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Identification of Proteins from Cap-Binding Complexes by Mass Spectrometry During Maize (*Zea mays* L.) Germination

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Dedicated to Dr. Estela Sánchez de Jiménez for her invaluable contributions to plant biochemistry

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Abstract. This work describes the identification of components in the Cap-binding complexes in non-germinated and 24-h-imbibed seeds using mass spectrometry. This approach revealed new components particularly present in the non-germinated seed. Among these, two heat shock proteins, HSP101 and HSP70, were detected as well as several proteins involved in carbohydrate metabolism. Between the new components of maize Cap-binding complexes, several proteins contain a motif that identifies them as potential direct interactors with eIF4E or eIF(iso)4E.Together with the major abundance of eIF(iso)4E at this developmental stage, our findings indicate clear differences between the translation initiation complexes that are available for protein synthesis right upon water imibition and those that are present once germination has been completed.
Key words: Cap-binding proteins, germination, eIF4E-binding motif, translation, *zea mays* L.

Resumen. En el presente trabajo se describe la identificación de componentes de los complejos de unión a Cap obtenidos de semillas de maíz sin germinar y embebidas en agua por 24 h utilizando espectrometría de masas. Mediante este procedimiento se encontraron nuevos componentes en los complejos de unión a Cap, presentes particularmente en las semillas sin germinar. Entre estos, se detectaron dos proteínas de choque térmico o chaperonas, HSP101 y HSP70, así como varias proteínas involucradas en metabolismo de carbohidratos. Entre los nuevos componentes de complejos de unión a Cap en maíz, varias proteínas presentan motivos de aminoácidos que los identifican como interactores directos potenciales de las proteínas eIF4E y eIF (iso)4E. Estos hallazgos indican que los complejos de inicio de la traducción difieren entre el inicio y término de la germinación de semillas de maíz, tanto por la abundancia de eIF(iso)4E como por su composición proteica.
Palabras clave: Germinación, motivo de unión a EiF4E, proteínas de unión a cap, traducción, *zea mays* L.

Introduction

Translation of mRNAs in eukaryotes initiates through their 5' end Cap structure (7mGpppN, where N is any nucleotide). Eukaryotic translation initiation factor (eIF) 4E of 25 kDa directly binds to the Cap and to a platform protein of 200 kDa, eIF4G. eIF4G interacts with the multi-subunit complex eIF3 (12 subunits; more than 500 kDa) and brings together the mRNA and the 43S pre-initiation complex formed by eIF3, the ternary complex (eIF2-Met-tRNA^{Met}-GTP), the 40S ribosomal subunit and other initiation factors [1]. eIF4G also recruits the RNA helicase eIF4A which unwinds secondary structures in the 5' untranslated region (5'UTR) of the mRNA during the scanning towards the initiation codon, and the poly(A) binding protein (PABP) allowing the mRNA circularization for efficient translation re-initiation. Translation is probably the most controlled event in protein synthesis and an important regulatory mechanism takes place during Cap recognition and the mRNA recruitment steps [2].

The Cap-binding protein eIF4E has a highly conserved amino acid sequence in all organisms allowing its direct contact with the Cap structure [3]. eIF4G interacts with eIF4E through an YXXXXLΦ motif (where X is any amino acid and Φ is a hydrophobic residue) and improves its union with the Cap, forming a stable eIF4F-mRNA complex [4, 5]. The interaction between Cap and the translational machinery is prevented by the binding of eIF4E to other cellular proteins through the same motif used for its interaction with eIF4G. By such means cells could modulate either their global translation levels, or specific mRNA recruitment [6]. During the last few years it became evident that through binding to specific proteins and the Cap of mRNAs, eIF4E participates in the nucleo-cytoplasmic transport, translational repression, and turnover of mRNA.

Multiple eIF4E family members have been identified in a wide range of organisms that include plants, flies, mammals, frogs, birds, nematodes, and fish. These members have been classified into three families: eIF4E-I, eIF4E-II and eIF4E-III [7]. Some eIF4E family members have altered its Cap-binding affinities or the interaction with eIF4G and other proteins, providing clues to their physiological roles. It has been suggested that each organism has at least one eIF4E that is ubiquitous and constitutively expressed to carry out general translation, and that the other family members are involved in specialized functions [2]. In addition, other proteins that do not belong to the eIF4E family, but are able to bind the Cap and perform a particular function during the RNA metabolism have been described. Such is the case of the nuclear Cap-Binding Complex (CBC) which participates in protection of the newly synthesized transcripts and their export to the cytoplasm, the De-Capping protein S (DcpS) involved in mRNA degradation, and the Argonaute protein (AGO) in animals which is part of small RNA regulatory pathways (for review see [2]). Therefore,

the proteic components of a Cap-binding complex may vary, depending upon the cellular conditions, growth and developmental requirements.

In plants, three eIF4E family members have been reported: eIF4E (ortholog of the mammalian eIF4E-1; class eIF4E-I) eIF(iso)4E (plant-specific; class eIF4E-I), and nCBP (novel Cap binding protein; class eIF4E-II). The eIF(iso)4E protein interacts with a particular eIF(iso)4G forming the unique plant eIF(iso)4F complex [8]. eIF4F and eIF(iso)4F complexes show selectivity in the recognition of mono and di-methylated Cap structures, as well as in *in vitro* translation of 5'UTR structured mRNAs. In most plant species, eIF(iso)4E shows about 50% amino acid identity with eIF4E and the relative abundance of each protein varies depending on the developmental stage and the plant tissue. In maize, the eIF(iso)4E protein is present at higher levels than eIF4E in non-germinated seeds [9]. The corresponding transcript is efficiently translated upon imbibition to maintain constant and high levels during the first 24 h of germination, whereas eIF4E levels increase toward germination completion. In addition, each Class I *Zea mays* eIF4E family member displays selective translational activity on the pool of mRNAs stored in the quiescent embryonic axes [10, 11].

Based on the above antecedents, in this work we aimed to identify the components of Cap-binding complexes at two particular developmental stages in maize: dry non-germinated (0h) and 24-h-imbibed germinated (24h) embryonic axes. Cap-binding proteins purified through affinity chromatography from 0h and 24h embryonic axes were separated on polyacrylamide gels, silver stained and identified by liquid chromatography-mass spectrometry (LC-MS-MS). This approach revealed a differential composition in the Cap-binding complexes from the two developmental stages, suggesting new roles for proteins as potential partners of the eIF4E family members.

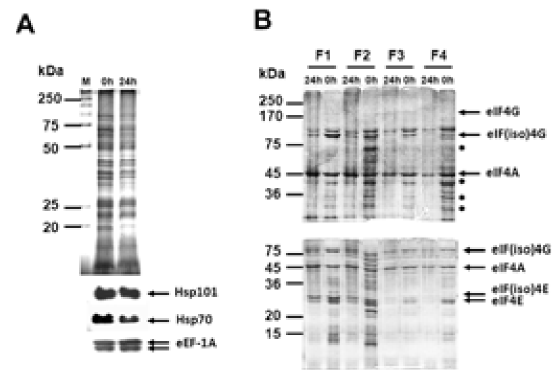

Fig. 1. Protein separation on denaturing polyacrylamide gels (SDS-PAGE) of maize Cap-binding complexes. (A) Total proteins were obtained from dry non-germinated (0h) and 24-h-imbibed germinated (24h) embryonic axes, quantified and resolved on 15% SDS-PAGE. The amounts of the heat shock proteins HSP101 and HSP70 as well as of the translation elongation factor (eEF) 1A, used as control to correct for protein loading, were analyzed by western blot with specific antibodies. (B) Equal amount of total protein extracts from 0h and 24h were incubated with m^7GTP-Sepharose and the m^7GTP-bound proteins were eluted in 5 fractions (200 µl each) with 100 µm m^7GTP. Equal volume of the first four fractions (F1-F4) was separated using 10% (B, upper panel) or 15% (B, lower panel) SDS-PAGE and stained with silver. Arrows correspond to proteins known as components of the maize Cap-binding complexes. Dots indicate unidentified bands that differ between 0h and 24h complexes.

Results and Discussion

Cap-binding protein patterns in dry (0h) and 24-h-imbibed (24h) embryonic axes

Maize Cap-binding complexes were purified from dry "non-germinated" (0h) and 24-h-imbibed (24h) "germinated" embryonic axes through m^7GTP-affinity chromatography. Equal amounts of total protein extracts were used for the m^7GTP-purification (Fig. 1A). After recovery of the m^7GTP-bound fractions, equal volumes of each eluted fraction (F1-F4) were separated on 10% or 15% denaturing polyacrylamide gels for protein resolution of 200-45 kDa (Fig. 1B, upper gel) and 45-15 kDa (Fig. 1B, lower gel), respectively. The silver stained protein patterns indicated several differences between 0h- and 24h- Cap-binding complexes. First, several proteins were preferentially detected in the m^7GTP-eluted fractions from 0h and were absent or decreased in the 24h embryonic axes (Fig 1A, dotted bands). Second, although the Cap-binding proteins in these complexes previously identified as eIF4E and eIF(iso)4E by western blot [9] were observed in both, 0h- and 24h- embryonic axes, their distribution in each eluted fraction was different, being eIF4E more tightly bound to the m^7GTP-Sepharose than eIF(iso)4E (Fig. 1B, fraction F4 from lower gel). On the other hand, eIF(iso)4E and its corresponding partner eIF(iso)4G were mostly present in fractions F1 and F2. Third, a doublet was present at the position of eIF(iso)4G, whereas by western blot usually only one band is detected for this protein (see Fig. 3). These results indicated that the composition and most importantly the affinity of the complexes bound to Cap vary between non-germinated and germinated maize seeds.

Protein identification in Cap-binding complexes from 0h and 24h embryonic axes

Several of the differential and conserved protein bands in the above purified Cap-binding complexes were selected for identification by mass spectrometry (Fig. 2; FT01-FT09). Proteins identified with the criteria of peptides with greater than 95% probability, and at least 2 identified peptides, are shown in Table 1. The complete information about the identified peptides is shwon in Tables 2 (0h) and 3 (24h). The analysis aimed to identify members of the translation initiation machinery and any associated proteins that may have potential translational regulatory roles during maize germination. It is important to notice, that there were silver-stained bands in the gel shown in Figure 2 not selected for identification by mass spectrometry in this study. These may include proteins present at both germination stages, or preferentially found at 24h. From the present analysis, eIF4E, eIF(iso)4E, and eIF(iso)4G proteins were identified in bands corresponding to their expected molecular

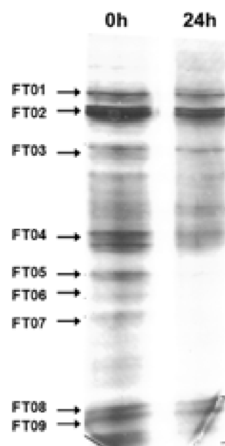

Fig. 2. Protein selection for Mass spectrometry identification. The second fraction (F2) from 0h- and 24h- Cap-binding complexes elution was separated on 10% SDS-PAGE and stained with silver. The indicated bands (FT01-FT09) were cut from the gel and processed for mass spectrometry. This selection included only proteins between 200 and 25 kDa.

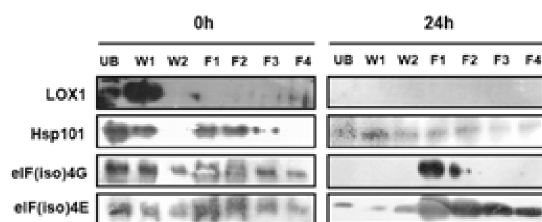

Fig. 3. Protein identification in maize Cap-binding complexes by immunoblotting. Non-germinated (0h) and germinated (24h) embryonic axes were used for the complexes purification. Equal volume of the first four fractions (F1-F4) of m^7GTP-eluate, as well as the m^7GTP-Sepharose unbound fraction (UB) and washes (W1, W2) were separated on 10% SDS-PAGE, transferred to PVDF membrane and detected with the corresponding antibody.

weight. Noticeably, eIF4E was detected with only one peptide in the 0h- and with three peptides in the 24h-sample, whereas eIF4G was not identified in any of the processed bands. This is in agreement with previous findings in our lab, showing that the eIF(iso)4F is the most abundant Cap-binding complex in the dry maize seed [9].

The upper band of the doublet found at 88 kDa, in both 0h- and 24h-embryonic axes, rendered the identification of the 80 kDa subunit of the nuclear Cap-binding complex CBC. Other translation factors, such as eIF3c, eIF4A, and eEF1A were also identified in bands that correspond to their reported molecular weight. According to the literature, these proteins are usually found in Cap-binding complexes from other organisms [12, 13]. The role of eIF3c and eIF4A is at the level of translation initiation, whereas eEF1A is the elongation factor that carries incoming aminoacylated tRNA to the A site of the ribosome. Since eIF4E and eIF4G proteins remain bound to the mRNA to allow efficient re-initiation of translation on a circularized mRNA, both initiation and elongation factors may be found within the Cap-binding complexes.

Recently, the protein degradation and synthesis machineries have been reported as complexes sharing several of their components [14]. In agreement, we found the 6A subunit of the 26S proteasome in the Cap-binding complex from 0h-embryonic axes. This protein is a component of the small subunit (19S) of the proteasome. According to the literature, several proteins from the eIF3 multisubunit complex may interact with proteins of the proteasome and are specifically targeted for degradation [14].

Interestingly, two heat shock proteins, HSP101 and HSP70, were identified as part of the Cap-binding complexes in the dry, but not in the 24-h-imbibed embryonic axes. These proteins were detected at similar levels in the total protein extracts from both samples (Fig. 1A), suggesting that a possible change of their interaction with components of the Cap-binding complex, instead of degradation, is taking place during maize germination. The chaperone HSP70 has been previously identified in Cap-binding complexes in *Drosophila melanogaster* [12]. This chaperone is usually associated with the polypeptide chains nascent from the ribosomal large subunit to assure correct folding early in protein synthesis. Its presence in the Cap-binding complex from 0h-embryonic axes may be indirect through the association with the nascent polypeptide chain or even more likely due to its requirement in assisting the folding of translation initiation factors. However, a closer look on the amino acid sequence of maize HSP70 revealed the YXXXXLΦ conserved motif of eIF4E interacting proteins (Table 4). This suggests that maize HSP70 may directly bind to eIF4E and regulate its function in mRNA recruitment. Supporting this, proteins from the HSP70 family have been shown to regulate translation and stability of mRNAs with AU-rich element (ARE) in their 3'UTRs [15]. However, the specific binding of HSP70 to any of the known Cap-binding translation factors must be further confirmed.

The other heat shock protein, HSP101, has been reported as a chaperone involved in disaggregation of large protein complexes and its expression is greatly induced under heat shock. Maize HSP101 is accumulated in the dry seed and is required to achieve thermotolerance in young germinating seedlings [16]. During the first 24 h after seed imbibition, its protein level remains unchanged, but after 72h of seed imbibition HSP101 is almost undetectable under normal temperature growth conditions (25-32 °C). Wheat HSP101 has been reported as translational regulator for specific mRNAs harboring particular sequences in their UTRs [17]. In maize, a null mutant for this chaperone displays accelerated root growth during germination under normal temperature conditions, but under heat shock, the successful seedling establishment is impaired [16]. Therefore, the presence of HSP101 in the 0h Cap-binding complexes may account for either protein disaggregation of translation factors needed to guarantee translation initiation of growth regulators as soon as the seed is imbibed, or for translation regulation of particular mRNAs during early germination.

Surprisingly, several proteins belonging to carbohydrate metabolic pathways were also detected in the 0 h-, but not in the 24 h- Cap-binding complexes. These included the 3-phos-

Table 1. Proteins identified by mass spectrometry as components of Cap-binding complexes in maize non-germinated (0 h) or germinated (24 h) seeds.

Band ID	Protein ID	NCBI Acc #	MW	MW_{obs}	Number of peptides[a]		% Protein Coverage
					0h	24h	
FT1	Heat shock protein HSP101	GI4584957	101	100	10	—	13
	Component of the nuclear Cap-binding complex (CBC), CBP80	GI108708115	99	100	4	4	4
	Component of the eukaryotic translation initiation factor 3 complex, eIF3c	GI113535264	104	100	3	—	3
FT2	Eukaryotic translation initiation factor gamma isozyme, eIF(iso)4G	GI113564790	88	90	7	7	7
	Lipoxygenase 1	GI12620877	96	90	3	2	4
FT3	Heat shock protein Hsp70	GI123593	71	70	22	—	32
	Leafbladeless 1	GI111559385	68	70	4	—	6
	Endoplasmic reticulum member of HSP70 family, cBiPe2	GI1575128	73	70	3	—	6
FT4	ATP-dependent RNA helicase, eIF4A	GI2341061	47	45	27	16	49
	Eukaryotic elongation factor eEF1A	GI2282584	49	45	16	6	35
	Homolog of the subunit 6 of proteasome 26S	GI3024431	39	45	6	2	13
FT5	3-Phosphoglycerate kinase	GI28172915	32	40	17	—	61
	Actin	GI1498388	37	40	16	—	38
	Alcohol dehydrogenase 1	GI113359	41	40	6	—	16
FT6	Fructose biphosphate aldolase	GI113621	39	36	23	—	55
FT7	Glyceraldehyde 3-phosphate dehydrogenase	GI1184772	37	32	11	—	48
	Malate dehydrogenase	GI18202485	36	32	3	—	11
FT8	Eukaryotic translation initiation factor-4E isozyme, eIF(iso)4E	GI3342823	28	28	8	3	33
FT9	Eukaryotic translation initiation factor-4E, eIF4E	GI3342821	26	26	—	3	14

[a] Only peptides with greater than 95% probability were considered. The shaded portion indicates proteins that were detected by mass spectrometry in the 0h but not the 24h Cap-binding complexes.

phoglycerate kinase, fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase, malate dehydrogenase and alcohol dehydrogenase1. According to the number of identified peptides for each of these proteins (Table 1), they appear as abundant in the Cap-binding complexes. One possibility might be that their synthesis is stopped during the desiccation process and hence they may co-purify as nascent polypeptides within translation complexes. On the other hand, they may form part of aggregates together with translation factors. Although translational functions for these proteins have not been described yet, in a recent work reporting the yeast translasome several metabolic enzymes co-purified with the eIF3 translation complex [18]. In addition, the glyceraldehyde 3-phosphate dehydrogenase has been associated to nuclear RNA export [19] suggesting that metabolic enzymes may have additional functions within protein synthesis. The presence of actin as part of the Cap-binding complexes is not unexpected, since translation complexes are bound to the cytosqueleton of the cell and this protein has been found as critical for normal protein synthesis in mammalian cells [20].

Maize Lipoxygenase 1 (LOX1) and the trans-acting siRNA (tasiRNA) biogenesis-related protein Leafbladeless 1 (LBL1) are not abundant proteins in the maize seed and were as well detected in the Cap-binding complexes of dry seeds (Table 1; FT02 and FT03). A member from the Lipoxygenase family, the *Arabidopsis thaliana* LOX2, was previously reported as eIF(iso)4E binding protein that contains a putative conserved YXXXXLΦ motif [21], whereas LBL1 has been found as meri-

Table 2. Peptide information for Cap-binding complexes obtained from maize embryonic axes at 0h of germination.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
heat shock protein 101	gi|4584957,gi|4928488	101,115.4	10	0.130%	13.0%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
ALVVAAQLSAR	95.0%	70.6	32.2	372	382
DLVMQEVR	95.0%	42.6	31.6	742	749
GDVPSNLLDVR	95.0%	58.4	32.2	229	239
IVRGDVPSNLLDVR	93.3%	26.0	31.1	226	239
LQMKDVAVR	95.0%	44.6	32.3	780	788
NNPVLIGEPGVGK	95.0%	51.3	31.3	203	215
QAITGASGGDGAAGDSFER	95.0%	141.0	32.1	48	66
SDILIQVPNSSTR	95.0%	50.5	32.0	877	889
VQLDSQPEEIDNLER	95.0%	53.8	31.0	406	420
VVGQTEAVSAVAEAVLR	95.0%	73.9	31.2	572	588
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eIF3c	gi|113535264,gi|113610340	106,143.0	3	0.0474%	3.31%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
ELLAQGVQQSR	95.0%	89.9	46.1	599	609
VEQLTFLTR	95.0%	46.9	45.7	280	288
VMAQLGLCAFR	95.0%	63.1	45.4	567	577
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
CPB80	gi|108708115,gi|113548568	98,875.3	4	0.0593%	4.15%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AENMQPVDR	95.0%	46.3	46.3	301	309
ISNMDMECR	95.0%	64.5	45.8	398	406
LLSNLAIVK	95.0%	42.6	46.4	603	611
VFVQEVLER	95.0%	65.9	46.1	443	451
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eIF(iso)4G	gi|113564790|dbj|BAF15133.1	88,477.2	7	0.228%	6.82%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AQVGPTPALIK	95.0%	40.2	45.5	192	202
DQLLELR	95.0%	41.5	46.8	80	86
INDTYFIQMK	95.0%	58.0	45.9	413	422
LTGPDQEMER	95.0%	52.0	46.1	326	335
RINDTYFIQMK	95.0%	42.9	45.5	412	422
SNNWVPR	92.9%	39.1	46.8	446	452
SWDNIREAK	92.9%	38.4	46.0	157	165

Table 2. Continue.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
lipoxygenase	gi|12620877|gb|AAG61118.1	96,461.0	3	0.0570%	3.59%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide stard index	Peptide stop index
AITQGIIPAVR	95.0%	33.6	31.0	277	287
MSDFLGYSIK	95.0%	35.1	31,9	267	276
TITLDDVPGR	95.0%	34.3	31.4	125	134
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
cBiPe2	gi|1575128|gb|AAC49899.1	73,068.8	2	0.0158%	6.33%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
DAGVIAGLNVAR	95.0%	44.4	31.1	190	201
IINEPTAAAIAYGLDK	95.0%	81.0	32.1	202	217
IINEPTAAAIAYGLDKK	95.0%	89.3	31.0	202	218
ITPSWVAFTDSER	95.0%	48.3	31.2	66	78
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
HSP70	gi|123593|sp|P11143	70,586.9	22	0.332%	31.6%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
ARFEELNMDLFR	95.0%	55.8	31.1	304	315
ATAGDTHLGGEDFDNR	95.0%	47.0	32.3	225	240
DAGVIAGLNVMR	95.0%	86.2	32.6	162	173
DISGNPR	93.3%	28.3	32.0	256	262
EIAEAYLGSTIK	95.0%	75.3	31.3	131	142
FEELNMDLFR	95.0%	61.7	31.1	306	315
IINEPTAAAIAYGLDK	95.0%	81.0	32.1	174	189
IINEPTAAAIAYGLDKK	95.0%	89.3	31.0	174	190
ITITNDKGR	95.0%	45.1	31.8	503	511
LSKEEIEK	95.0%	34.9	32.1	512	519
MDKSSVHDVVLVGGSTR	95.0%	69.7	31.0	330	346
MKEIAEAYLGSTIK	95.0%	57.7	30.5	129	142
MVNHFVQEFK	95.0%	32.1	30.9	241	250
NALENYAYNMR	94.8%	28.6	31.6	542	552
NAVVTVPAYFNDSQR	95.0%	33.2	30.3	143	157
NKITITNDKGR	61.2%	21.0	31.6	501	511
NQVAMNPTNTVFDAK	95.0%	87.1	31.6	59	73
NQVAMNPTNTVFDAKR	95.0%	70.1	32.3	59	74
QFAAEEISSMVLIK	95.0%	81.7	31.1	115	128
SSVHDVVLVGGSTR	95.0%	78.2	31.8	333	346
TTPSYVGFTDTER	95.0%	61.1	30.8	39	51
VEIIANDQGNR	95.0%	58.5	32.6	28	38

Table 2. Continue.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
leafbladeless1	gi|111559385|gb|ABH10613.1|	67,735.0	3	0.0237%	5.89%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
ELAALLEEELSR	95.0%	80.9	31.5	232	243
GTSVLPAGEQFGK	95.0%	31.2	31.6	245	257
NGPGAIDWYK	95.0%	39.4	31.6	201	210
NGSTSKVVK	94.4%	29.4	32.6	156	164
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eIF-4A	gi|2341061|gb|AAB67607.1|	46,964.7	27	1.25%	48.8%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
DELTLEGIK	95.0%	41.3	31.8	247	255
DIIMR	81.0%	24.8	32.7	320	324
ELAQQIEK	95.0%	43.5	32.0	120	127
FGRKGVAINFVTR	95.0%	38.1	31.2	374	386
FMNKPVR	95.0%	37.5	33.2	235	241
GIVPFCK	95.0%	31.9	32.2	71	77
GIYAYGFEKPSAIQQR	95.0%	59.8	31.2	55	70
GLDVIQQAQSGTGK	95.0%	35.5	32.6	78	91
GVAINFVTR	95.0%	66.2	31.8	378	386
ILASGVHVVVGTPGR	95.0%	43.0	32.3	155	169
IQVGVFSATMPPEALEITR	95.0%	30.3	30.2	215	233
IQVGVFSATMPPEALEITRK	95.0%	53.6	30.9	215	234
KGVAINFVTR	95.0%	57.7	32.0	377	386
KVDWLTDK	95.0%	39.9	32.3	292	299
MFVLDEADEMLSR	95.0%	95.4	31.7	186	198
MLFDIQK	95.0%	40.7	30.8	391	397
QFYVNVDKEDWK	95.0%	50.8	30.5	256	267
QSLRPDNIK	95.0%	33.2	30.7	177	185
RDELTLEGIK	95.0%	52.6	31.1	246	255
RKVDWLTDK	95.0%	43.0	32.2	291	299
RQSLRPDNIK	87.1%	26.0	32.4	176	185
VDWLTDK	81.2%	24.4	32.3	293	299
VFDMLR	95.0%	29.8	31.9	170	175
VFDMLRR	84.2%	24.4	31.6	170	176
VHACVGGTSVR	95.0%	50.9	31.8	140	150
VLITTDLLAR	95.0%	67.5	32.1	333	342

Table 2. Continue.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eE1alpha	gi|1321656|dbj|BAA08249.1|	49,215.7	7	0.107%	15.0%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
EVSSYLK	54.4%	19.2	31.3	173	179
IGGIGTVPVGR	95.0%	41.2	31.7	244	254
QTVAVGVIK	95.0%	31.8	31.6	419	427
STNLDWYK	95.0%	34.4	32.0	205	212
STTTGHLIYK	95.0%	33.1	31.6	21	30
YDEIVKEVSSYLK	77.9%	23.5	32.1	167	179
YYCTVIDAPGHRDFIK	95.0%	33.7	30.4	85	100
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
actin	gi|1498388|gb|AAB40105.1|	37,136.2	16	0.530%	37.8%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AGFAGDDAPR	95.0%	43.0	31.8	1	10
AVFPSIVGRPR	95.0%	60.3	31.4	11	21
CDVDIRK	95.0%	30.4	32.3	267	273
DAYVGDEAQSK	95.0%	48.9	32.1	33	43
DAYVGDEAQSKR	95.0%	56.1	31.5	33	44
DLTDSLMK	95.0%	44.7	32.2	166	173
EITALAPSSMK	95.0%	33.5	31.1	298	308
GILTLK	74.6%	24.5	33.7	45	50
GYSFTTTAER	95.0%	29.5	32.4	179	188
GYSFTTTAEREIVR	95.0%	31.5	32.2	179	192
IKVVAPPER	95.0%	32.7	30.4	309	317
LAYVALDYDQELENAK	93.8%	27.1	30.8	198	213
LDLAGR	65.4%	23.0	33.7	160	165
LDLAGRDLTDSLMK	95.0%	59.3	31.6	160	173
SYELPDGQVITIGAER	95.0%	98.5	30.8	221	236
VVAPPERK	94.6%	29.7	33.0	311	318
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
265 proteasome regulatory particle triple-A ATPase subunit1	gi|11094190|dbj|BAB17624.1|	47,665.1	2	0.0481%	5.87%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
FDDGVGGDNEVQR	95.0%	62.8	45.6	278	290
SVCTEAGMYAIR	81.1%	36.4	45.8	380	391
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
265 proteasome regulatory complex ATPase RPT3	gi|42602319|gb|AAS21759.1|	38,811.9	2	0.0171%	6.32%
Peptide sequence	Peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
ADTLDPALLR	95.0%	31.4	30.8	244	253
GVLLYGPPGTGK	95.0%	38.9	30.9	131	142

Table 2. Continue.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC2	gi|1184772|gb|AAA87578.1|	36,524.1	15	0.307%	48.1%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AASFNIIPSSTGAAK	95.0%	53.5	31.7	203	217
AGIALNDHFIK	95.0%	73.3	30.6	301	311
GASYEEIKK	95.0%	27.9	31.3	254	262
GIMGYVEEDLVSTDFTGDSR	95.0%	94.1	31.0	274	293
HSDIALKDSK	95.0%	41.1	31.0	58	67
IGINGFGR	95.0%	45.7	31.8	6	13
KVVISAPSK	95.0%	41.1	31.6	120	128
LTGMSFR	72.5%	21.3	32.1	230	236
LVSWYDNEWGYSNR	95.0%	43.0	30.1	312	325
SSIFDAK	95.0%	27.6	30.3	294	300
TLLFGEKPVTVFGIR	95.0%	44.4	32.3	68	82
VLPELNGK	95.0%	27.3	31.2	222	229
VPTVDVSVVDLTVR	95.0%	61.3	31.6	237	250
VVDLIR	73.3%	21.2	31.8	326	331
YDTVHGQWK	95.0%	33.9	32.4	49	57
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
Fructose-bisphosphate aldolase, cytoplasmic isozyme	gi|113621|sp|P08440|ALF_MAIZE	38,587.0	23	0.346%	54.6%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
ALQASTLK	95.0%	35.8	33.0	299	306
ANSEATLGTYK	95.0%	36.4	31.1	328	338
AWAGKVENLEK	95.0%	53.1	32.2	307	317
CAYVTETVLAACYK	95.0%	70.9	31.5	197	210
DGKPFVDVLK	95.0%	31.9	32.4	85	94
EGGVLPGIK	95.0%	35.4	31.2	95	103
EGGVLPGIKVDK	95.0%	46.3	31.6	95	106
GDAAADTESLHVK	95.0%	34.8	31.6	339	351
GILAADESTGTIGK	95.0%	75.1	30.9	25	38
GILAADESTGTIGKR	95.0%	81.5	32.3	25	39
GTIEVVGTDK	95.0%	64.5	32.4	107	116
GTIEVVGTDKETTTQGHDDLGKR	95.0%	58.4	31.0	107	129
IGPNEPSQLAIDLNAQGLAR	95.0%	69.6	30.1	149	168
KPWSLSFSFGR	95.0%	32.8	31.5	288	298
KVTPEVIAEYTVR	95.0%	36.7	32.5	237	249
LSSINVENVEENR	95.0%	74.2	32.1	40	52
LSSINVENVEENRR	95.0%	43.5	31.8	40	53
NAAYIGTPGK	95.0%	46.3	31.5	15	24
RLSSINVENVEENRR	83.0%	24.7	31.9	39	53
TKDGKPFVDVLK	95.0%	36.8	31.0	83	94
VDKGTIEVVGTDK	95.0%	43.3	31.2	104	116
VTPEVIAEYTVR	95.0%	73.2	31.9	238	249
YYEAGAR	95.0%	31.3	33.0	133	139

Table 2. Continue.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
Malate dehydrogenase, cytoplasmic	gi|18202485|sp|Q08062|MDHC_MAIZE	35,571.4	3	0.0225%	11.1%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
LNVQVSDVK	95.0%	27.7	31.6	172	180
MELVDAAFPLLK	95.0%	45.6	30.3	56	67
VLVVANPANTNALILK	95.0%	49.2	30.2	127	142
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
Cytosolic 3-phosphoglycerate kinase	gi|28172915|gb|AAO32643.1|	31,608.4	17	0.311%	61.2%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AKGVSLLLPTDIVVADK	83.0%	24.3	31.8	186	202
ELDYLVGAVANPK	95.0%	82.7	31.4	105	117
FAAGTEAIAK	95.0%	54.6	31.0	258	267
FAAGTEAIAKK	95.0%	31.1	31.6	258	268
FYKEEEKNEPEFAK	95.0%	51.4	31.1	49	62
GVSLLLPTDIVVADK	95.0%	64.2	30.3	188	202
GVTTIIGGGDSVAAVEK	95.0%	101.0	32.0	277	293
IGVIESLLAK	95.0%	48.1	32.4	133	142
KLAELTTTK	95.0%	44.9	32.5	268	276
KPFAAIVGGSK	95.0%	68.3	31.8	118	128
LAAALPEGGVLLLENVR	95.0%	62.7	31.3	32	48
LAELTTTK	95.0%	29.5	32.3	269	276
LASVADLYVNDAFGTAHR	95.0%	35.2	30.1	64	81
TFNEALDTTK	95.0%	37.4	31.4	233	242
TVIWNGPMGVFEFEK	95.0%	47.9	30.9	243	257
YLKPAVAGFLMQK	95.0%	30.9	30.7	92	104
YSLKPLVPR	95.0%	42.6	31.8	1	9
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eIF(iso)4E	gi|6016335|sp|O81482|IF4E2_MAIZE	24,086.1	8	0.328%	33.3%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AGVEPKWEDPECANGGK	95.0%	62.1	31.6	102	118
GDKLALWTR	95.0%	50.5	32.1	162	170
ITYTFHDDSK	95.0%	35.2	32.4	195	204
LSGNADFHLFK	95.0%	48.7	31.9	91	101
SGAAWGTSLK	95.0%	39.4	31.2	58	67
SGAAWGTSLKK	95.0%	44.5	32.0	58	68
TASNEAVQVNIGK	95.0%	79.9	31.4	171	183
TASNEAVQVNIGKK	95.0%	34.2	31.5	171	184

Table 3. Peptide information for Cap-binding complexes obtained from maize embryonic axes at 24h of germination.

Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
CBP80	gi|108708115|gb|ABF95910.1	98,875.3	5	0.284%	5.42%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot identity score	Peptide start index	Peptide stop index
AENMQPVDR	91.9%	42.9	46.5	301	309
ISNMDMECR	95.0%	51.2	46.0	398	406
LLSNLAIVK	95.0%	45.9	45.7	603	611
SFTHLITVLER	72.9%	32.8	45.9	548	558
VFVQEVLER	95.0%	56.6	46.0	443	451
EEEQGGGGGQKSR	55.4%	14.4	30.6	337	349
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eIF(iso)4G	gi|113564790|dbj|BAF15133.1	102,217.1	7	0.268%	6.39%
Peptide sequence	Best peptide identificaton probability	Best mascot ion Score	Best mascot ion score	Peptide start index	Peptide stop index
AEVPWSAR	57.5%	30.4	46.7	190	197
AQVGPTPALIK	95.0%	43.5	45.4	179	189
DQLLELR	95.0%	45.9	47.0	67	73
INDTYFIQMK	95.0%	79.6	45.9	400	409
LTGPDQEMER	95.0%	67.2	46.0	313	322
RINDTYFIQMK	95.0%	53.8	45.6	399	409
TVSLLEEYFGIR	92.5%	40.6	45.4	632	643
Protein name	Protein accession numbers	Protein molecular weight (Da)	Number of unique peptides	Percentage of total spectra	Percentage sequence coverage
eIF-4A	gi|2341061|gb|AAB67607.1	46,964.7	16	0.291%	36.5%
Peptide sequence	Best peptide identification probability	Best mascot ion score	Best mascot ion score	Peptide start index	Peptide stop index
ALGDYLGVK	95.0%	31.7	31.4	131	139
DELTLEGIK	95.0%	46.1	31.8	247	255
ELAQQIEK	76.2%	22.6	31.6	120	127
FGRKGVAINFVTR	91.6%	25.6	30.7	374	386
GIYAYGFEKPSAIQQR	95.0%	39.0	31.2	55	70
GLDVIQQAQSGTGK	95.0%	100	32.6	78	91
GVAINFVTR	95.0%	47.5	32.3	378	386
ILASGVHVVVGTPGR	95.0%	76.5	31.8	155	169
IQVGVFSATMPPEALEITR	95.0%	82.2	30.2	215	233
KGVAINFVTR	95.0%	54.0	32.5	377	386
KVDWLTDK	95.0%	38.0	32.3	292	299
MFVLDEADEMLSR	95.0%	74.0	32.0	186	198
MLFDIQK	95.0%	29.9	30.8	391	397
QSLRPDNIK	76.8%	21.6	30.5	177	185
RDELTLEGIK	95.0%	54.7	31.6	246	255
VLITTDLLAR	95.0%	32.7	32.1	333	342

Table 3. Continue.

<table>
<tr><td>Protein name</td><td>Protein accession numbers</td><td>Protein molecular weight (Da)</td><td>Number of unique peptides</td><td>Percentage of total spectra</td><td>Percentage sequence coverage</td></tr>
<tr><td>eEF1alpha</td><td>gi|1321656|dbj|BAA08249.1</td><td>49,272.7</td><td>6</td><td>0.162%</td><td>13.0%</td></tr>
<tr><td>Peptide sequence</td><td>Best peptide identification probability</td><td>Best mascot ion score</td><td>Best mascot identity score</td><td>Peptide start index</td><td>Peptide stop index</td></tr>
<tr><td>ARYDEIVK</td><td>95.0%</td><td>34.5</td><td>32.0</td><td>165</td><td>172</td></tr>
<tr><td>IGGIGTVPVGR</td><td>95.0%</td><td>67.4</td><td>32.0</td><td>244</td><td>254</td></tr>
<tr><td>QTVAVGVIK</td><td>95.0%</td><td>36.9</td><td>31.6</td><td>419</td><td>427</td></tr>
<tr><td>STNLDWYK</td><td>95.0%</td><td>34.0</td><td>32.3</td><td>205</td><td>212</td></tr>
<tr><td>STTTGHLIYK</td><td>95.0%</td><td>43.1</td><td>31.6</td><td>21</td><td>30</td></tr>
<tr><td>YYCTVIDAPGHR</td><td>95.0%</td><td>59.6</td><td>32.5</td><td>85</td><td>96</td></tr>
<tr><td>Protein name</td><td>Protein accession numbers</td><td>Protein molecular weight (Da)</td><td>Number of unique peptides</td><td>Percentage of total spectra</td><td>Percentage sequence coverage</td></tr>
<tr><td>eIF4E</td><td>sp|O81481|IF4E1_MAIZE</td><td>24,452.8</td><td>3</td><td>0.332%</td><td>13.8%</td></tr>
<tr><td>Peptide sequence</td><td>Best peptide identification probability</td><td>Best mascot ion score</td><td>Best mascot identity score</td><td>Peptide start index</td><td>Peptide stop index</td></tr>
<tr><td>IAIWTK</td><td>90.8%</td><td>31.7</td><td>33.6</td><td>167</td><td>172</td></tr>
<tr><td>LIVGADFHCFK</td><td>95.0%</td><td>40.6</td><td>31.7</td><td>94</td><td>104</td></tr>
<tr><td>NAANEAAQVSIGK</td><td>95.0%</td><td>68.8</td><td>32.4</td><td>173</td><td>185</td></tr>
<tr><td>Protein name</td><td>Protein accession numbers</td><td>Protein molecular weight (Da)</td><td>Number of unique peptides</td><td>Percentage of total spectra</td><td>Percentage sequence coverage</td></tr>
<tr><td>eIF(iso)4E</td><td>gi|6016335|sp|O81482|IF4E2_MAIZE</td><td>24,086.1</td><td>3</td><td>0.251%</td><td>11.1%</td></tr>
<tr><td>Peptide sequence</td><td>Best peptide identification probability</td><td>Best mascot ion score</td><td>Best mascot identity score</td><td>Peptide start index</td><td>Peptide stop index</td></tr>
<tr><td>SGAAWGTSLK</td><td>95.0%</td><td>32.5</td><td>31.2</td><td>58</td><td>67</td></tr>
<tr><td>TASNEAVQVNIGK</td><td>95.0%</td><td>58.6</td><td>31.4</td><td>171</td><td>183</td></tr>
<tr><td>TASNEAVQVNIGKK</td><td>95.0%</td><td>51.8</td><td>31.9</td><td>171</td><td>184</td></tr>
</table>

Table 4. Alignment of eIF4E Binding Motifs present in eIF4E Binding Proteins reported for Plants (At = *Arabidopsis thaliana*, Zm = *Zea mays*)[a]. Underlined amino acids correspond to the consensus sequence.

Protein	amino acid sequence	Reference
AteIF(iso)4GI	YTREQLL	[27]
AteIF(iso)4GII	FSREEIL	[27]
ZmeIF(iso)4G	YTRDQLL	this work
AtLOX2	YRKEELE	[21]
AtBTF3	STLKRIG	[28]
ZmLOX1	YRDDELR (1) YATRTLF (2)	this work
ZmLBL1	YMDLELE	this work
ZmHSP70	YSCVGLW	this work
Consensus	YXXXXLΦ	

stem specific protein involved in small RNA mediated gene silencing and abaxial/adaxial leaf fate definition [22]. Therefore, we searched the sequence of maize LOX1 and LBL1 for the YXXXXLΦ motif to find whether these are also potential eIF4E or eIF(iso)4E binding proteins. The analysis showed that both proteins presented the conserved motif (Table 4). Since eIF(iso)4G also uses this amino acid sequence to bind eIF(iso)4E and integrate a functional translation initiation complex on the mRNA, the presence of potential eIF(iso)4E binding proteins in the 0h Cap-binding complexes indicates that eIF(iso)4E may be a part of ribonucleoprotein particles not involved in translation initiation at this developmental stage.

HSP101 and LOX1 presence in the Cap-binding complexes from dry and 24-h-imbibed maize seeds

To test whether some of the newly identified proteins in Cap-binding complexes are indeed specifically eluted from the m^7GTP-chromatography, western blot with available antibodies was performed with extended washes before the specific elution with the m^7GTP ligand (Fig. 3). While the LOX1 protein was mostly detected in the first washes (W1) of the column, a small quantity was specifically retained and eluted with the ligand in the last fractions (F2-F4) from dry (0h) embryonic axes. This protein was not detected in the 24-h-imbibed axes neither in total protein extracts nor in the Cap-bound fractions, indicating that it is probably particularly expressed during seed maturation. A significant amount of HSP101 was also specifically retained in the column, but eluted in the first three fractions upon the ligand addition (F1-F3), when non-germinated embryonic axes were used. Interestingly, the protein was also detected in the 24-h-imbibed embryonic axes elution fractions, although to a lesser levels. This could correlate to previous data in wheat, where HSP101 was reported to bind to eIF4G and eIF3, but not to eIF4E proteins [23]. On the other hand, the elution pattern of LOX1 was similar to that observed for eIF(iso)4E, indicating a possible direct binding between these two proteins. These results support the notion that eIF(iso)4E, which is the major Cap-binding translation initiation factor in the dry non-germinated maize seed, forms different multiproteic complexes to regulate selective mRNA translation upon the germination onset.

Conclusions

The analysis of Cap-binding complexes at two different germination stages in the maize seeds indicated differential composition that may correlate with the translational requirements and regulatory mechanisms operating to achieve the appropriate protein synthesis patterns at each developmental stage. New components of the Cap-binding complexes in non-germinated seeds include the chaperones HSP101 and HSP70 as well as the lipoxygenase LOX1 and leafbladeless LBL1. HSP70, LOX1 and LBL1 are candidates to interact with members of the eIF4E family through an YXXXXLΦ motif.

Experimental

Material and Methods

Plant material

Maize (*Zea mays* L) seeds of a Mexican land race Tuxpeño, var. Chalqueño, were used for all experiments. Seeds were germinated by water imbibition on moisturized cotton, in the dark, at 25 ºC. Embryonic axes were manually excised from either dry (0h) or 24-h-imbibed (24h) seeds.

Cap-binding complexes purification

Cap-binding complexes were purified by m^7GTP-Sepharose affinity chromatography as previously reported [9], with some modifications. Briefly, 2.5 g of axes were macerated in liquid nitrogen and suspended in 25 mL Buffer "A" consisting of: 20 mM HEPES, pH 7.6; 100 mM KCl; 0.2 mM EDTA; 10 % glycerol; 1 % Triton X-100; 0.5 mM DTT; and Complete^{TM}, EDTA free protease inhibitors (Complete^{TM}, Roche Molecular Diagnostics, Pleasanton, CA, USA). The extract was clarified by 30 min centrifugation at 15,000 rpm and 4 °C in a Sorvall J-20 rotor. The supernatant was filtered through eight layers of cheesecloth and the protein amount was quantified. Approximately, 20 mg of total protein was incubated with 0.5 mL of m^7GTP-Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for one h at 4 °C. The slurry was poured onto a 10 mL Column (Bio-Rad Laboratories, Inc. Hercules, CA) and the resin was washed with 5 mL of Buffer "A", followed by 5 mL of Buffer "A" including 0.1 mM GTP. The bound proteins were eluted with 1 mL of Buffer "A" containing 100 µM m7GTP (Sigma-Aldrich Co., Saint Louis MO, USA) in 5 fractions of 200 µL each. To assess more-specific binding, washes with Buffer "A" were extended to 10 mL (20 bed volumes) divided in 5 mL each.

Electrophoresis and protein staining

Proteins from the purified Cap-binding complexes were resolved on either 10% or 15% (w/v) denaturing polyacrylamide (SDS-PAGE) gels. The silver staining procedure used was compatible with mass spectrometry analysis [24].

Mass spectrometry

The in-gel digest and mass spectrometry experiments were performed by the Proteomics platform of the Eastern Quebec Genomics Center, Quebec, Canada. Tryptic digestion was performed on a MassPrep liquid handling robot (Waters, Milford, USA) according to the manufacturer's specifications and to the protocol of [24] with the modifications suggested by [25]. Briefly, proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed using 105 mM of modified porcine trypsin (Sequencing grade, Promega, Madison, WI, USA) at 58°C for 1h. Digestion products were extracted using 1% formic acid, 2% acetonitrile followed by 1% formic acid, 50% acetonitrile. The recovered extracts were pooled, vacuum centrifuge dried and then resuspended into 8 µL of 0.1% formic acid and 4 µL were analyzed by mass spectrometry.

Peptide samples were separated by online reversed-phase (RP) nanoscale Capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES MS/MS). The experiments were performed with a Thermo Surveyor MS pump connected to a LTQ linear ion trap mass spectrometer (ThermoFisher, San Jose, CA, USA) equipped with a nanoelectrospray ion source (ThermoFisher). Peptide separation took place on a PicoFrit column BioBasic C18, 10 cm x 0.075 mm internal diameter (New Objective, Woburn, MA, USA), with a linear gradient from 2-50% solvent B (acetonitrile, 0.1% formic acid) in 30 min, at 200 mL/min (obtained by flow-splitting). Mass spectra were acquired using a data dependent acquisition mode using Xcalibur software version 2.0. Each full scan mass spectrum (400 to 2000 m/z) was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion (30 sec exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

Protein Identification

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2.0). Mascot was set up to search the ncbi_Zea_mays_20071004 database (10,023 entries) assuming the digestion enzyme non-specific. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.0 Da. Iodoacetamide derivative of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modification. Scaffold (version 3.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [26] and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Immunoblotting

For immunodetection, proteins were blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corp., Billerica, MA, USA), which was blocked with 5% (w/v) milk and incubated with the primary antibody for 2 h at room temperature. After several washes in Phosphate Saline Buffer (PBS), the membrane was incubated for 1 h with the appropriate secondary antibody at a 1:5000 dilution. Detection was performed with Immobilon Western Chemiluminescent HRP Substrate (Millipore Corp.). Primary antisera dilutions were as follows: antibodies against wheat eIF(iso)4E and eIF(iso)4G were kindly donated by Karen S. Browning, University of Texas, Austin, USA and were used at 1:5000 dilution; antibody against maize Hsp101 was kindly donated by Jorge Nieto-Sotelo, Instituto de Biotecnología, UNAM, Cuernavaca, Mexico and used at 1:1000 dilution; antibody against bean LOX2 was kindly donated by Helena Porta, Instituto de Biotecnología, UNAM, Cuernavaca, Mexico and used at 1:100 dilution.

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