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ARTICLE

Cloning and Bioinformatics Analysis of the *GlROP6* gene in *Glehnia littoralis*

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ABSTRACT

Rho-related GTPase from plants (ROP) proteins play an essential role in plant stress resistance. In this study, the full-length *GlROP6* gene was cloned based on *G. littoralis* transcriptome sequencing data acquired in response to salt stress. The protein sequence, conserved domains, secondary structure, three-dimensional structure, phylogenetic relationships, and expression pattern of the *GlROP6* gene were systematically analysed. Our results showed that the full-length *GlROP6* gene had an open reading frame of 606 bp, which encoded 201 amino acid residues with a relative molecular weight of 22.23463 kDa and a theoretical isoelectric point of 9.06. Amino acid sequence analyses indicated that the structure of the GlROP6 protein was conserved, and included five G-box motifs (G1–G5), an effector binding region, a Rho insert region and a C-terminal hypervariable region. According to our phylogenetic analysis, the GlROP6 protein was closely related to the ROP protein of *Daucus carota* subsp. *Sativus*. Our quantitative real-time PCR results revealed that *GlROP6* was highly expressed in flower, and *GlROP6* expression was significantly upregulated in *G. littoralis* roots treated with NaCl. This study will facilitate investigations into the function of *GlROP* genes in response to salt stress in *G. littoralis*.

KEYWORDS

GlROP6 gene; *Glehnia littoralis*; salt stress; expression analysis

1 Introduction

Glehnia littoralis Fr. Schmidt ex Miq. is an important medicinal halophyte that belongs to the Umbelliferae family. *Glehnia littoralis* grows on the seashores of countries such as China, Japan, the Korean Peninsula, Russia, and the United States [1]. Radix Glehniae is the dried root of *G. littoralis*, which has been used in traditional Chinese medicine for clearing away lung-heat, tonifying the stomach, nourishing yin and anti-inflammation [2]. *G. littoralis* exhibits strong salt-resistance. Its roots can penetrate deep into the sand layer of coastal beaches and mix with those of other plants to form coastal vegetation communities, which play important roles in coastal sand fixation and in improving saline-alkaline soils. Previous studies of *G. littoralis* have focused on its chemical components [2,3], pharmacological activities [4], and cultivation; however, few studies exploring the salt tolerance mechanisms and functional gene information of *G. littoralis* have been performed.

Small GTPases, having masses of 21 to 30 kD, are monomeric guanine nucleotide binding proteins related to the α subunit of heterotrimeric G proteins [5]. ROP proteins participate in an array of physiological processes including pollen tube growth [6,7], root hair development [8], vesicle trafficking [9], hormone signaling [10], and pathogen responses [11]. ROP-interactive CRIB motif-containing protein (RIC1) is microtubule-associated protein that is an effector of ROP. Recently, Li et al. [12] demonstrated that constitutively active ROP2 promoted both the reassembly of microtubules and the survival of *Arabidopsis* seedlings under salt stress via the ROP2-RIC1 pathway. In addition, Zhang et al. reported that *MaROP5g* overexpression enhanced salt tolerance in *Arabidopsis* by increasing root length, reducing membrane injury, and improving ion distribution [13].

Since the first *ROP* gene was isolated from pea plants, multiple *ROPs* have been described in numerous plant species: 11 in *Arabidopsis thaliana* [14], 9 in *Zea mays* [15], 7 in *Vitis vinifera* [16], 7 in *Oryza sativa* [17], 7 in *Medicago truncatula* [18], 9 in *Solanum lycopersicum* [19], and 17 in *Musa acuminata* [13]. To date, there have been no reports published describing *ROP* genes in *G. littoralis*. In this study, we identified and cloned the *GlROP6* gene based on previous *G. littoralis* transcriptome sequencing data acquired in response to salt stress [20]. We systematically analysed the protein sequence, conserved domains, secondary structure, three-dimensional structure, and phylogenetic relationships of GlROP6 using bioinformatics methods. Additionally, we performed quantitative real time polymerase chain reaction (qRT-PCR) analysis to examine *GlROP6* expression in various tissues and under salt stress. This comprehensive study of GlROP6 will aid future research into the salt tolerance mechanisms of *G. littoralis*.

2 Materials and Methods

2.1 Plant Materials

G. littoralis seeds were originally collected from Tannanwan Beach, Pingtan, Fujian Province, China (25°26′1.86″N, 119°45′14.4″E) and cultivated at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences, Nanjing, China. *G. littoralis* seedlings were grown in nutrient-enriched sandy soil under a 14-h light (26°C)/10 h dark (22°C) photoperiod. After 3 months of pot growth, the seedlings were treated with 200 mM NaCl for 0, 6, and 24 h. *G. littoralis* roots were sampled separately at various time points, as described previously [21]. Root, stem, leaf and flower tissues of *G. littoralis* were separately harvested throughout the same year to examine tissue-specific expression patterns of *GlROP6*. Duplicate samples were immediately frozen in liquid nitrogen and stored at –80°C for subsequent RNA extraction.

2.2 Total RNA Extraction and cDNA Synthesis

Total RNA of each *G. littoralis* sample was extracted using Trizol reagent (TaKaRa, Dalian, China) following the manufacturer's instructions. Complementary DNA was synthesised using a Prime Script™ RT Reagent kit with gDNA Eraser (TaKaRa, Dalian, China). cDNA products were stored at –20°C prior to qRT-PCR analysis.

2.3 Cloning and Sequencing of the GlROP6 Gene

Based on previous *G. littoralis* transcriptome sequencing data acquired in response to salt stress, we cloned the *GlROP6* gene. Specific primers were designed using the Primer Premier 5.0 software (Tab. 1). PCR amplifications were performed using KOD Plus neo enzyme (TOYOBO, Osaka, Japan). The PCR conditions used were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 1 min, with a final extension at 72°C for 8 min. The amplification products were tested by cloning into a pEASY-Blunt cloning vector (TransGen Biotech, Beijing, China) and sequenced.

Table 1: Primers used for PCR amplification

Gene name	Primer	Forward primer sequence	Reverse primer sequence
GlROP6	*GlROP6*	ACACACACACGCACACACTAGTTGGTG	GGAAGAAATCTTAGGGGAATCTTCATCG
GlROP6	*GlROP6-qRT*	TACAGTTGGGGATGGAGCTG	ACCAGCAGTATCCCACAGAC
GAPDH	*GAPDH-qRT*	ACCTTCTTTGCACCTCCCTT	GCTGTCTTTGGTTGCAGGAA

2.4 Bioinformatics Analysis of the GlROP6 Protein

The molecular weight, theoretical isoelectric point, and amino acid sequence of GlROP6 were predicted using ProtParam (http://web.expasy.org/protparam/). The GlROP6 and AtROP6 protein sequences were aligned using DNAMAN software. Protein hydrophobicity was predicted using ProtScale (http://web.expasy.org/protscale/). The secondary structure of GlROP6 protein was analysed using SOPMA (http://npsa-prabi.ibcp.fr/), and its tertiary structure was predicted using SWISS-MODEL (http://www.swissmodel.expasy.org/), which was then analysed with PyMOL software. The full-length GlROP6 amino acid sequence was aligned with the ROP protein sequences of 15 representative plant species using the ClustalW program. Amino acid sequences were acquired from the NCBI database. A Neighbour-Joining (NJ) phylogenetic tree was constructed using the MEGA5.0 program. Bootstrapping was performed with 1000 replications and other parameters remained default.

2.5 qRT-PCR Analysis

qRT-PCR amplification was performed using an Applied Biosystems StepOne™ Real-Time PCR System (Thermo Scientific, Waltham, MA, USA) with TB Green Premix Ex Taq (TaKaRa, Dalian, China). Primers used for the qRT-PCR experiment were designed using Primer3 Tools software (http://primer3.ut.ee/) (Tab. 1). The specificity of the primer pairs was assessed based on their melting curve analysis. The qRT-PCR conditions were as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. A melting curve analysis was performed following amplification. *GAPDH* were used as the internal control. Relative gene expression was examined using the $2^{-\Delta\Delta Ct}$ method [22]. Three biological replicates were performed for each sample.

3 Results

3.1 Cloning and Homology Analysis of the GlROP6 Gene

Based on the data acquired from a previous transcriptomics experiment, we identified and amplified the full-length *GlROP6* gene from *G. littoralis* via PCR. The *GlROP6* was named according to its strong sequence similarity to the *A. thaliana* gene *AtROP6*. The full-length *GlROP6* gene had an open reading frame (ORF) of 615 bp (Fig. 1). The GlROP6 protein sequence was aligned with homologous protein sequences of various plant species on NCBI. The results showed that GlROP6 shared 96.52%, 88.27%, 88.27%, 85.07%, 85.43% amino acid identity with homologous proteins of *Daucus carota* subsp. *Sativus* (XP_017244791.1), *Rhodamnia argentea* (XP_030516247.1), *Rhodamnia argentea* (XP_030553156.1), *Punica granatum* (PKI67829.1), and *Sesamum indicum* (XP_011093501.1), respectively.

3.2 Molecular Characteristics of the GlROP6 Protein

The molecular weight, theoretical isoelectric point, and amino acid number of GlROP6 were analyzed using ProtParam. We found that GlROP6 encoded 201 amino acid residues, which had a relative molecular weight of 22.23463 kDa and a theoretical isoelectric point of 9.06. GlROP6 was a hydrophilic protein.

The hydropathy plot revealed that GlROP6 exhibited the highest hydrophobicity at amino acid position 89 (score: 3.044) and the lowest hydrophobicity at amino acid position 191 (score: –3.489) (Fig. 2).

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  1 M  G  S  N  V  A  S  T  T  K  F  I  K  C  V  T  V  G  D  G  A  V  G  K  T
  1 ATGGGTAGTAATGTTGCAAGTACAACAAAGTTTATAAAATGTGTTACAGTTGGGGATGGAGCTGTTGGCAAGACT
 26 C  L  L  I  S  Y  T  S  N  T  F  P  T  D  Y  V  P  T  V  F  D  N  F  S  A
 76 TGTCTTCTTATTTCTTACACTAGCAACACCTTTCCTACTGATTATGTACCTACTGTGTTTGATAACTTTAGTGCA
 51 N  V  V  V  D  G  K  T  I  N  L  G  L  W  D  T  A  G  Q  E  D  Y  N  R  L
151 AATGTTGTTGTTGATGGGAAGACTATAAACCTGGGTCTGTGGGATACTGCTGGTCAAGAAGACTATAATCGGCTA
 76 R  P  L  S  Y  R  G  A  D  V  F  I  L  A  F  S  L  I  S  K  P  S  F  E  N
226 AGGCCTCTTAGTTACAGAGGAGCTGATGTTTTCATCCTTGCCTTCTCTCTCATTAGCAAGCCTAGCTTTGAGAAC
101 I  A  K  K  W  V  P  E  L  R  H  Y  A  P  S  I  P  I  V  L  V  G  T  K  L
301 ATTGCTAAAAAAATGGGTCCCAGAGCTTAGGCATTATGCTCCATCTATACCCATTGTTCTTGTGGGTACCAAACTA
126 D  L  R  E  D  K  Q  F  Q  L  D  Y  P  G  A  S  V  I  S  N  E  Q  G  E  E
376 GATTTGAGAGAGGATAAACAATTTCAGCTGGATTACCCAGGTGCATCTGTCATTTCCAATGAACAGGGTGAAGAA
151 L  K  K  Q  I  G  A  V  A  Y  L  E  C  S  S  K  T  Q  Q  N  V  K  A  V  F
451 TTAAAGAAGCAAATAGGTGCAGTGGCATACTTAGAGTGCAGCTCCAAGACACAGCAGAATGTGAAGGCTGTGTTT
176 D  A  A  I  K  V  V  L  H  P  P  K  T  K  K  E  K  R  K  H  K  G  C  Y  I
526 GATGCTGCAATCAAGGTGGTCCTTCATCCTCCGAAAACCAAAAAGGAGAAGCGAAAACATAAAGGTTGTTATATA
201 I  *
601 ATATGA
```

Figure 1: Full-length CDS of *GlROP6* gene

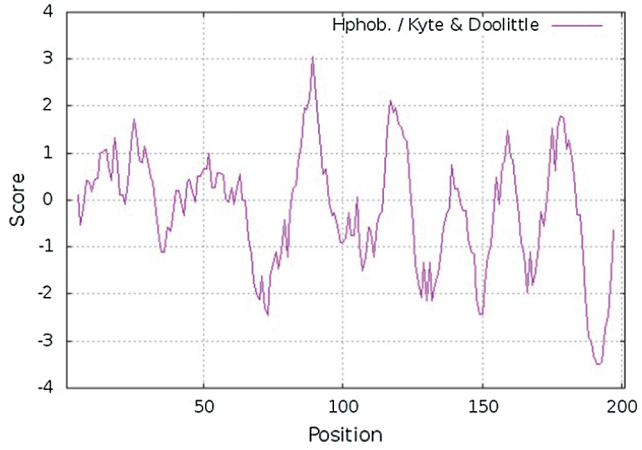

Figure 2: Hydropathy plot of GlROP6 protein

3.3 Conserved Domain Analysis of GlROP6

Both GlROP6 and AtROP6 contained five G-box motifs (G1–G5), an effector binding region, a Rho insert region, and a hypervariable region (HVR) (Fig. 3). G-box motifs are essential for binding of the nucleotide and the associated Mg^{2+} ion, as well as for GTP hydrolysis. Taking GlROP6 as an example, mutating the invariant glycine in G1 (G18 in GlROP6) or the glutamine in G3 (Q69 in GlROP6) could interfere with GTP hydrolysis, thus keeping the ROP in an active state (Constitutively active GlROP6, CA-GlROP6). Furthermore, substituting the threonine in G1 (T25 in GlROP6) or the aspartate in G4 (D126 in GlROP6) was thought to result in reduced nucleotide and increased GEF affinities (Dominant negative GlROP6, DN-GlROP6).

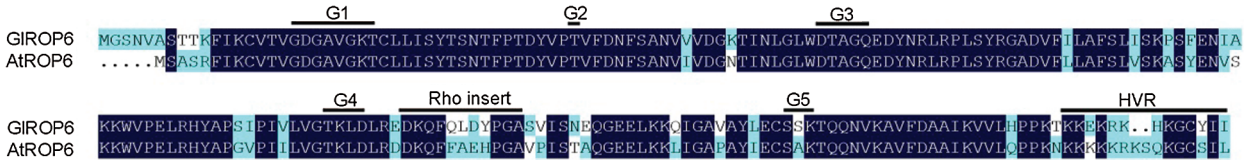

Figure 3: Alignment of GlROP6 and AtROP6 protein sequences

3.4 Phylogenetic Analysis of GlROP6

A comparison of GlROP6 with the ROP protein sequences of 15 representative plant species from NCBI database was performed by constructing a phylogenetic tree, which revealed that GlROP6 was closely related to the ROP protein of *Daucus carota* subsp. *Sativus* (XP_017244791.1). GlROP6 was distantly related to the ROP proteins of *Rosa chinensis* (XP_024174902.1) and *Cannabis sativa* (XP_030494758.1) (Fig. 4).

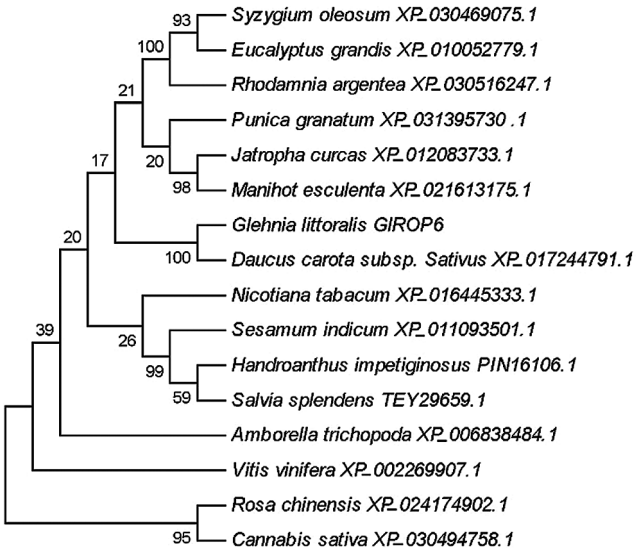

Figure 4: Phylogenetic analysis of ROP proteins. Protein sequences were aligned using ClustalW. The phylogenetic tree was constructed with MEGA 5.0 using the neighbor-joining (NJ) method with 1000 bootstraps replicates

3.5 GlROP6 Protein Structure Prediction

SOPMA online analysis showed that the secondary structure of GlROP6 comprised 32.84% alpha helices, 20.9% extended strands, 5.97% beta turns, and 40.3% random coils (Fig. 5).The tertiary structure prediction of GlROP6 exhibited 78.02% similarity to the 2j0v.2.A protein motif (Fig. 6).

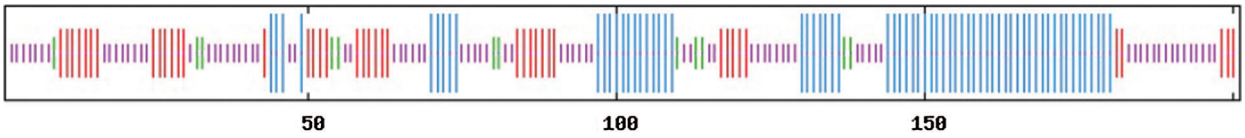

Figure 5: The secondary structure of GlROP6 was analysed using SOPMA. Blue lines indicate alpha helices, purple lines indicate random coils, red lines denote extended strands, and green lines represent beta turns

3.6 Analysis of GlROP6 Expression

Tissue-specific *GlROP6* expression patterns were investigated by qRT-PCR using *GlROP6*-specific primers. We found that *GlROP6* was expressed in root, stem, leaf and flower tissues, with some fluctuations. As shown in Fig. 7A, *GlROP6* was highly expressed in flowers, while its expression levels were lower in the other tissues examined.

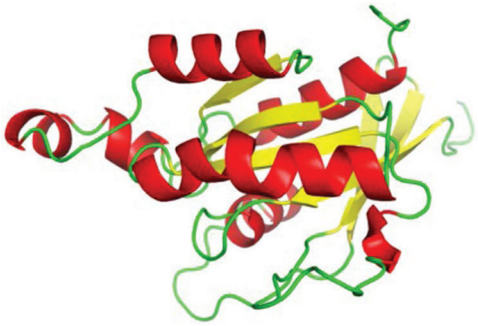

Figure 6: The tertiary structure of GlROP6 protein was constructed in PyMol. The alpha helices and beta turns are depicted in red and yellow, respectively

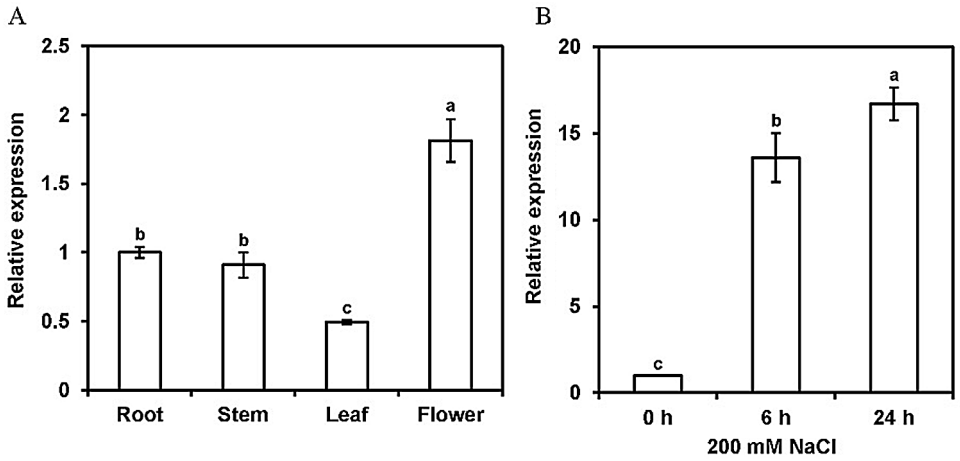

Figure 7: Analysis of *GlROP6* expression. The internal reference gene was *GAPDH*. (A) The relative expression of *GlROP6* in root, stem, leaf, and flower tissues. (B) Differential *GlROP6* expression in roots subjected to NaCl treatment for 0, 6 and 24 h. Error bars indicate means ± SD. Different letters indicate statistically significant difference in each treatment ($P < 0.05$, Duncan's multiple range test)

To determine the function of *GlROP6*, we examined its expression under NaCl treatment by qRT-PCR. As shown in Fig. 7B, *GlROP6* was significantly upregulated in *G. littoralis* roots in response to NaCl treatment, suggesting that *GlROP6* could be involved in the regulation of salt tolerance in *G. littoralis*.

4 Discussion

In this study, the *G. littoralis GlROP6* gene was cloned and compared with homologous sequences from other plant species. GlROP6 showed high amino acid sequence similarities to plant ROP proteins, ranging from 85.43% to 96.52%. We discovered that the GlROP6 protein has conserved domains characteristic of ROP family proteins, including five G-box motifs (G1–G5), an effector binding region, a Rho insert region, and a hypervariable region (HVR) (Fig. 3). GlROP6 was evolutionarily similar to the ROP of *Daucus carota* subsp. *Sativus* (Fig. 4). Our results indicate that *GlROP6* belongs to the *ROP* gene family.

At present, most salt tolerance studies in *G. littoralis* have focused on anatomical and morphological adaptations to high-salinity environments [23]. For example, a recent study revealed that the growth of *G. littoralis* seedlings treated with 100 mmol/L NaCl was similar to those of a control, and that the seedlings were able to tolerate 200 mmol/L NaCl stress. *G. littoralis* can adapt to NaCl stress due to complex regulatory mechanisms. When *G. littoralis* seedlings was treated with NaCl, the soluble sugar

content, peroxidase (POD) and catalase (CAT) activities were significantly increased at first and then decreased with the increase of the solution concentration [24]. However, there are few studies on the salt tolerance mechanisms and gene functional analyses in *G. littoralis*.

Our study has revealed the expression pattern of *GlROP6* during salt stress. This sheds light onto the function of *GlROP6* and its potential involvement in the regulation of salt stress signalling pathways. Thus, it would be worthwhile to investigate the function of *GlROP* genes in response to salt stress further.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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