# Molecular Cloning of Saslah3, a Novel Slow Anion Channel Slac/slah Family Gene, From Euhalophyte Suaeda Altissima (L.) Pall and Analysis of the Encoded Protein

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# Abstract

Cloning of the *SaSLAH3* gene (OQ271227.1), an *AtSLAH3* ortholog from the slow anion channels family (*SLAC/SLAH*), that was originally identified in *Arabidopsis thaliana*, has been carried out from the euhalophyte *Suaeda altissima*. Under hydroponic conditions, this species related to Amaranthaceae/Chenopodiaceae is capable to complete life cycle on nutrient solutions containing NaCl at high concentrations up to 1 M. Cloning of *SaSLAH3* was performed based on a putative similarity of *SaSLAH3* with the *SLAH3* homologs from the related species *Suaeda glauca* and *Suaeda fruticosa*, using transcriptomes assembled previously. Membrane topology, analyzed by *in silico* approach, the 3-D structure, presence of conservative phenylalanine-bearing motives and as well as MEME-generated sequence motif patterns of SaSLAH3, all of these features were found typical for the SLAC/SLAH channels and had validated SaSLAH3 belonging to this family. Analysis of SLAC/SLAH phylogenetic relationship demonstrated the evolutionary divergence of SLAC-like proteins into three distinct groups characteristic of this family with location of SaSLAH3 in SLAH2/3 clade. Investigation of the expression profile by quantitative Real-Time PCR revealed increasing *SaSLAH3* expression in roots and leaves upon elevation of NaCl concentration in nutrient solution under conditions of both sufficient nitrate supply and nitrate deficiency. SaSLAH3 is suggested to play an important role in enhancing chloride loading into root xylem and, thereafter in chloride delivery to shoot under salinity. Another physiological role of SaSLAH3 may be attributed to regulation of nitrate concentration in cytoplasm to ensure proper nitrogen nutrition of *S. altissima* plants under conditions of stiff competition of nitrate with chloride.


# Introduction

In recent years, the family of slow, or S-type anion channels, (SLAC/SLAH), attracts close attention of researchers. In *A. thaliana*, this family includes AtSLAC1 (slow anion channel-associated 1) and its four homologs, AtSLAH1 – AtSLAH4. AtSLAC1, AtSLAH2, and AtSLAH3 operate as anion-conducting efflux channels, while AtSLAH1 is a regulatory channel subunit; the function of AtSLAH4 currently remains unknown (Negi et al. 2008; Vahisalu et al. 2008; Dreyer et al. 2012; Qiu et. al. 2016; Hedrich and Geiger, 2017). Apart from *A. thaliana*, the members of SLAC/SLAH family have been identified and some of their functions were investigated in barley (*Hordeum vulgare*) (Liu et al. 2014), rice (*Oryza sativa*) (Sun et al. 2016), poplar (*Populus trichocarpa*) (Jaborsky et al. 2016), tobacco (*Nicotiana tabacum*) (Kurusu et al. 2013), pear (*Pyrus bretschneideri*) and other Rosaceae plant species (Chen et al. 2019).

All the proteins of this family were shown to be localized in plasma membrane (PM). AtSLAC1 and AtSLAH3 have been studied the most among proteins of the SLAC/SLAH family. The outflow of anions from guard cells during $CO_2$ and ABA-dependent stomatal closure occurs through AtSLAC1 and AtSLAH3 (Negi et al. 2008; Vahisalu et al. 2008; Geiger et al. 2009; 2011). AtSLAC1 transports both $Cl^-$ and malate (Negi et al. 2008). AtSLAH3 is significantly more specific for $NO_3^-$ rather than for $Cl^-$ (Geiger et al. 2011). The latter protein is found, apart from guard cells, in leaf mesophyll, root cortex and xylem parenchyma (Negi et al. 2008; Geiger et al. 2011; Zheng et al. 2015; Cubero-Font et al. 2016; Lhamo et. al,

2021). AtSLAH1 and extremely selective for $NO_3^-$ AtSLAH2 (Maierhofer et al. 2014a) are localized in the root central cylinder cells (Negi et al. 2008), where these proteins, along with AtSLAH3, control the ionic composition of xylem juice (Hedrich and Geiger, 2017). Both AtSLAC1 and AtSLAH3 are regulated by different kinase-phosphatase pairs involved in signal transduction under water, salinity and oxidative stresses (Geiger et al. 2009; Chen et al. 2010; Geiger et al. 2011; Sun et al. 2016; Hedrich and Geiger et al. 2017). AtSLAH3 is activated by $Ca^{2+}$-dependent kinases and also requires nitrate presence in extracellular medium for this activation (Geiger et al. 2011, Maierhofer et al. 2014a; 2014b; Hedrich and Geiger, 2017). It has been shown that AtSLAC1 and AtSLAH3 function as essential negative regulators of inward rectifying $K^+$ channels and stomatal opening in *A. thaliana* (Zhang et al. 2016). Two signaling pathway, the first underlying ABA-induced stomatal closure and the other participating in light-induced stomatal opening, were found to be interlinked through direct protein-protein interaction of AtSLAC1 and AtSLAH3 with the $K^+$ inward rectifying channels AtKAT1 and AtAKT2. Under drought stress and ABA action, interacting of these proteins led to an efficient inhibition of stomatal opening. AtSLAH3 has been shown to become activated upon complex formation with AtSLAH1 which is accompanied by a strong increasing AtSLAH3 chloride conductance (Cubero-Font et al. 2016). This protein was also found to be regulated by interacting with the lipid nanodomains (Demir et al. 2013). Along with participating in signal transduction in response to drought, salt, and oxidative stresses (Geiger et al. 2009; 2011; Sun et al. 2016; Hedrich and Geiger et al. 2017; Chen et al. 2019), AtSLAH3 is involved in the flooding-related stress response (Lehmann et al. 2021). Another known physiological function of the nitrate efflux channel AtSLAH3 is nitrate-dependent alleviation of ammonium toxicity and acidity tolerance increase (Zheng et al. 2015).

Since under saline conditions nitrate is absorbed and transported through the plant in stiff competition with chloride, one may expect unusual properties of halophyte nitrate transporters when compared with those of glycophytes. Though, these transporters are popular subjects in the studies of plant nitrogen nutrition, available information on halophyte orthologs of these proteins is scarce. Molecular and functional identification and characterization of halophyte anion transporters are therefore the important tasks for genetic engineering of salt tolerant plants. The main objective of the present study was cloning the *SaSLAH3* gene, an ortholog of *A. thaliana AtSLAH3*, from the euhalophyte *Suaeda altissima* related to Amaranthaceae/Chenopodiaceae. The genus *Suaeda* species belong to the most salt tolerant plants (Flowers, Colmer, 2008). Further, we analyzed structure of the encoded protein and its phylogenetic relationships using *in silico* approach. Expression profiles of the cloned *SaSLAH3* gene and some physiological characteristics of *S. altissima* plants grown under salinity and nitrate deficiency along with control conditions were also analyzed.

## Materials and methods

# Plant material

Seeds of *S. altissima* (L.) Pall, were collected from the plants growing in the wild on the shores of lake Elton, a salt lake located in Volgograd region, Russia. Seeds were stratified for 2–3 days. Plants were grown a phytotron chamber under controlled environmental conditions at 24°C and relative humidity of 60–70%. Plants were illuminated with high-pressure sodium lamps DNaZ-400 Reflux (Minimax, Saint Petersburg, Russia) with a photoperiod of 16 h/8 h (day/night) and a light intensity of 300 μmol $m^{-2}$•$s^{-1}$. Seeds were germinated in wet sand. Two weeks after emergence, the seedlings were transplanted into 3 L glass jars (6 plants in each jar) on the aerated Robinson-Downton nutrient solution (NS) (Robinson and Downton, 1985). To avoid salt shock, NaCl addition to the NS was performed gradually with increment of 50 or 100 mM every day. Two nitrate (0.5 and 15 mM) concentrations were used for different nitrate availability experiments. The plants were grown under the conditions described above. Fifty-five-day-old plants were used for sampling in all experiments. For total RNA extraction, leaves and roots were collected, frozen in liquid nitrogen, and stored at − 70°C.

# Primer Design

To amplify the *SaSLAH3* gene, a pair of primers SaSLAH3_inf_F2 and *SaSLAH3*_inf_R2 (Table S1, Fig. S1), containing the 5' and 3' flanking sequences of the *SfSLAH3* ORF, respectfully, were designed manually based on possible similarity of hypothetical gene *SaSLAH3* to putative ortholog genes from the halophytes *S. fruticosa* and *S. glauca*. These primers also contained 15 bp 5'-overhangs for Gibson assembly cloning as recommended by Gibson assembly kit manufacturer (New England Biolabs, USA). Primers for qPCR-RT experiments were designed using Light Cycler 2.0 Probe Design software (https://lifescience.roche.com/, accessed on 15 December 2021) or Primer Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 15 December 2021). Snap Gene Viewer software-generated sequence files (https://www.oligo.net/, accessed on 15 December 2021) were used for manual primer design. All primers used in this study are listed in Table S1, Fig. S1.

# Total RNA extraction and cDNA synthesis

Isolation of total RNA from the leaves and roots of 55-day-old plants was carried out using a hot phenol procedure based on a method described in (Yourieva et al. 2018). A total of 100 mg of plant tissue for RNA preparations was taken from finely chopped and then well-mixed leaves or roots of 18–24 individual plants. To check RNA integrity, the purified samples were analyzed on a 1% non-denaturing agarose gel containing 0.5 μg/ml ethidium bromide (EtBr). Genomic DNA impurities in RNA preparations were removed using DNAse I (Thermo Fisher Scientific, United States). First strand cDNA synthesis was performed using a MMLV RT kit (Evrogen, Russia) with oligo(dT) primer. The last two procedures were carried out according to the manufacturer's recommendations.

### SaSLAH3 amplification and cloning

A pair of primers, SaSLAH3_inf_F2 and SaSLAH3_inf_R2, bearing 5'- and 3'- flanking regions of *SfSLAH3* coding DNA sequences (CDS), was used to amplify open reading frame (ORF) of *SaSLAH3* gene from first strand cDNA template prepared from *S. altissima* total RNA. The *SaSLAH3* ORF was amplified in two rounds. First, PCR using Encyclo polymerase (Evrogen, Moscow, Russia) was performed to generate

1827 bp *SaSLAH3* amplicon. The PCR program included the following step: 94°C for 2 min, 30 cycles of 94°C for 30 s, 63°C for 25 s, 58°C for 25 s, and 72°C for 1 min 45 s. To increase the amount of the *SaSLAH3* fragment, a second round of amplification was performed. In this round, CloneAmp HiFi PCR Premix Polymerase (Takara Bio Inc. Shiga, Japan Cat, No. 639298) and 10–20 ng of *SaSLAH3* fragment as a template were used. The PCR program of the second round included 35 cycles of the following steps: 98°C for 10 s, 63°C for 10 s, 58°C for 10 s, and 72°C for 10 s. Cloning of the amplified fragment into methylotrophic yeast *Pichia pastoris* shuttle vector pVR2ADRHis was performed using Gibson Assembly Cloning Kit (New England Biolabs, USA) according to the manufacturer's recommendations. The vector was linearized by inverse PCR using the CloneAmp HiFi PCR Premix and a pair of primers pVR2_inf_F and pVR2_inf_R (Table S1). PCR program included 35 cycles of the following three steps: 98°C for 10 s, 55°C for 10 s, 72°C for 30 s. The resulting assembled mix was transformed into home-made *E. coli* XL1-Blue competent cells prepared according to the Eppendorf's instruction by electroporation using an Eppendorf instrument (Eppendorf, Germany), at a voltage of 1700 V and 5 ms pulse length. Screening of resulting clones for the presence of the *SaSLAH3* insert was carried out by colony PCR using 5X Screen Mix HS (Evrogen, Russia) and a pair of primers, pVR2_1639F20 and SaSLAH3_R1 (Table S1). Plasmid DNA was isolated from selected clones by modified alkaline lysis mini-prep method (Sambrook et al. 2012) and was subjected to restriction analysis. Plasmid DNA from positive clones was then sequenced at Evrogen (Moscow, Russia) to confirm the presence of the *SaSLAH3* fragment insert. The resulting recombinant construct was named pVR2-SaSLAH3 (Fig. S1).

# Quantitative Real-Time PCR

Expression of the *SaSLAH3* gene was analyzed by quantitative Real-Time RT-PCR (qRT-PCR) using Ready Mix for PCR qPCRmix-HS SYBRmix kit (Evrogen, Moscow, Russia) and a pair of primers, SaSLAH3_RT_F and SaSLAH3_RT_R (Table S1); the size of the resulting amplicon was 158 bp. Elongation factor 1 alpha from *S. altissima* (GenBank: MN076325.1) served as a reference gene using primers SaeELF1alfa_F1 and SaeELF1alfa_R1 (Table S1) yielding a fragment of 128 bp. From 30 to 100 ng (based on the amount of RNA taken for cDNA synthesis) of cDNA template synthesized on total RNA isolated from roots or leaves of *S. altissima* was added to the 25 µl sample. PCR program was created following the manufacturer recommendations of the Light Cycler 96 instrument (Roche, Switzerland) and the instructions for the PCR Ready mix (Evrogen, Moscow, Russia), and consisted of pre-denaturation at 95°C for 5 min, and 40 cycles of the following steps: 95°C for 20 s, 58°C for 20 s, 72°C for 20 s. The obtained data were analyzed by the $2^{-\Delta\Delta CT}$ method (Rao et. al. 2013) using Microsoft Excel and Sigma Plot software.

# Bioinformatics analysis of SaSLAH3 orthologs

Multiple sequence alignment (MSA) of *SaSLAH3* with the orthologs from *Suaeda fruticosa* (L.) Forssk. *Suaeda glauca* (Bunge), and *A. thaliana* was produced by Clustal omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/, accessed on 15 December 2021) and visualized in Jalview 2.11.1.4 program. Phylogenetic trees building was carried out with the maximum likelihood method (bootstrap test font-weight to a value of 1000) using MEGA X software (https://www.megasoftware.net/,

accessed on 15 December 2021). Virtual translation of the *SaSLAH3* gene was carried out by ExPASy (https://web.expasy.org/translate/, accessed on 15 December 2021), protein topology was predicted using Phobius (https://www.ebi.ac.uk/Tools/pfa/phobius/, accessed on 15 December 2021) and Phyre 2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index, accessed on 15 December 2021) software. Online Multiple Expectation Maximization for Motif Elicitation (MEME) was used for proteins alignment and conserved motifs discovery (http://meme.nbcr.net/meme/cgibin/meme.cgi, accessed on 15 December 2021). Phyre 2 and Swiss-Model software was also used for the protein 3D modeling. All nucleotide and protein sequences were downloaded from the NCBI database.

# Statistical analysis

Statistical analysis was performed using factorial analysis ANOVA with significant $P < 0.05$. Criteria of Romanovsky, Dixon and high-low lines were used for identification and rejection of outliers. Data shown are means ± SD from 3–5 independent experiments.

# Results

### Cloning of the SaSLAH3 coding sequence

At first, an *in silico* search for *AtSLAC/AtSLAH* gene family orthologs in two previously assembled transcriptomes of *Suaeda fruticosa* (L.) Forssk and *Suaeda glauca* (Bunge) that are closely related to *S. altissima* (Nedelyaeva et al. 2018; Shuvalov et al. 2021) has been carried out. For this purpose, the contigs of the assembled transcriptomes were translated into amino acids sequences and, in the obtained arrays, the search of the sequences related to the family SLAC1/SLAH proteins was accomplished. AtSLAC1, AtSLAH1, AtSLAH2, AtSLAH3, and AtSLAH4 proteins were used as queries. By doing so, in both *S. fruticosa* and *S. glauca* transcriptomes, contigs that included full-length sequences homologous to the *AtSLAH3* gene were found. The *AtSLAH3* homologs found in each of the two *Suaeda* species were named *SfSLAH3* and *SgSLAH3* based on their similarity to the *A. thaliana* gene. In addition, the *SfSLAH3* and *SgSLAH3* genes were found to be at high degree of identity between one another (Table S1). To amplify the entire *S. altissima* putative *SLAH3* ORF, which we named *SaSLAH3*, a primer pair, SaSLAH3_inf_F2 and SaSLAH3_inf_R2 (Table S1, Fig. S1), bearing the 5' and 3' flanking end sequences of the *SfSLAH3* CDS, respectfully, was designed. This choice of primer design was based on an assumption that hypothetical *S. altissima SLAH3* gene should be also closely related to the two genus *Suaeda* ortholog genes *SfSLAH3* and *SgSLAH3*, which are highly identical between themselves. These primers contained 5'-overhang tails for Gibson assembly cloning into pVR2ADRHis vector (Gerasimov et. al. 2012). With these primers, as expected, the 1827 bp amplicon was produced in a PCR using first strand cDNA from *S. altissima* as a template. This fragment was cloned into pVR2ADRHis vector yielding recombinant construct pVR2-SaSLAH3 that was then sequenced. The sequencing revealed that the cloned insert contained *SaSLAH3* ORF encoding a putative polypeptide consisting of 608 amino acids, with predicted molecular mass of 68.5 kDa and isoelectric point pI = 9.29. The length of the predicted SaSLAH3 protein was equal to the SgSLAH3 ortholog, but shorter than that of AtSLAH3 (641 a. a.).

### In silico characterization of SaSLAH3 protein

Multiple sequence alignment (MSA) showed a high degree of identity of the *SaSLAH3* CDS with the ortholog sequences from close related species *S. glauca* (92%) and *S. fruticosa* (96%). In sequence alignment of SaSLAH3 with *A. thaliana* ortholog AtSLAH3, however, only 71% identity was observed (Table S2). Predicted amino acid sequence of the SaSLAH3 protein was also aligned with those of the SLAH3 proteins from *S. glauca* and *S. fruticosa* as well as with the AtSLAH3 sequence from *A. thaliana.* Identity levels of the SaSLAH3 protein sequence with SgSLAH3 and SfSLAH3 were found to be 94% and 97%, respectively, while only 55% identity with AtSLAH3 amino acid sequence was seen (Fig. 1; Table S3). In addition, we found 60% identity between the SaSLAH3 and AtSLAH2 channels, and only 45% identity of the former protein with AtSLAC1, which probably reflects the closer relationship of SaSLAH3 to SLAH2 rather than to SLAC1 channels.

All channels of the SLAC/SLAH family are present in the cells in form of trimers. Each monomer of the trimer consists of ten transmembrane domains that form anion conducting path and contain five key conserved motifs, as well as conserved phenylalanine residues. The latter face to the pore center and participate in gating. These conserved phenylalanine-bearing motifs were shown to be a hallmark of all SLAC-like proteins (Dreyer et al. 2012). All these five main conserved motifs and phenylalanine residues we found in the sequence of the SaSLAH3 protein as well as in the other three channels SfSLAH3, SgSLAH3, and AtSLAH3 (Fig. 1).

Topology studies of the SaSLAH3 protein using either Phobius or Phyre 2 software have generated identical domain structure models. According to these models, SaSLAH3 as well as SgSLAH3, SfSLAH3, and AtSLAH3 homologs contained ten transmembrane domains with both N- and C-terminal sequences located in the cytoplasm (Fig. 2B). These results reveal principal similarity of our SaSLAH3 model with SLAC-like channel structures generated for proteins belonging to the same family earlier by the others (Dreyer et al. 2012). In addition, the modeling with Phyre 2 showed that SaSLAH3 protein contained 58% alpha-helices, 38% of which were transmembrane.

A 3D modelling performed by Swiss-Model software showed that three SaSLAH3 channel module subunits are assembled into a trimeric structure (Fig. 3). These results are in accordance with the SLAC-like anion channel structural model proposed earlier by Dreyer with coworkers (2012). In addition, the Phe-489 in motif 5 that corresponds to the Phe-450 of the *A. thaliana* AtSLAC1, presumably involved into gating function (Dreyer et al. 2012), is localized in this anion-conducting pathway (Fig. 3B).

To assess the structural conservativity of SaSLAH3 among SLAH3 proteins, we carried out MEME analysis and compared generated motif patterns of SaSLAH3 with those of SLAH3 channels from 12 other plant species (Fig. 4). From 11 to 15 common motifs were found in each of analyzed individual protein. Ten out of 13 investigated channels carried all 15 sequence motifs similarly located on amino acid sequences with the most conserved 9–7–2–5–12 motif location order for all plant species SLAH3 family members. Four motifs (5, 10, 13 and 15) were absent in 15% of proteins while location of three

others (1, 9 and 10) was variable. It is worth noting that the main, most conserved motif order 9–7–2–5–12 was common between glycophytes and halophytes.

Thus, all the bioinformatic analysis results outlined above strongly indicate that the SaSLAH3 protein belongs to the slow anion channels SLAC/SLAH family.

To find possible divergencies in SLAC/SLAH proteins molecular evolution, we carried out a phylogenetic analysis using MEGA X software (Fig. 5). All SLAC-type anion channels in the generated tree fall into three groups: SLAC1, SLAH2/3 and SLAH1/4. These results matched group SLAC/SLAH family structure obtained earlier by the others (Dreyer et al. 2012). The identity levels of channels within groups were equal to 61% for the SLAH2/3, 64% for the SLAC1, and 59% for the SLAH1/4 group, respectively. On the other hand, inter-group identity was 34% for SLAC1 and SLAH1/4, 52% for SLAC1 and SLAH2/3, and 37% for the SLAH2/3 and SLAH1/4 groups. Based on our results, we suggest that the majority of SLAC1 channels have a greater evolutionary similarity with the SLAH2/3 rather than with SLAH1/4 group of proteins.

To elucidate possible involvement of SaSLAH3 in *S. altissima* response to salinity and nitrate deficiency we studied plant growth, water content and the *SaSLAH3* gene expression in organs of the plants grown under various salinity and nitrate availability.

### Plant growth characteristics and water content in roots and leaves under diverse salinity and nitrate availability

Root and leaf fresh weight (FW) determination demonstrated that growth optimum of *S. altissima* plants lies at NaCl concentration of 250 mM under both sufficient nitrate supply conditions (15 mM) and nitrate deficiency (0.75 mM) (Fig. 6). At NaCl concentration of 750 mM as well as in NS lacking NaCl, FW of both organs was reduced when compared to that of plants grown at 250 mM NaCl, although, in case of nitrate deficiency, the difference between leaf FW at 0 mM NaCl and 250 mM NaCl was statistically insignificant. Under nitrate deficiency, FW values of both organs were far lower than those in plants grown in NS containing 15 mM NO3 – (Fig. 6).

We also determined water content in S. altissima roots and leaves when plants were grown at various NaCl concentrations and different levels of nitrate availability. At sufficient nitrate supply, water content was maintained at a level approximately of 92–93% in leaves in full concentration range of NaCl from 0 mM to 750 mM and in roots at NaCl concentrations up to 250 mM (Fig. 7). Nitrate deficiency had little effect on leaf water content both in the absence of NaCl and when the nutrient solution was salinized, but it increased significantly water loss in the roots under salinity conditions, to water content values below 80% (Fig. 7).

The results obtained in this series of the experiments as well as in our earlier studies which showed monotonic $Na^+$ and $Cl^-$ accumulation in tissues of *S. altissima* with an increasing external NaCl concentration (Balnokin et al 2004; 2005; Tsydendambaev et al. 2013; Nedelyaeva et al. 2019), indicate

that *S. altissima* belongs to a category of salt-acumulating halophytes (euhalophytes), with a maximal growth rate at 250 mM NaCl in NS. The plants of this species retained the ability of growing even at NaCl concentration of 750 mM and were capable of maintaining water content in leaves at NaCl concentrations exciding considerably growth maximum. Under salinity conditions combined with nitrate deficiency, *S. altissima* growth got retarded and water balance in roots disturbed (Fig. 6, 7).

### Analysis of the SaSLAH3 gene expression under various salinity and nitrate availability

To gain an additional insight into physiological role of SaSLAH3 protein, we investigated the expression of the gene encoding this protein in roots and leaves of *S. altissima* plants grown at various NaCl concentrations in the NS either at sufficient supply of nitrate (15mM) or at nitrate deficiency (0.75 mM) (Fig. 8) conditions. In both organs, expression levels of the gene increased strongly with NaCl concentration elevation in the growth medium. We also found that expression levels in the roots grew more than in the leaves with growth of NaCl concentration. The nitrate deficiency also stimulated the *SaSLAH3* gene expression in the organs. The stimulation was most noticeable at 750 mM NaCl in the medium. The results obtained may indicate an important role of the SaSLAH3 protein in *S. altissima* response to salt stress and its involvement in the processes underlying salt tolerance, as well as in nitrogen nutrition under salinity.

## Discussion

Coding sequence of *SaSLAH3*, a SLAC/SLAH family gene from *S. altissima* was cloned based on the high level of identity of this gene with SgSLAH3 and SfSLAH3, the genes of close related species *S. glauca* and *S. fruticosa*, and amino acid sequence of encoded protein was analyzed. The presence of five key conserved phenylalanine-bearing motifs (Fig. 1), membrane topology (Fig. 2), and 3-D structure (Fig. 3), MEME-generated sequence motif patterns (Fig. 4), collectively being typical for SLAC/SLAH validate belonging of SaSLAH3 to this family. Phylogenetic relationship analysis confirmed the evolutionary divergence of SLAC/SLAH proteins into three distinct groups characteristic of this family (Dreyer et al. 2012), with SaSLAH3 appearing in the SLAH2/3 group (Fig. 5). Organ fresh weight and water content determined at diverse salinity in NS (Fig. 6, 7) as well as ionic relations of *S. altissima* studied earlier (Balnokin et al. 2004; 2005; Nedelyaeva et al. 2019) indicate that this species is a high salt tolerant plant belonging to the group of the euhalophytes. In hydroponics conditions, *S. altissima* was able to complete the life cycle at NaCl concentration in NS up to 1 M, but was capable of doing that in the absence of NaCl as well (Balnokin et al. 2005). Accumulation of $Cl^-$ and $Na^+$ in plant organs grew upon increasing NaCl concentration in the medium (Balnokin et al. 2004; Nedelyaeva et al. 2019). Plant survival and growth on saline soils depends on ability of root anion-transporting system to absorb $NO_3^-$ from environment high in $Cl^-$, translocate absorbed nitrate to shoots through the xylem, and maintain concentrations of toxic $Cl^-$ and $Na^+$ ions in cytosol at low levels. For the translocation to shoot, $NO_3^-$ and $Cl^-$ ions have to be released from the root xylem parenchyma cells into the xylem vessels. The SLAC/SLAH family proteins are involved in this process and participate also in the regulation of

cytoplasmic $Cl^-$ and $NO_3^-$ homeostasis in diverse tissues (for the review see Hedrich and Geiger, 2017). AtSLAH3, the ortholog of SaSLAH3, has been shown to be localized in pericycle and xylem parenchyma cells and is permeable for both $NO_3^-$ and $Cl^-$ (Cubero-Font et al. 2013; Hedrich and Geiger, 2017; Lehmann et al. 2021). Upon heteromerization with S-type anion channel subunit AtSLAH1, AtSLAH3 gets more permeable for $Cl^-$ than for $NO_3^-$ and as a result chloride root-to-shoot translocation increases. Both salinity and ABA treatment were shown to lower transcript levels of both of these genes (Hedrich and Geiger, 2017). The authors suggested that the *AtSLAH3* and *AtSLAH1* expression lowering was required for preventing excess of chloride from entering the shoots; highly selective for $NO_3^-$ AtSLAH2 ensured nitrate translocation to shoot under these conditions (Hedrich and Geiger, 2017). *SaSLAH3* expression level, in contrast to that of *AtSLAH3*, was increased under salinity conditions. The increase was observed in both roots and leaves, either at sufficient supply with nitrate (Fig. 8) or at its deficiency (Fig. 8), which is in line with the ability of euhalophytes and, in particular, *S. altissima*, to accumulate $Cl^-$ ions in organs. Two organ-specific *AtSLAH1* homologs: *SaSLAH1.1*, expressed in leaves, and *SaSLAH1.2*, expressed in roots, were identified in *S. altissima* earlier (Shuvalov et al. 2019). In contrast to *SaSLAH3*, expression of the root-specific *SaSLAH1.2* dramatically decreased with increasing salinity at both nitrate concentrations used in the NS. Expression of leaf-specific *SaSLAH1.1* was also lowered as salinity increased, however, only at sufficient nitrate availability. Under nitrate deficiency conditions, it increased (Shuvalov et al. 2019). Based on the low expression level of *SaSLAH1.2* under salinity conditions, one can be suggested that in *S. altissima* root cells, a NaCl-induced elevation of SaSLAH3 $Cl^-$-permeability based on the SaSLAH3 and SaSLAH1.2 heteromerization is hardly likely. The heteromerization should rather be expected in leaves under conditions of nitrate deficiency. It is worth noting that, at low levels of *SLAH1* expression, NaCl-induced elevation of SLAH3 chloride conductance may occur via protein kinase-mediated phosphorylation of SLAH3 N-terminus in the presence of nitrate in growth medium (Geiger et al. 2011; Maierhofer et al. 2014b). It is conceivable that the modulation of *SLAH3* and *SLAH1* expression in response to salt stress is a salt tolerance mechanism that operates through the regulation of $Cl^-$ loading into xylem. However, there is no clear evidence to support this assumption. In almond (*Prunus dulcis*) rootstocks, transcript levels of *SLAH3* and *SLAH1* homologs of 14 cultivars differing in salt tolerance were studied after long-term watering with tap water either containing or lacking NaCl (Sandhu et al. 2020). Three of four of the most salt tolerant almond varieties demonstrated at least two-fold increasing in transcript levels of both *SLAH3* and *SLAH1* orthologs in response to NaCl treatment. In addition, the less salt-tolerant cultivars showed a general trend towards low levels of *SLAH3* and *SLAH1* transcripts both in the control and after NaCl treatment. However, in the experiments conducted on pepper, tomato, and eggplant varieties differing in salt tolerance, no correlation of NaCl-stimulated *SLAH3* expression in roots with salt tolerance of these varieties nor with increments of $Cl^-$ content in leaves was observed (Suarez et. al. 2021). To evaluate the contribution of NaCl-induced SLAH3 and SLAH1 expression modulations in salt tolerance, it is necessary to take into consideration whether the plant uses strategy of salt-accumulation or salt-exclusion in overcoming salt stress. For this purpose, plants contrasting in both salt tolerance and patterns of chloride distribution between organs are required to be investigated. It is possible that SaSLAH3 is involved, along with the loading anions into

xylem, in the regulation of $NO_3^-$ and $Cl^-$ content in cells of diverse tissues and in ensuring a proper $[NO_3^-]/[Cl^-]$ ratio in cytoplasm. Such regulation may play a particularly important role in halophyte cells, where nitrate is bound by transporters and enzymes being in stiff competition with chloride. Future studies are needed to address the precise roles that SaSLAH3 and its orthologs from other halophyte plant species may play in salt tolerance and nitrogen nutrition under salinity conditions.

# Conclusions

Coding sequence of *SaSLAH3*, a gene of the slow anion channel family (*SLAC/SLAH*) was cloned from the euhalophyte *S. altissima* and sequenced. The bioinformatic analysis of the encoded protein validated the belonging SaSLAH3 to this family. The increasing of *SaSLAH3* expression level in roots and leaves upon salinization at both sufficient nitrate supply and its deficiency may indicate the involvement of SaSLAH3 in mechanisms underlying salt tolerance. In future, the *SaSLAH* gene may be used in plant genetic modification to increase salinity tolerance via nitrate assimilation improvement under high salinity conditions.

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# Figures

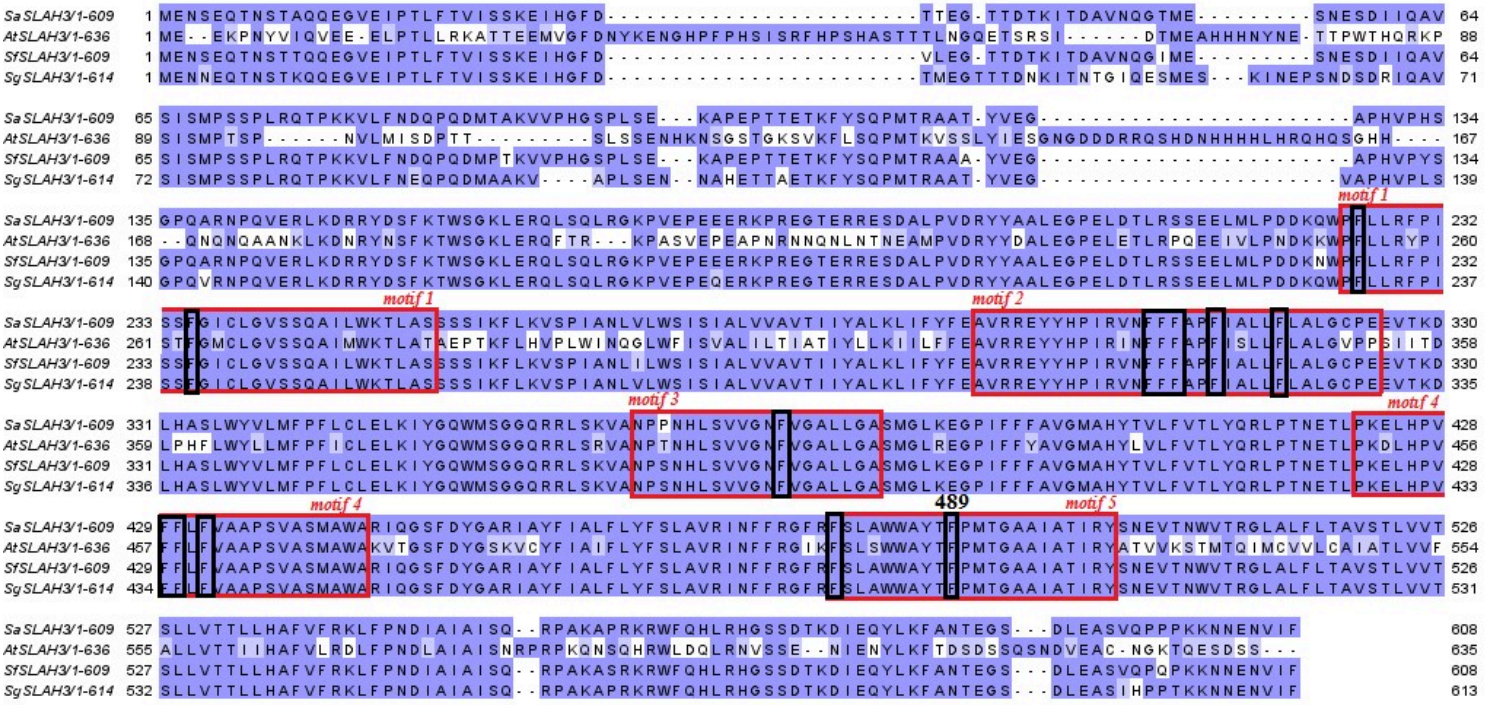
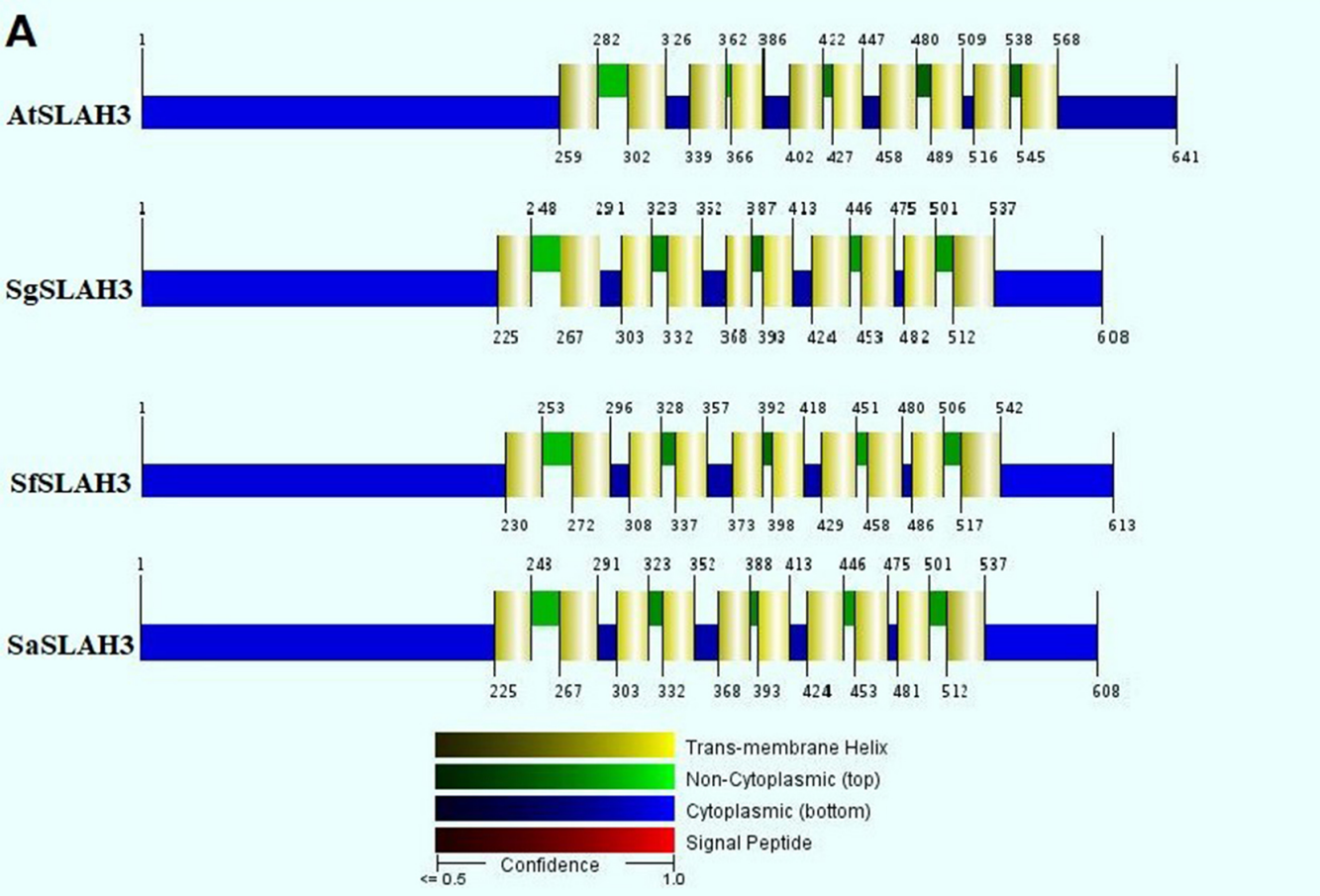
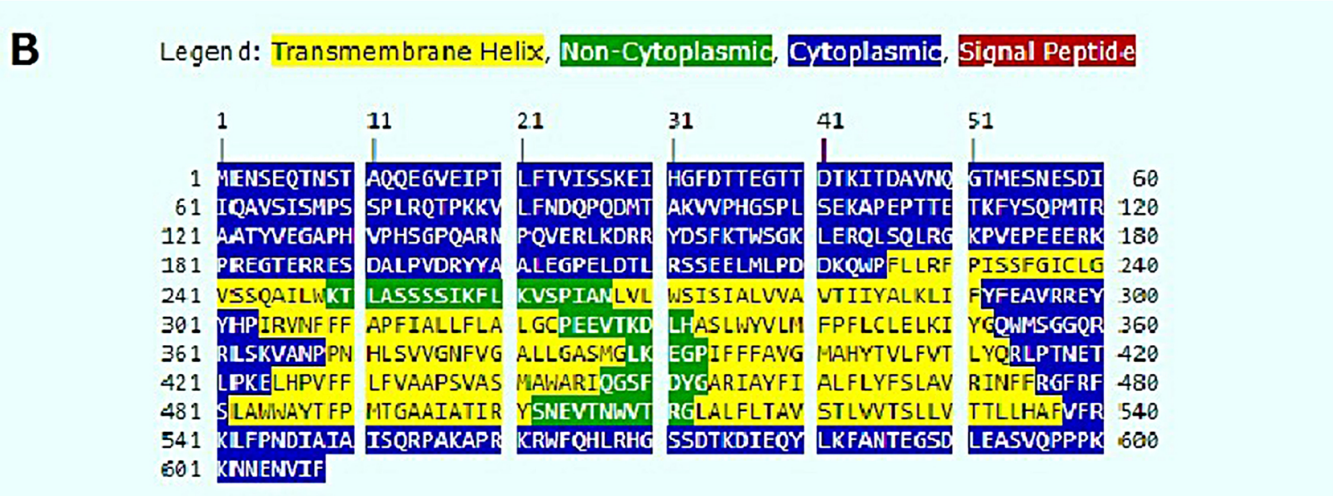



**Figure 1**

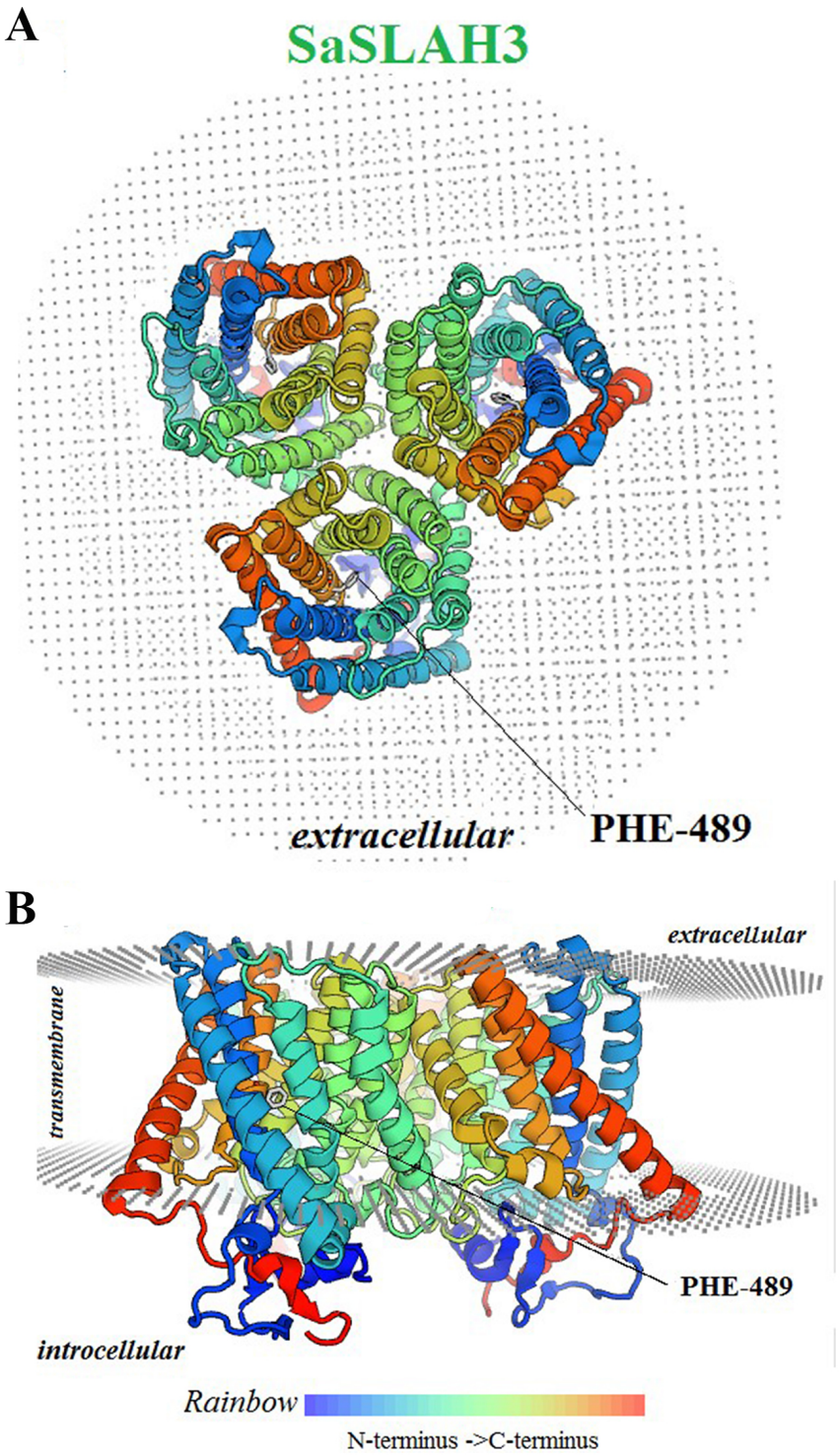
Multiple alignment of amino acid sequences of the SLAH3 proteins from *Suaeda altissima* (OQ271227), *Arabidopsis thaliana* (OAO96380.1), *Suaeda fruticosa* and *Suaeda glauca* (Nedelyaeva et al. 2018; Shuvalov et al. 2021). The alignment was generated and visualized in Jalview 2.11.1.4 software. The conserved motifs of SLAC-like channels are boxed by red and phenylalanine residues which may play a role in gating are boxed by black.



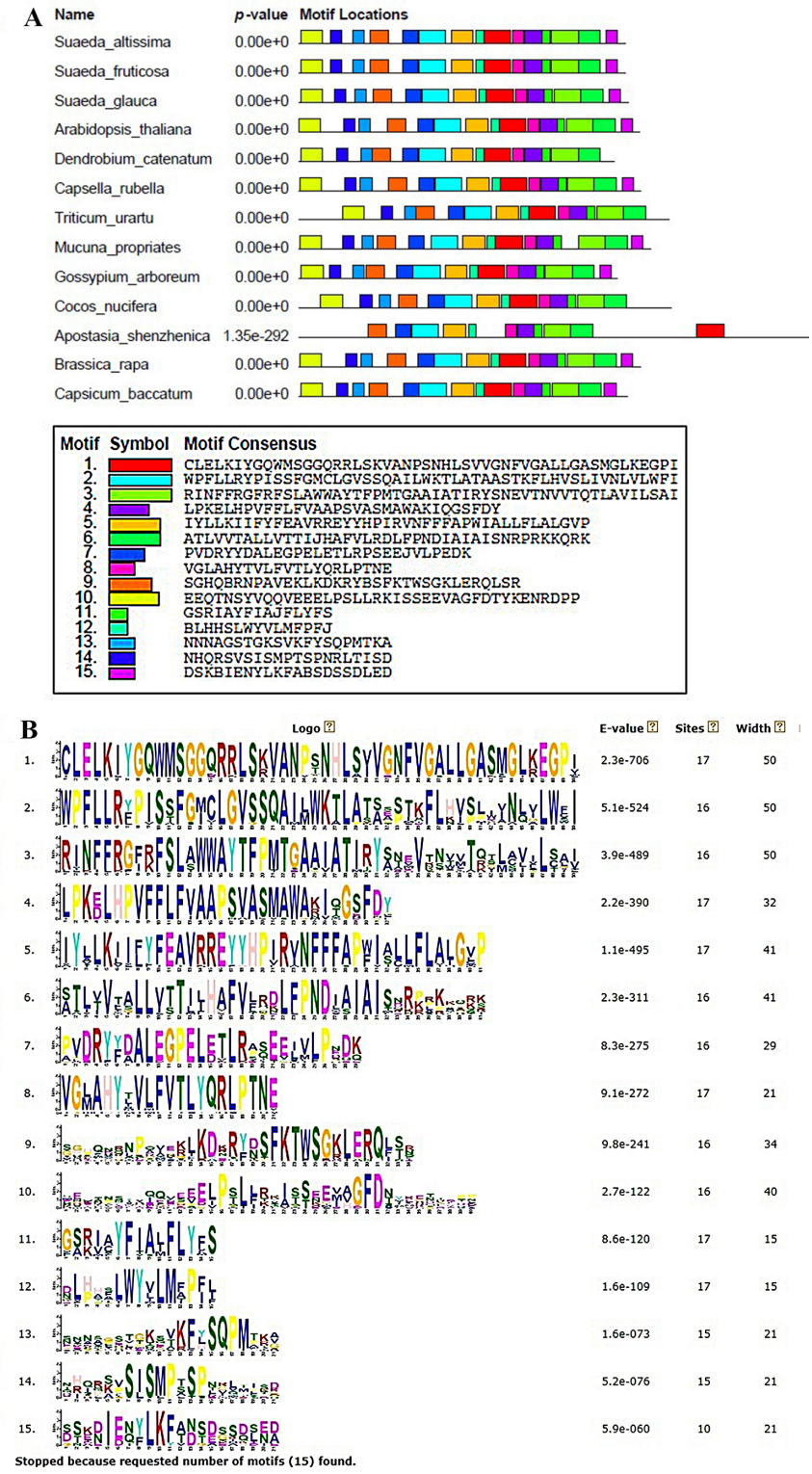

## Figure 2

Secondary structure of AtSLAH3 (1), SgSLAH3 (2), SfSLAH3 (3), and SaSLAH3 (4) proteins. (A) Topology of the SLAH3 homologs predicted by Philius and Phyre 2 software (visualized by Philius). (B) Primary structure of the SaSLAH3 protein with highlighted predicted localizations of particular sequences.




## Figure 3

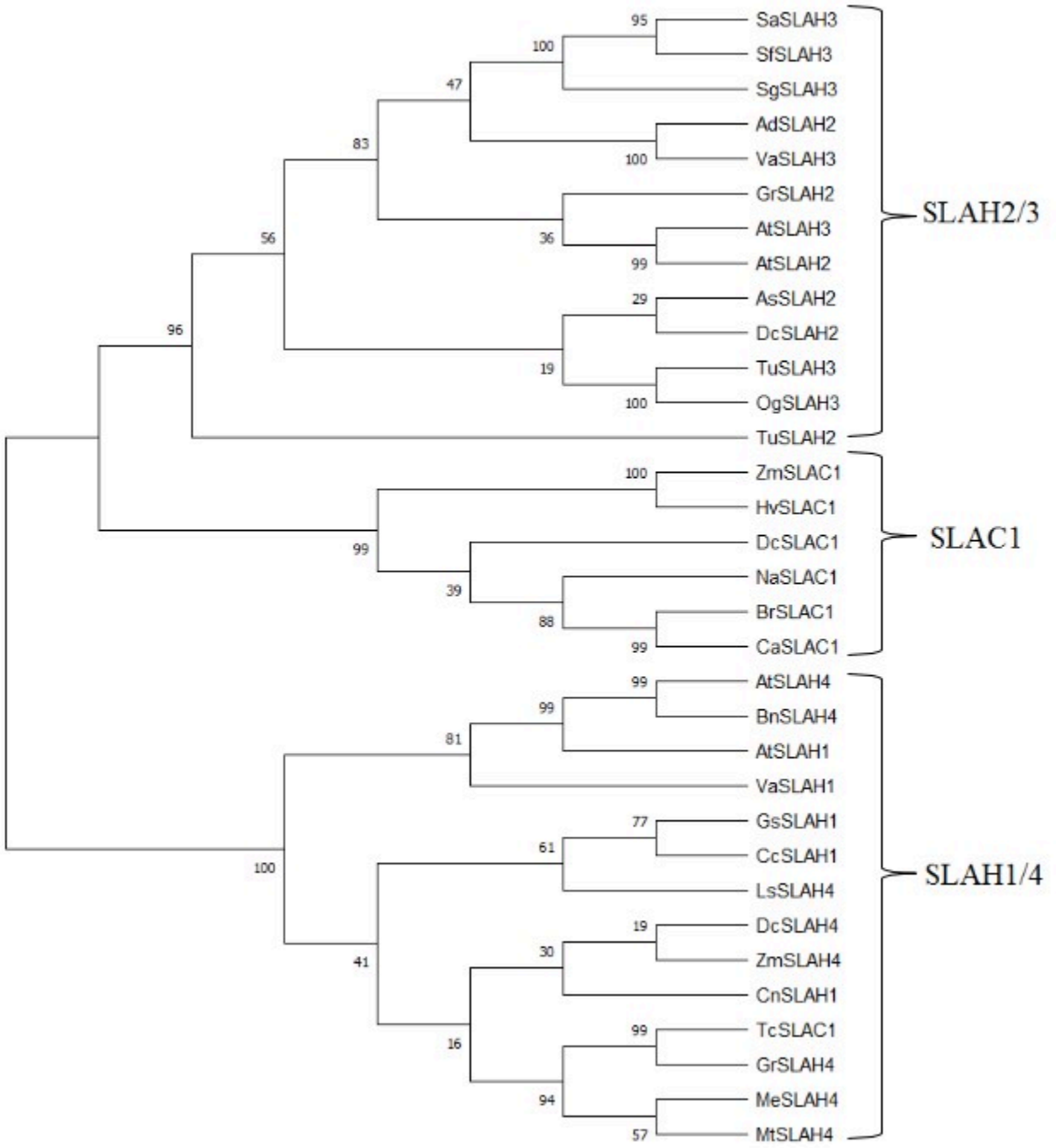
3D structural model of the SaSLAH3 protein predicted by Swiss-Model software. (A) The model seen from the extracellular side, with the side chains containing conserved Phe-489 involved presumable in gating. (B) The same as in (A), but shown as a side view. For simplicity, the key Phe-489 on only one out of three protein subunits is indicated.




## Figure 4

Plant SLAH3 protein motifs identified by MEME software. Parameters used for motif discovery: minimum width = 21, maximum width = 50, maximum number of motifs to find = 15. (A) Location of the identified SLAH3 motifs from the following plant species: *Suaeda altissima* (OQ271227), *Suaeda fruticosa*, *Suaeda glauca* (Nedelyaeva et al. 2018; Shuvalov et al. 2021), *Arabidopsis thaliana* (OAO96380.1), *Dendrobium catenatum* (PKU74009.1), *Apostasia shenzhenica* (PKA63678.1), *Gossypium*

*arboreum* (KHG12461.1), *Capsella rubella* (XP_006287257.1), *Triticum urartu* (EMS57353.1), *Mucuna propriates* (RDX71850.1), *Cocos nucifera* (KAG1335129.1), *Brassica rapa* (XP_009150932.1), *Capsicum baccatum* (PHT53212.1). (B) Frequency logos for identified motifs.




## Figure 5

Phylogenetic analysis of following 33 SLAC/SLAH family plant proteins: SLAC1 from *Zea mays* (PWZ36521.1), *Hordeum vulgare subsp vulgare* (AWG47875.1), *Dendrobium catenatum* (LOC110101857), *Nicotiana attenuata* (XP_019252311.1), *Brassica rapa* (XP_009148588.1), *Cucurbita argyrosperma* (KAG7028351.1), Theobroma cacao (EOY14904.1); SLAH1 from *Arabidopsis ofthaliana* (OAP13763.1), *Vigna angularis* (XP_017429404.1), *Glycine soja* (XP_028238287.1), *Citrus clementina* (LOC18051007), *Cocos nucifera* (KAG1347056.1); SLAH2 from *Arachis duranensis* (XP_015967327.1),

*Gossypium raimondii* (XP_012488569.1), *Arabidopsis thaliana* (OAO98701.1), *Apostasia shenzhenica* (PKA54459.1), *Dendrobium catenatum* (PKU75117.1), *Triticum urartu* (EMS54125.1); SLAH3 from *Suaeda altissima* (OQ271227), *Suaeda fruticosa*, *Suaeda glauca* (Nedelyaeva et al. 2018; Shuvalov et al. 2021), *Vigna angularis* (XP_017439103.1), *Arabidopsis thaliana* (OAO96380.1), *Triticum urartu* (EMS57353.1), *Oryza glaberrima* (XP_052141455.1); SLAH4 from *Arabidopsis thaliana* (A8MRV9.1), *Brassica napus* (XP_013714156.1), *Lactuca sativa* (XP_023745195.2), *Dendrobium catenatum* (PKU83271.1), *Zea mays* (PWZ32281.1), *Gossypium raimondii* (XP_012466162.1), *Manihot esculenta* (XP_021612946.1), *Medicago truncatula* (XP_013462190.2). All protein sequences were downloaded from the NCBI database. The phylogenetic tree was built in the MEGA X using the maximum likelihood method based on the Jones–Taylor–Thornton model, 1000 bootstrap cycles (Jones et. al. 1992).The tree could be divided into three subgroups: SLAH2/3. SLAC1 and SLAH1/4.

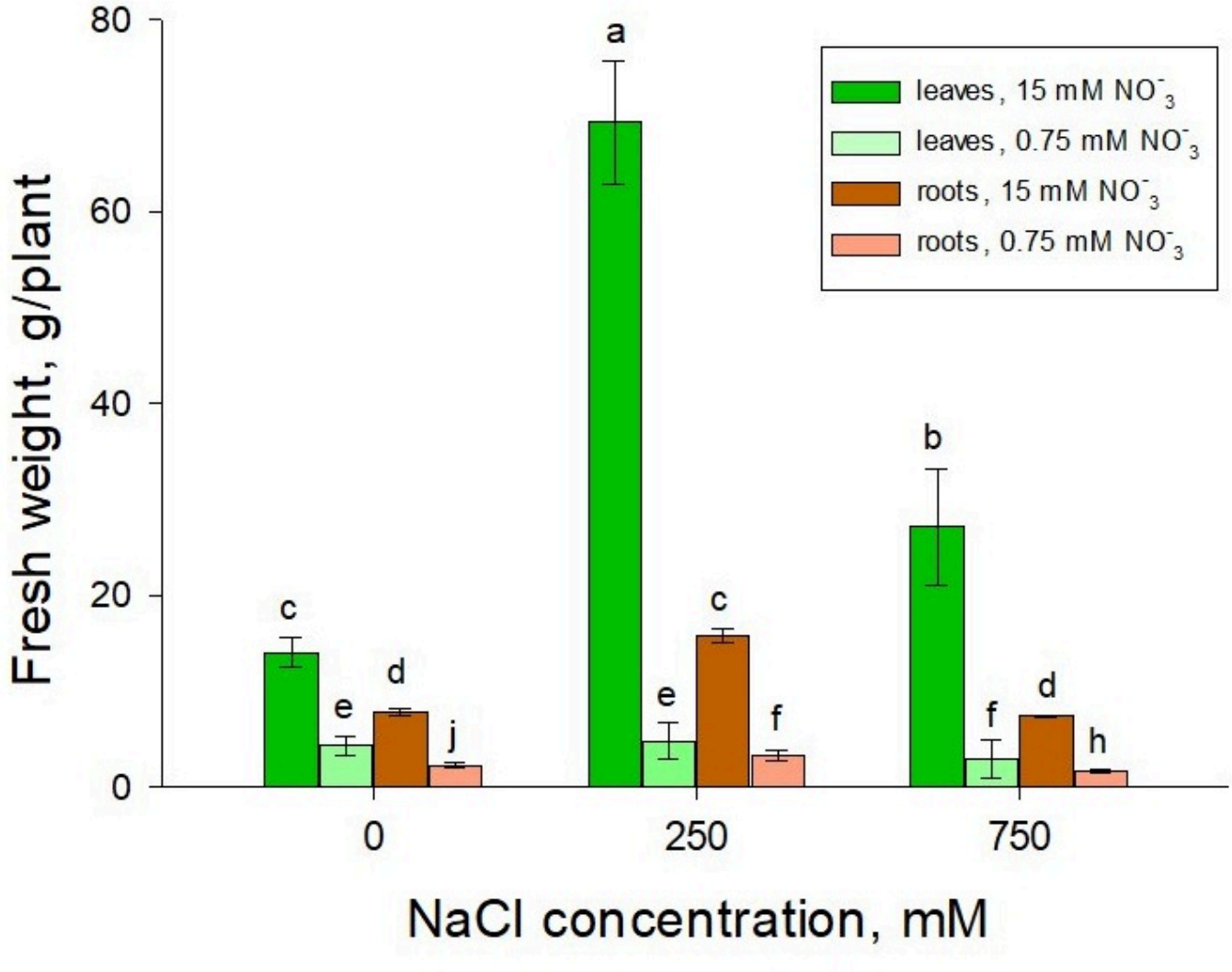



**Figure 6**

Root and leaf fresh weights of *S. altissima*plants grown in NS containing NaCl in the final concentrations from 0 to 750 mM at sufficient nitrate supply (15 mM) or nitrate deficiency (0.75 mM). Means ± SD are

given (n = 3 – 5, average weight of 20 plants in each repetition). Different letters indicate statistically significant differences (ANOVA; p< 0.05).

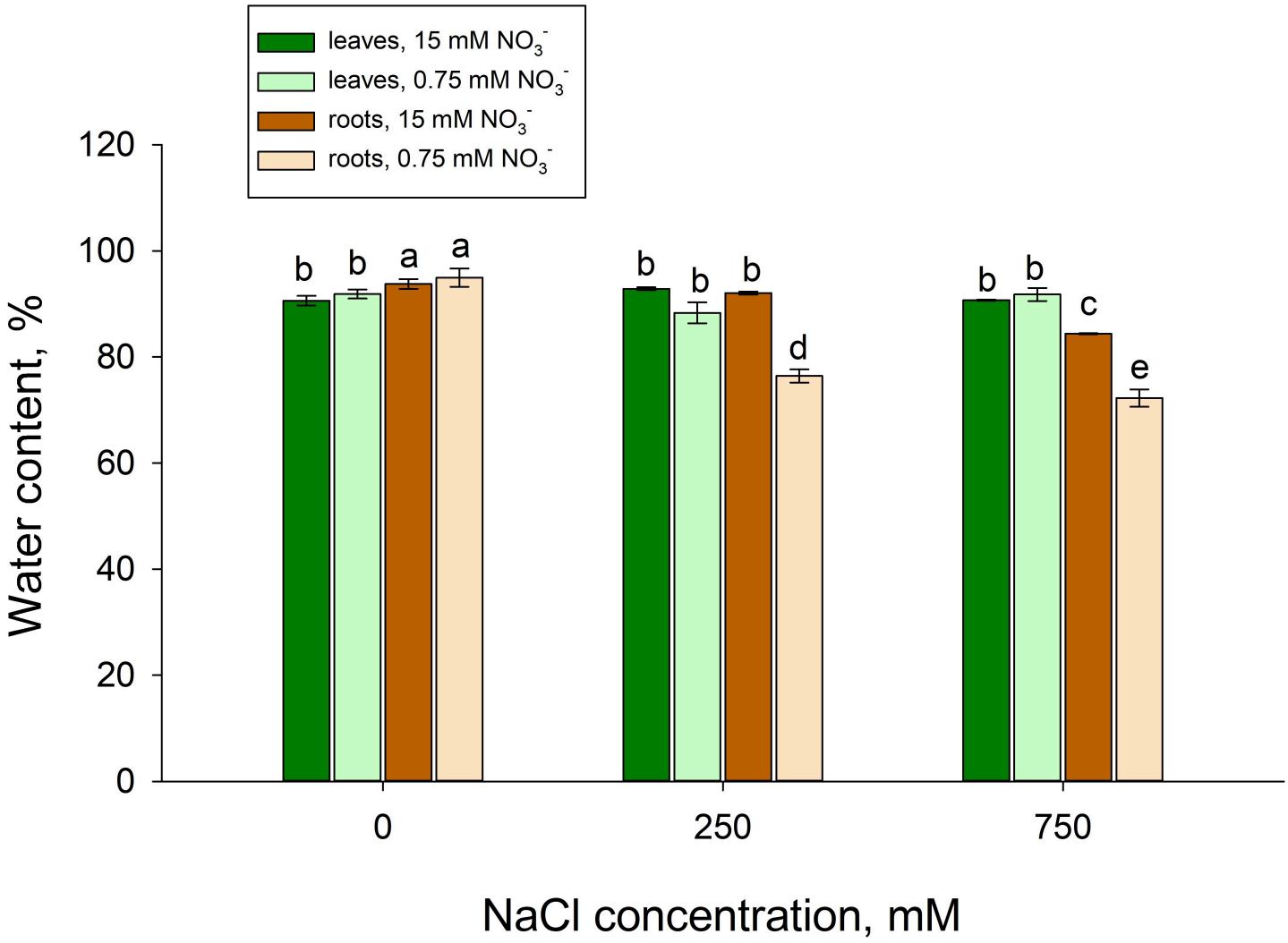



**Figure 7**

Water content in roots and leaves of *S. altissima* plants grown in NS containing NaCl in the final concentrations from 0 to 750 mM at sufficient nitrate supply (15 mM) or nitrate deficiency (0.75 mM). Means ± SD are given (n = 3 – 5, average water content of 20 plants in each repetition). Different letters indicate statistically significant differences (ANOVA; p< 0.05).

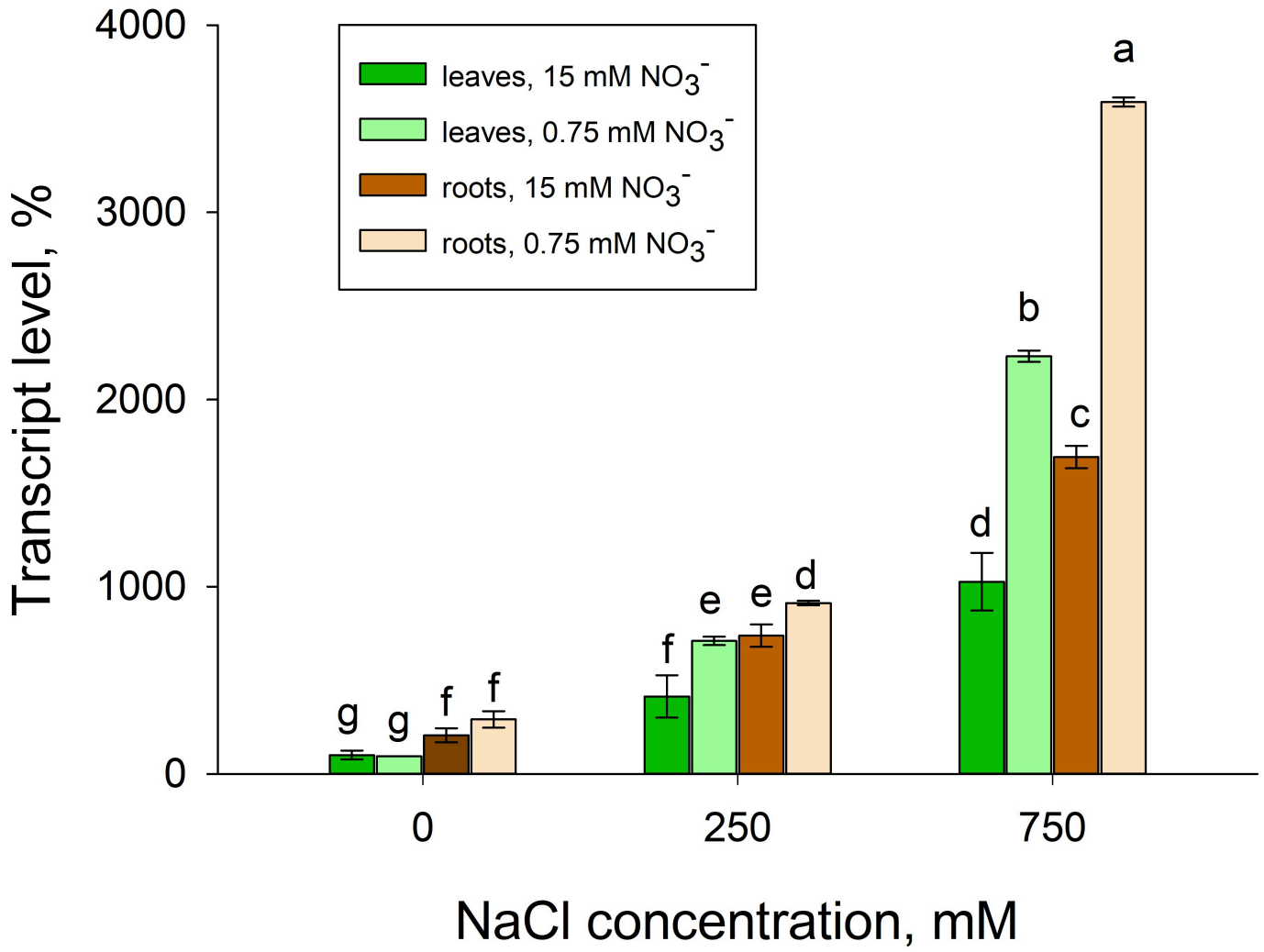



**Figure 8**

Expression of the *SaSLAH3* gene in roots and leaves of *S. altissima* plants grown in NS containing NaCl in the final concentrations from 0 to 750 mM at sufficient nitrate supply (15 mM) or nitrate deficiency 0.75 mM). The expression level of *SaSLAH3* in leaves under control conditions (without NaCl in NS) is taken as 100%. Means ± SD are given (n = 3 – 5). Different letters indicate statistically significant differences (ANOVA; p< 0.05).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytables.docx
- SLAH3geneandorthologsfromSuaedaspieces.docx