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Involvement of *SchRabGDI1* from *Solanum chilense* in endocytic trafficking and tolerance to salt stress

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ABSTRACT

Physiological responses of plants to salinity stress requires the coordinated activation of many genes. A salt-induced gene was isolated from roots of the wild tomato species *Solanum chilense* and named *SchRabGDI1* because it encodes a protein with high identity to GDP dissociation inhibitors of plants. These proteins are regulators of the RabGTPase cycle that play key roles in intracellular vesicular trafficking. The expression pattern of *SchRabGDI1* showed an early up–regulation in roots and leaves under salt stress. Functional activity of SchRabGDI1 was shown by restoring the defective phenotype of the yeast *sec19-1* mutant and the capacity of SchRabGDI1 to interact with RabGTPase was demonstrated through BiFC assays. Expression of *SchRabGDI*1 in *Arabidopsis thaliana* plants resulted in increased salt tolerance. Also, the root cells of transgenic plants showed higher rate of endocytosis under normal growth conditions and higher accumulation of sodium in vacuoles and small vesicular structures under salt stress than wild type. Our results suggest that in salt tolerant species such as *S. chilense*, bulk endocytosis is one of the early mechanisms to avoid salt stress, which requires the concerted expression of regulatory genes involved in vesicular trafficking of the endocytic pathway.

1. Introduction

Membrane trafficking in eukaryotes depends on the accurate targeting of transport vesicles to and from defined membrane-bound compartments whereby different proteins participate in distinct steps of the process. Among the proteins involved in this vesicular trafficking is the Rab/Ypt family (RabGTPases), which form the largest branch of the Ras superfamily of the small GTPases that exist in all eukaryotic cells [1]. Different members of the RabGTPase family localized in the cytoplasmic side of organelles have been shown to have specific roles in targeting and/or tethering transport vesicles during exocytosis and endocytosis in eukaryotic cells [1,2]. RabGTPases function depends on their interaction with accessory proteins and their capacity to bind and hydrolyze GTP, which translates in the alternation between "active" and "inactive" states. RabGTPases carrying a geranylgeranyl group require guanine nucleotide exchange factors (GEFs) that facilitate GDP dissociation, GTPase activating proteins (GAP) that stimulate GTP hydrolysis, and guanine dissociation inhibitors (GDI) that form soluble complexes with small GTPases by shielding their lipid group. GDI proteins play a critical role in regulating the recycling of RabGTPases, allowing their rapid recycling [3] and maintaining a cytosolic pool of available RabGTPases to be delivered to vesicle membranes [4].

Although specific GDIs for Rab GTPases (RabGDIs) have been well characterized in yeast and animals, in plants, only few genes that share homology with members of the GDI family have been reported. Whereas 57 RabGTPases members have been identified in the *Arabidopsis thaliana* genome [5], only three *RabGDI* homologues have been identified, *AtRabGDI1*, *AtRabGDI2* and *AtRabGDI3* [6–8]. Two cDNAs encoding RabGDI have been isolated from rice, *OsGDI1* and *OsGDI2* [9], one *GDI* has been cloned from tobacco [10] and chickpea [11], and three *RabGDIs* have been identified in grapevine [12]. Among all characterized RabGDI family members, five structurally and functionally sequence-conserve regions (SCRs) have been identified [4]. Mutations of residues within SCRs interrupt the binding of RabGDIs to RabGTPase proteins and may lead to a decreased RabGTPase recycling [13,14]. The ability of RabGDI to interact with distinct RabGTPases has

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been demonstrated, however different RabGTPase members present distinct binding affinity [15].

Plants display different mechanisms to tolerate salt stress including ion extrusion from the cell and ion sequestration in the vacuole to prevent excess accumulation in the cytosol [16,17]. The buildup of sodium into the vacuole is mediated by the action of Na^{+}/H^{+} antiporters located in the tonoplast [18,19]. In addition, a rapid increase in the root vacuolar volume leading to increased vacuolar salt content has been shown under salt stress [20]. During this process, endosomes are fused to the main vacuole, suggesting that vesicle trafficking might play an important role in the response to salt stress [21]. Overexpression of the *AtRabG3e* gene (encodes a RabGTPase) in *Arabidopsis* triggered accelerated endocytosis in roots, leaves, and protoplasts and resulted in accumulation of sodium in vacuoles and increased tolerance to salt and osmotic stresses [22]. Similar results have been observed with the overexpression of an *AtRabG3e* homologous gene, *PgRab7* from *Pennisetum glaucum* in tobacco [23]. Loss of function of RabA1 members involved in vesicle transport between the trans-Golgi network and the plasma membrane caused hypersensitivity to salt stress, most likely due to the participation of these proteins in the localization of cell-surface proteins, such as ion channels and pumps [24]. The involvement of RabGTPases in the response to salinity stress suggests that other interactive members controlling their function could also play a role in this response. Whereas one study has implicated an Arabidopsis RabGEF in mediating an endocytic pathway affecting stress tolerance [25], the role of RabGDIs in plant tolerance to salt stress has not been yet reported.

Cultivated species of the Solanaceae family are susceptible to a wide range of environmental stresses. For example, salinity is known to negatively affect seed germination, inhibit growth and decrease fruit productivity [26]. The Solanum section Lycopersicum includes *S. chilense*, a wild tomato species with a notable capacity to withstand salinity and drought [27]. When subjected to salt stress conditions, *S. chilense* activates the expression of a set of genes that may be associated with its capacity to adapt to its natural habitat [28–30]. Among the genes that are differentially expressed in salt treated *S. chilense* roots, we identified one encoding a protein with high homology to AtRABGDI1, hence named *SchRABGDI1*. Here, we analyzed the molecular function of SchRabGDI1 and its ability to bind RabGTPases *in vivo*. Expression of *SchRabGDI1* in *Arabidopsis thaliana* resulted enhanced tolerance to salt stress and in an increase of both the endocytosis rate in root cells and vacuolar Na^{+} content upon high salt exposure. Our results suggest that salt-induced expression of *SchRabGDI1* contributes to endocytic trafficking in *S. chilense* and to its natural salt tolerance.

2. Material and methods

2.1. Plant materials and growth conditions

Solanum chilense (Dunal) seeds were obtained from plants collected in Northern Chile at a 2500 m.s.n.m. 18° 26′ lat. S 69° 45′ long. Plants were clonally propagated in pots containing a mixture of perlite, vermiculite and peat moss (1:1:1 v/v) and grown under greenhouse conditions at 23–25 °C and a 16 h/8 h light/dark photoperiod. Plants were fertilized with commercial Hoagland's solution (1/4 strength) every 10 days. For gene expression analyses under non-stressed conditions organ samples including root (R), young and mature leaves (YL, ML), stem (S), flower bud (FB) and flower (F) were taken at flowering time. Salt stress in *S. chilense* was applied to 7-week-old plants grown in 2 l pots containing a mixture of perlite:vermiculite (1:1 v/v) and fertilized with Hoaglands solution by irrigating once with 400 ml of 300 mM NaCl. Leaves and roots samples were collected at 0, 3, 6, 12, 24, 48 and 72 h after salt treatment and immediately frozen in liquid nitrogen and stored at −80 °C.

Wild-type (Col-0) and transgenic *Arabidopsis thaliana* plants were grown in a chamber at 21 °C and a 16 h light/8 h dark photoperiod. For saline stress, 5-days-old Arabidopsis seedlings grown in solid half-strength MS (Murashige and Skoog, basal salt mixture) were transferred to half-strength MS medium containing 0 or 75 mM NaCl and maintained for 15 days. Then, biomass production (fresh weight) and leaf oxidative damage were evaluated. To assess germination rate under saline stress, seeds were surface sterilized with a 2% sodium hypochlorite solution. One-hundred seeds from wild-type and transgenic lines were sown in half-strength MS plates containing 0, 50 or 75 mM NaCl and kept at 4 °C in darkness for 3 days for seed stratification. Radicle emergence was examined every 24 h during 96 h. All treatments were done in three independent experiments.

2.2. RNA isolation

Total RNA was extracted from different organs of *S. chilense* and Arabidopsis leaves using the SV Total RNA Isolation System kit (Promega). All RNA extractions for gene expression assay were done in triplicate for each organ and condition. RNA integrity was visualized by 2% agarose gel electrophoresis and RNA concentration and purity (OD_{260}/OD_{280} ratio > 1.95) were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA samples were treated with RNase free DNase I (Ambion) to remove contaminant DNA traces.

2.3. Subtractive library construction

The Clontech PCR-Select cDNA Subtraction kit was used for the preparation of a subtractive cDNA library to identify differentially expressed genes in roots of *S. chilense* under salt stress (half-strength MS supplemented with 400 mM NaCl) in hydroponic cultivation. Double-stranded cDNA synthesis was carried out on total mRNA derived from roots of stressed (tester) and normal (driver) plants. The tester and driver cDNAs were then digested with RsaI yielding blunt end fragments of approximately 400 bp length on average and processed following the manufacturer's instructions with some modifications. The tester cDNA was aliquoted into two halves, and each half was ligated with different cDNA adaptors. Adapter ligation was followed by two rounds of hybridization with an excess of driver cDNA as per manufacturer's protocol. The resultant products were subjected to two cycles of PCR with adaptor targeting primers to amplify the differentially expressed sequences. Amplifications were performed on a Stratagene Mx3000P (Agilent Technologies). First PCR master mix contained 10x PCR reaction buffer, 0.2 mM dNTPs, 0.4 μM PCR primer 1 and Advantage cDNA polymerase (Clontech). PCR was performed under the following conditions: 94 °C (25 s) followed by 30 cycles each consisting of a denaturation step at 94 °C (10 s), an annealing step at 66 °C (30 s) and an elongation step at 72 °C (1.5 min). Before using the primary PCR products as templates for secondary PCR, these were diluted 10-fold with sterile water. The second PCR master mix contained 10x PCR reaction buffer, 0.2 mM dNTPs, 0.4 μM nested PCR primer 1, 0.4 μM nested PCR primer 2 and Advantage cDNA polymerase. PCR was run through 20 cycles each consisting of 94 °C for 15 s, 66° for 30 s and 72 °C for 1.5 min. cDNA molecules were cloned non-directionally into the pGEM-T-Easy Vector (Promega). The ligation products were used to transform the *E. coli* DH5α strain *via* electroporation. Positive clones were collected and used for plasmid isolation and sequencing.

2.4. Analysis of gene expression

Gene transcript levels were analyzed by quantitative PCR (qRT-PCR) using a Stratagene Mx3000P (Agilent Technologies) system and the Brilliant SYBR Green Master Mix (Stratagene). To prepare first-strand cDNA, 2 μg of total RNA were reverse transcribed in a 20 μl reaction using the oligo d(T) and AffinityScript QPCR cDNA Synthesis Kit (Stratagene) following manufacturer's instructions. For each sample (three biological replicates), qPCR was carried out in triplicate

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