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Five eIF4E isoforms from *Arabidopsis thaliana* are characterized by distinct features of cap analogs binding

Anna Kropiwnicka [a,b], Krzysztof Kuchta [a,c], Maciej Lukaszewicz [b], Joanna Kowalska [b], Jacek Jemielity [b,c], Krzysztof Ginalski [c], Edward Darzynkiewicz [b,c], Joanna Zuberek [b,*]

[a] College of Inter-Faculty Individual Studies in Mathematics and Natural Sciences, University of Warsaw, Zwirki i Wigury 93, 02-089 Warsaw, Poland
[b] Division of Biophysics, Institute of Experimental Physics, Faculty of Physics, University of Warsaw, Zwirki i Wigury 93, 02-089 Warsaw, Poland
[c] Centre of New Technologies, University of Warsaw, Banacha 2c, 02-097 Warsaw, Poland

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ABSTRACT

The assembly of the ribosome on majority of eukaryotic mRNAs is initiated by the recruitment of eIF4E protein to the mRNA 5′ end cap structure. Flowering plants use two eIF4E isoforms, named eIF4E and eIF(iso)4E, as canonical translation initiation factors and possess a homolog of mammalian 4EHP (or eIF4E-2) termed nCBP. Plants from *Brassicaceae* family additionally conserve a close paralog of eIF4E which in *Arabidopsis thaliana* has two copies named eIF4E1b and eIF4E1c. In order to assess the efficiency of plant non-canonical (eIF4E1b/1c and nCBP) and canonical (eIF4E and eIF(iso)4E) eIF4E proteins to bind mRNAs we utilized fluorescence titrations to determine accurate binding affinities of five *A. thaliana* eIF4E isoforms for a series of cap analogs. We found that eIF4E binds cap analogs from 4-fold to 10-fold stronger than eIF(iso)4E, while binding affinities of nCBP and eIF(iso)4E are comparable. Furthermore, eIF4E1c interacts similarly strongly with the cap as eIF4E, but eIF4E1b binds cap analogs ca. 2-fold weaker than eIF4E1c, regardless of the 95% sequence identity between these two proteins. The use of differentially chemically modified cap analogs in binding studies and a detailed analysis of the obtained homology models gave us insight into the molecular characteristic of varying cap-binding abilities of *Arabidopsis* eIF4E isoforms.

1. Introduction

The recognition of the cap (m^7GpppN, where N is any nucleoside) present at the 5′ end of eukaryotic mRNAs by eIF4F complex is a key point of gene expression regulation at the level of translation initiation. The eIF4F cap-binding complex consists of eIF4E protein, which recognizes the cap, and the scaffolding protein eIF4G that binds other factors to recruit the ribosome to the mRNA. In mammals eIF4F complex additionally contains eIF4A helicase which unwinds the mRNA 5′ secondary structure [1].

In many organisms multiple eIF4E homologs have been identified, which are grouped into three classes [2]. Flowering plants conserve two canonical translation initiation factors: eIF4E and eIF(iso)4E, both belonging to class I, and one non-canonical class II isoform termed nCBP. eIF(iso)4E together with its counterpart eIF(iso)4G make up a plant-specific isozyme form of the eIF4F complex named eIF(iso)4F [3]. Available data (including differential expression patterns for eIF4E and eIF(iso)4E [4–7]) suggests some mRNAs are preferentially translated using either eIF4F or eIF(iso)4F [8–11], which could contribute to selective translation of transcripts during plant growth and development.

In certain plant species additional paralogs of eIF4E or eIF(iso)4E are present, such as a close homolog of eIF4E: eIF4E1b, which is conserved in *Brassicaceae* and in *Arabidopsis* has diverged into two copies termed eIF4E1b and eIF4E1c [12]. *Arabidopsis thaliana* eIF4E1b, eIF4E1c and nCBP are able to bind m^7GTP-Sepharose, interact with plant 4G-type isoforms, and to promote translation initiation in yeast (eIF4E1b and eIF4E1c) and/or in wheat germ extracts (eIF4E1b, eIF4E1c and nCBP) [12,13]. However, based on their low levels of expression they do not seem to function as general translation initiation factors. Instead, *eIF4E1b* gene up-regulation in pollen and developing embryos [12] implies its possible role in processes of reproduction. Similarly, the increased expression of nCBP in early stages of cell growth [5] provides evidence for its yet unknown specialized function.

In this study we examine the ability of *A. thaliana* eIF4E isoforms (AteIF4E, AteIF4E1b, AteIF4E1c, AteIF(iso)4E and AtnCBP) to bind cap analogs and discuss them in relation to 3D protein structures obtained with homology modeling.

* Corresponding author. Fax: +48 225540771.
E-mail address: jzuberek@biogeo.uw.edu.pl (J. Zuberek).

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2. Materials and methods

2.1. Protein structure homology modeling

Homologs of *A. thaliana* eIF4E proteins were identified with PSI-Blast [14] searches (*E*-value threshold of 0.005) performed against the NCBI nonredundant protein sequence database using sequences of AteIF4E, AteIF4E1b, AteIF4E1c, AteIF(iso)4E, AtnCBP and mouse eIF4E as queries. Multiple sequence alignment was derived using PCMA program [15] followed by some manual adjustments. Secondary structures were predicted with PSIPRED [16]. The 3D models of *Arabidopsis* eIF4Es were constructed with MODELLER [17] using the closest homologs of known structure as templates [18]: *Pisum sativum* eIF4E (PDB: 2WMC) and *Mus musculus* eIF4E (PDB: 1EJ1) for AteIF4E, AteIF4E1b, AteIF4E1c and AteIF(iso)4E; *Homo sapiens* 4EHP (PDB: 2JGB), *P. sativum* eIF4E (PDB: 2WMC) and *M. musculus* eIF4E (PDB: 1EJ1) for AtnCBP.

2.2. Protein expression and purification

A. thaliana eIF4E proteins (Fig. S1) were prepared as recombinant proteins without any affinity tags and purified without a contact with cap analogs as described previously [19]. In the case of purification of AteIF4E1b, AteIF4E1c and AtnCBP the protocol was modified in order to remove co-purifying nucleic acids using benzonase, silica and ion-exchange chromatography during purification. The presence of nucleic acids was detected by absorption (Fig. S2). Proteins concentration was determined by absorption using extinction coefficients calculated by ProtParam tool (ExPASy server).

2.3. Fluorescence titrations

Fluorescence time-synchronized titrations [20] were carried out on a spectrofluorometer LS-55 (Perkin Elmer Co.) at 20 ± 0.3 °C in 50 mM HEPES/KOH pH 7.2, 134.5 mM KCl, 0.5 mM EDTA, 1 mM DTT. Equilibrium association constants (K_{AS}) for complexes of eIF4E with structurally modified cap analogs (Table S1) were determined as described previously [20]. The fitting procedure employing the non-linear least-squared regression analysis was performed using ORIGIN 6.0 (Microcal Software). The final K_{AS} was calculated as a weighted average of 3–8 independent titrations.

3. Results

3.1. Structures of Arabidopsis eIF4E proteins predicted by homology modeling

Several structures of eIF4E from human, mouse, fruit fly, pea, wheat and yeast have been solved, most often with various cap analogs. The eIF4E-like fold is characterized by the presence of an eight-stranded antiparallel β-sheet backed by α-helices (with β1αβ2αβ3β4αβ5β6αβ7αβ8 topology) [21,22]. The interatomic contacts between eIF4E and cap analogs can be divided into three classes: (i) sandwiching of the alkylated base between two tryptophans (in class I eIF4E isoforms) or a tryptophan and a tyrosine (in class II), (ii) hydrogen bonds and van der Walls contacts with N(7)-methylguanosine and with the second nucleoside, (iii) direct interactions and water-mediated contacts with the phosphate chain [20–22].

We built 3D homology models of *Arabidopsis* eIF4E isoforms to investigate differences in their structures (Fig. 1) and ability to bind the cap considering the sequence diversity (Fig. 1D) and the variety of contacts between protein and ligand. All analyzed proteins show the conserved eIF4E-like fold and the cap-binding pocket characteristic of eIF4E class I [21] and II [23] proteins. The sequence/structural differences that may influence the interaction with the cap are discussed below in relation to obtained results from the binding studies with cap analogs.

3.2. eIF(iso)4E has the lowest binding affinity for the cap among class I Arabidopsis eIF4E isoforms

All *Arabidopsis* eIF4Es, similarly to the mouse protein, exhibit quenching of tryptophan fluorescence upon binding of a cap analog (Fig. 2) and their binding affinities for the cap are from one to two orders of magnitude lower than those of mouse eIF4E (Table 1). The highest cap binding affinities are for AteIF4E and surprisingly for the non-canonical eIF4E isoform AteIF4E1c, and are comparable to values obtained for the yeast protein (Zuberek and Stelmachowska, unpublished result). In spite of 95% amino acid sequence identity between AteIF4E1b and AteIF4E1c (Fig. 1D), almost all cap analogs, except m^7GMP, have association constants (K_{AS}) for AteIF4E1b ca. 2-fold lower than for AteIF4E1c. The majority of residues that differ between these two proteins are located within or near the cap-binding region (Fig. 1A). The main possible reason for the difference in their binding affinity may be the replacement of large hydrophobic Phe147 and Thr145 in AteIF4E1c with Met147 and Ser145 in AteIF4E1b, which could result in weaker stabilization of the conserved arginine (Arg188 in AteIF4E1b/AteIF4E1c, equivalent to Lys162 in mammalian eIF4E) that binds cap's β- and γ-phosphate groups. In addition, Thr184 in AteIF4E1c, that may form hydrogen bonding (i.e. through water molecules) with the γ-phosphate, is replaced by Ala184 in AteIF4E1b.

Further unexpectedly, the second canonical translation initiation factor, AteIF(iso)4E protein, binds all cap analogs from 4-fold to 10-fold weaker than AteIF4E (Table 1), Probably, the poorer cap-binding efficiency of AteIF(iso)4E is mainly caused by the shortening of the long loop (that contains a short α-helix) between β-strands β7 and β8 (Fig. 1B). This loop is longer by six amino acids in AteIF4E, AteIF4E1b and AteIF4E1c, and forms the edge of the cap binding cavity, taking part in tight binding of the sugar and β- and γ-phosphate moieties of cap analogs. For instance, AteIF(iso)4E lacks a conserved aspartate (Asp226 in AteIF4E) from this region that interacts with the ribose of N(7)-methylguanosine.

All analyzed class I plant eIF4E proteins are specific to N(7)-monomethylated cap analogs because they interact poorly with either GTP or $m_3^{2,2,7}$GTP (Table 1) and this result contradicts the one previously reported [24] (see Section 4). Similarly to mammalian eIF4E K_{AS} increases with the extension of the cap's phosphate chain, while the addition of a second nucleoside to m^7GTP has a negative effect on interaction with *Arabidopsis* eIF4E isoforms.

3.3. nCBP binds cap similarly weakly as eIF(iso)4E

Arabidopsis class II eIF4E isoform, nCBP protein, interacts weakly with the cap, with the K_{AS} for m^7GTP 8.5-fold lower than that of AteIF4E (Table 1). In contrast to Ruud et al. paper [13] we obtained binding affinities of AtnCBP for N(7)-methylated cap analogs similar to those observed for AteIF(iso)4E, with the differences not exceeding 1.5-fold, and to values reported for human 4EHP protein [25]. The most prominent difference that may be responsible for the poor binding of the cap by AtnCBP seems to be the lack of the conserved arginine (Arg183 in AteIF4E, equivalent to Lys162 in mammalian eIF4E), that may interact with the cap's phosphate moiety, which is substituted in AtnCBP with isoleucine (Ile166, conserved in close homologs) (Fig. 1C). The lack of arginine in this position can be partially compensated in AtnCBP by Arg125 and Lys202, which are replaced by uncharged amino acids in AteIF4E, AteIF4E1b and AteIF4E1c (i.e. Thr142 and Ile217 in AteIF4E, respectively). However, AteIF4E, AteIF4E1b and AteIF4E1c possess also other positively charged residues that may take part in the binding

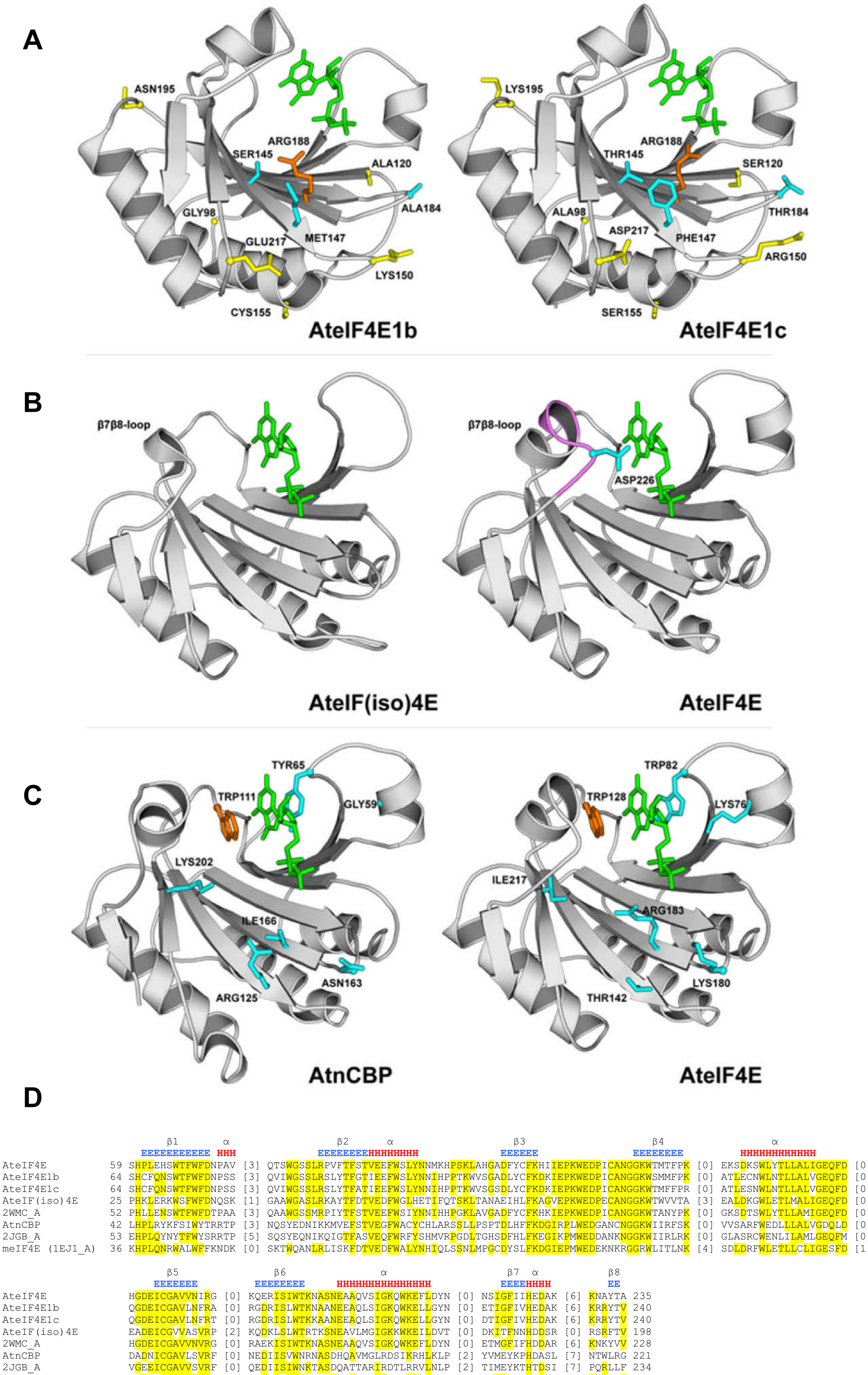
A
ASN195
ARG188
SER145
ALA120
ALA184
GLY98
MET147
GLU217
LYS150
CYS155
AteIF4E1b
LYS195
ARG188
THR145
SER120
THR184
ALA98
ASP217
PHE147
ARG150
SER155
AteIF4E1c
B
β7β8-loop
AteIF(iso)4E
β7β8-loop
ASP226
AteIF4E
C
TYR65
TRP111
GLY59
LYS202
ILE166
ASN163
ARG125
AtnCBP
TRP82
TRP128
LYS76
ILE217
ARG183
LYS180
THR142
AteIF4E
D
β1 α β2 α β3 β4 α
EEEEEEEEEEE HHH EEEEEEEEHHHHHHHH EEEEEE EEEEEEEE HHHHHHHHHHHH
AteIF4E 59 SHPLEHSWTFWFDNPAV [3] QTSWGSSLRPVFTFSTVEEFWSLYNNMKHPSKLAHGADFYCFKHIIEPKWEDPICANGGKWTMTFPK [0] EKSDKSWLYTLLALIGEQFD [0]
AteIF4E1b 64 SHCFQNSWTFWFDNPSS [3] QVIWGSSLRSLYTFGTIEEFWSLYNNIHPPTKWVSGADLYCFKDKIEPKWEDPICANGGKWSMMFPK [0] ATLECNWLNTLLALVGEQFD [0]
AteIF4E1c 64 SHCFQNSWTFWFDNPSS [3] QVIWGSSLRSLYTFATIEEFWSLYNNIHPPTKWVSGSDLYCFKDKIEPKWEDPICANGGKWTMFFPR [0] ATLESNWLNTLLALVGEQFD [0]
AteIF(iso)4E 25 PHKLERKWSFWFDNQSK [1] GAAWGASLRKAYTFDTVEDFWGLHETIFQTSKLTANAEIHLFKAGVEPKWEDPECANGGKWTWVVTA [3] EALDKGWLETLMALIGEQFD [0]
2WMC_A 52 PHLLENSWTFWFDTPAA [3] QAAWGSSMRPIYTFSTVEEFWSIYNNIHHPGKLAVGADFYCFKHKIEPKWEDPICANGGKWTANYPK [0] GKSDTSWLYTLLAMIGEQFD [0]
AtnCBP 42 LHPLRYKFSIWYTRRTP [3] NQSYEDNIKKMVEFSTVEGFWACYCHLARSSLLPSPTDLHFFKDGIRPLWEDGANCNGGKWIIRFSK [0] VVSARFWEDLLLALVGDQLD [0]
2JGB_A 53 EHPLQYNYTFWYSRRTP [5] SQSYEQNIKQIGTFASVEQFWRFYSHMVRPGDLTGHSDFHLFKEGIKPMWEDDANKNGGKWIIRLRK [0] GLASRCWENLILAMLGEQFM [0]
meIF4E (1EJ1_A) 36 KHPLQNRWALWFFKNDK [0] SKTWQANLRLISKFDTVEDFWALYNHIQLSSNLMPGCDYSLFKDGIEPMWEDEKNKRGGRWLITLNK [4] SDLDRFWLETLLCLIGESFD [1]
β5 β6 α β7 α β8
EEEEEEE EEEEEEEE HHHHHHHHHHHHHHH EEEEHHHH EE
AteIF4E HGDEICGAVVNIRG [0] KQERISIWTKNASNEAAQVSIGKQWKEFLDYN [0] NSIGFIIHEDAK [6] KNAYTA 235
AteIF4E1b QGDEICGAVLNFRA [0] RGDRISLWTKNAANEEAQLSIGKQWKELLGYN [0] ETIGFIVHEDAK [6] KRRYTV 240
AteIF4E1c QGDEICGAVLNFRT [0] RGDRISLWTKKAANEEAQLSIGKQWKELLGYN [0] DTIGFIVHEDAK [6] KRRYTV 240
AteIF(iso)4E EADEICGVVASVRP [2] KQDKLSLWTRTKSNEAVLMGIGKKWKEILDVT [0] DKITFNNHDDSR [0] RSRFTV 198
2WMC_A HGDEICGAVVNVRG [0] RAEKISIWTKNASNEAAQVSIGKQWKEFLDYN [0] ETMGFIFHDDAR [6] KNKYVV 228
AtnCBP DADNICGAVLSVRF [0] NEDIISVWNRNASDHQAVMGLRDSIKRHLKLP [2] YVMEYKPHDASL [7] NTWLRG 221
2JGB_A VGEEICGAVVSVRF [0] QEDIISIWNKTASDQATTARIRDTLRRVLNLP [2] TIMEYKTHTDSI [7] PQRLLF 234
meIF4E (1EJ1_A) YSDDVCGAVVNVRA [0] KGDKIAIWTTECENRDAVTHIGRVYKERLGLP [2] IVIGYQSHADTA [7] KNRFVV 217

Fig. 1. Comparison of *A. thaliana* eIF4E proteins. (A–C) Comparison of modeled structures. Cap analog (m^7GDP) taken from PDB: 1EJ1 structure (*M. musculus* eIF4E) is shown in green. Amino acids critical for the difference in the binding affinity for cap analogs are denoted in cyan. (A) All remaining residues that differ between AteIF4E1b and AteIF4E1c are presented in yellow. Conserved Arg188 is denoted in orange. (B) Insertion of six amino acids in the loop between β-strands β7 and β8 is denoted in violet. (C) Conserved tryptophan is colored orange. (D) Multiple sequence alignment for *A. thaliana* eIF4E isoforms and their closest homologs of known structure (pea eIF4E, PDB: 2WMC; mouse eIF4E, PDB: 1EJ1; human 4EHP, PDB: 2JGB). The numbers of excluded residues are specified in square brackets. Conserved residues (50% or greater) are highlighted in yellow. Locations of secondary structure elements (E, β-strand; H, α-helix) in PDB: 2WMC are marked above sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

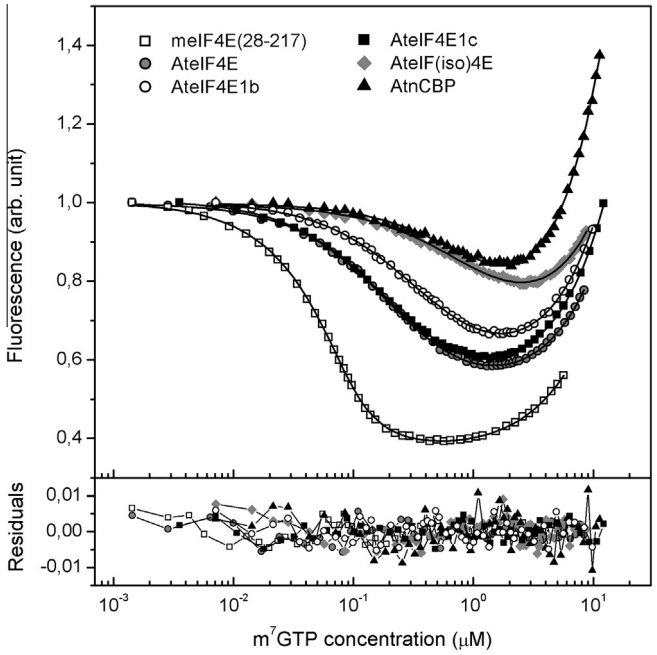

Fig. 2. Representative fluorescence titration curves for m^7GTP binding to *A. thaliana* eIF4E isoforms. Fitted curves (black lines) and experimental data points (upper panel) with fitting residuals (lower panel) for *A. thaliana* eIF4E (●), eIF4E1b (○), eIF4E1c (■), eIF(iso)4E (◆) and nCBP (▲) and mouse eIF4E (□). The increasing fluorescence signal at higher concentrations of m^7GTP originates from the fluorescence of a free cap analog in solution.

of cap's phosphate groups and which are not conserved in AtnCBP. This includes Lys76 and Lys180 in AteIF4E which are replaced in AtnCBP by Gly59 and Asn163.

Characteristic molecular feature of AtnCBP is its lower specificity toward monomethylation of guanosine at N(7), observed also for human 4EHP [25]. The difference between K_{AS} values for m^7GTP and GTP is only 10-fold for AtnCBP, whereas other plant proteins bind m^7GTP from 50-fold to 400-fold stronger than GTP (Table 1). This property is most likely not caused by the tryptophan-to-tyrosine substitution, that is specific to class II eIF4E proteins, at the residue involved in the sandwiching of the N(7)-methylguanine (Tyr65 in AtnCBP, equivalent to Trp82 in AteIF4E or Trp56 in mammalian eIF4E). Human eIF4E wild type and Trp56Tyr mutant proteins show similar cap-binding efficiencies [25].

3.4. Contribution of cap's phosphate groups into stabilizing the eIF4E-cap complex

Due to the location of basic amino acids at the entrance to the eIF4E cap-binding pocket, the more phosphate groups are linked to N(7)-methylguanosine, the tighter eIF4E binds the cap. When in the cap analog the phosphate chain is extended from one (m^7GMP) to four groups (m^7Gp_4), its binding affinity for mouse eIF4E increases 600-fold (Table 1). The changes in K_{AS} accompanying the lengthening of the cap's phosphate chain are less prominent for *Arabidopsis* eIF4E proteins. Yet, similarly to mouse eIF4E, the highest rise in the binding affinity is observed for the addition of the β-phosphate (m^7GMP → m^7GDP). Only AteIF4E1c binds the β-phosphate moiety comparably strongly as mouse eIF4E (20-fold increase in K_{AS}) while K_{AS} of AteIF4E and AteIF4E1b for m^7GDP are only 10-fold higher than for m^7GMP. However, the ca. −1.4 kcal/mol energetic gain of introducing the β-phosphate into the cap, evaluated as the change in the Gibbs free energy of binding ($\Delta\Delta G^o$, Table 2), suggests AteIF4E and AteIF4E1b, as well as AteIF4E1c, probably create two hydrogen bonds with the β-phosphate, similarly to mammalian eIF4E [22]. On the contrary, for AtnCBP the corresponding $\Delta\Delta G^o$ of ca. −0.5 kcal/mol is less than the value typical for a hydrogen bond [26], which indicates AtnCBP's interaction with the β-phosphate is probably indirect. In comparison, for human 4EHP the binding energy of the β-phosphate moiety equals ca. −0.7 kcal/mol [25] and corresponds to one direct hydrogen bond and one mediated by a water molecule [23]. The changes in the binding energy accompanying the extension of the cap's phosphate chain to a triphosphate (m^7GDP → m^7GTP) imply all *Arabidopsis* eIF4E isoforms except AteIF4E probably form one hydrogen bond with the cap's γ-phosphate group, similarly to mammalian eIF4E [22] and 4EHP [23]. AteIF4E interacts weakly with either γ- or δ-phosphate, while only for AtnCBP and mouse eIF4E the change in the binding energy which might correspond to the energy of forming one hydrogen bond with the δ-phosphate is observed.

3.5. Binding affinities of phosphorothioate cap analogs for Arabidopsis eIF4E isoforms

Previous studies investigated the properties of cap analogs in which one of non-bridging oxygens in the phosphate chain was substituted with a sulfur atom. It was found out these phosphorothioate cap analogs have strong affinity for mouse eIF4E and some of them are resistant to enzymatic cleavage by decapping enzymes (DcpS or Dcp2) [27,28]. Therefore, they act as strong inhibitors of translation and are able to increase stability and translational efficiency of transcripts [28–30]. In this work we examined the interaction of *Arabidopsis* eIF4E proteins with m^7GpppG analogs modified with the phosphorothioate moiety at the γ- or β-phosphate position, abbreviated m^7Gp_Sppg and m^7Gpp_SpG, respectively (Table 1). Due to a stereogenic P center in the phosphorothioate moiety each of the cap analogs exists as two diastereomers, termed D1 and D2. The presence of the phosphorothioate moiety at the γ-position in the diastereomer D1 (m^7Gp_Sppg (D1)) causes a 2-fold increase in K_{AS} for AteIF4E or AteIF(iso)4E,

Table 1
Equilibrium association constants (K_{AS}) of *A. thaliana* eIF4E proteins and mouse eIF4E in complex with cap analogs.

Cap analog	AteIF4E	AteIF4E1b	AteIF4E1c	AteIF(iso)4E	AtnCBP	meIF4E(28–217)
	K_{AS} (μM^{-1})					
m^7GMP	0.319 ± 0.016	0.120 ± 0.007	0.118 ± 0.003	<0.050	0.064 ± 0.004	0.82 ± 0.02
m^7GDP	3.54 ± 0.03	1.23 ± 0.03	2.47 ± 0.04	0.280 ± 0.019	0.148 ± 0.005	20.6 ± 1.9
m^7GTP	5.79 ± 0.13	3.12 ± 0.06	5.72 ± 0.10	1.07 ± 0.05	0.684 ± 0.014	81.5 ± 1.7
m^7Gp_4	6.9 ± 0.4	3.75 ± 0.07	9.02 ± 0.17	1.62 ± 0.04	1.81 ± 0.03	500 ± 30
m^7GpppG	2.08 ± 0.04	1.219 ± 0.012	2.48 ± 0.08	0.188 ± 0.011	0.263 ± 0.012	7.20 ± 0.18
m^7Gp_Sppg (D1)	4.69 ± 0.13	ND	ND	0.401 ± 0.008	0.229 ± 0.011	22.3 ± 0.4
m^7Gp_Sppg (D2)	2.29 ± 0.05	ND	ND	0.213 ± 0.007	0.295 ± 0.010	7.60 ± 0.19
m^7Gpp_SpG (D1)	2.7 ± 0.3	ND	ND	0.262 ± 0.007	0.350 ± 0.015	37.0 ± 1.7
m^7Gpp_SpG (D2)	2.68 ± 0.18	ND	ND	0.233 ± 0.003	0.326 ± 0.007	17.4 ± 1.1
GTP	<0.015	<0.053	<0.033	<0.015	0.079 ± 0.007	<0.030
$m_3^{2,2,7}$GTP	<0.040	<0.024	<0.038	<0.022	<0.050	ND

ND – not determined.

Table 2
Changes of standard Gibbs free energy ($\Delta\Delta G^o$) for the interaction of *A. thaliana* eIF4E isoforms with structurally modified cap analogs. $\Delta\Delta G^o$ values were calculated as the difference in the energy of binding (ΔG^o) of eIF4E to cap analogs which vary in single structural alteration. ΔG° values were obtained from K_{AS} values according to the standard equation: $\Delta G^\circ = -RT\ln K_{AS}$.

Modifications in cap structure	AteIF4E	AteIF4E1b	AteIF4E1c	AteIF(iso)4E	AtnCBP	meIF4E(28–217)
	$\Delta\Delta G^o$ (kcal/mol)					
Successive addition of the phosphate groups: $\Delta\Delta G^o = \Delta G^o(m^7Gp_{n+1}) - \Delta G^o(m^7Gp_n)$						
$m^7GMP \rightarrow m^7GDP$	−1.40 ± 0.03	−1.35 ± 0.05	−1.77 ± 0.02	>−1.0	−0.49 ± 0.06	−1.88 ± 0.07
$m^7GDP \rightarrow m^7GTP$	−0.287 ± 0.018	−0.54 ± 0.02	−0.49 ± 0.02	−0.78 ± 0.07	−0.89 ± 0.03	−0.80 ± 0.07
$m^7GTP \rightarrow m^7Gp_4$	−0.10 ± 0.05	−0.11 ± 0.02	−0.27 ± 0.02	−0.24 ± 0.04	−0.57 ± 0.02	−1.06 ± 0.04
Addition of the second nucleoside: $\Delta\Delta G^o = \Delta G^o(m^7Gp_nG) - \Delta G^o(m^7Gp_n)$						
$m^7GTP \rightarrow m^7GpppG$	0.60 ± 0.02	0.547 ± 0.017	0.49 ± 0.03	1.01 ± 0.06	0.56 ± 0.04	1.41 ± 0.03
Substitution of the phosphate moiety with the phosphorothioate: $\Delta\Delta G^o = (\Delta G^o(m^7Gp_SppG)$ or $\Delta G^o(m^7Gpp_SpG)) - \Delta G^o(m^7GpppG)$						
$m^7GpppG \rightarrow m^7Gp_SppG$ (D1)	−0.47 ± 0.03	ND	ND	−0.44 ± 0.05	0.08 ± 0.05	−0.66 ± 0.02
$m^7GpppG \rightarrow m^7Gp_SppG$ (D2)	−0.05 ± 0.02	ND	ND	−0.07 ± 0.05	−0.07 ± 0.05	−0.03 ± 0.03
$m^7GpppG \rightarrow m^7Gpp_SpG$ (D1)	−0.15 ± 0.08	ND	ND	−0.19 ± 0.05	−0.16 ± 0.05	−0.95 ± 0.04
$m^7GpppG \rightarrow m^7Gpp_SpG$ (D2)	−0.15 ± 0.05	ND	ND	−0.12 ± 0.04	−0.12 ± 0.04	−0.51 ± 0.05

ND – not determined.

while the diastereomer D2 is bound with a comparable affinity as the unmodified analog. No significant changes in K_{AS} are observed upon the oxygen-to-sulfur substitution at the γ-position for AtnCBP or at the β-position for any of *Arabidopsis* proteins. It was proposed that the increased polarizability of the phosphorothioate moiety, in comparison to the phosphate group, causes a shift of the negative charge from the sulfur to the oxygen. In consequence, when this oxygen is engaged in a hydrogen bond with the protein, i.e. for only one of diastereomers, the interaction is enhanced [27]. The lack of a significant increase in the binding affinity of AtnCBP for m^7Gp_SppG analogs suggests that oxygens from the γ-phosphate group of the cap (or α-phosphate in the mononucleotide cap) interact weakly with this protein, while AteIF4E and AteIF(iso)4E bind the γ-phosphate comparably strongly as mouse protein. The detected smaller changes in the binding affinity of AteIF4E, AteIF(iso)4E and AtnCBP for the cap upon its modification with the phosphorothioate at the β-position, in comparison to mouse eIF4E, are in accordance with the conclusion of a weaker interaction of these plant proteins with the cap's β-phosphate, which was based on the results for mononucleotide cap analogs.

4. Discussion

In this work we were able to successfully address in a quantitative manner the question of the ability of *A. thaliana* proteins from the eIF4E family to interact with the cap structure. Although there are reports of binding studies for plant eIF4Es with cap analogs or oligonucleotides [13,24,31], these studies are not free of methodological faults (discussed in [20]), the main one being the use of eIF4E protein samples which were contaminated with remnants of a cap analog applied during protein purification procedures. Here we obtained eIF4E samples free of any cap analog contaminants. In general, binding affinities of plant eIF4Es for the cap can be arranged in the rank of a diminishing affinity: AteIF4E ≈ AteIF4E1c > AteIF4E1b > AteIF(iso)4E ≈ AtnCBP. The result of a 4-fold to 10-fold difference in binding affinities between the two canonical translation initiation 4E-type factors, eIF4E and eIF(iso)4E, provokes an important question of how these two proteins are able to compete for the mRNA 5′-end *in vivo*. One plausible explanation is the reduction of the difference once these proteins are assembled into appropriate 4F-type cap-binding complexes, based on known data supporting the increase of the binding affinity for the cap of eIF4E upon interaction with eIF4G [32]. Moreover, eIF4G and eIF(iso)4G might vary in the extent of their influence on cap-binding because of an additional N-terminal domain in eIF4G which is not present in eIF(iso)4G [3]. Another explanation would be that other than cap specific elements of a transcript (such as secondary structure elements) could enhance the recruitment of either eIF4F or eIF(iso)4F to the mRNA.

Comparable binding affinities of AteIF4E and its closest two homologs: AteIF4E1b and AteIF4E1c, confirm AteIF4E1b and AteIF4E1c should be able to efficiently bind transcripts *in vivo*, which is in accordance with their ability to support translation in yeast [12]. Although AtnCBP binds cap analogs with similar strength as the second canonical factor, AteIF(iso)4E protein, and was shown to interact with eIF4G and eIF(iso)4G in wheat [13], in *Arabidopsis* it probably does not form a complex with eIF4G (Patrick and Browning, unpublished observation mentioned in [3]). Also AteIF4E1b and AteIF4E1c do not seem likely to form respective 4F-type complexes due to their very low affinity for eIF4G [12]. Therefore, it is more probable that eIF4E1b, eIF4E1c and nCBP interact with yet unknown mRNA-specific factors and function rather as translational regulators under specific developmental (i.e. the development of pollen and embryos for eIF4E1b [12]) or stress-related conditions, maybe in a manner similar to *Xenopus* oocyte-specific class I eIF4E1b [33], *Drosophila* testis-specific class I eIF4E3 [34], or mammalian and *Drosophila* 4EHP [35–39].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.032.

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