UDC 577.217.344

Original Article

CLONING OF CDNA-GENE OF *ARABIDOPSIS THALIANA* RIBOSOMAL PROTEIN S6, ITS EXPRESSION IN *ESCHERICHIA COLI* AND PURIFICATION OF *AT*RPS6B1 RECOMBINANT PROTEIN

Nadirova L.T.[1,2*], Beisenov D.K.[2], Stanbekova G.E.[2], Zhigailov A.V.[2], Ryabova L.A.[3], Iskakov B.K.[1]

[1] Al-Farabi Kazakh National University, Kazakhstan, Almaty, 050040, 71 al-Farabi Avenue
[2]M. Aitkhozhin Institute of Molecular Biology and Biochemistry, Kazakhstan, Almaty, 050012, Dosmukhamedov st., 80
[3]Institute of plant molecular biology, UPR 2357 CNRS, Strasbourg University, France, Strasbourg, 67084, Général Zimmer str, 12.
**leila.nadirova@gmail.com*

ABSTRACT

Recent studies highlight the significant role of ribosomal protein S6 in TOR/S6K signaling and its involvement in translation and protein biosynthesis. However, the mechanisms underlying the activation of cellular processes remain incompletely understood. The main function of RPS6 relates to regulating mechanisms controlling cell growth and division. RPS6 can be phosphorylated on specific serine and threonine residues by kinases like S6K1 and S6K2, activated by signaling pathways associated with mTORC1. This phosphorylation process plays a crucial role in regulating cell growth and protein synthesis, influencing translation initiation on ribosomes and thereby regulating cell size and division. Additionally, RPS6 can interact with other proteins, participating in various molecular interactions depending on cellular activity context.

Further exploration of RPS6 may unveil new insights into its molecular interactions, roles in cellular physiology and pathophysiology, and potential applications in enhancing plant biomass and crop yield. This study conducted cloning, site-directed mutagenesis, and expression of the second isoform of *At*RPS6 protein (*At*RPS6B). The obtained phosphomimetic and non-phosphorylated forms of this protein were expressed in E. coli ArcticExpress (DE3) cells, purified by metal chelate chromatography (IMAC), and confirmed for presence and purity via immunoblotting.

Keywords: *Arabidopsis thaliana*, *At*RPS6B1 cDNA gene cloning, phosphomimetic mutation, recombinant ribosomal protein S6 (*At*RPS6B1)

INTRODUCTION

During their lifetime, living organisms are forced to adapt their growth and development under the influence of external factors, such as stress and the availability of nutrients. Therefore, they have evolutionarily developed various regulatory pathways to improve the perception of the environment and accelerate the necessary metabolic changes [1, 2]. Conservative key proteins are involved in these pathways, which, as a result of stress and nutrient restriction, trigger anabolic and catabolic cellular processes [3, 4]. One of the most important pathways found in all eukaryotes is associated with the protein kinase TOR (Target of rapamycin). TOR is a large kinase that controls many biological processes, including the activation of S6K kinase [5], which, in turn, phosphorylates the S6 protein (RPS6) [6, 7]. Crystal structures reveal the specific location of RPS6 within the 40S ribosomal subunit and the structural features, such as the C-terminal helix, which contains multiple phosphorylation sites [8, 9]. Its main function is related to the regulation of mechanisms for controlling cell growth and division. In plants, eS6 is encoded by two conserved genes, and its activation and phosphorylation are closely linked to S6Ks [2, 5-6]. Recent studies [10-16] have drawn attention to the importance of phosphorylation of the S6 protein as a key event in signaling pathways associated with cell growth and survival factors, and this process is considered an important regulator of the initiation of translation and protein synthesis. The S6 protein can be phosphorylated on various serine and threonine residues by S6K kinase. Additional studies show that the S6 protein can interact with other molecules and proteins, expanding its functional roles in cell signaling.

It should be noted that further studies of the ribosomal protein S6 may provide new details about its molecular interactions, roles in cellular physiology and pathophysiology, as well as its potential applications in the development of plant biomass enhancement and yield.

In this study, cloning and site-directed mutagenesis of cDNA of the second isoform of the *At*RPS6 protein (*At*RPS6B) was carried out. Further, the obtained natural and phosphomimetic forms of this protein were expressed in *E.coli* ArcticExpress (DE3) cells, after which the proteins were purified by metal-chelate affinity chromatography (IMAC) [17], their presence and degree of purification were confirmed using Western blotting.

These results fit into the context of studies related to ribosomal protein S6. RPS6, as a key effector in the TOR signaling pathway, plays an important role in regulating ribosome biosynthesis by controlling transcription and translation processes. Phosphorylation of RPS6, reflecting the activity of S6K, is associated with cells actively undergoing the process of division. Contrary to this, the exact role of RPS6 in the regulation of ribosome biosynthesis remains the subject of further research.

The data obtained during the study on *At*RPS6B expand our understanding of the molecular mechanisms underlying the regulation of ribosomal biosynthesis. These findings may be an important contribution to understanding the relationships between signaling pathways, ribosome proteins and cellular metabolism, as well as creating new perspectives for therapeutic effects on cellular processes associated with ribosomes.

MATERIALS AND METHODS

The oligodeoxyribonucleotides used in the work (Table 1) were synthesized by Eurogentec.

Table 1 - Oligodeoxyribonucleotides used in the work

Name	Nucleotide sequence	Tm	Tm using
RPS6B-for-KpnI-NdeI	(5')TAGGTACCATATGAAGTTCAACGTCGCCAATCCG	61°	60°
RPS6B-rev-BamHI-SacI	(5')TTGAGCTCGGATCCTTAAGCAGCAACGGGTTTAGC	64°	
RPS6B-Ser/Glu231-for	(5')TCGCCGTAGTGAAGAATTGGCCAAGAAGAG	58°	56°
RPS6B-Ser/Glu231-rev	(5')CTCTTCTTGGCCAATTCTTCACTACGGCGA	58°	
RPS6B-Ser/Glu237-for	(5')TTGGCCAAGAAGAGGGAGAGACTCTCTTCTGCT	62°	60°
RPS6B-Ser/Glu237-rev	(5')GAGCAGAAGAGAGTCTGAGCCTCTTCTTGGCCAA	64°	
RPS6B-Ser/Glu240-for	(5')CCAAGAAGAGGGAGAGACTCGAGTCTGCTCCTGC	65°	62°
RPS6B-Ser/Glu240-rev	(5')GCAGGAGCAGACTCGAGTCTCTCCCTCTTCTTGG	65°	
RPS6B-Ser/Glu241-for	(5')CCAAGAAGAGGGAGAGACTCGAGGAGGCTCCTGC	65°	62°
RPS6B-Ser/Glu241rev	(5')GCAGGAGCCTCCTCGAGTCTCTCCCTCTTCTTGG	65°	

Computer analysis of nucleic acid and protein sequences was performed using SnapGene Viewer 4.0.4, VNTI-Viewer 11.5.1 and DNAman 4.03 programs. AtRPS6B1 cDNA nucleotide sequence (GeneBank: AT5G10360.1) was taken from the TOR database (https://www.arabidopsis.org).

Isolation of a total RNA preparation, reverse transcription reaction (RT) and polymerase chain reaction (PCR) were performed as described [18]

Cloning of *At*RPS6B1 cDNA of wild and mutant variants. Amplification of *At*RPS6B1 cDNA was conducted using RPS6B-for-*Kpn*I-*Nde*I and RPS6B-rev-*Bam*HI-*Sac*I primers. Amplification products were analyzed and eluted from the gel using a commercial Gel Extraction Kit (Thermo Fisher Sci.). Next, the DNA fragment was treated with *Nde*I and *Bam*HI restriction endonucleases and cloned into pET23c vector DNA, also treated with *Nde*I and *Bam*HI restrictases.

Screening of DNA clones was executed through PCR analysis using gene-specific primers and restriction analysis utilizing *Nde*I and *Bam*HI endonucleases.

Site-directed mutagenesis of cDNA *At*RPS6B1 was performed using additional pairs of primers for each mutation point. Pairs of primers were used sequentially starting from S231. After each PCR reaction, a full-length plasmid was obtained with the replacement of several nucleotides. After four such reactions, the plasmid pET23c-His-*At*RPS6B1-S231E,S237E,S240E,S241E (*At*RPS6B1ph) was obtained. To confirm the correctness of the synthesized sequence, cDNA sequencing was performed. Mutagenesis was carried out by Pfu Ultra High-fidelity DNA polymerase (Stratagene) under the following temperature regime: stage 1 – 95 ° C 30 sec; stage 2 – 95 ° C 30 sec, Tm using (is indicated for each pair of primers in Table 1) 1 min, 72 ° C 5 min – 30 cycles; stage 3 – 72 °C 7 min, 4 °C 5 min.

Sequencing of cloned sections of plasmids pET23c-*At*RPS6B1-His and pET23c-*At*RPS6B1ph-His was carried out using a commercial set of Big Dye® Terminator v.3.1 (Applied Biosystems) according to the manufacturer's methodology as described [18].

Expression of cDNA *At*RPS6B1 and *At*RPS6B1ph in bacterial cells, *purification* and *concentration* RPS6 protein were performed as described [17]. *E. coli* cells of the strain Arctic-Express (DE3) were transformed by pET23c-His-*At*RPS6B1 and pET23c-His-*At*RPS6B1ph DNA constructs. To purify the synthesized RPS6 protein containing «6xNis-tag» at the N-end, metal-chelate affinity chromatography was used [17], which allows recombinant proteins to be quickly and cleanly isolated from bacterial lysate. The target protein was purified under native conditions in a dissociating buffer with the addition of imidazole. For lysis, a dissociating buffer containing nonionic detergent Nonidet P40 was used to increase the yield of soluble proteins.

Immunoblotting was carried out as described [18] with Ist antibodies antiRPS6 and antiRPS6-P240-rabbit (Agrisera) at a dilution of 1:5000 and Anti-rabbit HRP-conjugate (Santa Cruz) IInd antibodies at a dilution of 1:4000.

General methods. Plasmid DNA isolation, spectrophotometric determination of nucleic acid concentration, preparation and transformation of competent *E. coli* cells and other procedures were performed according to standard methods [19]. The total protein concentration was determined by Bradford [20].

RESULTS AND DISCUSSION

Within the *A. thaliana* RPS6 genome, two genes, *At*RPS6A and *At*RPS6B, encode for the RPS6 proteins. These two proteins exhibit a high level of similarity in their amino acid sequences, and they demonstrate equal functional activity [21,22]. For the proper functioning of this protein within the cell, it undergoes exposure to the AtS6K kinase. This kinase phosphorylates serine and threonine residues, contributing to the correct conformation of the protein.

We hypothesized that replacing the triplets encoding serines at the C-terminus of this protein with a negatively charged glutamic acid codon would mimic phosphorylation (phosphomimetic mutations), thus the correct conformation of the protein would be preserved without the need for *At*S6K kinases. To confirm this hypothesis, *At*RPS6B1 cDNA was cloned and the *At*RPS6B1-S231E,S237E,S240E,S241E (*At*RPS6B1ph) sequence in the bacterial expression vector pET23c was obtained by site-directed mutagenesis, and two recombinant proteins were expressed and isolated.

5 pair primers were designed for amplification, cloning, and site-directed mutagenesis of the *At*RPS6B1 cDNA gene. Total RNA was extracted from *Arabidopsis thaliana* leaves, and reverse transcription polymerase chain reaction (RT-PCR) was performed. Electrophoretic analysis of the RT-PCR products revealed an approximately 750 bp amplification product, corresponding to *At*RPS6B1 cDNA (Fig. 1). Figure 1 displays the products of cDNA amplification after RT-PCR. As shown in the figure, an increase in the amount of cDNA in the PCR mixture from 2.5 µl to 5 µl did not significantly affect the results.

The gel-extracted PCR product and the pET23c vector were treated with *Nde*I and *Bam*HI restriction endonucleases then combined at a concentration of 1:2 and treated with a ligase. Then, *E.coli* cells were transformed with the resulting ligase mixture and grown on the selective antibiotic ampicillin overnight at +37°C. The verification of the grown clones was conducted in two stages. Initially, PCR was performed using the primers employed for cloning to confirm the presence of an insert. Subsequently, plasmids isolated from confirmed clones were subjected to digestion with the restriction enzymes *NdeI* and *BamHI*, which confirmed the presence of the appropriate restriction sites and the expected insert size. This process resulted in the successful creation of the pET23c-His-*At*RPS6B1 plasmid (Fig.2).

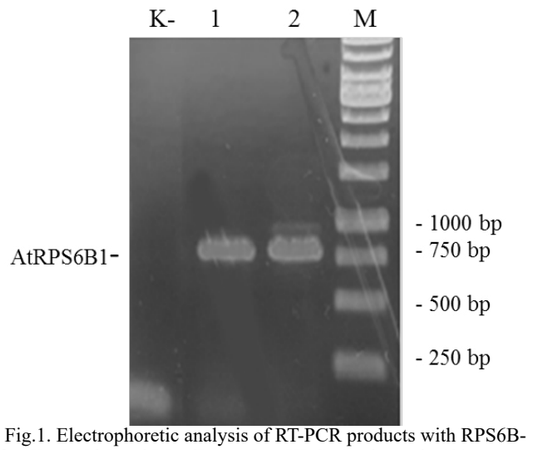

Fig.1. Electrophoretic analysis of RT-PCR products with RPS6B-for-*Kpn*I-*Nde*I and RPS6B-rev-*Bam*HI-*Sac*I primers in 1% agarose gel. «M» is a DNA ladder. Tracks: "K-» – negative control; 1 -2.5 µl of the mixture of mercury in the reaction; 2 -5 µl of the mixture of mercury in the reaction

Mutagenesis in vitro. To confirm our theory that by activating the RPS6 protein it is possible to influence the growth, development and productivity of cells, it was decided to create constructions with negatively charged glutamic acid (E). To accomplish this, a series of PCR reactions were conducted using the primers specified in Table 1. The method involves the use of overlapping primers that introduce a specific pair of complementary nucleotides at a precisely defined location, differing from the wild-type sequence. In this case, entire triplets (sequences of 3 consecutive nucleotides) encoding serine (AGT, TGT) were substituted for glutamic acid (GAA, GAG). Therefore, four serines at the C-terminus of the gene were substituted with glutamic acids. Subsequently, the newly formed plasmid containing the mutant *At*RPS6 gene was sequenced to confirm the sequence accuracy and to detect any errors that may have arisen from multiple amplifications. The schematic representation of the obtained plasmid is shown in Figure 2.

cDNA expression in E. coli cells and protein purification. The plasmid pET23c is designed for the expression of target proteins in a bacterial system (*E. coli*). It also contains a sequence of six histidine residues that bind to divalent metal ions (mostly nickel ions). Consequently, the proteins expressed in the cellular system can be subsequently purified using a method called metal-affinity chromatography. The selection of the *E. coli* ArcticExpress (DE3) strain was not random, as this strain is designed for proteins prone to misfolding and precipitation, which includes the target protein RPS6B and its mutant variant. Additionally, efforts were made to optimize the conditions for optimal protein expression, including temperature, incubation time, and induction method. The selected conditions were as follows: induction with 0.1% glucose and 125 µM IPTG, cell growth temperature of +37°C, and protein expression temperature of +20°C over a duration of 4 hours. As mentioned earlier, the presence of a histidine tag in the plasmid allows for the purification of the target protein using nickel ion affinity. For this purpose, a Ni-2+ charged column or Ni-NTA agarose can be used. In this study, agarose was utilized due to the large volume of the obtained cell extract. Incubation with Ni-NTA agarose was conducted overnight at +4°C. To elute the protein from the agarose, the

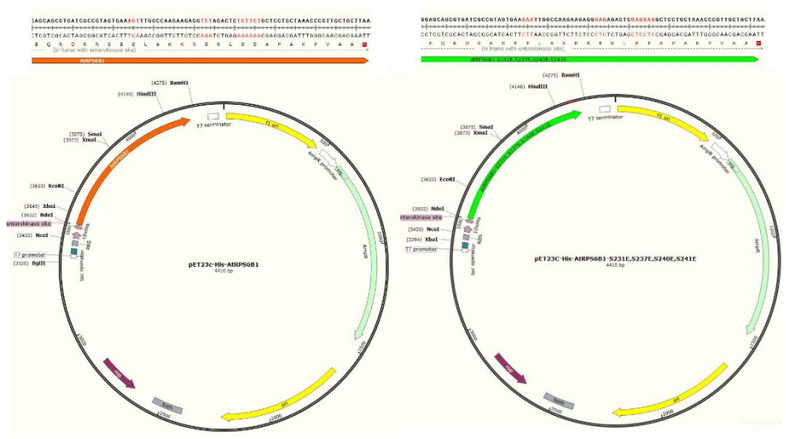

Fig.2. Schematic representation of the structure of plasmid pET23c with *At*RPS6B1 and *At*RPS6B1ph cDNA inserts.

imidazole concentration was increased to 250 mM, which competitively displaced the histidine ions from the immobilized nickel. The eluted protein was collected and analyzed using polyacrylamide gel electrophoresis. Figure 3 illustrates the supernatant and pellet fractions of total protein content from *E. coli* Arctic Express (DE3) cells transformed with the pET23c-His-*At*RPS6B1 and pET23c-His-*At*RPS6B1ph constructs. The use of protein-specific antibodies allows for clear visualization of protein distribution during expression. The majority of the target proteins are found in the supernatant; however, these proteins tend to form dimers and are susceptible to cleavage by cellular proteases, resulting in smaller fragments. Protein presence is also detected in the pellet fraction, where it remains in its original size, likely due to preservation within inclusion bodies.

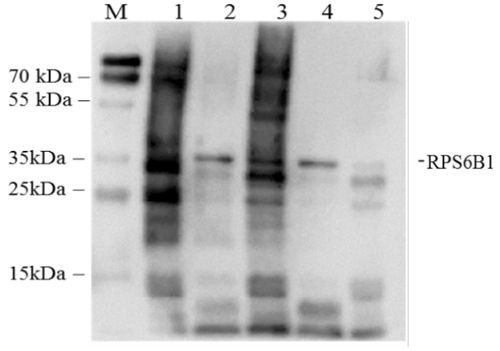

Fig.3. Western blot after SDS-PAGE of the fractions total cellular lysate content *E. coli*, using antibody to RPS6 (Anti-RPS6A *A.thaliana*, Agrisera). Tracks: M – protein ladder; 1 – ArcticExpress pET23c-His-*At*RPS6B1 supernatant, 2 – ArcticExpress pET23c-His-*At*RPS6B1 pellet, 3 – ArcticExpress pET23c-His-*At*RPS6B1ph supernatant, 4 - ArcticExpress pET23c-His-A*t*RPS6B1ph pellet, 5 - nontransformed ArcticExpress supernatant

Based on the results of this experiment, it was decided to perform protein extraction and purification from the supernatant, enabling the use of a gentler purification method under native conditions.

Figure 4 displays various fractions from the purification of RPS6B1ph protein, including the *E.coli* total protein preparation supernatant, pellet from this preparation, flow-through

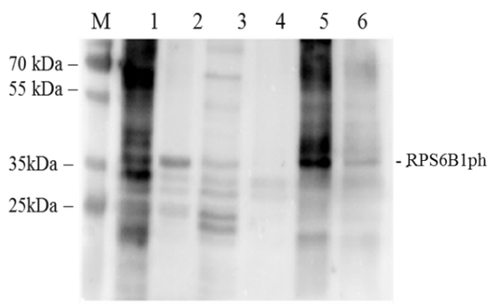

Fig. 4. Stages of RPS6B1ph protein purification. Western blot after SDS-PAGE of proteins isolated from *E. coli*, using antibody to RPS6. M – marker proteins; Tracks 1-6 purification fractions: 1 – supernatant, 2 – pellet, 3 – flow through, 4 – wash, 5 – elution1, 6 – elution 2.

and wash samples, and protein eluates collected in two rounds to maximize yield. The figure confirms that the protein binds strongly to negatively charged Ni-NTA agarose, remains bound during washing steps, and is effectively eluted with increased imidazole concentration, resulting in a sufficiently pure and concentrated protein preparation.

Figure 5a displays the purification results of the recombinant RPS6B1 protein using antiRPS6 antibodies. In Figure 5b is shown the purification of the RPS6B1ph protein and its interaction with antiRPS6-P240 antibodies (Agrisera), specific to the serine-240 phosphorylated form of RPS6 protein. Therefore, it can be concluded that the mutant RPS6 protein with phosphomimetic substitutions adopts a conformation that allows for recognition by phosphospecific antibodies.

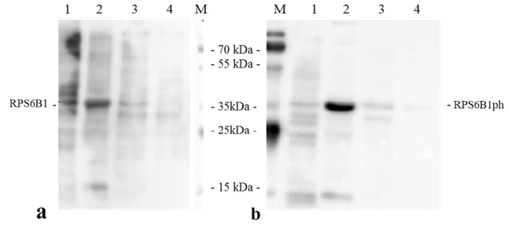

Fig. 5. RPS6B1 (a) and RPS6Bph (b) purification. Western blot after SDS-PAGE of proteins isolated from *E. coli*, using antibody to RPS6(a) and RPS6-P240 (b). M – protein ladder; Tracks 1-4 purification fractions: 1 – supernatant, 2 – pellet, 3 – elution1, 4 – elution 2.

After purifying the RPS6B1 and RPS6B1ph proteins by metal chelate affinity chromatography, the eluted protein fractions contained a significant amount of imidazole, which can interfere with proper protein conformation. To remove the excess imidazole and concentrate the protein preparations, additional purification was performed using Amicon Ultra 3K filters.

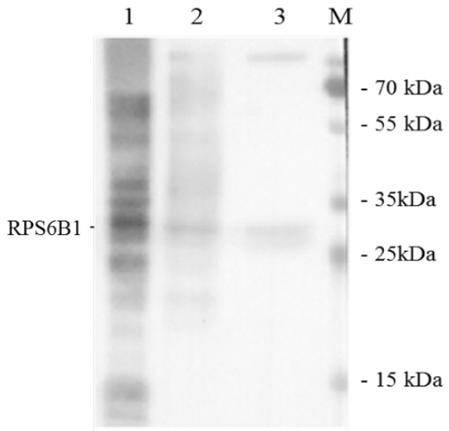

Fig. 6. RPS6B1 purification from imidazole. Western blot after SDS-PAGE using antibody to RPS6. M – protein ladder; Tracks 1-3 purification fractions: 1 – supernatant, 2 – elution1 after Ni-NTA agarose, 4 – elution 2 after purification from imidazole.

CONCLUSION

The results of this work were recombinant proteins of wild and phosphomimetic RPS6B1 and RPS6B1ph. Purification

of these proteins was performed by the IMAC method and confirmed by Western blot analysis. For the expression of recombinant proteins, the *E.coli* strain ArcticExpress (DE3) and plasmids containing cDNA constructs pET23c-His-*At*RPS6B1 and pET23c-His-*At*RPS6B1ph were used. Which were obtained by RT-PCR amplification from *Arabidopsis thaliana* total RNA, modified by site-directed mutagenesis method and cloned to expressional bacterial vector pET23c.

The study of the effect of the RPS6 protein on the degree of translation of cellular proteins, depending on the level of phosphorylation, has been conducted for a long time. But there is very little data on the mechanism of the cellular signaling pathway in plants, since all work is carried out mainly on mammalian cells.

Our idea is to utilize the resulting mutant cDNA variant, *At*RPS6B1ph, to construct new vectors based on the agrobacterial binary system for transformation and subsequent in vivo expression in laboratory plants. This study will enable us to monitor the level of protein biosynthesis when *At*RPS6B1ph cDNA is introduced into plant genotypes, leading to the constitutive expression of the active RPS6B1ph protein in plant cells without requiring additional activation by phosphorylation. If our hypothesis is correct and the RPS6ph protein influences the translation level of all cellular proteins, these constructs could be applied to economically important plants to accelerate growth and enhance productivity. Additionally, isolated recombinant proteins will be used to evaluate the activity of various kinases, representing an intriguing fundamental aspect of this research.

LITERATURE

1. Wolters H., Jürgens G. Survival of the flexible: hormonal growth control and adaptation in plant development // Nat. Rev. Genet. – 2009. – Vol. 10. – P. 305-317.

2. Dobrenel T, Mancera-Martínez E, Forzani C, Azzopardi M, Davanture M, Moreau M, Schepetilnikov M, Chicher J, Langella O, Zivy M, Robaglia C, Ryabova LA, Hanson J, Meyer C. The Arabidopsis TOR Kinase Specifically Regulates the Expression of Nuclear Genes Coding for Plastidic Ribosomal Proteins and the Phosphorylation of the Cytosolic Ribosomal Protein S6 // Front. Plant Sci. – 2016. – Vol. 7. – P. 1611.

3. **Scharf K.D., Nover L.** Control of ribosome biogenesis in plant cell cultures under heat shock conditions. II. Ribosomal proteins. Biochim Biophys Acta – 1987 – Vol. 909. – P. 44–57

4. Zhang J.Z., Creelman R.A., Zhu J.-K. From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops // Plant Physiol. – 2004. – Vol. 135. – P. 615-621.

5. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks // Biochem J. - 2012 – Vol. 441, No 1. – P. 1-21. doi: 10.1042/BJ20110892. PMID: 22168436.

6. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control // Nat Rev Mol Cell Biol – 2009. - Vol.10. - P. 307-318

7. Barbet N.C., Schneider U., Helliwell S.B., Stansfield I., Tuite M.F., Hall M.N. TOR controls translation initiation and early G1 progression in yeast // Mol. Biol. Cell. – 1996. - Vol – 7. - P.25-42

8. Holz M.K., Ballif B.A., Gygi S.P., Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events // Cell. – 2005. – Vol. 123. – P. 569–580.

9. Leader D.P.,Thomas A., Voorma H.O.The protein synthetic activity in vitro of ribosomes differing in the extent of phosphorylation of their ribosomal proteins. // Biochim et Biophys Acta. – 1981. – Vol. 656 – P. 69-75.

10. Yerlikaya S, Meusburger M, Kumari R, Huber A, Anrather D, Costanzo M, Boone C, Ammerer G, Baranov PV, Loewith R. TORC1 and TORC2 work together to regulate ribosomal protein S6 phosphorylation in Saccharomyces cerevisiae // Mol Biol Cell. – 2016. - Vol. 27, No 2. – P.397-409.

11. Ren M., Qiu S., Venglat P., Xiang D., Feng L., Selvaraj G., Datla R. Target of rapamycin regulates development and ribosomal RNA expression through kinase domain in Arabidopsis // Plant Physiol. – 2011. –Vol.155. – P. 1367-1382

12. Nandagopal N., Roux P.P. Regulation of global and specific mRNA translation by the mTOR signaling pathway // Translation. 2015. – Vol. 3, No. 1.

13. Henriques R., Bögre L., Horváth B., Magyar Z. Balancing act: matching growth with environment by the TOR signaling pathway // J Exp. Bot. – 2014. – Vol.65, No.10 – P. 2691-2701.

14. Rexin D., Meyer Ch., Robaglia Ch., Veit B. TOR signalling in plants // Biochem. J. – 2015. – Vol. 470. – P. 1–14.

15. Boex-Fontvieille E., Daventure M., Jossier M., Zivy M., Hodges M., Tcherkez G. Photosynthetic Control of Arabidopsis Leaf Cytoplasmic Translation Initiation by Protein Phosphorylation. // PLoS ONE. – 2013. – Vol.8

16. Nukarinen E., Nägele T., Pedrotti L., Wurzinger B., Mair A., Landgraf R., Börnke F., Hanson J., Teige M., Baena-Gonzalez E., et al. Quantitative phosphoproteomics reveals the role of the AMPK plant ortholog SnRK1 as a metabolic master regulator under energy deprivation // Sci Rep – 2016 – Vol. 6 - P. 31697.

17. Cheung RC, Wong JH, Ng TB. Immobilized metal ion affinity chromatography: a review on its applications. Appl Microbiol Biotechnol – 2012 – Vol.6 - P. 1411-1420.

18. Nadirova L.T., Beisenov D.K., Stanbekova G.E., Saparbaev M.K, Iskakov B.K. Cloning of cDNA-gene of Arabidopsis thaliana ribosomal protein S6, its expression in Escherichia coli and isolation of AtRPS6A1 recombinant protein // Exper Biol – 2022 - Vol. 91. P.138-147.

19. Sambrook J., Russel D.W. Molecular cloning: A laboratory manual: 3 volumes. – Third edition. – New-York: Cold Spring Harbor Laboratory Press, 2001.

20. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding // Anal. Biochem. – 1976. – Vol.7, No.72. – P. 248-254.

21. Creff A., Sormani R., Desnos T. The two Arabidopsis RPS6 genes, encoding for cytoplasmic ribosomal proteins S6, are functionally equivalent // Plant Mol. Biol. – 2010. –

Vol. 73. – P. 533-546.

22. Ruvinsky, I., and Meyuhas, O. Ribosomal protein S6 phosphorylation: From protein synthesis to cell size // Trends Biochem.Sci. – 2006. – Vol. 31. – P. 342–348.

REFERENCES

1. Barbet N.C., Schneider U., Helliwell S.B., Stansfield I., Tuite M.F., Hall M.N. TOR controls translation initiation and early G1 progression in yeast. // Mol. Biol. Cell. – 1996. - Vol. 7, P. 25-42, DOI:10.1091/mbc.7.1.25

2. Boex-Fontvieille E., Daventure M., Jossier M., Zivy M., Hodges M., Tcherkez G. Photosynthetic Control of Arabidopsis Leaf Cytoplasmic Translation Initiation by Protein Phosphorylation. // PLoS ONE. – 2013 - Vol.8. DOI:10.1371/journal.pone.0070692

3. Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. // Anal. Biochem.- 1976. - Vol.7(72), P. 248-254.

4. Cheung RC, Wong JH, Ng TB. Immobilized metal ion affinity chromatography: a review on its applications. // Appl Microbiol Biotechnol. – 2012. - Vol. 96(6), P.1411-20. DOI: 10.1007/s00253-012-4507-0, PMID: 23099912.

5. Creff A., Sormani R., Desnos T. The two Arabidopsis RPS6 genes, encoding for cytoplasmic ribosomal proteins S6, are functionally equivalent. // Plant Mol. Biol. – 2010. - Vol. 73. P. 533-546. DOI:10.1007/s11103-010-9639-y

6. Dobrenel T, Mancera-Martínez E, Forzani C, Azzopardi M, Davanture M, Moreau M, Schepetilnikov M, Chicher J, Langella O, Zivy M, Robaglia C, Ryabova LA, Hanson J, Meyer C. The Arabidopsis TOR Kinase Specifically Regulates the Expression of Nuclear Genes Coding for Plastidic Ribosomal Proteins and the Phosphorylation of the Cytosolic Ribosomal Protein S6 // Front. Plant Sci. – 2016. – Vol. 7. – P. 1611. DOI: 10.3389/fpls.2016.01611. PMID: 27877176.

7. Henriques R., Bögre L., Horváth B., Magyar Z. Balancing act: matching growth with environment by the TOR signaling pathway. // J Exp. Bot. – 2014. - Vol.65(10), P. 2691-2701. DOI:10.1093/jxb/eru049

8. Holz M.K., Ballif B.A., Gygi S.P., Blenis J. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. // Cell. – 2005 - Vol. 123, P. 569–580, DOI:10.1016/j.cell.2005.10.024

9. Laemmli U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. // Nature. – 1970 - Vol. 227, P. 680-685, DOI:10.1038/227680a0

10. Leader D.P., Thomas A., Voorma H.O. The protein synthetic activity in vitro of ribosomes differing in the extent of phosphorylation of their ribosomal proteins. // Biochim et Biophys Acta. - 1981 - Vol. 656, P. 69-75, DOI:10.1016/0005-2787(81)90028-9

11. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. // Nat Rev Mol Cell Biol. – 2009 - Vol.10, P. 307-318. DOI:10.1038/nrm2672

12. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. // Biochem J. - 2012. - Vol. 441(1), P. 1-21. DOI: 10.1042/BJ20110892. PMID: 22168436.

13. Nandagopal N., Roux P.P. Regulation of global and specific mRNA translation by the mTOR signaling pathway. // Translation. – 2015. - Vol. 3(1), DOI: 10.4161/21690731.2014.983402

14. Nukarinen E., Nägele T., Pedrotti L., Wurzinger B., Mair A., Landgraf R., Börnke F., Hanson J., Teige M., Baena-Gonzalez E., et al. Quantitative phosphoproteomics reveals the role of the AMPK plant ortholog SnRK1 as a metabolic master regulator under energy deprivation. Sci Rep. – 2016. - Vol. 6, P. 31697. DOI: 10.1038/srep31697

15. Ren M., Qiu S., Venglat P., Xiang D., Feng L., Selvaraj G., Datla R. Target of rapamycin regulates development and ribosomal RNA expression through kinase domain in Arabidopsis. // Plant Physiol. - 2011. - Vol.155, P. 1367-1382, DOI:10.1105/tpc.112.107144

16. Rexin D., Meyer Ch., Robaglia Ch., Veit B. TOR signalling in plants. Biochem. J. - 2015. - Vol. 470, P. 1–14, DOI:10.1042/BJ20150505

17. Nadirova L.T., Beisenov D.K., Stanbekova G.E., Saparbaev M.K, Iskakov B.K. Cloning of cDNA-gene of Arabidopsis thaliana ribosomal protein S6, its expression in Escherichia coli and isolation of AtRPS6A1 recombinant protein. Exper. Biol. - 2022. - Vol. 91, P.138-147, DOI:10.26577/EB.2022.V91.I2.012

18. Sambrook J., Russel D.W. Molecular cloning: A laboratory manual: 3 volumes. Third edition. New-York: Cold Spring Harbor Laboratory Press, 2001.

19. **Scharf K.D., Nover L.** Control of ribosome biogenesis in plant cell cultures under heat shock conditions. II. Ribosomal proteins. // Biochim Biophys Acta. – 1987. - Vol. 909, P. 44–57, DOI:10.1016/0167-4781(87)90045-5

20. Wolters H., Jürgens G. Survival of the flexible: hormonal growth control and adaptation in plant development. // Nat. Rev. Genet. – 2009. – Vol. 10. – P. 305-317. DOI:10.1038/nrg2558

21. Yerlikaya S, Meusburger M, Kumari R, Huber A, Anrather D, Costanzo M, Boone C, Ammerer G, Baranov PV, Loewith R. TORC1 and TORC2 work together to regulate ribosomal protein S6 phosphorylation in Saccharomyces cerevisiae. // Mol Biol Cell. – 2016 – Vol. 27(2) P. 397-409. DOI: 10.1091/mbc.E15-08-0594. PMID: 26582391.

22. Zhang J.Z., Creelman R.A., Zhu J.K. From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiol. – 2004 - Vol. 135, P. 615-621, DOI:10.1104/pp.104.040295

CONFLICT OF INTEREST.

All authors are familiar with the content of the article and have no conflict of interest.

FUNDING

The work was carried out in the framework of the scientific grant AP14869357 «The development of scientific basis for biotechnology for increasing the productivity of crops by modification of ribosomal protein S6 genes», funded by the Committee of Science of the Ministry of Science and Higher Education of the Republic of Kazakhstan.

КЛОНИРОВАНИЕ КДНК-ГЕНА РИБОСОМНОГО БЕЛКА S6 ИЗ ARABIDOPSIS THALIANA, ЕГО МОДИФИКАЦИЯ, ЭКСПРЕССИЯ В ESCHERICHIA COLI И ВЫДЕЛЕНИЕ РЕКОМБИНАНТНОГО БЕЛКА ATRPS6B1

Надирова Л.Т.[1,2*], Бейсенов Д.К.[2], Станбекова Г.Э.[2], Жигайлов А.В.[2], Рябова Л.А.[3], Искаков Б.К.[2]

[1] Казахский национальный университет им. Аль-Фараби, Казахстан, Алматы, 050040, пр. аль-Фараби, 71
[2]РГП «Институт молекулярной биологии и биохимии им. М.А. Айтхожина» КН МНВО РК, Казахстан, г.Алматы, 050012, ул.Досмухамедова,80
[3]Институт молекулярной биологии растений, UPR 2357 CNRS, университет Страсбурга, Франция, Страсбург, 67084, Генерал Циммер, 12.
** leila.nadirova@gmail.com*

АБСТРАКТ

Недавние исследования показывают большую важность рибосомального белка S6 в контексте передачи сигналов TOR/S6K и его роли в трансляции и биосинтезе белка. Но механизмы активации клеточных процессов до сих пор до конца не ясны. Его основная функция связана с регуляцией механизмов, контролирующих рост и деление клеток. Белок RPS6 может фосфорилироваться по различным остаткам серина и треонина с помощью киназ, таких как S6K1 и S6K2, которые, в свою очередь, активируются сигнальными путями, связанными с активацией молекулярного комплекса mTORC1. Процесс фосфорилирования белка RPS6 играет ключевую роль в регуляции роста клеток и синтезе белка. Активированный RPS6 влияет на инициацию трансляции, то есть запускает процесс синтеза белка на рибосоме. Таким образом, белок RPS6 связан с регуляцией размера клеток и их способности делиться. Кроме того, RPS6 может быть связан с другими белками и участвовать в различных молекулярных взаимодействиях, которые могут варьироваться в зависимости от контекста клеточной активности. В данном исследовании было проведено клонирование и сайт-направленный мутагенез кДНК второй изоформы белка *At*RPS6 (*At*RPS6B). Далее полученные фосформиметическую и нефосфорилированную формы этого белка экспрессировали в клетках *E.coli* ArcticExpress (DE3), белки очищали методом металл-хелатной хроматографии (IMAC), наличие и чистоту полученных белков подтверждали методом иммуноблоттинга.

Ключевые слова: *Arabidopsis thaliana*, клонирование кДНК-гена *At*RPS6B1, фосфомиметическая мутация, рекомбинантный рибосомный белок S6 (*At*RPS6B1)

ARABIDOPSIS THALIANA-ДАН S6 РИБОСОМАЛЫҚ АҚУЫЗЫНЫҢ ҚДНҚ ГЕНІН КЛОНДАУ, ОНЫҢ МОДИФИКАЦИЯСЫ, ESCHERICHIA COLI-ДЕН ЭКСПРЕССИЯСЫ ЖӘНЕ ATRPS6B1 РЕКОМБИНАНТТЫ АҚУЫЗЫН ОҚШАУЛАУ

Надирова Л.Т.[1,2]*, Бейсенов Д.Қ.[2], Станбекова Г.Е.[2], Жигайлов А.В.[2], Рябова Л.А.[3], Ысқақов Б.Қ.[2]

1Әл-Фараби атындағы Қазақ ұлттық университеті, Қазақстан, Алматы, 050040, әл-Фараби даңғылы, 71
2М.Айтхожин атындағы Молекулярлық биология және биохимия институты, Қазақстан, Алматы, 050012, Досмұхамедов көш, 80
3Өсімдіктердің молекулалық биология институты, UPR 2357 CNRS, Страсбург университеті, Франция, Страсбург, 67084, Генерал Циммер, 12.
** leila.nadirova@gmail.com*

ТҮЙІН

Соңғы зерттеулер TOR/S6K сигнализациясы контекстіндегі S6 рибосомалық ақуызының үлкен маңыздылығын және оның трансляция мен ақуыз биосинтезіндегі рөлін көрсетеді. Бірақ жасушалық процестерді белсендіру механизмдері әлі толық анық емес. Оның негізгі қызметі жасушаның өсуі мен бөлінуін бақылайтын механизмдерді реттеумен байланысты. RPS6 протеині әртүрлі серин және треонин қалдықтарында S6K1 және S6K2 киназалармен фосфорлануы мүмкін, олар өз кезегінде mTORC1 молекулалық кешенінің белсендірілуімен байланысты сигналдық жолдар арқылы белсендіріледі. RPS6 протеиннің фосфорлану процесі жасушаның өсуі мен ақуыз синтезін реттеуде шешуші рөл атқарады. Белсендірілген RPS6 трансляцияның басталуына әсер етеді, яғни рибосомадағы ақуыз синтезі процесін бастайды. Осылайша, RPS6 жасуша өлшемін және олардың бөлінуі қабілетін реттеумен байланысты. Сонымен қатар, RPS6 протеині басқа ақуыздармен байланысып, жасушалық белсенділік контекстіне байланысты әртүрлі молекулалық өзара әрекеттесулерге қатыса алады. Бұл зерттеуде *At*RPS6 ақуызының (*At*RPS6B) екінші изоформасының клондау және сайтқа бағытталған cDNA мутагенезі орындалды. Әрі қарай, осы ақуыздың алынған фосфомиметикалық және фосфорланбаған түрлері *E.coli* ArcticExpress (DE3) жасушаларында экспрессияланды, ақуыздар металл хелат хроматографиясы (IMAC) арқылы тазартылды және алынған ақуыздардың болуы мен тазалығы иммуноблоттау арқылы расталды.

Түйін сөздер: *Arabidopsis thaliana*, *At*RPS6B1 cDNA генінің клондалуы, фосфомиметикалық мутация, рекомбинантты рибосомалық ақуыз S6 (*At*RPS6B1)