Cloning and Characterization of Mitochondrial ATP Synthase 6kDa Subunit gene in Rice (*Oryza sativa.* L)

Xinxin Zhang[1], Tetsuo Takano[2] and **Shenkui Liu[1]**

[1]Alkali Soil Natural Environmental Science Center, Stress Molecular Biology Laboratory, The Northeast Forestry University, Harbin, 150040, People's Republic of China Email shenkuil@mail.nefu.edu.cn
[2]Asian Natural Environment Science Center, The University of Tokyo, Midori Chyou1-1-1, Tanashi City, Tokyo, 188, Japan Email takano@ims.u-tokyo.ac.jp

Abstract

Sodium carbonate ($NaHCO_3$、Na_2CO_3) causing stress for plants is called "carbonate stress" in this study. Some genes that related to carbonate stress were screened from rice (*Oryza sativa.* L) root cDNA library, rice mitochondrial ATP synthase 6kDa subunit gene (shortened form: RMtATP6 gene) is one of these genes. The encoded amino acid sequence has homology to mitochondrial ATP synthase 6kDa subunit (F_0 part of F_1F_0-ATP synthase) purified from potato (*Solanum tuberosum*) mitochondria by Jansch et al. (1996), but the function of this small protein is not clear and the gene has not been identified. In this report, the gene was cloned (Genebank accession no: **AB055076**), and its expression in plant and yeast under carbonate stress and subcellular targeting were characterized. Results suggested that the RMtATP6 gene encoding protein is rice mitochondrial ATP synthase 6kDa subunit. Southern analysis suggested that the RMtATP6 gene is a single-copy gene in the rice genome. Through analyzing the RMtATP6 gene expression in plant, and its expression in yeast under carbonate stress, we can infer that RMtATP6 gene is probably related to carbonate stress. Moreover, experimental data indicated the moderate expression of RMtATP6 gene in yeast can improve the ability against carbonate stress. RMtATP6 protein is targeted to mitochondria in yeast cells as shown by RMtATP6-GFP fusion protein.

Media summary heading

The RMtATP6 gene was cloned, and its expression in plant and yeast under carbonate stress and subcellular targeting were characterized.

Keywords

Rice mitochondrial ATP synthase 6kDa subunit gene; Carbonate stress; yeast; GFP; Subcelluar targeting;

Introduction

F_1F_0-ATP synthase complexes play a central role in the synthesis of ATP in all living organisms. It is a multimeric enzyme composed of a water-soluble portion, F_1, which performs the ATP synthesis and hydrolysis reactions; and a membrane portion, F_0, which mediates the proton transport. They are found in the plasma membranes of bacteria, thylakoid membranes of chloroplasts, and in the inner membrane of mitochondria (Lefebvre-Legendre *et al*., 2001). The plant mitochondrial ATP synthase displays the classical F_1 five subunits structure (Heazlewood *et al*., 2003). The F_0 structure of the plant mitochondrial F_1F_0-ATP synthase is more complicated. The mitochondrial F_1F_0-ATP synthase from beef heart mitochondria comprises 16 different subunits, five of which are part of F_1 (Collinson *et al*., 1994). The corresponding protein complex from plants, which had been isolated from spinach leaf mitochondria, could be resolved into 12 different polypeptides (Hamasur and Glaser, 1991; Hamasur and Glaser, 1992). Jansch et al. purified the mitochondrial F_1F_0-ATP synthase (complex V) from potato (*Solanum tuberosum*) mitochondria. It is resolved by BN-PAGE in two forms: the intact F_1F_0 complex (580 kDa) comprising six polypeptides. The potato F_0 section is composed of at least seven polypeptides with apparent molecular mass of 27, 23, 20, 20, 15, 6, and 6 kDa, one of the two 6 kDa proteins is the F_0 part of complex V, it does not exhibit significant similarity to any published primary structure (Jansch et al., 1996), but the

function of this small protein is not clear and the gene has not been identified. In this report, the gene was cloned and characterized.

Materials and methods

Plant Materials, Construction of Expression Vectors and media

Rice (*Oryza sativa*, cultivar: *Nipponbare*) plants were grown in soil-containing pots in the green house under controlled environmental conditions. Roots were harvested for construction of cDNA library; leaves were harvested for extraction of total DNA. RMtATP6, GFP and RMtATP6-GFP genes were respectively constructed into vector pYES2. The following media were used for growth of yeast cells: rich medium (YP) (1% yeast extract and 2% peptone) supplemented with different carbon sources, 2% glucose (YPD); 2% galactose and 1% raffinose (YPGR); 1.94% galactose, 0.06% glucose and 1% raffinose (YPGDR).

Construction and Screening of a cDNA Library

A cDNA library of rice root (cultivar: Nipponbare) was constructed by SMART cDNA Library Construction Kit (Clonthech, USA). The library was screened with cDNA fragments as probes.

DNA Sequence Analysis

DNA sequence was determined from DNA cycle sequencing reactions, which was performed with fluorescent dye terminators followed by product analysis on an automated model 373 DNA sequencer.

Nucleotides Blot Analysis

Nucleotides blot hybridization was performed with the DIG DNA labeling and detection kit (Boehringer Mannheim, Germany). Signal was detected under Luminescent Image Analyzer LAS-1000 plus (Fujifilm, Japan).

Staining of mitochondria and analysis by confocal microscopy

Yeast mid-log-phase cells were dyed with Mitotracker Red CmxRos (Molecular Probes) Confocal microscopy was performed with FV500 laser-scanning confocal imaging system (OLYMPUS).

Results

Cloning and characterization of gene expression

The length of RMtATP6 cDNA is 505 bp. The open reading frame encodes a predicted 58 amino acids protein with a calculated molecular mass 6.578kDa. Protein databases in DDBJ were searched for amino acid sequences with similarity to RMtATP6 encoding protein. The BLAST algorithm identified two amino acid sequences from *Arabidopsis thaliana* and potato (*Solanum tuberosum*) encoding proteins with greater similarity to RMtATP6 protein (Fig.1). RMtATP6 protein is most homologous to the *Arabidopsis thaliana* putative protein (accession no: **AL133298-15**), with 76% of the amino acids identical in total. Also RMtATP6 protein is more similar to ATP synthase 6kDa subunit in mitochondria of potato (*Solanum tuberosum*) (protein ID: **P80497**) with 73% of the amino acids identical in 25 length. According to these, we can predict that RMtATP6 protein and the two proteins is probably the identical protein.

The genomic organization of RMtATP6 was analyzed by Southern blot (Fig.2). Results suggested that RMtATP6 gene lies in rice genomic DNA and that RMtATP6 gene is a single-copy gene in rice. We tested the RMtATP6 gene expression in rice leaf and root under carbonate stress (leaf and root treated by 60mM $NaHCO_3$, respectively), results indicated the RMtATP6 gene was induced by this stress (Fig.3 (1~2)).

Role of the RMtATP6 gene against carbonate stress and subcellular targeting in yeast

Yeast cells containing the plasmid pYES2-RMtATP6 and pYES2 were used to investigate the growth in three media under carbonate stress condition. In YPD medium, transcription from the *GALI* promoter of yeast strain INVSc1 is repressed in the presence of glucose, so the pYES2-RMtATP6 recombinant gene expression was repressed and the growth rate was the same to pYES2 cells, both of them almost have the same ability against the carbonate stress (Fig.4A). In YPGR medium containing galactose with high concentration, the pYES2-RMtATP6 recombinant gene expressed a great lot, the pYES2-RMtATP6 cells have a reduced growth rate in comparison to that of the pYES2 cells and show the decreased ability against the carbonate stress (Fig. 4B). Consequently, we controlled the RMtATP6 gene expression through adding 0.06% glucose to medium (YPGDR medium) to improve the gene ability against the carbonate stress. In YPGDR medium, pYES2-RMtATP6 recombinant gene expressed moderately and the normal activity of yeast cells was not affected seriously, the growth rate of yeast cells is more rapidly than pYES2 cells and ability against carbonate stress was strengthened (Fig.4C).

We used the GFP protein to determine the localization of RMtATP6 gene encoding protein in vivo, we fused the yeast protein with the GFP at the C terminus. The mitochondria were visualized using a mitochondria-specific dye for living cells, Mitotracker. As controls, we used construct encoding GFP protein to study its targeting in yeast cell (Fig.5d-f). The green fluorescence of GFP protein is almost distributed throughout the yeast cell (Fig.5d), the localization signal is not consistent with the mitochondrial localization signal (red fluorescence area) (Fig. 5e-f). This result indicates the GFP protein is not targeting specific sites in yeast cell. The RMtATP6-GFP fusion protein is evenly distributed throughout the mitochondria, as can be observed by the RMtATP6-GFP/Mitotracker double staining (Fig.5a-c). The result of in vivo localization of RMtATP6-GFP protein maybe suggest that RMtATP6 gene of rice nucleus genome finishes transcription and translation to produce the RMtATP6 protein, which is localized in mitochondria.

Fig.1 Comparison of amino acid sequences among rice (*Oryza sativa*), *Arabidopsis thaliana* and potato (*Solanum tuberosum*).

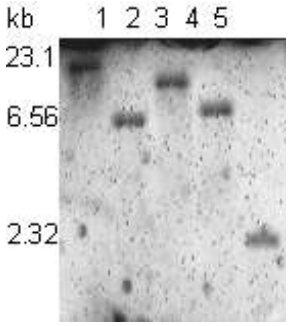


Fig.2 Southern blot analysis of RMtATP6 gene. Lane1-5: Rice (*Oryza sativa*)genomic DNA was digested respectively with *Eco*RI, *Eco*RV, *Hin*dIII, *Bam*HI and *Xba*I.

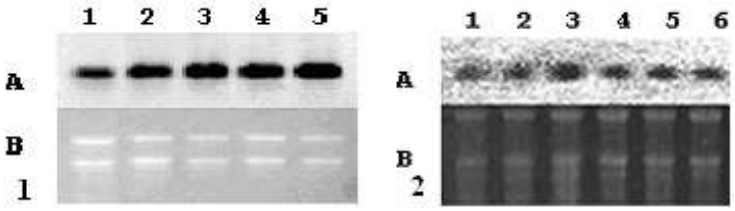
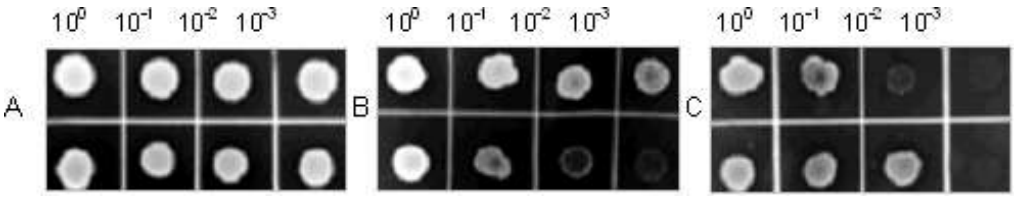


Fig.3 (1) Northern blot analysis of RMtATP6 gene expression in rice leaf treated by 60mM Na_2CO_3. A. Northern blot, B. Et-Br staining, Probe: RMtATP6 cDNA DIG labeling, lane1. control (pH7.0), lane2-5. treated respectively by 60mM Na_2CO_3 for 4, 8, 12, 16hours. (2) Northern blot analysis of RMtATP6 gene expression in rice root treated by 60mM Na_2CO_3. A. Northern blot, B. Et-Br staining, Probe: RMtATP6 cDNA



DIG labeling, lane1. control (pH7.0), lane2-6. treated respectively by 60mM Na_2CO_3 for 4, 8, 12, 16, 20hours.

Fig.4 The analysis of pYES2-RMtATP6 recombinant gene against carbonate stress. Yeast cells were grown in liquid medium at 30 ℃. The cultures were then normalized to the same OD600 before making serial dilutions and spotting them in 6 ?l droplets onto each medium. In every picture, the upper line represents the yeast cells containing pYES2-RMtATP6 plasmid; the lower line represents the pYES2-CV plasmid. A: YPD medium; B: YPGR medium; C: YPGDR medium.

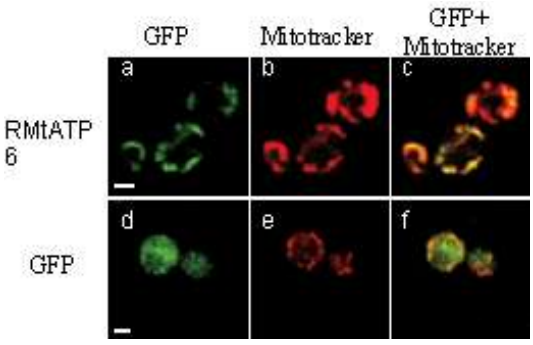


Fig.5 RMtATP6-GFP fusion protein is located at specific sites of mitochondrion in yeast. RMtATP6 has been cloned in frame at the C-terminal end with the GFP gene in the pYES2 vector and expressed in the yeast strain INVSc1. Live cells were incubated with Mitotracker as described in Materials and Methods, and the localization of RMtATP6-GFP was determined using a confocal microscope. a-c: expression of the RMtATP6-GFP fusion constructs in yeast; b-d: expression of the GFP protein in yeast. Scale bars, 2μm.

Conclusion

In this report, we cloned the investigated the expression of a 6 kDa mitochondrial ATP synthase subunit gene of rice, which most likely represents a plant specific subunit of this protein complex, in plant and yeast cells under carbonate stress. We found that this gene is related to the carbonate stress and

moderate expression of this plant protein improves yeast growth in the presence of stress. The protein is targeted to mitochondria in yeast cells as shown by a GFP fusion protein.

References

Collinson IR, Runswick MJ, Buchanan SK, Fearnley IM, Skehel JM, van Raaij MJ, Griffiths DE, Walker JE (1994) F_0 membrane domain of ATP synthase from bovine heart mitochondria: purification, subunit composition, and reconstitution with F1-ATPase. Biochemistry 33, 7971-7978.

Hamasur B, Glaser E (1991). F_1F_0-ATPase of plant mitochondria: isolation and polypeptide composition, *Biochemistry.* Biophys. Res. Commun 170, 1352-1358.

Hamasur B, Glaser E (1992). Plant mitochondrial F_1F_0-ATPase: Identification of the individual subunits and properties of the purified spinach leaf mitochondrial ATP synthase. Eur. J. Biochem 205, 409-416.

Lefebvre-Legendre L, Vaillier J, Benabdelhak H, Velours J, Slonimski PP, di Rago JP (2001). Identification of a nuclear gene (FMC1) required for the assembly/stability of yeast mitochondrial F_1 - ATPase in heat stress conditions. J. Biol. Chem 276, 6789-6796.

Heazlewood JL, Whelan J, Millar AH (2003). The products of the mitochondrial *orf*25 and *orf*B genes are F_0 components in the plant F_1F_0 ATP synthase. FEBS Letters 540, 201-205.

Jansch L, Kruft V, Schmitz UK, Braun HP (1996). New insights into the composition, molecular mass and stoichiometry of the protein complexes of plant mitochondria. Plant J 9, 357-368.