Short communication

AtGRP7 IS INVOLVED IN THE REGULATION OF ABSCISIC ACID AND STRESS RESPONSES IN ARABIDOPSIS

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Abstract: The Arabidopsis *AtGRP7* gene, encoding a glycine-rich RNA-binding protein, has been shown to be involved in the regulation of a circadian-regulated negative feedback loop. However, little is known about the role of *AtGRP7* in mediating abscisic acid (ABA) and stress responses. Here, we show that *AtGRP7* plays a role in both. *AtGRP7* was repressed by ABA, high salt and mannitol. Disruption of *AtGRP7* by T-DNA insertion led to hypersensitive responses to ABA in both seed germination and root growth assays. The *atgrp7-1* mutant was also hypersensitive to osmotic stress conditions, such as high salt and high concentrations of mannitol. In addition, the *atgrp7-1* mutant plants accumulated significantly higher transcript levels of two ABA- and stress-inducible genes, *RD29A* and *RAB18*, compared with the wild-type plants. Taken together, these results suggest that *AtGRP7* is involved in the regulation of ABA and stress responses.

Key words: *AtGRP7* gene*, Abscisic acid, Osmotic stress

INTRODUCTION

The plant hormone abscisic acid (ABA) regulates many agronomically important aspects of plant development and physiology, including seed maturation and dormancy, and responses to environmental stress conditions such as drought, salinity, and low temperature [1-3]. Gene regulation and the inhibition of seed germination provide useful bioassays for both forward and reverse genetic

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analyses that have revealed many molecular components in plant ABA signal transduction pathways [2, 4]. These components range from early signaling intermediates, such as G proteins and protein kinases/phosphatases, to transcription factors (TFs) and RNA metabolic proteins; thus, the modulation of ABA responses in plants involves a complex molecular network [2-9].

A family of proteins consisting of one amino-terminal consensus sequence RNA-binding domain and one carboxyl-terminal glycine-rich domain was isolated from cyanobacterium [10-11], plant [12-15], and human [16] cells. It is referred to as a glycine-rich RNA-binding protein (GR-RBP) family [13, 15]. There are eight members of the GR-RBP family in the Arabidopsis genome, including AtGRP7 (AT2G21660) [17], which has been shown to be involved in regulating a negative feedback loop through which it influences the circadian oscillations of its own transcript [18]. However, it remains unclear whether *AtGRP7* plays a role in mediating ABA and stress responses.

In this study, we showed that *AtGRP7* was repressed by ABA, salt (NaCl), and mannitol. This disruption of *AtGRP7* led to hypersensitive responses to ABA and osmotic stress conditions, such as high salt and high concentrations of mannitol, in both seed germination and root growth assays. Moreover, *atgrp7-1* mutant plants accumulated significantly higher transcript levels of two ABA- and stress-inducible genes, *RD29A* and *RAB18*, compared with the wild-type plants. Together, these results suggest that *AtGRP7* is involved in the regulation of ABA and osmotic stress responses.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of Arabidopsis (*Arabidopsis thaliana*) wild-type ecotype Colombia-0 and the *atgrp7-1* mutant were surface sterilized and plated on Murashige and Skoog (MS) media [19] containing 1% sucrose. The plates were stored for 3 days in the dark at 4ºC and then placed in a growth chamber set at 22ºC, with a light intensity of 100 µmol m^{-2} s^{-1} and a 16-h day.

For the germination assay, fifty seeds of each genotype (Wild type and *atgrp7-1*) were germinated and grown on growth media supplemented with different concentrations of ABA, NaCl and mannitol (Sigma, St. Louis, MO, USA), and incubated at 4ºC for 2 days before being placed at 22ºC under long-day conditions. Germination (the emergence of radicles) was scored 5 days after the treatments. For the root elongation assay, the root lengths were measured after seeds of wild-type and *atgrp7-1* mutant were germinated on growth media containing 0.1 µM ABA, 100 mM NaCl, and 200 mM mannitol for 5 days.

For the ABA, NaCl and mannitol treatments, two-week old wild-type seedlings grown on MS media were respectively treated with 100 µM ABA, 300 mM NaCl and 300 mM mannitol for 6 h, starting at 09:00, and then sampled for RT-PCR analysis.

Isolation of the *atgrp7-1* T-DNA insertional mutant allele
The *atgrp7-1* mutant is a sequence-indexed Arabidopsis T-DNA insertion mutant (SALK_113110; http://signal.salk.edu/) isolated by the Salk Institute Genomic Analysis Laboratory [20] and obtained by us from the Arabidopsis Biological Resource Center (ABRC; Ohio State University, Columbus, OH, USA).

RT-PCR analysis of gene expression
Seedlings were homogenized with a mortar and pestle in liquid nitrogen. RNA was extracted using Trizol Reagent (Invitrogen, CA), followed by chloroform extraction, isopropanol precipitation, and spectrophotometric quantification. cDNA was synthesized from DNase-treated RNA with Superscript reverse transcriptase (Invitrogen), following the manufacturer's instructions. The cDNA products were standardized for semi-quantitative RT-PCR using β-actin primers as a reference. For each transcript, sequence-specific 5' and 3' primers were designed with melting temperatures between 52ºC and 60ºC. The number of cycles was established empirivelly to amplify the target cDNA to allow the maximal detection of differences in transcript number. The following primers were designed for gene-specific transcript amplifications:
ACTIN11 (*ACTIN*, At3g12110): forward, 5'-GATTTGGCATCACACTTTCTA CAATG-3', and reverse, 5'-GTTCCACCACTGAGCACAATG-3';
RD29A (At5g52310): forward, 5'-ATCACTTGGCTCCACTGTTGTTC-3' and reverse, 5'-ACAAAACACACATAAACATCCAAAGT-3';
RAB18 (At5g66400): forward, 5'-GTGGTGGCTTGGGAGGAATGCTTCA-3' and reverse, 5'-ATGCGACTGCGTTACAAACCCTCA-3'.

Statistical analysis
The data is the mean ± S.E. of three replicates. The analyses of variance were computed on statistically significant differences ($P < 0.05$) determined based on the appropriate F-tests. The mean differences were compared utilizing Duncan's multiple range test.

RESULTS

Repression of *AtGRP7* expression by ABA, NaCl and mannitol
To determine whether *AtGRP7* is affected by ABA, NaCl or mannitol, the expression pattern of *AtGRP7* was analyzed in response to the three compounds. *AtGRP7* expression was regulated by the circadian clock, and the *AtGRP7* transcript levels in control plants have been shown to significantly increase from 09:00 to 15:00 [18]. Therefore, wild-type plants grown in long-day conditions were treated with ABA, NaCl and mannitol for 6 h, starting at 09:00. As shown in Fig. 1, ABA treatment significantly decreased the transcript level of *AtGRP7*. In addition, *AtGRP7* is also strongly repressed by NaCl and mannitol. These results suggest the possible involvement of *AtGRP7* in regulating ABA and stress responses.
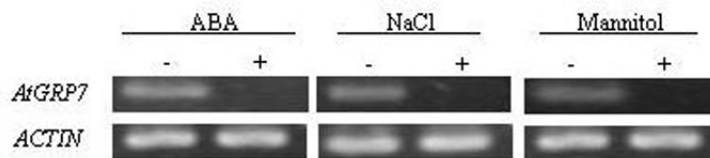
Fig. 1. Repression of *AtGRP7* expression by ABA, NaCl and mannitol in wild-type plants. Two-week old wild-type plants grown on MS medium at 22ºC were either not treated (-) or treated with 100 μM ABA (+), 300 mM NaCl (+), or 300 mM mannitol (+) for 6 h, starting at 9:00. The *ACTIN* gene was used as a loading control.

The *atgrp7-1* mutant is hypersensitive to ABA, NaCl and mannitol
To examine the function of the *AtGRP7* gene, we isolated a T-DNA insertional allele of this gene (*atgrp7-1*) from the collections of T-DNA-transformed Arabidopsis lines (SALK_113110, Arabidopsis Biological Resource Center). Homozygous mutant lines were established after selfing. The RT-PCR analysis showed that the insertion abolished the expression of *AtGRP7* (data not shown). To evaluate the consequences of *AtGRP7* gene disruption, we examined the mutant plants under normal growth conditions and found them to be indistinguishable from the wild type (Fig. 2A). However, the fact that the transcript of *AtGRP7* is repressed by ABA and abiotic stress signals prompted us to evaluate the effects of the *atgrp7-1* mutation on plant responses to ABA, NaCl and mannitol. When grown on media containing 0.2 μM ABA for 7 days, all the wild-type seeds were able to germinate and successfully develop healthy cotyledons and true leaves, whereas most of *atgrp7-1* seeds did not germinate (Fig. 2A). Hypersensitivity of the *atgrp7-1* mutant to ABA is further supported by the *atgrp7-1* mutant being more sensitive to all the tested ABA concentrations than the wild type was (Fig. 2B). Likewise, the *atgrp7-1* seeds were also more sensitive to all the tested concentrations of NaCl and mannitol than the wild-type seeds (Figs 3A and 4A).
We then examined the effects of ABA, NaCl and mannitol on the root growth of *atgrp7-1* seedlings. At 0.1 μM ABA, 100 mM NaCl and 200 mM mannitol, the respective root growths of wild-type seedlings were 26.3%, 78.9%, and 34.2% of their control rates, while those of the *atgrp7-1* seedlings were 10.5%, 26.5 %, and 16.2% of their control rates (Figs 2C, 3B and 4B). These results supported the conclusion that the *atgrp7-1* mutation renders the seedlings hypersensitive to ABA, osmotic stress, and high salt.
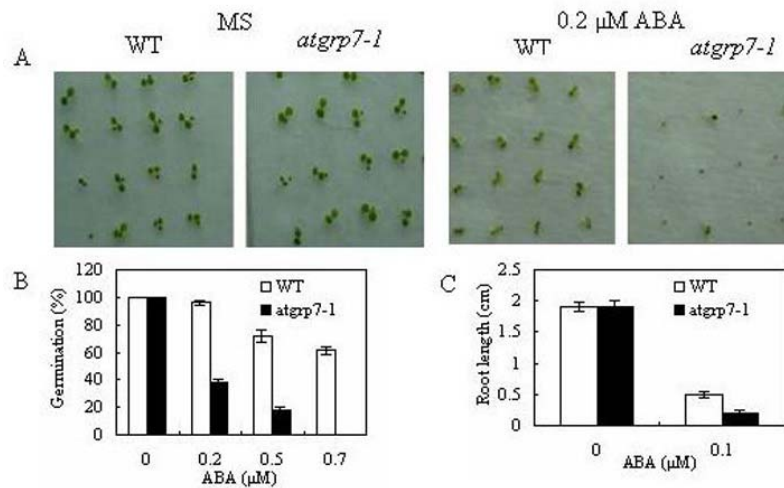
Fig. 2. The seed germination and ABA response of the *atgrp7-1* mutant. A. Germination of wild-type and *atgrp7-1* seeds grown on MS media without or with 0.2 μM ABA for 7 days. The experiments were repeated three times. Representative results are shown. B. Germination of wild-type and *atgrp7-1* seeds in response to ABA. Wild-type and *atgrp7-1* seeds were grown on MS media containing the indicated concentrations of ABA for 5 days. Error bars indicate ± SE (n = 3). C. The effect of ABA on the root growth of wild-type and *atgrp7-1* seedlings. The root lengths were measured after seeds of the wild-type and *atgrp7-1* mutants were germinated on growth media with or without ABA (0.1 μM) for 5 days. Error bars indicate ± SE (n = 3).
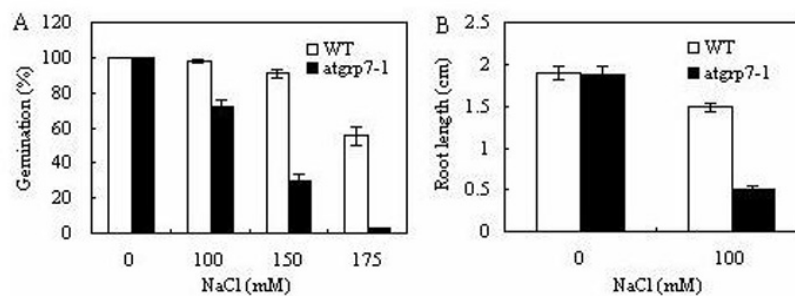

Fig. 3. Response of the *atgrp7-1* mutant to NaCl. A. Germination of wild-type and *atgrp7-1* seeds in response to NaCl. Wild-type and *atgrp7-1* seeds were grown on MS media containing different concentrations of NaCl for 5 days. Error bars indicate ± SE (n = 3). B. The effect of NaCl on the root growth of wild-type and *atgrp7-1* seedlings. The root lengths were measured after seeds of the wild-type and *atgrp7-1* mutant were germinated on growth media with or without NaCl (100 mM) for 5 days. Error bars indicate ± SE (n = 3).
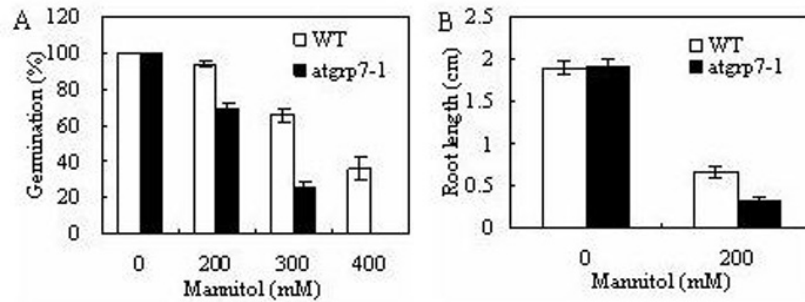
Fig. 4. Response of the *atgrp7-1* mutant to mannitol. A. Germination of wild-type and *atgrp7-1* seeds in response to mannitol. Wild-type and *atgrp7-1* seeds were grown on MS media containing different concentrations of mannitol for 5 days. Error bars indicate ± SE (n = 3). B. The effect of mannitol on the root growth of wild-type and *atgrp7-1* seedlings. The root lengths were measured after seeds of the wild-type and *atgrp7-1* mutant were germinated on growth media with or without mannitol (200 mM) for 5 days. Error bars indicate ± SE (n = 3).

The expression of ABA- and stress-responsible genes in *atgrp7-1* plants
The finding that the *atgrp7-1* mutant showed altered responses to ABA and stress suggests that the *atgrp7-1* mutation might alter the expression pattern of ABA- or stress-responsible genes. Two ABA- and stress-inducible genes, *RD29A* and *RAB18*, were used as markers to test this hypothesis. As shown in Fig. 5, higher transcript levels of *RD29A* and *RAB18* were detected in *atgrp7-1* plants than in wild-type plants in the absence or presence of ABA. These results suggest that the *atgrp7-1* mutation leads to up-regulation of ABA- and stress-induced genes.
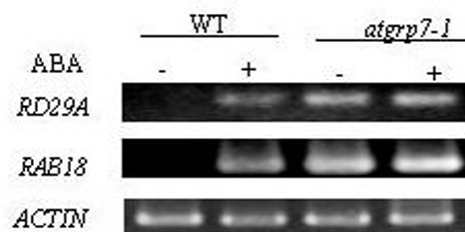

Fig. 5. RT-PCR analysis of two ABA- and stress-induced genes in wild-type and *atgrp7-1* plants. Two-week old wild-type and *atgrp7-1* plants were either not treated (-) or treated with 100 μM ABA (+) for 6 h, and then sampled for Rt-PCR analysis. The *ACTIN* gene was used as a loading control.

DISCUSSION

AtGRP7 has been studied before [18, 21], but information about it has been limited to its role in regulating a circadian-regulated negative feedback loop. In this study, we showed that *AtGRP7* plays a role in the regulation of ABA and stress responses. First, we found that the expression level of *AtGRP7* was strongly repressed by ABA, NaCl and mannitol treatments. Second, we showed that the *atgrp7-1* mutant is more sensitive to ABA, NaCl and mannitol compared with the wild type in both seed germination and root growth assays. Finally, we found that the *atgrp7-1* mutant plants accumulated significantly higher transcript levels of two ABA- and stress-inducible genes, *RD29A* and *RAB18*, compared with the wild-type plants.

The *AtGRP7* gene encodes a clock-regulated, glycine-rich, RNA-binding protein in Arabidopsis and shows a circadian variation in steady-state abundance [18, 21]. Constitutive overexpression of its product, AtGRP7, in transgenic Arabidopsis plants depresses the oscillations of the endogenous *AtGRP7* transcript, indicating that both the transcript and the protein are part of a clock-regulated negative feedback circuit [18]. In this study, we found that the expression level of *AtGRP7* was strongly repressed by ABA, NaCl and mannitol, which is consistent with the results of analysis of microarray data stored in the GENEVESTIGATOR database [22]. In addition, it was previously shown that, in mammalian cells, a mouse cold-inducible RNA-binding protein plays an essential role in the cold-induced growth suppression of mouse fibroblasts [23]. Moreover, some of the GR-RBP family members have been demonstrated to be induced by cold stress [15]. Interestingly, our unpublished data indicated that *AtGRP7* was also induced by cold stress, which is consistent with the results of analysis of microarray data stored in the GENEVESTIGATOR database [22]. Therefore, we are now conducting a study to examine the role of *AtGRP7* in mediating the cold stress response.

The mechanisms underlying ABA responses in plants have been intensively studied via biochemical and genetic approaches. Studies have identified a number of components in the molecular network linking the ABA signal to the cellular responses in plant cells. Such components are broadly defined into two large categories: signal transducers and transcription factors. The signal transducers include protein kinases and phosphatases, G proteins (both trimeric and small GTP-binding proteins), RNA metabolic proteins, phospholipases, and so on [2-3, 24]. It was shown that, of the RNA metabolism proteins, there are a number of regulators of ABA responses, including: HYL1, a double-stranded RNA-binding protein; ABH1, an mRNA cap-binding protein; and SAD1, an SM-like snRNP protein S [3]. Interestingly, it was recently determined that FCA, an RNA-binding protein, is an ABA receptor involved in RNA metabolism and in controlling flowering time [9]. In addition, GR-RBP4, one of eight members of the GR-RBP family, has been shown to be involved in mediating the germination and growth of Arabidopsis plants under various stress

conditions [17]. In this study, we identified a recessive T-DNA disruption mutant *atgrp7-1*. A detailed characterization of the *atgrp7-1* mutant, using both seed germination and root growth assays, demonstrated that the disruption of *AtGRP7* led to hypersensitive responses to ABA and osmotic stress conditions, such as high salt and high concentrations of mannitol, as well as the altered expression pattern of two ABA- and stress-inducible genes, *RD29A* and *RAB18*, suggesting that *AtGRP7* is involved in the regulation of ABA and stress responses. However, the analysis of the motif sequence revealed that the promoter of *AtGRP7* did not contain an ABRE (www.arabidopsis.leeds.ac.uk/ act/cislocator.php), suggesting that *AtGRP7* may be indirectly mediated by ABA. As hyperosmotic stress and high salt induce the production of ABA in plants [25, 26], hypersensitivity of the *atgrp7-1* mutant to these stress conditions could result from increased sensitivity to ABA, or from increased production of ABA, or both. In other words, altered stress sensitivity may be an ABA-dependent or -independent process. An inhibitor for ABA biosynthesis, norflurazon [5-6, 27], will help to distinguish these possibilities. In addition, it also remains to be determined whether AtGRP7 plays a role in the regulation of ABA-mediated stomatal closure.

In summary, to the best of our knowledge, this data is the first evidence that *AtGRP7* is involved in the regulation of ABA and stress responses.

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