Biochemical and Biophysical Research Communications 472 (2016) 353–359

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Overexpression of an *Arabidopsis* heterogeneous nuclear ribonucleoprotein gene, *AtRNP1*, affects plant growth and reduces plant tolerance to drought and salt stresses

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ARTICLE INFO

Article history:
Received 16 February 2016
Accepted 19 February 2016
Available online 26 February 2016

Keywords:
Heterogeneous nuclear ribonucleoproteins
AtRNP1
Growth and development
Abiotic stress
Proline

ABSTRACT

Heterogeneous nuclear ribonucleoproteins (hnRNPs) participate in diverse regulations of plant growth and environmental stress responses. In this work, an *Arabidopsis* hnRNP of unknown function, *AtRNP1*, was investigated. We found that *AtRNP1* gene is highly expressed in rosette and cauline leaves, and slightly induced under drought, salt, osmotic and ABA stresses. AtRNP1 protein is localized to both the nucleus and cytoplasm. We performed homologous overexpression of *AtRNP1* and found that the transgenic plants showed shortened root length and plant height, and accelerated flowering. In addition, the transgenic plants also showed reduced tolerance to drought, salt, osmotic and ABA stresses. Further studies revealed that under both normal and stress conditions, the proline contents in the transgenic plants are markedly decreased, associated with reduced expression levels of a proline synthase gene and several stress-responsive genes. These results suggested that the overexpression of *AtRNP1* negatively affects plant growth and abiotic stress tolerance.

1. Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) comprise of a large family of RNA-binding proteins (RBPs) associated with a wide range of functions such as DNA repair, chromatin remodeling, telomere biogenesis and cell signaling, and are involved in almost the entire life cycle of messenger RNAs (mRNAs) including transcription, 5′ capping, splicing, 3′ cleavage, polyadenylation, nuclear export, translation, storage, degradation and turnover [1,2]. Typical hnRNPs contain one of the following two RNA-binding structures, the RNA recognition motif (RRM) and the K homology (KH) domain, and may also possess other auxiliary structures such as the glycine-rich motifs and the arginine-glycine-glycine box [1,3]. Plants have a complex repertoire of hnRNPs. For example, there are 196 genes encoding RRM-RBPs and 26 genes encoding KH-RBPs in *Arabidopsis* genome [4], and approximately 55 of them encode for hnRNP-like proteins [3]. Plant hnRNPs have highly conserved RNA-binding motifs, distribute in nucleus or cytoplasm, or shuttle between them. In addition to their molecular roles as mRNA metabolic regulators, plant hnRNPs also perform plant-specific biologic functions such as development regulation and environmental stress response and tolerance [3,5].

AtRNP1 encoded by *At4G14300* contains two highly conserved RRMs and a glycine-rich C-terminus, showing high similarity to human hnRNP A1/A3 [6]. This hnRNP-like protein was first identified from a yeast two-hybrid screen with a truncated *Arabidopsis* ortholog of human transportin 1, AtTRN1. Both the full-length and C-terminus of *AtRNP1* interact with the C-terminus of AtTRN1 [6]. The glycine-rich C-terminus of *AtRNP1* contains an M9-like domain, which can be recognized as a nuclear targeting signal by transportin-like nuclear import receptors in diverse organisms [6–8]. Moreover, AtRNP1 was found to localize in both the nucleus and cytoplasm of tobacco protoplasts [6], indicating its role may be both nuclear- and cytoplasm-associated. However, the exact biological functions of AtRNP1 are still elusive. In the present study, we

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http://dx.doi.org/10.1016/j.bbrc.2016.02.081
0006-291X/

investigated the expression pattern of *AtRNP1* in different organs and under diverse stresses, and the subcellular localization of AtRNP1 in *Arabidopsis* protoplasts. We found that the overexpression of *AtRNP1* affects plant growth, and reduces plant tolerance to diverse abiotic stresses. We also found that the proline content and the expression levels of several stress-responsive genes are decreased in *AtRNP1* overexpression lines, under both normal and stress conditions. Our work is the first exploration on the biological functions of AtRNP1, and the results support the idea that plant glycine-rich hnRNPs perform plant-specific roles in growth regulation and stress tolerance [3,5].

2. Materials and methods

2.1. Plant materials and growth condition

Arabidopsis seeds of each genotype from Columbia-0 (Col-0) background were sterilized with 20% bleach and stratified at 4 °C for 3 d before sowed in soil or on Murashige and Skoog (MS) medium [9] (Phytotech) supplied with 1% sugar. All of the plants were grown at 22 °C under a 16-h light/8-h dark photoperiod.

2.2. RNA extraction and gene expression assays

Total RNAs were isolated using an RNAiso® plus reagent (TaKaRa) and reverse-transcribed into cDNAs with a RevertAid® First Strand cDNA Synthesis Kit (Thermo Scientific). The gene expression was analyzed by quantitative RT-PCR (qRT-PCR), which was performed with a SYBR® Premix Ex Taq™ II kit (TaKaRa) on a CFX96® Real-Time System (BIO-RAD). Primer pairs used in this work were listed in Table S1. The qRT-PCR quantifications were normalized to the expression of *TUBIN 2* (*TUB2*) and the relative expression level is calculated using $2^{-\Delta\Delta C_T}$ method [10].

2.3. Vector construction and plant transformation

The full length coding sequence (CDS) of *AtRNP1* (1233 bp, without stop code) was amplified with AtRNP1FLF1/R1 (Table S1), inserted into the binary vector pCAMBIA1300 and fused with an *eGFP* gene to its C-terminal to generate the *p35S*::*AtRNP1*::*eGFP* vector. Another full length CDS of *AtRNP1* (1236bp, with stop code) was amplified with AtRNP1FLF2/R2 (Table S1) and inserted into pCAMBIA1300 to generate the *p35S*::*AtRNP1* vector.

For transient expression, *Arabidopsis* leaf mesophyll protoplasts were enzymolysized and transformed with *p35S*::*eGFP* and *p35S*::*AtRNP1*::*eGFP* vectors as described previously [11]. Fluorescence signals were visualized and photographed using a LSM 510 META laser confocal microscope (Carl Zeiss). For stable overexpression of *AtRNP1*, the *p35S*::*AtRNP1* vector was transformed into *Agrobacterium tumefaciens* GV3101 strain and by which the WT plants were subsequently transformed using the floral dipping method described previously [12]. Positive transformants were screen by 20 mg/L hygromycin (Sigma) and selfed to homozygotes.

2.4. Abiotic stress treatments

The drought stress treatment was performed on adequately watered plants at 2-week-old by water withholding for 2 weeks. Plants were photographed before and after the water withholding. The survival rates were calculated one week after re-watering. The proline measurement (see below) and the expression analysis of stress-related genes were performed at 1 week after water withholding. More than 150 plants for each line were tested in three biological replicates. For salt, osmotic and ABA stress treatments, sterilized and stratified seeds were sowed on standard MS medium in the present or absent of 50, 100, 150 and 200 mM NaCl, 150, 300, 325 and 400 mM mannitol and 0.1, 0.3, 1 and 3 μM ABA. Seeds were photographed and the germination rate was calculated every day after sowing. More than 150 seeds for each line were tested in three biological replicates. For salt, osmotic or ABA treatment before gene expression analysis and proline measurement, 7-d-old seedlings grown on standard MS medium were transferred to MS medium containing 150 mM NaCl, 325 mM mannitol or 0.3 μM ABA, respectively, for 0 h, 1 h or 1 d, and then subjected to gene expression analysis and proline measurement.

2.5. Proline measurement

Proline measurement was performed as described previously [13] with slight modifications. Briefly, plant samples were homogenized in 2 ml of 3% (w/v) sulfosalicylic acid and incubated in water bath at 100 °C for 10 min. After cooling, the homogenate was centrifuged at 3000 rpm for 10 min. 0.4 ml of the supernatant was mixed with 0.4 mL double distilled water, 0.2 mL acetic acid and 0.6 mL 2.5% (w/v) acid ninhydrin reagent (Sigma) and incubated in water bath at 100 °C for 40 min. After cooling, 0.4 ml toluene was added and the mixture was adequately vortexed, and the absorbance of the supernatant was measured at 520 nm. Final proline concentration was obtained from a standard curve. Three biological replicates were performed for each measurement.

3. Results

3.1. The expression pattern and subcellular localization of AtRNP1

The function of a protein is tightly correlated with its expression pattern and subcellular localization. First, using qRT-PCR, we investigated the expression level of *AtRNP1* in different organs. We found that the expression level of *AtRNP1* is relatively higher in rosette and cauline leaves, and is relatively lower in cotyledons and flowers (Fig. 1A). Next, we questioned whether the expression of *AtRNP1* is responsible to diverse abiotic stresses. Results showed that the expression level of *AtRNP1* was up-regulated after 1 week of drought treatment, and it was also up-regulated after 1 or 24 h of salt (150 mM NaCl), osmotic (325 mM mannitol) and ABA (0.3 μM) treatments (Fig. 1B). However, all of these upregulations are relatively weak, within 2-fold (Fig. 1B). These data indicated that *AtRNP1* is responsible to drought, salt, osmotic and ABA stresses. We also investigated the subcellular localization of AtRNP1 in *Arabidopsis* mesophyll protoplasts using transient expression of an AtRNP1::eGFP fusion protein. We found that the eGFP control protein is widely distributed in the nucleus, cytoplasm and plasma membrane, whereas the AtRNP1::eGFP fusion is localized to both the nucleus and cytoplasm (Fig. 1C). This is in accord with the previously observed result in tobacco protoplasts [6].

3.2. Overexpression of AtRNP1 affects plant growth

To investigate the biological function of *AtRNP1*, the full length coding sequence of this gene was cloned and overexpressed in WT *Arabidopsis* under control of the *CMV 35S* promoter. Six typical transgenic lines, designated OE1, OE7, OE8, OE11, OE12 and OE13, were selected for further research. The expression levels of *AtRNP1* in these transgenic lines were elevated from about 12-fold to more than 60-fold (Fig. 2A). Interestingly, all of these transgenic lines exhibited pleiotropic alterations in morphology during various developmental stages. In seedling stage, the primary root of *AtRNP1* overexpression lines were shortened, especially in OE1, OE8, OE11 and OE12 (Fig. 2B and Table 1). The vegetative growth period of all these *AtRNP1* overexpression lines were also shortened, resulting in