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CLONING OF cDNA-GENES OF *ARABIDOPSIS THALIANA* TRANSLATION INITIATION FACTOR 2 α-, β-, and γ-SUBUNITS, THEIR EXPRESSION IN *ESCHERICHIA COLI* AND ISOLATION OF RECOMBINANT PROTEINS AteIF2α, AteIF2β, AteIF2γ

Zhigailov A.V.[1*], Stanbekova G.E.[1], Beisenov D.K.[2], Kislitsin V.U.[1], Alexandrova A.M.[1], Krasnoshtanov V.K.[3], Iskakov B.K.[2]

[1]M.A. Aitkhozhin Institute of Molecular Biology and Biochemistry,
86, Dosmukhamedov str., Almaty, 050012, Kazakhstan
[2]Institute of plant biology and biotechnology,
45, Timiryazev str., Almaty, 050040, Kazakhstan
[3]Kazakh Scientific-Research Institute of oncology and radiobiology,
91, Abai ave., Almaty, 050022, Kazakhstan
**andrzhig@yandex.ru*

ABSTRACT

The eukaryotic translation initiation factor 2 (eIF2) consists of three non-identical subunits, denoted α, β and γ; α-subunit performs a regulatory function. This factor is strictly required for initiation of eukaryotic mRNA translation. In animal cells, phosphorylation of the α-subunit of the meIF2 factor by specific kinases dramatically inhibits the initiation of translation of the majority of mRNAs during various stresses. In plants, the role of phosphorylation of homologous factor (peIF2α) in regulation of protein biosynthesis remains unclear.

The cDNA genes of α, β and γ subunits of eIF2 factor from *A. thaliana* were amplified by RT-PCR (*AteIF2α, AteIF2β* and *AteIF2γ,* respectively). These cDNA genes were cloned in *E. coli* cells in the expression vector pET19b. Recombinant proteins AteIF2α, AteIF2β and AteIF2γ were isolated by affinity chromatography, dialyzed and concentrated. They all had correct dimensions.

These preparations were used to assemble a full factor of three recombinant subunits. At ionic strength corresponding to 100 mM KCl, the subunits could form united, but not stable complex, which dissociated already at 150 mM, whereas the native factor can remain stable even at 350 mM KCl. The assembly of plant factor from individual subunits makes it possible to elucidate the role of peIF2α phosphorylation in regulation of various mRNAs translation in plant *in vitro* system and also in the *in vivo* system permits constructing cellular factor peIF2 from various modified subunits.

Key words: *Arabidopsis thaliana,* eukaryotic translation initiation factor 2 (eIF2), cloning, recombinant subunits, phosphorylation.

INTRODUCTION

The eIF2 factor is necessary for the initiation of translation of virtually all mRNAs in eukaryotic cells. It consists of three nonidentical subunits {α•β•γ} and forms a triple complex {GTP●eIF2●Met-tRNA$_i^{Met}$}, which then binds to the 40S ribosomal subunit. Together with the eIF1, eIF1a and eIF5 factors, eIF2 plays an important role in the selection of the correct starting codon [1]. At first, eIF2 attaches GTP to GDP/GTP nucleotide-binding site of its γ-subunit. Then, acylated methionine initiator tRNA (Met-tRNAiMet) is attached to this binary complex. β-subunit significantly stimulates this process, but the binding site of initiator tRNA is also located on the γ-subunit [1, 2]. Although the α-subunit also stimulates ternary complex formation, its main role is in the regulating of protein synthesis level. Phosphorylation of α-subunit of meIF2 factor at amino acid residue Ser51 by specific protein kinases during stress or physiological conditions resulting in rapid and deep suppression of protein synthesis in mammalian cells, and protein synthesis returns to baseline after the dephosphorylation of meIF2αP. [1-3].

The meIF2 factor is released as a strong complex {meIF2*GDP} after each initiation cycle. The GDP molecule bound to meIF2 must be exchanged for GTP to make meIF2 be able to participate in the following cycle. This exchange can not proceed independently, since affinity of meIF2 to GDP is two orders greater than its affinity to GTP. Thus it can only be done by an auxiliary factor meIF2B, that forms a complex with {meIF2*GDP}, from which GDP readily dissociates. The mechanism of regulation of protein biosynthesis by phosphorylation of the α-subunit of eIF2 in mammalian and yeast cells is well known and was described in detail in the literature [1-3].

At present, the view is accepted that plants do not use the mechanism of inhibition of protein synthesis through the phosphorylation of peIF2α, which is significantly different from the analogous mechanism in animals [4-7].

At the same time, there is another point of view that such a mechanism can still function in plants that are in different physiological states and under stressful conditions, although not as distinctly as in animals [8-10]. Four protein kinases in animal cells (mHRI, mPKR, mPERC, mGCN2) can phosphorylate meIF2α in response to various biotic and abiotic stresses. The plants have only one pGCN2-kinase, which phosphorylates peIF2α in response to certain, but not all types of stresses. Thus, peIF2α phosphorylation was not observed in case of saline stress (250 mM NaCl), oxidation stress (1 mM H_2O_2), viral infections and heat shock (2h at 42°C) [8, 11-12]. Moreover, *A. thaliana* mutants with defective *gcn2* gene are quite viable [11]. Recently, it has been shown that adaptation to stress conditions in plants is achieved through the activation of NOXY7/pGCN1 in some cases, but not through the activation of pGCN2 [13, 14]. The details of this mechanism in plants have not been clarified, and additional experimental studies are required for their full understanding, which in the future will allow the creation of plants with increased resistance to abiotic stresses.

The aim of this work was to obtain recombinant α, β and γ subunits of eIF2 factor from *A. thaliana*, as well as polyclonal antibodies to these proteins. To do this, we cloned cDNA genes of three subunits of this factor. These cDNA-genes were expressed separately in *E. coli* strain BL-21(DE3); synthetized recombinant proteins were isolated and purified. Specific polyclonal antisera to the purified recombinant proteins were obtained.

Then we attempted to assemble a whole factor from individual subunits *in vitro*. The assembly of the plant factor from individual subunits allows model experiments in a plant system *in vitro* to elucidate the role of eIF2α phosphorylation in the regulation of protein biosynthesis in plants *in vitro* using specific protein kinases such as PKR. Such an approach will avoid the use of complex process of isolating the natural peIF2 factor.

It will also allow constructing the cellular peIF2 factor from various modified (mutated) subunits in *in vivo* systems. At the same time, no one has ever managed to construct a stable and active peIF2 factor from individual recombinant subunits.

MATERIALS AND METHODS

We used reagents produced by "Sigma", "Serva", "Merck", "Fermentas", "Roche", "Promega" and "BioLabs" companies. All used oligodeoxyribonucleotides were synthesized by "Sigma": "eIF2-NdeI-FW" (5')GAATCATATGACCATGGCGAATCCTGCTCCGAATCTAGAATGTCGTATGT, "eIF2-BamHI-Rev" (5')TGCGGATCCTTTTGTTCATTCAATTATCCCGCT ACCTCCATCGATATC, "eIF2b-Nde-FW": (5')TACACATATGGCTGATGAAATCAATGAGATAAGGGAAGAG, "eIF2b-Xho-Rev": (5')TTACCTCGAGTAATTTCTCAAGTCTTCCTGCGACTAACACGAGCA, "eIF2g-Nde-Fw": (5')ATATCATATGTCGAGGAACAAGGGTTTGGC TGAGCAAGAT, "eIF2g-Bam-Rev": (5')AATAGGATCCATAGCTTAGAAAGGTGAAGGAGGAACTTCGATGGT.

Computer analysis of nucleotide sequences before inserting them into vector DNA was performed using Vector NTI 11.5 software. Primer design was performed using RNA-structure 3.5 and Blast (http://www.blast.genome.jp) programs. Nucleotide sequences of cDNA-genes were taken from the NCBI database (www.ncbi.nlm.nih.gov). Densitometry analysis was performed using the ImageJ v.1.42q computer program.

Isolation of RNA. The total preparation of RNA was isolated from the *A. thaliana* leaves using the *PARIS kit* ("Ambion").

The reverse transcription (RT) reaction was performed using the QuantiTect® Reverse Transcription Kit ("Qiagen") according to the manufacturer's protocol. The following oligonucleotides were used as primers: "eIF2-BamHI-Rev" – for *AteIF2α* cDNA; "eIF2b-Xho-Rev" – for *AteIF2β* cDNA; "eIF2g-Bam-Rev" – for *AteIF2γ* cDNA.

Amplification of cDNAs was performed using high-precision polymerase Pwo ("Roche") in case of RT-PCR, as well as Taq-polymerase ("Fermentas") – in case of PCR-screening. We used "eIF2-NdeI-FW"/"eIF2-BamHI-Rev" primer pair in case of *AteIF2α* cDNA; "eIF2b-Nde-FW" / "eIF2b-Xho-Rev" pair – in case of *AteIF2β* cDNA, and "eIF2g-Nde-Fw"/"eIF2g-Bam-Rev" – in case of *AteIF2γ* cDNA. Temperature regime: stage 1 – 30 sec. at 94°C – 1 cycle; Stage 2 – 30 sec. at 94°C, 30 seconds. at 54°C, 1 min. 30 seconds at 72ºC – 30 cycles; Stage 3 – 5 min. at 72°C – 1 cycle.

Ligation. Eluted from agarose gel RT-PCR-products were digested by *Nde*I and *BamH*I endonucleases followed by cloning into pET19b vector using T4 DNA ligase ("Fermentas") at the same restriction sites according to the manufacturer's protocol.

Sequencing of DNA was performed using the *Big Dye Terminator v.3.1 kit* ("Applied Biosystems") according to the manufacturer's protocol. Capillary electrophoresis was performed on a genetic analyzer 310 ("Applied Biosystems"). The analysis of obtained data was carried out using "Sequencing Analysis 5.2" software.

Expression and isolation of recombinant proteins. Cells of expression strain *E. coli* BL21(DE3) were transformed with pET19b-vector derived plasmids by heat shock method [15]. Transformed cells were grown in 200 ml of LB liquid culture containing ampicillin at 30°C until the culture reached an optical density at 600 nm (OD^{600}) of 0.5. Then cells were precipitated at 4000 g for 3 minutes, re-suspended in 200 ml of fresh LB medium containing IPTG to a final concentration of 1 mM, followed by 4 hours incubation at 30°C. The isolation of recombinant proteins containing His-tag was

carried out by immobilized metal ion affinity chromatography (IMAC) in native conditions using the *PerfectPro Ni-NTA Agarose kit* ("5-Prime") according to the manufacturer's protocol.

Electrophoresis of proteins was carried out in a polyacrylamide gel (T = 12.5%, C = 0.5%) in the presence of 0.1% SDS using the standard Laemmli method [16]. Gels were stained with 0.125% Coomassie solution of brilliant blue G-250 ("Serva").

Immunoblotting. The semi-dry transfer of proteins from the PAAG to the nitrocellulose membrane was carried out in transfer buffer (102 mM glycine, 25 mM Tris, 20% (v/v) ethanol) at a current of 0.8 mA/cm^2 and 20V for 1 hour. Membrane was washed twice for 10 minutes in TBS buffer (20 mM TrisHCl (pH 7.6), 140 mM NaCl), twice for 10 min - in TBST buffer (TBS + 0.05% Tween-20) and incubated in blocking buffer (5% skimmed milk powder of "Sigma" in TBST) overnight at 4°C. Membrane after blocking was incubated with first antibodies (Penta-His mouse antibodies of "5-Prime" in a dilution of 1: 2000 in blocking buffer) for 1 hour at 25°C. After three washings in TBST for 20 minutes, the membrane was incubated with anti-mouse HRP-conjugate ("5-Prime") in a 1: 2000 dilution in blocking buffer) for 1 hour at 25°C. After washing the membrane twice for 20 min in TBST and washing twice for 10 min in TBS, its development was carried out by a chemiluminescent substrate for peroxidase of "Promega", according the manufacturer's manual.

Dialysis of recombinant proteins was performed in dialysis bags ("Sigma") against dialysis buffer (20 mM TrisAc pH 7.6, 90 mM KAc, 2 mM $Mg(OAc)_2$) at 4°C for 12 hours. Concentration of the protein after dialysis was carried out by centrifugation of the samples in columns *10000 MWCO HY* ("Sartorius") with a semi-permeable membrane according to the manufacturer's manual.

Ion exchange chromatography of peIF2 factor from recombinant subunits. The dialyzed and concentrated recombinant subunits (AteIF2α, AteIF2β and AteIF2γ) were pooled in equimolar amounts and incubated for 1 hour in buffer A (20 mM Tris (pH = 7.6), 4 mM $MgCl_2$, 5 mM Na_2EDTA, 5 mM DTT, 10% glycerol) containing 100 mM KCl and a protease inhibitor (*UltraCruzTM Protease Inhibitor Cocktail Tablet*, "Santa-Crus"). After incubation, the mixture was applied to a Q-Sepharose column (5 ml of resin) pre-equilibrated with the same buffer. The column was washed with buffer A containing 100 mM KCl, followed by elution of the resin-retained proteins using a step gradient of KCl concentrations (150 mM, 225 mM, 300 mM, 350 mM, 425 mM, 500 mM). The collected fractions were precipitated with ethanol and analyzed by electrophoresis followed by immunoblotting using specific anti-5His-tag antibodies.

Preparation of polyclonal antisera to recombinant subunits.

Recombinant proteins His-AteIF2α, His-AteIF2β and His-AteIF2γ were treated with enterokinase ("Sigma") to get rid of His-tag sequence according to manufactures' protocol followed by electrophoresis in PAA-gel. Strips corresponding to the target proteins were cut from the gel, ground in liquid nitrogen, homogenized with equal volume of PBS buffer and used as antigens. Immunization was carried out in the vivarium of the RSE "Kazakh Scientific-Research Institute of oncology and radiobiology", Ministry of Health, Republic of Kazakhstan. 5 month-old sexually mature white laboratory rats (males, 270-300 g.) were immunized once with 100 μg of antigen (AteIF2α, AteIF2β or AteIF2γ). Antigens were emulsified with complete Freund's adjuvant, and 1.0 ml was injected intraperitoneally. Two weeks later, animals were reimmunized with 100 μg of the same antigen emulsified with incomplete Freund's adjuvant. Two weeks after the second injection, a third immunization was given with the homologous antigen emulsified with the same adjuvant. Seven days after the last immunization, blood from the tail veins was collected (1.5 ml); animals were not killed.

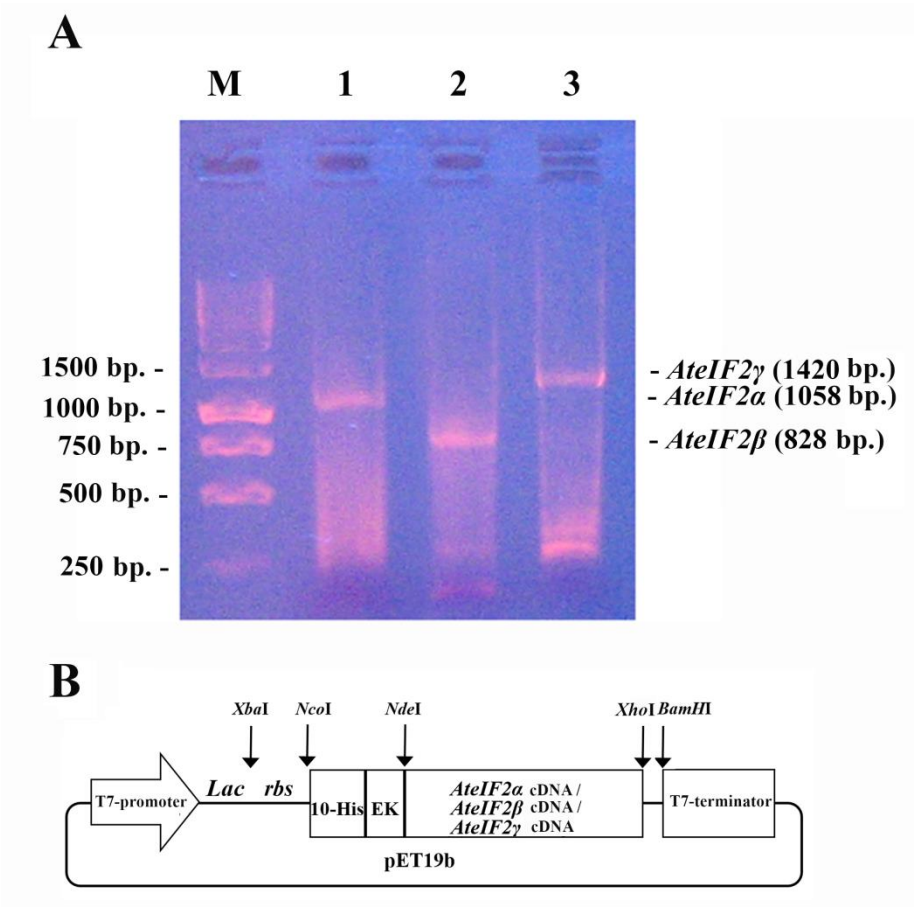
Sera preparations obtained from whole blood samples were used as the first antibodies to correspondent proteins.

A protein concentration in solutions was estimated by the Bradford protein assay in three replicates with the calculation of the arithmetic mean (X_{cp}) and the arithmetic mean error (m).

Common methods were performed according the standard procedures [15].

RESULTS AND DISCUSSION

The nucleotide sequences of the mRNAs encoding AteIF2α, AteIF2β and AteIF2γ subunits (GenBank #: NM_120629.3, AF353095.1 and AF353097.1, respectively) were used to select the appropriate primers for amplification and cloning of these cDNAs into the pET19b vector. The results of RT-PCR and a schematic representation of obtained constructs AteIF2α-pET19b, AteIF2β-pET19b and AteIF2γ-pET19b are shown in fig. 1. The functional regions of the resulting DNA-constructs were sequenced. According to their nucleotide sequences, cloned cDNAs fully corresponded to the open reading frames (ORFs) of the mRNAs encoding AteIF2α, AteIF2β and AteIF2γ.

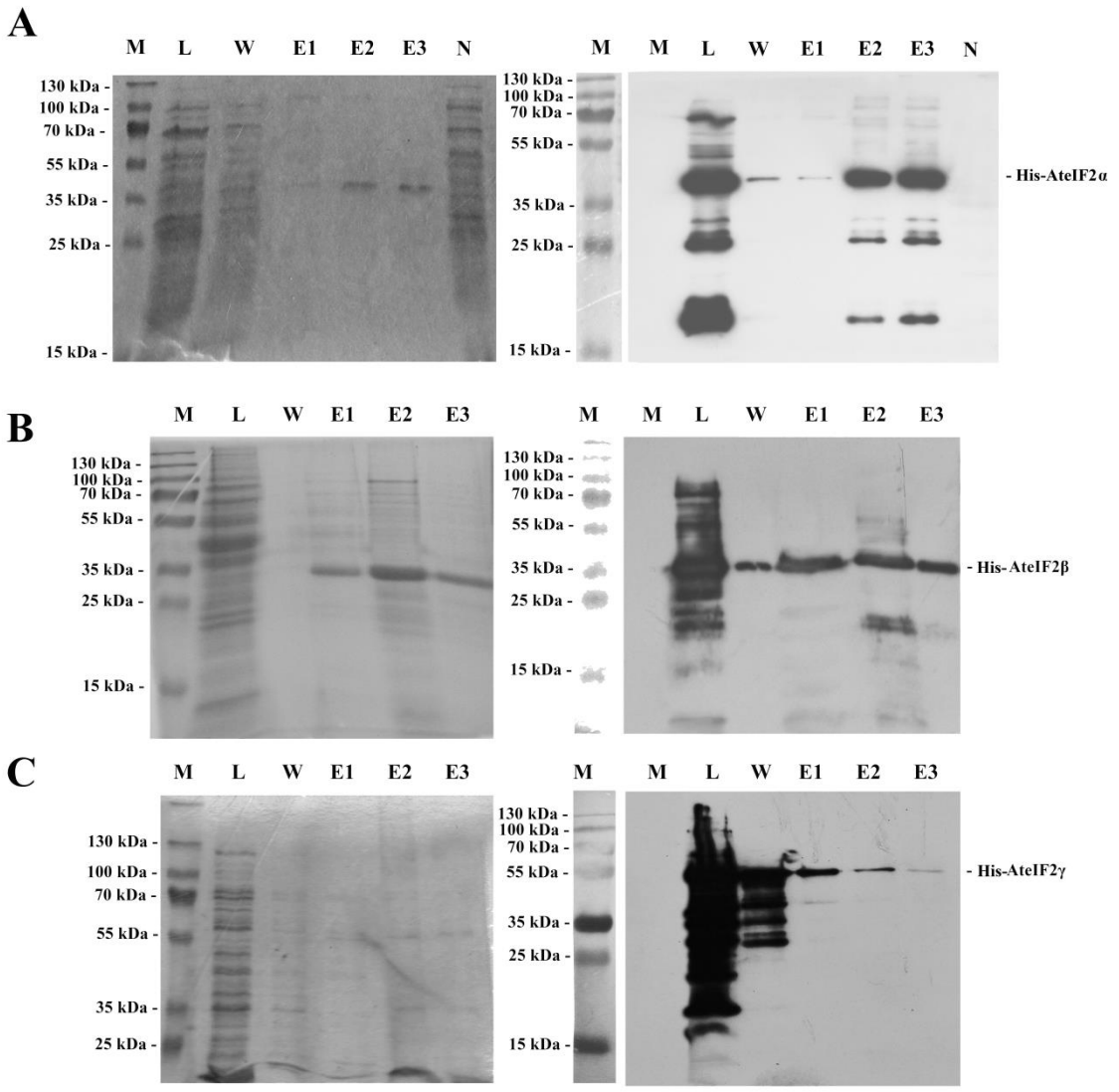


(А): M – *GeneRuler™ 1kb DNA ladder* (“Thermo”); 1 – *AteIF2α* RT-PCR product; 2 – *AteIF2β* RT-PCR product; 3 – *AteIF2γ* RT-PCR product;

(Б)*: Lac* – lac-operator; *rbs* – ribosome binding site; 10-His – sequence encoding 10 histidine residues; EK – sequence encoding enterokinase recognition site for cleavage of 10His-tag from recombinant protein.

Fig. 1. Electrophoretic analysis in 1% agarose gel of RT-PCR products (A) and schematic presentation of obtained DNA-constructs *AteIF2α-pET19b, AteIF2β-pET19b* and *AteIF2γ-pET19b* (B)

The *E. coli* cells of the expression strain Bl21(DE3) were transformed with AteIF2α-pET19b, AteIF2β-pET19b and AteIF2γ-pET19b DNA-constructs. After the expression of the recombinant genes, the synthesized proteins were isolated on 10His-tag by the IMAC method. The results of Western blot analysis of different fractions during the isolation process of recombinant protein is shown in fig. 2.



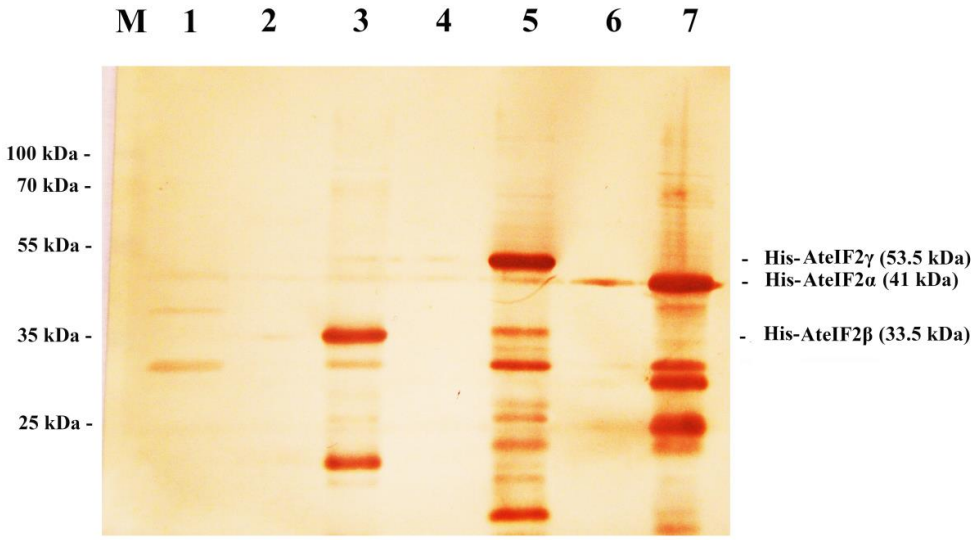
(A): His-AteIF2α, (B): His-AteIF2β; (C): His-AteIF2γ. Left panels present 12.5% PAA-gels stained with Coomassie G250; right panels present immunoblotting membranes ("Penta-His Ab" were used as the first antibodies).

Tracks: M – *PageRuler Plus* protein marker ("Life science"); N – negative control (lysate of bacteria that contains empty pET19b vector); L – lysate of bacteria synthesizing recombinant proteins before binding to Ni-NTA agarose; W – proteins eluted from column with washing buffer; E1, E2 и E3 – proteins eluted from Ni-NTA agarose column with buffer containing 20 мМ of imidazole (3 fractions).

Fig. 2. Electrophoregrams and western-blot analyses of recombinant proteins His-AteIF2α, His-AteIF2β and His-AteIF2γ fraction obtained after IMAC

The preparations of the recombinant subunits were dialyzed followed by concentration by centrifugation using columns semi-permeable membranes. The results of Western blot

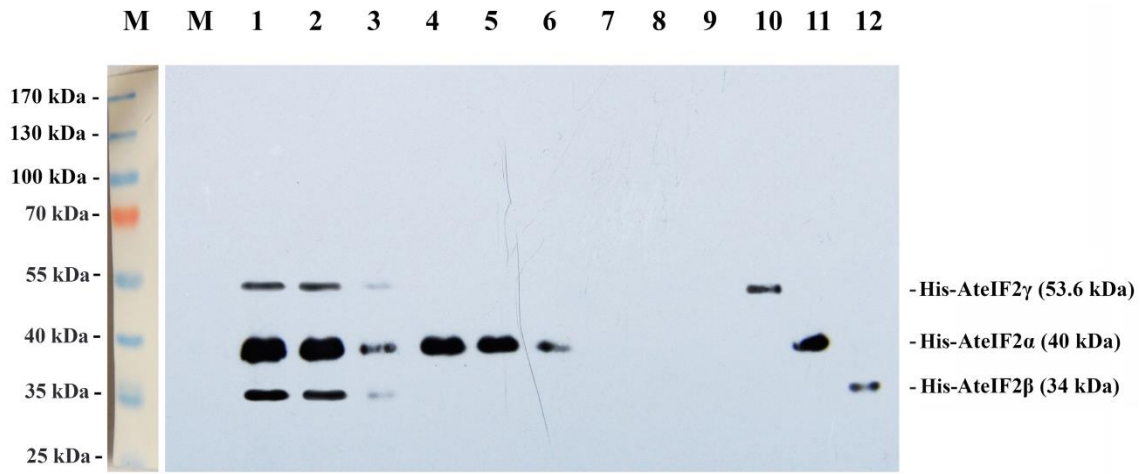
analysis of purified recombinant AteIF2 subunits before and after concentration are shown in fig. 3. A protein preparation from *E. coli* strain Bl21(DE3) cells transformed with the empty pET19b vector that was isolated underwent the same conditions as recombinant AteIF2 subunits was used as a negative control.



Tracks: M – *PageRuler Plus* protein marker ("Life science"); 1 – negative control; 2 – His-AteIF2β after dialysis; 3 – His-AteIF2β after dialysis and concentrating; 4 – His-AteIF2γ after dialysis; 5 – His-AteIF2γ after dialysis and concentrating; 6 – His-AteIF2α after dialysis; 7 – His-AteIF2α after dialysis and concentrating. "Penta-His Ab" were used as the first antibodies.

Fig. 3. Western blot analysis of isolated, purified and concentrated recombinant proteins

In order to validate the physiological state of the isolated and purified subunits, we have attempted to collect a whole peIF2 factor from them. The native eF2 factor from wheat germs (WGeIF-2) elutes from Q-Sepharose at 250-300 mM concentrations of KCl at pH 7.0 [4]. We suggested that the AteIF2 factor, assembled from the individual recombinant subunits AteIF2α, AteIF2β and AteIF2γ, will elute approximately under the same conditions. After the assembly phase, during which three subunits were co-incubated with each other, the reaction mixture was passed through a Q-Sepharose column and washed with a KCl step gradient (fig. 4).

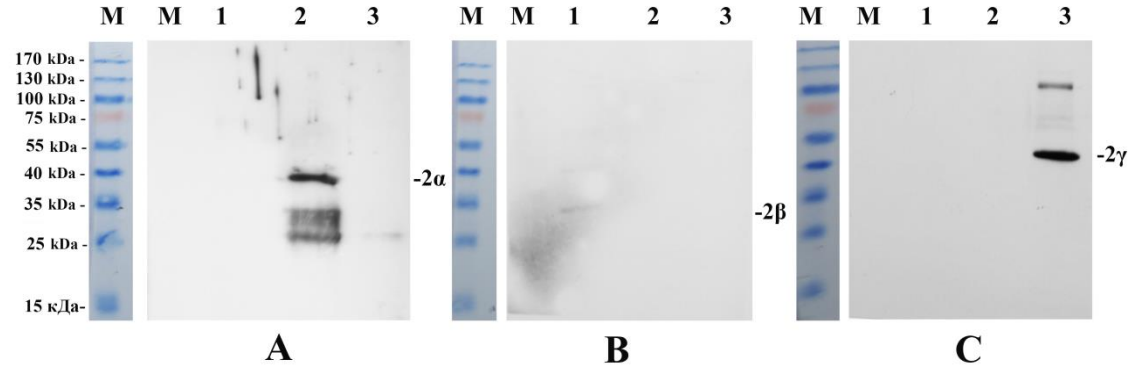


Tracks: M – *PageRuler Plus* protein marker ("Life science"); Fractions eluted from the column with buffer A containing the following KCl concentrations: 1-3 – 100 mM KCl; 4 – 150 mM KCl; 5 – 225 mM KCl; 6 – 300 mM KCl; 7 – 350 mM KCl; 8 – 425 mM KCl; 9 – 500 mM KCl. Tracks with purified recombinant subunits: 10 – 10 μg of His-AteIF2γ; 11 – 10 μg of His-AteIF2α; 12 – 10 μg of His-AteIF2β. "Penta-His Ab" were used as the first antibodies.

Fig. 4. Western blot analysis of fractions from ion exchange Q-sepharose chromatography of the products of His-AteIF2α, His-AteIF2β and His-AteIF2γ recombinant subunits joint incubation that where eluted with a stepped gradient of KCl concentrations

The results shown in Fig. 4 show that at an ionic strength corresponding to 100 mM KCl (lanes 1-3 in Fig. 4), all subunits behave as a single complex, since the quantitative content of all subunits decreases proportionally with the elution at a given ionic strength. It should be noted that this complex is not very strong and breaks down already at 150 mM KCl. In the concentration range from 150 to 300 mM KCl, only the acidic AteIF2α subunit with isoelectric point (pI) 5.1 is retained on the Q-Sepharose column (see lanes 4-6 in fig. 4). The more basic subunits of AteIF2β (pI 7.1) and especially AteIF2γ (pI 8.8) dissociate from the complex and are not observed in the eluate even at 150 mM KCl.

A possible reason for the low stability of recombinant AteIF2 factor, assembled from individual recombinant subunits, may be the presence of a sequence of ten histidines ("10His-tag") at the N-termini of the isolated recombinant proteins, which could interfere with the formation of the tri-subunit eIF2 complex. In gained pET19b-derived DNA-constructs, a nucleotide sequence encoding the enterokinase cleavage site was placed between the nucleotide sequence encoding "10His-tag" and the inserted ORF of the target cDNA-genes (see fig. 1B). This allowed us to obtain recombinant subunits of AteIF2α, AteIF2β and AteIF2γ free of "10His-tag" sequence using enterokinase. In order to preserve the possibility of their detection, polyclonal antisera were obtained for these proteins from the blood of immunized rats. Immunoblot analysis using gained antisera in the optimal dilution for the detection is shown in fig. 5.



A – antiserum against AteIF2α in 1:4000 dilution; B – antiserum against AteIF2β in 1:500 dilution; C – antiserum against AteIF2γ in 1:1000 dilution. Tracks: M – *PageRuler* protein marker ("Life science"); 1 – 10 μg AteIF2α (2α); 2 – 10 μg AteIF2β (2β); 3 – 10 μg AteIF2γ (2γ).

Fig. 5. Testing of sensitivity and specificity of polyclonal antisera against AteIF2α, AteIF2β and AteIF2γ recombinant proteins

Antibodies to the AteIF2β subunit had the lowest sensitivity and specificity (Fig. 5B). With an increase in the concentration of these antibodies in the blocking buffer, non-specific binding to the AteIF2α and AteIF2γ subunits, as well as to the accompanying

minor polypeptides appeared (data not shown). Therefore, it was not possible to detect small amounts of AteIF2β in the peIF2 factor using the obtained antisera. In addition, the target polypeptides were significantly degraded during the treatment of preparations of recombinant proteins with enterokinase (data not shown). These circumstances led to the impossibility of repeating the experiment of assembling the tri-subunit AteIF2 factor from separate recombinant subunits lacking the "10His-tag" segment.

Isolated and purified recombinant subunits of AteIF2 factor will be used subsequently to study the role of phosphorylation of peIF2 in plants using recombinant PKR kinase that we have isolated previously [17]. Optimization of the AteIF2 factor assembly process from individual recombinant subunits will be continued. Obtained earlier mutated AteIF2α(S51D) and AteIF2α(S51A) subunits [18] will be used to construct hybrid forms of recombinant AteIF2 containing phosphomimetic and non-phosphorylatable variants of AteIF2α.

CONCLUSION

The cDNA genes encoding AteIF2α, AteIF2β and AteIF2γ subunits of translation initiation factor 2 from *A. thaliana* (AteIF2) are cloned. These cDNA genes were expressed in *E. coli* cells, and synthesized corresponding proteins containing "10His-tag" at their N-termini were isolated by immobilized metal ion affinity chromatography. The resulting AteIF2α, AteIF2β and AteIF2γ subunits are dialyzed and concentrated. Specific polyclonal antisera to the purified recombinant proteins are obtained.

After the simultaneous co-incubation of all three recombinant subunits at 100 mM KCl, a single three-subunit complex AteIF2 is formed, the fact of which is confirmed by ion exchange chromatography on Q-Sepharose. However, the complex formed *in vitro* was unstable and break down at 150 mM KCl.

Since eIF2 is the key factor involved in the regulation of protein biosynthesis in eukaryotes during stress conditions, its assembly *in vitro* from separate subunits may be useful in studying the mechanisms of protein biosynthesis regulation in plants. The AteIF2α, AteIF2β and AteIF2γ subunits are needed to study the role of phosphorylation of AteIF2 in the regulation of mRNA translation in plant systems *in vitro*. Cloned cDNA-genes may be useful for constructing the cellular plant eIF2 factor *in vivo* from modified subunits containing definite amino acid substitutions.

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