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SHORT COMMUNICATION

N-Terminal His-Tagged AtTPR7 Interactions with Hsp70 and Hsp90 Proteins

ANANDAYU PRADITA[1*], REGINA SCHWEIGER[2], SERENA SCHWENKERT[2], JÜRGEN SOLL[2]

[1]School of Life Science and Technology, Institute of Technology Bandung, Jalan Ganesha 10, Bandung 40131, Indonesia
[2]Department of Biology I, Botany, Ludwig-Maximilians-Universität München, Großhaderner Strasse 2–4, D-82152 Planegg-Martinsried, Germany

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Post-translational protein import into organelles is an important process to maintain cellular functions. During preprotein transport in the cytosol, chaperones, such as heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90), are functioning to prevent aggregation and to maintain the correct protein folding of preproteins. This research was conducted in order to understand the chaperone-mediated, post-translational import of preproteins into the endoplasmic reticulum of *Arabidopsis thaliana.* AtTPR7 (*Arabidopsis thaliana* Tetratrico Peptide Repeat 7) is found in the endoplasmic reticulum and contains TPR domain, which mediates protein interaction with cytosolic Hsp70 and Hsp90. In this study, recombinant AtTPR7 was expressed in *E. coli* BL21 (DE3)-*RIPL* cells and purified using an N-terminal His-tag. In order to study the interactions of the protein with the chaperones, we used pulldown and Western blot assays. We could thereby show that the N-terminally His-tagged AtTPR7 protein interacted with Hsp70 and Hsp90.

Key words: AtTPR7, Chaperones, Hsp90, Hsp70, Pull down assay

INTRODUCTION

Arabidopsis thaliana tetratrico peptide repeat 7 (AtTPR7) is a novel protein found to act as a chaperone docking protein in *Arabidopsis thaliana* (Schweiger *et al.* 2012). The AtTPR7 contains a TPR domain, which consists of three tandem TPR motifs with 34 amino acid repeats, with no strictly conserved residues (Brinker *et al.* 2002). The function of the TPR domain is to recognize chaperones. Similar chaperone docking proteins are found in other organelles and contain one or more TPR motif facing the cytosol (Loeffellholz *et al.* 2011; Kriechbaumer *et al*. 2012).

In the chaperone mediated, post-translational import pathway, TPR domains of some receptor proteins facilitate interaction with pre-proteins. Most studies on post-translational import in plants have been conducted in the mitochondria and in chloroplasts; however, little is known about post-translational import into endoplasmic reticulum (ER). For example, the TPR domain of Toc64 outer envelope receptor in the chloroplast, mediates interaction with Hsp90 bound preproteins and transfers them to the Toc translocon (Qbadou *et al.* 2006). On the other hand, in mitochondria, the C-terminal TPR motif of Tom70 directly binds to internal targeting signals of mitochondrial pre-proteins (Young *et al.* 2003).

TPR proteins may contain a domain that binds to the ER membrane. So far this domain has only been identified in yeast (Harada *et al.* 2011). For example Sec63 is an integral protein that has been shown to anchor to the ER. This protein interacts with other Sec proteins, such as Sec61-Sec62 and Sec71-72. Complete interactions will activate the Sec61 as a translocon, which then will transport the pre-protein into the ER (Harada *et al.* 2011). Apart from this basic understanding of the function of Sec63 in yeast, not much information is available about the ER system in plants, although the major components of the Sec translocon show high homology between yeast and mammals (Harada *et al.* 2011).

AtTPR7 has been shown to be located in the ER membrane of plants. It has a TPR domain facing the cytosol, and interacts with Hsp70 and Hsp90 (Schweiger *et al.* 2012). AtTPR7 is the third chaperone docking protein in plants, sharing the

*Corresponding author. Phone: +62-22-2511575, Fax: +62-22-2534107, E-mail: anandayu@me.com

same function as the two others Toc64 and Tom70. It could functionally replace the TPR domain containing Sec72 in yeast, which is involved in post-translational import. Moreover, AtTPR7 associates with components of the Sec translocon of *Arabidopsis* to transfer pre-proteins to the channel Sec61 (Schweiger *et al*. 2012). However, some conflicting evidence comes from Loeffelholfz *et al.* (2011) whose research indicated that AtTPR7 (OEP61) specifically recognizes Hsp70 but cannot recognize Hsp90.

Schweiger *et al.* (2012) conducted their experiment by adding the His-tag in the C-terminus of the AtTPR7, whereas Loeffelholfz *et al.* (2011) used an N-terminally tagged AtTPR7. This study examined whether the N-terminal His-tag interferes with Hsp90 binding to AtTPR7. Furthermore, the results of this study could confirm the role of AtTPR7 as a chaperone binding protein, which mediates post-translational sorting in plant cells.

MATERIALS AND METHODS

N-Terminal His-Tagged AtTPR7 Construct. The method for cDNA of AtTPR7 used in this study was replicated from a previous study (Schweiger *et al.* 2012). Amplification of the cDNA of AtTPR7 (1500 bp) lacking the transmembrane domain was performed in 25 µL of PCR mixture containing 1 µL 10 mM forward AtTPR7 *Nde*I for (5'- CTA GCA TAT GTT TAA CGG GTT AAT GG 3'), 1 µL 10 mM reverse primer AtTPR7 *Xho*I -TM (5'-CGA TCT CGA GGG GAG AAA GAG AAG CCA TG-3'), dNTPs (1 mM each), 1 µL *Taq* Buffer 10x, 1 unit/µL *Taq* polymerase, and water, adding up to a total volume of 25 µL for each sample (all reagents were from *Fermentas*). AtTPR7 was amplified via primary denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C 30 sec denaturation, 55 °C 30 sec annealing and 2 min elongation in 72 °C and ended by 72 °C for 7 min for final elongation. PCR samples were then visualized by using electrophoresis with 1% (w/v) agarose gel followed by DNA purification using a QIAquick (QIAGEN) procedure.

Purified DNA was then digested using *Nde*I and *Xho*I (NEB). Digestion with both enzymes was performed in accordance with the manual provided. In a 1.5 mL microcentrifuge tube, a total volume of 20 µL digestion mixture was created, containing: 2 µg of PCR product, 2 µL *Nde*I/*Xho*I (1 unit/µL each), 2 µL NEB buffer 4 and 2 µL water. The mixture was then incubated for 1 h at 37 °C in an incubator. AtTPR7 *Nde*I/*Xho*I was then ligated into a pET-14b vector by using T4 DNA ligase. The ligation component consisted of 1.5 µL digested pET14b vector (*Nde*I/*Xho*I), 1 µL T4 DNA ligase, and 1 µL T4 buffer 10x, which was added into a PCR tube. Samples were incubated for 1-2 h at room temperature (RT). The ligated plasmid was then transferred/inserted into *E. coli* TOP10 cells using heat-shocked method.

Confirmation of the His-AtTPR7 construct was done via colony PCR and sequencing. The grown colonies were amplified using the same primers and cycles used for amplification. The sequencing was performed at the LMU sequencing service using T7 primer forward 5'-TAATACGACTCACTATAGGG-3' and AtTPR7 reverse primer 5'-CGATCTCGAGGGGAGAAA GAGAAGCCA TG-3'. Then DNA sequencing results were analyzed by using Bioedit, VecScreen, and Basic Local Alignment Searching Tools (BLAST) to evaluate the similarity of the resulting sequence with the corresponding gene in the GenBank database from NCBI (http://www.ncbi.nlm.nih.gov).

Over-Expression and Purification of N-Terminal His-Tagged AtTPR7. Over-expression of His-AtTPR7 was conducted using *E. coli* BL21-CodonPlus (DE3)-*RIPL* competent cells grown in LB broth medium, supplemented with ampicilin 100 µg/mL. One mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added into the LB broth medium to induce the expression of the protein of interest, and the mixture was incubated at 37 °C overnight. The cells were then lysed using a French Press, and purified using a Ni-NTA column. The concentrate of purified protein was then measured using the Bradford and Lowry test. Gels were visualized by using 10% SDS-PAGE followed by staining with Coomasie Brilliant Blue.

N-Terminal His-Tagged AtTPR7 Interaction Studies with Chaperones. Interactions of His-AtTPR7 with chaperones were observed by using the Pull-down technique followed by SDS PAGE, Western blot, and autoradiography. His-AtTPR7 proteins were incubated with Strep-tagged chaperones for 1 h at room temperature. His-AtTPR7 was subsequently purified by incubation with Ni-NTA for 1 h at room temperature, and proteins were eluted with 300 mM imidazole and probed with Hsp70 or Hsp90 antibodies. Resulting signals were visualized by ECL (GE).

RESULTS

Successful cloning of the N-terminal His-tagged AtTPR7 construct was confirmed using colony PCR and sequencing. The results showed that His-AtTPR7 had been successfully cloned with 1500 bp insert. The

BLAST result shows 99% similarity with At5G21990 (data not shown). This demonstrates that N-terminal His-AtTPR7 was correctly inserted into the pET14b vector. Over-expression of AtTPR7 in *E.coli,* and purification of N-terminal His-tagged AtTPR7, indicated that the protein was highly over-expressed and could be visualized by coomasie staining after purification.

Interaction of His-AtTPR7 with Hsp70 was tested using pull-down assay followed by immunodetection. AtTPR7 was bound to Ni-NTA and incubated with chaperones. Proteins were eluted and chaperones were detected by immunostaining. Interaction was tested with the N-terminally tagged AtTPR7 generated in this work, as well as with C-terminally tagged AtTPR7, for which chaperone binding had already been shown. As a control, empty beads without bound AtTPR7 were incubated to detect background binding. Moreover, purified chaperones were loaded to serve as a control for the immunostaining. Figure 1 shows successful interaction of Hsp70 with both AtTPR7 constructs. The results of interaction of His-AtTPR7 and Hsp90 are shown in Figure 2. The bands at 90 kDa indicate that there is also an interaction between Hsp90 and both AtTPR7 constructs. We therefore conclude that neither the N-terminal nor the C-terminal tag interfere with chaperone binding.

DISCUSSION

The TPR motif has a helical structure and functions in a wide range of cellular processes including the cell cycle, splicing, transcription, neurotransmitter release, phosphate turnover, signal transduction, and chaperone-binding regulation (Blatch & Lassle 1999). The TPR domains analyzed in this study interact with molecular chaperones in order to mediate delivery of pre-proteins into an organelle (Loeffelholz *et al*. 2011).

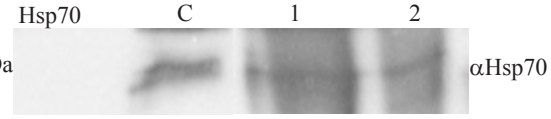

Figure 1. Western blot result of various AtTPR7 proteins interactions with HSP70. C = Control, 1 = AtTPR7-His, 2 = His-AtTPR7, Hsp70 = 0.2% of pure Hsp70.

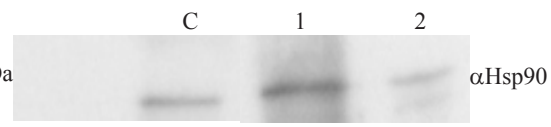

Figure 2. Western blot result of various AtTPR7 proteins interactions with HSP90. C = Control, 1 = AtTPR7-His, 2 = His-AtTPR7, Hsp90 = 0.2% of pure Hsp90.

The result of this study shows that the N-terminal His-tagged AtTPR7 and the control protein C-terminaly tagged AtTPR7-His-tag were both able to interact with Hsp70. As stated in Loeffelholz *et al.* (2011) that the presence of a TPR clamp domain of *Arabidopsis thaliana* OEP61/AtTPR7, binds specifically to Hsp70. The same result occurred in our research, in both of N-terminal and C-terminal His-tagged AtTPR7 treated with Hsp90. This contrasts with the results of Loeffelholz *et al.* (2011) who found that Hsp90 could not bind with His-OEP61. Using our setup, our results showed that the N-terminal His-tagged AtTPR7 does not interfere with Hsp90 binding. Indeed, the first view on the function of AtTPR7 in *Arabidopsis thaliana* not only represents a milestone in the translocation field, but also serves a starting point for future studies that are designed to overcome present barriers in our understanding of protein translocation.

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