

PHOSPHORYLATION OF MAIZE eIF5A BY CK2: IDENTIFICATION OF PHOSPHORYLATED RESIDUE AND INFLUENCE ON INTRACELLULAR LOCALIZATION OF eIF5A

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Maize eukaryotic translation initiation factor 5A (ZmeIF5A) co-purifies with catalytic α subunit of protein kinase CK2 and is phosphorylated by this enzyme. Phosphorylated ZmeIF5A was also identified after separation of maize leaf proteins by two-dimensional electrophoresis. Multiple sequence alignment of eIF5A proteins showed that in monocots, in contrast to other eukaryotes, there are two serine/threonine residues that could potentially be phosphorylated by CK2. To identify the phosphorylation site(s) of ZmeIF5A, the serine residues potentially phosphorylated by CK2 were mutated. ZmeIF5A and its mutated variants Ser2Ala and Ser4Ala were expressed in *E. coli* and purified. Of these recombinant proteins, only ZmeIF5ASer2Ala was not phosphorylated by maize CK2. Also, *Arabidopsis thaliana* and *Saccharomyces cerevisiae* eIF5A Ser2Ala mutants were not phosphorylated despite effective phosphorylation of wild-type variants. A newly developed method exploiting the specificity of thrombin cleavage was used to confirm that Ser2 in ZmeIF5A is indeed phosphorylated. To find a role of the Ser2 phosphorylation, ZmeIF5A and its variants mutated at Ser2 (Ser2Ala and Ser2Asp) were transiently expressed in maize protoplasts. The expressed fluorescence labeled proteins were visualized by confocal microscopy. While wild-type ZmeIF5A and its Ser2Ala variant were distributed evenly between the nucleus and the cytoplasm, the variant with Ser2 replaced by aspartic acid, which mimics a phosphorylated serine, was sequestered in the nucleus. These results suggests that phosphorylation of Ser2 plays a role in the regulation of nucleocytoplasmic shuttling of eIF5A in plant cells.

Protein kinase CK2 (formerly known as casein kinase 2) is a ubiquitously expressed serine/threonine protein kinase present in all eukaryotes that displays extraordinary evolutionary conservation (for review see Ref. 1). In contrast to most other protein kinases, CK2 is constitutively active, not regulated by a second messenger, and can use both ATP and GTP as phosphate donors. CK2 is most often present as a tetramer composed of two catalytic α and two regulatory β subunits (for review see Ref. 2). CK2 is a pleiotropic kinase with a growing list of more than 300 substrates (for review see Ref. 3). A characteristic feature of CK2 phosphorylation sites are multiple acidic residues located mostly downstream of the phosphorylatable amino acid (4). The striking variety of its cellular targets and its absolute requirement for cell viability suggest that CK2 plays essential role(s); it has been implicated in many cellular functions with special reference to proliferation, gene expression, and RNA and protein synthesis (for review see Ref. 3).

Studies concerning functions of plant CK2s have shown that this enzyme is involved in light-regulated gene expression and growth control (5), cell division and cell expansion (6-8). CK2 phosphorylates transcription factors (G-box-binding and bZip factors) involved in photomorphogenesis (9-11). Opaque2, a basic leucine zipper transcriptional activator which binds to the G-box of promoters, is phosphorylated by CK2 during maize seed development. Phosphorylation of Opaque2 is light-dependent and is under diurnal control (12). Also the transcription factors CCA1 (circadian clock-associated 1) and LHY (late elongated hypocotyl) are phosphorylated by CK2 and are essential for the regulation of the endogenous circadian clock in *Arabidopsis* (13). Only phosphorylated CCA1 is able to bind promoters and regulate the transcription of target genes in

the nucleus, whereas the unphosphorylated protein is sequestered in the cytoplasm (14). The stability of transcription factor HY5 (Long Hypocotyl 5), which is involved in auxin signaling and photomorphogenesis (15), is regulated both by interaction with a negative regulator of photomorphogenesis (COP1) and by phosphorylation by CK2 (9). It has been demonstrated that the maize abscisic acid responsive protein Rab17, a late embryogenesis abundant protein involved in plant responses to stress, is highly phosphorylated by CK2. Rab17 specifically interacts with CK2 β (16). In *Zea mays*, three cDNA clones encoding CK2 β and three encoding CK2 α (*ZmCK2 α -1*, *ZmCK2 α -2*, and *ZmCK2 α -3*) have been isolated and characterized (17-19), which indicates that in plants several different tetrameric forms of CK2 may be present. Also enzymatically active monomeric forms of CK2 catalytic α subunits have been identified in maize (20, 21). A group of low molecular weight proteins were found to co-purify with the monomeric form of CK2. Those proteins migrating at 17-19 kDa were highly phosphorylated by CK2 (22). The present work was undertaken to search for and characterize new substrate(s) of maize CK2 and to investigate the role of their phosphorylation.

Experimental Procedures

Materials – Staurosporine, heparin, phosphocellulose, HSRT 100 kit were purchased from Sigma. Glutathione-agarose beads, source 15Q, Sephadex G-100, Superdex G-200 10/300 GL column for FPLC, and pGEX-4T-1 expression vector were obtained from Amersham Pharmacia Biotech. TOPO[®] vector was from Invitrogen. TRI[®] Reagent was obtained from MRC, Cincinnati, OH. Antibodies against ZmeIF5A were purchased from BioGenes. All other chemicals were of highest grade available.

Plant growth – Maize seeds (*Zea mays* cv. Mona), after overnight soaking in water at room temperature, were grown for 72 h at 26°C on wetted paper in the dark. Etiolated apical parts of the seedlings were harvested, immediately frozen in liquid nitrogen and stored at -80°C. For leaves, maize plants were cultivated hydroponically for 2 weeks in a growth chamber with a daily cycle of 16 h light at 25°C and 8 h dark at 20°C.

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were sown onto soil and maintained

at 4°C for 3 days, and afterwards were transferred to a growth chamber with a daily cycle of 16 h light at 23°C and 8 h dark at 21°C. Rosette leaves of 2-week old plants were harvested, frozen in liquid nitrogen and stored at -80°C.

Saccharomyces cerevisiae strain W303 was grown overnight at 30°C in YPD medium.

Purification of native CK2 from maize seedlings – All purification steps were performed at 4°C. Frozen maize seedlings (40 g) were ground with liquid nitrogen in a mortar. Proteins were extracted by homogenization for 15 min with 80 ml of Buffer 1 (50 mM Tris-HCl, pH 7.8, 6 mM MgCl₂, 10 mM β -mercaptoethanol (β -ME), 0.1 mM PMSF, 250 mM (NH₄)₂SO₄). After sonication (3 times for 20 s), the suspension was centrifuged at 10 000 rpm for 10 min and filtered through glass wool. Proteins were precipitated from the supernatant with (NH₄)₂SO₄ (up to 60% of saturation). After 90 min of gentle stirring, the suspension was centrifuged at 12 000 rpm for 45 min. The supernatant was discarded and the collected precipitate was dissolved in Buffer 2 (50 mM Tris-HCl, pH 7.8, 6 mM MgCl₂, 10 mM β -ME, 0.1 mM PMSF, 10% (v/v) ethylene glycol) to the conductivity of the same buffer containing 40 mM (NH₄)₂SO₄. After equilibration, 120 ml of phosphocellulose resin (pre-equilibrated with 10 volumes of Buffer 2 containing 40 mM (NH₄)₂SO₄) was added. Binding was carried out for 1 h with constant stirring. Then, the phosphocellulose resin with bound proteins was washed with 1200 ml (3 x 400 ml) of Buffer 2 containing 0.1 M NaCl. The beads were packed into a column and a linear gradient of 0.1-1 M NaCl (2 x 150 ml) in Buffer 2 was applied. Fractions containing CK2 activity, of a total volume of 80 ml, were collected, concentrated by Amicon ultrafiltration to 20 ml (Amicon Ultra, Millipore) and dialyzed against Buffer 3 (50 mM Tris-HCl, pH 7.8, 10 mM β -ME, 0.1 mM PMSF, 10% (v/v) ethylene glycol), 3 x 1 l for 15 h. The obtained sample was loaded on a 5-ml Source 15Q column pre-equilibrated with 10 volumes of Buffer 3 and the column was washed with 50 ml of Buffer 3. A linear gradient of 0-0.6 M NaCl (2 x 30 ml) in Buffer 3 was applied for enzyme elution. Active fractions were collected (30 ml), concentrated by Amicon ultrafiltration to 1 ml, adjusted to a final salt concentration of 0.5 M NaCl, loaded on a Sephadex G-100 column (1.7 x 86.5 cm) and eluted with Buffer 4 (100 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.01% Brij 35, 0.05 mM PMSF); 1-

ml fractions were collected. Two peaks with CK2 activity (named I and II, 8.0 ml each) were separately collected, concentrated eight-fold by Amicon ultrafiltration and dialyzed against Buffer 5 (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.05 mM PMSF) for 4 h (2 x 1 l). Glycerol was added to the final concentration of 10% to the enzyme preparations and the samples were frozen in liquid nitrogen.

Separation of maize leaf proteins by two-dimensional gel electrophoresis (2-DE) and identification of phosphoproteins - Total protein was prepared from 2-week old maize leaves according to a modified phenol-based procedure as described previously (23). The extracted proteins were precipitated by 100% ethanol containing 0.1 M ammonium acetate, and after centrifugation the obtained pellet was dissolved in isoelectrofocusing extraction buffer (8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) Triton X-100, 50 mM dithiothreitol). For the first dimension, sample was loaded on a 13-cm strip (pH 3-10) and focused on Multiphor II apparatus (Amersham Pharmacia Biotech). For the second dimension, the strip was equilibrated, placed on an SDS-polyacrylamide gel and run as described previously (23). For phosphoprotein detection, the gel was stained with Pro-Q[®] Diamond phosphoprotein gel stain (Pro-Q[®] DPS) according to (24) and imaged using a Molecular Imager PharoFX system (Bio-Rad). For detection of all proteins the gel was stained with colloidal Coomassie Brilliant Blue G-250. Phosphoprotein spots were cut out from the gel and analyzed by mass spectrometry (MS).

Mass Spectrometry - Protein bands or spots were excised from the SDS-polyacrylamide gel, reduced, alkylated, digested overnight with trypsin and then analyzed by tandem mass spectrometry using a Q-ToF 1 mass spectrometer (Micromass, Manchester, UK) combined with a nano-HPLC system (LC Packings, Amsterdam, The Netherlands). The MaxEnt3 program (Micromass) was used to identify the obtained peptide sequences.

Immunoblot analysis - Proteins were separated on 12% SDS-polyacrylamide gels and transferred to PVDF membranes (ImmobilonTMP, Millipore, USA) overnight at 15 V using 25 mM Tris-HCl, 192 mM glycine. The membranes were blocked for 2 h at room temperature in TBST buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) containing 5% dry milk, and incubated for 1.5 h in TBST buffer with antibody against eIF5A at a

1:5000 dilution. Anti-eIF5A antibodies were raised against a specific peptide (LTSDGNTKDDLRLPT). After removing the unbound antibodies by washing (5 times for 5 min each) with TBST buffer, the blots were incubated with alkaline phosphatase-conjugated anti-rabbit antibodies at a 1:2000 dilution. After washing (as above) with TBST buffer, immunoreactive proteins were visualized by reaction with 0.3 mg/ml NBT (nitroblue tetrazolium) and 0.16 mg/ml BCIP (5-bromo-4-chloro-indolyl-phosphate) in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 5 mM MgCl₂.

Cloning, expression and purification of ZmCK2 α -1 - The pGEX-4T-1 expression vector was used to express CK2 α -1 in *E. coli*. Plasmid pT7-7 for maize full-length CK2 α -1 as a template and two primers 1 and 2 (for sequences of primers see Supplementary Table 1) were used for PCR. The PCR product was cloned into the TOPO[®] vector for amplification and subsequently subcloned into the SmaI/SalI sites of pGEX-4T-1 expression vector. The GST:ZmCK2 α -1 construct was transformed into *E. coli* BL21(DE3). For induction of GST:ZmCK2 α -1 expression, 1 mM IPTG was added for 4 h at 37°C. The expressed protein was purified using glutathione-Sepharose 4B resin according to manufacturer's instruction. To remove the GST tag the fusion protein bound on the glutathione affinity resin was treated with thrombin. After overnight cleavage, pure ZmCK2 α -1 without the GST tag was separated from the resin by centrifugation. The yield was 0.1 mg from 1 l of bacterial culture.

Cloning of ZmeIF5A (Z. mays), AteIF5A (A. thaliana) and SceIF5A (S. cerevisiae) - Total RNA was isolated from *Z. mays* seedlings or *A. thaliana* leaves using TRI[®] Reagent according to the manufacturer's instruction and used as a template for eIF5A cloning. Total RNA was reverse transcribed using HSRT 100 kit. The reaction was performed for 50 min at 47°C in a final volume of 20 μ l containing 1 U of enhanced avian reverse transcriptase, 500 μ M each dNTP, 1 U of RNase inhibitor, and 3.5 μ M oligo (dT) primer. Two microliters of the reverse transcription reaction was used for PCR in a 20 μ l volume containing 0.4 U of JumpStart AccuTaq LA DNA polymerase, 100 μ M each dNTP, 1.5 mM MgCl₂, and 300 nM of primers 3 and 7 for *ZmeIF5A* or 15 and 17 for *AteIF5A*. PCR conditions were: 3 min, 94°C (first cycle);

30 s, 94°C; 30 s, 55°C; 1 min, 68°C (25-35 cycles), and 10 min, 68°C (final cycle). The PCR product was cloned into TOPO[®] vector. For amplification of *ScelIF5A* genomic DNA isolated from *S. cerevisiae* according to (25) and primers 12 and 14 were used for PCR at the above conditions. The obtained product was cloned into TOPO[®] vector.

Site-directed mutagenesis of ZmeIF5A, AteIF5A and ScelIF5A. – The TOPO[®] vector containing the *eIF5As* was used as templates for site-directed mutagenesis. Two constructs of ZmeIF5A were generated with the following substitutions: ZmeIF5ASer2Ala and ZmeIF5ASer4Ala. Primers 4 and 7, and 5 and 7 were used in the PCR for the Ser2Ala and Ser4Ala mutation, respectively. To generate the Ser2Ala substitution of AteIF5A and ScelIF5A primers 16 and, 17 and 13 and 14 were used, respectively.

To create Δ Met-ZmeIF5A, primers 6 and 7 were used (Fig. 1S panel A, rows 1, 3). (Deletion of the first Met from the ZmeIF5A sequence moves Ser2 to position +2 in the thrombin cleavage site in the GST- Δ Met-ZmeIF5A fusion protein.) The PCR product was cloned into TOPO[®] vector.

Expression and purification of ZmeIF5A, AteIF5A, ScelIF5A and their mutated variants – The pGEX-4T-1 expression vector was used to express *eIF5As* and its mutated variants in *E. coli*. ZmeIF5Awt, ZmeIF5ASer2Ala, ZmeIF5ASer4Ala, AteIF5Awt, AteIF5ASer2Ala, ScelIF5Awt and ScelIF5ASer2Ala were subcloned from TOPO[®] vector into the EcoRI/SalI or EcoRI/XhoI sites, whereas Δ Met-ZmeIF5A was subcloned into the BamHI/SalI sites of pGEX-4T-1 (see Supplementary Table 1). The constructs were transformed into *E. coli* BL21(DE3), expressed and purified using glutathione-Sepharose 4B according to manufacturer's instruction. For induction of expression, 0.5 mM IPTG was added for 4 h at 37°C. Cleavage of GST from the recombinant proteins using thrombin was carried out overnight at 4°C in Thrombin Cleavage Buffer (100 U/ml thrombin, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2.5 mM CaCl₂). GST bound to the resin was removed by centrifugation. Glycerol was added to the final concentration of 10% and the obtained proteins were frozen in liquid nitrogen. The yield was about 1 mg from 1 l of bacterial culture. When cleavage by thrombin was used to check for Ser2 phosphorylation, glutathione-Sepharose (500 μ g) with bound GST- Δ Met-ZmeIF5A was divided

into halves which were separately incubated for 1 h at 37°C: in 20 mM Tris HCl, pH 7.5 containing 20 mM MgCl₂ (nonphosphorylated); and the second half in the same buffer with ATP (50 μ M) and recombinant ZmCK2 α -1 (15 μ g) (phosphorylated). After incubation both portions of the resin, containing, respectively, nonphosphorylated and phosphorylated GST- Δ Met-ZmeIF5A, were separately washed 5 times with 1 ml of 50 mM Tris-HCl, pH 8, 100 mM NaCl and 2.5 mM CaCl₂. Cleavage by thrombin (100 U/ml) was carried out overnight at 4°C in the above buffer.

Constructs of wild type and mutated ZmeIF5A tagged with EYFP – Reverse primer 9 and forward primers 3, 4 or 8 were used for amplification of full-length cDNA of ZmeIF5Awt, ZmeIF5ASer2Ala, and ZmeIF5ASer2Asp, respectively. For amplification of full-length EYFP cDNA primers 10 and 11 were used. The PCR products were cloned into TOPO[®] vector. ZmeIF5Awt, ZmeIF5ASer2Ala, or ZmeIF5ASer2Asp were separately connected with EYFP by the restriction site for HindIII and inserted into the pLNU expression vector using EcoRI and XhoI/SalI sites. pLNU is a pUC-based vector (<http://www.dna-cloning.com/index.php>) carrying a constitutively active maize ubiquitin *Ubi-1* promoter with the first *Ubi-1* intron (Fig. 2S, panel A). The *Ubi-1* intron was used to enhance transgene expression in monocots (26). The *Ubi-1* intron sequence ends with a PstI restriction site containing 3' splice junction. The nucleotide sequence of the 3'-region of the *Ubi-1* intron and the 5'-polylinker sequence of pLNU vector is presented in Supplementary data (Fig. 2S, panel B). The *ZmeIF5A-EYFP* sequence begins with ATG, therefore the expression of ZmeIF5A-EYFP protein starts from the native methionine codon.

All constructs were sequenced to confirm the correctness and the introduced changes.

Sequences of primers 1-17 used for cloning are given in Supplementary Table 1S.

The numbering of amino acid residues in recombinant *eIF5A* is in accordance with the numbering of the encoded amino acid sequence of native ZmeIF5A. Detailed information concerning the additional N-terminal residues coming from the vector is provided in Supplementary data (Fig. 1S, panel A).

Protoplast transient expression assay – The preparation and electroporation of maize mesophyll protoplasts was performed according

to the protocol described previously (27). Maize protoplasts (about 2×10^5) were transformed separately with 50 μg of plasmid DNA (*ZmeIF5Awt-EYFP*, *ZmeIF5ASer2Ala-EYFP*, and *ZmeIF5ASer2Asp-EYFP*) and about 50 μg of carrier DNA. The transformed protoplasts were incubated at 22°C for 16 h, and the fluorescent labeled proteins visualized by confocal microscopy (Eclipse TE200-E, Nikon).

In-vitro phosphorylation assay – Recombinant proteins: *ZmeIF5Awt*, *ZmeIF5ASer2Ala*, *ZmeIF5ASer4Ala*, *AteIF5Awt*, *AteIF5ASer2Ala*, *SceIF5Awt*, *SceIF5ASer2Ala* or casein as a positive control (3 μg of each) were separately incubated for 20 min at 30°C with recombinant *ZmCK2 α -1* at the concentration of the enzyme giving V_{max} (0.1 μg of protein) and [γ - ^{32}P]ATP (50 μM , 100-200 cpm pmol^{-1}) in 50 μl of 20 mM Tris-HCl, pH 7.5, 20 mM MgCl_2 . After incubation, 40 μl of the assay mixture was spotted onto a square (2 x 2 cm) of 3MM Whatman paper, which was then immediately immersed in cold 5% (w/v) trichloroacetic acid containing 0.3% *o*-phosphoric acid, and washed five times for 10 min each. Then the squares were washed in 96% ethanol and allowed to dry. The radioactivity was quantified using a liquid scintillation counter. Efficiency of phosphorylation was calculated from the specific radioactivity of ^{32}P incorporated into the given amounts of proteins, and was expressed as mol of phosphate/mol of *ZmeIF5As* and mol of phosphate/mol of casein. Alternatively, the reaction products were subjected to SDS-PAGE, visualized by Coomassie Brilliant Blue and analyzed by autoradiography.

Gel filtration of ZmeIF5A – Filtration was performed at 4°C on a Superdex G-200 10/300 GL FPLC column calibrated with molecular mass markers. Recombinant *ZmeIF5A* (100 μg) was applied on the column previously equilibrated with 100 mM Tris-HCl, pH 7.5, 0.01% Tween, 1 mM EDTA containing 0.3 M NaCl. Filtration was performed with the same buffer.

RESULTS

Identification of maize eIF5A (ZmeIF5A).

After gel filtration, the last step of purification of native CK2 from maize seedlings (described in the Experimental Procedures), two peaks exhibiting CK2 activity were eluted: the first

peak of about 130 kDa (designated I_{130}), and the second one of about 40 kDa (designated II_{40}) (Fig. 1A). After electrophoresis of proteins in 12% SDS-PAGE, gel fragments with proteins from both peaks (stained with Coomassie Brilliant Blue) were cut out and the proteins were identified by MS (Table I). The analysis demonstrated that peak I_{130} contains three CK2 β (β_1 , β_2 , β_3) and one isoform of CK2 α . The calculated mass of the eluted proteins and MS results indicate that holoenzyme of CK2 ($\alpha_2\beta_2$) is present in this peak. In peak II_{40} , containing proteins of lower molecular masses, three isoforms of the CK2 α (α_1 , α_2 , and α_3) were detected, but CK2 β , which confirmed the previous supposition (20) that in plants CK2 may be present also as an active free CK2 α .

For identification of endogenous CK2 α substrates, the proteins that co-purified with CK2 α in peak II_{40} were allowed to undergo phosphorylation by endogenous protein kinase(s) using [γ - ^{32}P]ATP as the phosphate donor. After separation of the phosphorylated proteins by SDS-PAGE, the gel was dried and exposed to autoradiography. A protein of about 18 kDa was phosphorylated. To demonstrate specificity of the phosphorylation, the following inhibitors of protein kinases were added to the phosphorylation assay (see Legend to Fig. 1): heparin (28) and 4, 5, 6, 7 - tetrabromobenzotriazole (TBBt) (for review see Ref. 29), which selectively inhibit CK2 activity; and staurosporine, an inhibitor of many protein kinases but not CK2 (30). The phosphorylation of the protein(s) was not decreased by staurosporine, whereas heparin and TBBt abolished the phosphorylation completely (Fig. 1B). The results clearly indicate that the phosphorylation is catalyzed by CK2. Fragment of the gel which contained the protein(s) of 18 kDa was cut out from the wet gel as indicated in Fig. 1C and subjected to MS analysis. Two high mobility group (HMG) proteins characterized before as CK2 substrates were identified: HMGB1 and HMGc1 (Acc. nos. CAA46876 and CAA69605, respectively). Phosphorylation of cytosolic HMG homologues of the animal HMG-1 subgroup by CK2 was observed previously in broccoli (31). Phosphorylation of HMGB1 by CK2 modulates its stability and DNA binding properties (32). Among the 18-kDa proteins, we also identified eukaryotic translation initiation factor 5A (eIF5A) by the presence of two peptides (VVEVSTSK and LPTDETLVAQIK).

Maize *eIF5A* (Acc. no. YO7920) consists of 480 nucleotides encoding 160 amino acids.

To check whether ZmeIF5A is phosphorylated *in vivo* total proteins from maize leaves were separated by 2D-electrophoresis (Fig. 2). Among the phosphorylated proteins detected by Pro-Q[®] DSP staining (Fig. 2B) we identified one spot containing a protein of about 18 kDa and a pI of about 5.5. This spot was analyzed by MS and five peptides of ZmeIF5A sequence were identified (Table 2). The result indicates that ZmeIF5A is phosphorylated *in vivo*.

A comparison of eIF5A sequences from different organisms showed that eIF5A proteins are highly conserved but have lower amino acid similarity at the terminal positions (Fig. 3). It should be stressed that in nine analyzed plants, worm (*C. elegans*) and yeast (*S. cerevisiae*), but not in another yeast (*S. pombe*) or animals (*D. melanogaster*, *H. sapiens*), eIF5As contain a serine residue at position 2. Moreover, in all five analyzed monocots, an additional phosphorylatable residues (serine or threonine), surrounded by acidic amino acids, is present at position 4 (Fig. 3).

Phosphorylation of eIF5A by CK2 and identification of phosphorylated residue by site directed mutagenesis. Because eIF5A had not been identified before as a CK2 substrate, to investigate its phosphorylation by CK2 α in an *in vitro* system, recombinant ZmeIF5Awt, AteIF5Awt, SceIF5Awt, and ZmCK2 α -1 proteins were produced in *E. coli* (see Experimental Procedures). To identify which serine residue(s) (Ser2 and/or Ser4) is phosphorylated in eIF5As, these serine residues were individually mutated to alanine, which cannot undergo phosphorylation, and the resulting mutated proteins ZmeIF5ASer2Ala, ZmeIF5ASer4Ala, AteIF5ASer2Ala and SceIF5ASer2Ala were expressed in bacteria. Wild and mutated variants of eIF5A were phosphorylated by recombinant ZmCK2 α -1, using casein as a positive control. ZmeIF5Awt and casein were phosphorylated efficiently. The efficiency of ZmeIF5Awt phosphorylation was 0.88 mol of phosphate/mol of protein indicating that wild type ZmeIF5A is a good substrate for CK2 α . The protein with Ser2 converted to Ala was not phosphorylated by CK2 (Fig. 4). The lack of phosphorylation of the mutated Ser2Ala variant indicates that only Ser2, and no other Ser/Thr residue present in ZmeIF5A, undergoes phosphorylation by CK2. The protein with Ser4 converted to Ala was phosphorylated less

efficiently (0.29 mol of phosphate/mol of ZmeIF5ASer4Ala) than the wild type of ZmeIF5A. The decreased incorporation of phosphate into the Ser4Ala mutated variant may indicate that the presence of the hydrophilic, hydroxyl group of Ser4 facilitates accessibility of Ser2 for CK2. Similarly to ZmeIF5Awt, eIF5As from the other organisms (*A. thaliana* and *S. cerevisiae*) were efficiently phosphorylated by CK2, while their mutated variants (Ser2Ala) were unable to accept phosphate groups (Fig. 5). The above results demonstrate that Ser2 present in plant and yeast of eIF5A is phosphorylated by CK2. To verify the site of phosphorylation, these phosphoproteins were analyzed by MS. However we were unable to detect any phosphorylation (data not shown), most probably because the phosphorylated residue is located at the terminal position of the protein. The failure to identify by MS an N-terminal peptide containing modified residue(s) has prompted us to elaborate a new method to confirm the phosphorylation of Ser2 in ZmeIF5A.

Identification of phosphorylated serine by exploiting the specificity of thrombin. The method elaborated for this purpose exploits the specific proteolytic cleavage by thrombin. The minimal consensus recognized by thrombin is BBPR_{-4 -3 -2 -1}↓UU_{+1 +2} (B - hydrophobic amino acid, P - proline, R - arginine, U - non-acidic amino acid), where the cleavage occurs after the arginine. In the GST-ZmeIF5A fusion protein the C-terminal part of the GST-tag (LVPRG) and the N-terminal amino acid of ZmeIF5A (Met) create a sequence recognized and cleaved by thrombin. After thrombin cleavage the recombinant protein contains an additional glycine residue derived from the GST-tag at the N-terminus (GlyMetSer). For thrombin to be used as a probe of Ser2 phosphorylation, the assumption was as follows. Removal of methionine from GST-ZmeIF5Awt (GST- Δ Met-ZmeIF5A) moves serine 2 to a critical for thrombin cleavage position +2 which has to be occupied by a non-acidic amino acid. When this serine is not modified, thrombin will recognize and cleave the sequence. However, if there is the negatively charged phosphoserine at position +2, the site no longer fulfils the consensus criteria for thrombin and the protease should not be able to cleave the sequence (Fig. 6A). Therefore, the construction of the modified fusion protein containing at position +2 a residue susceptible to phosphorylation should allow distinguishing

between a nonphosphorylated (cleaved by thrombin) and a phosphorylated (not cleaved by thrombin) protein.

After thrombin treatment, phosphorylated and nonphosphorylated GST- Δ Met-ZmeIF5A were analyzed by SDS-PAGE. In the experiment where GST- Δ Met-ZmeIF5A was not phosphorylated, thrombin action produced two proteins, as expected: GST and Δ Met-ZmeIF5A (Fig. 6A and C). When eIF5A was phosphorylated, only one protein band of higher molecular mass (45 kDa) was detected (Fig. 6B and C) indicating that the phosphorylated fusion protein was resistant to thrombin. This approach employing the specificity of protease cleavage can be applied for confirmation of postulated phosphorylation sites also in other proteins.

Dimerization of ZmeIF5A. To check whether ZmeIF5A assumes a monomeric or oligomeric form, gel filtration of the recombinant protein was carried out on Superdex G-200 in a buffer containing 0.3 M NaCl to preclude non-specific ionic interactions (Fig. 7A). ZmeIF5A was detected in the eluted fractions by its phosphorylation by recombinant ZmCK2 α -1 (Fig. 7B) and by Western blotting using anti-eIF5A antibodies (Fig. 7C). ZmeIF5A was present in fraction 15 containing proteins of about 40 kDa (Fig. 7). This indicates that ZmeIF5A occurs in a dimeric form.

Role of ZmeIF5A phosphorylation by CK2. To shed light on the physiological significance of phosphorylation of Ser2 in ZmeIF5A, transient expression of wild type eIF5A and its variants mutated at position 2 of the encoded amino acid sequence was performed in maize protoplasts. In one mutated form the serine was substituted by the neutral alanine, and in the second, by aspartic acid which mimics phosphoserine. As a reporter of expression Enhanced Yellow Fluorescent Protein (EYFP) was used either alone (as a control) or as a C-terminal tag of ZmeIF5A. Four constructs in pLNU vector were prepared individually (Supplementary Table 2S). After expression of the proteins in maize protoplasts (see Experimental Procedures), the intensity of fluorescence in the nucleus and cytoplasm was measured by confocal microscopy in optical sections of the protoplasts (Fig. 8A). EYFP was located both in the nucleus and in the cytoplasm (for which the ratio of fluorescence intensity was 1:1). Also ZmeIF5Awt-EYFP (ratio 1:1.21, SD 0.23) and ZmeIF5ASer2Ala-EYFP (ratio 1:1.09, SD 0.29) were evenly distributed between the

nucleus and the cytoplasm. In contrast, the ZmeIF5ASer2Asp-EYFP protein exhibited an about 2-3-fold higher fluorescence in the nucleus (ratio 1:2.91, SD 0.61) than in the cytoplasm (Fig. 8B). The acidic group of aspartic acid that mimics phosphorylated serine seems to inhibit the nuclear-cytoplasmic shuttling, leading to preferential ZmeIF5A accumulation in the nucleus. The obtained results suggest that phosphorylation of Ser2 plays a role in determining the intracellular distribution of ZmeIF5A.

DISCUSSION

Eukaryotic translation initiation factor 5A (eIF5A) is a highly conserved 18-kDa protein present in all eukaryotic cells (33), involved in a broad spectrum of cellular functions, including proliferation and cell cycle control (for review see Refs 34, 35). eIF5A is the only known cellular protein that contains the unusual positively charged amino acid hypusine, a derivative of lysine. Hypusination is essential for eukaryotic cell proliferation and apoptosis (for review see Ref. 34).

Currently available results indicate that in plants, like in other eukaryotic organisms, eIF5A is involved in many cellular functions. Three isoforms of eIF5A have been identified in *Arabidopsis* (36). Isoform 1 (AteIF-5A1) plays a role in xylem formation (37), whereas isoform 2 (AteIF-5A2) regulates programmed cell death caused by pathogen infection (38). Reducing the level of eIF5A in *Arabidopsis* has dramatic effects on the plant's growth and development. The developmental defects are associated with abnormal cell division and cell growth (39). In tomato (*Lycopersicon esculentum*), a correlation has been found between expression of *eIF5A* and programmed cell death (40). In rice (*Oryza sativa*), two cDNA clones encoding eIF5A have been characterized. Analysis of mRNA level showed that *OseIF5A-1* and *OseIF5A-2* were expressed in leaves and panicles, and relatively high amounts of both transcripts were detected in old leaves. In addition, both *OseIF5A-1* and *OseIF5A-2* levels were regulated during leaf development, sugar starvation and environmental stresses (41). In maize, eIF5A is expressed in most tissues investigated. Unfertilized egg cells of maize in G0 phase contain a high level of the *eIF5A* transcript. The transcript is strongly induced during the G1 phase, but continuously decreases during the S, G2 and M phases (42).

The authors suggest that unfertilized egg cells of maize are prepared for selective mRNA translation that is quickly triggered after fertilization. The triggering could be regulated by reversible phosphorylation known as the key modification of proteins involved in the regulation of cellular activities including gene expression, proliferation and differentiation (43). The N-terminal sequence of plant eIF5A contains a consensus motif for CK2 ((S/T)X₀X₊₁Z₊₂₊₃, where S/T is serine or threonine, X - non-basic amino acid, and Z – an acidic or phospho-amino acid), suggesting that this enzyme might be involved in the eIF5A regulation by phosphorylation. Indeed, we have shown here that phosphorylation of ZmeIF5A occurs *in vivo* in maize leaves. Moreover, our results indicate that Ser2 is the target of CK2 not only in the case of ZmeIF5A, but also in eIF5A from other organisms (*A. thaliana* and *S. cerevisiae*). It is worthy to add that these serine residues are absent in human, insect and another yeast (*S. pombe*) eIF5As. Interestingly, in monocots an additional potentially phosphorylatable 4 residue (Ser/Thr) is present in position 4, although the CK2 consensus for this residue is predicted to be weak (3). In case of ZmeIF5A, *in vitro* phosphorylation of Ser4 by CK2 was not observed. The role of Ser/Thr4 in monocots might be in enhancing Ser2 phosphorylation by CK2. Ser/Thr4 is located at position +2 in the consensus of Ser2 phosphorylation by CK2, were acidic amino acids (Asp, Glu, P-Tyr, P-Ser, P-Thr) predominate (3). One can assume that phosphorylation of Ser4 by other protein kinases could also enhance the phosphorylation of Ser2 by CK2. Other eukaryotic proteins containing CK2-phosphorylated Ser2 are known, e. g., translation initiation factor 2β (eIF2β, MSGDEMI...) or CK2β (MSSSEEV...) (44, 45).

Transfection of HeLa cells with human eIF2β (carrying the HA-tag) or its phosphorylation site mutants showed Ser2 as the main site for constitutive eIF2β phosphorylation by CK2 (44). In CK2β Ser2, and to a lesser extent Ser3, are phosphorylated; both fulfill the CK2 consensus sequence requirements (3). The N-terminal region of CK2β also contains several docking sites for proteins which are involved in many essential functions, like cellular localization, control of translation and protein degradation (46). These results suggest that the N-terminus of

the CK2β reflects the supramolecular organization of the native protein complex, in which phosphorylation plays an important regulatory role (45). This suggestion was partially confirmed by Bodenbach et al. (47), which showed that in the recombinant holoenzyme of CK2 (expressed in a tandem arrangement without any tag), Ser2 and Ser3 are phosphorylated. We have demonstrated that CK2 can phosphorylate Ser2 of recombinant ZmeIF5A irrespective of additional residues located upstream (Supplementary data Fig. 1S, panel B).

All three CK2β (β1, β2, β3) identified in maize are able to interact with CK2α subunits as the dimer, which results in the formation of a stable tetrameric form of the holoenzyme (19). Similarly to the CK2β, the dimeric form of ZmeIF5A could play a role of a scaffold protein assembling specific partners. Interestingly, such binding ability has been shown for eIF2β (48). Llorens and coworkers have demonstrated that eIF2β, besides serving as a substrate for CK2 holoenzyme, binds free CK2α and affects its enzymatic properties. This suggests that eIF2β might act as a CK2 anchoring protein, locating the kinase in close vicinity of its targets and helping to maintain some components of the translation machinery in a functional conformation (48).

The role of eIF5A phosphorylation in plants and yeast is not known. Previous results (49) indicate that the phosphorylated and non-phosphorylated forms of yeast eIF5A exhibit virtually identical activities in the mammalian assay for methionyl-puromycin synthesis. However, it is possible that the *in vitro* assay system used for translation was not sufficiently sensitive to detect any differences, or that phosphorylation of eIF5A regulates other cellular processes. The N-terminal region of eIF5As has a high content of acidic residues, whereas the classical nuclear localization signals (NLS) are rich in basic residues (50). Using confocal microscopy and different constructs encoding eIF5A-related sequences fused to GFP, those authors have shown that the N-terminal region of mammalian eIF5A proteins is essential for the localization of the protein to the nucleus. Since the N-terminal region of most eukaryotic eIF5As studied is rich in acidic residues and does not contain phosphorylatable residue(s), those authors (50)

did not consider the role of phosphorylation in eIF5A localization. It is known that exportin 4 (Exp4) functions as a nuclear export receptor for eIF5A (51). eIF5A can specifically assemble into a trimeric eIF5A-Exp4-RanGTP export complex, which results in efficient nuclear export. The complex is disassembled when cytoplasmic RanBP1 and RanGAP are both present. The positively charged amino acid hypusine improves eIF5A binding to Exp4, thereby enhancing its nuclear export. It is possible that phosphorylation may cause an opposite effect - the sequestration of eIF5A in the nucleus by inhibiting its interaction with the exporting protein complex. Intramolecular masking of NLSs within cargo proteins to prevent IMP/EXP recognition is a common mechanism of regulation of the efficiency of nuclear transport. It can be effected by phosphorylation close to the target sequence to prevent IMP/EXP binding (for review see Ref. 52). There is evidence that phosphorylation by CK2 is mechanistically important in NLS recognition and enhances nuclear import by facilitating recognition by the transport receptor (53). Maize CK2 catalytic

subunits α contain a single functional NLS, consisting of a basic stretch of 20 amino acids (16) sufficient to target the protein to the nucleus of plant cells (19). The CK2 trafficking to the nucleus is a signal-mediated process (54, 55), whereas eIF5A can enter the nucleus via passive diffusion, as revealed for a human isoform (56). We suggest that eIF5A can easily enter into the nucleus, but its transport from the nucleus is arrested by the acidic residue of phosphoserine, which in our experiment was mimicked by Asp. The Ser2Asp mutation increased the rate of accumulation of eIF5A in the nucleus because it could not be dephosphorylated.

In the case of ZmeIF5A, phosphorylation of Ser2 blocks its export from the nucleus by preventing interaction with the nuclear exporting complex; whereas P-Ser2 dephosphorylation would reactivate nuclear export of ZmeIF5A. It is possible that modulation of nucleocytoplasmic shuttling of eIF5A by phosphorylation may be a physiological mechanism unique to plants and yeasts regulating specific nuclear export of eIF5A-bound mRNAs.

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FOOTNOTES

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The abbreviations used are: CK2 - casein kinase 2/protein kinase CK2, CK2 α - catalytic α subunit of CK2, CK2 β - regulatory β subunit of CK2, eIF5A - eukaryotic translation initiation factor 5A, Exp4 - exportin 4, EYFP - enhanced yellow fluorescent protein, GFP - green fluorescent protein, GST - glutathione S-transferase, IPTG - isopropyl β -D-1-thiogalactopyranoside, NLS - nuclear localization signal, TBBt - 4,5,6,7-tetrabromo-1H-benzotriazole, 2-DE - two-dimensional electrophoresis, MS - mass spectrometry, Pro-Q[®]DPS - Pro-Q[®] Diamond phosphoprotein gel stain, wt - wild type (without mutations), Δ Met - means lack of the first methionine in a protein.

FIGURE LEGENDS

Fig. 1. Analysis of CK2 activity in maize seedlings. A. Separation on Sephadex G-100 of two peaks (I₁₃₀ and II₄₀) of the kinase activity. CK2 activity was determined as described in Experimental Procedures using casein as a substrate (see *In vitro* phosphorylation assay). B. Phosphorylation of proteins in peak II₄₀ was performed in 50 µl of reaction mixture containing 50 µM ATP, 20 mM Tris HCl pH 7.5, 20 mM MgCl₂; alone (lane 1), in the presence of 10 µM staurosporine (lane 2), 10 µM heparin (lane 3) or 50 µM TBBt (lane 4). [γ -³²P]ATP was used for detection of phosphorylation whereas non - radioactive ATP for MS analysis. After 20 min of incubation at 30°C the reaction was stopped by addition of Laemmli buffer, heated at 95°C for 5 min and subjected to SDS-PAGE. In the case of proteins phosphorylated with [γ -³²P]ATP, the gel was dried and exposed to autoradiography. C. SDS-PAGE of proteins from peak II₄₀. For MS analysis appropriate protein band (18 kDa) stained with Coomassie Brilliant Blue was cut out from wet gel.

Fig. 2. Two-dimensional gel electrophoresis of proteins from maize leaves. A. Gel stained with Coomassie Brilliant Blue. B. Phosphoproteins visualized with Pro-Q[®] DPS. ZmeIF5A is indicated by white arrow.

Fig. 3. Alignment of eIF5A sequences from different kingdoms. The sequences are derived from: *Z. mays* (Y07920), *O. sativa* (NP0011051336), *T. aestivum* (AAZ95173), *S. sylvestre* (ABB29987), *D. sinicus* (ABW78939), *A. thaliana* (AAF87023), *M. trunculata* (ACJ85877), *N. tabacum* (CAA45105), *S. tuberosum* (BAA20876), *S. cerevisiae* (NP010880), *C. elegans* (NP495807), *S. pombe* (NP596130), *H. sapiens* (NP001961), *D. melanogaster* (NP611878). Identical residues are indicated by black background, dash indicates gap introduced to maximize alignment.

Fig. 4. Phosphorylation of ZmeIF5Awt and its mutated variants. Efficiency of phosphorylation is shown relative to casein phosphorylation taken as 100%. Values (in mol of phosphate/mol of protein) are given above the bars. *In vitro* phosphorylation was performed in three independent experiments as described in Experimental Procedures.

Fig. 5. Phosphorylation of recombinant eIF5A from *Z. mays*, *A. thaliana* and *S. cerevisiae*, and their Ser2Ala mutants by CK2.

Fig. 6. Effect of GST-ΔMet-ZmeIF5A phosphorylation on thrombin cleavage. Consensus sequence recognized by thrombin is in grey. Construct encoding GST-ΔMet-ZmeIF5A fusion protein was obtained and expressed as described in Experimental Procedures. A. Predicted effect of thrombin action on nonphosphorylated GST-ΔMet-ZmeIF5A. B. Predicted effect of thrombin action on phosphorylated GST-ΔMet-ZmeIF5A. C. SDS-PAGE visualization of thrombin treatment of nonphosphorylated (lane 1) and phosphorylated (lane 2) GST-ΔMet-ZmeIF5A.

Fig. 7. Gel filtration of recombinant ZmeIF5A. A. Elution profile of ZmeIF5A on Superdex G-200 FPLC column. Fractions of 0.5 ml were collected. B. Phosphorylation of ZmeIF5A. Each fraction was incubated with recombinant ZmCK2 α -1 and [γ -³²P]ATP. After SDS-PAGE, phosphorylated eIF5A was detected by autoradiography. C. Immunodetection. Western blotting using anti-eIF5A antibodies. For details see Experimental Procedures.

Fig. 8. Localization of ZmeIF5A-EYFP and its mutated variants in maize protoplasts. A. Optical sections by confocal microscopy. B. Relative intensities of fluorescence in the nucleus (taken as 1) and cytoplasm. Constructs are described in Supplementary Table 2S. The results are representative of eight independent experiments.

Table 1.
Identification of CK2 subunits in peaks I₁₃₀ and II₄₀ by MS

Accession No.	Name	Mascot score	Queries matched
Peak I₁₃₀			
gi 11526999	ZmCK2 β -1	152	3
gi 11527001	ZmCK2 β -2	130	4
gi 11527003	ZmCK2 β -3	160	5
gi 22116	ZmCK2 α -1	66	2
Peak II₄₀			
gi 22116	ZmCK2 α -1	1035	44
gi 3821792	ZmCK2 α -2	882	33
gi 162463502	ZmCK2 α -3	797	31

Table 2

Identification of maize eIF5A peptides by MS

Peptide sequence	Localization of peptide in ZmeIF5A sequence	Mascot score	Queries matched
TYPQQAGTVR	20-29	64	94
PTDETLVAQIK	119-131	99	121
AMGEEQICALK	145-155	67	128
LPTDETLVAQIK	118-131	53	141
DLVVTVQSAMGEEQICALK	137-155	101	228

Fig. 1

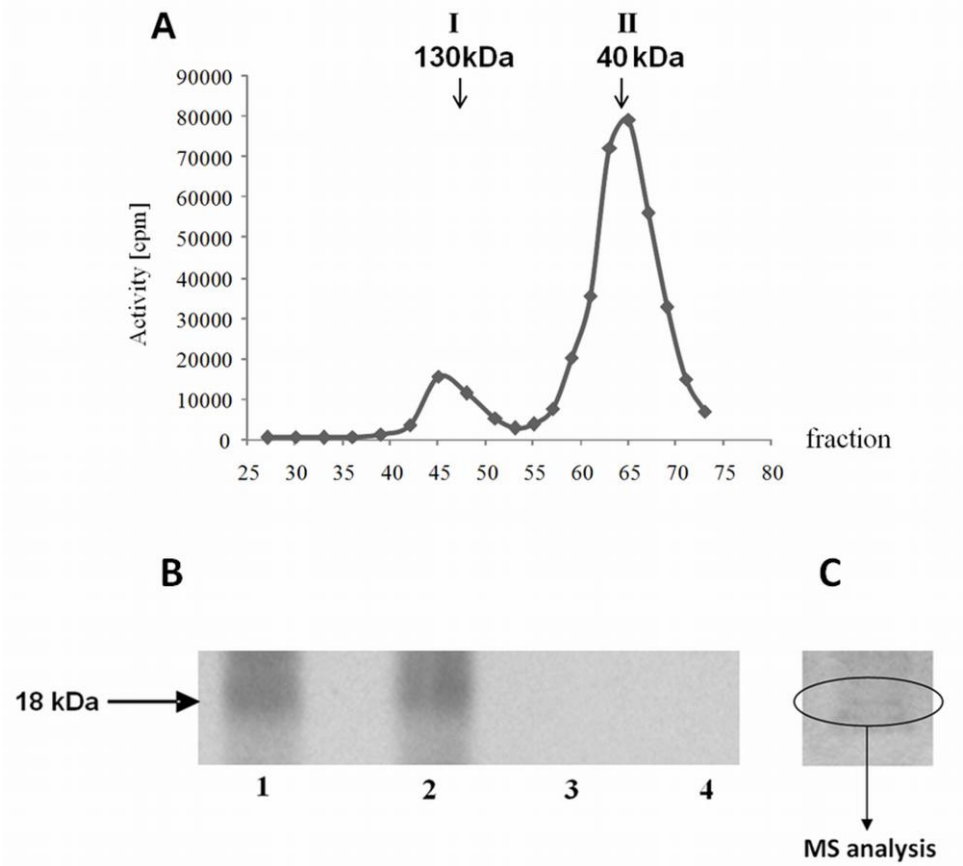


Fig. 2

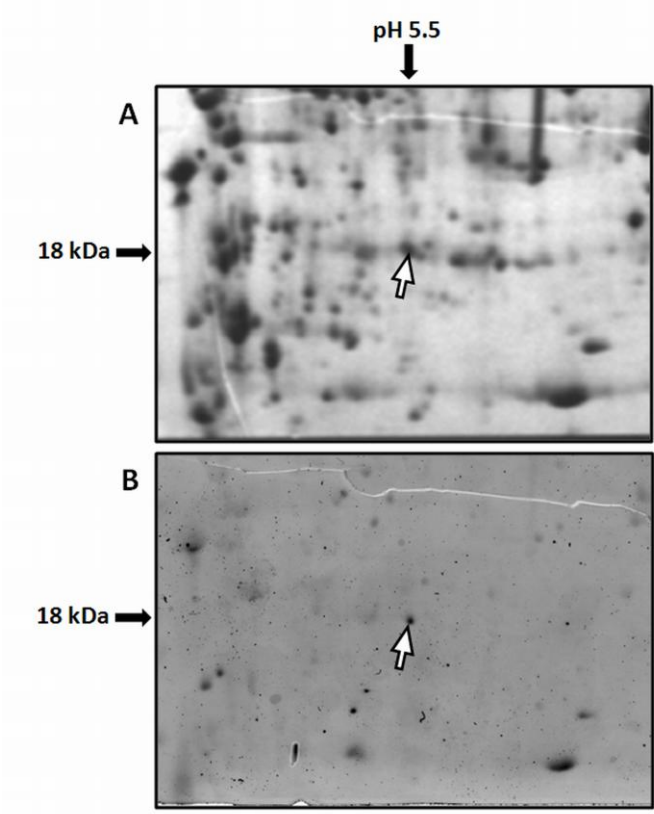


Fig. 3

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Zea mays : MSDS--BEHHESKADAGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 82
Oryza sativa : MSDS--BEHHESKADAGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 82
Triticum aestivum : MSDT--DEHHESKADSGASKIYDQAGAIRKGGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 82
Secale sylvestre : MSDT--DEHHESKADSGASKIYDQAGAIRKGGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 82
Dendrocalamus sinicus : MSDS--BEHHESKADAGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 82
Arabidopsis thaliana : MSD---DEHHESA-SESGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 80
Medicago truncatula : MSD---BEHHESKADAGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 81
Nicotiana tabacum : MSD---BEHHESKADAGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 81
Solanum tuberosum : MSD---BEHHESKADAGASKIYDQAGTIRKNGHIVIKNRPKVVEVSTSKTGKKGHAKCHEVAIDIFNGKKLEDIVPSSHN C : 81
Saccharomyces cerevisiae : MSD---BEHTRET-ADGSSAIFYMOCASLRKNGFVVLKSRPKIVDMSTSKTGKKGHAKVHLVAIDIFNGKKLEDIVPSSHN C : 80
Ceanorhabditis elegans : MSDHHDDEHHT-GDGGAAATPKQCSALRKNHVMIKSRPKIVDMSTSKTGKKGHAKVHLVAIDIFNGKKLEDIVPSSHN C : 83
Schizosaccharomyces pombe : MAB---EBHVDEG-GEAGASLTFMOCASLRKNGHIVIKNRPKIVDMSTSKTGKKGHAKVHLVAIDIFNGKKLEDIVPSSHN C : 81
Homo sapiens : MAD---DLD-RET-GDAGASLTFMOCASLRKNGFVVLKSRPKIVDMSTSKTGKKGHAKVHLVAIDIFNGKKLEDIVPSSHN C : 79
Drosophila melanogaster : MADM--DDH-RET-TDGSASSIFYMOCASLRKNGFVVLKSRPKIVDMSTSKTGKKGHAKVHLVAIDIFNGKKLEDIVPSSHN C : 80

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Zea mays : DVEHVNRTDYQ-LIDISEDGEVSLIT-ESGGTKDDLRLP TDETL-VAQIKEGEESGK-DIVVTVQSAMGSEQICALKDVGPK-- : 160
Oryza sativa : DVEHVNRTDYHYLLIISDGEVSLIT-ESGGTKDDLRLP SDEAL-LTQIKDGFAGK-DLIVTVMSAMGSEQICALKDVGPK-- : 162
Triticum aestivum : DVEHVNRTDYQ-LIDIDDDGVVSLIT-ESGNTKDDLRLP TDDVL-LGQIKTGFADGK-DLIVLSVMSAMGSEQICAVKIGGGK-- : 161
Secale sylvestre : DVEHVNRTDYQ-LIDIDDDGVVSLIT-ESGNTKDDLRLP TDDVL-LGQIKTGFADGK-DLIVLSVMSAMGSEQICAVKIGGGK-- : 161
Dendrocalamus sinicus : DVEHVNRTDYQ-LIDISEDGEVSLIT-ENGNTKDDL-LE TGRALCILLQKDGEPKPK-DLIGNVMSVRGSEQICALKDVGPK-- : 160
Arabidopsis thaliana : DVEHVNRTDYQ-LIDISEDGEVSLIT-DSGGTKDDLRLP TDDGL-TAQLRKGDEGK-DLIVSVMSAMGSEQICAVKIGGGK-- : 159
Medicago truncatula : DVEHVNRTDYQ-LIDISEDGEVSLIT-ESGGTKDDLRLP TDETL-LSQIKDGFAGK-DLIVSVMSAMGSEQICGLKDVGPK-- : 159
Nicotiana tabacum : DVEHVNRTDYQ-LIDISEDGEVSLIT-ENGNTKDDLRLP TDDNL-LALIKDGFAGK-DLIVLSVMSAMGSEQICAVKIGGGK-- : 159
Solanum tuberosum : DVEHVNRTDYQ-LIDISEDGEVSLIT-ENGNTKDDLRLP TDDAL-LNQLKGFAGK-DLIVLSVMSAMGSEQICAVKIGGK-- : 160
Saccharomyces cerevisiae : EHVVKRREYL-LMAI--DGGVSLMN-MDGDVKKAP EGEL--GDSIQTADEGK-DLIVTVMSAMGSEARAIKFAARTD-- : 157
Ceanorhabditis elegans : DVEHVNRTDYQ-LVNI--DGGVSLMN-TDGTIKDDLRLP TDEL--GQQRDAVEKDEGSVIVQVVAIGBEAILGWVSTKE-- : 161
Schizosaccharomyces pombe : DVEHVNRTDYQ-LVNI--DGGVSLMN-TDGTIKDDLRLP TDEL--GNEHEGFEAGK-DLIVTVMSAMGSEALACROAPSA-- : 157
Homo sapiens : DVENKRNDFQ-LTGTQD-GVLSLLQ-DSGVEKDDLRLP EGDL--GKBEQKYOCCG-DLIVTVMSAMTSEAAVAIKAMAK--- : 154
Drosophila melanogaster : DVENKRNDFQ-LTGTQD-GVLSLLQ-EGDLEKDDLRLP TDEL--GQQRDAVEKDEGSVIVQVVAIGBEAILGWVSTKE-- : 159

```

Fig. 4

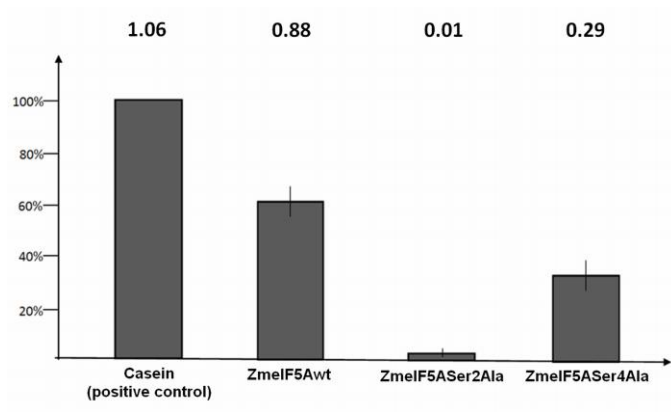


Fig. 5

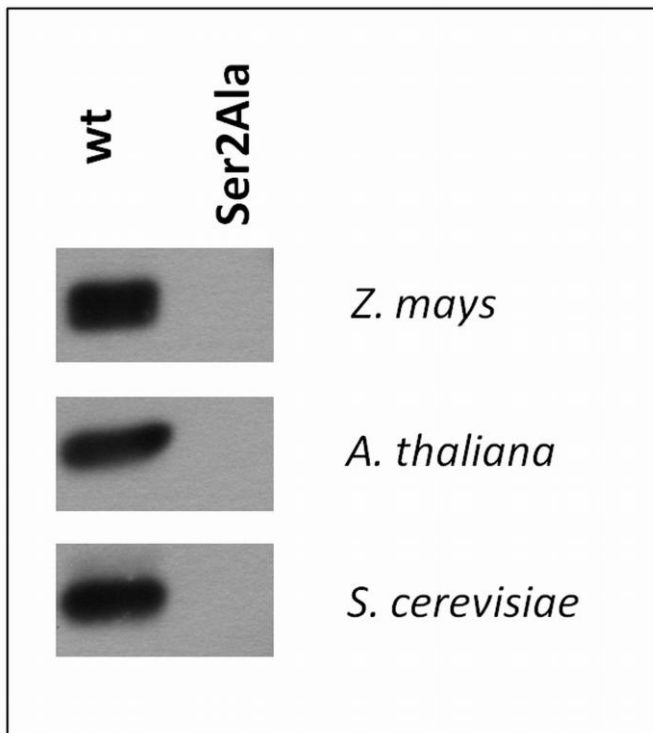


Fig. 6

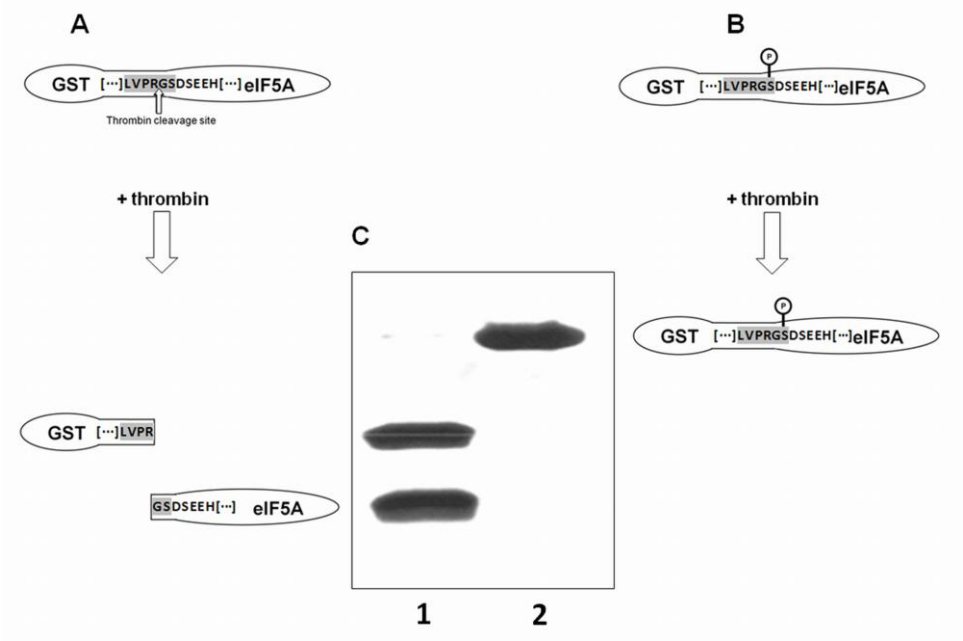


Fig. 7

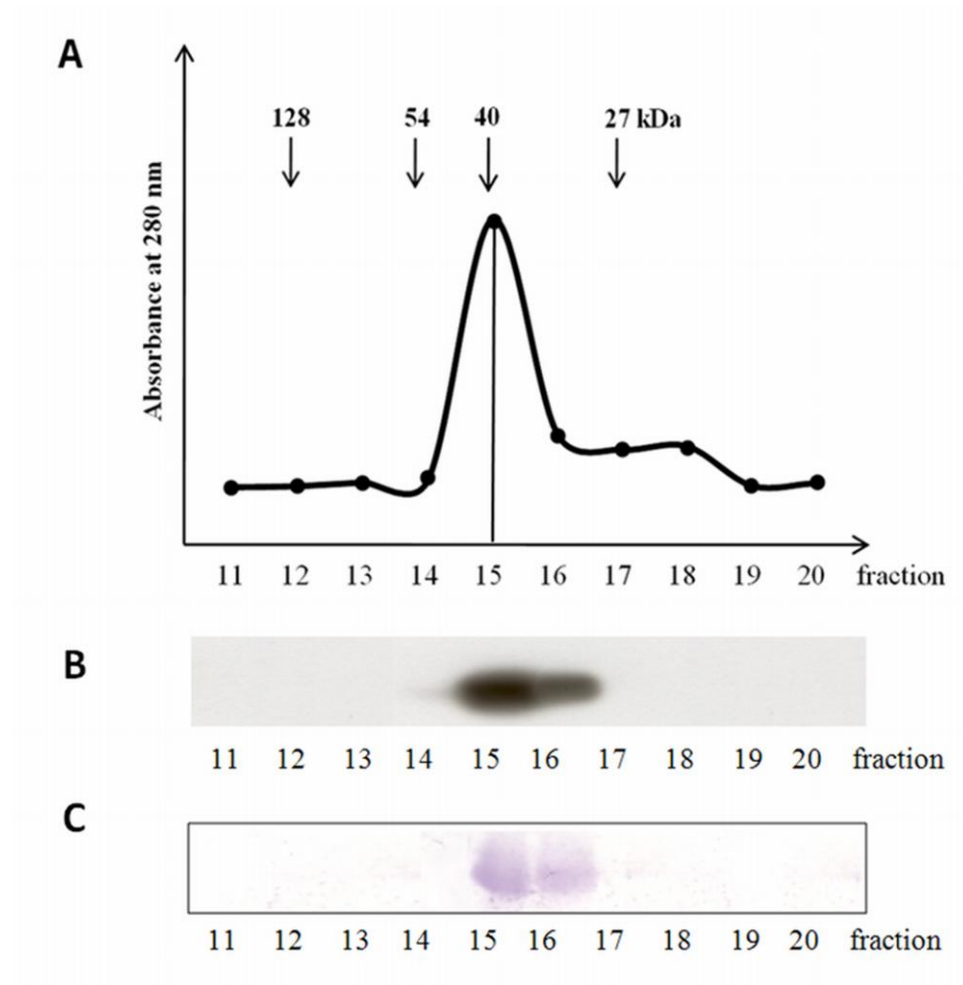


Fig. 8

