Molecular Cloning and Characterization of SaCLCd, SaCLCf, and SaCLCg, Novel Proteins of the Chloride Channel Family (CLC) from the Halophyte Suaeda altissima (L.) Pall

Olga I. Nedelyaeva *, Larissa G. Popova, Vadim S. Volkov * and Yurii V. Balnokin

K.A. Timiryazev Institute of Plant Physiology RAS, 127276 Moscow, Russia; lora_gp@mail.ru (L.G.P.); balnokin@mail.ru (Y.V.B.)
* Correspondence: olga.nedelyaeva@yandex.ru (O.I.N.); vadim.s.volkov@gmail.com (V.S.V.)

Abstract: Coding sequences of the CLC family genes SaCLCd, SaCLCf, and SaCLCg, the putative orthologs of Arabidopsis thaliana AtCLCd, AtCLCf, and AtCLCg genes, were cloned from the euhalophyte Suaeda altissima (L.) Pall. The key conserved motifs and glutamates inherent in proteins of the CLC family were identified in SaCLCd, SaCLCf, and SaCLCg amino acid sequences. SaCLCd and SaCLCg were characterized by higher homology to eukaryotic (human) CLCs, while SaCLCf was closer to prokaryotic CLCs. Ion specificities of the SaCLC proteins were studied in complementation assays by heterologous expression of the SaCLC genes in the Saccharomyces cerevisiae GEF1 disrupted strain Δgef1. GEF1 encoded the only CLC family protein, the Cl\(^-\) transporter Gpf1p, in undisrupted strains of this organism. Expression of SaCLCd in Δgef1 cells restored their ability to grow on selective media. The complementation test and the presence of both the “gating” and “proton” conservative glutamates in SaCLCd amino acid sequence and serine specific for Cl\(^-\) in its selectivity filter suggest that this protein operates as a Cl\(^-\)/H\(^+\) antiporter. By contrast, expression of SaCLCf and SaCLCg did not complement the growth defect phenotype of Δgef1 cells. The selectivity filters of SaCLCf and SaCLCg also contained serine. However, SaCLCf included only the “gating” glutamate, while SaCLCg contained the “proton” glutamate, suggesting that SaCLCf and SaCLCg proteins act as Cl\(^-\) channels. The SaCLCd, SaCLCf, and SaCLCg genes were shown to be expressed in the roots and leaves of S. altissima. In response to addition of NaCl to the growth medium, the relative transcript abundances of all three genes of S. altissima increased in the leaves but did not change significantly in the roots. The increase in expression of SaCLCd, SaCLCf, and SaCLCg in the leaves in response to increasing salinity was in line with Cl\(^-\) accumulation in the leaf cells, indicating the possible participation of SaCLCd, SaCLCf, and SaCLCg proteins in Cl\(^-\) sequestration in cell organelles. Generally, these results suggest the involvement of SaCLC proteins in the response of S. altissima plants to increasing salinity and possible participation in mechanisms underlying salt tolerance.

Keywords: Suaeda altissima; anion transporters; chloride channel family; CLC family; halophytes; molecular cloning; salt tolerance; SaCLCd; SaCLCf; SaCLCg

1. Introduction

Soil salinization is a significant problem in agriculture. Salt-affected soils occupy more than 6% of the earth’s land surface (800 million hectares) and, according to various estimates, 20–50% of irrigated land [1–3]. The annual losses from salinization in the world currently exceed US $27 billion [4]. The decrease in yield caused by salinity is due to the fact that the vast majority of agricultural crops are salt-sensitive plants, also known as glycophytes [5–8]. NaCl in soil results in disturbances in plant–water relations and causes Na\(^+\) and Cl\(^-\) accumulation up to toxic levels in the cytoplasm [7,9–11].

Halophytes are plants of saline habitats that have evolved mechanisms to adequately regulate Na\(^+\) and Cl\(^-\) concentrations in cytoplasm and acquire nutrients, in particular...
While the functions and physiological roles of their products have been extensively investigated, the halophyte orthologs of CLC family proteins remain barely studied. In this study, the anion selectivity of SaCLCd, SaCLCf, and SaCLCg was examined in complementation assays by heterologous expression of their genes in the euhalophyte Suaeda altissima, the putative orthologs of A. thaliana. Seven genes of the CLC family have been cloned from A. thaliana, namely AtCLCa–e. While the functions and physiological roles of their products have been extensively investigated, the halophyte orthologs of CLC family proteins remain barely studied. The molecular cloning and functional characterization of proteins from halophytes are important for elucidating the mechanisms underlying plant salt tolerance and improving crop resistance to soil salinity by genetic manipulations.

Recently, we cloned SaCLCa1 and SaCLCc1, the putative orthologs of AtCLCa and AtCLCc encoding NO\textsuperscript{−}3/H\textsuperscript{+} and Cl\textsuperscript{−}/H\textsuperscript{+} antiporters of A. thaliana, respectively, from the euhalophyte Suaeda altissima [51,52]. In the present work, we describe the cloning of other genes of the CLC family from S. altissima, namely SaCLCd, SaCL Cf, and SaCLCg, the putative orthologs of AtCLCd, AtCL Cf, and AtCLCg, and investigate anion selectivity of the encoded proteins. The anion selectivity of SaCLCd, SaCL Cf, and SaCLCg was examined in complementation assays by heterologous expression of their genes in the Saccharomyces cerevisiae GEF1 disruption mutant Gef1\textsuperscript{-}. GEF1 is the only gene from the CLC family in S. cerevisiae, and the protein Gef1p is characterized by Cl\textsuperscript{−} specificity [53]. Relative SaCLCd, SaCL Cf, and SaCLCg transcript levels as well as Cl\textsuperscript{−} content in organs and their biomass were also measured for S. altissima plants grown at various NaCl concentrations.
2. Materials and Methods

2.1. Plant Material

Seeds of *S. altissima* (L.) Pall. were collected from plants growing in the wild on the shores of Lake Elton, a salt lake located in Russia (Volgograd region). The seeds were germinated in wet sand at 21–23 °C. After three weeks, the seedlings were transplanted into a 3 L glass container (4 plants per container) on an aerated Robinson and Downton [54] nutrient solution, supplemented with 250, 500, and 750 mM NaCl or without salt. Plants were then grown in a growth chamber under controlled environmental conditions in water culture at 24 °C and air relative humidity of 60–70%. The 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 photons/(m²·s). Plants that were 45 days old were used in the experiments. For total RNA extraction, leaves and roots of *S. altissima* were sampled (approximately 1 g fresh weight of each sample) and frozen in liquid nitrogen for further use.

2.2. Total RNA Extraction and First-Strand cDNA Synthesis

Total RNA samples from *S. altissima* organs were obtained by the hot phenol procedure of Yourieva et al. [55] and used as templates for first-strand cDNA synthesis. For the amplification of 3′- and 5′-ends of CLC transcript sequences using Step-Out RACE technology, synthesis of first-strand cDNA was carried out with Mint reverse transcriptase (“Evrogen”, Moscow, Russia) according to the protocol from the manufacturer. For cloning cDNA of CLC family genes and quantitative analysis of *SaCLCd*, *SaCLCf*, and *SaCLCg* transcripts in *S. altissima* organs, synthesis of first-strand cDNA was carried out using total RNA, (dT)₁₅ primer, and MMLV reverse transcriptase (“Evrogen”, Moscow, Russia).

2.3. Amplification of *SaCLCd*, *SaCLCf*, and *SaCLCg* cDNA Partial Sequences

First, we performed an *in silico* search for the sequences homologous to the *AtCLCd*, *AtCLCf*, and *AtCLCg* genes in the *Suaeda fruticosa* (L.) Forssk, which is a closely related species of *S. altissima* [51,56]. To do this, the contigs of the assembled transcriptomes were translated into amino acid sequences and search for the sequences related to the CLC family proteins was accomplished in the obtained arrays. *AtCLCd*, *AtCLCf*, and *AtCLCg* proteins were used as queries. The primers for amplification of partial cDNA fragments of *S. altissima* homologous genes (Table S1) were then designed using the contigs identified in the assembled *S. fruticosa* transcriptomes and encoding partial sequences of putative chloride channels/transporters. With these primers, the partial cDNAs of *S. altissima* CLC genes were amplified from cDNA template using Encyclo DNA polymerase (“Evrogen”, Moscow, Russia) and sequenced.

2.4. Cloning of the Full-Length *SaCLCd*, *SaCLCf*, and *SaCLCg* cDNA Sequences

Based on the partial *SaCLCd*, *SaCLCf*, and *SaCLCg* sequences obtained, the forward and reverse primer sets were designed for amplification of the 3′- and 5′-end fragments (Table S1). With these primers, we amplified the 3′- and 5′-end fragments of *SaCLCd*, *SaCLCf*, and *SaCLCg* (~1000–1500 bp) by 3′- and 5′-rapid amplification of cDNA ends (3′- and 5′-RACE) using the Step-Out RACE technology and cloned them into vector pAL2-T (“Evrogen”, Moscow, Russia). Cloned 3′- and 5′-ends fragments of *SaCLCd*, *SaCLCf*, and *SaCLCg* cDNA were then sequenced. Partial sequences (central segments, the 5′- and 3′-end fragments of *SaCLCd*, *SaCLCf*, and *SaCLCg*) were then combined *in silico*, and the resulting complete coding sequences for *SaCLCd*, *SaCLCf*, and *SaCLCg* contained open reading frames (ORFs) for proteins of 793, 587, and 776 amino acids (aa), respectively. Experimentally, the full-size *SaCLCd*, *SaCLCf*, and *SaCLCg* cDNA sequences were amplified with a CloneAmpPCR PreMix kit (“TaKaRa”/Takara Bio Inc., Shiga, Japan; cat # 638916) using pairs of the forward and reverse primers (Table S1) and total first-strand cDNA as a template. The amplified *SaCLCd*, *SaCLCf*, and *SaCLCg* cDNAs were cloned into shuttle vector pMB1, which are designed for expression of proteins in yeast cells [57], under the control of the strong
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constitutive promoter GPD1. A linear form of pMB1 was amplified using the pair of primers (Table S1). The recombinant plasmids pMB1–SaCLCd, pMB1–SaCLCf, and pMB1–SaCLCg were obtained by fusion of SaCLCd, SaCLCf, and SaCLCg cDNAs and the linear form of pMB1 using a Gibson Assembly Cloning kit (“New England Biolabs”, Ipswich, MA, USA). The cloned SaCLCd, SaCLCf, and SaCLCg were sequenced, and the obtained sequences were deposited in GenBank.

2.5. Heterologous Expression of the SaCLCd, SaCLCf, and SaCLCg Genes in ∆gef1 Yeast Mutant

*S. cerevisiae* mutant strain ∆gef1 that was created by us earlier [51,52] was transformed with constructs pMB1–SaCLCd, pMB1–SaCLCf, and pMB1–SaCLCg using the lithium protocol [58]. To explore the growth characteristics of the mutant strain ∆gef1 and the transformants, yeast cells were plated on a number of agarized (2%) selective media described in [59], namely (1) rich YPD medium consisting of 1% yeast extract, 2% peptone, and 2% dextrose (as a fermentable carbon source); (2) rich YPEG medium consisting of 1% yeast extract, 2% peptone, 2% ethanol, and 2% glycerol (as a nonfermentable carbon source); (3) minimal synthetic medium SD [60] supplemented with 2% dextrose and buffered with 50 mM Mes-Tris, pH 7.0; and (4) minimal synthetic medium SR supplemented with 2% raffinose as a nonfermentable carbon source and buffered with 50 mM Mes-Tris, pH 7.0. Yeast cells were left to grow on the selective media for two days (YPD), three days (YPD, YPEG, and SD) or four days (SR) at 28 °C. To study the effect of Mn^{2+} on yeast cell growth, MnCl₂ or MnSO₄ were added to the media at final concentrations of 2 or 3 mM. To set up iron deficiency, ferrosin, which is an iron chelator, was added to the media at a final concentration of 1 mM.

2.6. Quantitative Analysis of SaCLCd, SaCLCf, and SaCLCg Transcripts in *S. altissima* Organs

The cDNA templates for SaCLCd, SaCLCf, and SaCLCg fragment amplification were synthesized on the templates of total RNAs isolated from roots and leaves of *S. altissima* plants grown on nutrient media with various NaCl concentrations. Quantitative analysis of SaCLCd, SaCLCf, and SaCLCg transcripts was performed by the qRT-PCR method using a LightCycler® 96 system (Roche Diagnostics Corporation, Indianapolis, IN, USA). A reaction mixture with intercalating dye SYBR Green I (“Evrogen”, Moscow, Russia) was used. To amplify the SaCLCd, SaCLCf, and SaCLCg fragments, the pairs of primers were used (Table S1). Target gene mRNA expression levels were normalized for the *S. altissima* actin gene SaAct7 (GenBank, acc. no. MK615596.1) and the elongation factor 1 alpha gene SaeEF1alpha (GenBank, acc. no. MN076325.1). To amplify the SaAct7 and SaeEF1alpha fragments, the primer pairs were used (Table S1). Results were based on three to five biological replicates. The results obtained were processed by LightCycler 96SW 1.1 software. The expression of the selected reference genes was quite stable with fold changes not exceeding 0.4 under the chosen experimental conditions.

2.7. Primer Design

Primers for qPCR-RT experiments were designed by Light Cycler96 Probe Design software (https://lifescience.roche.com/, accessed on 15 December 2021). Other primers were designed using Oligo 7 software (https://www.oligo.net/, accessed on 15 December 2021) or Primer Blast software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 15 December 2021). The primers are listed in Table S1.

2.8. Bioinformatic Analysis of Amino Acid Sequences

Multiple alignment of amino acid sequences of CLC proteins was performed by MAFFT software using on-line service (https://www.ebi.ac.uk/Tools/msa/mafft/, accessed on 15 December 2021). The phylogenetic tree of plant CLC family proteins was created by Molecular Evolutionary Genetic Analysis (MEGA) 11 software (https://www.megasoftware.net/, accessed on 15 December 2021) using the maximum likelihood method based on the Jones–Taylor–Thornton model [61] (1000 bootstrap replication performed).
Protein topology was predicted by MEMSAT-SVM software (http://bioinf.cs.ucl.ac.uk/software_downloads/memsat/, accessed on 15 December 2021). Intracellular localization of the proteins was predicted with the DeepLoc 1.0 software’s eukaryotic protein subcellular localization predictor (http://www.cbs.dtu.dk/services/DeepLoc-1.0/index.php, accessed on 15 December 2021).

2.9. Determination of Chloride Content in S. altissima Organs

Water extracts from S. altissima roots and leaves were prepared by incubating samples that were dried for 1 day at 90 °C and then ground in boiling deionized water for 10 min. Concentrations of Cl\(^{-}\) in the extracts were determined by titration with Hg\(^{2+}\) using a Top Buret H digital burette (Eppendorf, Wesseling-Berzdorf, Germany).

2.10. Statistical Analysis

Statistical analysis of the data was made by one-way analysis of variance (ANOVA). A \(p\)-value < 0.05 was considered to be statistically significant. * \(p \leq 0.05\); ** \(p \leq 0.01\); *** \(p \leq 0.001\). Standard deviations are given. Correlation coefficients were calculated in the Excel program.

3. Results

Coding nucleotide sequences of SaCLCd, SaCLCf, and SaCLCg, genes from the halophyte S. altissima, were determined based on the putative similarity of these genes to homologous genes from the halophyte S. fruticosa. As a result of in silico searches of sequences related to the CLC family in the de novo assembled transcriptome of S. fruticosa [51,56], the contigs containing the partial coding regions of three sequences homologous to the A. thaliana CLC genes were designated by us as SfCLCd, SfCLCf, and SfCLCg. The contigs from S. fruticosa served as a base for identification of the full-size coding sequences of the target S. altissima genes by a rapid amplification of 3′- and 5′-cDNA ends. The cDNAs of the SaCLCd, SaCLCf, and SaCLCg genes thus obtained were then cloned and sequenced. The cloned cDNAs of SaCLCd (GenBank, acc. no. OK626332), SaCLCf (GenBank, acc. no. OK626333), and SaCLCg (GenBank, acc. no. OK626334) genes contained open reading frames (ORFs) encoding polypeptides consisting of 793, 587, and 776 amino acids, with calculated molecular masses of 87.6, 62.3, and 85.8 kDa, respectively. SaCLCd and SaCLCg were of molecular masses close to those of most plant and animal CLC proteins [37,62–65]. SaCLCf was noticeably smaller than the other two proteins. It should be noted that similar but smaller CLC proteins have been found in other plants. The AtCLCf gene encodes two forms of the AtCLCf protein, one with molecular mass of 83.5 kDa (781 a.a., At1g55620.2) and a shorter one with molecular mass of 62.5 kDa (586 a.a., At1g55620.1) [37] (Figure S1). Moreover, shortened CLCf transcripts with corresponding shortened proteins were revealed in transcriptomes of grape (Vitis vinifera) (GenBank: NP_001268117.1), pistachio (Pistacia vera) (GenBank: XP_031257549.1), and alfalfa (Medicago truncatula) (GenBank: KEH32883.1).

Each of the three proteins identified in S. altissima contained three conserved motifs (Figure 1) that are a distinctive feature of all CLC proteins. In the amino acid sequences of the SaCLC proteins, the motifs occupied the positions given in Table S2. The motifs of two proteins, SaCLCd and SaCLCg, were found to match the next sequences, namely (1) GxGxE, (2) GXXGPxxH, and (3) PxxGXLF revealed earlier in A. thaliana [32]. The three homologous motifs identified in SaCLCf, namely (1) SSKSSQ, (2) GPEGPSVD, and (3) AVAGCFF, differed from those of SaCLCd and SaCLCg and were almost identical to the motifs of AtCLCf (Figure S1).

According to [66], the conserved motifs in CLC proteins are involved in the formation of the anion-conducting pathway through membrane, in determination of channel ionic selectivity, and in gating of anion-conducting pathway. The motif GSGIPE and its putative homolog SSKSSQ (Figure 1) are functional as selectivity filters [66,67]. The amino acid occupying the second position in the motifs has been shown to be responsible for anionic
specificity of the CLC protein, namely proline (P) for NO$_3^-$ and serine (S) for Cl$^-$ [68,69]. The GSGIPE (SSKSSQ in SaCLCf) motif of the *S. altissima* CLC proteins identified in the current study included serine in the second position, thus suggesting involvement of these proteins in chloride transport.

**Figure 1.** Alignment of the amino acid sequences of CLC proteins from *S. altissima*: SaCLCa1 (GenBank, acc. no. ANG09048.1), SaCLCc1 (GenBank, acc. no. AVQ93350.1), SaCLCd (GenBank, acc. no. OK626332), SaCLCf (GenBank, acc. no. OK626333), and SaCLCg (GenBank, acc. no. OK626334). The alignment was performed in the MAFFT program and visualized in Jalview 2.11.1.4 program [70]. The conserved amino acid motifs (GxGxPE, GKxGPxxH and PxxGxLF) are framed. GxGxPE motif is a selective filter. Eg and Ep are the key glutamates of the CLC family proteins. The intensity of staining for amino acid residues depicts the degree of their identity (percentage identity). CBS1 and CBS2 domains are underlined.
Two conserved glutamates, Eg ("gating" glutamate) and Ep ("proton" glutamate), play key roles in the functioning of anion/H\(^+\) antiporters of the CLC family \[36,71\]. Eg participates in the gating of the transmembrane anion path, whereas Ep is necessary for H\(^+\) translocation \[72\]. We found both conserved glutamates inherent in CLC anion/H\(^+\) antiporters in only the SaCLCd amino acid sequence. Only "gating" glutamate (Eg) was found in SaCLCf, and only "proton" glutamate (Ep) was found in SaCLCg (Table S2). This suggests that the ion transport mechanisms differ from anion/proton antiport for SaCLCf and SaCLCg.

Like other CLCs, SaCLCd, SaCLCf, and SaCLCg contain the regulatory cystathionine beta synthase (CBS) domains CBS1 and CBS2 in the hydrophilic region at the C-terminus (Figure 1; Table S2).

According to the topology models predicted by the MEMSAT-SVM software, SaCLCd, SaCLCf, and SaCLCg are integral membrane proteins. They form 11, 7, and 9 transmembrane domains, respectively, with the N- and C-ends of the protein located on opposite sides of the membrane.

Phylogenetic analysis (Figure 2) revealed a similarity between SaCLCd, SaCLCf, and SaCLCg and CLC family representatives from other plants. Therefore, we named the cloned genes of \textit{S. altissima} CLC proteins based on their similarity to \textit{A. thaliana} CLC proteins characterized earlier.

The known CLCs can be divided into two subfamilies. One of them is characterized by a higher homology to eukaryotic (mostly human) CLCs; the representatives of the other are closer to prokaryotic CLCs \[37,45\]. Accordingly, in our cladogram of plant CLCs, the proteins were also divided into two clusters (Figure 2). We found SaCLCd and SaCLCg in the first "eukaryotic" cluster and SaCLCf in the second "prokaryotic" one. Interestingly, according to predictions obtained with the DeepLoc 1.0 software, "eukaryotic" SaCLCd and SaCLCg as well as "prokaryotic" SaCLCf were more likely to be localized to the vacuolar membrane (P\(_{\text{SaCLCd}} = 0.59\); P\(_{\text{SaCLCf}} = 0.34\); P\(_{\text{SaCLCg}} = 0.64\)) than to the plasma membrane (P\(_{\text{SaCLCd}} = 0.39\); P\(_{\text{SaCLCf}} = 0.18\); P\(_{\text{SaCLCg}} = 0.32\)). However, the "prokaryotic" SaCLCf gave indications of localization in mitochondria (P\(_{\text{SaCLCf}} = 0.19\)), endoplasmic reticulum (P\(_{\text{SaCLCf}} = 0.11\)), plastids (P\(_{\text{SaCLCf}} = 0.11\)), or Golgi network (P\(_{\text{SaCLCf}} = 0.05\)), which could be linked to the symbiogenetic (or partially symbiogenetic) origin of the organelles.

To elucidate the transport functions of SaCLCd, SaCLCf, and SaCLCg proteins, we used the previously generated \[51,52\] knockout mutant strain \(\Deltagef1\) of \textit{S. cerevisiae}. Such mutants have successfully been used earlier for clarifying anion selectivity of CLC proteins from diverse organisms \[24,59,73–76\]. The Gef1p protein, encoded by \textit{GEF1}, transports chloride and is the single member of the chloride channel family in \textit{S. cerevisiae}. Knockout mutation of the \textit{GEF1} gene caused disturbances in a range of cellular processes and led to corresponding phenotypic manifestations \[53,59\]. The growth of \(\Deltagef1\) was suppressed on rich media containing nonfermentable carbon sources (glycerol, ethanol, acetate, lactate, and raffinose) and on media with fermentable carbon sources (glucose, fructose, and mannose) at pH 7.0. As the solubility of iron salts decreases upon moderate alkalization, the availability of iron for yeast cells was also lowered to pH \(\geq 7.0\). Suppressed growth of \(\Deltagef1\) was also found on media containing cations (Li\(^+\), Mn\(^{2+}\), Ca\(^{2+}\), Mg\(^{2+}\), and tetramethylammonium\(^+\)) in toxic concentrations.

We transformed the yeast mutant strain \(\Deltagef1\) by the constructs pMB1–\textit{SaCLCd}, pMB1–\textit{SaCLCf}, and pMB1–\textit{SaCLCg} created on the basis of the shuttle vector pMB1 and containing sequences \textit{SaCLCd}, \textit{SaCLCf}, and \textit{SaCLCg} under control of the strong constitutive \textit{GPD1} promoter. To determine the phenotype of the transformants, the strains obtained were plated on the agarized selective diagnostic media described above (Figure 3). According to Gaxiola et al. \[59\], \(\Deltagef1\) cells fail to grow on Fe-deficient medium YPEG with nonfermentable carbon sources and on both SD and SR synthetic media at pH 7.0. In our experiments, growth of \(\Deltagef1\) cells failed under an Fe-deficient setup when Fe chelator ferrozine was added to YPEG medium with ethanol and glycerol as carbon sources. The same was the case when the pH was adjusted to 7.0 for SD and SR media. The growth of the
mutant strain ∆gefI on rich YPD medium was also inhibited by Mn$^{2+}$ ions at concentrations of 2 or 3 mM (Figure 3).

Figure 2. Phylogenetic tree of CLC family proteins of A. thaliana, O. sativa, N. tabacum, and S. altissima. AtCLCa (NP_198905.1), AtCLCb (NP_189353.1), AtCLCc (NP_199800.1), AtCLCd (NP_197996.1), AtCLCe (NP_567985.1), AtCL Cf (NP_564698.1), AtCLCg (NP_198313.2), OsCLC1 (XP_015633162.1), OsCLC2 (XP_015622009.1), OsCLC3 (XP_015626588.1), OsCLC4 (AAA019370.1), OsCLC5 (XP_015636607.1), OsCLC6 (XP_015650515.1), OsCLC7 (XP_015620662.1), NtCLCc (NP_001312418.1), NtCLCb (NP_001312163.1), NtCLCd (XP_016512457.1), NtCLCe (XP_016461326.1), NtCL Cf (XP_009787963.1), NtCLCg (XP_016468444.1), SaCLCa1 (ANG09048.1), SaCLCc1 (AVQ93350.1), SaCLCd (OK626332), SaCL Cf (OK626333), and SaCLCg (OK626334). All protein sequences were taken from the protein database (NCBI). Subgroup I is the “eukaryotic” branch, and subgroup II is the “prokaryotic” branch. The phylogenetic tree was built in the MEGA 11 using the maximum likelihood method based on the Jones–Taylor–Thornton model. The number of bootstrap replicates was 1000; the values of bootstrap support are indicated near the nodes. Scale: 0.5 substitutions per site.
Figure 3. The growth of the yeast mutant $\Delta gef1$ transformed with $SaCLCd$, $SaCL Cf$, and $SaCL Cg$ genes. Controls: wild-type W3031A and the mutant $\Delta gef1$ transformed with vector pMB1. Selective media: lanes 1—YPD (YPD medium: 1% yeast extract, 2% peptone, and 2% dextrose; 2 days of cells growth); 2—YPD + 2 mM MnCl$_2$ (2 days); 3—YPD + 3 mM MnCl$_2$ (2 days); 4—YPD + 2 mM MnSO$_4$ (3 days); 5—YPD + 3 mM MnSO$_4$ (3 days); 6—YPEG (rich YPEG medium: 1% yeast extract, 2% peptone, 2% ethanol, and 2% glycerol; 3 days); 7—YPEG + 1 mM ferrozine (Fe$^{3+}$ chelator) (3 days); 8—SD, pH 7.0 (minimal synthetic medium [60] supplemented with 2% dextrose; 3 days); 9—SR, pH 7.0 (minimal synthetic medium supplemented with 2% raffinose; 4 days). Approximately $10^5$ of the yeast cells were plated on selective media.

Expression of $SaCL Cf$ or $SaCL Cg$ did not restore the growth of $\Delta gef1$ colonies on the selective media. However, growth restoration of $\Delta gef1$ occurred when the mutant strain was transformed with the construct pMB1–$SaCL Cd$ (Figure 3), indicating the recovery of Cl$^-$/H$^+$ exchanger function in the mutant cells.

To gain further insight into the putative physiological functions of $SaCLCd$, $SaCL Cf$, and $SaCL Cg$, we investigated expression of genes encoding these proteins in the roots and leaves of $S. altissima$ plants grown under increasing NaCl concentrations in the nutrient solution. We also determined the growth characteristics of $S. altissima$ plants and the contents of chloride in $S. altissima$ organs under these conditions. The growth of $S. altissima$ was stimulated for both roots and shoots at 250 mM NaCl and stimulated for roots even at 500 mM NaCl, while inhibition (compared to 0 mM NaCl) only started at 750 mM NaCl (Figure 4a). Chloride was accumulated in $S. altissima$ organs under salinity. The contents of chloride in the root and leaf tissues of $S. altissima$ linearly increased with increasing NaCl concentration in the nutrient solution, and the increase was more pronounced in the leaves than in the roots (Figure 4b). With NaCl addition, concentration of chloride in the leaves were only slightly lower than that in the nutrient solution and much higher than that in the roots. At 750 mM NaCl in the nutrient solution, the concentration of chloride in the roots was less than half that in the leaves and in the nutrient solution.
Figure 4. Fresh weights (a) and chloride contents (b) of leaves and roots of S. altissima grown at various NaCl concentrations in the nutrient medium. A p-value < 0.05 was considered to be statistically significant. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001. Standard deviations are given.

The expression of SaCLCd, SaCLCf, and SaCLCg genes showed different patterns for roots and leaves of the eukalyptus under increasing salinity (Figure 5), although changes in the transcript levels were similar for all three genes. In the leaves, the relative quantity of SaCLCd, SaCLCf, and SaCLCg transcripts grew linearly as the salt concentration in the medium increased. Correlation coefficients (R^2) between chloride content and the level of expression of SaCLCd, SaCLCf, and SaCLCg genes were 0.946, 0.975 and 0.951, respectively. In the roots, the relative quantity of the CLC transcripts did not change significantly with increasing salinity. The minor changes observed in the expression of CLCs genes in the roots were in good agreement with smooth and relatively moderate changes in the Cl^- content in this organ (Figure 4b). In the leaves, a significant accumulation of Cl^- ions, observed from the elevation of NaCl concentration in the medium, corresponded to a noticeable increase in the expression of SaCLCd, SaCLCf, and SaCLCg, indicating the possible participation of CLC proteins that were presumably residing in the tonoplast and mediating vacuolar ion accumulation.
Figure 5. Relative abundance of the *SaCLCd* (a), *SaCL Cf* (b), and *SaCL Cg* (c) transcripts in the roots (dark bars) and leaves (light bars) of *S. altissima* plants grown at various NaCl concentrations in the plant growth medium. The actin gene *SaAct7* was used as internal reference gene. Similar results were obtained with *SaAct7* and *SaeEF1alpha* as reference genes, so the data are presented for the actin gene only. Data shown are means ± SD from three independent experiments. The results were deduced from three biological replicates, and each of them were performed in three analytical replicates. A *p*-value < 0.05 was considered to be statistically significant. * *p* ≤ 0.05; ** *p* ≤ 0.01.
4. Discussion

In this work, we cloned SaCLCd, SaCLCf, and SaCLCg genes of CLC family, the putative orthologs of AtCLCd, AtCLCf, and AtCLCg genes, from the euhalophyte S. altissima. The primary structure, membrane topology, and phylogenetic analysis of the cloned genes confirmed that they belong to the chloride channel family and have common properties both with representatives of this family from other plant species and with each other. At the same time, the three cloned S. altissima CLC proteins displayed some differences.

SaCLCd, like the homologous A. thaliana gene AtCLCd, belongs to the “eukaryotic” subfamily of CLC family genes (Figure 2) [37,45,52]. Amino acid sequences of both SaCLCd and AtCLCd contained the two conserved glutamates Eg and Ep (Figure 1), a hallmark of the “eukaryotic” subfamily of CLC proteins [71,77]. Serine in the second position of GSGxPE motif (Figure 1) indicates a preference for Cl\(^{-}\) over NO\(_3\)\(^{-}\) in anionic specificity of both these proteins [45,59]. Bioinformatic predictions located the corresponding protein for SaCLCd to tonoplast or plasma membrane with combined probability of 0.98.

In complementation analyses, AtCLCd is often used as a positive control for heterologous expression of CLC genes from diverse organisms in the Saccharomyces cerevisiae GEF1 disrupted strain Δgef1. Expression of AtCLCd in the Δgef1 strain invariably rescues the growth defect phenotype of the latter [37,52,59,78]. Like AtCLCd, the expression of SaCLCd gene also complemented the growth defect phenotype of the Δgef1 yeast mutant. The cells of Δgef1 transformed with the construct pMB1–SaCLCd, in contrast to Δgef1 cells transformed with empty vector pMB1, demonstrated indistinguishable growth from that of WT S. cerevisiae cells on selective media (Figure 3). Altogether, these results indicate that SaCLCd operates as an anion/H\(^{+}\) exchanger rather than an anion channel.

SaCLCf belongs to the “prokaryotic” subfamily of CLC family genes (Figure 2). AtCLCf, the homolog of SaCLCf protein, is currently the least studied protein of the AtCLC family, and its subcellular localization, transport functions, and physiological role are not yet fully clarified. The available data concerning its localization and functions are contradictory. For yeast grown on selective media [59], complementation of the growth defect phenotype of Δgef1 was observed in the mutant cells expressing AtCLCf [37]. However, Lv and coworkers [78] did not obtain complementation of the Δgef1 mutant phenotype in response to the expression of this gene. According to [37], AtCLCf mainly resides in cis-Golgi compartments and, to a lesser extent, in the trans-Golgi network. However, a recent work [45] demonstrated that the relation of AtCLCf is predominantly to the trans-Golgi network/early endosomes (TGN/EE), where it colocalizes with V-ATPase (VHA-a1) as well as AtCLCd. The authors suggested that AtCLCd and AtCLCf transfer Cl\(^{-}\) into the endosomal lumen and, together with V-ATPase, are likely involved in regulation of pH and chloride concentration in the lumen of the trans-Golgi network and in late endosomes. Our bioinformatic predictions located the homolog of AtCLCf protein, SaCLCf, to tonoplast or plasma membrane with a combined probability of 0.52. Arguably, SaCLCf is equally likely to be located in the Golgi network or endosome membranes, similar to the AtCLCf protein.

We failed to complement the growth defect phenotype of the yeast mutant strain Δgef1 by expressing SaCLCf in its cells (Figure 3). Given the presence of serine in the second position of the SaCLCf conserved motif SSKSSQ (Figure 1), which indicates chloride specificity of this protein [66,67], the complementation failure could be because SaCLCf functions as a chloride channel and not as a Cl\(^{-}/H^{+}\) antiporter. In line with the result, amino acid sequence of SaCLCd did not contain Ep, the conserved glutamate (Figure 1), playing a key role in the H\(^{+}\) transport through membrane [71,77]. In relation to these data, it should be noted that “neutralization” of “proton” glutamate E203 in prokaryotic CLC-ec1 by mutation E270Q abolished H\(^{+}\) coupling to Cl\(^{-}\) transport [72]. However, the suggestion that CLCs act as chloride channels did not find support in a recently proposed hypothesis [45]. It was hypothesized that in members of the “prokaryotic” subfamily AtClCe and AtClCi, the function of “proton” glutamate might be performed by a glutamate other than the Ep of the “eukaryotic” CLC subfamily and that these “prokaryotic” representatives of the CLC...
family are likely antiporters [45]. Generally, the operation mechanism of SaCLCf, like that of AtCLCf, remains to be elucidated.

The third gene SaCLCg, the homolog of AtCLCg, was a member of the “eukaryotic” subfamily of S. altissima CLCs. The presence of serine in the SaCLCg protein in the second position of the conserved motif GSGIPE, canonic for the “eukaryotic” CLC subfamily, indicated chloride selectivity of SaCLCg. Expression of SaCLCg in cells of the yeast mutant strain Δgef1 did not complement its growth defect phenotype (Figure 3) like the expression of the AtCLCg homolog [78]. This fact, together with the missing “gating” glutamate in its amino acid sequence (Figure 1), indicates that SaCLCg, like its homolog from Arabidopsis, is a chloride channel rather than a Cl\(^-\)/H\(^+\) antiporter. The importance of Eg for functioning “eukaryotic” anion/H\(^+\) antiporters is highlighted by the fact that mutating the “gating” glutamate E203 to alanine in AtCLCa expressed in Xenopus oocytes resulted in uncoupled anion conductance [71]. AtCLCg, most likely a Cl\(^-\) channel [33,38,71], is expressed in mesophyll, phloem, and hydathode cells of mature leaves as well in root cells; the protein was shown to localize in tonoplast [46,78]. AtCLCg was suggested to be involved in sequestering Cl\(^-\) ions in vacuoles, phloem recirculation, guttation, and xylem loading, thereby providing tolerance to salt stress [46]. Minimizing xylem loading and subsequent transport of Cl\(^-\) to the shoot might contribute to salt tolerance by keeping photosynthetic tissues away from Cl\(^-\) overaccumulation [9]. The euhalophytes have evolved another strategy for growth under salinity. These plants translocate absorbed Na\(^+\) and Cl\(^-\) to the shoots and accumulate them preferentially in leaf vacuoles [14,79,80]. This maintains cytoplasmic Na\(^+\) and Cl\(^-\) concentrations at nontoxic levels and contributes to water potential gradient setup in the system, namely soil–root–shoot, which promotes continuous water flow in the ascending direction [81]. Greater Na\(^+\) and Cl\(^-\) accumulation in S. altissima leaves than in the roots and the growth stimulation in response to increase in NaCl concentration in the medium (Figure 4) are in line with patterns of SaCLCd, SaCLCf, and SaCLCg expression in these organs under saline conditions (Figure 5). Results of the study of SaCLC gene expression in the roots and leaves of plants grown at different NaCl concentrations in the medium showed that abundance of SaCLCd, SaCLCf, and SaCLCg transcripts did not change significantly in the roots with increasing salinity in the nutrient solution (Figure 5). However, in the leaves, a substantial increase in SaCLCd, SaCLCf, and SaCLCg transcript levels was observed under these conditions, which implies the participation of the proteins encoded by these genes in Cl\(^-\) accumulation in leaf organelles.

5. Conclusions

In this study, we cloned coding sequences of three novel chloride channel family genes SaCLCd, SaCLCf, and SaCLCg, the putative orthologs of A. thaliana AtCLCd, AtCLCf, and AtCLCg genes, from the euhalophyte S. altissima. The growth of this euhalophyte was stimulated by 250 mM NaCl, while inhibition only started from 750 mM. This is an extremely unusual feature for most plants, including agricultural ones that are glycophytes. However, global climate change and soil salinization have made us consider the importance of halophytes. In plants, members of the chloride channel family transport Cl\(^-\) and NO\(^3\) across membranes of intracellular organelles and account for a number of physiological functions. The results of a complementation assay using yeast expression system as well as bioinformatic analyses of the proteins encoded by these genes indicate that SaCLCd protein is a Cl\(^-\)/H\(^+\) antiporter, while the SaCLCf and SaCLCg proteins are likely Cl\(^-\) channels. The results of qRT-PCR analyses showed that expression of all three genes was activated in the leaves with increase in NaCl concentration in the growth medium, suggesting the involvement of the SaCLCd, SaCLCf, and SaCLCg proteins in the response of S. altissima to NaCl. All three encoded proteins share common properties with the proteins of CLC family representatives from glycophytes, particularly the presence of conserved motifs, conserved glutamates, and regulatory CBS domains in amino acid sequences. However, differences between the proteins of S. altissima and their putative orthologs from glycophytes remain to be elucidated. Future investigations of the proteins, such as studies aimed at revealing their
intracellular localization and distribution in whole plant, functional studies employing heterologous expression systems, and examination of the physiochemical and structural protein features, could clarify differences between halophyte and glycophyte proteins and give clues to understanding the processes underlying salinity tolerance and ways to improve them by methods of molecular genetics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants11030409/s1, Figure S1: Alignment of the amino acid sequences SaCLCf (GenBank, acc. no. OK626333), AtCLCf_long (GenBank, acc. no. NP_564698.1), and AtCLCf_short (GenBank, acc. no. NP_849813.1) performed in the MAFFT program and visualized in Jalview 2.11.1.4 program [70]. The intensity of the staining of amino acid residues corresponds to the degree of their identity (Percentage Identity), Table S1: List of the primers used in the study, Table S2: Conserved amino acids motifs and residues in SaCLCd, SaCLCf and SaCLCg sequences and their coordinates.

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