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ORIGINAL RESEARCH

Characterization, *in silico* analysis and expression of *DREB2* and *VPase* from *Suaeda edulis*

Caracterización, análisis *in silico* y expresión de genes *DREB2* y *VPasa* de *Suaeda edulis*

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ABSTRACT

Soil salinity severely affects plants and specially to crop plants. Halophytes are resistant to salinity stress since they have developed several gene mechanisms of resistance, such as the enzymatic Na^+ compartmentalization into vacuoles by vacuolar Pyrophosphatase (VPase) and the up-expression of transcription factors such as Dehydration-sensitive Element-Binding proteins (DREB). Halophytes are a potential source of salt-resistance genes and are considered as a study model for this type of stress. In this study, the

halophyte *Suaeda edulis* was used for the identification, characterization, *in silico* analysis and expression of the DREB and VPase genes. Deduced amino acid sequences of SeDREB and SeVP showed >80% similarity with their homologous and contain conserved domains and motifs characteristic of these proteins. Phylogeny showed that SeDREB is in the subgroup A2 that is expressed in salinity, while SeVP is in the group of the K-dependent vacuolar proteins. *In silico* coexpresion analysis showed the interaction with several proteins related with salinity and drought. The expression of genes was higher in roots and leaves in wild plants than in *in vitro* plants. The soil inhabited by *S. edulis* has a pH 9.2, an EC=4.2 dS m^{-1}, as well as a translocation factor for sodium of 3.4, which indicates a higher adsorption of this metal by the plant

Keywords: Genic expression, halophytes, ionic cytotoxicity, saline-sodic soil

RESUMEN

La salinidad del suelo afecta severamente a las plantas y en especial a las de importancia agrícola. Las halófitas son resistentes a estrés por salinidad ya que han desarrollado varios mecanismos génicos de resistencia, como es la compartimentación enzimática de Na+ en vacuolas mediante la pirofosfatasa vacuolar (VPasa) y la sobreexpresión de factores de transcripción como las proteínas de Unión a Elementos Sensibles a la Deshidratación (DREB; siglas en Inglés). Las halófitas son una fuente potencial de genes de resistencia a la salinidad y son consideradas como modelo de estudio ante este tipo de estrés. En este estudio, se utilizó el halófito *Suaeda edulis* para la identificación, caracterización, análisis *in silico* y expresión de los genes DREB y VPase. Las secuencias deducidas de aminoácidos de SeDREB y SeVP muestran una similitud mayor al 80% con sus homólogos y presentan los dominios y motivos conservados. La filogenia mostró que SeDREB pertenece al subgrupo A2 que se expresa por salinidad, mientras que SeVP está en el grupo de las K-dependientes. El análisis de coexpresión *in silico* muestra la interacción con varias proteínas relacionadas con la salinidad y la sequía. La expresión de los genes fue mayor en raíces y hojas en plantas silvestres que las crecidas *in vitro*. El análisis de suelo del hábitat de *S. edulis* mostró un pH de 9.2, un EC=4.2 dS m^{-1}, así como un factor de translocación para sodio de 3.4, lo que indica una mayor adsorción de este metal por la planta.

Palabras clave: Expresión génica, halófitas, citotoxidad iónica, suelo salino-alcalino.

1. INTRODUCTION

Drought, salinity, and extreme temperatures are stresses that affect plant growth and development and crop yields. However, some plants can tolerate these conditions, such as the halophytes, they can survive and grow in these stresses because they carry out to biosynthesis of osmoprotectors, sodium partitioning, up-regulate of transcription factors, cell wall modifications, and the regulation of water transport across the membrane

(Flowers & Colmer 2008; Liang *et al*., 2018). For this reason, the halophytes represent an excellent model for the study of salinity tolerance and are a potential source of discovery for resistance genes for conventional crop plants (Liang *et al.*, 2018; Rahman *et al*., 2021). Flowers and Colmer (2008) define halophytes as those plants capable of surviving and reproducing in environments where salt concentration (NaCl) is above 200 mM; they represent approximately 1% of the global flora diversity. In the Amaranthaceae family, there are succulent halophytes that are highly tolerant to salt, among which the genera *Suaeda* and *Salicornia* stand out for their participation in saline agriculture. Particularly the genus *Suaeda* Forssk. ex Scop. (Flowers *et al*., 2015; Wang *et al*., 2022; Yu *et al*., 2022). Some species of the genus has been used as models for the analysis of gene response to saline stress due to their salinity tolerance. *Suaeda edulis* is distributed in the central region of Mexico; in this region a crater (crater la Olla; Valle de Santiago, Gto.) is located with saline-sodic soil formed by the accumulation of NaCl and sodium carbonate (Na_2CO_3)/bicarbonate sodium ($NaHCO_3$) and salts ions, where *S. edulis* also grows (Brandt *et al*, 2015; Noguez *et al*., 2013). Saline-sodic soil can be determined in their percentage of exchangeable sodium (PES), measuring the electrical conductivity (EC) in the saturation extract and evaluating its basicity using its pH *(Ghnaya et al*., 2015). On the other hand, soil salt ions or metals can be removed by taking from roots and translocated to other plant organs, to this process is called translocation factor (TF) (Mirza *et al*., 2014). TF is a value that indicated if some plants has the capacity to be used in the process of phytoremediation. For example, *Suaeda salsa* has been used to reclaim saline soil in Chine (Rahman *et al*., 2021), and others differents species of *Suaeda* have been successfully evaluated in soil phytoremediation (Mirza *et al.,* 2014; Yu *et al*., 2022). Due to its characteristics, *S. edulis* could be used for phytoremediation, especially in saline soils.

In recent years, studies of genes and proteins related to tolerance to drought and salinity stress in halophyte plants have been carried out, especially in Suaeda species such as *S. salsa*, *S. japonica*, *S. glauca*, *and S. corniculata*. Among these genes and proteins are the Dehydration-sensitive Element-Binding proteins (DREBs) and Vacuolar Membrane Proton Pyrophosphatase protein (VPPase) (Yu *et al*., 2022; Song *et al*., 2022; Xu *et al*., 2017).

The DREB transcription factor plays a key role in the regulation of many genes related to abiotic stress. DREB transcription factors interact with cis-acting elements present in the promoter region of various related genes with several types of stress (Eckardt, 2019). *DREBs* are members of AP2/ERF subfamily and contain the domain AP2 that has conserved DNA-binding motifs and amino acids sequences; this domain has an important role in biotic and abiotic stress responses in plants (Allen *et al.*, 1998; Sazegari *et al*., 2015, Eckardt 2019; Chen *et al*., 2020). Several *DREBs* genes have been isolated from different plants and *Suaedas* species to study stress tolerance. As such, *Oryza sativa* and *Salicornia brachiata* up-regulate *OsDREB2A* and *SbDREB2A*, respectively, in response to saline stress (Dubouzet *et al*., 2003; Song *et al*., 2022).

Other tolerance mechanisms are active ion transport by a selective membrane to maintain ionic homeostasis (Na^+, K^+, and Ca^{+2}); in this process the vacuolar pyrophosphatase (*VP*) gene has important participation. The Na^+/K^+ pump carries out the transport of Na^+ from the cytoplasm to the vacuole to avoid ionic cytotoxicity, which is dependent on V-ATPase and V-PPase activity. These enzymes establish a H^+ electrochemical gradient in the tonoplast that energizes Na^+ transport against the concentration gradient (Maathuis *et al*.,

2014), allowing it to be vacuole stored. Protein sequence shows [DE]X7KXE is a catalytic motif common to both soluble and membrane-associated H^+-PPase, and works as a binding site and/or substrate (PPi) hydrolysis on the membrane surface from the cytoplasmic side (Drozdowicz & Rea, 2001). Genes that encode these enzymes have significantly increased expression in *Suaeda salsa* (Wang *et al*., 2001) and *Salicornia europaea* in response to high NaCl concentrations (Lv *et al*., 2012).
Since DREB and VPase genes contain conserved domains, these characteristic facilities their molecular identification. For example, DREB proteins contains a short sequence of amino acid residues to binding to the promoter region of the target gene (Sun *et al.,* 2014). While the VPase protein contains several transmembrane domains such as [DE]X7KXE that participates directly in substrate binding; this conserved domain has been used as a recognition sequence by polyclonal antibodies PABTK and PABHK (TKAADVGADLVGKIE and HKAAVIGDTIGDPLK, respectively) (Drozdowicz & Rea, 2001), and could be identification by PCR analysis too. So, with the design of suitable primers in these regions, these genes can be identified by PCR and different molecular and *in silico* analyzes can be carried out in *S. edulis*.
In previous studies, we propagated *S. edulis in vitro,* and we analyzed expression levels of genes involved in osmoprotectant biosynthesis as a tolerance salinity mechanism (Cerrillo-Rojas *et al*., 2020). Therefore, *in vitro* plants were used to some experiments and the objective of this work was the analysis of the soil of the natural habitat of *S. edulis*, the presence of ions and their translocation factor, as well as the identification and *in silico* analysis of the VPase and DREB2 genes and the expression levels in leaves and roots of plants *in vitro* and *ex vitro*.

2. MATERIAL AND METHODS

2.1. Collection of samples

Complete Plants were collected from la Olla soil crater (20.422195796559237°, 101.24784444030067°) in Rincon de Parangueo, Valle de Santiago, Guanajuato, Mexico. The plants were placed in cool containers and transferred to the laboratory, rinsed twice with sterile water, and washed carefully with 1% benzalkonium chloride (Antibenzil®). Roots and leaves taken from this material were subsequently pulverized with liquid nitrogen and stored at -80°C until use, the same was done with the *in vitro* plants. *In vitro* cultures were carried out on basal medium MS (Murashige & Skoog, 1962) at 25 °C ± 1 °C and 16 h light/8 h dark, as done by Cerrillo-Rojas *et al*., (2020).

A

B

Fig. 1. Natural habitat and sampling site of *S. edulis*. A) Panorama of the crater. B) Plants of *S. edulis*.

2.2 Plant and soil analysis

The soil was analyzed following the protocols of the Official Mexican Norm (NOM-021-SEMARNAT-2000). A saturation extract was obtained by vacuum filtration of a soil paste made with distilled water to analyze. pH and EC were measured with a pH and conductivity meter (OAKTON Ph/CON 610 series). Soluble cations were determined by

atomic absorption (Ca and Mg) and atomic emission spectroscopy (Na+ and K+). Soluble anions were determined by volumetric titration (chlorides, carbonates, bicarbonates), and by turbidimetry (sulfates).

Samples of root and leaves were prepared in triplicate following the protocols of Ghnaya *et al*., (2015). The samples were analyzed by atomic absorption spectroscopy (GBC SavantAA Σ). The translocation factor (TF) was calculated using the equations of

TF= [metal concentration in leaves and stems] / [metal concentration in root]

2.3 RNA extraction and RT-PCR

Pulverized and frozen samples of root and stem *in vitro* and *ex vitro* plants of *S. edulis* were taken for total RNA extraction. The extraction was carried out using PureLink® Plant RNA Reagent kit (Ambion Life Technologies, Carlsbad, CA, USA). The integrity of the molecule was visualized by electrophoresis and the concentration and purity by spectrophotometry using a Nanodrop Spectrophotometer (Thermo Scientific). For the cDNA synthesis, 1 µg of total RNA from each sample was used, following SuperScript™ (Invitrogen, Carlsbad, CA, USA) protocol. cDNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific), and the concentration was adjusted to 100 ng/µl. Specific oligonucleotides were designed with DNAMAN program [http://www.lynnon.com/dnaman.html], for VPase primers, an alignment of the *S. corniculata* VPase sequence (HQ412514) was performed with other sequences of the genera Halostachys (EF471358), Salicornia (AEI17665), Kalidium (ABK91685) which included the conserved TKAADVGADLVGKVE motif. For DREB2 the primers were designed from the DREB sequences of *S. brachiata* (ADE35085.1)*, Chenopodium quinoa* (XP_021769255) and *Beta vulgaris* (XP_010692920).
The oligonucleotides used were DREB2-For 5- ATACCCTTTGATTACACCAGGAA-3´ and DREB2-Rev 5´-CATCAAACATTTCATCCATTGTT-3´; VP-For 5´-ACTATGGTGATGATTGGGAAGGT-3´ and VP-Rev 5´-GGCCCATAACCCAACAGCAA-3´. GoTaq® Flexi DNA Polymerase (Promega Corp.) commercial kit was used for the gene amplification along with 50 ng of DNA, 0.4 mM of each oligonucleotide, and 0.2 mM of each dNTP, in a final volume of 25 µl. The amplification conditions were initial denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, ending with a final extension at 75° C for 5 min. Amplificated fragments were sequenced in the Laboratorio Nacional de Biotecnología Agrícola, Médica y Ambiental of the Instituto Potosino de Investigación Científica y Tecnológica (LANBAMA-IPICyT) in San Luis Potosí, Mexico.
For real-time quantitative reverse transcriptional PCR (qRT-PCR), oligonucleotides for *VP* and *DREB2* were designed from the specific sequence of *S. edulis*: SeVP-F 5-GGTCTTGGAGGATCTTCCAT-3 and SeVP-R 5-CTTCTCAGTTGAATGTTGGTCA-3; SeDREB2-F 5-TAAATTCCGAGGAGTTAGACAG-3 and SeDREB2-R 5-TCTTCAAACAACGATGAAGGG-3. Quantification was normalized respect with β-tubulin for which the primers are as follows: β-tub-F 5-CTTATTCCATTCCCCAGGCTTC-3 and β-tub-R 5-CATCTGCTCATCAACCTCCTTTGTGC-3 (Cao *et al*., 2016).
Amplification reactions were carried out in 96-well plates in a StepOne™ Real-Time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems).

The data were analyzed using the Applied Biosystems StepOne™ Real-Time PCR System Software (Applied Biosystems, Mulgrave, AUS). The normalized relative expression was calculated by the $2^{-\Delta\Delta ct}$ method (Livak and Scmittgen, 2001). Three biological replicates were analyzed. For each biological replica, the samples were evaluated in triplicate. A single-factor analysis of variance (ANOVA) was performed to detect differences in each gene's relative quantification.
Tukey test determined the significance of the differences between each value obtained. Differences with $p<0.05$ were considered significant. These analyses were performed with Minitab v16 software (State College, PA, USA).

2.4 Bioinformatics analysis

The partial amplified sequences of *DREB* and *VPase* of *S. edulis* were subjected to bioinformatic analysis starting with the search of homologous sequences in the NCBI database using NCBI BLAST [http: //blast.ncbi.nlm.nih.gov/Blast.cgi] and UNIPROT (https://www.uniprot.org/). Deduced amino acid sequences were obtained with Translate tool from ExPASy (https://web.expasy.org/translate/). The conserved domains were determined in CDART platform (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi).
The modeling of the amino acid sequences was carried out through two programs: 1) SWISS-MODEL 3.1.0 [https://swissmodel.expasy.org/] (Waterhouse *et al*., 2018) to obtain the PDB file and 2) and Chimera 1.8 (Pettersen *et al.,* 2004) to obtain the three-dimensional model. QMEAN server [https://swissmodel.expasy.org/qmean/] (Pascal *et al.,* 2009) was used to analyze SeVP and SeDREB2 predicted protein model.
Multiple alignments in amino acids were carried out with UniProt software and the construction phylogenetic analyses was carried out using MEGA7 software with the UPGMA method and 1000 bootstraps (Kumar *et al.,* 2016). STRING 11.0 (https://string-db.org/) carried out to identification of protein-protein interaction. Phylogenetic analysis of the DREB proteins was performed with several homologous amino acid sequences deposited at NCBI and Uniprot databese; the sequence selection was according to Sakuma *et al.*, (2002). For phylogenetics analysis of VPase proteins, the above-mentioned method was followed, except that K-dependent and K-independient proteins were selected.

3. RESULTS

Crater soil pH was 9.2, EC=4.2 dS m^{-1}, and contains soluble cations and anions; for cations there is a greater amount of sodium and anions for carbonates, Table 1.
In plants of *S. edulis*, the highest amount of sodium was found in the leaves with 80,470.00 mg/kg, while in the root it was 23,610.70 mg/kg (Table 2). TF indicates that both sodium and manganese have the facility to be translocated very easily from root to leaves (Table 2).

Table 1. Cations and anions found in the soil of the Olla crater of Rincón de Parangueo crater.

Soluble cation	meq/L	Soluble anion	meq/l
Ca	0.9	Carbonates	20
Mg	2.6	Bicarbonates	7
Na	34.8	Clorures	13.5
K	2.3	Sulfates	6.9

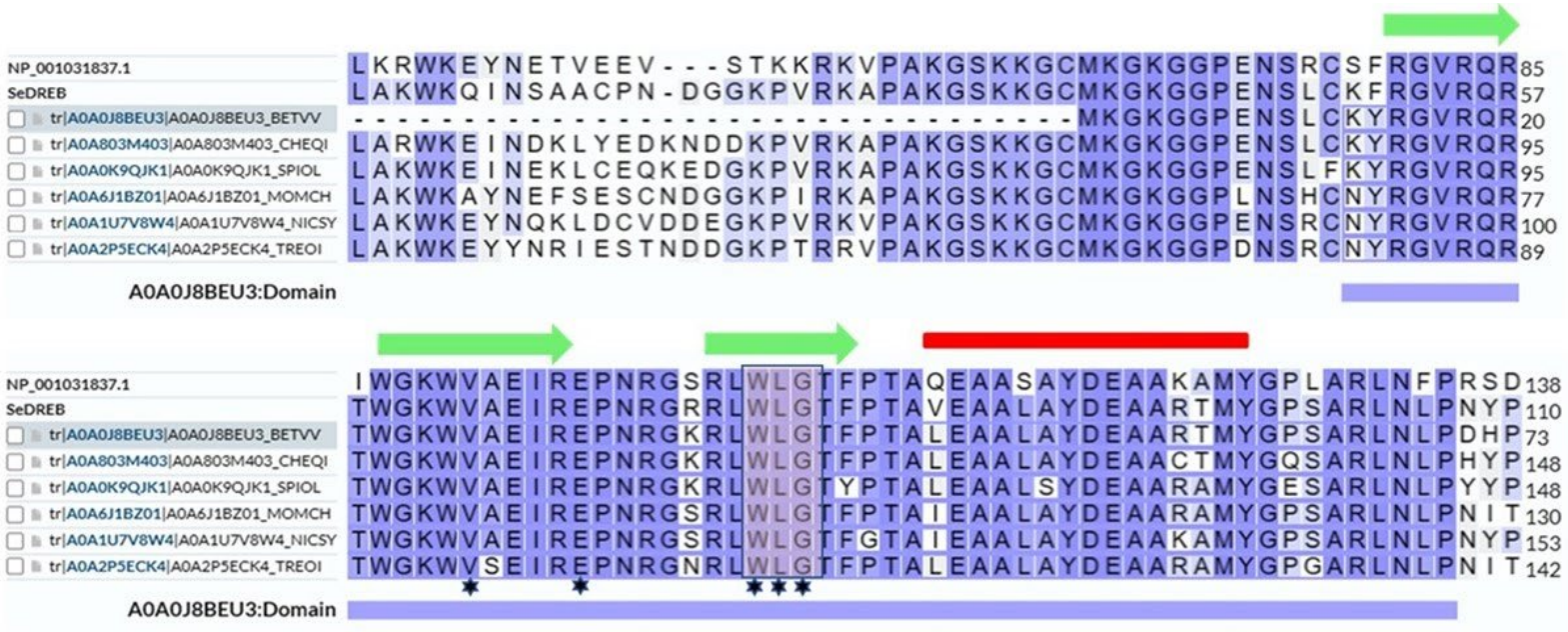
Table 2. Elements found in leaves and root of *S. edulis* from the crater.

Element	Leaves	Root	TF
P	0.11%	0.9%	0.122
K	1.64%	2.0%	0.82
Ca	0.28%	1.03%	0.271
Mg	2.49%	3.535%	0.704
Na	80,470 mg/kg	23,610.70 mg/kg	3.4082
Fe	117.41 mg/kg	138.84 mg/kg	0.845
Mn	28.75 mg/kg	10.32 mg/kg	2.785
Zn	13.66 mg/kg	21.91 mg/kg	0.623
Cu	4.41 mg/kg	4.46 mg/kg	0.988
TF= Translocation Factor			

With the oligonucleotides designed for the *DREB* and *VPase* genes, 750 and 560 bp fragments were obtained respectively as expected and were named as SeDREB2 and SeVP.

The deduced amino acid sequence of SeDREB2 is of 240 aa and multiple alignment in Uniprot database with other DREB protein sequences (including DREB2 of *Arabidopsis thaliana*), showed that it contains the AP2 domain, which is within the AP2/ERF subfamily, in addition to the conserved amino acids V (14) and E (19) and the WLG motif (Fig. 2A). The hypothetical modeling was generated with SWISS-MODEL, this program used as template 1GCC (PDB) of *Arabidopsis thaliana*. This model presents the form of 3 β sheets connected in an antiparallel manner with α loops and one helix (Fig. 2B) and has 90% identity with 1GCC domain (Fig. 2C). Overlapping of AtERF2 (1GCC) and SeDREB2 structures showed the presence of a DNA-binding domain (Fig. 2D). The analysis of predicted models carried out by QMEAN4 server, showed the global qualities estimates. QMEANDisCo global was 0.55 and QMEAN was -3.59.

A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tr|A0A6J1BZ01|A0A6J1BZ01_MOMCH
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tr|A0A0K9QJK1|A0A0K9QJK1_SPIOL
tr|A0A6J1BZ01|A0A6J1BZ01_MOMCH
tr|A0A1U7V8W4|A0A1U7V8W4_NICSY
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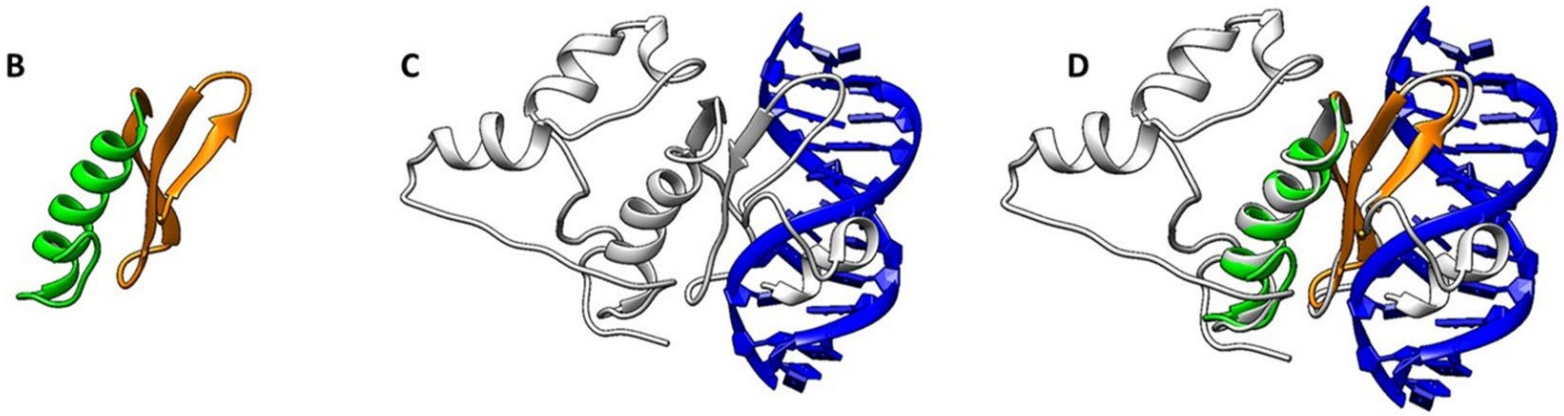


Fig. 2. SeDREB2 multiple alignment and hypothetical modeling. A) SeDREB2 Alignment with other proteins that have the domain AP2/ERF: NP_001031837.1 **(**DREB2 *Arabidopsis thaliana*), A0A0J8BEU3_BETVV (***Beta vulgaris subsp. vulgaris***), A0A803M403_CHEQI (***Chenopodium quinoa*****),** A0A0K9QJK1_SPIOL (***Spinacia oleracea*****)**, A0A6J1BZ01_MOMCH (*Momordica charantia*), A0A1U7V8W4_NICSY (***Nicotiana sylvestris*****)**, A0A2P5ECK4_TREOI (*Trema orientale*). The blue bar represents the A0A0J8BEU3: Domain (AP2/ERF), the green arrows represent the alpha helices and red bar the beta sheets, the star indicates conserved amino acids, and the shaded box is the WLG motif. B) hypothetical modeling of SeDREB2, C) Modeling of 1GCC of PDB with DNA union and D) SeDREB2 overlapping with the structure of 1GCC.

The phylogenetic tree was based on multiple alignment of SeDREB2 with various sequences of DREBs and showed six clusters (A1-A6), where SeDREB2 is in cluster A2 (Fig. 3).

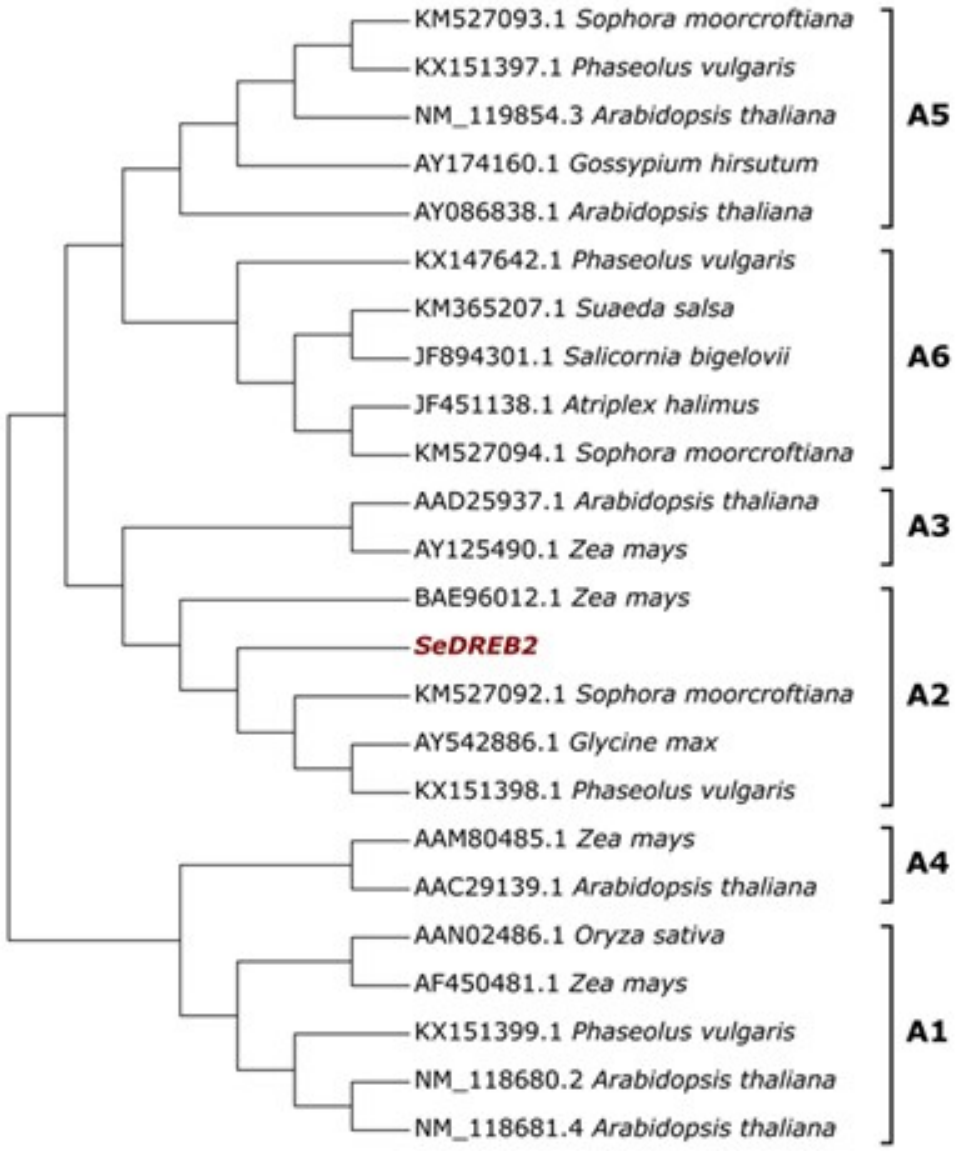


Fig. 3. Phylogenetic analysis of SeDREB2 (*Suaeda edulis*). Multiple sequence alignment and phylogenetic tree were conducted with MEGA7. A-1 to A-6 indicate subgroups proposed by Sakuma *et al*., (2002). SeDREB is shown in red. DREB protein sequences were retrieved from GenBank.

The protein–protein interaction (PPI) analysis was carried out using the STRING platform and we used the *A. thaliana* genome because the genome of *S. edulis* has not yet been sequenced. We obtained the DREB2A protein with >80% similarity with SeDREB2. So,

SeDREB2 were represented by *A. thaliana* DREB2A for coexpression analysis. This analysis showed a forecasted confidence scores of 0.973–0.866 which indicated the functional network among the set of proteins of the organism under study. The representative model was predicted to be interacting with ten proteins: HSFA3, DRIP2, PFT1, ABF2, DRIP1, LTI78, RCD1, ABF4, LTI65, and AP2 (Fig. 4A). The network stats showed that DREB2A PPI comprised 11 nodes connected with 24 different edges.

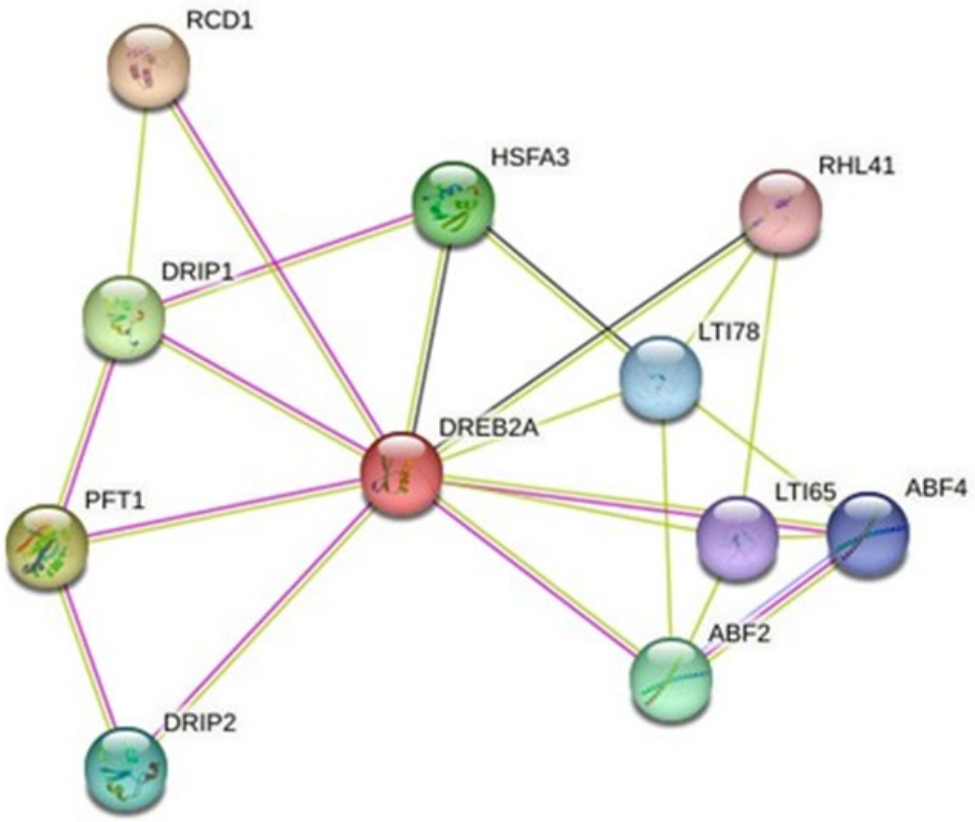
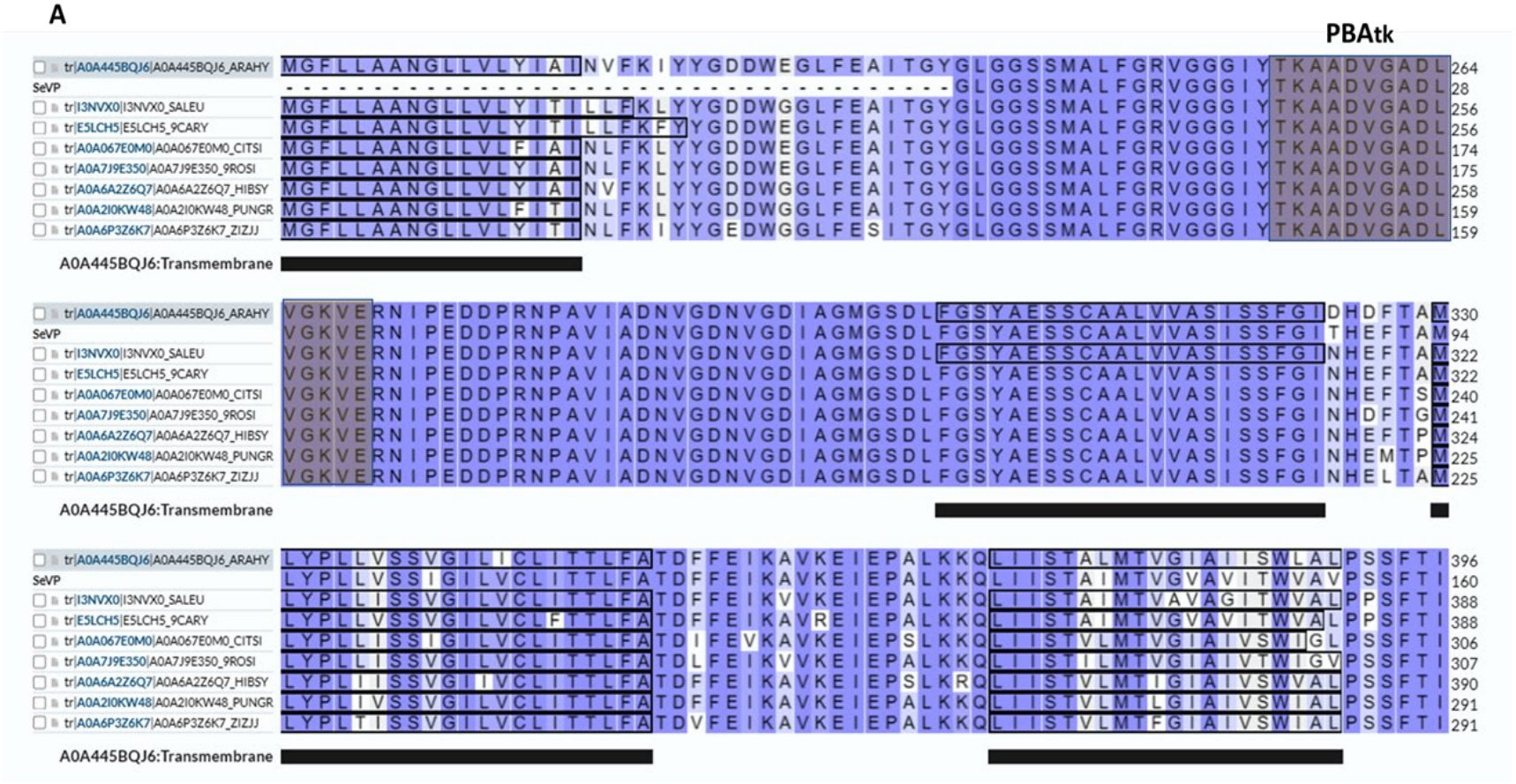


Fig. 4. Map of protein–protein interactions. DREB2A represent to SeDREB2 and the PPI network shows that all proteins interact with each other.

Putative protein SeVP showed 94 % similarity to the V-PPase of *Suaeda corniculata* and *Kalidium foliatum*. Multiple uniprot alignment of SeVP (partial sequence) with other H(+)-exporting diphosphatase proteins shows that it contains the conserved PBATK motif, and within this the DVGADLVGKV motif, plus three helical transmembrane regions of the 13 or 16 typically contained by these proteins (Fig. 5A). The hypothetical modeling was generated with SWISS-MODEL program based on the crystal structure of the H-translocating Pyrophosphatase (4A01) template from *Vignia radiata* (Fig. 5B). The overlapping of both structures is shown in figure 5C. The analysis of predicted models

carried out by QMEAN4 server, showed the global qualities estimates. QMEANDisCo global was 0.67 and QMEAN was -3.03.



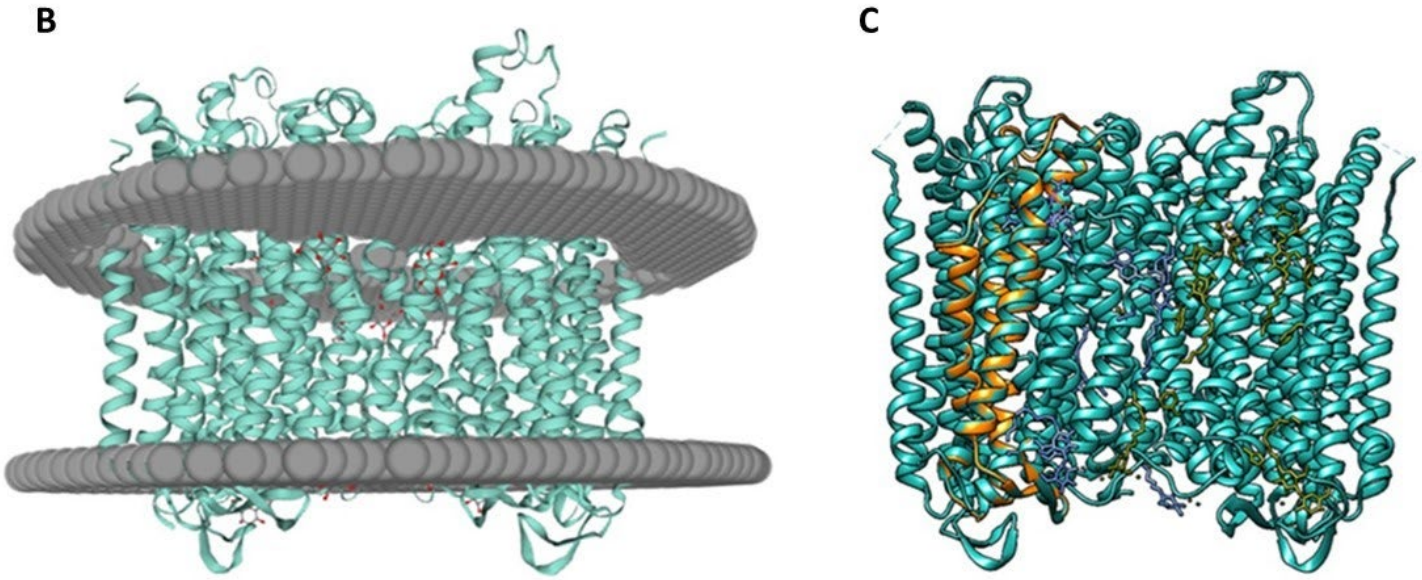


Fig. 5. SeVP multiple alignment and hypothetical modeling. A) SeVP Alignment with other homologs proteins: A0A445BQJ6_ARAHY (*Arachis hypogaea*), I3NVX0_SALEU (*Salicornia europaea*), E5LCH5_9CARY (*Suaeda corniculata*), A0A067E0M0_CITSI (*Citrus sinensis*), A0A7J9E350_9ROSI (*Gossypium trilobum*), A0A6A2Z6Q7_HIBSY (*Hibiscus syriacus*), A0A2I0KW48_PUNGR (*Punica granatum*), A0A6P3Z6K7_ZIZJJ (*Ziziphus jujuba*). In the gray box is the conserved PBATK motif, and within this the DVGADLVGKV motif, the black bar represents three helical transmembrane (A0A445BQJ6: Transmembrane) for SeVP. B) modeling (4A01) template from *Vignia radiata* C) SeVP overlapping with the structure of 4A01.The phylogenetic tree was based on the alignment of SeVP with several VPAs proteins from different species of plants and allowed its classification into K-dependents and K-independent groups (Fig. 6); in the K-dependent group is SeVP.

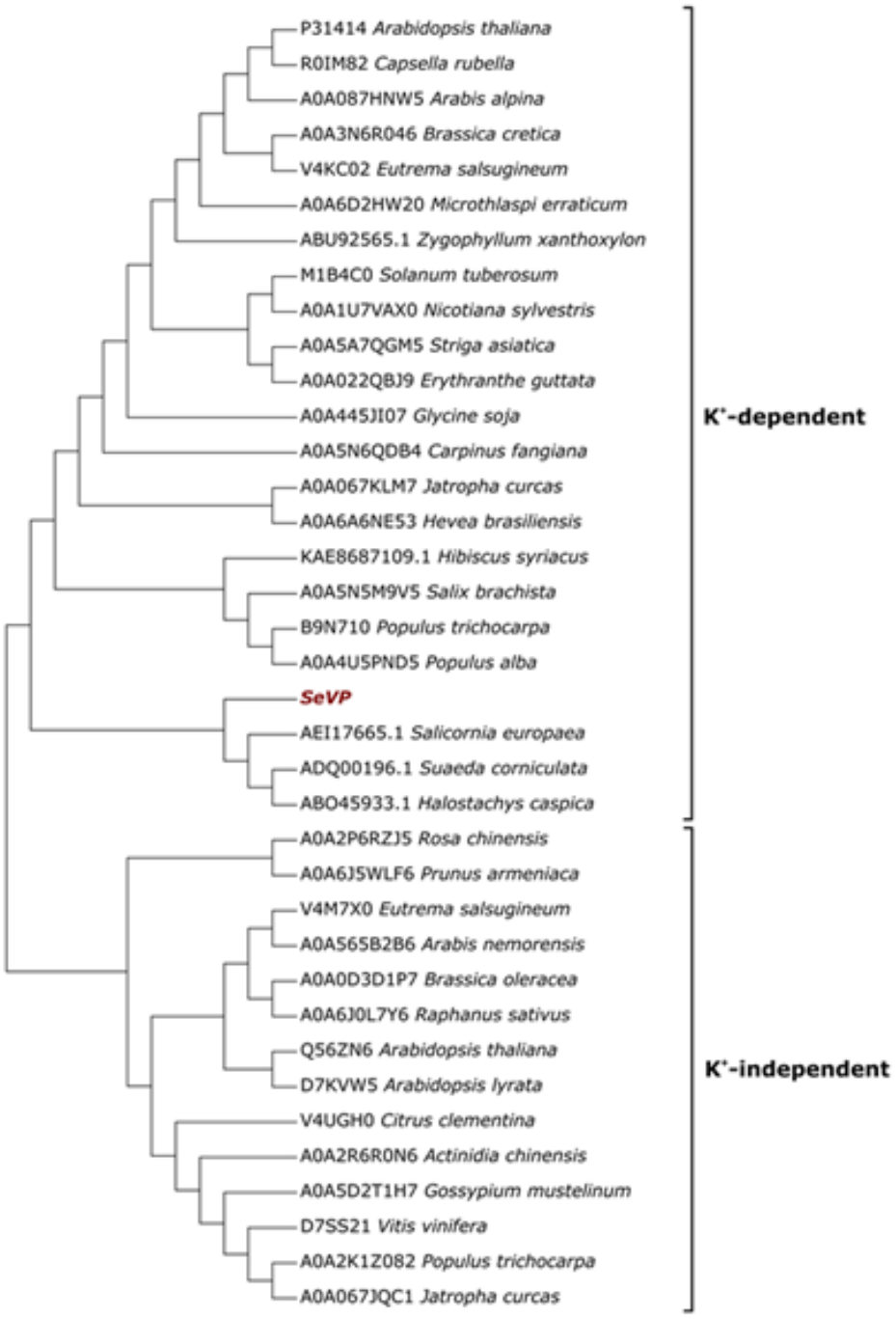


Fig. 6. Phylogenetic analysis of SeVP with others vacuolar H+ pyrophosphatase. Multiple sequence alignment and phylogenetic tree were conducted with MEGA7. SeVP is shown in red and is in the group of K+dependent. Protein sequences were retrieved from GenBank

The PPI of SeVP was represented by AVP1 from *Arabidopsis thaliana* and forecasted confidence scores of 0.959–0.814. The representative model was predicted to be interacting with ten proteins: NHX1, VHA-A3, VHA-A2, AT2G20050, AT2G25610, HA5, NHX3, VHA-C3, HA1, and SOS1 (Fig. 7). The network stats showed that AVP1 PPI comprised eleven nodes connected with 26 different edges.

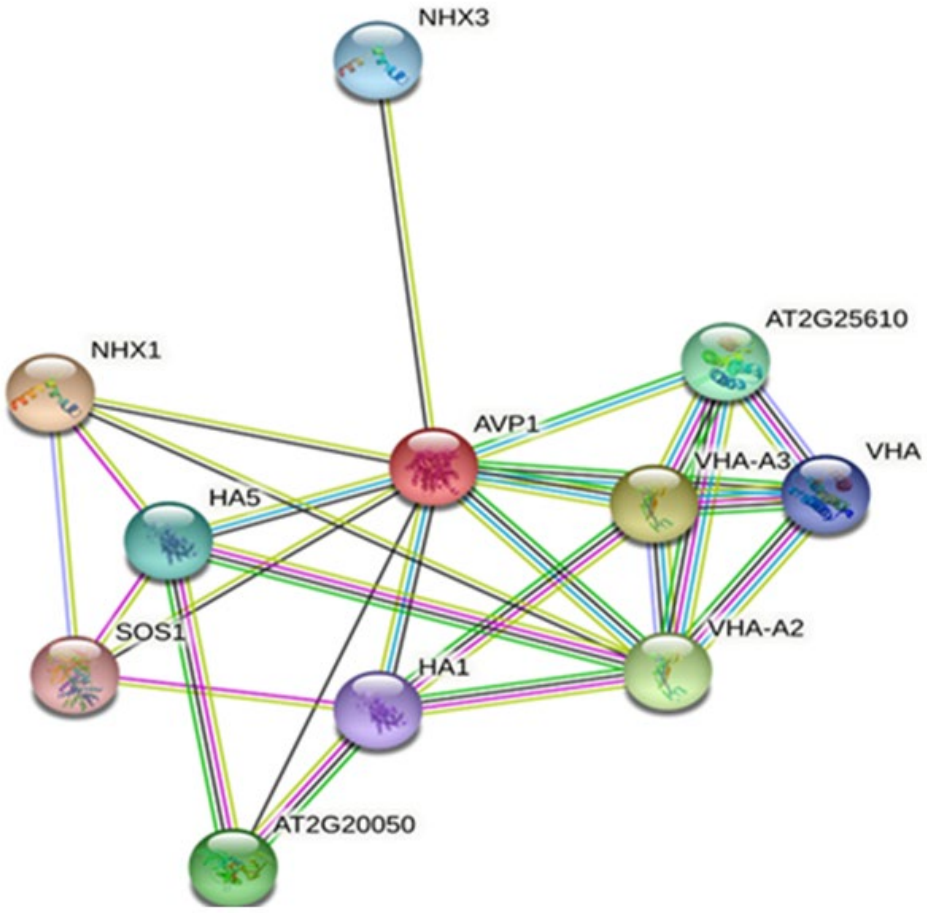


Fig. 7. Map of protein–protein interactions. AVP1 from *A. thaliana* represent to SeVP and the PPI network shows that all proteins interact with each other.

Real-time expression analysis of *SeVP* and *SeDREB2* showed statistically significant changes in their expression levels (Fig. 8). *SeDREB2* showed expression levels almost 10 times higher in the roots of *S. edulis* taken from the crater respect to those grown *in vitro*; in leaf, the increase was nearly 38 times more in crater plants. Se*VP* showed expression levels 14 times higher in the roots of *S. edulis* taken from the crater, concerning those grown *in vitro*; in leaf, the increase was only 7 times in crater plants than those cultivated *in vitro*.

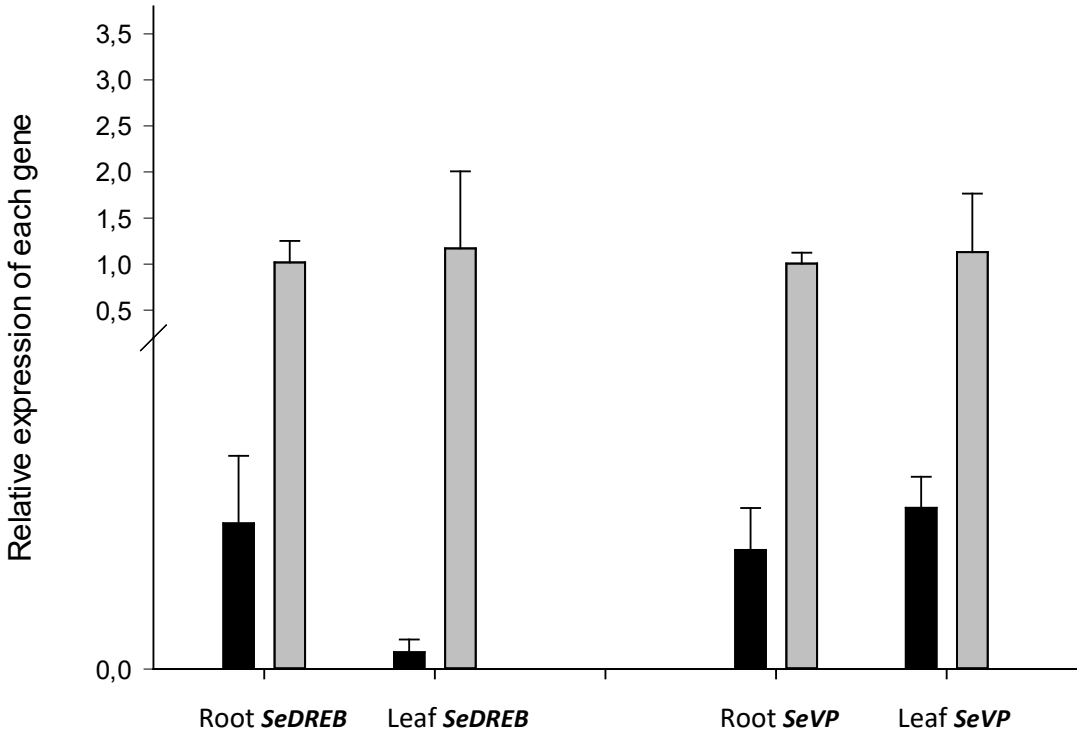


Fig. 8. Quantitative RT-PCR analysis of *SeDREB2*, and *SeVP*. The amount of cDNA templates in each sample was normalized by the amplification of *ß-tubulin*. Comparation between root and leaf from crater (gray bar) and from *in vitro* (black bar) cultures. Error bars represent the standard deviations with three replicates.

4. DISCUSSION

Salinity is one of the main problems for crops production throughout the world since it affects the osmotic potential of water in the soil and reduces its availability to plants (Gul *et al*., 2022). The excessive absorption of sodium and chloride in saline soils by plants impairs their growth and development due to the cytotoxicity of these ions and there is generally oxidative stress due to the generation of reactive oxygen species (ROS) (Isayenkov & Maathuis, 2019).

Halophytic plants have been useful for treating salinity-affected soils because they have salinity-resistant genes. This property may help plant breeders and molecular biologists to increase the salt tolerance of conventional crop plants in the future. Hence *S. edulis* could be used as a model plant for the study of genes resistant to salinity and for phytoremediation in saline soils.

Regarding phytoremediation, it is very important to know the amount of salts or metals in the soil and analyze the ability of plants to absorb them.

To determine the salinity of the soil, several parameters are analyzed, such as: 1) electrical conductivity, 2) pH; 3) soluble cations and anions; percent base saturation (PSB), sodium adsorption ratio (RAS) and percentage of exchangeable sodium (PSI)

(NOM-021-SEMARNAT-2000). The soil is considered saline if the EC value is greater than 4.1 and if it contains high concentrations of sodium, carbonates and sulfates; if the pH is higher than 8.5, the soil is strongly alkaline (NOM-021-SEMARNAT-2000). The results of the soil analysis from the habitat of *S. edulis* indicates that it is a saline-alkaline soil, which indicates that *S. edulis* has genes resistant to salinity. Figs. 1A-B shows *S. edulis* in its natural habitat.

The analysis of elements in the plant showed that there is more sodium in the leaf than in the root. The TF values for Na and Mn indicate that these elements are mobilized to the aerial part of the plant, unlike others. It has been reported that several species of *Suaeda* can carry sodium to their leaves and stems, and because they are succulent, they can dilute it, a mechanism that allows them to adapt and have a high tolerance to salt (Rahman *et al*., 2021). Different species of *Suaeda* have been successfully evaluated in soil phytoremediation (Mirza *et al.,* 2014; Yu *et al*., 2022). Due to its characteristics, *S. edulis* could be used for phytoremediation, especially in saline soils.

In recent years different *Suaeda* species such as *S. salsa, S. japonica, S. glauca, S. corniculate* have been studied regarding genes and proteins related to drought stress tolerance and salinity. Among the genes and proteins discovered are those related to ion regulation and compartmentalization, those involving osmoprotector synthesis and transcription factors, among others (Rahman *et al.,* 2021; Song *et al*., 2022; Yu *et al*., 2022; Xu *et al*., 2022).

In this work, we focused on the study of two genes: 1) the *DREB2* gen and 2) a vacuolar membrane proton pyrophosphatase protein (*V-+H Pasa* o *VPA*).

The deduced amino acid sequence of SeDREB2 features the AP2 DNA-binding and containing the conserved valine (14) and glutamic acid (E19) residues; these amino acids are conserved in the AP2/ERF domain and play a crucial role in DREB binding specificity (Allen *et al*., 1998; Chen *et al*., 2020). Transcription factors possessing this domain interact with cis-acting elements present in the promoter region of several stress response genes and thus switch on networks of genes at the same time to give a response (Sakuma *et al.,* 2002; Chen *et al*., 2020).

The phylogenetic tree presents six subgroups (A1-A6) with subgroup A2 containing SeDREB2. These results are similar to those reported by Sakuma *et al*., (2002) with the DREB genes of *A. thaliana* where the classification was based on the number of repetitions and sequence of the AP2 domain. They reported six subgroups where the DREB1/CBF and DREB2 proteins are classified in subgroups A1 and A2, respectively. DREB2 is mainly induced by dehydration and salt stress so SeDREB2 has the same characteristics while on the other hand DREB1 is induced by low temperatures (Sakuma *et al*., 2002).

PPI networks were used to identify the complex mechanisms and gain basic knowledge of molecular pathways implicated in the tolerance salinity processes in plants. SeDREB2 showed 62.5 % similarity with DREB2A of *A. thaliana* with one AP2 domain. The closest annotated interacting protein was DRIP2 E3 ubiquitin-protein ligase that acts as a negative regulator of the response to water stress (Phukan *et al*., 2017). ABF2 increases drought tolerance in vegetative tissues and is a leucine zipper transcription factor that binds to the abscisic acid (ABA)-responsive element (ABRE) motif in the promoter region of ABA-inducible genes (Phukan *et al.,* 2017). DRIP1, another ubiquitin-protein ligase that acts as a negative regulator of the response to water stress (Phukan *et al., 2017*). RCD1 protein acts under high salt or oxidative stress is found in the nucleus and cytoplasm

(Lindemose *et al.,* 2013). From PPI network analysis, it can be predicted that SeDREB2 may be a part of a tolerance system to salinity stress in *S. edulis*. Similar coexpression studies have been carried out, but with DREB gene promoters; in these there is a high rate of similarity with several post-drought and salinity stress response genes (Sazegari *et al*., 2015).

We also identified a partial fragment of a gene coding for inorganic vacuolar pyrophosphatase (H^+V-PPase). SeVP shows the [DE]X7KXE catalytic motif common to both soluble and membrane-associated H^+-PPase and works by binding hydrolyzing substrates on the membrane surface from the cytoplasm side (Lin *et al*., 2012). Universal polyclonal antibodies such as PABTK [TKAADVGADLVGKIE] and PABHK [HKAAVIGDTIGDPLK] have been generated which identify V-PPase polypeptides (Drozdowicz & Rea 2001). SeVP contains the PABTK region recognized by these antibodies. Another way to identify these polypeptides is by PCR amplification, including one or more of the five motifs present in all the V-PPase.

The phylogenetic alignment showed that SeVP is contained within the K-dependent group. In this regard, it has been shown that plant H+V-PPases have a high degree of similarity (80-93 %) and play an important role in the maintenance of cytoplasmic and vacuole pH; a process carried out by the translocation of protons into the vacuole through the hydrolysis of PPi (Lin *et al.,* 2012). These proteins are divided into two subfamilies: type I (K1-dependent) and type II (K1-independent). Type I are vacuolar while type II are found in the Golgi apparatus (Zhang *et al*., 2020).

In the protein-protein interaction, SeVP showed 77.5 % similarity with the pyrophosphate-energized vacuolar membrane proton pump 1 (AVP1) of *A. thaliana*. AVP1 encodes an H^+-translocating (pyrophosphate-energized) inorganic pyrophosphatase H^+-PPase located in the vacuolar membrane. The closest annotated interacting protein is NHX1; a sodium/hydrogen exchanger that acts in low-affinity electroneutral exchange of protons for cations Na^+ or K^+ across membranes involved in vacuolar ion compartmentalization necessary for cell volume regulation and cytoplasmic Na^+ detoxification (Wu *et al*., 2015). With NHX3, Na^+/H^+ (sodium hydrogen) exchanger may act in low-affinity electroneutral exchange of protons for cations such as Na^+ or K^+ across membranes (Wu *et al*., 2015; Fang *et al*., 2021). SOS is a sodium proton exchanger that functions in the extrusion of toxic Na^+ from cells and is essential for plant salt tolerance (Chen *et al*., 2015; Fang *et al*., 2021). It can be estimated from PPI network analysis that SeVP may be part of a tolerance salinity system through vacuolar ions compartmentalization to avoid Na^+ cytoplasmic toxicity.

The expression of the *DREB* genes has been investigated in several crops and with different types of abiotic stress. In this investigation, *SeDREB2* showed expression levels almost ten times higher in the roots of *S. edulis* taken from the crater to those grown in vitro; in leaf, the increase was nearly 38 times more in crater plants. In this regard, the overexpression of *DREB* in *S. salsa* was significantly induced by salinity and drought, and *SsDREB* was used to generate transgenic tobacco plants. The transgenic plants showed better growth, higher chlorophyll content, a net rate of photosynthesis, and a higher level of proline and soluble sugars, even in the presence of salinity and drought (Zhang *et al*., 2015).

SeVP showed 14 times higher expression levels in roots, and seven times in leaves of plants from the crater, concerning *in vitro* plants, probably because plants in the crater are in contact with high salts concentrations and carbonates. The H^+V-PPases raise

tolerance to salinity in various plant species, including *S. salsa,* by maintaining the ions Na^+ inside the vacuoles (Dubouzet *et al*., 2003). In studies with *S. corniculata*, another halophyte native to saline-alkaline soil, transcripts of these genes increased more in response to carbonates than NaCl (Rahman *et al.,* 2021).
In this work, the primers used are effective for the identification of members of this family of DREB y VPase genes in halophytic plants. With the partial sequences of both genes, a bioinformatic analysis could be carried out, which showed the high similarity with other homologous proteins reported in the database. The translocation factor showed that *S. edulis* can be useful in phytoremediation processes. Therefore, study of these genes and others related with saline stress is essential for future research to carry out work on the mechanisms of tolerance in plants of agricultural interest, such as been done in tree tomato (*Solanum betaceum*) (Jaramillo *et al*., 2018)

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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