Prokaryotic Expression, Protein Purification and GTP-binding Assay of Rice *rab5A* Gene*

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Abstract: *Osrab5A*, a *rab5A* gene cloned from Rice (*Oryza sativa* L.), encodes a putative Rab protein with high similarity to mammalian Rab5A, which was localized on the early endosome and in the cytoplast. Rab5A was identified in mammalian cells as a rate-limiting factor for homotypic endosome fusion. The complete coding sequences of *Osrab5A* cDNA clone were amplified and inserted into the prokaryotic expression vector pGEX4T1. The resulting recombinant plasmid with correct reading frame, pG-5AE, was introduced into *E. coli*. BL21 (DE3), The GST tagged fusion protein, GST-OsRab5A was induced by IPTG induction and purified on a GSTrapTM column. The GTP-binding assay of this protein showed the GST tagged OsRab5A protein has GTP-binding ability *in vitro*.

Key words: rice (*Oryza sativa L.*); Rab protein; GTP—binding assay
CLC number: Q78 **Document code**: A **Article ID**: 0529-6579 (2002) 03-0060-04

Extensive studies on mammals and yeast indicated that Rab/Ypt proteins, which belong to small GTP-binding protein superfamily, play important roles in the intracellular membrane traffic, including secretory pathway from endoplasm reticulum to Golgi and to plasma membrane then, and endocytosis pathway from plasma membrane to lysosome, Golgi or other subcellular membrane compartments[1—4]. Three isoforms of Rab5 proteins in mammalian cells, named Rab5A, Rab5B and Rab5C, were demonstrated to control early endosome fusion cooperatively[5,6]. Many *rab5A* genes were cloned form various plants, including *Nicotiana tabacum*[7], *Vicia faba*[8], *Arabidopsis thaliana*[9], *Lotus japonicus*[10], *etc*. However, little is known about their subcellular localization and function. To understand the functional properties of the rice counterpart of mammalian Rab5A protein, we isolated a *rab5A* cDNA clone from rice (*Oryza sativa*L.). The nucleotide sepuences of it has been deposited in DDBJ/EMBL/GenBank with accession anmber AJ292320. Here we describe the construction of expression vector, protein purification and GTP-binding assay in regards to rice Rab5A protein.

1 Materials and Methods

1.1 Materials

E. coli BL21 (DE3), plasmid vector pGEX4T1 (Amersham Pharmacia), and recombinant plasmid pMD—5A were stored in our laboratory.

1.2 Reagents

Restriction endonuclease, T_4 ligase, DNA and protein molecular weight standard, α-^{32} PGTP, oligonucleotide (Genecore), Nitrocellulose.

1.3 Construction of expression vector

The complete Coding sequences (CDS) of *Osrab5A* cDNA clone were amplified with primes 5AE1 and 5AE2. The sequences of these primers were 5'—CGG AAT TCA TGG CGG CCA ACC CCG GC—3' and 5'—CCG CTC GAG ATT ATG AGC AGC ATG AAG—3', with restriction endonucleases *Eco*R I and *Xho* I site, respectively. The PCR profile was as following: initial denaturing at 94 ℃ for 3 min, then 25 cycles of denaturing at 94 ℃ for 45 s annealing at 60 ℃ for 45 s, and elongation at 72 ℃ for 90 s, and then followed by an extra elongation reaction at 72 ℃ for 6 min. The PCR products with

* 收稿日期: 2002—03—30
基金项目: 国家转基因植物研究与产业化专项资助项目 (J00—A—009); 中科院华南植物所所长基金项目 (10—00—312)
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anticipated size were digested with *Eco*RⅠ and *Xho* Ⅰ, and then inserted into the prokaryotic expression vector pGEX4T1 according to standard procedure[11]. The resulting recombinant plasmid was introduced into *E. coli* BL21 (DE3). The in-frame CDS were confirmed by sequencing. The resulting clone was named pG-5AE.

1.4 Sequence analysis

DNA sequencing was performed on ABI PRIME™ 377. The sequences were aligned through CLUSTAL W program[12].

1.5 Prokaryotic expression of GST-fused protein

An isolated pG-5AE colony was inoculated into 3 mL 2× YT liquid medium with appropriate amount of ampicillin and incubated at 37 ℃ with vigorous shaking at 200 r/min. A 0.8 mL of overnight culture was inoculated into 80 mL fresh 2× YT liquid medium with the same culture condition for 2 h. The culture was then cooled to room temperature before addition of isopropyithio β-galactoside (IPTG) to a final concentration of 0.3 mol/L. The culture was incubated at 30 ℃ for 3 h at 200 r/min[11]. The induced cells were collected by centrifugation. The cell pellet can be stored at −70 ℃ for short period.

1.6 SDS-PAGE

SDS-PAGE was performed according to Lameli[13].

1.7 Purification of GST-tagged fusion protein

The pellet of induced cells was resuspended with 20 mL of 1× PBS. 0.5 mL of 20 mg/mL lysozyme was added to the resuspension. The resuspension was ice-bathed for 20 min. After addition of DTT and PMSF to final concentration of 5 mol/L and 1 mol/L, respectively, the cells were lysed by sonication with a Φ 5 mm head for 80 cycles of 4-second-on- and 4-second-off. The lysates were centrifuged at 12 000× g for 30 min at 4 ℃. The supernatant was filterd through a 0.45 μm filter, and then applied onto a GSTrap™ column (Amersham Pharmacia). Purification was performed according to the manufacturer's instruction. The eluate was collected and analyzed by 12% SDS-PAGE.

1.8 GTP-binding assay

Protein samples were fractionated by 12% SDS—PAGE and electro-blotted onto a nitrocellulose filter (Schleicher and Schuell). The filter was incubated in buffer A (20 mmol/L Tris-HCl 1 mol/L $MgCl_2$ 5 mol/L DTT 0.3% Tween20) at 30 ℃ for 10 min, and then transferred to a 30 mL of fresh buffer A plus 0.3% BSA and 5 μL of α^{-32}PGTP (3 000 Ci·mmol^{-1}, 10 mCi·mL^{-1}), After incubation at 30 ℃ for 30 min, the filter was washed with fresh buffer A three times, each for 5 min. The filter was air-dried and autoradiolabelled[14].

2 Results

2.1 Construction of prokaryotic expression vector pG-5AE

The recombinant vector for expression of fusion protein GST-OsRab5A was constructed as Figure 1 indicated. Each step was described in detail in the section of Materials and Methods. The resulting recombinant plasmid pG-5AE was introduced into *E. coli* BL21 (DE3) and confirmed by agarose electrophoresis (Figure 2) and sequencing.

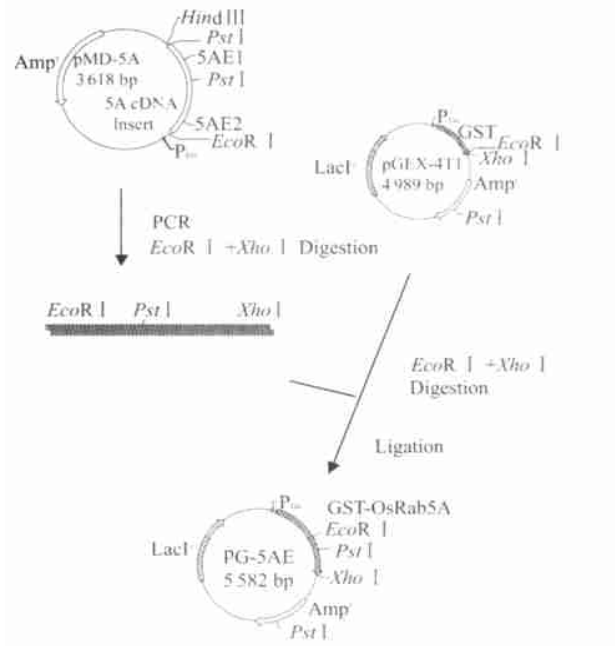

Fig. 1 Flow chart of construction of expression vector of pG-5AE

(5AE1 and 5AE2 indicated the position of primer 5AE1 and 5AE2 respectively)

2.2 Expression of fusion protein GST-OsRab5A

Transformed cells with plasmid pG-5AE were induced by IPTG and characterized by SDS-PAGE. 500 μL of culture incubated with addition of IPTG was collected and washed by 50 μL of STE. The pellet was resuspended with 50 μL of 50 μL of 2× SDS-PAGE protein loading buffer and boiled for 3 min. 20 μL aliquots were fractioned by 12% SDS-PAGE. Compared with the controls, the encoded products of pG-5AE were synthesized with expected molecular weight (Figure 3).

2.3 Purification of fusion protein GST-OsRab5A

Purification of fusion proteins GST-OsRab5A with affinity column GSTrap™ was performed according to the manufacturer's instruction. SDS-PAGE and image-scanning analysis indicated more than 95% of the eluates were the target proteins (Figure 3). The fusion protein also could be found in the flow-through (data not shown).

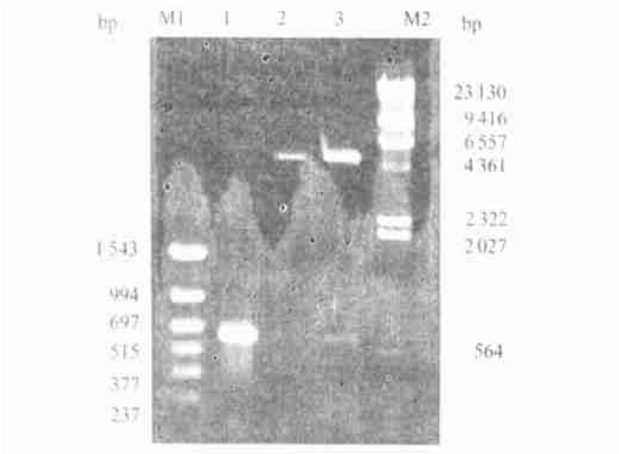

Fig. 2 Restriction endonuclease analysis of expression vector pG-5AE

M1, M2 DNA molecular weight standards;
1 PCR amplified target fragment;
2 linear pGEX-4T1;
3 digested products of plasmid pG-5AE with endonucleases of *Eco*RI and *Xho*I

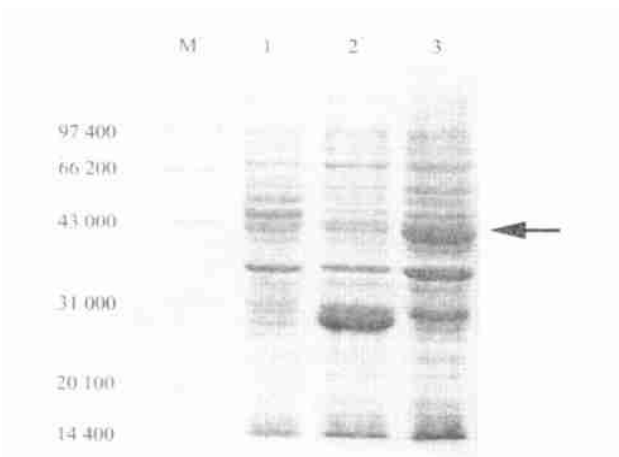

Fig. 3 SDS-PAGE analysis of expression of pG-5AE

M protein molecular standard;
1, 2 the induced total bacterial protein with expression of pG-5AE;
3 GST control;
4 total bacterial protein control

(The arrow shows the target fusion protein, GST-OsRab5A)

The reasons for it may accredit to the binding efficiency of the affinity column and sample overloading.

2.4 GTP-binding assay of fusion protein GST-OsRab5A

The purified fusion protein was electro-blotted onto nitrocellulose filter after fractionation by SDS-PAGE. Total bacterial proteins with synthesis of GST or fusion protein GST-OsRab5A were loaded on the same gel as control. The filter was then subjected to GTP-binding assay. The autoradiograph showed

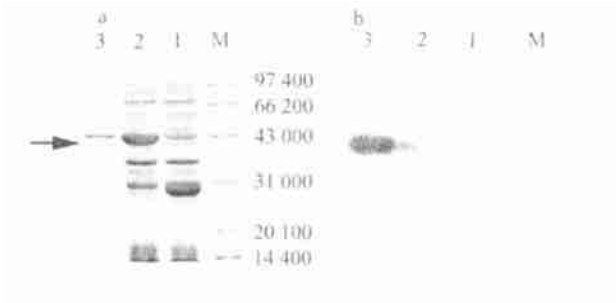

Fig. 4 SDS-PAGE analysis and GTP-binding assay of fusion protein of GST-OsRab5A

a SDS-PAGE analysis of fusion protein of GST-OsRab5A.
M protein molecular standard;
1, 2 total bacterial protein with induced expression of pGEX-4T1 and pG-5AE respectively;
3 purified fusion protein of GST-OsRab5A marked by the arrow
b GTP-binding assay of fusion protein of GST-OsRab5A.
The protein was blotted from a, so lanes M, 1, 2 and 3 on panel B are the counterparts illustrated on panel a

clearly that the heterologous fusion protein has GTP-binding ability. There were faint noises on the lane of total bacterial protein plus fusion protein.

3 Discussion

In summary, we constructed a recombinant vector for the expression of GST-fused OsRab5A protein. The fusion proteins were synthesized in *E. coli* cells and purified by affinity chromatography. The GTP-binding assay against the fusion protein indicated OsRab5A protein had GTP-binding ability *in vitro*. However, There were faint noise signal on the autograph of GTP-binding assay on the lane containing unpurified fusion protein. To study what caused these noises, we had designed controll experiments. Those investigations indicated that the faint noises were most probably due to very small amount of by-products of truncated fusion proteins. These truncated proteins may be produced in the synthesis process (incorrectly stop, for example) and over-sonication (data not shown). Anyway, the autograph suggested that the Osrab5A gene is a functional gene. Based on these work, we will further characterize other biochemical and biophysical features of rice Rab5A protein.

Acknowledgements: We are grateful to Dr. Liu Qiu-yun for his critical reading and helpful suggestion for this manuscript.

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水稻小 GTP 蛋白 OsRab5A 的表达、纯化和 GTP 结合分析

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摘　要：小 GTP 结合蛋白是一类在细胞内的运输过程中重要作用的蛋白。它们通过结合子 GTP 而激活，当 GTP 被水解为 GDP 后处于非活性状态。哺乳动物中的研究表明，Rab5A 蛋白是细胞内的形成早期内吞体 (early endosome) 的限速因子。证实了所克隆的水稻 *rab5A* 基因编码的蛋白具有 GTP 结合功能。将 *Osrab5A* 基因的编码序列按正确读码框重组到 pGEX4T1 载体中，转化大肠杆菌 BL21 (DE3)，电泳判定插入片段大小无误后，进一步测序鉴定其读码也无误。将该克隆命名为 pG-5AE。以 IPTG 诱导 pG-5AE 所编码的融合蛋白 GST-OsRab5A 的表达。SDS-PAGE 电泳与无外源质粒和表达谷胱甘肽硫转移酶 (GST) 的对照相比，得到了预计大小的融合蛋白。以 GSTrapTM 亲和柱纯化融合蛋白。在不同的泳道分离纯化的融合蛋白和作为对照的含 GST 以及无外源蛋白的细菌总蛋白，电转移到硝酸纤维膜上。将该膜与 α-^{32}PGTP 温育，洗膜，放射自显影后，获得了融合蛋白结合 GTP 的结果，这表明在原核中表达出的水稻 Rab5A 蛋白能在体外结合 GTP。

关键词：水稻；Rab 蛋白；GTP 结合
中图分类号：Q78