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Full Length Research Paper

Ectopic expression of a vesicle trafficking gene, OsRab7, from *Oryza sativa*, confers tolerance to several abiotic stresses in *Escherichia coli*

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Rab7 is a small GTP-binding protein involved in intracellular vesicle trafficking from late endosome to the vacuole. In this study, the gene *OsRab7* was isolated from *Oryza sativa*. Over-expression of *OsRab7* gene in *Escherichia coli* increased the resistance to heat, cold and salt stress. In addition, subcellular localization of OsRab7 protein in *E. coli* and onion cells all revealed that OsRab7 protein is specifically present in the cytoplasm. These results showed that *OsRab7* plays an important role in stress tolerance and probably develop abiotic stress tolerance in *E. coli* and planta with similar mechanism.

Key words: Vesicle trafficking, rice, *OsRab7, Escherichia coli,* abiotic stresses.

INTRODUCTION

Small GTP-binding protein are largely delivered among eukaryotes, which are involved in a wide variety of cellular processes in eukaryotic cells including signal transduction, cell proliferation, vesicular transport and cytoskeletal organization. Small GTP-binding proteins can be subdivided into five families: Ras, Rho, Arf, Ran and Rab. Rab proteins constitute the largest subfamily of small GTPase and play an important role in intracellular vesicle trafficking and in the organization of membranes (Zerial and McBride, 2001). In animal and yeast cells, Rab7 GTPase is generally thought to be required for the formation of lytic compartments (Bucci et al., 2000; Rosales et al., 2009) and the Rab7 protein was found localized to late endosomes and lysosomes/vacuoles (Bruckert et al., 2000; Schimmoller and Riezman, 1993; Balderhaar et al., 2010). In plant, Rab7-related protein appears to be located on the vacuolar membrane and regulate the vesicle fusion with the vacuole in *Arabidopsis* (Mazel et al., 2004). While in soybean, the presence of Rab7-related protein was found on both endosomes and tonoplast, indicating that Rab7 multi-vesicular bodies are participating in endocytosis pathway (Limpens et al., 2009).

Endocytosis has been viewed traditionally as a constitutive housekeeping function in organism, however, the information about the intracellular vesicle trafficking in resistance to environmental stress have been reported recently. In *Escherichia coli,* mutations that caused increased vesiculation enhance bacterial survival upon challenge with stress agents or accumulation of toxic misfolded proteins (McBroom and Kuehn, 2007). In yeast, the vesicle trafficking between cytol and the plasma membrane was inhibited by oxidative stress (Levine et al., 2001). In *Arabidopsis thaliana*, the transgenic plant which knocked down the expression of an autophagy-related gene *AtATG18a* was more sensitive to salt and osmotic stress (Liu et al., 2009). Many plant *Rab7* gene were determined to be affected by environmental stressors, and transgenic experiments demonstrated that over-expression of the *AtRab7* and *PgRab7* could increase stress tolerance in transgenic *A. thaliana* and transgenic tobacco, respectively (Agarwal et al., 2008; Mazel et al., 2004). However, the biological functions of ectopic expression of such a gene are seldom studied.

In this study, we introduced a vesicle trafficking gene from rice, *OsRab7*, into *E. coli*, and we found that over-expression of *OsRab7* gene enhances heat, cold and salt tolerance of *E. col i* cells. In addition, the subcellular

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localization of OsRab7 protein in *E. coli* and onion cells revealed that OsRab7 protein is specially localized in the cytoplasm.

MATERIALS AND METHODS

Oryza sativa seeds were kindly provided by Key Laboratory of MOE for Plant Developmental Biology in Wuhan City, *E. coli* DH5α and BL21 (DE3) strains were kept in Key Laboratory of Molecular and Gene Engineering in Nanchang City.

Isolation of *OsRab7* CDNA

Total RNA was isolated from rice seedling with Trizol Reagent (Invitrogen). The reverse transcription was performed using MMLV Reverse Transcriptase (Invitrogen) according to the manufacturer's directions. To get the full length *OsRab7*, a pair of primers was designed according to the *OsRab7* CDNA sequence. The primer sequence was forward: 5'- CCGGAATTCATGGCTTCGCGCCGC-3', reverse: 5'- GC ACTCGAGCTAGCA GCAGCCTGATGATCTTG-3'.

Plasmid construction

OsRab7 sequence was ligated into the pET-28a vector digested with *Eco*R1 and *Xho*I to construct the plasmid *pET-OsRab7*. The coding sequence of EGFP was PCR amplified from plasmid pEGFP-C1 (Clontech) using primers: sense, 5'-GGGATCCG TGAGCAAGGGCGAGGAGCTG-3'; antisense, 5'-GCGAGCTCTTA CTTGTACA GCTCGTCCATG-3'. EGFP sequence were ligated into the *pET-28a* vector digested with the same enzymes from an intermediated construct named *pET-28a-GFP*, and then the *OsRab7* sequence was ligated into the plasmid *pET-28a-GFP* digested with Nde1 and BamH1, the resulting construct was designated *pET-OsRab7-GFP.*

Expression of the recombinants

The plasmids, pET-28a and *pET-28a-OsRab7* were transformed into *E. coli* strain BL21, respectively. The cells were grown at 37°C in 20 ml LB medium containing kanamycin (50 mg/L) with shaking (250 rpm). IPTG was added at 1 mM when the OD600 value was 0.6. After incubated for 4 h, the cell proteins were analyzed by 12% (W/V) SDS-PAGE.

Western blot

The recombinant proteins were separated by 12% SDS-PAGE gel, and then transferred onto an PVDF transfer membrane (PVDF type; Millipore). A polyclonal antibody against OSRAB7 protein purified from recombinant *E. coli* was raised in rabbits. The anti-OSRAB7 antiserum used at 1:5000 and a goat anti-rabbit IgG conjugated with alkaline phosphatase (AP) with a dilution of 1:2000 were used for Western blot. Finally, signal was detected with a method described in Li et al. (2004) and Yue et al. (2004) with brief modifications: the membrane was washed with AP 7.5 buffer (0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$) twice, and once with AP 9.5 buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 10 min each. Then, the membrane was incubated with 2.5 mg nitroblue tetrazolium (NBT; Promega) and 1.25 mg 5-bromo-4-chloro-3–indolyl phosphate (BCIP; Promega) in 7.5 ml AP 9.5 buffer at room temperature until the signal appeared. Finally, TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) was added to stop the reaction.

Abiotic stress-resistance assays

To test the temperature stress tolerance of *OsRab7* transformant, the cell cultures of BL/pET-28a and BL/*pET-OsRab7* were induced by 1 mM of IPTG for 4 h and adjusted to OD600 at 0.8 in spectrophotometer (Pharmacia, USA) and exposed at -20°C for 24 h or 52°C for 45 min, respectively. Then the cultures were diluted serially (1:10) and 10 µl of each sample was spotted onto the LB plates. These plates were incubated overnight at 37°C overnight. To assay the salt stress tolerance of BL/pET-OsRab7, the cell cultures were induced and adjusted to OD600 at 0.8 as described earlier and then diluted serially (1:10). 10 µl of each sample was spotted onto the LB plates in the presence or absence of 1000 mM NaCl. Then the plates were incubated at 37°C overnight. To test the growth curve of these transformants, the cultures were grown at 37°C in LB medium in the presence or absence of 1000 mM NaCl, and the cell density (OD_{600}) of these cultures were monitored by withdrawing aliquots at various times.

Subcellular localization in onion cells and *E. coli*

The CDS of *OsRab7* were cloned into plant binary vector *pCAMBIA-1302*; the constructs were transiently transformed into onion epidermal cells on agar plates by a helium-driven accelerator (PDS/1000; Bio-Rad). Bombardment parameters were as follows: 1100 p.si. bombardment pressure, 1.0 um gold particles, a distance of 9 cm from macrocarrier to the samples, and a decompression vacuum of 88,000 Pa. After culture for 1 day, the bombarded epidermal cells were imaged with a scanning confocal microscope (Olympus FV 1000). Excitation was 488 nm for visualization of GFP. Subcellular localization of OsRab7 in *E .coli* was observed the same way.

RESULTS AND DISCUSSION

Transformants construction and expression of *OsRab7* in *E. coli*

Expression cassette of *pET-OsRab7* was transformed into *E. coli*. We extracted the total protein of transfomants after induction and then carried out SDS-PAGE and western blot assay. As shown in Figure 1A, the specific bands of 24 KD was detected in the total protein of *pET-OsRab7* transformed *E. coli* on Coomassie blue gels (Figure 1A).The expression of OsRab7 was further confirmed by western blot assay (Figure 1B). A band of about 24 KD was detected in the total protein of *pET-OsRab7* transformed *E. coli*, no signal was either detected in the empty plasmid (*pET-28a*) transformed *E. coli* or the empty *BL21* (DE3) strains and *pET-OsRab7* transformants without IPTG been induced.

The temperature tolerance of *E. coli* recombinants expressing the OsRab7 protein

To determine the effect of the over-expression of the OsRab7 protein on the growth of *E. coli* recombinants

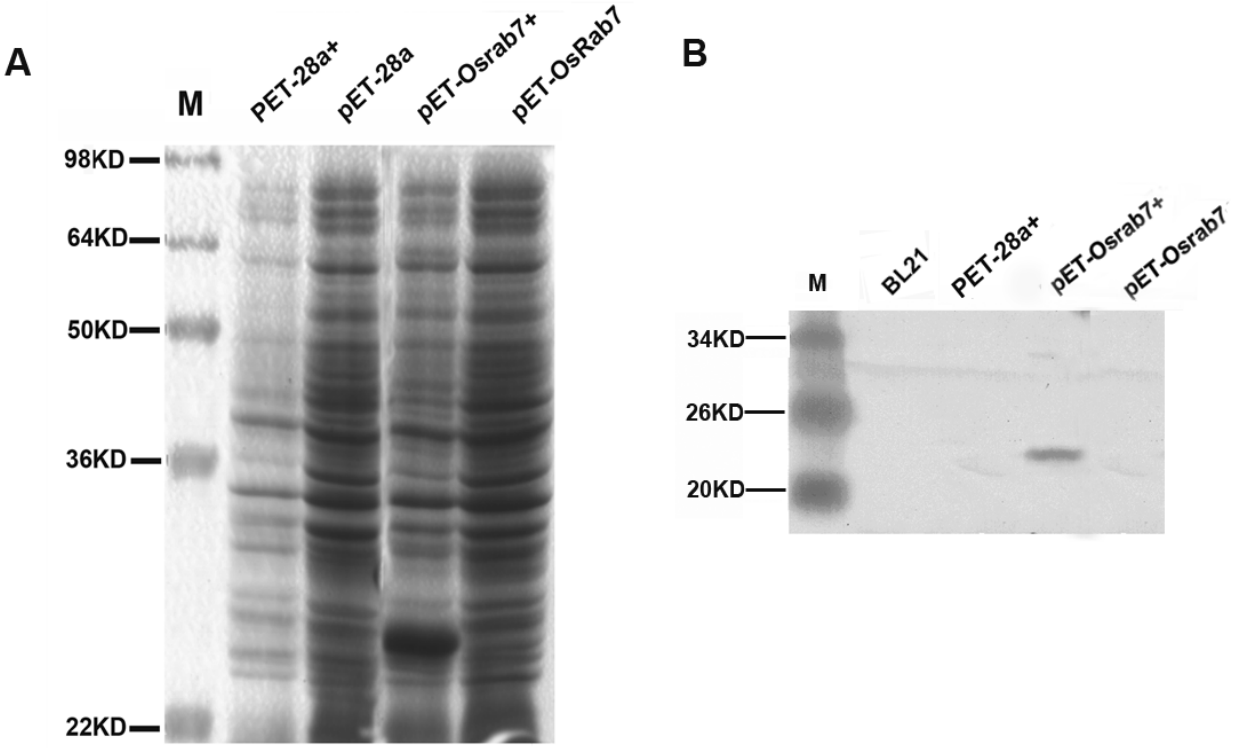

Figure 1. Expression of *OsRab7* gene in *E. coli*. A: SDS-PAGE analysis of total protein from *E. coli*; B: western blot analysis of the expression of OsRab7. M: protein molecular weight markers; +: with IPTG induced; BL21, BL21 (DE3) strains.

under different temperature stresses, cultures of BL/*pET-28a* and BL/*pET-OsRab7* recombinants were induced by IPTG, and then the spot assay were performed. The spot assay showed that there were no obvious difference in growth between BL/*pET-28a* and BL/*pET-OsRab7* transformants at 37℃, indicating that expression of the OsRab7 protein did not inhibit the growth of *E. coli*. When the recombinants were subjected to the temperature of -20 and 52℃, respectively, the numbers of BL/*pET-OsRab7* colonies were much greater than those of BL/*pET-28a* (Figure 2), suggesting that the expression of *OsRab7* increased the heat and cold stress tolerance in *E. coli*.

Expression of the *OsRab7* gene increased the salt stress tolerance in *E. coli*

In order to assess the function of expressed OsRab7 protein in salt stress condition, the effect of high concentration of NaCl on the growth of BL/p*ET-OsRab7* and BL/*pET-28a* were examined. As shown in Figure 3, transformants of *pET-28a* and *pET-OsRab7* exhibited similar viability on the normal LB plate, while on the LB plate supplemented with 1000 mM NaCl, the *pET-OsRab7* recombinant revealed increased cell viability when compared with the control, *pET-28a* transformant (Figure 3A). Meanwhile, we also preformed experiments using liquid medium to monitor growth curve of these transformants (Figure 3B) and got the same result that the expression of OsRab7 protein enhanced the high salt stress tolerance in *E. coli.*

Subcellular localization of GFP fused OsRab7 in *E. coli* and onion cells

Ectopic expression of OsRab7 protein can increase the stress tolerance in *E. coli* just like its function in planta, which inspired us to focus on the subcellular localization of OsRab7 protein in *E. coli* and plant cells. Thus, the *OsRab7* gene was fused to the GFP, and the OsRab7 GFP fusion constructs were translated to *E. coli* and onion cell, respectively, and GFP localization was determined by confocal microscope. As shown in Figure 4, in comparison with the uniform distribution of green fluorescence in control (Figure 4C, D and F), GFP fluorescence of OsRab7 transformants occurred specifically in the cytoplasm, both in *E. coli* and onion cells (Figure 4A, B and E).

Over the past few years, some of the *Rab* genes were found to be responsive by environmental cues. Overexpression of the *AtRab7* could increase salt and osmotic tolerance in transgenic *Arabidoposis* (Mazel et al., 2004).

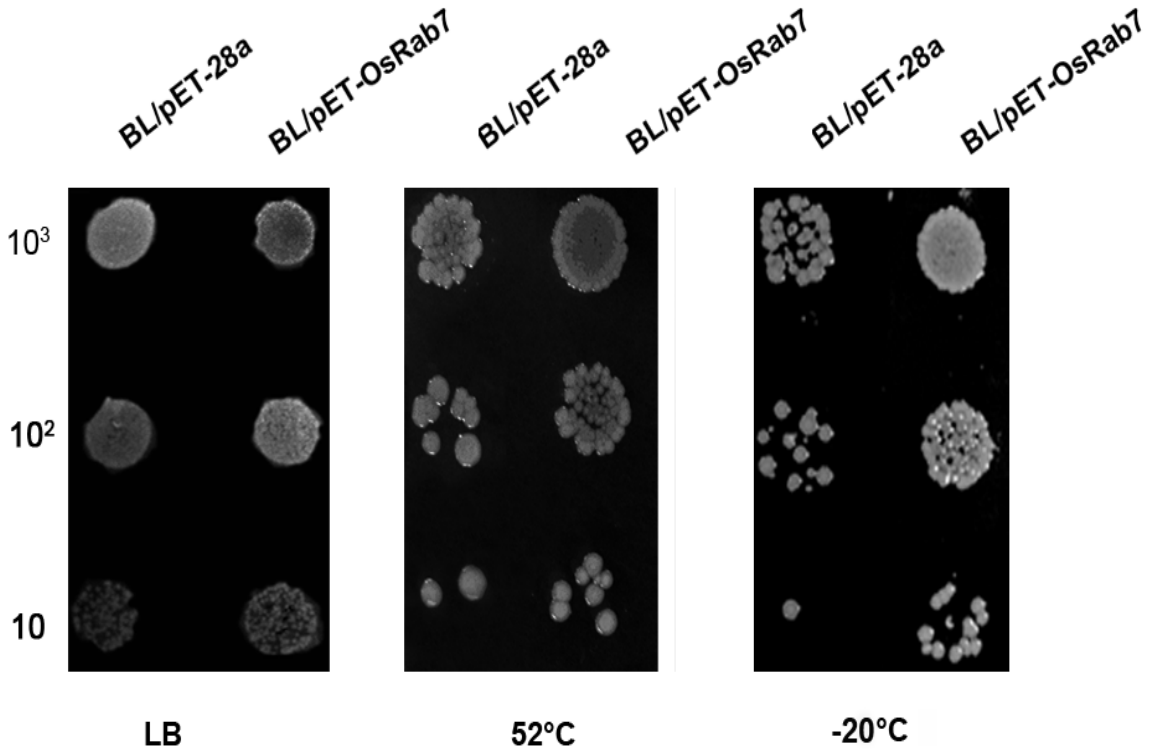

Figure 2. Spot assay of BL/pET-28a and BL/pET-OsRab7 recombinants. The *E. coli* cells were subjected to -20℃ for 24 h or 52℃ for 45 min and then spoted.

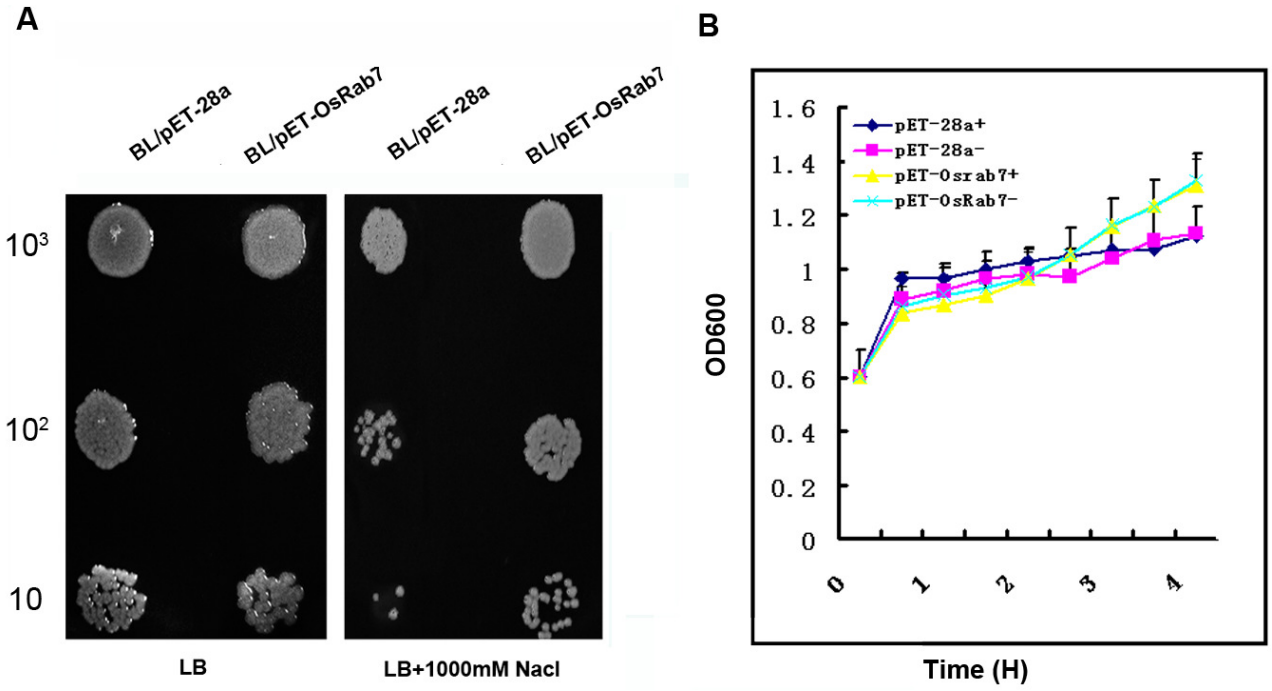

Figure 3. The growth performance of BL/OsRab7 and BL/pET-28a transformants under salt stress. A: Analysis of the transformants growth under high salt stress. IPTG was added to the cultures of BL/pET28 and BL/pET-Osrab7 to induce the recombinants expressing aimed protein. The cultures were adjusted to OD600 = 1.0, and then ten microliters of the serially diluted bacterial suspension was spotted onto normal LB plant or LB plate containing 1000 mM NaCl, respectively. The cells were grown at 37℃ for 1 day. B: Growth curve of tansformants in liquid culture supplemented with 1000 mM NaCl. Data were presented as mean ± SE of triplicate.

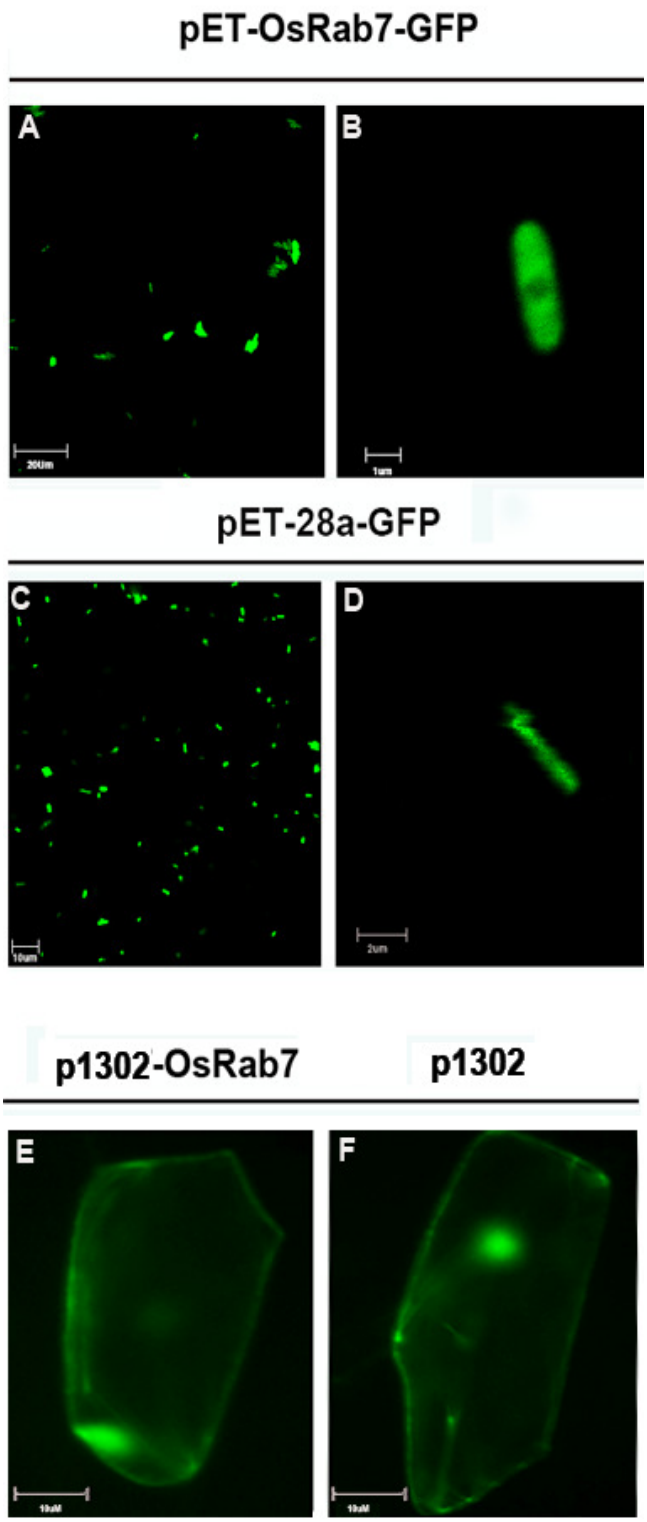

Figure 4. Analysis of the subcellular localization of GFP fused OsRab7 in *E. coli* and onion cells. A and B: Confocal micrographs illustrating the subcellular localization of OsRab7 protein in *E. coli*. Bar = 20 uM (A) 1 uM (B). C and D: Confocal images of the control GFP subcellular localization in *E. coli*. Bar =10 uM (C), 1 uM. E: Confocal images of the subcellular localization of OsRab7 protein in onion cell. Bar = 10 uM. F: Confocal images of the control GFP subcellular localization in onion cell. Bar = 10 uM.

Agarwal et al. (2008) also reported that over-expression *PgRab7* could confer salinity and dehydration tolerance in transgenic tobacco. However, report on this kind of genes heterologously expressed in *E. coli* cells has not been seen. In this study, we demonstrated that over-expression of OsRab7 protein enhanced the tolerance of *E. coli* recombinants diverse stress: heat, cold and high salinity.

In a previous study, Mazel et al. (2004) showed that over-expressing the *Arabidopsis Rab7* gene conferred stress tolerance in plant which is highly correlated with the membrane endocytosis, and subcellular localization of OsRab7 revealed that OsRab7 protein is specifically present in cytoplasm both in *E. coli* and onion cells, indicating that expression of such a gene contributes to increase of stress tolerance of the bacteria host cells probably associated with the membrane endocytosia pathway. Therefore, our findings here may provide a new clue to get some information about the similarity in regulation of stress response between prokaryote and eukaryote via endocytosis pathway.

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