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ORIGINAL ARTICLE

Identification and characterization of the EXPA7, EXPA18 and EXT10 genes in *Turbinicarpus lophophoroides* (Werderm.) Buxb. & Backeb; and their expression analysis in the root under abiotic stress

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Abstract

Expansin and extensin are proteins involved in resistance to various abiotic stresses by processes of cell wall modification and in the formation and elongation of the hairy root. They are located in several organs of the plant included root epidermis. *Turbinicarpus lophophoroides* is a cactus model to studies these genes in adventitious and transformed roots. In this study, we identified and characterized the expansin7, expansin18 and extensin10 genes in *T. lophophoroides*. Bioinformatic analysis indicated that the expansin sequences contained the motifs: HTFYG, HFD, YRR, VPC and YW; and certain conserved cysteine (C) residues. Regarding extensin10, the sequence contains the conserved SPPPP (SP4), YYS and YV motifs. The expression analysis in adventitious and transformed roots under osmotic stress (300 mM mannitol), heat (37 °C) and cold (4 °C); shows a higher expression of *TlExpA18* in both roots, a decrease in *TlExpA7* in transformed roots and a null expression in *TlExt10* in both roots. In addition, a morphological comparison of the maturation/differentiation zone, meristem and cap between adventitious and transformed roots by SEM was performed, finding differences in the quantity and length of the hairy roots and the shape of the root cap. Overall, the study concluded that *TlExpA18* and *TlExpA7* belong to expansin family and *TlExt10* belong to extensin family. The expression characteristics of *TlExpA18*, *TlExpA7* and *TlExt10* will facilitate the investigation of its function in stress response and other physiological processes in *T. lophophoroides*.

Keywords *Cactaceae* · Hairy roots · Abiotic stress · SEM

Introduction

Plants have evolved to detect subtle changes and cope with different types of stress, mainly abiotic ones. These can alter their metabolism and lead to adverse effects on their growth, development and productivity [21]. In general, two stress response strategies are recognized: resistance and tolerance. Both mechanisms involve morphological, physiological and biochemical changes, characteristic in each species [33]. These changes can manifest in different organs. The development of broader and deeper root systems has been observed, as well as an increase in the density of trichomes, the suppression of cell growth and the reprogramming of gene expression [6]. The gene products of these responses can be classified into two groups: (1) Chaperones, LEA proteins, osmotines, antifreeze proteins, aquaporins, osmolytes, proline and sugar transporters, detoxifiers and various proteases. (2) Regulatory proteins, transcription factors, phosphatases, kinases and signaling molecules [25].

Growing roots need cell expansion, and modulation of cell wall extensibility plays a central role in this phenomenon. Therefore, cell wall modifier proteins play an essential role in controlling cell wall plasticity/rheology; expansin and extensin are a couple of examples of these types of proteins [38].

Expansins are proteins that induce extensibility and relaxation of pH-dependent plant cell wall tension. Expansins belong to a protein superfamily divided into four families: α-expansins (EXPA), β-expansins (EXPB), α-expansin-like proteins (EXLA) and β-expansin-like proteins (EXLB) [38]. These proteins participate in cellular

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processes where the extension of the cell wall is crucial. They are located in radical and apical meristems, stem, growth zones and root epidermis [7, 22]. Some members of the expansin family are involved in root development and growth, for example; AtEXPA7 and AtEXPA18 [7], and GmEXPB2 [13]. Others are involved in the formation and elongation of the hairy root, such as GmEXP1 [23]. And others participate in cell elongation and lateral root generation, as well as in the formation of lateral cells in the root cap, for example: AtEXLA2 [4]. Abiotic stress conditions positively or negatively regulate the transcription of some expansin gene members, such as heat [47], water deficit [15], and phosphate (Pi) and iron (Fe) deficiency [13].

Another type of proteins involved in the modification of the cell wall in the root are extensins. These are glycoproteins whose function is found at a structural level, giving shape and size to the cell [24]. The amino acid sequences of extensins contain multiple sequence repeats such as: Ser-$(Pro)_3$, Ser- $(Pro)_4$ or O-glycosylated Ser- $(Pro)_5$, cross-linked Tyr (Y) motifs and an O-glycosylated arabinogalactan motif (AG) [32]. Extensins participate in the development of the hypocotyl, the stem [37] and hairy roots [46]. Furthermore, they participate in the defense against abiotic stress [41]. Hydroxyproline rich glycoprotein (HRGP) genes, including extensins, are known to be involved in hair root morphogenesis [3].

Cacti have metabolic, physiological and anatomical characteristics related to the extreme conditions that often form part of their habitats; such as low water availability, poor nutrient soils and high-temperature variations [2]. Among the species with these characteristics is *Turbinicarpus lophophoroides*, which is located mainly in the north-central region of México. They are small, globose or cylindrical plants, with ribs containing small tubers from which the areolas grow. They have variable spines, white or pink flowers and have two thickened primary roots [45]. They grow mainly in heavily drained rocky areas at altitudes between 300 and 3300 m above sea level [2]. Unfortunately, due to its low growth rate and the predation suffered by its populations due to anthropogenic causes, it is subject to special protection by Mexican regulations [2]. For these reasons, *in vitro* micropropagation schemes have been carried out to conserve the species and for research [8]. Among these studies, the induction and propagation of hairy roots [5] and analysis of secondary metabolites in transformed roots [42] have been analyzed.

In other plant species, it was observed that genes expressed in roots are involved in resistance to different abiotic factors. For example, in the soybean root system, the expression of the *GmExpB2* gene was analyzed; it codes for a β-expansin and is positively regulated by the deficiency of water, phosphate (Pi) and iron (Fe) [13]. Also, *IbEXPL1* and *IbExp1* in *Ipomoea batatas* showed modifications in their expression, altering root growth under cold stress [34].

In this study, the identification, characterization and bioinformatic analysis of the expansin7, expansin18 and extensin10 genes in *Turbinicarpus lophophoroides* was carried out. Their expression and morphological effects in transformed adventitious roots under different conditions of abiotic stress (osmotic stress, heat and cold) were analyzed.

Materials and methods

Plant material, growth conditions and treatments

T. lophophoroides seedlings obtained *in vitro* were incubated at 25 °C with a 16/8 h light-dark photo-period; and specimens were selected in relation to the generation of adventitious roots. Adventitious roots were separated from the seedling, inoculated on liquid MS medium and incubated in the dark with agitation (80 rpm). Samples from adventitious roots were collected after 35 days, according to the growth kinetics reported by Solis-Castañeda et al. [42]. Roots were collected and subjected to stress treatments, as described below.

For the transformed roots, *in vitro* cultures already established in the Plant Biotechnology Laboratory of the Universidad Autónoma de Aguascalientes, México were selected. The transformed roots were generated by *Agrobacterium rhizogenes* A4 agropine-type strain that contains the wild-type plasmid pRiA4, which confers the hairy root phenotype, and the binary vector pESC4, that contains the nptII gene and the gus gene in the T-DNA region [5]. The *in vitro* multiplication process of these roots was done in a 250 mL flask with liquid MS medium, without growth regulators at 25 °C under darkness and constant stirring at 80 rpm. Roots were collected at random to verify their transformation by PCR (looking for the presence of the *NtpII* and *GUS* genes) and by the GUS histochemical test. Once the transformation is verified, the roots were collected and subjected to stress treatments, as indicated below.

Both types of roots were subjected to stress treatment by osmotic shock, heat and cold [19]. For osmotic stress, 250 mg of roots were inoculated into150 mL of liquid MS medium supplemented with 300 mM mannitol in a 250 mL flask. This medium was kept at 25 °C for 12 h before being used for all the experiments. The experiment was carried out at 25 °C and samples were taken in triplicate at 0.5, 12, and 24 h under continuous agitation at 80 rpm. In cold stress, 250 mg of roots were inoculated on 150 mL of liquid MS medium in a 250 mL flask at 4 °C for 0.5, 12, and 24 h. And for heat stress, 250 mg of roots were inoculated into 150 mL of liquid MS medium in a 250 mL flask at 37 °C for 0.5, 12, and 24 h in an incubator with shaking at 80 rpm. The liquid

MS medium used was kept previously for 12 h at 37 °C. As controls, we used adventitious and transformed roots generated as mentioned above, without being subjected to any abiotic stress treatment. Each assay was done in triplicate. All samples were stored at −80 °C until RNA extraction.

Nucleic acid extraction

DNA extraction from adventitious roots in culture (not subjected to any type of stress) was followed the protocol described by Tel-Zur et al. [44] with modifications (polyvinylpyrrolidone (PVPP) in the extraction buffer and β-mercaptoethanol elimination). For total RNA extraction, the plants subjected to osmotic stress, heat and cold treatments were used. This was carried out with a commercial PureZOL kit (BIO-RAD, USA) according to the manufacturer's specifications. DNA and RNA integrity were confirmed by 1.0% agarose gel electrophoresis. The concentration and purity were analyzed by spectrophotometry with a NanoDrop 2000 spectrometer (Thermo Scientific, USA). cDNA synthesis was performed using the iScript Advanced cDNA Synthesis kit for RT-qPCR (BIO-RAD, USA), according to the manufacturer's specifications.

Identification and sequencing of the EXPA7, EXPA18 and EXT10 genes in *T. lophophoroides*

PCR was carried out from DNA extracted from *T. lophophoroides* roots with the GoTaq DNA Polymerase kit (Promega). The primers used for the amplification were: Exp7 For (5′-GCGGCGCTAAGCACGACAT-3′), Exp7 Rev (5′-ATAAAGCCGGGCCACCACAA-3′), Exp18 For (5′-GGCGCCCTCAAGAAAACAGA-3′), Exp18 Rev (5′-GTAAGAGGTGAGCCGGAACGAGA-3′) and Ext10 For (5′GGAGAAGAGCAAAGGCAACAAGAC-3′), Ext10 Rev (5′GGAAATCACGTAGGGCAGAAGAGT-3′). The amplification conditions were: 1 cycle (94 °C, 4 min), 35 cycles (94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min), and 1 cycle (72 °C, 5 min) (BioRad Gene Cycler). The amplified product was purified using a commercial PCR Clean-Up System kit (Promega) according to the manufacturer's specifications. Once purified, products were ligated into the Promega pGEM T-Easy cloning vector. The clones 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.

Bioinformatic analysis

The obtained nucleotide sequences were translated to amino acid sequences in the EXPASY platform (https://web.expasy.org/translate/) [11]. The search for homologous amino acid sequences was done with the BLASTP program in the NCBI platform (https://blast.ncbi.nlm.nih.gov/Blast.cg) [39]. The multiple sequence alignments were done with the UNIPROT tool - ClustalW method - in the European Institute of Bioinformatics (EMBL-EBI) platform (https://www.uniprot.org/align/) [31]. The search for domains in the putative amino acid sequences was done using the following databases: PROSITE (Database of protein domains, families and functional sites) (https://prosite.expasy.org/scanprosite/), PFAM (Protein Data Base) (https://pfam.xfam.org/search) and InterPro from EMBL-EBI (https://www.ebi.ac.uk/interpro/protein/) [11, 31]. Phylogenetic analysis of TlEXPA7 was performed with the expansin7 amino acid sequences of *G. raimondii* (XP_012488711.1), *R. chinensis* (XP_024174835.1), *O. sativa* (XP_015631937.1), *V. radiata* (XP_014506528.2), *B. rapa* (AGM16349.19), *Osmanthus fragrans* (AVT44074.1), *Capsella rubella* (XP_006303220.1), *B. nivea* (AVG44218.1), *M. notabilis* (XP_010108063.1) and *A. thaliana* (sp | Q9LN94). Two sequences of β expansins from *S. arundinaceum* (A0A2I6SQK7_9POAL) and *Z. mays* (NP_001105643.1) were used as an external group. Phylogenetic analysis of TlEXPA18 was performed based on expansin18 sequences from *A. thaliana* (NC_003070.9), *O. sativa* (NC_029258.1), *S. lycopersicum* (NC_015443.3), *B. rapa* (NC_024803.1), and *D. catenatunm* (0A2I0X7N5). The sequence of β-expansin18 from *Zea mays* (A0A3L6G8Q6) was used as the external group. Phylogenetic analysis of TlEXT10 was performed based on *A. thaliana* (OAO95970.1, NP_849895.1, NP_173553.1, AEE28829.1, AEE33968.2) and *B. napus* (AAM88422.1) extensin sequences. Two sequences of leucine-rich extensins, members of the of the hydroxyproline-rich protein (HRGP) superfamily, from *V. radiata* (XP_014506341.1) and *M. truncatula* (XP_024641590.1) were used as the external group. The evolutionary history was inferred using the maximum likelihood method, based on the Whelan and Goldman model. The tree concensus was calculated with an inferred bootstrap (1000 repetitions). Evolutionary analyzes were performed in MEGA7 [43].

Real-time PCR (qPCR)

For expression analysis, the Maxima SYBR Green/ROX Qpcr Master Mix 2X kit (Thermo Scientific) was used according to manufacturer's specifications. The primers used were: Exp7tr For (5′-GAGTGCCATGCCAAAGGAGTG-3′), Exp7tr Rev (5′-TGTAAGAAGTGACCCGGAAAGAGA-3′), Exp18tr For (5′CTATCGGCAGTTGCCTGGTT-3′), Exp18 tr Rev (5′-CTCCCATAGTTGCGCTGC-3′), Ext10tr For (5′-AGTCCTCGCCACTACCTTACT-3′) Ext10tr Rev (5′-AGCCGGGGACTGTACTAAAC-3′). The 25S ribosomal subunit was used as a reference gene with the primers:

F25S (5′-CGTAAGGCGTAAGGAAGCTG-3′) and R25S (5′-TCGGAGGGAACCAGCTACTA-3′). The reactions were run in a BioRad CFX96 Real-time System Thermal Cycler. The normalized relative expression was calculated by the $2^{-\Delta\Delta ct}$ method [29]. Statistical analyzes were carried out with the GraphPad Prism 6.0 program. To assess the significance of the observed differences a one-way ANOVA and a Tukey-Kramer test (α 0.05) were performed.

Morphological comparison of transformed and adventitious roots in *T. lophophoroides* by scanning electron microscopy

Samples were taken from both types of roots and fixed in 1.5% glutaraldehyde for 4 h and washed with 1X PBS. They were dried in a Smadri Tousimis critical drying point apparatus. Subsequently, they were mounted and coated with Gold with a Denton Vacuum Desk II device. The samples were analyzed in a JEOL JSM-5900LV scanning electron microscope with an acceleration voltage of 20 KV, and SEM-EDS RX 650X magnification.

Results

Identification and bioinformatic analysis of the *TlExpA7*, *TlExpA18* and *TlExt10* genes in *T. lophophoroides* roots

A 609 pb fragment was amplified for *TlExpA7*, a 309 pb fragment for *TlExpA18* and a 275 pb fragment for *TlExt10*. The sequences were deposited in the NCBI database with accession numbers: *TlExpA7* (MN990670) and *TlExt10* (MT017919). The *TlExpA18* sequence was not stored in the database because it did not meet the sequence nucleotide minimum requested by NCBI.

In the search for amino acid homologous sequences for the TlEXPA7 putative sequence, it was found that there is a 76.5% similarity with AtEXPA7 from *A. thaliana* (Q9LN94), 72.9% with *Brassica campestris* (A0A3P5YKR5) and 74.7% with *Capsella rubella* (R0IH20). Multiple sequence alignments with various plant expansin7 sequences (XP_018438451.1, XP_012488711.1, XP_024174835.1, XP_015631937.1, XP_014506528.2, sp.|Q9LN94, XP_006303220.1, XP_013732375.1, AVG44218.1, XP010 XP_010054792.1) show that there is a high degree of conservation (Fig. 1a). The putative amino acid sequence of TlEXPA7 presented the highly conserved HFD motifs, as well as four of the six cysteine residues (C) that forming disulfide bonds and tryptophan (W) residues present in all expansins (Fig. 1a). With respect to conserved domains, the presence of a domain I fragment present in expansins was found towards the amino-terminal end; and a domain II fragment was found near the carboxyl-terminal end (Fig. 1a). The phylogenetic analysis showed two perfectly defined clades between α and β expansins7. The obtained sequence forms a 100% supported clade together with expansin7 from *A. thaliana* (Fig. 1b).

For the TlEXPA18 putative amino acid sequence, a 90.9% similarity was found with AtEXPA18 from *A. thaliana* (NC_003070.9). A multiple sequence alignment with various expansins18 sequences (*A. thaliana* (NC_003070.9), *O. sativa* (NC_029258.1), *S. lycopersicum* (NC_015443.3), *D. catenatum* (NC_024803.1), *B. rapa* (A0A2I0X7N5)) showed very high similarity. Likewise, the conserved motifs HTFYG y TMG present in expansins were found (Fig. 2a). The phylogenetic tree showed two perfectly defined clades between α and β expansins18. The sequences obtained form two well-supported clades, one where only the sequence obtained from *T. lophophoroides* is included, and in the other, the rest of the analyzed sequences. Furthermore, the cladogram shows our sequence as one of the first to diverge (Fig. 2b).

For the TlEXT10 putative amino acid sequence an 83.3% similarity was found with the AtEXT10 of *A. thaliana* (OAO95970.1). A multiple sequence alignment (*A. thaliana* (OAO95970.1), *C. rubella* (XP_023633611.1), *C. sativa* (XP_010480949), *A. thaliana* (AEE28829.1)) shows the presence of highly conserved SPPPP (SP4), YYS and YV motifs (Fig. 3a). The phylogenetic tree shows two perfectly defined clades conformed by proline-rich extensins and leucine-rich extensins. The cladogram shows four well-supported clades, where TlEXT10 shares a clade with *A. thaliana* extensin 10, and in the remaining clades the rest of the extensins are grouped (Fig. 3b).

Expression analysis of the *TlExpA7*, *TlExpA18* and *TlExt10* genes

The expression analysis showed that for *TlExpA7* in adventitious roots, there is no expression under any of the treatments tested in this study (data not shown). In the transformed root, expression levels decreased in a general way in the three treatments and in all their times. The heat stress analysis showed a decrease in expression progressively from 24 h to 0.5 h with a significant decrease in expression at 0.5 h (Fig. 4, 1a). Regarding cold stress treatment, the analysis showed a significant decrease in expression at 12 and 24 h (Fig. 4, 1b), and under osmotic stress at all times compared to the transformed control root (Fig. 4, 1c).

TlExpA18 expression in adventitious roots subjected to heat (37 °C) showed an increase in the expression for all times with respect to the control (not subjected to stress). The highest expression was at 24 h, while at 12 h and 0.5 h

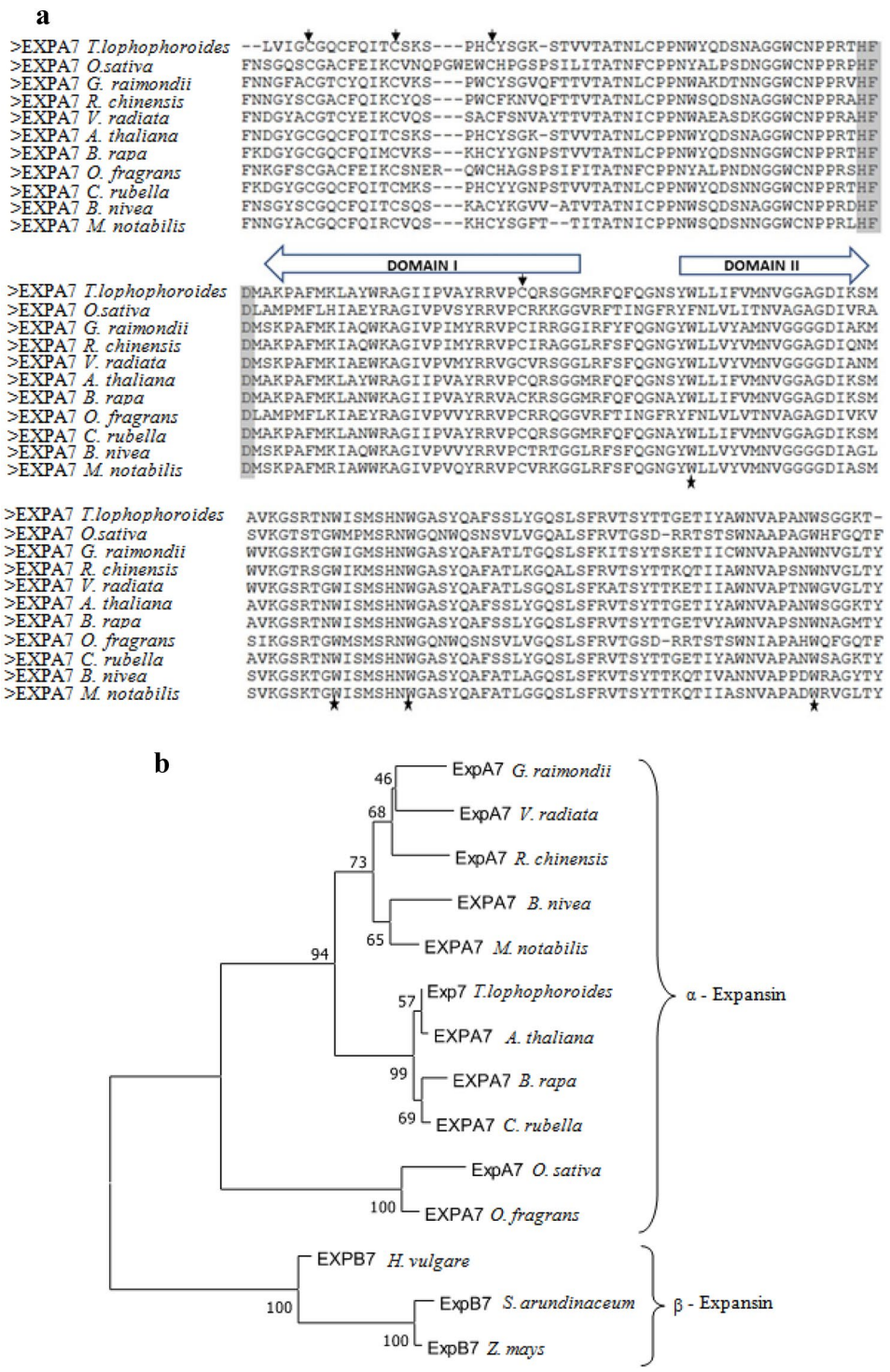


Fig. 1 Multiple sequence alignment and phylogeny of the putative TlEXPA7 partial sequence. (**a**) Multiple sequence alignment - Clustal method W, where the conserved residues between sequences are indicated, (**b**) Maximum Likelihood Phylogenetic tree, (Whelan and Goldman model), with a 1000 repetition Bootstrap. Two defined clades: α-expansins7 and β-expansins7 are evident. The arrowheads show four conserved cysteines in domain I, the stars indicate conserved Tryptophan residues in domain II, and the shaded area indicate the conserved HFD motif

there were no significant differences (Fig. 4, 2a). Regarding the cold treatment (4 °C), it was observed that expression increases as time progresses, with a maximum expression level at 24 h (Fig. 4, 2b). Finally, the osmotic stress treatment (300 mM mannitol) showed its maximum expression at 24 h, while at 0.5 h and 12 h there were no significant differences (Fig. 4, 2c). On the other hand, the transformed roots showed highly significant increases for all treatments at all times in general. The heat stress analysis showed an increase in expression progressively from 0.5 h to 24 h with substantial differences in relation to the control (Fig. 4d). Regarding cold stress treatment, the analysis showed the

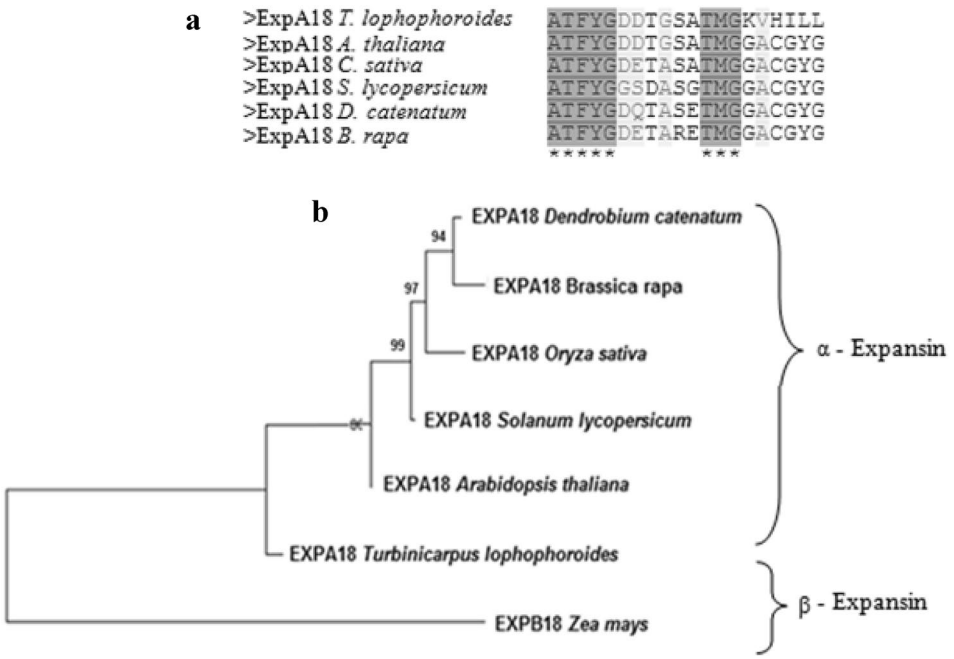


Fig. 2 Multiple sequence alignment and phylogeny of the putative TlEXPA18 partial sequence. (**a**) Multiple sequence alignment - Clustal method W where the conserved residues between sequences are indicated, (**b**) Maximum Likelihood phylogenetic tree (Whelan and Goldman model) with a 1000 repetition Bootstrap. Two defined clades: α-expansins18 and β-expansins18 are evident. Asterisks show the conserved ATFYG and TMG motifs characteristic of expansins

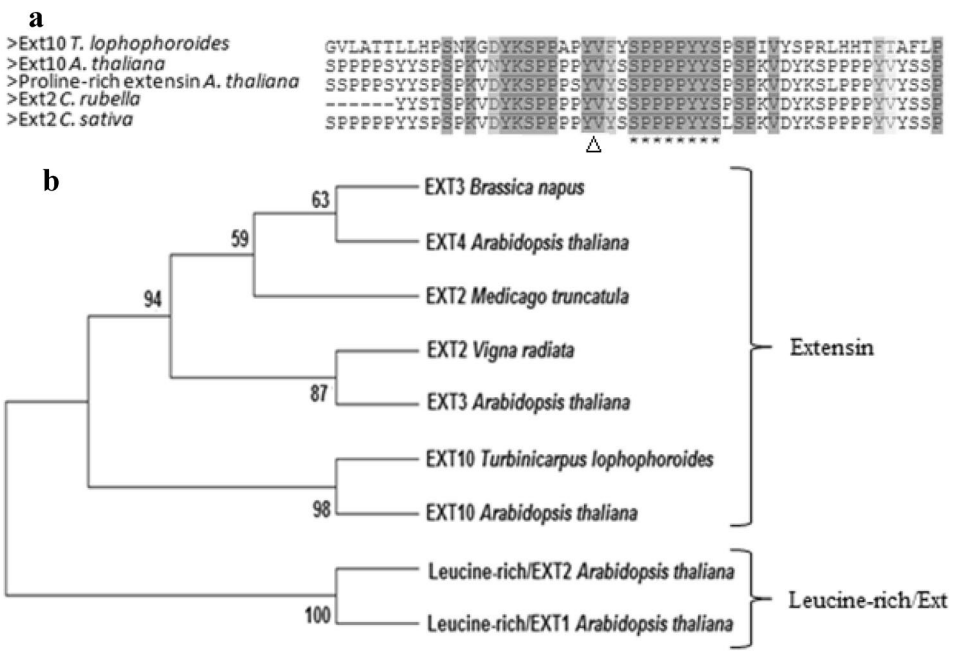


Fig. 3 Multiple sequence alignment and phylogeny of the TlEXT10 partial sequence. (**a**) Multiple sequence alignment - Clustal method W where the conserved residues between sequences are indicated, (**b**) Maximum Likelihood phylogenetic tree (Whelan and Goldman model) with a 1000 repetition Bootstrap. Two defined clades: extensins and Leucine-rich extensins are evident. The asterisks show the conserved SPPPP and YYS motifs and the triangle show YV motif conserved of extensins

highest peak at 30 m with a downward trend at 12 and 24 h, respectively (Fig. 4e). In the osmotic stress treatment, the highest expression level was achieved at 0.5 h, and as time progressed, the expression decreased (Fig. 4e).

Finally, the expression level of the *TlExt10* gene in adventitious and transformed roots was determined under the treatments mentioned earlier. In no type of roots or under any treatment was detected the expression of *TlExt10*.

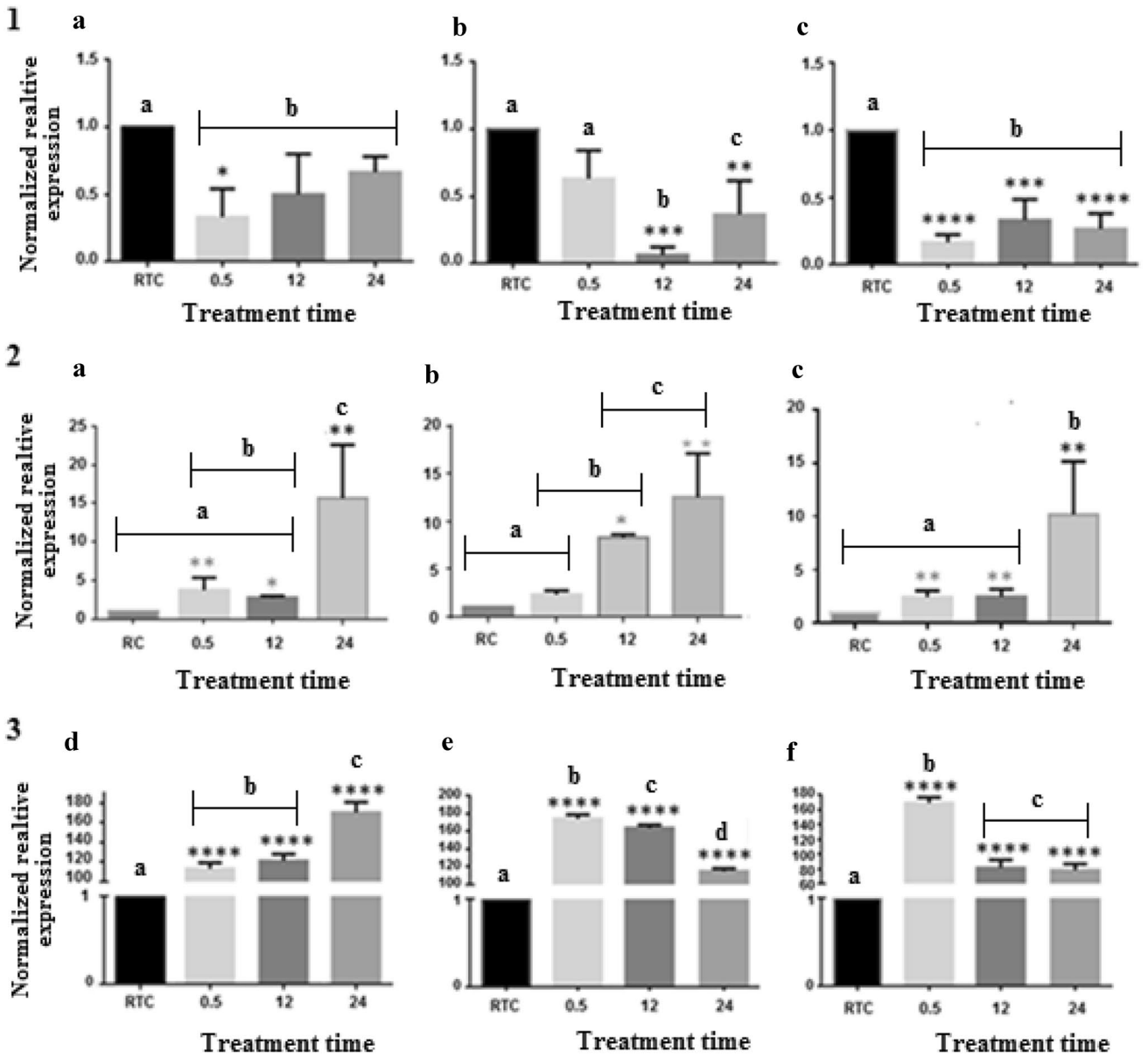
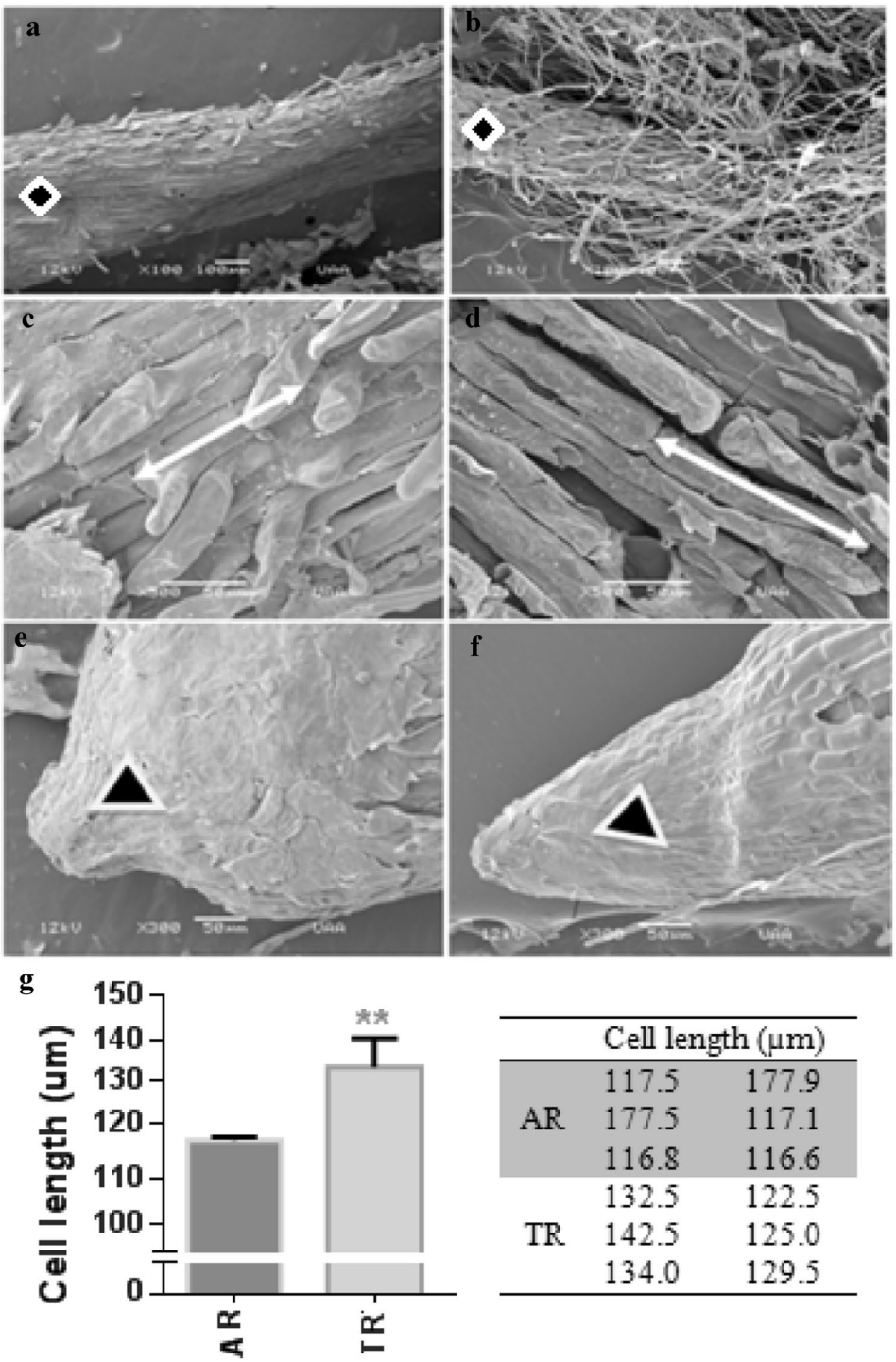


Fig. 4 Analysis of expansins expression in transformed roots in *T. lophophoroides* under abiotic stress. (1) Transformed root (*TlExpA7)*, (2) adventitious root (*TlExpA18*), (3) transformed root (*TlExpA18*), (**a** and **d**) heat stress (37 °C), (**b** and **e**) cold stress (4 °C), and (**c** and **f**) osmotic stress (300 mM mannitol) at 0.5 h, 12 h, and 24 h. The normalized relative expression was calculated by the $2^{-\Delta\Delta ct}$ method. Variability between treatments was determined with a one-way ANOVA test and a Tukey-Kramer test (α 0.05). (RC) Adventitious root control, (RTC) transformed root control. N = 3

Morphological comparison of the maturing/differentiation zone, meristem, and cap of transformed and adventitious roots in *T. lophophoroides* by scanning electron microscopy (SEM)

In the maturation/differentiation zone, the initiation of the hairy root is seen in both cases, although in greater quantity and length in the transformed root (Fig. 5a, b). Likewise, its cellular comparison shows an increase in the length of the transformed root (Fig. 5c, d). In the cap of the transformed roots, a more pointed and elongated column and an extended lateral zone is observed. The meristematic region and the cap appear flat (Fig. 5e, f).



	Cell length (µm)	
AR	117.5	177.9
	177.5	117.1
	116.8	116.6
TR	132.5	122.5
	142.5	125.0
	134.0	129.5

Fig. 5 Morphological comparison of adventitious and transformed roots in *T. lophophoroides* by SEM. Maturation/differentiation zone of (triangle), (**a**) Adventitious root, and (**b**) Transformed root, Maturation/differentiation zone at the cellular level of, (**c**) Adventitious root, and (**d**) Transformed root, Meristematic zone and cap of (diamond), (**e**) Adventitious root, and (**f**) Transformed root, (**g**) Table and graph where it is found that there is a significant difference between the length of the cells in the maturation/differentiation zone between adventitious and transformed roots. (AR) adventitious roots, (RT) transformed roots. In general, a greater number and length of hairy roots, and cellular and cap elongation are observed in the transformed roots compared to the adventitious ones

Discussion

The putative TlEXPA7 amino acid sequence showed a fragment from domain I towards the amino terminus, homologous to the catalytic domain of members of the 45 family of glucoside hydrolases (GH45), and part of domain II, towards the carboxyl terminus; homologous to group II grass pollen allergens (CBM63) [38] (Fig. 1a). The fragment containing domain I has six cysteine residues (C) and the HFD motif. These cysteines are essential for the formation of disulfide bridges, which favor the folding of the six-strand DPBB (Double Psi Beta Barrel) structure. The HFD motif, together with the DPBB structure, form a groove for substrate binding, suggesting this is the active site of the protein [38]. Members of the EXLA and EXLB families do not possess the HFD motif [38].

Domain II presents aromatic amino acids Y, and W residues. Expansins are characterized by the presence of highly conserved polar and aromatic amino acids (two tryptophan residues and one tyrosine residue) that form a flat platform that could favor polysaccharide binding [38]. Domain II has a β-sandwich fold formed by two covers of four antiparallel β sheets each; this is the most common folding in carbohydrate-binding modules that generally bind to substrates such as crystalline cellulose or chitin [17]. Phylogenetic analysis grouped this sequence with the rest of α expansins 7 from other species (Fig. 1b); this shows that the obtained sequence has similarities with sequences of the same gene in different plant species.

On the other hand, the multiple sequence alignment of the TlEXPA18 putative amino acid sequence (Fig. 2a) showed the conserved ATFYG motif. This motif is found in domain I, after the signal peptide of all α and β-expansins, and is accompanied by conserved cysteine residues [26, 38]. Furthermore, in the search for homologs, the maximum similarity was found with *A. thaliana's* AtEXPA18. TlEXP18 phylogenetic analysis grouped the sequence with the rest of α expansins18 from other plant species (Fig. 2b). These results suggest that TlEXP18 is an α-expansin 18, although a complete sequence is necessary to achieve a complete characterization of the TlEXP18 gene and protein.

In the case of TlEXT10, the putative amino acid sequence contains the highly conserved motifs Ser-Pro-Pro-Pro-Pro (SPPPP, SP4), YY, and YV. In general, extensins are proteins that contain multiple Ser- (Pro) 3-5 repeats, Ser-Pro-Ser-Pro (SPSP) and Tyr (Y) motifs [32]. Ser-Pro's rigid hydrophilic repeating motifs undergo post-translational modifications; they are converted to Hyp and are O-glycosylated to give molecular rigidity and ability to move. Furthermore, the YxY and V – Y – L hydrophobic motifs ("x" = Lys (L), T, Leu (L), or Val (V)) give it the potential to generate cross-links, hydrophobicity and molecular rigidity [18]. Furthermore, the phylogenetic analysis grouped the sequence with *A. thaliana* AtEXT10, separating it from the rest of the extensins and other members of the superfamily (Fig. 3b). With these results, it can be concluded that the amplified fragment corresponds to an extensin 10 in *T. lophophoroides*.

Due to the importance of expansins and extensins in root systems and their contribution to the generation and morphogenesis of hairy roots, an expression analysis of the *TlExpA7, TlExpA18* and *TlExt10* genes and a morphological comparison between the transformed and adventitious roots in *T. lophophoroides* were carried out. In the expansin expression analysis, in the three analyzed treatments, there was no *TlExpA7* expression in the adventitious roots, while there was a decrease in the transformed roots (Fig. 4, 1). These results contrast with those found in hairy roots in *A. thaliana* in which *Exp7* is specifically overexpressed in hairy root cells [7, 16, 28]. Otherwise, an increase in expression was observed in both types of roots for *TlExpA18* (Fig. 4, 2). These results are consistent with those observed in hairy roots from *A. thaliana*, where there was an overexpression of this gene specifically in hairy root cells [7, 28]. Kim et al. [20] showed that both genes have almost identical spatiotemporal expression patterns in hairy root morphogenesis, something not observed in this study for *T. lophophoroides*.

Several studies have found a relationship between expansin expression changes in plants, induced by abiotic stress. For example, the *PpExp1* gene in transgenic tobacco plants led to a better tolerance and adaptation to heat (35 °C) [48] and *ExpA5* in *B. napus* plants subjected to heat stress was negatively regulated ten-fold [51]. Furthermore, during cold acclimatization, the expression of expansin genes was negatively regulated in sweet potato [34] and positively in *O. sativa* and *A. thaliana* [14, 49]. Also, the overexpression of *TaExpB23* and *RhExpA4* in transgenic plants conferred greater tolerance to drought stress [27, 30]. Hence, these results indicate species specificity and/or expansin isoforms in response to different types of abiotic stress.

TlExt10 did not express itself under the abiotic stress treatments tested here. These results indicate that its participation in response to these types of stress in *T. lophophoroides* roots is probably null. Compared with the results of other studies, it was observed that genes that encode cell wall proteins were positively regulated up to 2–3 times after their first exposure to high-temperature conditions (37 °C), including extensins [50]. Seki et al. [40] showed that genes encoding for extensins were down-regulated in *Arabidopsis* under a cold stimulus. Furthermore, differences in the expression of extensins were reported in cold affected *Solanum tuberosum* (4 °C), these differences were associated to increased cell wall stiffness and resistance to cell collapse [35]. Together these results show that the expression of extensin 10 is dependent on plant species and extensin type.

A morphological comparison between transformed and adventitious roots in *T. lophophoroides* was made (Fig. 5). In the transformed root (Fig. 5b, d, f), differences were observed in the quantity and length of the hairy roots; and in the cell length and the shape of the cap. This was due to the insertion of the rol genes (*rolA*, *rolB*, *rolC*, *rolD*) and auxin biosynthesis (indoleacetic acid (IAA)) from the T-DNA of the Ri plasmid from *A. rhizogenes* [12]. It is important to consider that the analysis results of *TlExpA7* and *TlExpA18* expression could be due to the hormonal regulation of auxins, especially IAA. It has been established that IAA has a significant effect on the diameter and length of roots in cacti [1], and in the number of adventitious roots produced [10]. Those increases are due to growth induction by rapidly stimulating the synthesis of cell wall components. Several

authors have confirmed the specific role of auxins, including IAA, in the activation or repression of genes that degrade the cell wall in *F.ananassa* [9]. For example, the repression of the *FaExp1* and *FaExp2* genes in *F. ananassa* and the activation of *FaExp5* in *F. chiloensis* [9]. Furthermore, it is also likely that auxins can regulate the activity of expansins at the post-transcriptional level through their effects on the cell wall pH [36].

Conclusion

A fragment of the genes for expansin7, expansin18 and extensin10 was identified and characterized in *T. lophophoroides*. Expression analysis showed that *TlExpA18* increased its expression levels in adventitious and transformed roots under osmotic, heat, and cold stress at all observed times. The *TlExpA7* gene did not show expression in adventitious roots, although it showed a decrease in transformed roots. Regarding the *TlExt10* gene, it did not show expression in any type of roots or under any treatment.

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Authors contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juan Pablo Martínez Vázquez, Abraham Loera Muro, Yenny Adriana Gómez Aguirre and José Francisco Morales Domínguez. The first draft of the manuscript was written by Juan Pablo Martínez Vázquez and José Francisco Morales Domínguez and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflicts of interest We declare that we do not have conflicts of interest.

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