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Phosphorylation of the alpha-subunit of plant eukaryotic initiation factor 2 prevents its association with polysomes but does not considerably suppress protein synthesis

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Abstract

Phosphorylation of the α-subunit of eukaryotic initiation factor 2 (eIF2α) and subsequent inhibition of protein synthesis is a major survival response to different stresses in animal and yeast cells. However, the role of this regulatory mechanism in plants is not unambiguously established to date. Here we describe a slight reduction of polysome abundance in *Nicotiana benthamiana* after the transient expression of a cDNA, *AteIF2α(S56D),* encoding a phosphomimetic form of *Arabidopsis thaliana* eIF2α. In contrast, the expression of a cDNA, *AteIF2α(S56A),* that encodes a non-phosphorylatable form of *At*eIF2α caused slightly elevated polysome formation compared to the control. Recombinant *At*eIF2α(S56A) was detected in association with 40S ribosomal subunit-containing complexes and also in the polysomal fraction, while recombinant *At*eIF2α(S56D) was detected mainly in complex with 40S subunits. Intentional phosphorylation of *Ta*eIF2α induced by L-histidinol in a wheat germ (*Triticum aestivum*) cell-free extract did not reduce the abundance of polysomes. Interestingly, the phosphorylated *Ta*eIF2(αP) was not detected in the polysomal fraction, similar to *At*eIF2α(S56D) in the *in vivo* experiment. Using mRNAs with a 'Strepto-tag' in the 3′ untranslated region, the 48S pre-initiation complexes isolated from histidinol-treated wheat germ extracts were shown to contain phosphorylated *Ta*eIF2(αP). Thus, the phosphorylation of plant eIF2 does not greatly affect its ability to participate in the initiation of mRNA translation, in contrast to animals and yeast, in which eIF2α phosphorylation results in profound suppression of protein synthesis.

Key words: Arabidopsis (*Arabidopsis thaliana*) phosphomimetic and non-phosphorylatable *At*eIF2α; transient expression in tobacco; polyribosomes formation; wheat germ cell-free system; GCN2; 48S preinitiation complex.

1. Introduction

The process of protein synthesis is conserved among all eukaryotes [1] but significant differences exist in its regulation [2-4]. Eukaryotic translation initiation factor 2 (eIF2), in the form of ternary complex (TC = [GTP∗eIF2∗Met-tRNA$_i^{Met}$]), is needed to deliver Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit during the initiation step of mRNA translation. Factor eIF2 contains three unequal subunits (α, β and γ), and reversible phosphorylation of the α-subunit, eIF2α, is a well-known mechanism of regulating protein biosynthesis that has been carefully described for mammalian, protozoan, and yeast cells exposed to different stress conditions [5, 6]. Factor eIF2 can bind either GDP or GTP, but is only able to initiate mRNA translation when bound to GTP. Following attachment of the TC to the 40S subunit, the GTP is hydrolyzed to GDP and P_i and eIF2 is released as an inactive binary complex [eIF2∗GDP]. In animals and yeast, phosphorylation of eIF2α prevents the exchange of GDP for GTP mediated by the guanine nucleotide exchange factor, eIF2B, thus resulting in the inhibition of translation. Since mammalian eIF2α (meIF2α) is in 3- to 5-fold excess over meIF2B, even slight changes in the levels of meIF2(αP) have a significant impact on overall mRNA translation level [7]. It should be noted, however, that even in yeast and mammalian cells, the translational depression induced by some stress responses is independent of eIF2α phosphorylation. This is true for UV irradiation, for example [8], even though UV irradiation induces strong eIF2α phosphorylation. For a long time, it was believed that a mechanism for regulating protein synthesis through eIF2α phosphorylation also operated in plants [9]. However, while plant eIF2α (peIF2α) is certainly subject to reversible phosphorylation, the functional relevance of this regulatory mechanism in response to stress in plants has not been unambiguously demonstrated [2]. Indeed, factor eIF2B, the central participant of such a mechanism of translation inhibition in animals and fungi, is not necessary for the cyclic functioning of plant peIF2 [10, 11], and may not be present in plants at all, since no evidence has ever been presented for the existence of the protein itself, its biochemical activity or the genes that encode its subunits [2, 12].

In mammals, phosphorylation of meIF2α can be accomplished by any one of four distinct protein kinases, mGCN2, mPKR, mPERK, and mHRI (see Abbreviations), each of which is activated in response to different stress stimuli [13]. In contrast, only one equivalent protein kinase, pGCN2, has been found in plants [14]. Plant pGCN2 is activated under some but not all types of abiotic stress [2, 15, 16], and does phosphorylate peIF2α. However, we have shown previously that this does not essentially reduce mRNA translation in the wheat germ cell-free system [16].

In the present study, we used phosphomimetic and non-phosphorylatable versions of *At*eIF2α, transiently expressed in *Nicotiana benthamiana*, and investigated their effect on the level of

protein synthesis. The use of such variants of proteins that are regulated by phosphorylation has proved to be a powerful tool [17]. We also investigated the phosphorylation status of peIF2α present in 48S pre-initiation complexes following the activation of endogenous pGCN2 in a wheat germ cell-free protein synthesizing system.

2. Materials and Methods

2.1. Plant material and growth conditions

Tobacco (*N. benthamiana*) plants were grown on solid Murashige and Skoog (MS) medium containing 1% sucrose (w/v) and 1% agarose or in soil. Cultivation conditions: 16-h-light/8-h-dark cycle at 22°C. Wheat embryos isolated from dry grains (*T. aestivum* L., Kazakhstanskaya-10 cultivar) were used to prepare wheat germ extract (WGE) for mRNA translation *in vitro* [16].

2.2. Mutagenesis of AteIF2α cDNA

Cloning of cDNA encoding *At*eIF2α (NM_120629.3) into the pET-19b vector, resulting in pET19b-AteIF2α plasmid, was described previously [18]. The introduction of the S56D substitution (encoding an aspartate instead of serine residue at position 56 of the amino acid sequence) in the wild type *AteIF2α* cDNA was performed by overlapping PCR, using primers: eIF2_Nde_F/eIF2-D-Rev (Table 1) for a 183 bp 5′-fragment, and eIF2-D-FW/eIF2-X-B-R (Table 1) for a 885 bp 3′-fragment. Amplification of both 5′- and 3′- DNA-fragments together resulted in mutated *AteIF2α(S56D)* cDNA (Fig 1). Mutated *AteIF2α(S56A)* cDNA (encoding an alanine instead of serine residue at position 56) was obtained in the same way using primers eIF2-A-FW and eIF2-A-Rev (Table 1) instead of eIF2-D-FW and eIF2-D-Rev, respectively.

2.3. 'Deconstructed vector' for expression of AteIF2α cDNA in plants

To develop a vector based on the *Grapevine virus A* (GVA) genome for the transient expression of mutated *AteIF2α* cDNAs in plants, plasmids V2TaCPE, V2TaCPEgva and pCAMgva were used; these had been developed earlier [19]. A DNA fragment encoding 6His-tag formed by 6His-F and 6His-R oligonucleotides (Table 1) and phosphorylated by T4 polynucleotide kinase (Thermo Fisher) was inserted into 'V2TaCPE' plasmid digested with *Nde*I and treated with calf intestinal alkaline phosphatase (Thermo Fisher Scientific) resulting in V2Ta-6H-CPE plasmid. Amplified, mutated *AteIF2α(S56D)* and *AteIF2α(S56A)* cDNAs digested with *Nde*I and *Xho*I

were then ligated into V2Ta-6H-CPE vector digested by the same restriction enzymes, resulting in V2Ta-6HAteIF2α(S56D)-E and V2Ta-6HAteIF2α(S56A)-E plasmids, respectively. After that, the DNA-cassette consisting of S35-promoter and first ORFs of pCASSgva vector (developed by Dr. Galiakparov, N.; GenBank: AF007415.2) carrying the full GVA genome, was cloned into V2Ta-6HAteIF2α(S56D)-E and V2Ta-6HAteIF2α(S56A)-E plasmids at *Pst*I sites. Modified parts of the GVA genome were then inserted into the pCAMgva vector at *Aat*II and *Sal*I sites, resulting in pCAM2T-6HAteIF2α(S56D)-E (Fig. 1) and pCAM2T-6HAteIF2α(S56A)-E plasmids, respectively.

2.4. Agroinfection of plants

Co-agroinfection of *N. benthamiana* with the pCAMgva vector and plasmids pCAM2T-6HAteIF2α(S56D)-E or pCAM2T-6HAteIF2α(S56A)-E was performed as described [19]. To produce the soluble protein fraction, homogenates were centrifuged for 5 min at 4°C and 8000 × g. To obtain mitochondria-free cytoplasmic extracts (S23), the resulting supernatants were centrifuged for 20 min at 4°C and 23,000 × g as described earlier [16].

2.5. Purification of recombinant His-AteIF2α(S56) protein

Escherichia coli strain BL21(DE3) cells transformed with the pET19b-AteIF2α plasmid were grown in LB medium at 30°C to an A_{600} of 0.5. The expression of recombinant protein was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 4 h. Isolation of His-tagged *Ate*IF2α was performed by immobilized metal ion affinity chromatography using PerfectPro Ni–NTA resin suspension (5-Prime) according to manufacturer's instructions.

2.6. Western blotting analysis

Proteins were separated by 12.5% SDS-PAGE according to Laemmli [20]. The separated proteins were transferred to a nitrocellulose membrane (GVS) or stained in PageBlue Protein Staining Solution (Thermo Fisher Scientific). For immunodetection of His-tagged proteins, membranes were probed using Penta-His mouse antibody (5-Prime, 1:2,000 dilution). After incubation with a horseradish peroxidase-coupled anti-mouse secondary antibody (Santa Cruz, 1:2,000 dilution), immunoblots were developed by using chemiluminescent peroxidase substrate-3 detection reagents (Sigma Chemical Co.). The phospho-eIF2α(S51) antibody against human phosphorylated eIF2α (Cell Signaling Technology, 1:1000) and a horseradish peroxidase-

coupled anti-rabbit secondary antibody (ECL, 1:2000 dilution) were used for the detection of phosphorylated *Ta*eIF2(αP).

2.7. Densitometry analysis

Optical densitometry analysis of the bands and analyses of polyribosome and ribosome profiles were done using the ImageJ 1.42q software (https://imagej.nih.gov/ij/). The obtained semi-quantitate data were statistically processed. Student's t-test was utilized to evaluate the mean values differences; p-values below 0.05 were considered statistically significant.

2.8. Sedimentation analysis of polysomes

Reaction mixtures after translation *in vitro* or S23 extracts from *N. benthamiana* were layered (10 A_{254} units per tube) onto linear 10% - 50% sucrose density gradients prepared in buffer A (20 mM Tris(Ac), pH 7.6; 90 mM K(Ac), 2.5 mM $Mg(Ac)_2$, 1.6 mM DTT) and centrifuged at 4°C for 3 h at 35000 rpm in a Beckman SW41 rotor. Fractions were collected from the bottom of the tube and the absorbance at 254 nm was monitored in a continuous flow.

2.9. Plasmid construction for in vitro *experiments*

All constructs for *in vitro* experiments were based on the `pl-GUS' plasmid, encoding bacterial β-glucuronidase (GUS) [21], which was kindly provided by Dr D.R. Gallie. Y-GUS, **Ω**-GUS and (3xARC1)-GUS DNA constructs have been described previously [22]. DNA constructs encoding short mRNAs containing a 'Strepto-tag' sequence in their 3′-UTRs were constructed using these plasmids by overlapping polymerase chain reaction (PCR) using primers: T7-FW/GUS-Kpn-Rev (Table 1) for a 204-310 nt 5′-fragments, and Strep-Kpn-FW/Strep-EcoR-R (Table 1) for a 66 nt 3′-fragment. Amplification of both 5′- and 3′- DNA-fragments together resulted in 254-363 nt PCR-products that were digested with *Hin*dIII and *Eco*RI and ligated into pl-GUS plasmid digested by the same restriction enzymes resulted in T7-5′PVY-ORF_{87}-Strep, T7-5′**Ω**-ORF_{87}-Strep and T7-5′(3xARC1)-ORF_{87}-Strep DNA-constructs.

2.10. RNA synthesis and translation in vitro

T7-promoter containing plasmids were linearized at an *Eco*RI site, and uncapped synthetic mRNAs were *in vitro* transcribed using T7 RNA-polymerase (Thermo Fisher Scientific) according to manufacturer's instructions. Extract S23 from wheat embryos was obtained as described

previously [23]. The reaction mixture for *in vitro* translation contained: 1 × buffer A (20 mM Tris(Ac), pH 7.6, 90 mM K(Ac), 2.5 mM $Mg(Ac)_2$, 1.6 mM DTT); 1 mM ATP; 0.2 mM GTP; 10 mM creatine phosphate; 0.12 mg/ml creatine phosphokinase; 0.1 mM spermine; 0.1 mM of amino acids; 35% (v/v) of WG S23 extract. To activate the *Ta*GCN2-kinase contained in S23 extracts, L-histidinol (HisOH) was added to the reaction mixture in final concentration of 2 mM, followed by 30 min pre-incubation at 26°C prior to addition of mRNA [16]. The control reactions were similarly pre-incubated without addition of HisOH. After supplementing 2 µg of transcribed mRNA (Table 2), the translation mixtures were further incubated for 1 hour at 26°C.

2.11. Isolation of 48S preinitiation complexes

Dihydrostreptomycin attachment to epoxy-activated Sepharose 6B was performed according to the described procedure [24]. Isolation of 48S preinitiation complexes (48S PICs) was carried out by the method of Lokker [25] with slight modifications. Translational mixtures (500 µl) containing 180 µl of S23 WG extract, 1 mM guanosine 5′-[β,γ-imido]triphosphate (GMP-PNP, a non-hydrolyzable analog of GTP), 2 mM L-histidinol and 0.08 mg/ml 'Strepto-tag'-containing mRNAs were incubated for 30 min at 26°C, chilled on ice and passed at 4°C through a streptomycin-Sepharose 6B column, equilibrated with the Strepto-tag buffer (20 mM Tris(Ac), pH 7.6, 100 mM K(Ac), 2.5 mM $Mg(Ac)_2$, 5% glycerol, 5 mM DTT). The column was washed with three volumes of the same buffer at 4°C. Elution of 48S PICs was carried out with Strepto-tag buffer, containing 20 µM streptomycin at 26ºC. The eluate was collected and precipitated with three volumes of ethanol, followed by centrifugation at 24000 × g for 40 minutes.

3. Results

3.1. Expression of mutated AteIF2α cDNA in N. benthamiana

To study the role of peIF2α phosphorylation *in vivo*, the *Arabidopsis thaliana* cDNA, *AteIF2α,* was cloned and overlapping PCR used to generate two mutation variants of the phosphorylation site (serine-56). These were *AteIF2α(S56D),* encoding a phosphomimic form of the protein in which the serine residue at position 56 was replaced with an aspartic acid residue; and *AteIF2α(S56A),* encoding a non-phosphorylatable form of the protein in which the same serine was replaced with an alanine. Nucleotide sequence analysis confirmed the fidelity of each eIF2α cDNA variant.

Plant viral expression vectors are advantageous for rapid functional characterization studies of genes due to their capability for rapid, high-level, transient expression of proteins [26]. For the transient expression of mutated *AteIF2α* cDNAs in plants, a 'deconstructed' vector based on the *Grapevine virus A* (GVA) genome was engineered so that the coat protein gene was replaced by the target cDNAs (Fig 1). The vector was designed so that the recombinant *At*eIF2α variants would be expressed with a His-tag. We have previously shown that vectors based on the GVA genome can be used for transient expression of heterologous proteins in agroinfected plants [19]. GVA-based vectors are particularly suitable because GVA cannot move between cells in its non-encapsidated form. Leaves of *N. benthamiana* were co-agroinfiltrated with the GVA-based binary vectors containing the mutated *AteIF2α* cDNAs along with the pCAMgva vector, which contains the full, unmodified GVA genome. The transcription of the target genes in the agroinfiltrated *N. benthamiana* plants was confirmed by RT-PCR analysis, and synthesis of the recombinant proteins was confirmed by western blot analysis using a His-tag-specific antibody. The His-*At*eIF2α(S56) protein was also expressed in *E. coli* and the purified protein was used as a control for assessing the level of *AteIF2α(S56A)* and *AteIF2α(S56D)* expression in *N. benthamiana.* This was achieved by semi-quantitative densitometry analysis of blots using the ImageJ program. The yield of *At*eIF2α(S56D) and *At*eIF2α(S56A) recombinant proteins was approximately 0.91% and 0.88%, respectively, of total soluble protein. Tobacco (*N. benthamiana*) was selected as the expression system because it has been shown to be suitable for the efficient transient expression of a variety of proteins, it is well suited for infiltration, especially by virus-based recombinant vectors, and it has lower natural protease activity than many other plants.

3.2. Involvement of phosphomimetic and non-phosphorylatable variants of plant eIF2 in the formation of polysomes

On the third day after agroinfiltration of *N. benthamiana* with pCAMgva-based vectors, pCAM2T-6HAteIF2α(S56D)-E or pCAM2T-6HAteIF2α(S56A)-E, the cytoplasmic extracts of tobacco leaves were fractionated in sucrose density gradients. Analysis of the polysome profiles demonstrated that expression of the phosphomimetic protein, *At*eIF2α(S56D), caused a slight reduction in the polysome/non-polysome (P/NP) ratio (Fig 2A). This ratio is derived by analyzing the sedimentation profile of polysomes, mono-ribosomes, and ribosomal subunits, calculating the area under the combined 40S + 60S + 80S peaks, compared with peaks of polysomes, and reflects the level of total protein synthesis at the moment [27]. Expression of the non-phosphorylatable protein, *At*eIF2α(S56A), on the other hand, resulted in a modest increase in the P/NP ratio compared to the control (Fig 2A, see Discussion). Student's t-test (n = 3)

revealed that the difference in the P/NP ratio was significant in both cases: $p < 0.05$ for *Ate*IF2α(S56A) and < 0.01 for *Ate*IF2α(S56D).

Sedimentation distribution of ribosome-containing structures and subsequent SDS-PAG electrophoresis and western-blot analysis of sucrose gradient fractions demonstrated that the recombinant *Ate*IF2α(S56A) and *Ate*IF2α(S56D) proteins were associated mainly with complexes containing the 40S ribosomal subunit (Fig 2B, 2C, tracks 8), suggesting their involvement in mRNA translation initiation. None of the recombinant proteins was present in the 60S-80S fractions (Fig 2B, 2C, tracks 6-7). At the same time, the initiation factor that contained non-phosphorylatable subunit *Ate*IF2α(S56A) was also detected in the polysomal fraction (albeit in a small amount: Fig 2B, right panel, tracks 1-4), while the factor containing the phosphomimetic subunit *Ate*IF2α(S56D) was not detected in association with polysomes (Fig 2C, right panel).

Using optical densitometry of blots with ImageJ 1.42q software, we performed a quantitative evaluation of the joint amount of non-phosphorylatable subunit AteIF2α(S56A) in the polysomal fractions (Fig 2B, tracks 1-4) with respect to that contained in the 40S complexes (track 8) and estimated it to be approximately 15%. It is noteworthy that both recombinant *Ate*IF2α(S56A) and *Ate*IF2α(S56D) proteins were not detected in ribosome-free fractions (Fig 2B, 2C, tracks 9) suggesting that they eventually incorporate into a hybrid factor peIF2(*At*-α, *Nb*-β, *Nb*-γ) that correctly functions in the formation of the ternary complex and in delivering it to the 40S ribosomal subunit.

It should be mentioned that the expression of recombinant α-subunit of plant factor *Ta*eIF2 from wheat germ has already been performed successfully in a very systematically distant heterologous system, mammalian BSC-40 cells [3]. Both native *Ta*eIF2α and its non-phosphorylatable variant *Ta*eIF2α(S51A) were correctly expressed from a vaccinia virus-based vector, incorporated into the composition of the host cellular eIF2, and performed physiological functions.

3.3. Effect of pGCN2-mediated peIF2α phosphorylation on polysome formation in vitro

Phosphorylation of peIF2α by the protein kinase pGCN2 has been reported in Arabidopsis plants subjected to amino acid deprivation [14]. The embryos of wheat (*Triticum aestivum*) (wheat germ, WG) are a well-known plant model system for *in vivo* and *in vitro* studies. Indeed, most plant translation factors were first isolated from WG-cytoplasmic extracts and their function and regulation were studied in the WG-cell-free system (WG-CFS). L-histidinol (HisOH), a competitive inhibitor of $tRNA^{His}$ charging, has been shown to induce pGCN2 activation in WG-CFS [16]. In the present study, L-histidinol was used to investigate the effect of *Ta*eIF2α

phosphorylation by activated *Ta*GCN2 on the formation of polysomes in WG-CFS. The addition of L-histidinol to the *in vitro* translation reaction did not lead to appreciable changes in the sedimentation profiles (Fig 3A): the polysome to non-polysome ratio (P/NP) was 0.638 ± 0.048 with activated pGCN2 versus 0.712 ± 0.014 in the control, with the difference being statistically insignificant ($p = 0.269817$). This substantially differs from the effect of eIF2α phosphorylation in mammalian, protozoan, and yeast cells, where phosphorylation of eIF2α by specific protein kinases leads to a rapid and deep suppression of protein synthesis [2, 7].

The amino acid sequences surrounding the target sites for phosphorylation by GCN2-kinases are very conservative in human *Hs*eIF2α (Ser51) and in wheat *Ta*eIF2α (Ser51) [28], so commercial monoclonal antibodies against the *Hs*eIF2α(P) are suitable for detection of wheat phosphorylated form, *Ta*eIF2(αP) [14-16]. Western-blot analysis confirmed that the addition of L-histidinol to a WG S23 extract prior to *in vitro* mRNA translation led to efficient peIF2α phosphorylation (Fig. 3B, track S23). It is appropriate to recall here that the phosphorylated form of *Ta*eIF2α is not detected at all in wheat germ extract until the addition of histidinol [16].

In mammalian systems, phosphorylated meIF2(αP) may accumulate on free 60S ribosomal subunits and on the 60S subunits in the composition of 80S ribosomes and subsequently be released by the action of the GDP→GTP exchange factor, meIF2B [29]. In our experiments, however, phosphorylated *Ta*eIF2(αP) accumulated mainly in the 40S fraction (Fig. 3B, track 8) and was not detected either in polysomes (Fig. 3B, tracks 1-5) or the 80S and 60S fractions (Fig. 3B, tracks 6-7). A small amount of *Ta*eIF2(αP) was located in ribosome-free fractions (Fig. 3B, tracks 9-10), where the factor may be present in its free form or in the composition of a ternary complex (TC) [GTP∗eIF2∗Met-tRNA$_i^{Met}$]. In the 40S fraction, the *Ta*eIF2(αP) may also be present in the TC as part of 43S and/or 48S preinitiation complexes, which are the intermediates of translation initiation. Anyway, the presence of *Ta*eIF2(αP) in complex with 40S subunits means that the plant factor is able to form the TC and function correctly in delivering TC to 40S subunits in the course of translation initiation even when phosphorylated at the α-subunit.

3.4. Analysis of TaeIF2 phosphorylation status in isolated 48S preinitiation complexes

The mRNA-containing 48S preinitiation complex (PIC) is the closest one to the 80S initiation complex, which forms when the start codon is recognized. This is accompanied by the eIF5-mediated hydrolysis of eIF2-bound GTP, with concomitant dissociation of [eIF2*GDP] and other factors (eIF5, eIF1, eIF3, eIF4A, eIF4G) and joining of the 60S ribosomal subunit. Finally, the hydrolysis of eIF5B-bound GTP occurs, and factors eIF1A and GDP-bound eIF5B are released from the assembled elongation-competent 80S ribosomes [2]. Replacing eIF2-bound GTP with

GMP-PNP, a non-hydrolyzable analog of GTP, stalls ribosome assembly at the 48S PIC stage by blocking GTP hydrolysis and 60S joining [24, 25].

By using mRNAs that contain a streptomycin-binding RNA-aptamer ('Strepto-tag') in the 3′-untranslated region (3′UTR), it is possible to isolate the formed 48S PICs by affinity chromatography on Streptomycin-Sepharose and to study their composition [24]. This method has been used successfully to isolate animal 48S PICs [25]. Such an approach permits the separation of 48S from 43S PICs, as well as from unbound ternary complexes, freeing peIF2 and enabling the analysis of whether phosphorylated peIF2 is able to participate throughout translation initiation.

For the formation of 48S PICs, three *in vitro*-transcribed uncapped reporter mRNAs containing various 5′-untranslated regions (5′UTRs) were used. All of them contained the same short open reading frame (ORF_{87}, encoding short polypeptide of 28 amino acids) derived from the 5′ coding region of the *uidA* gene, as well as the same 'Strepto-tag' sequence in their 3′UTRs (Strep), so differing only in their 5′UTRs (Table 2). The 5′UTRs were as follows: 5′UTR of Tobacco mosaic virus (TMV) genomic (g)RNA (5′Ω); 5′UTR of Potato virus Y gRNA (5′PVY), and artificial translation enhancer (3xARC1). All these 5′UTRs can provide efficient cap-independent translation initiation of the reporter mRNAs (22).

Each of the reporter mRNAs ("5′PVY-ORF_{87}-Strep", "5′Ω-ORF_{87}-Strep", or "5′(3xARC1)-ORF_{87}-Strep") was added to the WG-CFS in the presence of GMP-PNP and the histidinol that activates the endogenous protein kinase, *Ta*GCN2, as described [16]. Then 48S PICs were isolated using affinity chromatography on a streptomycin-Sepharose 6B column (Fig 4A). Figure 4B shows the electrophoregram of isolated 48S PIC (track 48S-i) formed with the use of "5′Ω-ORF_{87}-Strep" reporter mRNA. The western blot analysis using anti-*Hs*eIF2(αP) antibody demonstrates that a substantial amount of phosphorylated factor, *Ta*eIF2(αP), occurs in the composition of purified 48S PICs (Fig 4C, right panel, track 2). Essentially the same results were obtained when the "5′PVY-ORF_{87}-Strep" or "5′(3xARC1)-ORF_{87}-Strep" mRNAs were used for the formation of 48S PICs (not presented). The relatively large amount of *Ta*eIF2(αP) that occurs in the flow-through fractions (Fig 4C, right panel, track 1) compared to the affinity fractions eluted with the buffer containing 20 μM streptomycin (Fig 4C, right panel, track 2) indicates that a substantial amount of *Ta*eIF2(αP) in the reaction mixtures may be present in the form of 43S PICs, while some may exist in 40S-free form (see also Fig. 3B, tracks 9, 10). In addition, it is possible that the capacity of the streptomycin-Sepharose 6B column was insufficient, resulting in some 48S PICs being present in the flow-through fraction

4. Discussion

Repression of overall protein synthesis under stress conditions is an efficient means to conserve energy and resources, which are greatly consumed during the biogenesis of the translational apparatus and the process of mRNAs translation. However, mechanisms of global protein synthesis inhibition at the level of reversible phosphorylation of translation factors may be significantly different in plants and animals [2, 4, 10, 14, 16]. Indeed, the mammalian mechanism that regulates the availability of factor eIF4E through the phosphorylation of eIF4E-binding proteins (eIF4E-BPs) by TOR-kinase is not similarly reproduced in plants [2, 4]. Though several plant-specific eIF4E-interacting proteins were discovered and characterized recently, their structure and mode of functioning differ from that of eIF4E-BPs from mammalian cells [30, 31]. Also, a new mechanism of translation inhibition that is specific to plants has recently been discovered, which is mediated by phosphorylation of peIF4E and peIFiso4E by a different protein kinase, SnRK1 [27]. However, the universality of such a mechanism requires additional investigation because none of *At*eIF4E, *At*eIFiso4E or *At*eIFiso4G undergo any phosphorylation when plants are subjected to light (with a high level of protein synthesis) or dark (low level of protein synthesis) [32].

Additionally, the mechanism of translation suppression by eIF2 phosphorylation, which definitely operates in mammalian cells, apparently does not function in plants [10, 16]. Plants do contain one kind of protein kinase, pGCN2, that phosphorylates peIF2α; however, the *gcn2*-mutant of Arabidopsis lacking *At*GCN2 activity is quite viable, although it does demonstrate somewhat higher sensitivity to herbicides that disrupt the synthesis of amino acids and purines [14, 15]. Moreover, pGCN2 becomes activated only under certain types of abiotic stress: no phosphorylation of peIF2α has been observed when plants were subjected to heat shock [2, 16], osmotic or oxidative stress [15, 16], for example, despite the fact that the level of protein synthesis decreases significantly under these conditions [2, 4].

Concerning biotic stress, it has been shown previously that pGCN2-mediated peIF2 phosphorylation is induced by the presence of the whitefly *Bemisia tabaci* in tobacco plants [33], bacterial infection by *Pseudomonas syringae* in Arabidopsis [34], fungal infection by *Botrytis cinerea* spores, or just by the presence of chitin [35]. Chitin at a rather high concentration (400 mg/L) induced a moderate reduction in global protein synthesis [35] whereas heat shock (HS, 38 °C for 30 min) caused much stronger inhibition, other than for HS-proteins, despite HS not causing peIF2α phosphorylation [2, 16, 36].

There is some evidence that expression of the human gene encoding *Hs*PKR (another protein kinase that phosphorylates eIF2α) in transgenic tobacco plants leads to a reduction of viral symptoms during infection by plant RNA viruses, such as *Cucumber mosaic virus* and *Potato*

virus Y [37]. The authors of that study tentatively connected this reduction with the phosphorylation of peIF2α and the inhibition of general protein synthesis. However, later it was established that infection of Arabidopsis plants by different RNA viruses (*Turnip yellow mosaic virus* or *Turnip crinkle virus*) is not accompanied by activation of endogenous *At*GCN2 or *At*eIF2α phosphorylation [14]. Additionally, the intense artificial phosphorylation of wheat factor *Ta*eIF2α by either heterologous *Hs*PKR or homologous *Ta*GCN2 did not essentially inhibit translation of different mRNAs in WG-CFS [16].

So, many examples in the literature indicate that in plants an increase in peIF2 phosphorylation does not necessarily correlate with an inhibition of overall protein synthesis [2, 15, 16] and, *vice versa*, a strong decrease in protein synthesis may not be accompanied by peIF2 phosphorylation [2, 32, 36]. The decrease in general protein synthesis under different stress circumstances could be partially explained by other mechanisms, such as the activation of the mRNA degradation system in stress granules (P-bodies) that is triggered by, for example, heat shock, osmotic stress, viral and fungal infections, and prolonged deficiency of light [38, 39].

The data presented in this paper demonstrate that phosphorylation of *Ta*eIF2α *in vitro* by *Ta*GCN2 as a result of activation with histidinol is not accompanied by a decrease in the number of polysomes (Fig 3A) and the ratio of polysomes to non-polysomal material does not change significantly. Sedimentation distribution shows that factor *Ta*eIF2(αP) with phosphorylated α-subunit is present mainly in complex with the 40S subunit in the composition of 43S and 48S PICs (Fig 3B). This means that *Ta*eIF2(αP) is able to correctly form the ternary complex and then deliver it to 40S ribosomal subunit. The experiments with reporter mRNAs, which contained 'Strepto-tag' sequences in their 3′UTRs, demonstrate that *Ta*eIF2(αP) is competent to function throughout the formation of 48S PICs (Fig 4); i.e. till the last stages of translation initiation.

The notable result of this study is the modest increase of the proportion of polysomes versus non-polysomes during the expression in *N. benthamiana* of the transgene encoding the non-phosphorylatable variant, *At*eIF2α(S56A) (Fig 2A). This is unlikely to be associated with an increase in the overall amount of peIF2 in the transfected plant cells since the synthesis of the other two subunits (*Nb*eIF2β and *Nb*eIF2γ) of this factor is expected to remain unchanged. At the same time, the level of recombinant *At*eIF2α(S56A) subunit (approximately 1% of total soluble protein) compared with the maximal relative concentration of peIF2 in plant systems (~0.5% of total soluble protein in WG-extract [40]) means that a large amount of hybrid peIF2 containing the non-phosphorylatable recombinant α-subunit should have formed. Very likely, the inability of hybrid peIF2 to be phosphorylated caused the slight increase in the polysome pool that was observed.

Regarding the detection of *Ate*IF2α(S56A) subunit in the fraction of polysomes (Fig 2B), this event may have several explanations. For instance, the same mRNA that is being translated by 80S ribosomes can simultaneously associate at the 5′-end with the next 43S PICs that contain *Ate*IF2α(S56A). The circumstance that the phosphomimetic subunit, *Ate*IF2α(S56D), which differs by just a single substitution, was not found in the polysomes may suggest that 48S PICs containing phosphomimetic (or phosphorylated) peIF2α are not able to pass into the elongation stage being selectively incorporated into stress granules. If so, this should be accompanied by a large decrease in the amount of polysome and the extent of protein synthesis. However, this explanation seems unlikely since we did not observe a considerable decrease of polysomes either in plants expressing *Ate*IF2α(S56D) (Fig 2A) or in WG-CFS with *Ta*eIF2α(P) that was intensely phosphorylated by histidinol-activated *Ta*GCN2 (Fig 3A) [16].

Another explanation for the association of recombinant *Ate*IF2α(S56A) subunit with polysomes is based on the long-known fact that unphosphorylated mammalian factor meIF2 can physiologically bind to 60S subunits in the composition of polyribosomes [29]. After phosphorylation of meIF2α in hemin-supplemented lysate of rabbit reticulocytes and disaggregation of polyribosomes, phosphorylated factor meIF2(αP) may accumulate on free 60S subunits and 80S ribosomes [17, 29]. However, in our experiments, we never observed the association of non-phosphorylatable, phosphomimetic or phosphorylated forms of peIF2 with free 60S subunits or 80S ribosomes (Fig 2, 3).

A more appropriate explanation of the polysomal association of hybrid peIF2 with the *Ate*IF2α(S56A) subunit (and possibly with unphosphorylated *Nb*eIF2) involves the participation of a new plant factor: reinitiation stimulating protein (RISP) [41]. RISP may bind both the 60S ribosomal subunit *via* the ribosomal protein (rp) eL24 and the 40S subunit *via* connection with rp eS6, as well as the initiation factors peIF2 (*via* β-subunit) and peIF3 (*via* α-subunit) [42], so opening up the possibility of retention of initiation factors on translating ribosomes even after completion of the initiation stage. Such retention may promote reinitiation of mRNA translation. Possibly, after phosphomimetic substitution (or phosphorylation) of peIF2α, this factor may lose connection with RISP and hence the ability to associate with polysomes. A possible consequence of this may be that plant eIF2 with phosphorylated (or phosphomimetically substituted) α-subunit becomes unable to support translation reinitiation while still being able to function in the course of 48S PIC formation on the first start codon of mRNA.

In conclusion, we have shown that the replacement of the serine-56 residue with a glutamic acid residue abolished the ability of *Ate*IF2α to associate with polysomes when expressed in tobacco (*N. benthamiana*), leading to a slight but statistically significant reduction of polysome abundance. Replacement of the same residue with an alanine residue led to a slight increase in

polysome formation, and *At*eIF2α(S56A) was able to associate with polysomes. Histidinol-induced phosphorylation of *Ta*eIF2α in WG-CFS did not cause a significant decrease in the number of polysomes. Moreover, unlike mammalian eIF2(αP) [17, 29], the phosphorylated *Ta*eIF2(αP) of wheat was not detected in 60S, 80S, or polysomal fractions, but was present in 43-48S PICs.

We did not observe a strong suppression of mRNA translation in plants in response to phosphorylation of plant eIF2, either *in vivo* (Fig 2) or *in vitro* (Fig 3). In agreement with this, it was found recently using *A. thaliana* mutants that, while *At*GCN2 does mediate phosphorylation of *At*eIF2α, the formation of *At*eIF2(αP) is not linked to general protein synthesis arrest [43]. Moreover, in spite of *At*eIF2α phosphorylation being detected in Arabidopsis under both salt and cold stress (obviously *via At*GCN2 activation), no significant decline of polysome content in wild-type or *gcn2* mutant plants was observed [44]. These data completely agree with our results and once again indicate that phosphorylation of peIF2α in plants does not necessarily cause the inhibition of global protein synthesis. Similarly, phosphorylation of *Ta*eIF2α has very little effect on mRNA translation in the WG-CFS [16]. Therefore, in plants, the phosphorylation of eIF2α may be involved in regulating the translation of certain mRNAs rather than the global, profound suppression of protein synthesis seen in yeast and animal cells.

Abbreviations

eIF2, eukaryotic initiation factor 2; meIF2, mammalian eIF2; peIF2, plant eIF2; TC, ternary complex [GTP∗eIF2∗Met-tRNA$_i^{Met}$]; *Ta*eIF2, eIF2 of wheat (*Triticum aestivum*); WGE, wheat germ cytoplasmic extract (= S23); WG-CFS, WG cell-free system; eIF2α, the α-subunit of eIF2; eIF2(αP), eIF2 phosphorylated in the α-subunit; eIF2α(P), phosphorylated α-subunit of eIF2; [eIF2∗GDP], binary complex of eIF2 and GDP; GCN2, general control nonderepressible 2 – kinase of eIF2α activated by deficiency of amino acids, nucleotides and some kinds of stress; PKR, eIF2α kinase activated by double-stranded (ds)RNA; PERK, PKR-like endoplasmic reticulum eIF2α kinase; HRI, heme-regulated inhibitor, eIF2α kinase activated by heme deficiency; HisOH, L-histidinol; GMP-PNP, a non-hydrolyzable analog of GTP; 43S-PIC, preinitiation complex, containing 40S ribosomal subunit, initiation factors eIF3, eIF1,eIF1A, TC, eIF5; 48S-PIC, containing 40S ribosomal subunit, initiation factors eIF3, eIF1,eIF1A, TC, eIF5, eIF4A, eIF4B, eIF4E (-iso4E), eIF4G (-iso4G), poly(A)-binding protein, mRNA; GVA, Grapevine virus A; ORF, open reading frame; 3′UTR, (5′UTR), 3′(5′)-untranslated region of mRNA; (Ac), acetate; BSA, bovine serum albumin; DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside.

Author Contributions

AVZ: conceptualization, investigation, engineering recombinant constructs, “Strepto-tag” experiments, writing original draft. GES: investigation, transient expression of mutated genes. ASN: *in vitro* transcription and translation of mRNAs. NNG, DAG: methodology, constructing viral vector. NSP: methodology, sucrose gradient analysis. NGH: conceptualization, writing review, editing. BKI: conceptualization, supervision, writing review, editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Scheme of 'deconstructed vector' construction for the expression of *AteIF2α(S56D)* cDNA in plants

Abbreviations: ORF1-5, open reading frames of *Grapevine virus A* genome; aCP, coat protein gene of *Apple chlorotic leafspot virus*; 35Sprom and 35Ster, the *Cauliflower mosaic virus* 35S RNA promoter and terminator of transcription, respectively; T7prom and T7ter, T7 bacteriophage promoter and terminator respectively; T2A, self-cleavage peptide from *Thosea assigna* 2A virus; E2A, self-cleavage peptide from *Equine rhinitis A* virus; 6His and 10His, sequences encoding 6 or 10 histidine residues, respectively.

Figure 2. Analysis of polysomes formation in *Nicotiana benthamiana* plants transfected with constructs encoding the phosphomimetic and non-phosphorylatable variants of AteIF2a subunit

(A) Sedimentation analysis in 10-50% sucrose gradient of ribosome-containing complexes of S23-extracts prepared from *N. benthamiana* that were subjected to agroinfection with pCAMgva vector ('Control', red line), pCAM2T-6HAteIF2α(S56A)-E (blue line) plasmid, and pCAM2T-6HAteIF2α(S56D)-E (green line) plasmid. Presented are absorbance profiles at 254 nm. Positions of 80S ribosomes, 60S- and 40S ribosomal subunits are indicated by vertical arrows. P/NP – polysome/non-polysome (80S+60S+40S) ratio (arithmetic mean ± standard error). Significant differences are indicated by * [$p < 0.05$] or ** [$p < 0.01$].

(**B**) Western-blot analysis after SDS-PAG-electrophoresis of gradient fractions with the non-phosphorylatable variant 6H-AteIF2α(S56A). (**C**) Western-blot analysis after SDS-PAG-electrophoresis of gradient fractions with the phosphomimetic variant 6H-AteIF2α(S56D). Left panels represent Ponso-S stained blot membranes; right panels – blot membranes developed using anti-His-tag antibodies. Tracks 1-9 correspond to fractions of sucrose gradients; track S23 – S23 extract (10 μg of protein); track Rs – the material of combined non-polysomal fractions (prepared separately); M – marker proteins. The horizontal arrows on the right indicate the positions of His-tagged *At*eIF2α polypeptides (~ 42 kDa).

Figure 3. The effect of *Ta*eIF2*α* phosphorylation by endogenous *Ta*GCN2 kinase activated by L-histidinol on polysomes formation *in vitro*

(**A**) Sedimentation analysis in 10-50% sucrose gradient of ribosome-containing complexes formed in WG-CFS. Reaction mixtures were pre-incubated for 30 min at 26º C in the absence ('Control', red line) or presence ('Histidinol', black line) of 2 mM L-histidinol. Then mRNA was

added and the translation reactions continued for 60 min. Presented are absorbance profiles at 254 nm. Positions of 80S ribosomes, 60S- and 40S subunits are indicated by vertical arrows. P/NP – polysome/non-polysome (80S+60S+40S) ratio (arithmetic mean ± standard error).
(**B**) Western-blot analysis after SDS-PAG-electrophoresis of gradient fractions of the histidinol-treated extract using antibody against phosphorylated human eIF2α (*Hs*eIF2α(P)). Upper panel represents Coomassie G-250 stained electrophoregram in 12.5% SDS-PAG. Lower panel – western blot membrane probed by anti-*Hs*eIF2α(P) antibody. Tracks 1-10 correspond to fractions of sucrose gradients; track S23 – wheat germ S23 extract (10 μg of total protein); M – marker proteins with known molecular masses that are indicated on the left. The position of phosphorylated *Ta*eIF2α (~ 42 kDa) is indicated on the right.

Figure 4. Phosphorylated *Ta*eIF2*α* can participate in the formation of 48S preinitiation complexes formed in wheat germ cell-free system

(**A**) Chromatographic profile of 48S preinitiation complexes (48S PICs) assembled in WG-CFS applied to a streptomycin affinity column. Plot of absorbance at 254 nm is shown.
(**B**) Analysis of isolated 48S PICs in 1% agarose gel stained with ethidium bromide. Tracks: L – DNA-ladder; S23 – reaction mixture for *in vitro* "5′Ω-ORF_{87}-Strep" reporter mRNA translation in WG-CFS that contained 1 mM GMP-PNP and 2 mM L-histidinol before passing through a column with streptomycin-sepharose 6B; 40S-i – isolated wheat germ 40S ribosomal subunits; 60S-i – isolated wheat germ 60S ribosomal subunits; 48S-i – wheat germ 48S PICs isolated by affinity chromatography on streptomycin-sepharose 6B column.
(**C**) Western-blot analysis after SDS-PAG electrophoresis of proteins contained in isolated 48S PICs using anti-*Hs*eIF2α(P) antibody (right panel) and the related fragment of blot-membrane stained with Ponso-S (left panel).
Tracks: M – marker proteins; 1 – flow-through fraction from the streptomycin-Sepharose 6B column; 2 – affinity-purified wheat germ 48S PICs eluted from the column with the buffer containing 20 μM streptomycin. The position of phosphorylated *Ta*eIF2α (~ 42 kDa) is indicated on the right where arrows point to non-specific bands in the flow-through fraction.

Table 1. Primers used in cloning and *in vitro* mutagenesis

Primer name	**Nucleotide sequence (5′→3′)***
T7-FW	CGTAATACGACTCACTATAG
GUS-Kpn-Rev	TGCGATCCGGTACCTCAACAGTTTTCGCGATCCAGA
Strep-Kpn-FW	TTGAGGTACCGGATCGCATTTGGACTTCTGCCCAGGGTGGCA
Strep-EcoR-R	TGCGATCCGGTACCTCAACAGTTTTCGCGATCCAGA
eIF2_Nde_F	AATCATATGGCGAATCCTGCTCCGAATCTAGAATGTCGT
eIF2-X-B-R	TGCGGATCCTACTCGAGTATCCCGCTACCTCCATCGATATCGA
eIF2-D-FW	CTCCGAGCTC**GAT**CGCCGTCGGATTGGTAGTAT
eIF2-D-Rev	CCGACGGCG**AT**CGAGCTCGGAGAACAGGATCATT
eIF2-A-FW	CTCCGAGCTC**GCG**CGCCGTCGGATTGGTAGTAT
eIF2-A-Rev	CCGACGGCG**CG**CGAGCTCGGAGAACAGGATCATT
6His-F	TATCCACCACCACCACCACCA
6His-R	TATGGTGGTGGTGGTGGTGGA
*Mutated nucleotides are shown in bold	

Table 2. Segments of 'Strepto-tag' - containing mRNAs

Reporter mRNAs	5′UTRs	ORF_{87} [d]	3′UTR[e]
5′PVY-ORF_{87}-Strep	(5′)GCCCUAAGCUUAAUUAAAACAACUCAAUACAACAUAAGAAAAACAACGCAAAAACACUCAUAAACGCUCAUUCUCACUCAAGCAACUUGCUAAGUUUCAGUUUAAAUCAUUUCCUUGCAAUUCUCUAGAACAAUAUUGGAAACCAUUUCAACUCAACAAGCAAUUUCAUCACUUCCAACCAAUUUCAGAUCCACC-[a]	AUGGUACGUCCUGUAGAAACCCCAACCCGUGAAAUCAAAAAACUCGAC	GGUACCGGAUCGGCAUUUGGACUUCUGCCCAGGGUG
(3xARC1)-ORF_{87}-Strep	(5′)GCCCUAAGCUUACAAAUACUCCCCCACAACAGCUUACAAAUACUCCCCCACACAGCUUACAAAUACUCCCCCACAAGCUUGUCGACC-[b]	GGCCUGUGGGCAUUCAGUCUG	GCACCACGGUCGGAU
5′Ω-ORF_{87}-Strep	(5′)GCCCUAAGCUUCAUUUUUACAACAAUUACCAACAACAACAAACAACAAACAACAAACAACAUUACAAUUACUAUUUACAACAAGAGUCGACC-[c]	GAUCGCGAAAACUGUUGA-	CCG(3')

[a] The leader sequence of PVY gRNA is underlined with a dashed line.

[b] The 3xARC1 sequence is underlined with dotted line.

[c] The leader sequence of TMV gRNA is underlined with a double line.

[d] Nucleotides corresponding to start and stop codons in ORF_{87} are highlighted with gray.

[e] 'Strepto-tag' sequence is underlined with a solid line.

Fig. 1.

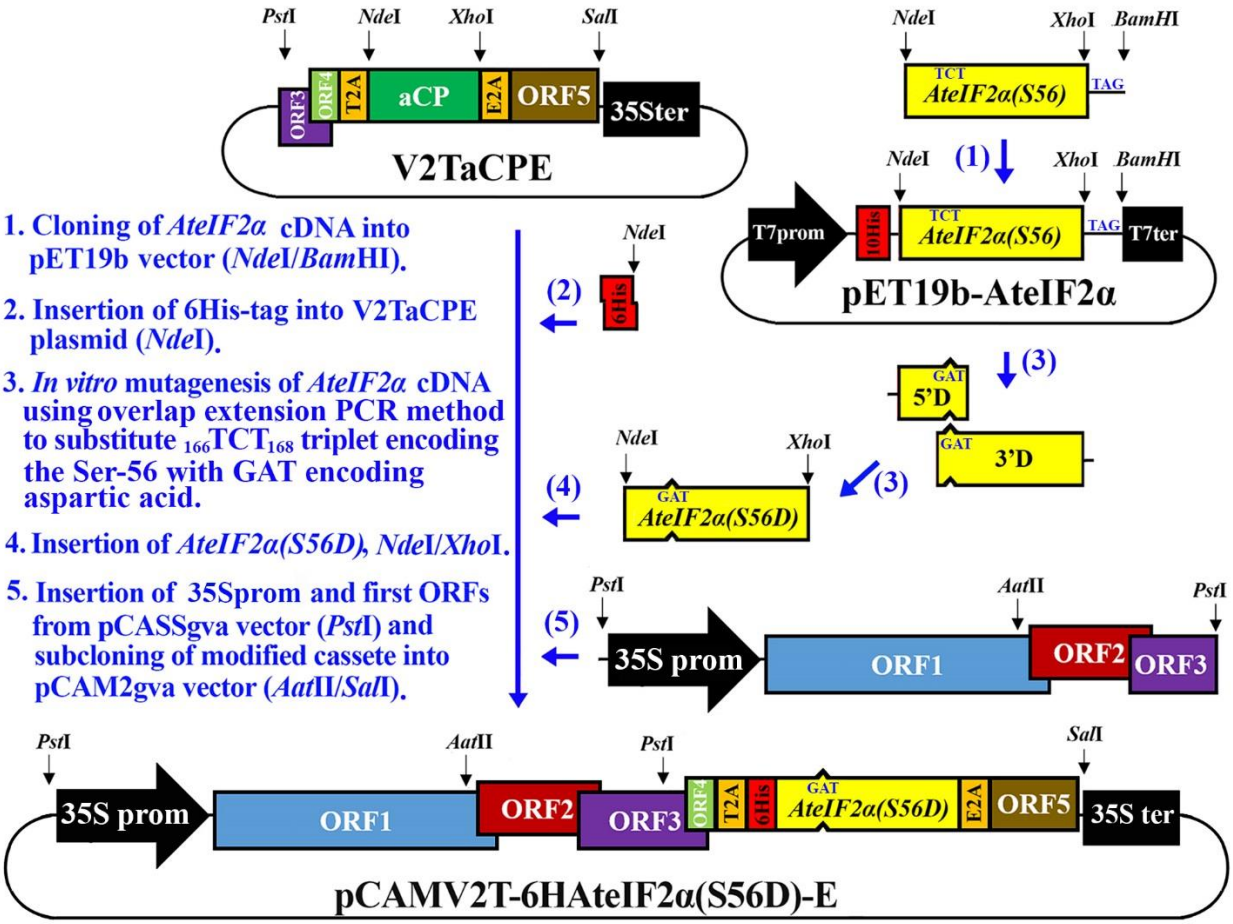

PstI
NdeI
XhoI
SalI
ORF3
ORF4
T2A
aCP
E2A
ORF5
35Ster
V2TaCPE
NdeI
XhoI
BamHI
TCT
AteIF2α(S56)
TAG
(1)
T7prom
10His
T7ter
pET19b-AteIF2α
1. Cloning of AteIF2α cDNA into pET19b vector (NdeI/BamHI).
2. Insertion of 6His-tag into V2TaCPE plasmid (NdeI).
3. In vitro mutagenesis of AteIF2α cDNA using overlap extension PCR method to substitute 166TCT168 triplet encoding the Ser-56 with GAT encoding aspartic acid.
4. Insertion of AteIF2α(S56D), NdeI/XhoI.
5. Insertion of 35Sprom and first ORFs from pCASSgva vector (PstI) and subcloning of modified cassete into pCAM2gva vector (AatII/SalI).
(2)
6His
(3)
GAT
5'D
3'D
(4)
AteIF2α(S56D)
(5)
AatII
35S prom
ORF1
ORF2
ORF3
6His
E2A
35S ter
pCAMV2T-6HAteIF2α(S56D)-E

Fig. 2.

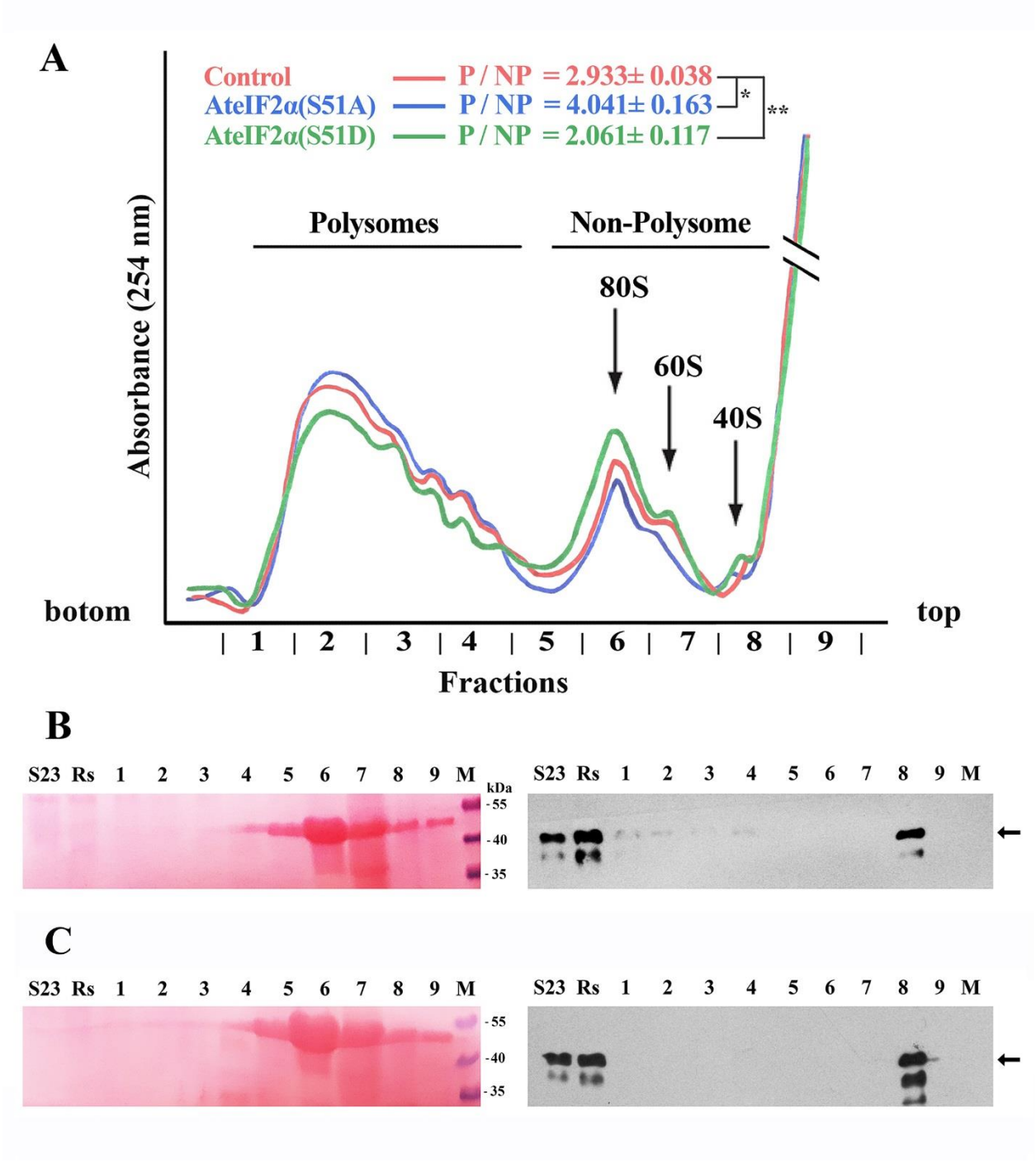

A
Control
AteIF2α(S51A)
AteIF2α(S51D)
P / NP = 2.933± 0.038
P / NP = 4.041± 0.163
P / NP = 2.061± 0.117
*
**
Polysomes
Non-Polysome
80S
60S
40S
Absorbance (254 nm)
botom
top
1 2 3 4 5 6 7 8 9
Fractions
B
S23 Rs 1 2 3 4 5 6 7 8 9 M
kDa
55
40
35
S23 Rs 1 2 3 4 5 6 7 8 9 M
C
S23 Rs 1 2 3 4 5 6 7 8 9 M
55
40
35
S23 Rs 1 2 3 4 5 6 7 8 9 M

Fig. 3

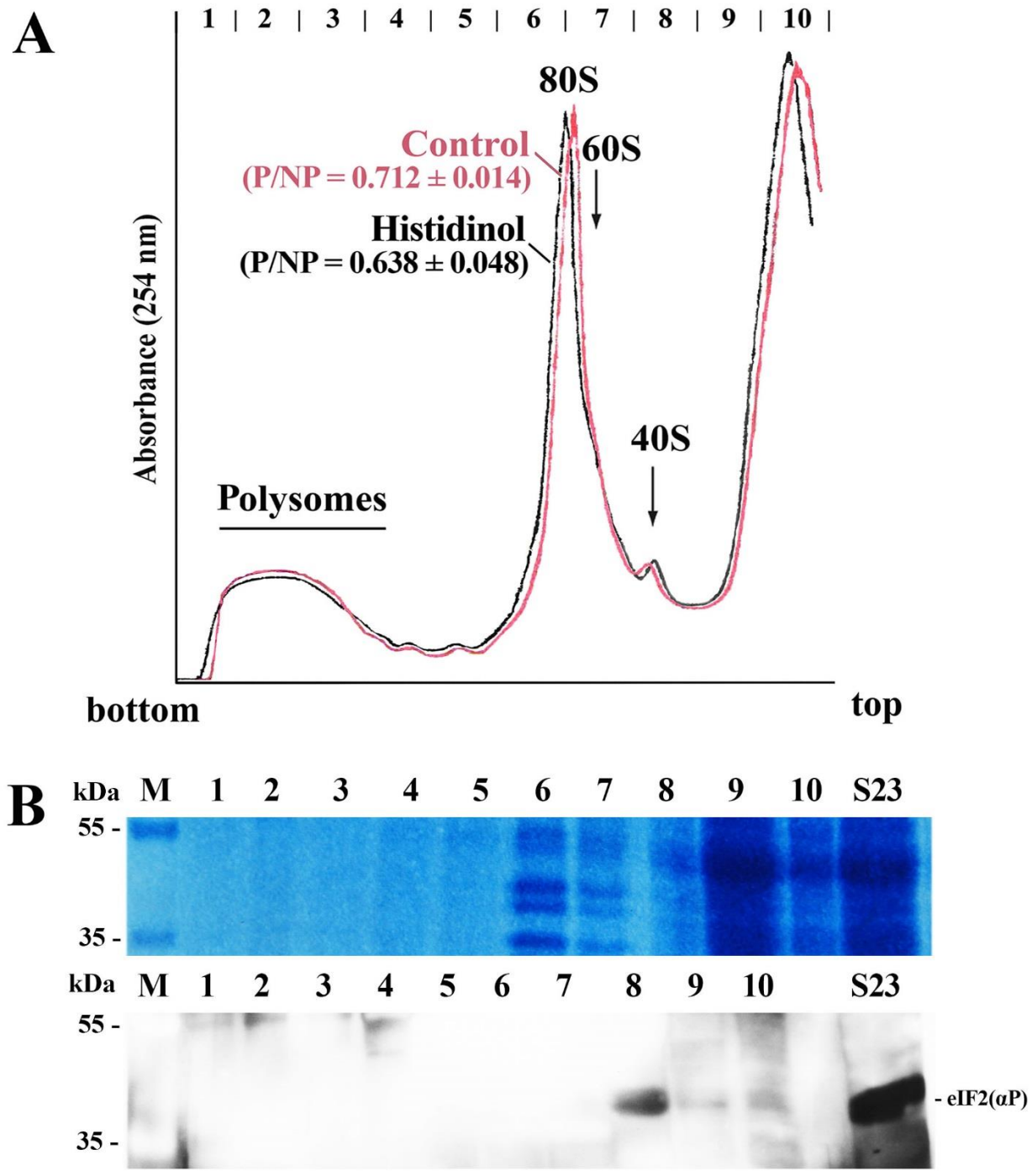
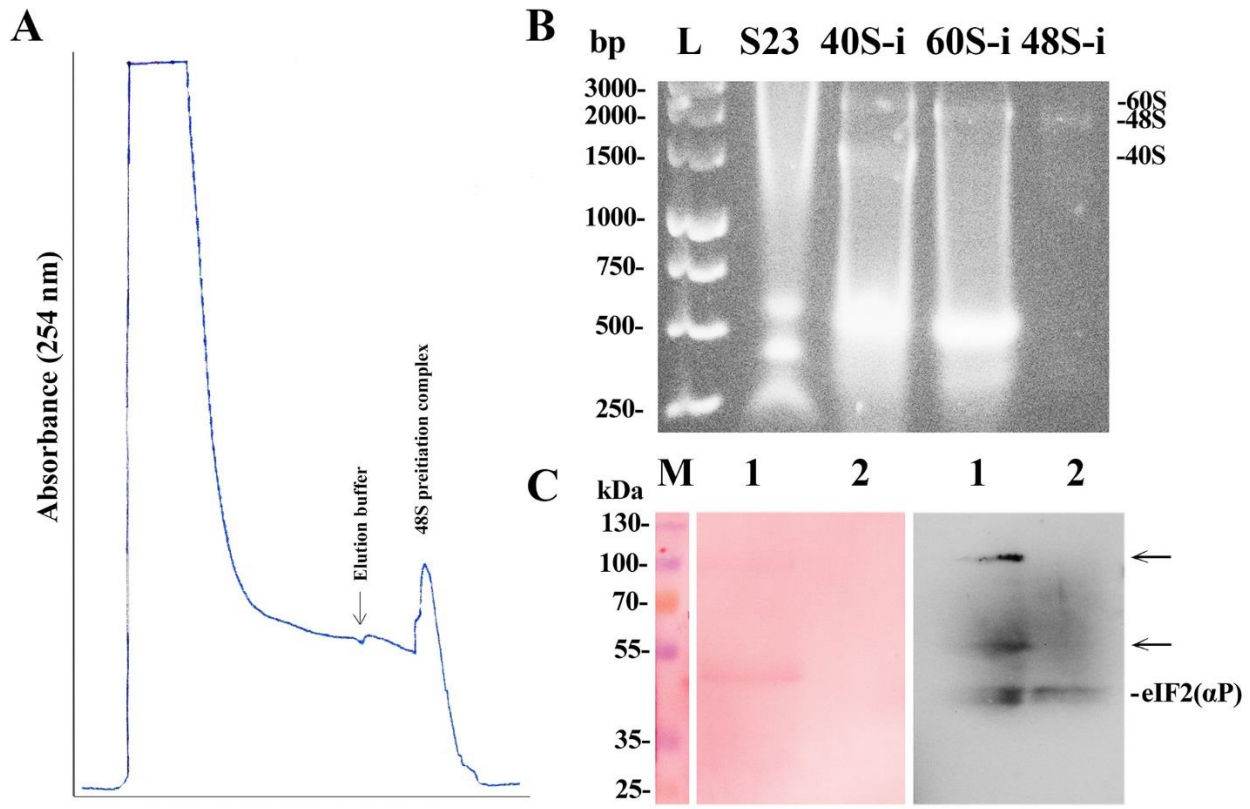

A
1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
80S
Control
(P/NP = 0.712 ± 0.014)
60S
Histidinol
(P/NP = 0.638 ± 0.048)
40S
Polysomes
Absorbance (254 nm)
bottom
top
B
kDa M 1 2 3 4 5 6 7 8 9 10 S23
55 -
35 -
kDa M 1 2 3 4 5 6 7 8 9 10 S23
55 -
35 -
- eIF2(αP)

Fig. 4.


A
Absorbance (254 nm)
Elution buffer
48S preitiation complex
B
bp
L
S23
40S-i
60S-i
48S-i
3000-
2000-
1500-
1000-
750-
500-
250-
-60S
-48S
-40S
C
kDa
M
1
2
1
2
130-
100-
70-
55-
35-
25-
-eIF2(αP)