

SeAMT1;2a and SeAMT1;2b from the halophyte *Salicornia europaea* play dual roles in ammonium transport and salt tolerance

Jinbiao Ma

Zitong Ou

Umashankar Chandrasekaran

Yinan Yao

Xinlong Xiao

xiaoxl@ms.xjb.ac.cn

Xinjiang Institute of Ecology and Geography <https://orcid.org/0000-0002-6456-7976>

Research Article

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Abstract

Aims

Ammonium (NH_4^+) is an essential nitrogen nutrient for plant growth, but excessive NH_4^+ from fertilizer application and aquaculture wastewater poses eutrophication risks in waterbodies. The halophyte *Salicornia europaea* can efficiently remove NH_4^+ from wastewater, yet the molecular mechanisms governing its ammonium uptake remain largely elusive. This study aimed to identify and functionally characterize ammonium transporter (AMT) genes in *S. europaea* and to assess their roles in NH_4^+ uptake and salt tolerance.

Methods

We identified six AMT genes—*SeAMT1;1*, *SeAMT1;2a*, *SeAMT1;2b*, *SeAMT1;3*, *SeAMT1;4*, and *SeAMT3;1*—in *S. europaea*, determined their expression patterns and subcellular localization, and characterized their functions in ammonium transport and salt tolerance using transgenic yeast and Arabidopsis.

Results

Among the six AMT genes, *SeAMT1;1* and *SeAMT1;3* were shoot-predominantly expressed genes, whereas *SeAMT1;2a* and *SeAMT1;2b* were root-expressed and transcriptionally induced by both nitrogen starvation and salt stress. Functional analyses revealed that *SeAMT1;2a* and *SeAMT1;2b* are the two major AMTs mediating high-affinity and low-affinity ammonium transport in *S. europaea*. Moreover, these two transporters also confer salt tolerance independent of NH_4^+ uptake, with *SeAMT1;2a* exhibiting K^+ transport activity that likely contributes to maintaining cellular K^+ homeostasis under salinity. Furthermore, the high NH_4^+ affinity of *SeAMT1* transporters, which enables them to function effectively despite Na^+ competition, allows *S. europaea* to sustain nitrogen acquisition even under highly saline conditions.

Conclusions

Our findings provide new insights into *SeAMT*-mediated ammonium transport and salt tolerance in this halophyte, with promising potential for phytoremediation.

Introduction

Water eutrophication stemming from industrial discharges, aquaculture operations, and agricultural fertilizer runoff remains a pervasive and intractable issue (Zhang et al. 2021). This underscores an urgent demand for efficient, cost-effective treatment systems capable of handling wastewater with high nutrient loads. Phytoremediation, in which nutrient or heavy metal ions are taken up and removed by

specialized plants, is a highly promising approach (He et al. 2026; Webb et al. 2012). Ammonium (NH_4^+), as an essential nitrogen nutrient, promotes plant growth at safe concentrations but becomes toxic when supplied in excess (Xiao et al. 2023). NH_4^+ uptake by plants depends primarily on ammonium transporters (AMTs). The AMT gene family has been characterized in various plant species: *Arabidopsis thaliana* contains 6 AMT members, including 5 AMT1 genes (AtAMT1;1–1;5) and one AMT2;1 gene (Yuan et al. 2007); *Oryza sativa* contains ten members, with three each for OsAMT1, OsAMT2, and OsAMT3, and one for OsAMT4 (Suenaga et al. 2003); *Chenopodium quinoa* contains twelve AMT members, comprising ten AMT1 and two AMT3 members (Wang et al. 2024). The functions of AMT transporters differ among members. In *Arabidopsis*, AtAMT1;2, AtAMT1;3, and AtAMT1;5 were identified as the three major ammonium transporters, a triple mutant *amt1;2/1;3/1;5* reduces root high-affinity NH_4^+ influx by $\sim 90\%$, demonstrating that these AMTs are the main entry route for external ammonium (Gazzarrini et al. 1999; Neuhäuser et al. 2009; Yuan et al. 2009; Yuan et al. 2007). Moreover, AMT-dependent ammonium uptake is conserved among plants and yeast, indicating that it is a fundamental biological process. Heterologous expression of *Arabidopsis* AMT genes is sufficient to restore NH_4^+ uptake in yeast mutants. Furthermore, overexpression of AMT1 genes in crops, including rice, maize, and cotton, enhances ammonium uptake as well as biomass or yield under low-nitrogen conditions (Fan et al. 2017; Gu et al. 2013; Ranathunge et al. 2014; Sun et al. 2019). Screening hyperaccumulating plants and identifying key ammonium transporter genes contribute to unraveling the mechanistic basis of phytoremediation and offer a reference for improving nitrogen uptake efficiency in crops.

Salicornia europaea, like other species in the *Salicornia* L. genus such as *S. virginica* and *S. bigelovii*, are commonly used as an alternative bioenergy crop for NASA fuel production (Cardenas-Perez et al. 2021). *S. europaea* is an extremely salt-tolerant halophyte with fleshy and succulent shoot that can tolerate up to 1000 mM NaCl in the soil (Lv et al. 2012). It has been cultivated in harsh environments such as saline soils and aquaculture wastewater for phytoremediation to remove salts or excess nutrients (Quinta et al. 2015; Yasseen and Al-Thani 2022). Phytoremediation through *S. europaea* cultivation is an efficient, cost-effective approach for treating harsh soils or wastewater while simultaneously producing industrial fuel oil. It has been reported that *S. europaea*-based filter bed can efficiently remove ammonium (NH_4^+) loading via hyperaccumulation or metabolism. Although high concentrations of NH_4^+ are often toxic to plants, *S. europaea* can tolerate 50 mM NH_4Cl in the presence of 200 mM NaCl. While NH_4^+ concentration in the shoots of *S. europaea* increased with the supplementation of 200 mM NaCl, NaCl significantly reduced NH_4^+ concentration in the roots, possibly by enhancing the activity of nitrogen metabolism-related enzymes such as GS and GSH (Ma et al. 2020). Potassium (K^+) can alleviate NH_4^+ toxicity in species such as rice, barley, and *Arabidopsis*, likely due to competitive uptake between K^+ and NH_4^+ (Szczerba et al. 2008; ten Hoopen et al. 2010). A similar phenotype has been observed in *S. europaea*, where an increased NH_4^+ concentration significantly reduces K^+ content in both shoots and roots (Ma et al. 2020). However, the molecular mechanisms underlying ammonium uptake in *Salicornia* and the interactions between NH_4^+ and Na^+/K^+ remain largely unknown.

Salt stress impairs plants mainly by producing osmotic stress and disrupting ion homeostasis. The interplay between salt stress and nitrogen metabolism has been reported in many plants. Ammonium

assimilation-related genes (OsGS1;1, OsGOGAT) are rapidly upregulated by salinity treatment in rice (Wang et al. 2012). In sorghum, NH_4^+ enhances salt tolerance by restricting Na^+ accumulation and thereby improving the K^+/Na^+ ratio; in addition, NH_4^+ regulates important metabolites to activate photosynthetic efficiency to alleviate salt toxicity (Miranda et al. 2013; Paula-Marinho et al. 2025). Ammonium uptake is primarily mediated by ammonium transporters (AMTs). In *Populus simonii*, the high-affinity transporter PsAMT1;2 facilitates NH_4^+ uptake under salt stress and enhances tolerance by promoting root nitrogen assimilation (Li et al. 2021). Furthermore, in *Arabidopsis*, the kinase SOS2 phosphorylates AtAMT1;1 to enhance ammonium uptake activity, while elevated ammonium promotes SOS2 phosphorylation in turn and activates downstream salt tolerance pathways (Liang et al. 2025). These studies imply the links between AMT-mediated ammonium transport and salt tolerance in plants.

To elucidate the molecular mechanisms of ammonium uptake in *Salicornia europaea*, we cloned six full-length CDS sequences of AMT genes from its transcriptome and systematically characterized them. We identified root-expressed SeAMT1;2a and SeAMT1;2b as two major AMTs exhibiting both high- and low-affinity ammonium uptake. Notably, these two genes confer salt tolerance independently of ammonium, likely by promoting ion homeostasis under saline conditions. This study elucidates the molecular basis of ammonium uptake and provides new insights into AMT-mediated plant adaptation to saline environments.

Materials and Methods

Plant material and culture conditions

Salicornia europaea seeds were collected from Xinjiang Institute of Ecology and Geography, CAS. The seeds were grown in plastic pots filled with sand and four-week-old seedlings were transferred to hydroponic culture under controlled conditions: constant temperature of 22°C, 16 h light/8 h dark photoperiod, and a relative humidity of $50 \pm 10\%$. The *Arabidopsis* quadruple ammonium-transporter mutant *qko* (*amt1;1 amt1;2 amt1;3 amt2;1*; Yuan et al., 2007), yeast strains including the ammonium-transport-defective mutant *31019b* (*mep1Δ mep2Δ mep3Δ*; Marini et al., 1997), and the potassium-uptake-defective strain *CY162* (*trk1Δ trk2Δ*) (Anderson et al. 1992) were kindly provided by the authors in previous studies.

Phylogenetic and motif analysis of SeAMT family

RNA-Seq data for *S. europaea*, including sequences and gene expression values, were obtained from previously published studies (Ma et al. 2018; Ma et al. 2013). Ammonium transporter genes of *S. europaea* were further assembled based on the expression pattern analysis of Unigenes in the transcriptome (Xiao et al. 2015a; Xiao et al. 2014). A phylogenetic tree of AMT proteins from *Arabidopsis thaliana*, *Oryza sativa*, *Salicornia europaea*, and *Chenopodium quinoa* was constructed with MEGA 11.0 and subsequently visualized and refined through the Chiplot web server (<https://www.chiplot.online/>). Conserved domains within AMT proteins were identified using NCBI Batch CD-search (<https://api-www-ncbi.laibokeji.com/Structure/bwrpsb/bwrpsb.cgi>). Conserved motifs of *Salicornia europaea* AMTs were

detected with the MEME online suite (<https://meme-suite.org/>), specifying 12 motifs based on empirical optimization. Transmembrane topologies of six SeAMT proteins (SeAMT1;1, SeAMT1;2a, SeAMT1;2b, SeAMT1;3, SeAMT1;4, SeAMT3;1) were predicted using TMHMM 2.0 (<https://services.healthtech.dtu.dk/services/TMHMM-2.0/>). The AMT protein sequences of *Arabidopsis thaliana*, *Oryza sativa*, and *Chenopodium quinoa* used in this study, along with their corresponding NCBI version numbers, are listed in Supplementary Table S1.

SeAMT genes induction assay by real-time qPCR

For nitrogen deficiency treatment, four-week-old *S. europaea* seedlings were grown in hydroponic solution containing 2 mM NH_4NO_3 , 1 mM KH_2PO_4 , 1 mM MgSO_4 , 0.25 mM K_2SO_4 , 0.25 mM CaCl_2 , 0.1 mM Na-Fe-EDTA, 50 μM H_3BO_3 , 5 μM MnSO_4 , 1 μM ZnSO_4 , 1 μM CuSO_4 , and 1 μM NaMoO_4 (pH 6.0). Subsequently, seedlings were transferred to fresh hydroponic solution either with or without 2 mM NH_4NO_3 , and harvested after three days. Shoots and roots were collected separately in triplicate. For NaCl treatment, four-weeks old *S. europaea* seedlings were grown in the same complete hydroponic solution, then transferred to fresh solution supplemented with or without 200 mM NaCl. Samples were collected at 0 h (control), 0.5 h, 2 h, and 4 h after transfer, with shoots and roots harvested in triplicate for each treatment. Total RNA was extracted from shoots and roots using the RNeasy Plant Kit (Qiagen), and 1 μg of RNA was reverse-transcribed according to the kit instructions (YESEN). Using cDNA as the template, real-time PCR was performed on a CFX96 real-time PCR detection system (Bio-RAD). CAC and UBC genes were used as the reference genes (Xiao et al. 2015b). The primers used in real-time quantitative PCR are listed in Supplementary Table S2.

Gene cloning and plasmid construction

For stable transformation of the *Arabidopsis* mutant *qko*, the full-length coding sequences (CDSs) of SeAMT genes were amplified by PCR and cloned into the binary vector pCAMBIA1300 under the control of the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter. For transient expression assays in tobacco leaves, the SeAMT CDSs were similarly amplified and cloned into the pCAMBIA1300 vector, fused in-frame with a C-terminal green fluorescent protein (GFP) tag. For yeast transformation, the SeAMT CDSs were amplified and cloned into the yeast expression vector pYES2.

Subcellular localization of SeAMT-GFP fusion proteins

For subcellular localization, 3-weeks old *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* strain GV3101 carrying the pCAMBIA1300-GFP vector. After 48 h in the growth chamber, the infiltrated regions were excised, mounted, and imaged directly under a laser confocal microscope to capture green fluorescent signals.

Functional analysis of SeAMT genes by transgenic *Arabidopsis*

Agrobacterium tumefaciens strain GV3101 harboring various SeAMT constructs was used to transform the *Arabidopsis* quadruple ammonium-transporter mutant *qko* via the floral dip method (Clough and Bent

1998). Homozygous T_2 transgenic lines were selected for subsequent ammonium uptake and salt tolerance assays. For NH_4^+ and methylamine (MeA) uptake assays, seeds from two independent overexpression lines for each *SeAMT* gene were germinated and grown on solid medium containing 5 mM KNO_3 , 1 mM KH_2PO_4 , 1 mM MgSO_4 , 0.25 mM K_2SO_4 , 0.25 mM CaCl_2 , 0.1 mM Na-Fe-EDTA, 50 μM H_3BO_3 , 5 μM MnSO_4 , 1 μM ZnSO_4 , 1 μM CuSO_4 , and 1 μM Na_2MoO_4 , supplemented with 1.5% (w/v) sucrose and 0.8% (w/v) agar, with 5 mM KNO_3 as the sole nitrogen source. The *qko* mutant transformed with the empty vector served as the control. One week after germination, seedlings were transferred to medium in which the 5 mM KNO_3 was replaced with either 0.25 mM or 0.5 mM NH_4^+ (as the sole nitrogen source). In parallel, seedlings were also transferred to medium supplemented with 10 mM or 20 mM MeA (methylamine, an NH_4^+ analog that is transported by AMTs and is cytotoxic). For salt tolerance assays, one-week old seedlings were transferred to the same medium supplemented with 0 or 100 mM NaCl. Growth phenotypes and stress responses were analyzed to evaluate the roles of *SeAMT* genes in ammonium uptake and salt tolerance.

Analysis of AMT-mediated ion transport interactions in transgenic yeast

For yeast transformation, competent cells of strains 31019b and *CY162* were prepared, and plasmid introduction was performed using the Frozen-EZ Yeast Transformation II Kit (Zymo Research). Transgenic yeast monoclonal lines were selected on SC-U (synthetic complete medium lacking uracil) plates and confirmed by PCR. The empty pYES2 vector without *SeAMT* gene insertion served as the negative control. For NH_4^+ uptake assays, yeast cultures were normalized to equal optical density at 600 nm (OD_{600}), and 2 μL of each cell suspension was spotted onto YNB (yeast nitrogen base) solid medium (containing 1% agar and 2% galactose) supplemented with various concentrations of NH_4^+ (0.25, 0.5, 1, 2, and 20 mM) as the sole nitrogen source. Plates were incubated at 30°C for 2–4 days, and yeast growth was monitored. For salt tolerance assays, the YNB medium was supplemented with 2 mM arginine to ensure adequate nutrition for yeast growth, and various concentrations of NaCl (0, 10, 20, 50, 100, and 200 mM) were added to assess salt tolerance. To investigate the effects of Na^+ or K^+ on AMT-mediated NH_4^+ uptake, yeast cells were spotted onto YNB medium containing 0.25 mM or 2 mM NH_4^+ as the sole nitrogen source, or 10 mM or 20 mM MeA (methylamine, a cytotoxic NH_4^+ analog transported by AMTs). These media were further supplemented with various concentrations of Na^+ or K^+ (up to 50 mM) to evaluate their competitive effects on NH_4^+ uptake, as reflected by yeast growth phenotypes.

Results

Identification of *SeAMT* gene family members in *Salicornia europaea*

Six coding sequences (CDS) putatively encoding AMT homologs were assembled and identified from the *Salicornia europaea* transcriptome, and were designated as *SeAMT1;1*, *SeAMT1;2a*, *SeAMT1;2b*, *SeAMT1;3*, *SeAMT1;4*, and *SeAMT3;1* (Fig. S1). To infer the evolutionary relationships of these putative transporters, a phylogenetic tree was constructed using the Neighbor Joining (NJ) method, incorporating AMT protein sequences from *Arabidopsis thaliana*, *Oryza sativa*, *S. europaea*, and *Chenopodium quinoa*.

As shown in Fig. 1A, all SeAMT members, with the exception of SeAMT3;1, were resolved within the AMT1 clade and exhibited close orthology with their corresponding counterparts in *Chenopodium quinoa* a finding consistent with the fact that both *S. europaea* and *C. quinoa* belong to the family Amaranthaceae. The nomenclature of each SeAMT gene was assigned accordingly based on this phylogenetic topology. Conserved domain analysis of the AMT proteins included in the phylogenetic dataset revealed three major domain architectures, namely the Amt domain, the Ammonium_transp superfamily domain, and the AmtB domain. Notably, SeAMT3;1 was the only member harboring the Ammonium_transp superfamily domain, whereas all other SeAMT proteins contained the Amt domain (Fig. S2A). In addition, SeAMT3;1 possessed only three conserved motifs, substantially fewer than the 11–12 motifs identified in the remaining five SeAMT members (Fig. 1B). These marked differences in domain composition and motif distribution suggest that SeAMT3;1 may have undergone functional divergence from the other five SeAMT paralogs. To further corroborate their identity as membrane-localized transporters, transmembrane helix prediction was performed using the TMHMM 2.0 server. All six SeAMT proteins were predicted to contain 9 or 11 transmembrane helices (Fig. S2B), a feature consistent with their expected plasma membrane localization and putative ammonium transport activity.

Transcript tissue expression and protein subcellular localization of SeAMTs

To determine the tissue-specific expression patterns of the six SeAMT genes, RT- qPCR analysis was performed on shoot and root tissues. As shown in Fig. 2A,B, *SeAMT1;1* and *SeAMT1;3* were predominantly expressed in the shoot, whereas *SeAMT1;2a*, *SeAMT1;2b*, and *SeAMT3;1* exhibited pronounced root specific expression. In contrast, transcript levels of *SeAMT1;4* were extremely low in both tissues. RNA-Seq results were consistent with the qPCR results that showed *SeAMT1;2a* and *SeAMT1;2b* having the highest expression in root, while *SeAMT1;1* showing the highest expression in shoot (Fig. S3).

To investigate the subcellular localization of SeAMT proteins, four representative members (*SeAMT1;2a*, *SeAMT1;3*, *SeAMT1;4*, and *SeAMT3;1*) were transiently expressed as GFP fusion proteins in *Nicotiana benthamiana* leaves via Agrobacterium mediated infiltration. Confocal microscopy analysis revealed that all four fusion proteins were localized to both the plasma membrane (PM) and the endoplasmic reticulum (ER). Due to the reticular network of the ER surrounding the nucleus, the ER appears as a green hollow circle (Fig. 2C). Notably, in addition to PM and ER localization, *SeAMT3;1*-GFP also produced spherical fluorescent puncta in the cytoplasm that were not observed for other SeAMT members; these puncta morphologically resembled peroxisomes. As a negative control, free GFP expressed from the empty vector exhibited a broad subcellular distribution, with fluorescence detected throughout the nucleus, cytoplasm, and PM. Collectively, the PM localization of SeAMTs supports their role in mediating the uptake of extracellular ammonium into the cell. The concurrent ER retention of these proteins, however, raises the possibility of a regulatory mechanism that governs the trafficking of ammonium transporters from the ER to the PM. Given that *SeAMT1;2a* and *SeAMT1;2b* exhibited the highest transcript abundance in roots, these two AMT1 members are likely to function as the primary transporters responsible for root ammonium acquisition in *S. europaea*.

SeAMT1;2a and SeAMT1;2b are induced by nitrogen starvation

To investigate the nitrogen deficiency responses of the six SeAMT genes, *S. europaea* seedlings initially grown under NH_4NO_3 -supplied conditions were then transferred to nitrogen-free medium for 3 days of nitrogen starvation. RT-qPCR analysis was performed on both shoot and root tissues. In agreement with the tissue-specific expression patterns shown in Fig. 2A, SeAMT1;1 and SeAMT1;3 were predominantly expressed in shoots, whereas SeAMT1;2a, SeAMT1;2b, and SeAMT3;1 were mainly root-localized (Fig. 3). The shoot-specifically expressed genes SeAMT1;1 and SeAMT1;3 were not responsive to nitrogen deficiency (Fig. 3A,D). SeAMT1;4 was expressed in both shoots and roots but exhibited generally low transcript levels (high Ct values), with no significant changes in shoots and root under nitrogen deficiency (Fig. 3E). SeAMT3;1, which is predominantly root-expressed, was not induced by nitrogen starvation (Fig. 3F). By contrast, transcripts of SeAMT1;2a, which is primarily root-specific, were induced by nitrogen starvation in both roots and shoots, representing up to 2-fold and 2.5-fold increases, respectively, compared with the control (N+) (Fig. 3B). Similarly, SeAMT1;2b, which is also root-specifically expressed and exhibits the highest overall expression abundance among all SeAMT genes (Fig. 2A,B), was upregulated by nitrogen starvation, to approximately 1.5-fold levels of control (Fig. 3C). The upregulation of root-specifically expressed SeAMT1;2a and SeAMT1;2b under nitrogen starvation suggests their critical roles in the adaptive response to nitrogen deficiency stress.

SeAMT1;2a and SeAMT1;2b are predominant NH_4^+ transporters in the SeAMT family

To evaluate the ammonium transport capacity of the *S. europaea* AMT family, the CDSs of SeAMT genes were heterologously expressed in the ammonium-transport-deficient yeast strain 31019b and the *Arabidopsis* quadruple knockout mutant *qko*, respectively. In the yeast assay, the mutant strain 31019b was able to grow normally when the medium was supplied with up to 20 mM NH_4^+ (Marini et al. 1997). As shown in Fig. 4A, with the exception of SeAMT3;1, all five SeAMT1 subfamily members significantly rescued the growth of 31019b, indicating their capacity for effective NH_4^+ acquisition. These five SeAMT1 transporters exhibited high-affinity transport characteristics, as they mediated efficient NH_4^+ transport at concentrations below 1 mM. By contrast, SeAMT3;1 appeared incapable of ammonium uptake. Among the SeAMT1 members, SeAMT1;2a was the most efficient ammonium transporter, as evidenced by its extremely significant rescue of NH_4^+ transport in the yeast mutant (Fig. 4A).

To further verify the role of SeAMT1;2a and SeAMT1;2b in ammonium transport, these two genes, with other two SeAMTs (SeAMT1;4, and SeAMT3;1), were individually transformed into the *Arabidopsis* quadruple knockout mutant *qko* (*amt1;1 amt1;2 amt1;3 amt2;1*). This mutant retains only 5–10% of wild-type ammonium uptake capacity and exhibits poor growth when NH_4^+ was supplied as the sole nitrogen source (Yuan et al., 2007). Transgenic lines with high and consistent transient expression were identified and selected for phenotypic assay (Fig. S4A,B). As shown in Fig. 4B, when NO_3^- was provided as the sole nitrogen source, no significant differences in shoot biomass (fresh weight) were observed between *qko* and the SeAMT-transgenic lines. However, upon transfer to medium containing NH_4^+ as the sole nitrogen source, the shoot biomass of *qko* was significantly reduced at both 0.25 mM and 0.5 mM NH_4^+

compared with that under NO_3^- -supplied conditions. Notably, transformation with *SeAMT1;2a* significantly rescued the shoot biomass of *qko* under these conditions, confirming its role in NH_4^+ transport for nitrogen supply (Fig. 4B,C). This result is consistent with the yeast assay and together supports that *SeAMT1;2a* is a key ammonium transporter in *S. europaea* (Fig. 4A,B). To examine the low-affinity characteristics of these *SeAMT* transporters, *qko* and transgenic seedlings were subjected to treatment with 0 mM (control), 10 mM, and 20 mM MeA. Notably, *SeAMT1;2b*-expressing seedlings exhibited substantially higher MeA uptake compared with other transgenic lines. This transporter displayed low-affinity ammonium transport characteristics, with nonsaturable uptake kinetics at high substrate concentrations. As a result, *SeAMT1;2b*-expressing seedlings suffered the most severe toxicity upon MeA treatment (Fig. 4D,E). *SeAMT1;2a* and *SeAMT1;4* also exhibited low-affinity features, as their transgenic seedlings showed significantly reduced growth under high MeA concentrations due to enhanced MeA uptake (Fig. 4D,E). Collectively, through complementary functional analyses in yeast and *Arabidopsis*, our results demonstrate that *SeAMT1;2a* and *SeAMT1;2b* which possess features of both high- and low-affinity transporters are the two most important and powerful ammonium transporters central to nitrogen acquisition in *S. europaea*.

SeAMT1;2a and *SeAMT1;2b* confer salt tolerance in transgenic yeast and *Arabidopsis*

Interestingly, several *SeAMT* genes were found to respond to NaCl treatment. The transcript level of the shoot-expressed gene *SeAMT1;1* decreased progressively with prolonged NaCl treatment, whereas the three root-expressed genes *SeAMT1;2a*, *SeAMT1;2b*, and *SeAMT3;1* were upregulated under NaCl stress (Fig. 5A). To evaluate whether these *SeAMT* genes confer salt tolerance, the transgenic yeast lines of *31019b* used in Fig. 4A were cultured in YNB medium supplemented with 2 mM arginine as the nitrogen source to eliminate the differences of NH_4^+ uptake among different transformants. Additionally, various concentrations of NaCl were included in the growth medium. The yeast strain *31019b* began to exhibit salt stress when the NaCl concentration exceeded 50 mM. Notably, compared with yeast lines expressing other *AMT* genes, those expressing *SeAMT1;2a* and *SeAMT1;2b* exhibited markedly enhanced salt tolerance under 100 mM and 200 mM NaCl stress (Fig. 5B).

To further confirm the salt tolerance function of *SeAMT1;2a* and *SeAMT1;2b*, the transgenic lines of *Arabidopsis* shown in Fig. 4B were subjected to salt stress assays. Considering the potential influence of ammonium (NH_4^+) transport mediated by *SeAMTs* in the various transgenic lines, the medium contained 5 mM KNO_3 as the sole nitrogen source for each treatment. Under 100 mM NaCl stress, the root length of the *qko* mutant was significantly reduced; however, transgenic seedlings expressing *SeAMT1;2a* or *SeAMT1;2b* showed significant root elongation compared with the *qko* and transgenic lines expressing *SeAMT1;4* or *SeAMT3;1* (Fig. 5C,D). The enhanced salt tolerance conferred by *SeAMT1;2a* and *SeAMT1;2b* in both yeast and *Arabidopsis* suggests that these genes have versatile roles not only in ammonium transport but also in salt tolerance.

Versatile *SeAMT* genes confer functions in ion uptake and antagonism

Salicornia europaea typically grows in saline environments. To investigate whether Na^+ affects SeAMT-mediated NH_4^+ transport, transgenic yeast line *31019b* expressing various SeAMT genes was cultured on YNB medium supplemented with either 0.25 or 2 mM NH_4^+ , with 2 mM arginine serving as the control. Consistent with the results shown in Fig. 4A, all five SeAMT1 genes conferred NH_4^+ transport function to the *31019b* strain (Fig. 6A,B). Since NaCl concentrations below 50 mM did not impair yeast growth, 0–50 mM NaCl was introduced into the medium containing 0.25 or 2 mM NH_4^+ . Notably, even when the $\text{Na}^+/\text{NH}_4^+$ concentration ratio reached up to 200 (Fig. 6A), NaCl had no significant effect on SeAMT1-mediated NH_4^+ transport. Although both Na^+ and NH_4^+ carry a monovalent positive charge, the SeAMT1 transporter exhibits much higher transport affinity for NH_4^+ than for Na^+ (Fig. 6A,B). Under treatment with 50 mM MeA (a cytotoxic NH_4^+ analog transported by AMTs), with 2 mM arginine as the sole nitrogen source, SeAMT1;2a- and SeAMT1;2b expressing in yeast cells showed severe growth inhibition due to the uptake of toxic MeA (Fig. 6C). These results were consistent with the observation that SeAMT1;2a- and SeAMT1;2b expressing *Arabidopsis qko* mutants also exhibited growth inhibition under MeA treatment (Fig. 6C and Fig. 4D). Together, these two independent assays strongly support that SeAMT1;2a and SeAMT1;2b are the predominant NH_4^+/MeA transporters. Furthermore, the addition of varying concentrations of NaCl to the medium failed to rescue the growth of SeAMT1;2a or SeAMT1;2b-expressing yeast, indicating that exogenous NaCl could not inhibit SeAMT1;2a- or SeAMT1;2b-mediated MeA uptake (Fig. 6C). However, supplementation with exogenous KCl significantly alleviated the cytotoxic effect when the K^+/MeA concentration ratio reached 1, suggesting that competitive uptake between K^+ and MeA may occur through the AMT transporters, thereby reducing MeA uptake (Fig. 6D).

To validate whether SeAMT1;2a or SeAMT1;2b mediates K^+ uptake, these two genes were introduced into the potassium-uptake-defective yeast strain *CY162*, which requires up to 100 mM KCl for normal growth (Anderson et al. 1992). The transformants were cultured in AP medium (a potassium-deficient complete medium) supplemented with either 1 mM or 100 mM KCl to represent low-potassium stress or normal conditions, respectively, for *CY162* growth. Compared with SeAMT1;2b and SeAMT1;3, only SeAMT1;2a rescued the growth of *CY162* on 1 mM KCl treatment, demonstrating that SeAMT1;2a confers K^+ transport function (Fig. 6E). It is well established that *S. europaea* possesses remarkable salt tolerance. In our studies, we found that SeAMT1 exhibits high-affinity NH_4^+ transport that is not inhibited by NaCl, even when the NaCl concentration is 200-fold higher than that of NH_4^+ . Robust NH_4^+ acquisition and maintenance of nitrogen supply under salt stress are critical for the growth of *S. europaea* in saline environments. On the other hand, Na^+ competitively suppresses K^+ acquisition in plants, leading to disruption of K^+ homeostasis and substantial inhibition of plant growth and development. Intriguingly, SeAMT1;2a from *S. europaea* provides an alternative route for K^+ uptake, which may partially alleviate ionic imbalance by helping sustain Na^+/K^+ equilibrium.

Discussion

The halophyte *S. europaea* possesses fleshy and succulent shoot that accumulate large amounts of ions to promote its growth. Owing to its high biomass productivity and substantial nitrogen removal capacity, this species is considered a promising candidate for phytoremediation of industrial or aquaculture

wastewater. Previous studies have shown that *S. europaea*-based filter beds can remove more than 90% of influent NH_4^+ under ambient nutrient loading conditions (Webb et al. 2012), indicating a high capacity for ammonium uptake. To elucidate the molecular basis of ammonium transport in *S. europaea*, we systematically identified six ammonium transporter genes in this halophyte and characterized each member in terms of expression patterns and inducible regulation. Furthermore, through functional analysis of these genes, we revealed clear functional redundancy and divergence among SeAMT members, with SeAMT1;2a and SeAMT1;2b playing predominant roles in both ammonium uptake and salt tolerance.

Functional complementation assays in yeast confirmed that all five SeAMT1 genes possess high-affinity ammonium transport activity. The functional redundancy among these SeAMT genes likely underpins the robust adaptability of *S. europaea* to diverse environmental conditions. However, pinpointing the predominant SeAMT gene responsible for ammonium transport is of particular importance for targeted genetic improvement of nitrogen acquisition in this halophyte. Previous studies have established that AtAMT1;1, AtAMT1;2, and AtAMT1;3 function as the principal ammonium transporters among the six AMT members in *Arabidopsis thaliana* (Yuan et al. 2007) and similarly, AtPHT1;1 and AtPHT1;4 play dominant roles in phosphate acquisition (Shin et al. 2004). In the present study, we observed marked differences in ammonium transport capacity among SeAMT members: SeAMT1;2a exhibited the strongest transport activity, whereas SeAMT3;1 showed no detectable NH_4^+ transport. Sequence and protein motif analyses revealed that SeAMT3;1 diverges considerably from the SeAMT1 subfamily proteins (Fig. 1B), suggesting functional diversification among these homologs. SeAMT3;1 is an ortholog of the rice OsAMT3 family members. Among these, OsAMT3;2 positively regulates ammonium uptake in rice (Wu et al. 2024), whereas OsAMT3;1 may be involved in the mycorrhizal nitrogen uptake pathway (Pérez-Tienda et al. 2014), and OsAMT3;3 is predominantly expressed in old leaves (Li et al. 2012). However, there is currently no evidence supporting a direct role for either OsAMT3;1 or OsAMT3;3 in ammonium transport. On the other hand, the distinct tissue-specific expression patterns of SeAMT genes imply additional functional differentiation. SeAMT1;1 and SeAMT1;3 were expressed in shoots, suggesting their involvement in ammonium transport or distribution within aboveground tissues. In contrast, root-specific genes such as SeAMT1;2a and SeAMT1;2b are likely responsible for ammonium uptake from the external environment. Notably, only SeAMT1;2a and SeAMT1;2b were transcriptionally induced by nitrogen starvation, indicating their potential roles in low-nitrogen adaptation. Functional complementation in both yeast and *Arabidopsis* validated their robust ammonium uptake capabilities. Collectively, our findings provide compelling evidence that *S. europaea* root-expressed SeAMT1;2a and SeAMT1;2b constitute the two major genes mediating external ammonium uptake.

In previous work, we uncovered an interplay between NH_4^+ and Na^+ treatments, in which NaCl alleviates ammonium stress by enhancing nitrogen metabolism in *S. europaea* (Ma et al. 2020). Notably, in this study, the ammonium transporters SeAMT1;2a and SeAMT1;2b also play roles in salt tolerance (Fig. 5B,C). Furthermore, a recent study reported that ammonium positively regulates SOS2 (known salt tolerance-related proteins) kinase activity to enhance salt tolerance in *Arabidopsis*, while SOS2 phosphorylates and modulates AMT1;1 activity to improve ammonium uptake (Liang et al. 2025). This

positive feedback loop “SOS2 – AMT1;1 (phosphorylation) – ammonium uptake – SOS2” provides a compelling explanation for AtAMT1;1-dependent ammonium uptake that enhances salt tolerance. However, in our salt tolerance assays of *SeAMT1;2a* and *SeAMT1;2b* in transgenic yeast and *Arabidopsis*, the growth media were devoid of NH_4^+ ; instead, arginine or NO_3^- served as the sole nitrogen source, respectively (Fig. 5B,C). This indicates that *SeAMT1;2a*- and *SeAMT1;2b*-mediated salt tolerance is independent of ammonium uptake. In addition, Ser-450 of AtAMT1;1 is the essential phosphorylation site targeted by SOS2. *SeAMT1;1* and *SeAMT1;2b* possess a conserved ‘Ser-450’ at the corresponding position, but *SeAMT1;2a* contains an ‘Arg’ substitution, suggesting that *SeAMT1;2a* may not be subject to phosphorylation regulation at this position (Fig. S5). As transporters, we consider *SeAMT1;2a* and *SeAMT1;2b* to be versatile in ion uptake, protecting against single-ion toxicity. We have provided evidence supporting K^+ transport activity for *SeAMT1;2a* (Fig. 6E). This dual NH_4^+/K^+ transport capability of *SeAMT1;2a* likely contributes to the salt-tolerant phenotype by helping to maintain cellular K^+ homeostasis alongside ammonium absorption. Furthermore, the high affinity of *SeAMT1* transporters for NH_4^+ enables *S. europaea* to sustain nitrogen acquisition even under high $\text{Na}^+/\text{NH}_4^+$ ratio conditions. Collectively, our findings provide new insights into *SeAMT*-mediated salt tolerance in the halophyte species.

Owing to its remarkable ion-accumulating capacity, the halophyte *S. europaea* has been employed not only for the desalination of saline soils but also for the phytoremediation of industrial or aquaculture wastewater (Webb et al. 2012; Yasseen and Al-Thani 2022). Phytoremediation provides an efficient and cost-effective strategy for treating wastewater with high nutrient or heavy metal pollution. In the present study, we identified two major ammonium transporters, *SeAMT1;2a* and *SeAMT1;2b*, from the roots of *S. europaea*. These transporters function as high-affinity ammonium transporters capable of efficiently acquiring NH_4^+ at low concentrations. In addition, they exhibit low-affinity transporter features that sustain increasing NH_4^+/MeA transport rates even under high-substrate conditions (Fig. 4D and Fig. 6C). In the context of eutrophic wastewater phytoremediation, *S. europaea* benefits from the activity of these low-affinity transporters to achieve high-rate ion accumulation. Our findings uncover the genes involved and their underlying molecular mechanisms, revealing how *S. europaea* efficiently removes NH_4^+ from eutrophic wastewater. More importantly, these two genes represent promising targets for transgene- or gene-editing-based breeding strategies aimed at crop improvement, while the cultivation of *S. europaea* in saline soils and wastewater for bioenergy seed oil production could contribute to sustainable development.

Conclusions

In this study, six ammonium transporter genes (*SeAMT1;1*, *SeAMT1;2a*, *SeAMT1;2b*, *SeAMT1;3*, *SeAMT1;4*, and *SeAMT3;1*) were identified and characterized in the halophyte *Salicornia europaea*. Five members belong to the AMT1 subfamily, while *SeAMT3;1* diverged in domain and motif composition. *SeAMT1;1* and *SeAMT1;3* were mainly expressed in shoots, whereas *SeAMT1;2a*, *SeAMT1;2b*, and *SeAMT3;1* were root-localized. Subcellular localization assays indicated that *SeAMTs* were localized to both the plasma membrane and the ER. Functional assays in yeast and *Arabidopsis* showed that all five *SeAMT1*

members mediate high-affinity NH_4^+ transport. Notably, *SeAMT1;2a* and *SeAMT1;2b* were induced by nitrogen starvation and exhibited both high-affinity and low-affinity ammonium transport characteristics. In addition, these two genes enhance salt tolerance through a mechanism independent of ammonium uptake. Importantly, *SeAMT1;2a* mediated K^+ uptake, providing an alternative route for K^+ acquisition to resist salt stress.

Collectively, *SeAMT1;2a* and *SeAMT1;2b* are the predominant ammonium transporters in *S. europaea*, coupling efficient NH_4^+ acquisition with salt tolerance. These findings provide new insights into ammonium transport in halophytes and offer promising targets for improving crop nitrogen use efficiency and salt tolerance, as well as for phytoremediation of eutrophic wastewater.

Declarations

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