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Plant RelA/SpoT Homolog Confers Salt Tolerance in Escherichia coli and Saccharomyces cerevisiae

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To analyze the mechanisms of salt tolerance in the halophyte Suaeda japonica, Escherichia coli was used as a host organism to undertake functional screening of cDNAs encoding proteins that may play an important role for the salt-tolerance mechanisms. A transformant expressing RelA/SpoT homolog (Sj-RSH) was found to have enhanced salt tolerance. In E. coli, RelA/SpoT controlled the amount of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), which are the effectors of the bacterial stringent response. Complementation analysis using the relA mutant of E. coli showed that Sj-RSH conferred the phenotype associated with (p)ppGpp synthesis. Furthermore, expression of Sj-RSH driven by the GAL1 promoter also gave rise to enhanced salt tolerance in yeast. Northern blot analyses of the yeast transformant revealed that the transcriptional levels of stress responsive genes including GPD1, VMA6, BMH1, HYP1 and HOG1 were clearly enhanced in the Sj-RSH transformant when compared with an empty vector transformant under stress-free and 1.5 M NaCl stress conditions. These results suggest that (p)ppGpp synthesis mediated by plant RelA/SpoT homologs plays a critical role for the transcriptional induction of several stress responsive genes, directly or indirectly in yeast, and that the conserved stress-resistance system may exist in higher plants.

Keywords: Guanosine tetraphosphate — RelA — Salt-tolerance — SpoT — Suaeda japonica.

Abbreviations: ppGpp, guanosine tetraphosphate; pppGpp, guanosine pentaphosphate.
The nucleotide sequence reported in this paper has been submitted to EMBL/GenBank/DDBJ database under accession number AB079577.

Introduction

Salt-stress is one of the most serious factors limiting plant growth and productivity in the world (Boyer 1982). To enhance salt tolerance in higher plants, numerous key genes have been cloned, e.g. late-embryogenesis abundant proteins (Xu et al. 1996), P5CS (Kishor et al. 1995), DREB1A (Kasuga et al. 1999) and AtNHX1 (Apse et al. 1999). Suaeda japonica, a member of the family Chenopodiaceae, is a halophyte that grows on the shores of the Ariake Sea in Japan. This plant species can grow in the presence of 0.7 M NaCl (Yokoishi and Tanimoto 1994, Tanimoto et al. 1997). Finding the key genes for salt tolerance in this plant may greatly assist agricultural productivity in the future. We therefore constructed a cDNA library and undertook functional screening for genes that may be required for salt-tolerance mechanisms, using Escherichia coli as the host organism. This screening isolated a transformant with enhanced salt tolerance that expressed RelA/SpoT homolog (Sj-RSH).

In bacteria, RelA/SpoT determines the level of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) (Cashel et al. 1996). These unusual nucleotides were produced under starvation and other stress conditions, and altered the expression of a large number of gene products in order to adjust cellular metabolisms under the stress conditions. This physiological phenomenon is called the stringent response (Cashel et al. 1996). (p)ppGpp has been found not only in bacteria but also in lower eukaryotes including fungi and yeasts (Hamagishi et al. 1981). However, its physiological functions in eukaryotes are still an enigma. Recently, three plant RelA/SpoT homologs (At-RSH1, 2 and 3) were found in Arabidopsis thaliana (van der Biezen et al. 2000). Phylogenetic analysis of (p)ppGpp synthetase domains of the RelA/SpoT family indicated that At-RSH2 and 3 were clustered, and that At-RSH1 had a distinct amino acid sequence. Functional analysis showed that At-RSH1 confers several distinct phenotypes associated with (p)ppGpp synthesis in E. coli and Streptomyces coelicolor (van der Biezen et al. 2000). The functions of At-RSH2 and 3 are still unknown. Furthermore, the role of eukaryotic RelA/SpoT homologs in stress resistance has not been described previously.

In this study, we found that Sj-RSH, which showed the highest homology with At-RSH2 and 3, enhanced salt tolerance in E. coli and yeast. Furthermore, expression of the Sj-RSH gene in yeast enhanced transcription of several stress responsive genes. Sj-RSH may play a critical role in salt- and osmotic-stress tolerance in E. coli and yeast. This functional characterization of Sj-RSH implicates the existence of a stress...
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resistance system in higher plants, analogous to the bacterial stringent response.

Results

Screening of S. japonica cDNAs

Of one million E. coli transformants expressing S. japonica cDNAs, five showed remarkable growth in the presence of 450 mM NaCl. Analysis of the nucleotide sequences in these transformants showed the presence of five different cDNAs, encoding a full-length RelA/SpoT-like protein, a partial length of phosphoethanolamine N-methyl transferase homolog, and three unknown proteins. In this study, we focused on the cDNA encoding the RelA/SpoT homolog. The length of the S. japonica RelA/SpoT homolog (Sj-RSH) cDNA is 2,716 bp and it encodes 709 amino acid residues. The sequence was deposited in the EMBL/GenBank/DDBJ database under accession no. AB079577. Database searches with the BLAST program (Altschul et al. 1997) revealed that the amino acid sequence of Sj-RSH showed the highest homology with Arabidopsis thaliana RelA/SpoT homologs 2 and 3 (At-RSH2 and At-RSH3), which are similar proteins. The identities between the amino acid sequences of the Sj-RSH and At-RSH2 or At-RSH3 were each 61%. To analyze the functions of Sj-RSH, Sj-RSH cDNA and the empty vector were reintroduced into E. coli, and the salt- and osmotic-stress tolerances of the respective transformants were tested with the spot test (Fig. 1). All transformants grew on normal 2YT medium (86 mM NaCl). The empty vector transformants did not grow on 400 mM NaCl, nor on 1,000 mM sorbitol. In contrast, the Sj-RSH transformant grew in the presence of 400 mM NaCl, or 1,000 mM sorbitol, and it was concluded that this gene may play an important role in the salt- and osmotic-stress tolerance in E. coli.

Phylogenetic analysis

RelA and SpoT are proteins with similar amino acid sequences and play central roles in the bacterial stringent response. A phylogenetic analysis was conducted of the (p)ppGpp synthetase domains of bacterial RelA/SpoT proteins and of the corresponding regions of Sj-RSH and other plant RelA/SpoT homologs (Fig. 2). (p)ppGpp synthetase domains were determined as described previously (van der Biezen et al. 2000). With the exception of three RelA/SpoT homologs in A. thaliana, only two plant RelA/SpoT homologs and a chloroplast RelA/SpoT homolog were found in the protein databases. Plant RelA/SpoT homologs were divided into two groups. Sj-RSH was clustered with At-RSH2, 3 and two other plant RelA/SpoT homologs, and was clearly distinct from At-RSH1. The N- and C-terminal portion of Sj-RSH, excluding the (p)ppGpp synthetase domains, displayed little homology to the corresponding portions of At-RSH1. At-RSH1 confers phenotypes associated with (p)ppGpp synthesis in E. coli and Streptomyces coelicolor (van der Biezen et al. 2000). On the other hand, the functions of plant RelA/SpoT homologs in the At-RSH2 and 3 cluster have not been investigated previously.

Complementation analysis

The primary functions of RelA and SpoT are (p)ppGpp synthesis and degradation, respectively, and SpoT also is capable of (p)ppGpp synthesis under conditions of energy limitation. E. coli relA mutants are unable to grow on minimal agar medium supplemented with the amino acids Ser, Met and Gly (SMG medium) (Ojha et al. 2000). In order to analyze (p)ppGpp synthetase activity in Sj-RSH, Sj-RSH was expressed in the E. coli relA mutant, and growth on SMG agar plates was investigated (Fig. 3). As a control, a transformant with an empty vector was also tested. All transformants, including relA-positive and -negative strains, grew on LB agar plates. The growth of the vector transformant of the relA-negative mutant was strongly inhibited on the SMG agar plate. In contrast, significant growth was observed in Sj-RSH expressing relA-negative strain on the SMG agar plate. Thus, it can be postulated that Sj-RSH has (p)ppGpp synthetase activity, as was previously reported in At-RSH1 (van der Biezen et al. 2000).

Expression of Sj-RSH in yeast

S. cerevisiae was used to investigate the effect of Sj-RSH expression on salt- and osmotic-stress tolerance because of its utility as a model organism in the analysis of eukaryotic genes (Lee et al. 1999, Swire-Clark and Marcotte 1999, Zhang et al. 2000). Fig. 4 shows the growth curves of S. cerevisiae express-
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ing Sj-RSH in SC-uracil medium (Sherman 1991) under stress-free conditions or in the presence of 1.5 M NaCl or 1.35 M sorbitol. Sj-RSH mRNA was detected in the Sj-RSH transformant S. cerevisiae (Fig. 4D). An empty vector transformant was used as a control. Microscopic analyses indicated that there were no significant differences in the cell size and the morphology among these transformants under the stress-free and the various stress conditions. Under stress-free conditions, the growth of Sj-RSH and the control transformants was almost the same (Fig. 4A). Under the salt-stress conditions, the initial growth rate of the transformant expressing Sj-RSH was improved compared with the control (Fig. 4B). Similarly, osmotic stress damage was slightly reduced in Sj-RSH transformants (Fig. 4C). These results suggested that Sj-RSH played an important role in the yeast salt- and osmotic-stress tolerance, as was demonstrated in E. coli (Fig. 1).

Northern analyses

In S. cerevisiae, large numbers of salt-stress responsive genes were identified by DNA chip analysis (Posas et al. 2000). We cloned some of the stress responsive gene fragments, including glycerol-3-P dehydrogenase (GDPI), the 36-kDa subunit of vacuolar H+-ATPase (VMA6), high osmolarity glycerol responsive (HOG1) mitogen-activated protein (MAP)
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kinase, a homolog of mammalian 14-3-3 protein (BMH1), translation initiation factor eIF-5A (HYP2), and heat shock protein 104 (SED5). Fig. 5 shows the effect of Sj-RSH expression in S. cerevisiae on the transcription of several salt-stress responsive genes under stress-free conditions, salt-stress conditions (1.5 M NaCl), and osmotic-stress conditions (1.35 M sorbitol) by Northern blot analysis. The empty vector transformant was used as a control. Under stress-free conditions, transcriptions of GPD1, VMA6, HOG1, BMH1, and HYP2 were enhanced in the Sj-RSH transformant. Furthermore, these enhancement effects were strongly displayed under salt-stress conditions. Under osmotic-stress conditions, without HOG1, significantly enhanced transcriptions of these stress responsive genes were not observed in the Sj-RSH transformant. SED5 is known as a salt-stress responsive gene (Posas et al. 2000). However, transcription of SED5 was not enhanced under all conditions in both transformants. Transcription of SED5 might be transiently enhanced by the addition of NaCl, and therefore enhanced transcription of SED5 was not detected.

All of the data obtained suggested that Sj-RSH-mediated (p)ppGpp synthesis may regulate the expression of several stress responsive genes in S. cerevisiae.

Discussion

In the present study, the RelA/SpoT homolog Sj-RSH was successfully isolated from an S. japonica cDNA library by the E. coli functional screening method, and the role of Sj-RSH in salt- and osmotic-stress tolerance was investigated using E. coli and yeast as host organisms. In general, RelA and SpoT transfer pyrophosphate groups from ATP to 3' positions of GDP and GTP, resulting in the rapid accumulation of (p)ppGpp in bacteria (Cashel et al. 1996). The complementation analysis shown in Fig. 3 strongly suggests that Sj-RSH has (p)ppGpp synthetase activity, as well as bacterial RelA and At-RSH1. Thus, the enhanced salt- and osmotic-stress tolerance in E. coli expressing Sj-RSH may be caused by the (p)ppGpp-dependent transcription of several genes essential for stress tolerance.

To analyze the role of RelA/SpoT homologs in eukaryotes, Sj-RSH was expressed in S. cerevisiae and salt- and osmotic-stress tolerance was investigated. S. cerevisiae could synthesize ppGpp, although neither the RelA/SpoT homolog nor any other ppGpp synthetase was found in this organism (Hamagishi et al. 1981). Surprisingly, enhanced salt- and osmotic-stress tolerance was observed in the S. cerevisiae expressing Sj-RSH (Fig. 4). Northern blot analyses indicate that transcriptions of several stress responsive genes were enhanced in the Sj-RSH transformant under stress-free, salt-stress and osmotic-stress conditions (Fig. 5). Recently, the transcriptional response of S. cerevisiae to salt stress was investigated by genome-wide DNA chip analysis (Posas et al. 2000). This analysis suggested that the transcriptional induction of most genes that are strongly responsive to salt stress is highly or fully dependent on the presence of HOG1. Some data have also suggested that the HOG1-mediated signaling pathway plays a key role in global gene regulation under salt-stress conditions (Schuller et al. 1994, Proft and Serrano 1999, Sunnarborg et al. 2001). In this study, enhanced transcription of HOG1 was found in S. cerevisiae expressing Sj-RSH under stress-free, salt-stress and osmotic-stress conditions, when compared with the expression in each control transformant. Therefore, it can be postulated that Sj-RSH-mediated (p)ppGpp synthesis may regulate expression of several stress responsive genes, directly or indirectly, in S. cerevisiae.

In order to analyze the expression of Sj-RSH in S. japonica, Northern blot analysis was conducted (data not
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shown). As with RelA/SpoT expression in bacteria (Cashel et al. 1996), Sj-RSH mRNA in S. japonica calluses was constitutively expressed in the presence of 150 mM NaCl or in stress-free conditions. Sj-RSH may play an important role in adapting to sudden stress conditions in a similar manner to bacterial RelA/SpoT. Further studies are needed to quantify (p)ppGpp in the plant under stressed or non-stress conditions.

All of these data suggest that the functional characterization of Sj-RSH implicates the existence of a stress resistance system in yeast and higher plants, analogous to the bacterial stringent response.

Materials and Methods

cDNA library synthesis and functional screening

Seeds of S. japonica Makino were collected at Higashi-yoka, Saga Prefecture, Japan. The seeds were sown on vermiculite. Approximately 5-cm lengths of whole plants, which were grown in the presence of 250 mM NaCl, were used to make the cDNA library. This cDNA library was successfully constructed with 1×10^6 independent clones using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA). After construction of the cDNA library, functional screening was conducted as described previously (Yamada et al. 2002a, Yamada et al. 2002b, Yamada et al. 2002c). The cDNA library was transformed into E. coli (SOLR) using an in vivo excision system (Stratagene). One million clones of E. coli transformants expressing the S. japonica proteins were obtained and plated on a 2YT-agar plate containing 400 mM NaCl, 50 μg/ml kanamycin, 50 μg/ml ampicillin, and 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The host strain was unable to grow under this salt-stress condition. To confirm the salt-stress effect more clearly, plasmids from the putative high-salt-tolerant clones were extracted and reintroduced into E. coli (SOLR). The transformants were grown to exponential phase in liquid 2YT medium containing 0.05 mM IPTG, 50 μg/ml ampicillin and 50 μg/ml kanamycin. Serial dilutions, 1 : 10, were made from these E. coli transformants, whose cell density was adjusted to 0.1 (A_600). A 15-μl aliquot of each dilution was spotted on 2YT agar plates supplemented with 86 mM NaCl (control) or 400 mM NaCl, 50 μg/ml kanamycin, 50 μg/ml ampicillin and 0.05 mM IPTG. The spotted agar plates were cultivated at 37°C.

Fig. 5 Effect of Sj-RSH expression in S. cerevisiae on the transcriptional responses to NaCl and sorbitol. The band intensities corresponding to stress responsive genes and ribosomal RNA were determined using a Bioimage analyzer (BAS-1500, Fuji Film, Japan) and Typhoon 8600 (Amer-sham Bioscience Corp., U.S.A), respectively.
Phylogenetic analysis

The amino acid sequences of bacterial RelA/SpoT and the homologs in higher plants were aligned using the CLUSTALW algorithm (Thompson et al. 1994) and phylogenetic analysis was conducted with the neighbor-joining method (Saitou and Nei 1987).

Complementation analysis

E. coli DH5a and the derived relA mutant, ME9038, which is available from the E. coli culture collection in The National Institute of Genetics (Japan), were used for the complementation analysis. SJ-RSH cDNA in EcoRI/XhoI sites of pBluescript SK was introduced into E. coli DH5a and ME9038, respectively. As a control, empty vector transformants were also tested. These transformants were pre-cultured in 2YT medium supplemented with 50 μg/ml ampicillin and 50 μM IPTG. The initial growth phase of these transformants was collected and washed twice with SMG medium. After the wash, cells were resuspended in SMG medium and the concentrations were adjusted to 0.1 (A600). Serial dilutions of these cell suspensions were spotted on LB and SMG agar plates containing 50 μg/ml ampicillin and 50 μM IPTG and incubated at 37°C.

Yeast strains, transformation and growth conditions

S. cerevisiae YM4271, purchased from Clontech (Palo Alto, CA, U.S.A.), was used as a host organism to test the salt-tolerant effect of SJ-RSH expression in yeast. YM4271 was grown on SC-uracil (Sherman 1997) solid medium containing 2% glucose, or in liquid medium containing 1% raffinose and 1% galactose. In order to express Sj-RSH in YM4271, the whole Sj-RSH cDNA was first cloned into the BamHI/XhoI site of a high-copy-number shuttle vector pYES2 (Invitrogen, CH Groningen, The Netherlands) that contains the GALI promoter. The constructed plasmid was then transformed into YM4271 by electroporation. For a control, the empty vector was also introduced into YM4271.

For growth tests, these transformants were pre-cultured in the SC-uracil liquid medium. After the transformants were grown up to 2.0 (A600), cells were inoculated in SC-uracil liquid medium or the medium supplemented with 1.5 M NaCl, or 1.35 M sorbitol and the initial cell concentrations were adjusted to 0.1 (A600). All cultures were grown at 30°C. The liquid cultures were agitated at 200 rpm.

PCR amplification of yeast gene fragments

PCR was conducted to amplify the several salt-stress responsive gene fragments (Posas et al. 2000) using S. cerevisiae YM4271 genome as a template. The following primers were used.

GPD1: 5'-CTGTCTCTTGGAAAAGGCTGCAGGAAAGCC-3', 5'-CAGGCCAGTGTTCTCAAAATCATGC-3'

VMA6: 5'-CTTCTCTGAGGTACGACCGTATTG-3', 5'-CGTGCAGAGGCCGAGGTCATCA-3'

HOCl: 5'-GACACTTCGCAACAGACCGCCTTG-3', 5'-CAGCAACTTGTCATCAACACGTGGCC-3'

BMH1: 5'-CAAACCGTGCAGGATATCTTGTGACC-3', 5'-GAATACCGCTCAACAAGGTCGTCGG-3'

HYP2: 5'-GTGCTCGGAAAGGATACTCCATTTT-3', 5'-CGGGTCATGAGACGAAGAGGATCC-3'

SED5: 5'-CATTGATATGACTGAGGAGGAC-3', 5'-GGGCAACCATCCTCCTCCTGAGC-3'

The PCR products obtained were cloned into the EcoRV endo-nuclease site of pBluescript SK.

Northern analyses

For Northern blot analyses, the yeast transformants were cultured as described in the legend to Fig. 4. The initial growth phase (A600 = 0.75 – 1.00) of each transformant, which was grown in SC-uracil liquid medium or the medium supplemented with 1.5 M NaCl, or 1.35 M sorbitol, was collected immediately. The total RNAs were extracted by the guanidine thiocyanate/CsCl method (Kingston 1991). Twenty micrograms of total RNA was separated by agarose gel electrophoresis and transferred to a nylon transfer membrane, Nytran supercharge (Schleicher & Schuell GmbH, Dassel Germany). The RNA blots were hybridized to yeast stress responsive gene fragments labeled randomly with 32P.

Acknowledgments

This work was supported in part by the Salt Science Research Foundation and Grants-in-Aid for Encouragement of Young Scientists to A.Y. (no. 11740438) from the Ministry of Education, Science, Sports and Culture, Japan.

References


(Received June 18, 2002; Accepted October 13, 2002)