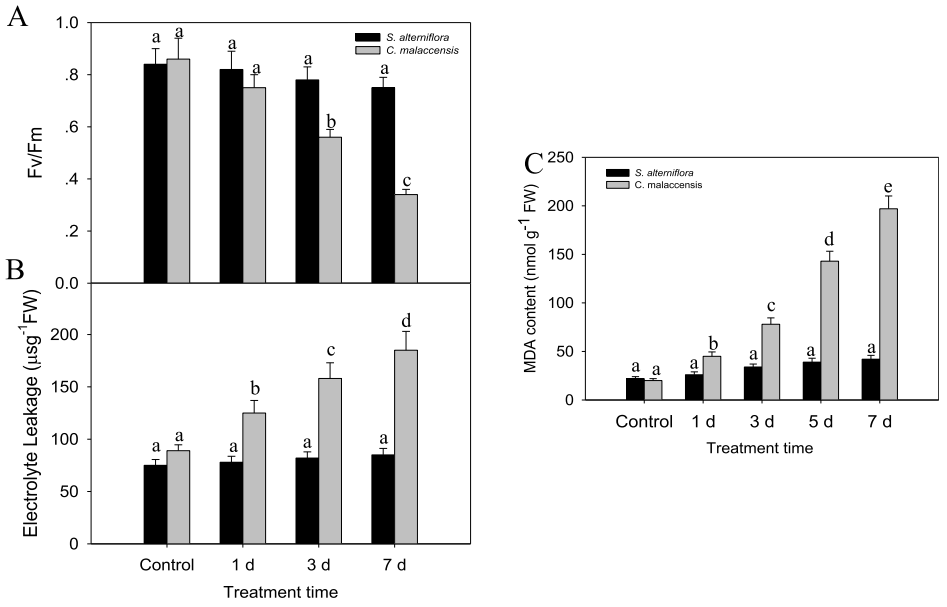

Figure 1. Effects of salt stress on the leaf photosynthesis capability and electrolyte leakage. *S. alterniflora* and *C. malaccensis* were collected and cultivated in a greenhouse for 7 days, and then treated with 300 mM NaCl. At the indicated time points, the average F_v/F_m values (A), electrolyte leakage (B), and MDA content (C) were determined using whole leaves. Data represent mean ± SE of three replicate experiments. Means denoted by different letters show significant differences at $p < 0.05$ according to Tukey's test.

of rice to salt stress or mercury toxicity.[19,20] Salt stress can induce L- activity and raise the production of endogenous H_2S in plant *Medicago sativa*. Furthermore, exogenous H_2S treatment was shown to minimize the salt toxicity effects by modulating the activities of antioxidant enzymes, maintaining K^+/Na^+ homeostasis, and preventing K^+-efflux triggered by NaCl.[21] H_2S also alleviates heat stress in maize, arsenate toxicity in pea, and cadmium toxicity in *Brassica* through the regulation of the ascorbate−glutathione cycle and ROS signaling.[21] However, the mechanism by which H_2S interacts with other signals to modulate plant adaptation to environmental stress is yet to be elucidated. The H_2S signal increases the activity of plasma membrane NADPH oxidase, which generates hydrogen peroxide (H_2O_2) to control stomatal closure.[22]

Spartina alterniflora is a perennial rhizomatous grass which is a native species in the Atlantic and Gulf coasts of North America. It is considered to be an important "environmental engineer" in terms of silting land, repairing wetlands, and fixing carbon dioxide. However, since *S. alterniflora* was introduced to South China in the 1970's, it has become established as an invasive species due to its rapid growth, changing the biodiversity of the region, and disrupting the mangrove ecosystem.[23−28] The biomass of *S. alterniflora* could be five times greater than those of native species such as *Suaeda salsa* in the coastal region of the Yangtze River Basin.[29] Although *S. alterniflora* has the potential to increase the soil organic carbon content and primary productivity (as well as improving the carbon sequestration capability of the flora), as a pioneer plant, it has successfully adapted to environmental stresses including flooding, high salinity, and sediment burial. However, there is a dearth of literature in deciphering the mechanism by which *S. alterniflora* has adapted to the saline environment.

Mass spectrometry-based chemical proteomics approaches have been used in several aspects of small molecular drug research, including identifying drug targets, verifying drug to target interaction affinity, and selectivity profiling. It has also been used to profile enzyme activity at a systemic level across different samples, becoming an integrated research engine that removes the hurdle of identifying protein targets of biologically active small molecules with unknown modes of action.[30−32]

In this study, the physiological and chemico-proteomics comparisons focused on the role of H_2S in salt tolerance were studied in order to systemically understand the adaptation of *S. alterniflora* to salt stress, as compared with the native species *Cyperus malaccensis* in southern China. Therefore, an inhibitor experiment was designed to explain the effects of H_2S signaling on salinity tolerance in *S. alterniflora*. Our study therefore demonstrated that H_2S is a novel signal mediating tolerance to salinity stress in the invasive plant *S. alterniflora*, providing important clues to control this species and curb its encroachment on native plant species.

2. RESULTS

2.1. Physiological Response of *S. alterniflora* and *C. malaccensis* Induced by Salt Treatment and the Salt-Related Dynamic Protein Profiling in *S. alterniflora*. To decipher the mechanism underlying *S. alterniflora* tolerance to NaCl stress, the responses of the invading *S. alterniflora* and the domestic *C. malaccensis* to different concentrations of salt were researched. A salt concentration of 100 mM reduced the F_v/F_m ratio and increased ion leakage in *C. malaccensis*, while a salt concentration of over 300 mM only somewhat decreased the F_v/F_m ratio and increased the ion leakage in *S. alterniflora* (Figure S1); when the salt concentration exceeded 500 Mm, the damage inflicted by salt toxicity was more severe, and possibly caused side effects such as unhealthy growth, extremely wilted leaves, and so forth. Thus, a salt concentration of 300 mM was selected to challenge *S. alterniflora* in subsequent experiments, in which *S. alterniflora* plants were exposed for 1, 3, and 7 days. As the control, the native species *C. malaccensis* was simultaneously treated with 300 mM NaCl. The leaf photosynthesis capability, ion leakage, and malondialdehyde (MDA) content, important criteria to estimate the

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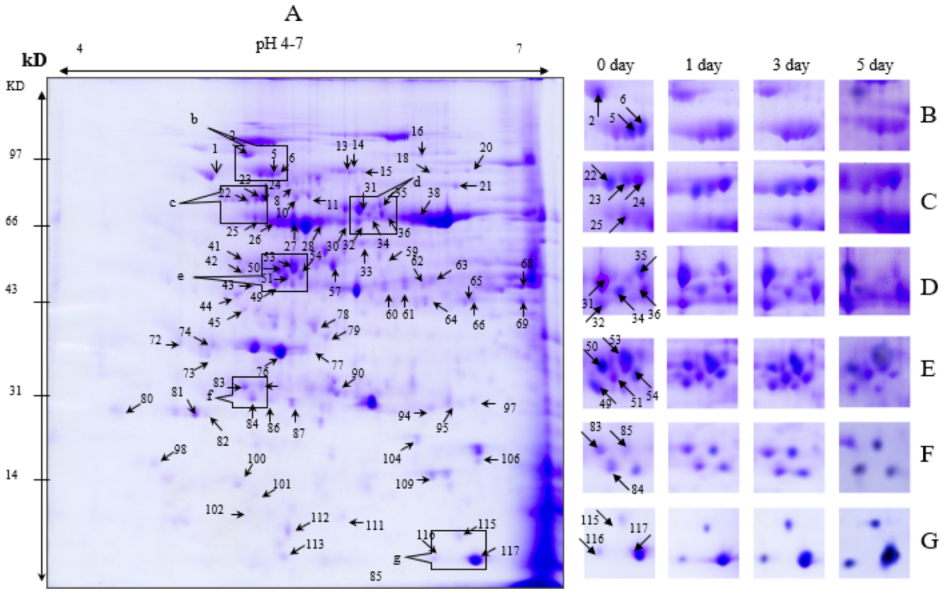

Figure 2. Changes in protein spots derived from the leaves of *S. alterniflora* after treatment with 300 M NaCl. Plant samples were collected at different time points [0 day (control) and 1, 3, and 5 days] and 1 mg of total protein was extracted and loaded onto gels. (A) Representative BR-20-stained 2-D gel of the total leaf protein at 0 days showing protein spot position. (B−G) Enlarged windows from panel (A) showing spot changes from samples collected after different periods of salt treatment.

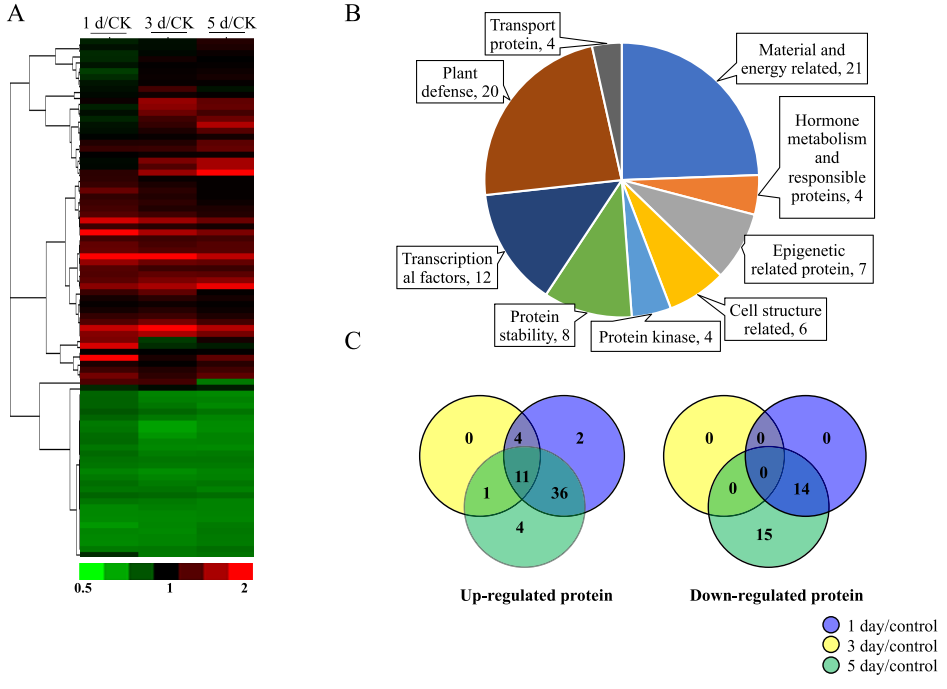

Figure 3. Protein species expression profiles from the leaves of *S. alterniflora* after exposure to salt stress. Hierarchical clustering (A), functional classification, and (B) Venn diagram analysis of protein species expression profiles in *S. alterniflora* leaf samples obtained after different periods of salt stress treatment. The hierarchical cluster analysis was conducted using Cluster 3.0 and Treeview software (http://bonsai.ims.u-tokyo.ac.jp/?mdehoon/software/cluster). The different colors correspond to the log-transformed values of the protein species change-fold ratio shown in the bar at the bottom of the figure (C). Data represent mean ± SE of three replicate experiments.

leaf damage degree, were measured. As shown in Figure 1A−C, NaCl treatment for different periods did not markedly affect F_v/F_m, leaf ion leakage and MDA degree in *S. alterniflora*, but markedly reduced F_v/F_m, and increased leaf ion leakage and MDA content in the native species *C. malaccensis* after 3 or 5 days of NaCl treatment. These data support the notion that *S. alterniflora* has a higher tolerance for NaCl stress than does *C. malaccensis*.

In order to further explore the mechanism underlying salt stress response in *S. alterniflora*, the comparative proteomics approach was used to monitor the protein profile in *S. alterniflora* after 1, 3, and 5 days of 300 mM NaCl stress. A total of 86 proteins were successfully identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF)/TOF and changed significantly in response to salt stress ($p < 0.05$) compared with the control (Figures 2A,B and 3A).

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These identified proteins were divided into nine and seven groups based on the annotated and putative biological functions, respectively (Figure 3B,C and Table S1). Most of these proteins were metabolism- and energy-related proteins, followed by defense proteins and transcription factors. Proteins associated with epigenetics, protein stability, and hormone metabolism were also detected (Figure 3B). In addition, we found that proteins involved in the H_2S metabolism, such as protein spot 65 (putative D-cysteine desulfhydrase 2) and protein spot 79 (cystathionine γ-lyase/cysteine synthase), and proteins involved in autophagy, such as spot 44 (autophagy protein 5) and spot 50 (cysteine protease ATG4), were regulated differentially after salt stress (Table S1), suggesting the critical role of H_2S during the response to NaCl stress in *S. alterniflora*.

2.2. Comparing the Chemico-Proteomics Characteristics in *S. alterniflora* and *C. malaccensis* Induced by Salt Treatment. *2.2.1. H_2S Biosynthesis and the Main Responsible Enzyme (L-CD) Activity.* H_2S is a signal in the plant defense response.[9] Proteomics data in this study showed that some proteins involved in the H_2S biosynthesis coordinately accumulated after NaCl treatment (Table S1), indicating the potential role of the H_2S signal in *S. alterniflora* tolerance to salt stress. Thus, the endogenous H_2S content in the leaves of *S. alterniflora* was measured and found that NaCl gradually induced the generation of H_2S, peaking after 5 days of NaCl treatment. NaCl treatment also induced H_2S generation in the leaves of *C. malaccensis*, but to a lesser extent than in *S. alterniflora* (Figure 4). Consistent with H_2S generation, the enzyme activity of L-CD was also higher in the leaves of *S. alterniflora* than in those of *C. malaccensis* after NaCl treatment (Figure 4). These data show that NaCl treatment induced higher levels of H_2S generation in *S. alterniflora* than in *C. malaccensis*, and indicate the potential role of H_2S in enhancing *S. alterniflora* tolerance to salt stress.

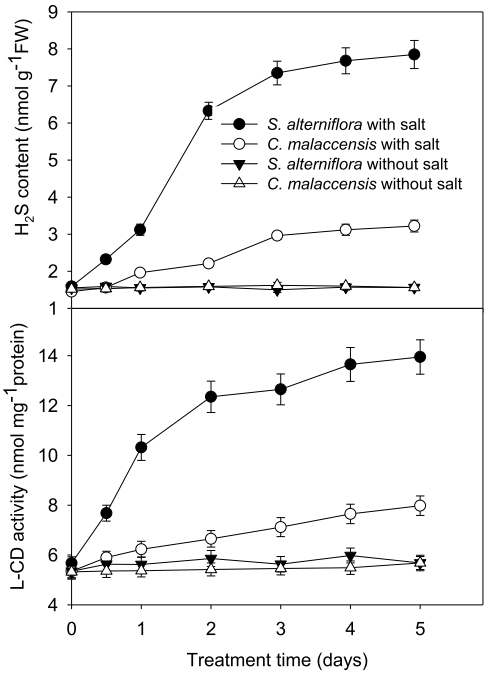

Figure 4. Comparing the generation of H_2S in *S. alterniflora* and *C. malaccensis* induced by salinity. *S. alterniflora* and *C. malaccensis* plants were treated with 300 mM NaCl. At the indicated time points, the leaves of *S. alterniflora* and *C. malaccensis* plants were collected, the H_2S content (upper) and L-CD activity (bottom) were measured. Data represent mean ± SE of three replicate experiments.

2.2.2. H_2O_2 and O_2^- Generation and the Activities of Related Antioxidant Enzymes [Ascorbate Peroxidase (APX) and Superoxide Dismutase (SOD)]. Plants have evolved protective mechanisms such as the antioxidant enzyme system to sustain the cytosolic redox balance.[33−35] In this study, a series of antioxidant enzymes, such as spot 54 (thioredoxin reductase NTRC), spot 57 (glutathione synthetase), spot 77 (cysteine synthase), spot 84 (SOD), spot 85 (APX), and spot 87 (glutathione S-transferase 1), were found and their differential regulation after salt stress indicated the possible role of antioxidant enzymes in the response to salt stress in *S. alterniflora*. To investigate the functions of these antioxidant enzymes, in situ H_2O_2 and O_2^- generation by histochemical diaminobenzidine (DAB) staining (for detecting H_2O_2, dark brown color) and nitro-blue tetrazolium (NBT) staining (for detecting O_2^-, purple blue color) were first monitored. The leaves of *C. malaccensis* plants exhibited more purple blue and dark brown staining after NaCl treatment than those of *S. alterniflora* (Figure 5A), indicating that the H_2O_2 and O_2^- contents of the *C. malaccensis* leaves were higher (Figure 5B,C). H_2O_2 and O_2^- changes suggested that more ROS accumulated in the *C. malaccensis* leaves than in the *S. alterniflora* leaves following exposure to salt stress. Besides, the activities of antioxidant enzymes, including APX and SOD, gradually increased after salt treatment was also found in this study (Figure 6A,B). These results indicate that these antioxidant enzymes have a protective role by scavenging ROS after exposure to salt stress.

2.2.3. S-Nitrosothiols (SNO) Content and the Related S-Nitrosoglutathione Reductase (GSNOR) Activity. Besides H_2O_2, NO also acts as a signal that regulates the cellular redox balance. NO-derived RNS, such as peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), and nitrogen dioxide (NO_2^-), reacts with cellular GSH to form GSNO, which then transfers the NO group to cellular protein thiols to form the longer-lived SNOs through protein S-nitrosylation.[36,37] A previous study showed that the metabolism of GSNO could be catalyzed by the evolutionarily conserved GSNOR. The proteomic data also showed that the accumulation of GSNOR (spot 69) was upregulated by salt stress (Figure 3A and Table S1), indicating a potential role of GSNOR in controlling SNOs in *S. alterniflora* after exposure to salt stress. Here, the level of SNOs in *S. alterniflora* and *C. malaccensis* after exposure to salt stress was compared, and the result suggested that salt stress increased SNO accumulation in both *S. alterniflora* and *C. malaccensis*, with the SNO level in *S. alterniflora* being markedly lower than that in *C. malaccensis* (Figure 6C). Consistent with the proteomic data, the enzyme activity of GSNOR was upregulated in *S. alterniflora* after exposure to salt stress. While such an increase in activity was also observed in *C. malaccensis* after exposure to salt stress, the increase was not as striking as in *C. malaccensis* (Figure 6D). These data suggest that GSNOR catalyzes the SNO metabolism in plants after exposure to abiotic stress.

2.2.4. Na^+/K^+ Ratio and K^+ Efflux. Cellular ion homeostasis is impaired in plants exposed to salt stress, and the cytosol of cells of salt-tolerant plants typically sustain high levels of K^+ and low levels of Na^+ under salt stress.[38,39] Plasma membrane H^+-ATPase and Na^+/H^+ antiporter play key roles in maintaining cellular Na^+/K^+ homeostasis.[40,41] In this study,

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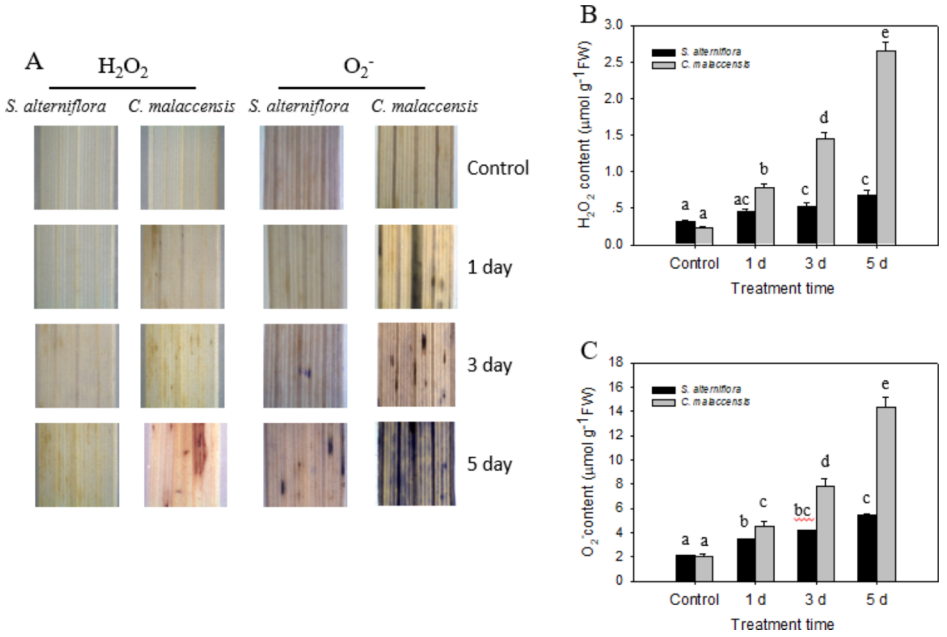

Figure 5. Comparing the generation of H_2O_2 and O_2^- in *S. alterniflora* and *C. malaccensis* induced by salinity. *S. alterniflora* and *C. malaccensis* plants were treated with 300 mM NaCl for 7 days. At the indicated time points, leaves were collected for H_2O_2 staining [(A), left panel] by DAB, or O_2^- staining (a, right panel) by NBT. The H_2O_2 content (B) and O_2^- content (C) were measured. Data represent mean ± SE of three replicate experiments.

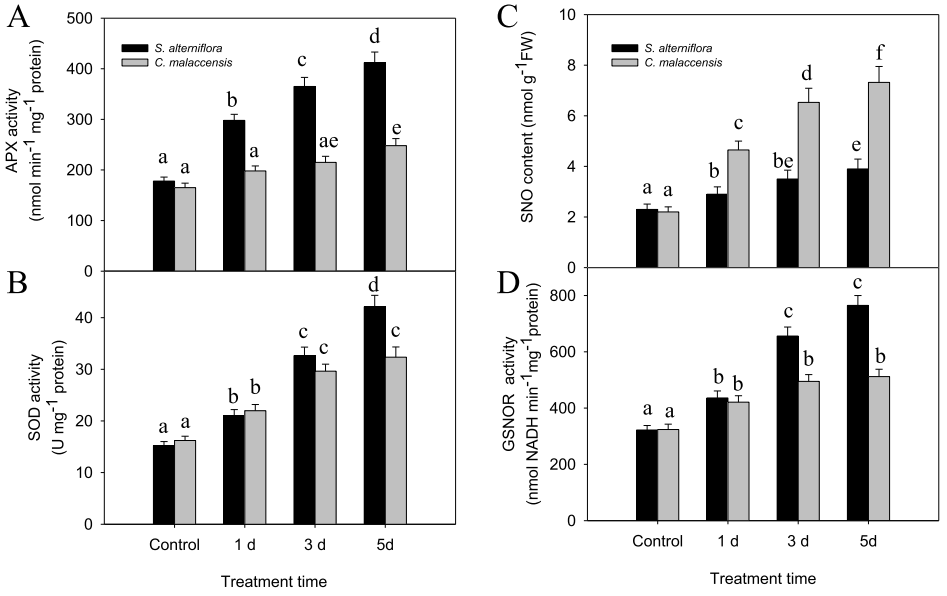

Figure 6. Comparing the activities of antioxidant proteins in *S. alterniflora* and *C. malaccensis* induced by salinity. *S. alterniflora* and *C. malaccensis* were treated with 300 mM NaCl for 7 days. At the indicated time points, *S. alterniflora* and *C. malaccensis* leaves were collected and APX activity (A), SOD activity (B), SNO content (C), and GSNOR activity (D) were measured. Data represent mean ± SE of three replicate experiments. Means denoted by different letters show significant differences at $p < 0.05$ according to Tukey's test.

proteomics data showed that the accumulation of protein spot 16 encoding H^+-ATPase and spot 111 encoding V-type proton ATPase subunit G1 were increased, indicating that they function in sustaining ion homeostasis in *S. alterniflora* after exposure to salt stress. To investigate cellular Na^+/K^+ homeostasis following exposure to salt stress, Na^+ content, K^+ content, and Na^+/K^+ ratio in the leaves of *S. alterniflora* and *C. malaccensis* plants were then examined under salt stress. Salt stress increased the concentration of Na^+ and decreased K^+, resulting in a sharp increase in the Na^+/K^+ ratio in the *C. malaccensis* leaves (Figure 7A). The increase in Na^+ percentage and Na^+/K^+ ratio and decrease in K^+ percentage were not as obvious in *S. alterniflora* (Figure 7A), possibly due to the increased PM H^+-ATPase activity.

To further characterize how NaCl modulates K^+ homeostasis in *S. alterniflora* and *C. malaccensis* upon salt stress, noninvasive microtest technology (NMT) was used to detect dynamic changes in K^+ in the *S. alterniflora* and *C. malaccensis* roots after exposure to salt stress. NaCl stress led to a large K^+ efflux in *C. malaccensis* roots but not in *S. alterniflora* roots (Figure 7B,C), suggesting that the lower K^+ efflux in *S. alterniflora* contributes to its salt tolerance.

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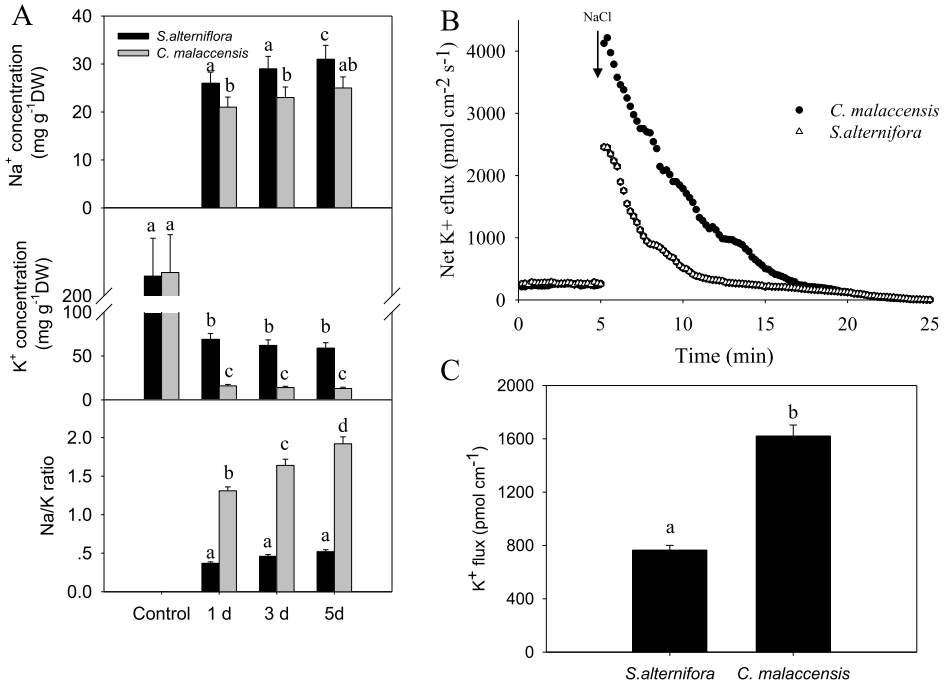

Figure 7. Comparing the ion homeostasis and potassium efflux in *S. alterniflora* and *C. malaccensis* induced by salinity. *S. alterniflora* and *C. malaccensis* plants were treated with 300 mM NaCl for 7 days. The leaves were collected at the indicated time points, and the Na^+ and K^+ contents and Na^+/K^+ ratio were measured (A). Time-course analysis of the transient net K^+ efflux (B) was performed using the noninjuring technique after the addition of salt. The average K extrusion during 20 min of NaCl stress was also determined (C). Data represent mean ± SE of three replicate experiments. Means denoted by different letters show significant differences at $p < 0.05$ according to Tukey's test.

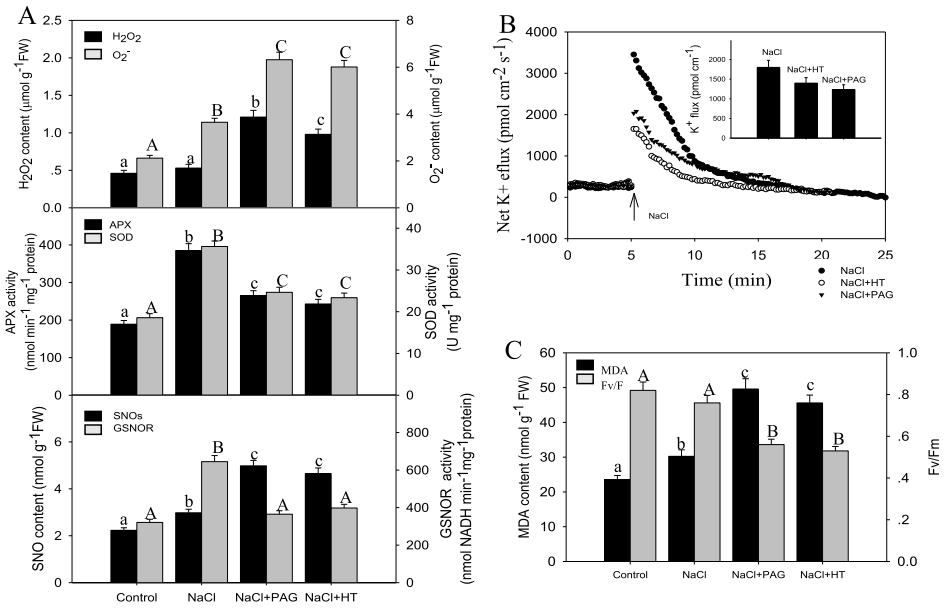

Figure 8. H_2S signal and GSNOR activity modulated ROS and RNS generation, antioxidant enzyme activities, ion homeostasis, and leaf viability in *S. alterniflora* after exposure to salt stress. Seven-day-old *S. alterniflora* plants were treated with 300 mM NaCl with or without the H_2S biosynthesis inhibitor PAG (2 mM) or scavenger HT (100 μM). After 3 days of treatment, the accumulation of H_2O_2 and O_2^-, the activities of APX and SOD, the generation of SNOs, and GSNOR activity were measured (A). The effect of HT and DA on the net K^+ efflux was monitored by the noninjuring technique after the addition of salt (B). The inset shows the mean efflux rates 0−20 min after treatment. Furthermore, the effects of PAG and HT on the MDA content and F_v/F_m ratio were evaluated in *S. alterniflora* leaves (C). Data represent mean ± SE of three replicate experiments. Means denoted by different letters show significant differences at $p < 0.05$ according to Tukey's test.

2.3. H_2S Signal Functions in Salt Tolerance in *S. alterniflora*. Through the chemico-proteomics comparison between invasive plant *S. alterniflora* and native plant *C. malaccensis* induced by salinity stress, we found that salt stress induced higher levels of H_2S and the activity of the responsible enzyme (L-CD), activities of the activated antioxidant enzymes (APX and SOD), as well as of the enzyme GSNOR in *S. alterniflora*. To further understand the potential role of H_2S in sustaining *S. alterniflora* tolerance to salt stress, *S. alterniflora* was pretreated with a H_2S inhibitor and then exposed to salt

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stress. DL-propargylglycine (PAG) inhibits L-DES activity and hypotaurine (HT) scavenges H_2S. In this experiment, NaHS was used as the exogenous H_2S donor. Pretreatment of *S. alterniflora* with different concentrations of PAG or HT reduced the salt-induced H_2S accumulation (Figure S2), and this reduction was more obvious at high concentrations of PAG (2 mM) and HT (100 μM). Thus, these concentrations were used to investigate the role of H_2S signaling in mediating the salt stress response in *S. alterniflora*.

First, the redox status of *S. alterniflora* under salt stress with or without PAG or HT pretreatment were compared. As shown in Figure 8A, PAG or HT treatment aggravated salt-induced ROS accumulation and suppressed salt-induced antioxidant APX or SOD enzyme activities. These findings agreed with a previous study[35] and suggest that salt-induced H_2S is the signal that activates the antioxidant enzyme activity to reduce ROS accumulation during salt stress. Similarly, a NMT analysis showed that PAG or HT pretreatment increased the K^+ efflux, resulting in an increase in the Na^+/K^+ ratio compared with plants not subjected to a PAG or HT pretreatment (Figure 8B). This suggests that suppressing the H_2S signal affected Na^+/K^+ homeostasis in *S. alterniflora* under salt stress. PAG or HT pretreatment significantly reduced the F_v/F_m ratio and increased the MDA content in *S. alterniflora* leaves after 3 days of exposure to salt stress (Figure 8C), in contrast to samples lacking PAG or HT pretreatment. These data suggest that H_2S mediates tolerance to salt stress in *S. alterniflora*.

Pretreatment with PAG or HT also aggravated NaCl-induced SNO content and reduced GSNOR activity in *S. alterniflora* (Figure S3), suggesting that salt-induced H_2S also regulates the NO metabolism and GSNOR activity. To determine the cross-talk between H_2S and GSNOR in *S. alterniflora* after exposure to salt stress, *S. alterniflora* was pretreated with the GSNOR inhibitor dodecanoic acid (DA) and it was found that DA pretreatment increased salt-induced MDA degree and reduced salt-induced F_v/F_m (Figure S4), suggesting that GSNOR activity modulates salt tolerance in *S. alterniflora*. However, the DA treatment did not obviously affect salt-induced H_2S generation (Figure S5). These data indicate that H_2S acts upstream of the NO signal to enhance *S. alterniflora* tolerance to salt stress. H_2S can buffer the overaccumulation of NO that may cause plant damage by leveraging GSNOR activity, which serves as a protective shelter for *S. alterniflora* under a high salinity environment.

3. DISCUSSION

In China, *S. alterniflora* is regarded as an invasive species based on its aggressive growth capability and tolerance to salt stress. However, the mechanism underlying salt stress tolerance in *S. alterniflora* was hitherto unknown. In this study, *S. alterniflora* was more tolerant to salt stress than *C. malaccensis* was, and further proteomic analysis showed that a series of proteins associated with sugar signaling, such as spot 14 (sucrose synthase), spot 30 (soluble starch synthase 1), spot 42 (β-amylase), and spot 43 (α-amylase isozyme 2A), were differentially upregulated, suggesting that sugar signaling may mediate the salt stress response in *S. alterniflora*. In addition to providing energy, sugar has been reported to act as a signal mediating many physiological processes, such as iron deficiency and plant lifespan.[42,43] It is possible that the sugar signal regulates intracellular osmotic levels. Other plant hormone-response-related proteins, such as spot 49 (ninja-family protein 8), spot 62 (gibberellin 20 oxidase), spot 63 (S-adenosylmethionine synthase), and spot 109 (auxin-responsive protein SAUR40) were also differentially regulated after exposure to salt stress; several hormone-responsive transcriptional factors, such as spot 23 (transcription factor MYC2), spot 25 (ETHYLENE INSENSITIVE 3-like 3 protein), spot 95 (ethylene-responsive transcription factor 13), and spot 97 (GRF1-interacting factor 3) presented differential accumulation, suggesting the possible role of jasmonic acid, gibberellin, and auxin signaling in response to salt stress in *S. alterniflora*. Furthermore, salt stress modulated protein stability through the protein ubiquitination system, as proteins associated with this system, including spot 13 (ATP-dependent zinc metalloprotease FTSH 9), spot 21 (BTB family PROTEIN 1), spot 80 (ubiquitin-conjugating enzyme E2), spot 86 (proteasome subunit alpha type-2), spot 104 (probable E3 ubiquitin-protein ligase XERICO), and spot 117 (ubiquitin-40S ribosomal protein S27a), were markedly modulated after exposure to salt stress. Proteins associated with cell autophagy, such as spot 44 (autophagy protein 5) and spot 50 (cysteine protease ATG4), were also reduced by salt stress, possibly through the H_2S signal. A previous study demonstrated that H_2S is the signal that represses cell autophagy in *Arabidopsis*.[44,45] The proteomic data also showed that proteins related to splicing or epigenetic modification components, such as spot 2 (isoform 5 of nuclear poly(A) polymerase), spot 5 [poly (ADP-ribose) polymerase], spot 6 (SWI/SNF complex subunit SWI3C), spot 20 (probable DNA helicase MCM8), spot 27 (U4/U6 small nuclear ribonucleoprotein PRP4-like protein), and spot 76 (PHD finger protein ALFIN-LIKE), were also differentially expressed after exposure to salt stress, indicating their role in modulating *S. alterniflora* tolerance to salt at the splicing or epigenetic level. These proteomics data suggest that *S. alterniflora* uses multiple strategies to enhance its tolerance to salt stress.

Among these differentially expressed proteins, antioxidant enzymes and defense proteins presented high expression after exposure to salt stress, suggesting that they enhance the *S. alterniflora* tolerance to salt stress. In agreement with this notion, salt treatment efficiently increased the activities of antioxidant enzymes in the leaves, which scavenge excess H_2O_2 and O_2^-, thus limiting ROS damage, while salt stress induced the rapid accumulation of ROS because of the reduced activities of antioxidant enzymes. Compared with *S. alterniflora*, the F_v/F_m ratio of *C. malaccensis* was also lower. These data suggest that *S. alterniflora* efficiently promoted antioxidant enzyme activity, and thereby enhanced the plant's ability to adapt to salt stress.

NO also plays a central role in regulating various physiological processes, such as flowering, stomatal closure, germination, root development, gravitropism, and the response to abiotic and biotic stresses.[36−52] However, NO is a short-lived signal that reacts with the antioxidant glutathione to form GSNO, which then transfers its NO group to the cys thiol to form SNOs. This process, called protein S-nitrosylation, is a key redox-based post-translational modification in plants.[53] Cellular SNO levels are also regulated by GSNOR. This enzyme function is conserved among bacteria, plants, and animals and plays an important role in plant de-nitrosylation.[54,55] As demonstrated before, GSNOR efficiently removes excess SNOs to mitigate chilling damage to poplar or salt stress to alga.[44] Here, the proteomics data showed that salt stress induced the accumulation of GNSOR, indicating its

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role in sustaining the RNS status in *S. alterniflora* in response to salt stress. Consistent with this, salt stress in this study also increased the activity of the GSNOR enzyme, which would limit damage to the leaves by removing excess SNOs. The finding that exposure to salt stress caused SNOs to accumulate and resulted in lower levels of GSNOR indicates that these enzymes mediate tolerance to saline stress in *S. alterniflora*.

While a previous study demonstrated the critical role of H_2S in plant adaptation to environmental stress, its role in invasive plant adaptation was unknown.[56] Here, the proteomic data revealed that several proteins associated with the H_2S metabolism were upregulated after the plants were exposed to salt stress. Further analysis showed that salt stress induced the activity of L-CD and the generation of H_2S in *S. alterniflora*, and removing endogenous H_2S accumulation by pretreatment with a H_2S scavenger resulted in the salt stress causing more damage to the leaves, as indicated by the increased ion leakage and MDA content and reduced F_v/F_m ratio. These data suggest that H_2S enhances the tolerance to salt stress in *S. alterniflora*. Compared with *C. malaccensis*, the rates of K^+ efflux were lower in *S. alterniflora*. This lower efflux of K^+ would result in higher levels of cytosolic K^+ and maintain the Na^+/K^+ balance in *S. alterniflora*. In agreement with this, suppressing H_2S generation increased the K^+ efflux and the Na^+/K^+ ratio, and reduced salt-induced antioxidant enzyme activity to increase ROS accumulation in the leaves, which possibly caused the lower F_v/F_m ratio in the leaves. Furthermore, H_2S-mediated salt-induced GSNOR activity reduced SNO damage in *S. alterniflora* after exposure to salt stress, and suppressing H_2S generation reduced salt-induced GSNOR activity increases SNO generation. This finding is in agreement with the results of a previous study[31] and supports the conclusion that H_2S enhances *S. alterniflora* tolerance to salt stress through increasing GSNOR activity.

4. CONCLUSIONS

In this study, physiological and chemico-proteomics approaches were applied to explore the mechanism underlying salt stress tolerance in the invasive plant species *S. alterniflora* compared with the native species *C. malaccensis*. Additionally, the effects of H_2S signaling on *S. alterniflora* were also determined through an inhibitor experiment. The results demonstrated that H_2S signaling plays a central role in enhancing *S. alterniflora* tolerance to salt stress. Salinity stress triggers L-CD enzyme activity to rapidly induce H_2S generation in *S. alterniflora*; the H_2S signal activates antioxidant enzymes (APX and SOD), thereby limiting ROS accumulation, and also activates GSNOR, thereby reducing RNS damage. H_2S also improves *S. alterniflora* tolerance to salinity stress by reducing the K^+ efflux and thereby maintaining an intracellular Na^+/K^+ homeostasis. Thus, the findings provide insights into the H_2S mechanism underlying salt stress tolerance in the invasive plant species *S. alterniflora* and show that H_2S has an important role in this process by modulating the ROS and RNS status of the stressed cells. The findings also provide clues as to how to control this species and curb its encroachment on the native plant species by reducing H_2S signaling.

5. MATERIALS AND METHODS

5.1. Material and Salt Treatment. *S. alterniflora* was collected in the Shanyutan wetland in the Min River Estuary, Southeast China (119°34′12″−119°40′40″ W; 26°00′36″−26°03′42″ N; ca. 3120 ha) (Figure S6). In this region, *S. alterniflora* invaded the native species *C. malaccensis* that dominated the marsh in 2004. *C. malaccensis* occupies from the intertidal area to near the bank.[57] In this experiment, the collected plants were about 25 cm in height and then transplanted into 4 L pots filled with 1 L sandy soil and irrigated with 3 L of one-fourth strength Hoagland's nutrient solution, pH 6.5. The Hoagland's nutrient solution was changed every three days. The plants were grown under greenhouse conditions with an average air temperature of 25/18 °C (day/night), an air relative humidity ranging from 65 to 90%, and an average irradiance of 300/500 μmol m^{-2} s^{-1} for 7 days, and then were subjected to salt treatment. We chose a 7 day growth of plants because the roots, stems, and leaves of both plants were in a withered state after 7 days. For the salt or chemical inhibitor treatment, sodium chloride (NaCl) was dissolved in the Hoagland's nutrient solution at the indicated concentrations (100, 300, 500, and 700 mM) and used to irrigate the sandy soil. For the chemical inhibitor treatment, PAG and HT were dissolved in 1% dimethyl sulfoxide at 1 M as the stock solution which was then diluted into the indicated concentration with the Hoagland's solution before being used to irrigate the plants. The treated leaves were collected at various time points for further analysis. Three to five replicates were conducted and each replicate comprised six or ten individual plants. Samples not exposed to salt or chemical treatment were used as the controls.

5.2. Analysis of Chlorophyll Fluorescence. A pulse-amplitude modulated chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) was used to measure chlorophyll fluorescence.[58] After adapting the leaves in darkness for 30 min, the maximum quantum yield of PSII (F_v/F_m) was obtained by analyzing the entire leaf. Maximal fluorescence (F_m) and minimum fluorescence (F_o) were obtained using a 0.8 s pulsed light at 4000 μmol s^{-1} m^{-2} and the weak measuring pulses, respectively.

5.3. H_2O_2 and O_2^- Detection and Quantification. As reported, in situ detection of H_2O_2 and O_2^- was tested with some modifications.[59] Three leaf discs exposed to different salt treatments were punched out and vacuum infiltrated with 10 mL of 1 mg mL^{-1} of DAB solution for 2 h. After infiltration, using boiling ethanol (95%), the leaves were cleaned for 30 min and H_2O_2 was detected using DAB staining. The accumulation of O_2^- in leaves after different periods of treatment was determined using 10−2 M NBT reduction. The leaf discs were then vacuum infiltrated for 2 h using 10 mL of NBT and cleaned for 30 min using boiling ethanol (95%).

H_2O_2 content was determined based on the method described by Hu et al.[60] The leaves (1 g) after different treatments were ground into powder using liquid nitrogen and then homogenized with 5 mL of 0.2 M $HClO_4$ at 4 °C. After keeping the extract on ice for 5 min and then centrifuging at 10,000*g* for 10 min at 4 °C, the supernatant obtained was stored in a refrigerator at −70 °C until further analysis. At room temperature for 1 h, a total of 100 μL of the supernatant was added to 1 mL of reaction buffer containing 0.25 mM $(NH_4)_2SO_4$, 0.25 mM $FeSO_4$, 1.25 M xylenol orange, 25 mM H_2SO_4, and 1 mM sorbitol. H_2O_2 levels were calculated via measuring absorbance at 560 nm by reference to standards.

Mixing 1 mL of the supernatant, 1 mL of 50 mM potassium phosphate buffer (pH 7.0), 10 mM hydroxylamine hydrochloride, 2 mL of 7 mM α-naphthyl and 2 mL of 17 mM sulphanilic acid, and then measuring the absorbance of the

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pink phase at 530 nm. The O_2^- content was determined by comparison with a standard curve according to the method previously described by Hu et al.[60]

5.4. H_2S Measurements. The leaf tissue collected under different treatment conditions was ground into fine powder in liquid nitrogen, and after vortexing for 1 min in distilled water, the content of H_2S was recorded by a micro sulfide ion electrode (LIS-146AGSCM; Lazar Research Lab. Inc., Los Angeles, CA, USA) at 25 °C.[61] Each measurement is repeated thrice.

5.5. Measurement of Lipid Peroxidation. Lipid peroxidation was measured using the thiobarbituric acid-reacting substance method with MDA as a standard.[62]

5.6. Relative Ion Leakage Measurement. Relative ion leakage was determined as reported.[58] The fresh leaf sample (0.5 g) and 10 mL of deionized water were kept in a petri dish at 25 °C for 2 h. The first conductivity of the bathing solution was tested after incubation (C_1). The second conductivity was determined after boiling the bathing solution continuously for 15 min (C_2). The relative ion leakage was calculated as: $C_1/C_2 \times 100$.

5.7. APX and SOD Enzyme Activity Analysis. Leaf samples (1 g), 5 mL of extraction medium containing 50 mM Tris-HCl (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2% (v/v) Triton X-100, 2 mM ascorbate, and 1 mM phenylmethylsulfonyl fluoride were homogenized. APX activity was determined by the decreasing of the absorbance of a reaction medium containing 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid-NaOH (pH 7.6), 0.25 mM ascorbate, 0.1 mM H_2O_2, and 10−40 μL of extract under 290 nm. The SOD enzyme activity was calculated by the 50% inhibition of the photochemical reduction of NBT.

5.8. SNO Content and GSNOR Measurement. The total SNO content was measured using the gas-phase chemiluminescence method based on the NO release from the reductive decomposition of nitrogen species in an iodine/triiodide mixture. A nitric oxide monitor 410 (2B Technologies, Boulder, CO, USA) was used to determine the released NO content.

GSNOR activity was tested at 25 °C via recording the reduced nicotinamide adenine dinucleotide (NADH) oxidation at 340 nm as reported.[30] Samples exposed to different treatments were collected and quickly homogenized in liquid nitrogen, using extraction buffer (20 mM Tris-HCl (pH 8.0), 0.2 mM NADH, and 0.5 mM EDTA) at 4 °C. After centrifuging the homogenates at 3000*g* for 10 min at 4 °C, the supernatants were then collected for GSNOR enzyme activity assays. Nanomoles NADH consumed per minute per milligram of protein (e340 6.22 mM^{-1} cm^{-1}) represented the GSNOR activity.

5.9. L-Cysteine Desulfhydrases Enzyme Activity Measurement. The activities of L-CDs were measured as reported.[63] The leaf tissue (1 g) exposed to different salt treatments was collected and ground to a fine powder using liquid nitrogen. The sample was vortexed after adding 1.5 mL of cold extraction buffer [20 mM Tris-HCl (pH 8.0), 0.1% (w/v) dithiothreitol and 0.2% (w/v) sodium ascorbate]. The homogenate was centrifuged at 13,000*g* for 15 min at 4 °C and the resulting supernatant was collected for analysis of the activities of L-CDs enzyme.

5.10. Protein Extraction and 2D Electrophoresis. Protein extraction and 2D separation were performed according to the method previously described by Yang et al. and Ma et al.[64,65]

5.11. In-Gel Digestion. We excised manually the protein spots which significantly changed in expression from colloidal CBB-stained 2DE gels, and then performed protein digestion with trypsin based on the method described by Yang et al. and Ma et al.[64,65]

5.12. MALDI-TOF/TOF Analysis and Database Search. The lyophilized peptide samples were completely dissolved in 0.1% trifluoroacetic acid. A MALDI-TOF/TOF mass spectrometer 4800-plus proteomics analyzer (Applied Biosystems; Massachusetts, USA) was used for the MS analyses.[64] (see the details for above mentioned measurements in Supporting Information Text).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01275.

> Detailed methodology; dose effect of salt on photosynthetic F_v/F_m (A) and ion leakage and PA and HT on salt-induced H2S generation; effects of PAG (2 mM) and HT (100 μM) on GSNOR activity and SNO content under salt stress; effect of DA on the MDA content and F_v/F_m ratio and H_2S generation; detailed information on sample site; and identification of *S. alterniflora* proteins that are differentially expressed by more than 1.5-fold or less than 0.6-fold after salt treatment using MALDI-MS/MS analysis (PDF)

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Author Contributions

Z.Y., S.C., and J.Z. contributed equally to this work. J.L. conducted most of the experiments and completed the writing, S.C. supervised the experiments and revised the manuscript, Z.Y. and J.Z. conducted the experiment. Z.W. planned and designed the research, R.X. conceived the project and wrote the article.

Notes

The authors declare no competing financial interest.

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