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Research article

AtObgC-AtRSH1 interaction may play a vital role in stress response signal transduction in *Arabidopsis*

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ABSTRACT

The interaction of Obg (Spo0B-associated GTP-binding protein) GTPase and SpoT, which is a bifunctional ppGpp (guanosine 3′,5′-bispyrophosphate) hydrolase/synthetase, is vital for the modulation of intracellular ppGpp levels during bacterial responses to environmental cues. It has been recently reported that the ppGpp level is also inducible by various stresses in the chloroplasts of plant cells. However, the function of the Obg–SpoT interaction in plants remains elusive. The results from the present and previous studies suggest that AtRSH1 is a putative bacterial SpoT homolog in *Arabidopsis* and that its transcription levels are responsive to wounding and salt stresses. In this study, we used a yeast two-hybrid analysis to map the regions required for the AtObgC–AtRSH1 interaction. Moreover, protein–protein docking simulations revealed reasonable geometric and electrostatic complementarity in the binding surfaces of the two proteins. The data support our experimental results, which suggest that the conserved domains in AtObgC and the N terminus of AtRSH1 containing the TGS domain contribute to their interaction. In addition, quantitative real-time PCR (qRT-PCR) analyses showed that the expression of AtObgC and AtRSH1 exhibit a similar inhibition pattern under wounding and salt-stress conditions, but the inhibition pattern was not greatly influenced by the presence or absence of light. Based on *in vivo* analyses, we further confirmed that the AtRSH1 and AtObgC proteins similarly localize in chloroplasts. Based on these results, we propose that the AtObgC–AtRSH1 interaction plays a vital role in ppGpp-mediated stress responses in chloroplasts.

1. Introduction

The stringent response is a wide-spread bacterial adaptation system for global adjustments in response to nutrient limitation and adverse environmental stresses. This response is mediated by the alarmone nucleotide guanosine 3′,5′-bispyrophosphate (ppGpp) (Potrykus and Cashel, 2008). In *Escherichia coli*, RelA synthesizes ppGpp, and SpoT either synthesizes or degrades ppGpp (Mittenhuber, 2001). However, the SpoT hydrolase activity is essential to bacterial cells because high levels of ppGpp disrupt the cell cycle (Xiao et al., 1991). It was recently reported that the *Vibrio cholera* G protein Obg functions as a repressor of the stringent response by interacting with SpoT to maintain low ppGpp levels under a nutrient-rich environment (Raskin et al., 2007). Obg (Spo0B-associated GTP-binding protein) (Wout et al., 2004) is a member of the Obg GTPase subfamily, which broadly exists in both prokaryotes and eukaryotes. In addition, it has been reported that bacterial Obg homologs (from *Caulobacter crescentus*, *Streptomyces coelicolor*, and *Vibrio harveyi*) are essential for cell growth, morphological differentiation, and DNA replication (Maddock et al., 1997; Okamoto and Ochi, 1998; Slominska et al., 2002).

Abbreviations: ACT, aspartate kinase-chorismate mutase-TyrA; At, *Arabidopsis thaliana*; BiFC, bimolecular fluorescence complementation; CTD, C-terminal domain; DS 3.0 software, discovery studio version 3.0 software; ESP, electrostatic potentials; MEGA 5.05, molecular evolutionary genetics analysis; NTD, N-terminal domain; Obg, Spo0B-associated GTP-binding protein; ObgC, chloroplast-targeting Obg GTPase; ONPG, o-nitrophenyl-β-D-galactopyranoside; ppGpp, guanosine 3′,5′-bispyrophosphate; qRT-PCR, quantitative real-time PCR; RD29A, responsive to desiccation 29A; RSH, RelA/SpoT homolog; RT-PCR, reverse transcription PCR; TGS, threonyl-tRNA synthetase-GTPase-SpoT proteins; TP, transit peptide; VDW, van der Waals; VSP2, vegetative storage protein 2.

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ppGpp is found in the chloroplasts of plant cells and is markedly elevated by plant hormones and biotic and abiotic stresses (Takahashi et al., 2004). Plant RelA/SpoT homolog (RSH) proteins have also been found in the chloroplasts of *Arabidopsis* (vanderBiezen et al., 2000), tobacco (Givens et al., 2004), rice (Tozawa et al., 2007), and *Chlamydomonas reinhardtii* (Kasai et al., 2002). In the model plant *Arabidopsis*, four RSHs (AtRSH1, AtRSH2, AtRSH3, and AtCRSH) are exclusively nuclear-encoded proteins and are located in the chloroplasts (vanderBiezen et al., 2000; Masuda et al., 2008). These proteins display different ppGpp synthetase activities, organ specificity, and circadian rhythms-dependent expressions (Mizusawa et al., 2008). Furthermore, the induction of *AtRSH1* and *AtRSH2* transcripts is responsive to wounding and salt stresses (Mizusawa et al., 2008). The detailed molecular mechanism and additional components of this process are still unknown in *Arabidopsis*. Nevertheless, our recent finding that AtObgC strongly interacts with AtRSH1 suggests that AtObgC may be a novel regulatory factor in ppGpp signaling in chloroplasts (Bang et al., 2012).

In some previous reports, AtObgC, as a chloroplast-targeted Obg homolog GTPase, was found to be indispensable for embryogenesis (Bang et al., 2009; Chigri et al., 2009), plastid ribosome biogenesis (Bang et al., 2012), and thylakoid membrane formation (Garcia et al., 2010) during chloroplast development. The chloroplasts of algae and land plants are considered to be originated from ancient cyanobacteria-like prokaryotes. Therefore, chloroplasts conceivably reserve adaptive stress-responsive functions mediated via ppGpp signaling (Braeken et al., 2006). In this study, we used yeast two-hybrid assays and computational docking simulations to demonstrate that all of the conserved domains of AtObgC (Obg fold, G domain, and OCT domain) and the N terminus of AtRSH1 that contains the TGS domain are essential for their interaction. We also found that the expression of *AtObgC* and *AtRSH1* genes is suppressed in a similar manner upon exposure to wounding and salt stress. Taken together, the results obtained in this study suggest that plant AtObgC may play a significant role in the stress-responsive ppGpp signaling pathway by interacting with AtRSH1 in chloroplasts, similarly to bacterial Obgs.

2. Results and discussion

2.1. Three domains of AtObgC$_{208–681}$ are required for interaction with AtRSH1$_{78–883}$

Although RelA and SpoT share high similarity in their amino acid sequences, in *E. coli* and *V. cholera*, Obg only interacts with SpoT and not RelA (Raskin et al., 2007; Wout et al., 2004; Jiang et al., 2007). Based on their primary structures, of the four AtRSHs in *Arabidopsis*, only AtRSH1, which contains the conserved TGS (threonyl-tRNA synthetase-GTPase-SpoT proteins) and ACT (aspartate kinase-chorismate mutase-TyrA) domains, is equivalent to bacterial RelA/SpoT (Atkinson et al., 2011). To identify the *Arabidopsis* homolog of *E. coli* SpoT, we performed a phylogenetic analysis using the amino acid sequences of all four *Arabidopsis thaliana* AtRSHs and *E. coli* SpoT and RelA. The phylogenetic analysis also displays that AtRSH1 rather than AtRSH2, AtRSH3, and AtCRSH is closer to *E. coli* SpoT, which further supports the finding that AtRSH1 is an *Arabidopsis* homolog of the bacterial SpoT protein and may possess specific ppGpp hydrolysis activity, similarly to *E. coli* SpoT (Fig. S1). The finding that AtObgC strongly interacts with AtRSH1 and that its OCT domain is required for this interaction appears to be well consistent with our previous report (Bangetal., 2012).

To reveal the role of all of the conserved domains of AtObgC in its interaction with AtRSH1$_{78–883}$, four truncated and three G-domain mutated *AtObgC* constructs were generated (Fig. 1A). Compared with *AtObgC*(*208–681*) (Fig. 1Ba), the truncated constructs showed significantly lower interaction with AtRSH1$_{78–883}$ (Fig. 1Bb–e), which indicates that the Obg fold, the G domain, and the OCT domain of AtObgC are necessary for this interaction. In this experiment, 1–206 amino acids of the AtObgC N terminus were not used for the interaction analysis because these consists of a chloroplast transit peptide (TP) and a disordered region containing many glutamate and aspartate residues that induce autonomous activity. In addition, to examine which type of guanosine nucleotide binding forms among the GTP-binding, GDP-binding, and nucleotide-free forms is appropriate for the interaction, three point mutants of the G domain were constructed according to a previous

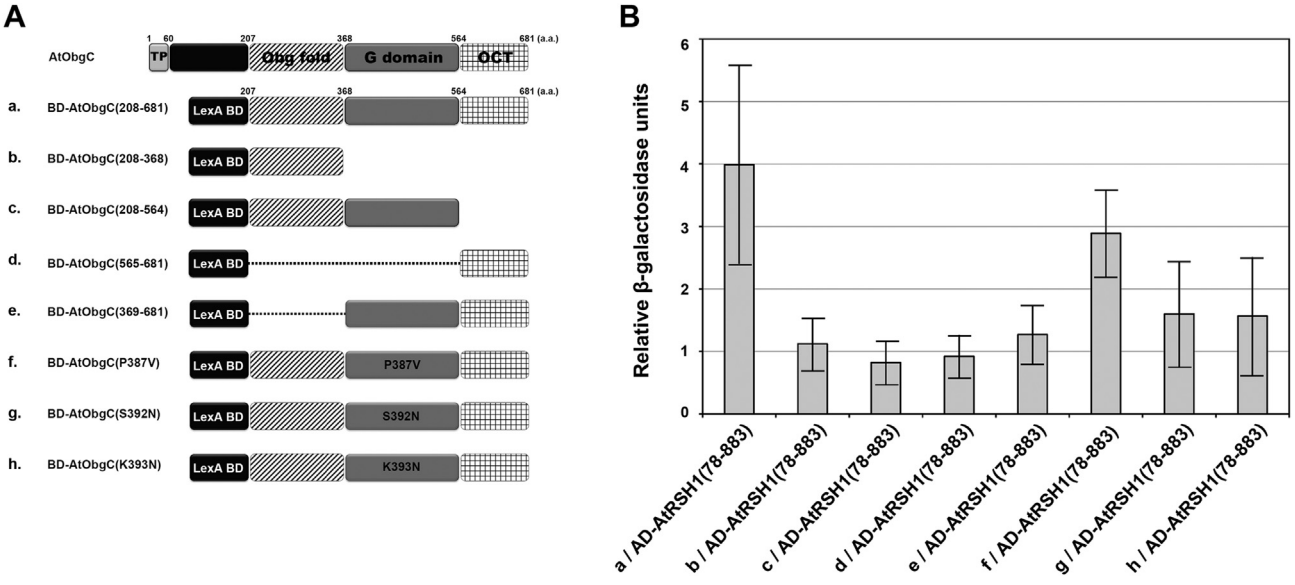

Fig. 1. Dissection of the AtObgC domains that interact with AtRSH1. (A) Functional domain structure of the full-length AtObgC (at top), as described in 'Materials and Methods'. BD and AD used in this assay indicate the LexA DNA binding domain and the B42 activation domain, respectively. Yeast strains containing various *AtObgC* mutant constructs (a–h) with *AtRSH1(78-883)* were tested for *LacZ* activity using liquid ONPG as the β-galactosidase substrate (B). Each β-galactosidase activity, which represents the interaction of various AtObgC mutants with AtRSH1(78–883), was divided by the activity of the corresponding bait construct with the prey vector only. For reproducibility, at least triplicate analyses using three transformants were performed. The error bars indicate the standard deviations ($n = 3$).