Identification and characterization of a salt tolerance-responsive gene (*AtGRP9*) of *Arabidopsis* *

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Abstract Soil salinity is one of the important limiting factors for plant growth and development. A cDNA clone encoding a glycine-rich protein (designated *AtGRP9*) was identified from *Arabidopsis* by functional expression of the plant cDNA library in the fission yeast *S. pombe*. Yeast cells overexpressing *AtGRP9* displayed significantly enhanced salt tolerance. Northern analysis showed that expression of *AtGRP9* in *Arabidopsis* was induced by NaCl and plant hormone abscisic acid (ABA). These results suggest that *AtGRP9* may be involved in the salt stress response in *Arabidopsis*.

Keywords: ***arabidopsis*, glycine-rich protein, salt tolerance-responsive genes, *S. pombe*.**

Study on the mechanisms by which plants respond to salinity stress not only is important in study of gene regulation and signal transduction of plants but also offers genetic information for improving salt tolerance of the crop plants[1,2]. Considerable progress has been made in understanding the molecular bases of plant salt stress responses, the discovery of Ca-dependent SOS pathway for the regulation of ion homeostasis and plant salt tolerance in *Arabidopsis* stands for a good example in this aspect[2]. However, owing to the complex nature of the plant salt tolerance, the existing data are insufficient yet to elucidate the mechanisms that control this process. For this reason, identification of salinity stress-related genes needs to be continued in order to fill in the gaps in the known regulatory pathways and to reveal new components in the signaling networks involved in salt stress responses[2,3].

Because genetic studies to the yeast, the single eukaryotic cells, are more amenable than to higher plants, yeast has been used as a simple and efficient system to identify and characterize functionally conserved plant genes. It is believed that some cellular responses to salinity stress are similar in yeast and plant cells[4~6], since several plant genes have been shown to be able to increase the yeast salt tolerance and to functionally complement the yeast salt-tolerant mutants upon overexpression[7~9]. Thus, yeast is thought to be an ideal system for isolating certain salt tolerance-related genes of higher plants.

Although *Arabidopsis* is not a halophytic plant, a lot of evidence proved that all higher plants have genes for salt tolerance in their genomes[3]. Therefore, *Arabidopsis* is also a model organism for salt tolerance studies[3]. In this work, we have isolated a cDNA clone encoding a glycine-rich protein from *Arabidopsis* (designated *AtGRP9*) by functional expression of the plant cDNA library in the fission yeast *S. pombe*. Effect of *AtGRP9* overexpression on yeast salt tolerance was analyzed, and the expression levels of *AtGRP9* in *Arabidopsis* seedlings subjected to NaCl and abscisic acid (ABA) treatments were measured. The results suggest that *AtGRP9* may be involved in the response to salt stress in *Arabidopsis*.

1 Materials and methods

1.1 Materials

Arabidopsis thaliana ecotype Columbia was grown in soil in the greenhouse or on K1 medium containing Murashige and Skoog salts supplemented with vitamins, 1% sucrose and 0.8% agar.

Escherichia coli strain DH5α was a stock of this laboratory; *Schizosaccharomyces pombe* strain Q-01 was purchased from Stratagene; plasmid pREP1 was kindly provided by Dr. K Maundrell.

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Media for *S. pombe* and *E. coli* were YE, MM[10] and LB[11], respectively. Restriction enzymes and T4 DNA ligase were purchased from Gibco; [α-^{32}p]dCTP was supplied by Yahui Company.

1.2 Transformation of *S. pombe* cells with the *Arabidopsis* cDNA library

A whole-plant cDNA library of *Arabidopsis thaliana* was constructed in the *S. pombe* expression vector pREP1 containing a thiamine repressible promoter *nmt-1*[7] (Fig. 1). The cDNAs in the library were transformed into *S. pombe* cells by electroporation. Colonies grown on minimal medium (MM) containing 200 mmol/L NaCl were replicaplated onto MM plates supplemented with increasing NaCl up to 500 mmol/L. The clones grown on 500 mmol/L NaCl were selected for further analysis. Plasmids were isolated from these candidate clones and re-transformed into *S. pombe* to confirm the phenotype.

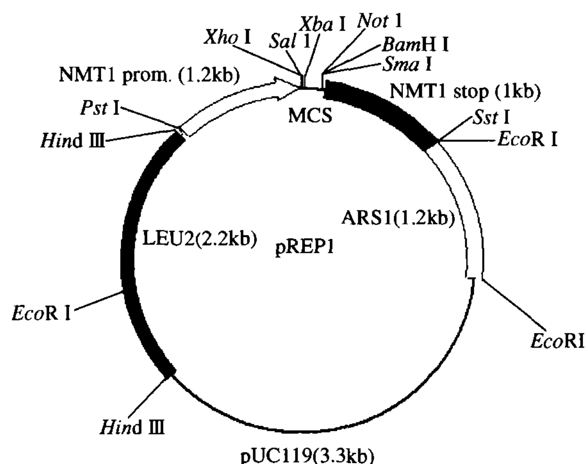

Fig. 1. *S. pombe* expression vector pREP1.

1.3 DNA sequencing and protein analysis

DNA sequence was determined by an automated DNA sequencer (Genecore, Shanghai). ORF and motif analysis was carried out with DNAMAN and ANTHEPROT programs.

1.4 Salt stress and ABA treatments

Salt and ABA treatments were performed with 10-day-old seedlings. For salt stress treatment, plantlets were placed on the filter paper moistened with MS medium containing increasing amounts of NaCl for 12 h. For ABA treatment, plantlets were placed on the filter paper moistened with MS medium containing 10 μmmol/L ABA for different time intervals. Treated seedlings were harvested, immersed in liquid nitrogen and stored at −80 ℃ for RNA preparation.

1.5 Northern analysis

Total RNAs were extracted from yeast and plant cells as described in Refs. [10] and [12]. Twenty micrograms of total RNA from each sample were denatured and separated by electrophoresis on 1.2% formaldehyde gels. The RNAs were transferred onto nylon membranes (Hypond-N +, Amersham) by capillary transfer and UV cross-linked to the membrane. After pre-hybridization for 2 h at 65 ℃, the blots were hybridized with ^{32}P -labelled *AtGTP9* cDNA probe for 12 h, washed at 65 ℃ and exposed to X-ray films for autoradiography according to the standard protocol[11]. The probes were stripped off from the membrane that was subsequently hybridized with 18S rDNA probe to normalize the RNA loadings.

2 Results

2.1 Isolation of *AtGRP9* cDNA

Based on the conservatism of some salt stress-responsive genes of eukaryotes, we attempted to use the fission yeast as a simple experimental system to isolate putative plant genes related to salt stress response through functional overexpression of plant cDNAs in yeast cells. For this purpose, a whole-plant cDNA library of *Arabidopsis thaliana* was constructed in the yeast expression vector pREP1 and the cDNAs were transformed into *S. pombe* cells by electroporation. Since the wild type yeast cells can not survive in the medium containing 150 mmol/L NaCl, transformants were first selected for their ability to grow in the presence of 200 mmol/L NaCl. Approximately 1×10^3 clones were obtained, which were subsequently subjected to a stepwise selection procedure. Four clones were found able to propagate in the MM medium containing 500 mmol/L NaCl (Fig. 2(a)). The pREP plasmids were isolated from these clones and used to retransform *S. pombe* in order to confirm that the increased salt tolerance of the yeast cells were due to the transformation of *Arabidopsis* cDNAs rather than a self-mutation of *S. pombe*. After sequencing of the plant cDNAs, the clone encoding a glycine-rich protein (designated *AtGRP9*) was chosen for further analysis.

To see the expression level of *AtGRP9* in the yeast cells, Northern analysis was performed with the transformants. As shown in Fig. 2(b), *AtGRP9*

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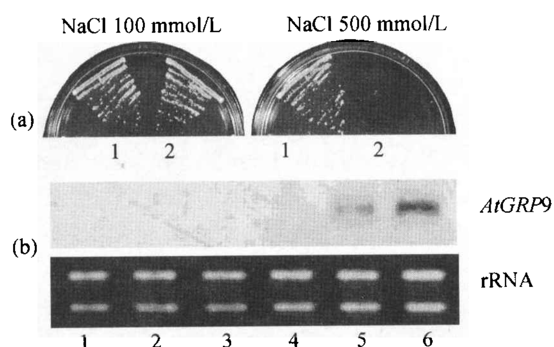

Fig. 2. Salt tolerance enhancement of yeast transformants and induced expression of *AtGRP9* cDNA. (a) Growth of yeast transformants on the MM medium containing NaCl. 1, yeast cells transformed with pREP1-*AtGRP9* recombinant plasmid; 2, yeast cells transformed with plasmid pREP1. (b) Northern analysis of *AtGRP9* expression in yeast cells. 1, wild type; 2 ~ 4, wild type, pREP1 and pREP1-*AtGRP9* transformants grown in the presence of 100 mmol/L NaCl, respectively; 5 and 6: pREP1-*AtGRP9* transformants grown in the medium containing 200 and 500 mmol/L NaCl, respectively.

mRNA levels increased parallel with the elevation of NaCl concentration in the transformed cells. While it is almost undetectable in the wild type cells, pREP1-(empty plasmid) transformed cells and untreated *AtGRP9*-transformed cells, *AtGRP9* transcripts were obviously present in the salt stress-treated cells, with the highest accumulation occurred in the cells treated with 500 mmol/L NaCl. This result showed clearly that the expression of *AtGRP9* could be induced by NaCl stress in the yeast cells, and the yeast salt tolerance was positively correlated with the expression level of *AtGRP9*.

2.2 Structural features of *AtGRP9* protein

The amino acid composition and the primary structure of the protein deduced from *AtGRP9* cDNA were analyzed. The 650 bp cDNA comprises an open reading frame that encodes 154 amino acids (Fig. 3).

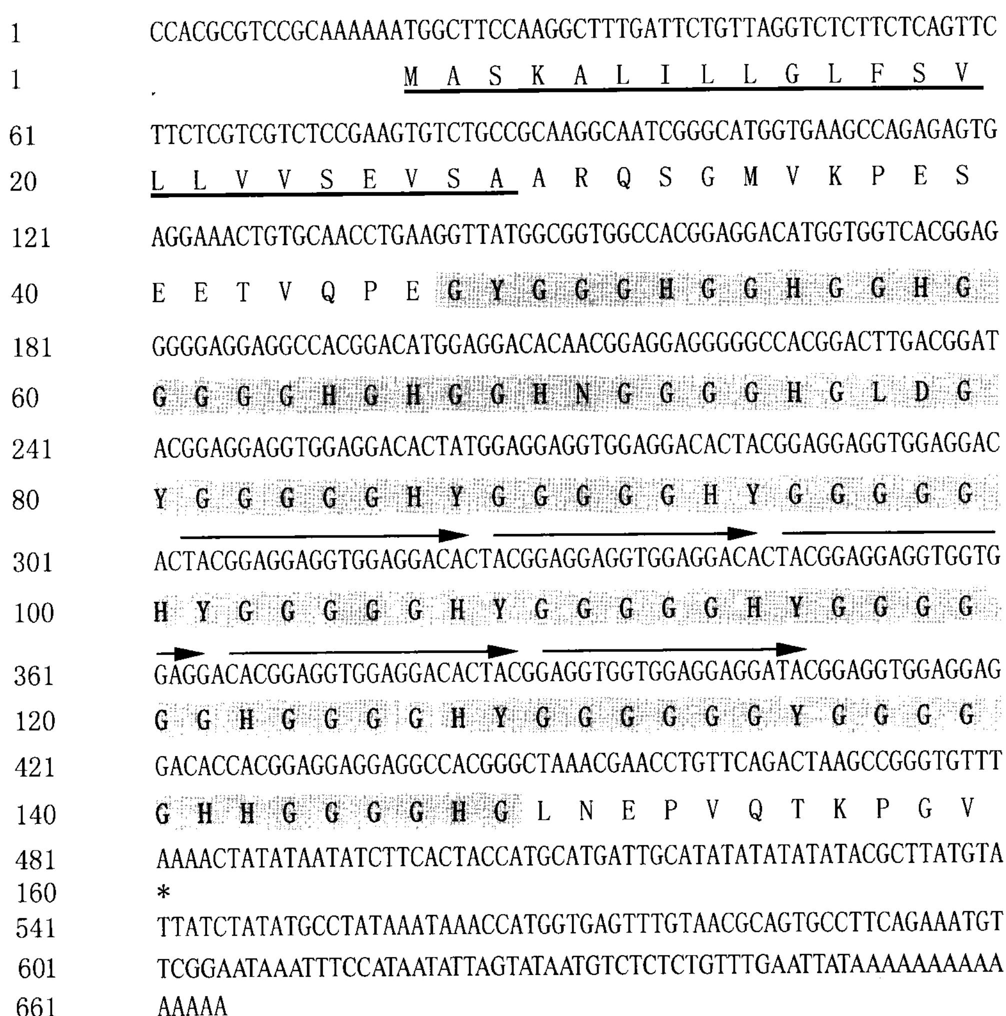

Fig. 3. Nucleotide sequence of *AtGRP9* cDNA and the predicted amino acid sequence (GenBank accession No. At2g05440). The signal peptide is underlined; the shadow area marks glycine-rich domain; the arrows indicate the direct GGGGGHY repeats.

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Two striking structural features were found in *AtGRP9* protein: (1) the N-terminal portion of the protein contains a predicted endoplasmic reticulum (ER) signal peptide (23 amino acids long) which is present in most of the glycine-rich proteins (GRPs) identified up to now[13]; (2) the glycine content is extremely high (50% of all amino acids) and there exists five direct GGGGGHY repeats. Apart from glycine, *AtGRP9* is also rich in histidine (11%) and tyrosine (6%), these amino acids residues are believed to be important in the formation of macromolecular complex[14,15].

2.3 Expression of *AtGRP9* under salt stress

To investigate the expression of *AtGRP9* gene in *Arabidopsis* subjected to salt stress, 10-day-old seedlings were treated on the filter paper moistened with MS medium containing 0, 50, 100, 150, 200, 250 mmol/L NaCl, respectively for 12 h. Total RNAs were isolated from these plantlets and Northern analysis was conducted using *AtGRP9* cDNA as a hybridization probe. As shown in Fig. 4, expression levels of *AtGRP9* were enhanced in the seedlings subjected to salt stress treatment, with the highest expression occurred in the plantlets treated with 200 mmol/L NaCl, then decreased rapidly. This result indicates the expression of *AtGRP9* can be induced by salinity stress in *Arabidopsis*.

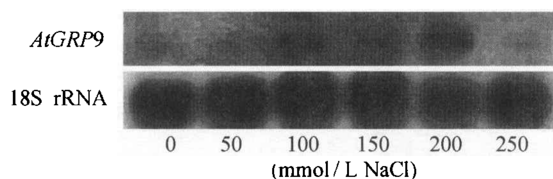

Fig. 4. Expression of *AtGRP9* under salt stress.

2.4 Expression of *AtGRP9* under ABA treatment

There is numerous evidence showing that expression of many plant stress-responsive genes are induced by the plant hormone abscisic acid. To see whether expression of *AtGRP9* is also mediated by ABA, 10-day-old *Arabidopsis* seedlings were treated on filter papers moistened with MS medium containing 10 μmol/L ABA for 0, 1, 3, 6, 9, 12 h, respectively. Total RNAs were isolated from these seedlings and Northern analysis was performed using *AtGRP9* cDNA as a hybridization probe. Fig. 5 shows that the expression of *AtGRP9* was induced by ABA indeed. The expression levels increased along with the duration of ABA treatment, and reached to the top level at 9 h, then decreased rapidly. This result suggests that *AtGRP9* may be involved in the salt stress response mediated by ABA. This ABA-induced expression pattern of *AtGRP9* resembles many other plant GRPs[13].

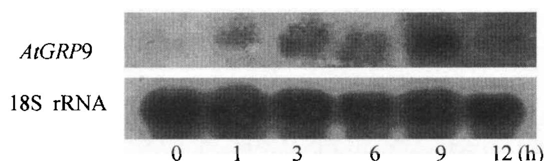

Fig. 5. Effect of ABA treatment on *AtGRP9* expression.

3 Discussion

Glycine-rich proteins are a set of proteins containing glycine repeat domains. In plants, *GRP* genes have been identified from various plant species such as *Arabidopsis*, tobacco, petunia and maize. These plant *GRP* genes exhibit several distinct features: (1) expression pattern of the genes is highly tissue-specific but diverse, and the sub-cellular localisation of different GRP groups is distinct; (2) expression of the genes is regulated developmentally; (3) expression of the genes is induced by various environmental factors such as osmotic stress, plant hormones, wound and pathogens. These characteristics of plant GRPs indicate that they are implicated in several independent physiological processes. For these reasons, it is thought that GRP proteins can be useful as markers and/or models to study different aspects of plant biology[13].

Although the large spectrum of sub-cellular localisation, and of the expression pattern and modulation of GRP genes point out the importance of GRPs in various physiological processes, the exact biological function of these proteins is still obscure. The existing data demonstrated that GRPs may have very diverse functions. For example, bean GRP-1.8 and petunia PtGRP-1 are proposed to have structural functions, presumably acting as scaffold or agglutinating agent for deposition of cell wall constituents[13]; *Arabidopsis* AtGRP3 has been found to interact with the cell wall-associated kinase Wak1 and negatively regulate the function of the enzyme[16], and AtGRP5 is though to be a linker protein mediating membrane-cytoskeleton interconnection[13]. Moreover, some GRPs contain RNA-binding sequences, these proteins may be involved in RNA processing, maturation or control of gene expression[13]. Although our knowledge on the global functions of

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GRPs is limited at present, these observations have shed light on the understanding of the cellular roles of this set of plant proteins.

Database search reveals that there exist forty-one genes encoding GRP or putative GRPs in *Arabidopsis* genome. Up to now, at least eight of these *AtGRP* genes have been reported[13,17]. The *GRP* isolated in our study is a first description on this gene, thus we designated it *AtGRP9*. Overexpression of *AtGRP9* in *S. pombe* enhanced significantly the salt tolerance of the yeast cells, suggesting that *AtGRP9* is a salt stress-responsive gene. In addition, this result also suggests that the mechanism of salt stress response involving *AtGRP9* may be similar in plant and in yeast. In *Arabidopsis*, expression of *AtGRP9* was induced by NaCl as well as by ABA. These data further support the estimation that *AtGRP9* is a salinity stress-responsive gene since the expression of many stress-responsive plant genes have been shown to be mediated by ABA.

The signal peptide observed in the N-terminal portion of AtGRP9 is a common motif in most of the GRPs identified. The presence of this ER signal peptide implies that AtGRP9 may be a protein secreted to cell wall or membrane[13]. AtGRP9 contains almost 50% of glycine residues including five direst GGGGGHY repeats, this kind of glycine-rich region has been proposed to benefit the interaction of GRPs with other proteins[16]. Thus, AtGRP9 may be involved in the salt stress response through binding to some other related proteins in *Arabidopsis*.

We are currently testing the function of AtGRP9 in salt stress responce through transgenic approaches. The preliminary results showed that sense-AtGRP9 transgenic plants were more tolerant to the salinity stress than the wild type *Arabidopsis*. We are also conducting more detailed studies of AtGRP9 in the aspects of sub-cellular location, interacting proteins and its application in genetic engineering of crop salt tolerance.

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