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Evaluation of Translational Control Mechanisms in Response to Oxygen Deprivation in Maize*

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Abstract—Numerous changes in gene expression occur in response to flooding (oxygen deprivation, i.e., anoxia and hypoxia) in seedling roots of maize (*Zea mays* L.) and other plants. Increased *de novo* transcription of anaerobic polypeptide (ANP) genes is responsible, in part for increased production of glycolytic and fermentative enzymes, such as alcohol dehydrogenase-1. There is increasing evidence that regulation of mRNA translation plays an important role in the expression of ANP genes during oxygen deprivation. By quantitative analysis of ribosomal complexes, we demonstrated a dramatic decrease in polysomes and an increase in monosomes in maize seedling roots deprived of oxygen, indicative of regulation of translational initiation. We report that oxygen deprivation causes dynamic changes in the phosphorylation status of the eukaryotic initiation factors (eIF) eIF4E, eIF4A, and eIF4B. By affinity purification of initiation complexes with ^{7m}GTP and poly(A) resins, we demonstrate that a reduction in pH, which occurs in the cytosol in response to this stress, affects the assembly of mRNA 5'-cap and 3'-tail-binding complexes. We also describe oxygen deprivation-induced changes in phosphorylation of ribosomal protein S6, ribosomal 12-kD P-proteins and eukaryotic elongation factor-2 (eEF2). A model is presented that considers the implication of modifications in translational machinery in the interactions between the 5'-cap and 3'-tail of the mRNA that facilitate initiation and eEF2 GTPase activity which promotes elongation.

Key words: Zea mays - ribosome - eukaryotic initiation factor - eIF4 - ribosomal protein S6 - eukaryotic elongation factor - poly(A)-binding protein - ribosomal P-protein

INTRODUCTION

Specific changes in gene transcription and protein accumulation are attributed to adaptation, tolerance, or resistance of plants to a number of abiotic and biotic stimuli [1–3]. Stress-induced changes in gene expression are frequently attributed to differential regulation of transcription. However, there is increasing evidence of rapid and reversible regulation of gene expression at the level of mRNA translation in response to transient environmental fluctuations in water availability, temperature and light intensity [4, 5]. Translation can be regulated at a global level or a message-specific level, where translation of an individual mRNA or a subset of cellular mRNAs is positively or negatively modulated [6, 7].

There is considerable evidence that mRNA translation is regulated in response to oxygen deprivation (hypoxia and anoxia) in seedling roots of maize (*Zea mays*) and other plants. The global regulation of translation can be monitored by centrifugation of cell extracts or pre-pelleted ribosomes through sucrose density gradients followed by spectrometric analysis of ribosomal subunits (40S and 60S), monosomes (80S), and polysomes (> 80S). Such polysome analyses demonstrated that oxygen deprivation (hypoxia followed by anoxia or sudden anoxia) causes a rapid disaggregation of polysomes and an accumulation of monosomes in cultured cells of soybean (*Glycine max* Merr.) and seedling roots of maize [8, 9]. Evidence of differential translation of individual mRNAs can be obtained by comparison of the spectrum of proteins synthesized *in vivo* to those produced *in vitro* by translation of purified mRNA. This approach made apparent post-transcriptional regulation in roots of maize and soybean following oxygen deprivation [10, 11]. In both cases, the complexity of proteins produced in the stressed roots was significantly lower than expected, even when detergents were used to efficiently extract proteins labeled *in vivo* (Bailey-Serres, unpublished). The translation of an individual mRNA can be examined by determination of the density of polysomes formed on that transcript. When maize roots were deprived of oxygen, mRNAs

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Abbreviations: eIF—eukaryotic initiation factor; eEF—eukaryotic elongation factor; dNTP—deoxynucleotide triphosphate; ORF—open reading frame; RPS6—ribosomal protein S6; PABP—poly(A)-binding protein; PCR—polymerase chain reaction; PMSF—phenylmethylsulfonyl fluoride; TEV—tobacco etch virus; UTR—untranslated region.

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that encode anaerobic polypeptides (ANPs, also referred to as stress proteins) (e.g., alcohol dehydrogenase-1 (ADH1), alcohol dehydrogenase-2 (ADH2), sucrose synthase-1 (SH1), sucrose synthase-2 (SH2), aldolase, and enolase) were associated with large polysomes, whereas mRNAs that encode nonstress cellular proteins (e.g., actin, mitochondrial adenine nucleotide translocator, eukaryotic initiation factor 4A (eIF4A), and ribosomal protein P2a) were maintained but show dramatically reduced association with polysomes [12, 13]. There is also evidence of decreased elongation of translation of mRNAs under anoxia. In oxygen-deprived maize roots *SH1* mRNA was associated with polysomes but inefficiently translated [13–15]. Similarly, in oxygen-deprived potato tubers wound-induced mRNAs were associated with polysomes but poorly translated [16]. These examples led to the hypothesis that the global down-regulation of translation as a consequence of oxygen deprivation is circumvented by a subset of cellular mRNAs, evidenced by the maintained translation of *ADH1* and other stress protein mRNA.

To unravel the molecular basis of translational regulation in response to oxygen deprivation we have developed tools to quantitatively evaluate mRNA translation, assess modifications in proteins involved in translation, and characterize preinitiation complexes. Here we report on dynamic changes in phosphorylation status of initiation factors that form the cap-binding complex, eukaryotic elongation factor 2 (eEF2) and several ribosomal proteins. We propose that the observed changes impact the physical interaction between the 5'- and 3'-ends of the mRNA that facilitates initiation and ribosome-mediated GTPase activation that promotes elongation.

MATERIALS AND METHODS

Plant material and oxygen deprivation treatments. *Zea mays* L. (inbred line B73, gift of Pioneer HiBred, Johnston, IA, United States) kernels were surface sterilized with 0.025% (v/v) sodium hypochlorite for 15 min and germinated on moist paper towels in trays in the dark for 5 days. Oxygen deprivation treatment was by complete submersion of 50 g of intact seedlings, taken directly from covered trays, in 3 l of induction buffer (0.5 mM Tris–HCl, pH 8.0, 7.5 μg/ml chloramphenicol) in a closed 1 gallon Mason jar into which 99.995% (v/v) argon or a gas mixture of 5% oxygen/95% argon continuously flowed. Positive pressure was maintained by allowing gas to exit through tubing into water in a 250-ml flask. The gas was released through a second hole in the stopper of the 250-ml flask. After 6 h of bubbling with 99.995% (v/v) argon, the oxygen concentration in the buffer had decreased from 274 to 13 μM. Seedlings were removed from the treatment chamber, root tips were quickly frozen on a metal plate standing on dry ice, and the apical 2 cm of the root tip was excised, placed in liquid nitrogen, pulverized, and stored at –80°C.

Polysome profile analysis from crude cell extracts. Polysome profile analysis was performed using a scaled down and modified version of an earlier protocol [12]. Approximately 0.2 g of frozen pulverized tissue was hydrated in an Eppendorf tube with 1 ml of polysome extraction buffer (200 mM Tris–HCl, pH 7.5, 200 mM KCl, 25 mM EGTA, 35 mM $MgCl_2$, 100 μM 2-mercaptoethanol, 50 μg/ml cycloheximide, 50 μg/ml chloramphenicol, 1% (v/v) Triton X-100, 1% (v/v) Brij 35, 1% (v/v) Tween-40, 1% (v/v) Nonidet P-40). Cell debris was removed by centrifugation at 14000 *g* for 2 min at 4°C and 350 μl of the supernatant was loaded onto a 4.5 ml 20 to 60% (w/v) sucrose gradient. Gradients were centrifuged for 90 min at 275000 *g* and analyzed with an ISCO Model 185 gradient fractionator (Lincoln, United States) and an UA5 detector (A_{254} nm) attached to a CIO-DAS08/Jr-AO-based (Computer Boards, United States) data acquisition system (for system details see, http://cepceb.ucr.edu/resources/links.htm#8). The gradient baseline was determined from the absorbance of a gradient loaded with 350 μl polysome extraction buffer and subtracted from the sample absorbance, samples were normalized to equal absorption (equal optical density) per gradient, and the areas under the monosome (80S ribosome and one ribosome per transcript), and large polysomes (>5 ribosomes per transcript) of the profile were calculated. The areas of the monosome and large polysome regions were calculated as a percentage of the total area under the profile, with the lower boundary defined at the base of the 40S subunit peak and the upper boundary defined as the bottom of the gradient.

Production of recombinant eIF4A and ribosomal protein S6 by overexpression in E. coli. Maize eIF4A and RPS6 were overexpressed in *E. coli* by insertion of the *eIF4A* (GenBank accession U17979) or *RPS6* (GenBank accession U92045) gene coding sequence into the pRO-EX vector (Gibco-BRL, United States), which provides a 25 residue amino-terminal extension including a His_6-tag and tobacco etch virus (TEV) protease cleavage site. For RPS6, the fusion protein was produced as described by Williams *et al.* [61]. For eIF4A, *Ehe*I, and *Sal*I sites were added to the 5' and 3' ends of the open reading frame (ORF) by use of polymerase chain reaction (PCR) and custom primers (*Ehe*I: 5'-GGGGCGCCATGGCAGGATTGGCACCA-GAAGGG-3' and *Sal*I: 5'-GGGGGTCGACCTA-GAGAAGGTCGGCGACATT-3'). PCR mix contained 5 mM KCl, 10 mM Tris–HCl, pH 9.0, 0.01% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 1 μM of each primer, 50 ng of plasmid DNA, 0.2 mM dNTPs, and 2.5 units of Taq polymerase and was performed for 30 cycles (2 min, 96°C; 1.5 min, 42°C; 2 min, 72°C). The PCR product was digested with *Ehe*I and *Sal*I and ligated into the vector pPRO-EX. Bacterial DH5α cells, in an exponential growth state carrying the eIF4A-ORF or the RPS6-ORF recombinant plasmid, were induced to transcribe the *LacZ* promoter by addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for eIF4A and 2 mM

IPTG for RPS6. For purification of recombinant eIF4A, cells were grown for 3 h at 37°C, lysed in Buffer A (50 mM Tris–HCl, pH 8.5, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C by sonication in Microson XL2005 sonicator (Heat Systems, United States). For purification of recombinant RPS6, cells were lysed in Buffer B (8 M urea, 100 mM sodium phosphate, 10 mM Tris–HCl, pH 8.0, 20 mM imidazole) for 2 h at room temperature. After lysis, cell debris was removed by centrifuged at 10000 *g* for 10 min. For purification of eIF4A, the supernatant was loaded onto a Ni-NTA bead (Qiagen, United States) column containing 1 ml of resin equilibrated in Buffer D (50 mM potassium phosphate buffer, pH 6.0, 300 mM KCl, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol). The column was washed 10 times with Buffer D, and His-tagged protein was eluted using Buffer E (50 mM potassium phosphate, pH 6.0, 300 mM KCl, 100 mM imidazole, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol). Ten 0.5 ml fractions of elutant were collected and an aliquot of each fraction was examined by fractionation through a Laemmli 12% SDS-PAGE gel followed by staining with Coomassie blue R250. The His_6-tag was removed from the overexpressed eIF4A-His_6 protein by cleavage with the addition of 30 units of TEV protease (Gibco-BRL) in 50 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol. For purification of RPS6, Ni-NTA beads equilibrated in Buffer B were added to the supernatant and stirred at room temperature for 1 h. The slurry was packed into a 1 ml column and washed five times in Buffer B, five times in Buffer C (8 M urea, 100 mM sodium phosphate, 10 mM Tris–HCl, pH 6.3, and 20 mM imidazole) and eluted using Buffer C containing 250 mM imidazole. Protein quantification was by comparison to BSA (0.5, 1 μg) standards on a 12% Laemmli SDS-PAGE gel and staining with Coomassie blue R250.

Production of antisera and affinity purification of antibodies. Polyclonal antiserum was produced against recombinant eIF4A and His_6-TEV-tagged RPS6 in rabbits by injection of 1 mg of protein in Freund's adjuvant. Antibodies against recombinant maize eIF4E [17] or recombinant maize eIF4A were affinity purified using as described by Sambrook *et al.* [18]. Antisera against recombinant maize RPS6 [61], native wheat eIFiso4F and eIF4B (kindly provided by K. Browning, University of Texas, Austin, United States) and native wheat PABP (kindly provided by D. Gallie, University of California, Riverside, Unites States) were used without purification.

Two-dimensional protein gel immunodetection analyses. An extract of total soluble protein was obtained by hydration of frozen pulverizing maize 100 to 200 root tips in 10% (w/v) TCA and 0.05% (v/v) 2-mercaptoethanol in acetone. The suspension was held at –20°C for 40 min and total soluble protein was isolated by centrifugation at 16000 *g* for 10 min. The protein precipitate was washed twice with 0.05% (v/v) 2-mercaptoethanol in acetone and allowed to air dry. Protein concentration was determined using the Bradford reagent. Total soluble protein (120 μg) from aerobic and 6-h oxygen-deprived seedling roots were loaded on isoelectric focusing (IEF) tube gels (3.6% acrylamide, 0.25% N',N'-methylene-*bis*-acrylamide, 9 M urea, 2% (v/v) ampholytes (0.5% pH 3–10, 1.5% pH 5–7) (BioRad, United States) and 2% (v/v) Nonidet-P40) that had been prerun at 200 V for 10 min, 300 V for 15 min and 400 V for 15 min using the BioRad Mini-Protean II gel apparatus. For fractionation of eIF4A isoforms, the sample was loaded at the anode end of the apparatus and electrophoresis was from anode to cathode for 500 V for 5 h. For fractionation of eIF4B and eEF2 isoforms, the sample was loaded at the cathode end of the apparatus and electrophoresis was from cathode to anode for 550 V for 5 h. Proteins were fractionated in the second dimension by standard 12% Laemmli SDS-PAGE (12% (w/v) acrylamide, 0.3% (w/v) *bis*-acrylamide, 0.1% SDS (w/v)) and transferred to NitroPure nitrocellulose membranes (MSI, United States) in transfer buffer (50 mM Tris–base, 39 mM glycine, 20% (v/v) methanol) using a BioRad Mini-Protein II western transfer apparatus at 250 mA for 2.5 h. Membranes were blocked with 5% (w/v) nonfat dry milk (NFDM) (Nestle, United States) in PBST buffer (13.7 mM NaCl, 0.27 mM KCl, 1.0 mM Na_2HPO_4, 14 mM KH_2PO_4, 0.1% (v/v) Tween-20) for 2 h, washed twice for 15 min in PBST. For immunodetection, western blots were incubated with antibodies in 1% (w/v) NFDM in PBST (antiserum against eIF4B (1 : 5000), eIFiso4F for recognition of eIFiso4G and eIFiso4E (1 : 1000)), PABP (1 : 2000) and RPS6 (1 : 2000)), and affinity purified primary antibodies against eIF4A (1 : 2000), eIF4E (1 : 50)). After 2 h of incubation with the primary antibody, membranes were washed twice for 15 min in PBST and incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate (1 : 25000) in 1% (w/v) NFDM in PBST for 1 h. Membranes were washed twice for 30 min in PBST and developed with 4 ml of enhanced chemiluminescence detection ECL reagent (Amersham Pharmacia, United States). Membranes were exposed to X-ray film and images recorded with a Scanjet 5p (Hewlett Packard, United States) scanner.

Extraction and two-dimensional gel analysis of ribosomal proteins. Ribosome isolation and protein extraction was as follows: frozen pulverized root tissue (1 to 2 g fr wt) was hydrated and thoroughly resuspended in extraction buffer (200 mM Tris–HCl, pH 7.5, 200 mM KCl, 25 mM EGTA, 36 mM $MgCl_2$, 1 mM sodium molybdate, 1 mM dithiothreitol, 50 μg/ml cycloheximide, 50 μg/ml chloramphenicol, 80 mM β-glycerophosphate, 1% (v/v) Triton X-100, 1% (v/v) Brij 35, 1% (v/v) Tween-40, 1% (v/v) Nonidet P-40) at 4°C. The extract was centrifuged at 10000 *g* for 15 min at 4°C, and the supernatant was layered over a sucrose cushion (1.3 M sucrose, 400 mM Tris–HCl, pH 7.5, 200 mM KCl, 5 mM EGTA, 36 mM $MgCl_2$, 1 mM

sodium molybdate, 1 mM dithiothreitol, 50 μg/ml cyclohexamide, 50 μg/ml chloramphenicol, 80 mM β-glycerophosphate). A pellet of ribosomes was obtained by centrifugation overnight at 149000 *g*, followed by resuspension in buffer (20 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM sodium molybdate, 1 mM dithiothreitol) by rotation of the sample for 1 h at 4°C [19]. For each gel sample, 0.5 mg ribosomes, where, $A_{254}/11.1$ is proportional to μg/μl ribosomes, were diluted in 0.1 volume 1 M $MgCl_2$ and two volumes of fresh glacial acetic acid (Sigma, United States), vortexed for 1 h at 4°C, and centrifuged 10 min at 4°C, at 14000 *g* to remove rRNA. Five volumes of acetone were added to precipitate ribosomal proteins with a brief centrifugation at 14000 *g*. The sample was washed twice with acetone over 24 h, twice with ethanol for 1 h, and then dried in air.

The ribosomal proteins were solubilized in 15 μl of fresh sample buffer (8 M urea, 40 mM Tris–HCl, pH 8.6, 2.3 mM EDTA·Na_4, 0.06% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED), 52 mM boric acid, 5% (v/v) 2-mercaptoethanol) at room temperature for 20 min before electrophoresis. Separation in the first dimension was in a basic-urea polyacrylamide gel (6 M urea, 8% (w/v) acrylamide, 0.3% (w/v) bis-acrylamide, 21 mM EDTA·Na_4, 520 mM boric acid, 200 mM Tris–HCl, pH 8.6, 0.1% (w/v) ammonium persulfate, 0.1% (v/v]) TEMED) in fresh running buffer (6.5 mM EDTA·Na_4, 156 mM boric acid, 120 mM Tris–HCl, pH 8.6) [20] in tube gels (130 × 3.5 mm) (Hoefer Scientific, United States) for 26 h at 143 V, with migration towards the cathode. The first dimension gel was removed from the glass tube, incubated for 15 min in SDS buffer (0.05 M Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 0.1% (w/v) Bromophenol Blue) and embedded onto the top of the second-dimension 12% Laemmli SDS-PAAG with SDS agarose (0.05 M Tris–HCl, pH 6.8, 5% (v/v)) 2-mercaptoethanol, 10% (v/v) glycerol, 2% (w/v) SDS, 1% (w/v) agarose). Electrophoresis was performed using the BioRad Mini-Protean II gel apparatus for 90 min at 180 V and proteins were stained with Coomassie blue R250.

Affinity chromatographic purification of cap-binding complexes with ^{7m}GTP-sepharose and poly(A)-agarose. Frozen pulverized root tips (0.5 ml packed volume) was hydrated in 1 ml of buffer B-90 (200 mM Hepes–KOH, pH 7.6, 90 mM KCl, 0.1 mM EDTA, 2 mM sodium molybdate, 1 mM dithiothreitol, 10% (v/v) glycerol, 5 mM PMSF, 3 μg/ml leupeptin, 10 μg/ml pepstatin, 0.1 mg/ml soybean trypsin inhibitor, 10 μl/ml protease inhibitor cocktail for plant extracts (Sigma)), on ice, vortexed briefly, and centrifuged twice at 10000 *g* for 10 min at 4°C. The supernatant was transferred to a 1.5 ml microfuge tube that contained 1 mg of ^{7m}GTP-sepharose 4B (Amersham Pharmacia) or 6 μg of poly(A)-agarose (Sigma) that had been equilibrated with buffer B-90. The sample was mixed for 30 min at 4°C, centrifuged at 1000 *g* for 1 min, and the pellet was washed four times with buffer B-90. Proteins bound to the resin were eluted in 10 μl in 4X SDS loading buffer (20 mM Tris–HCl, pH 6.8, 2% (v/v) 2-mercaptoethanol, 8% (w/v) SDS), fractionated by 12% Laemmli SDS-PAGE and analyzed by western blot analysis, as described above.

Table 1. Effect of oxygen deprivation on the levels of large polysomes in maize root tips

Treatment condition	Percentage large polysomes
Aerobic	32.84 ± 0.61
Anoxia 3 h	13.12 ± 0.91*
Anoxia 6 h	10.03 ± 0.57*
Hypoxia 1 h, anoxia 3 h	17.18 ± 0.40*
Hypoxia 1 h, anoxia 6 h	13.93 ± 0.30*

Note: Data are the mean and standard error of at least three independent biological replicates.

* Indicates that value is significantly different from the aerobic sample ($P \leq 0.05$).

RESULTS AND DISCUSSION

Anoxia and Hypoxia Impair the Initiation of mRNA Translation

Our studies had indicated that oxygen deprivation results in a reduction in the initiation of mRNA translation [9, 12]. We developed a procedure to measure changes in initiation of translation that uses small quantities of starting material (e.g., 0.2 g fr wt root tips), in order to quantitatively assess adjustments in protein synthesis in response to oxygen deprivation treatments of different severity and duration. Crude cell lysates were prepared by detergent extraction in a buffer that stabilizes polysomes, fractionated by centrifugation through an exponential sucrose density gradient, analyzed by use of a UV spectrophometer, and levels of ribosomal subunits, monosomes, small polysomes and large polysomes (> 5 ribosomes per mRNA) were quantified on the basis of equal OD (A_{254}) per sample. This method revealed a dramatic decrease in polysomes and an increase in monosomes, indicative of a decrease in the initiation of translation (Fig. 1). Translation was down-regulated by anoxia and anoxia preceded by 1 h of hypoxia (5% oxygen for 1 h prior to 3 or 6 h of anoxia) (Table 1). Anoxia resulted in a more severe reduction in large polysomes, with a 2.5-fold decrease within 3 h of stress. Pretreatment of seedlings with 5% oxygen prior to anoxia dampened the loss of large polysomes. However, both treatment regimes resulted in significant loss of large polysomes, most likely because of the depletion in nucleotide triphosphates that are required to drive initiation and elongation of translation. The observation that a greater number of polypeptides are synthesized *de novo* under hypoxia than anoxia [10, 21] may indicate that the degree of inhibition of protein synthesis differentially affects the translation of individual mRNAs. In other words, the trans-

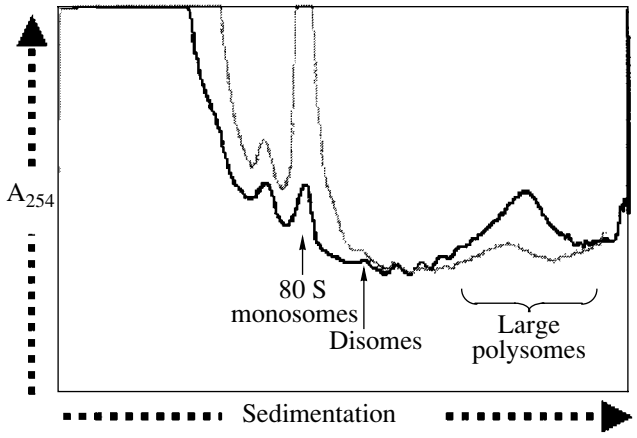

Fig. 1. Polysome analysis of crude cell lysates from root tips of seedlings.

Detergent-treated cell lysates from root tips of 5-day-old seedlings were fractionated on 20 to 60% (w/v) sucrose gradients and analyzed by measurement of absorbance at 254 nm. The baseline absorbance values from a gradient loaded with extraction buffer was subtracted and profiles from the two samples were normalized to equal optical density absorption units. The black line is the aerobic polysome profile and the gray line is that of 6-h oxygen-deprived root tips.

lational constraints under hypoxia may be less severe than that under anoxia. However, the polysome profile analysis indicates that oxygen deprivation, whether imposed as anoxia or hypoxia, leads to dramatic and progressive loss in protein synthesis.

Translational Regulation at Initiation

The mRNA cap-binding complex. Most documented examples of translational regulation in plants occur at the initiation phase, when the 43S preinitiation complex is recruited to a 5'-^{7m}Gppp-capped and 3'-polyadenylated mRNA [4, 5]. The first step in this recruitment process involves binding of a small cap-binding protein (eIF4E) and a large scaffold protein (eIF4G) to the 5'-cap of the mRNA. An important distinction between the translational apparatus of plants and animals is that plants possess at least two heterodimeric cap-binding complexes [22]. These include the eIF4E–eIF4G complex present in animals and the eIFiso4E–eIFiso4G complex that is limited to plants. eIFiso4E, a 28-kD cap-binding protein, is evolutionarily related to eIF4E, whereas eIFiso4G, an 86-kD scaffold protein, is evolutionarily distinct from eIF4G. These two complexes are functionally redundant in a cell-free translation system (e.g., wheat germ translation extract) but discriminate between mRNAs based on the presence or absence of the 5'-cap, as well as secondary structure near the 5'-end [22, 23]. The eIFiso4E-eIFiso4G complex preferentially translates 5'-capped and 3'-poly(A) tailed mRNAs that lack a structured 5'UTR, whereas the eIF4E-eIF4G complex efficiently translates 5'-capped and 3'-poly(A) tailed mRNAs as well as transcripts with a stem-loop structure near to the 5'-cap, mRNAs lacking a 5'-cap, and the downstream ORF of uncapped dicistronic mRNAs [23, 24]. Both eIF4G and eIFiso4G interact with eIF4A (a weak ATPase and α-helicase) and eIF4B (an RNA-binding protein) to form an ATP-dependent RNA α-helicase complex that assists the scanning process of the 43S preinitiation complex. The scaffold protein eIF4G (or eIFiso4G) also interacts with eIF3, a ten subunit complex [25], which tethers the 40S ribosomal subunit to the eIF2–GTP–$tRNA_{met}$ ternary complex. eIF1 and eIF1A are also involved in the ATP hydrolysis and α-helicase process that facilitates scanning of the 43S preinitiation complex for the initiation codon [26], but have not been studied in plants. Hence, there is extensive potential for intricate regulation of the mammoth initiation complex. In animal cells, a primary mechanism for regulation of initiation is through regulation of the phosphorylation of eIF4E [27]. Phosphorylation of eIF4E stabilizes its binding to the mRNA 5'-cap and is strongly correlated with cell growth [27, 28]. Nonphosphorylated eIF4E is sequestered by nonphosphorylated 4E-binding protein (4E-BP). At least two distinct signal transduction pathways regulate phosphorylation of mammalian eIF4E and 4E-BP [29]. Another distinct mechanism for regulation of initiation is through modulation of levels of intact eIF4G, required for initiation of translation of most mRNAs. Cell infection by piconoviruses and apoptosis result in caspase-dependent cleavage of eIF4G and leads to translation via 5'-cap-independent mechanism [29, 30].

The role of the cap-binding complex components in the regulation of initiation in plants is poorly understood. The establishment and maintenance of interactions between the 5'- and 3'-termini of an mRNA appears to be a major factor. A functional interaction between the two ends of the mRNA was shown by the synergistic stimulation of translation by the presence of both a 5'-cap and 3'-poly(A) tail [31]. eIF4G and eIFiso4G physically interact with poly(A) binding protein (PABP) bound to the mRNA 3'-end in plants, yeast and animals [32–34]. The eIF4G–PABP interaction results in the "circularization" of the mRNA transcript and stimulates the ATP-dependent RNA α-helicase activity of the scanning 43S preinitiation complex [35, 36]. In plants, the 5'-cap and 3'-tail interaction also includes binding of one or multiple eIF4B molecules to eIFiso4G and PABP [33]. The formation and maintenance of the 5'-cap and 3'-tail initiation complex may be important in the initial initiation event and/or subsequent reinitiation by recycling ribosomes. This cross-talk between the 5'-cap and 3'-tail appears to be disrupted in plant cells in response to heat shock [37]. Interestingly, a number of plant viral mRNAs may manage to facilitate 5'-cap and 3'-tail interactions via novel means [4, 5]. The finding that chimeric maize *ADH1*–GUS mRNAs that possess both *ADH1* 5'UTR/coding and 3'UTR sequences are efficiently

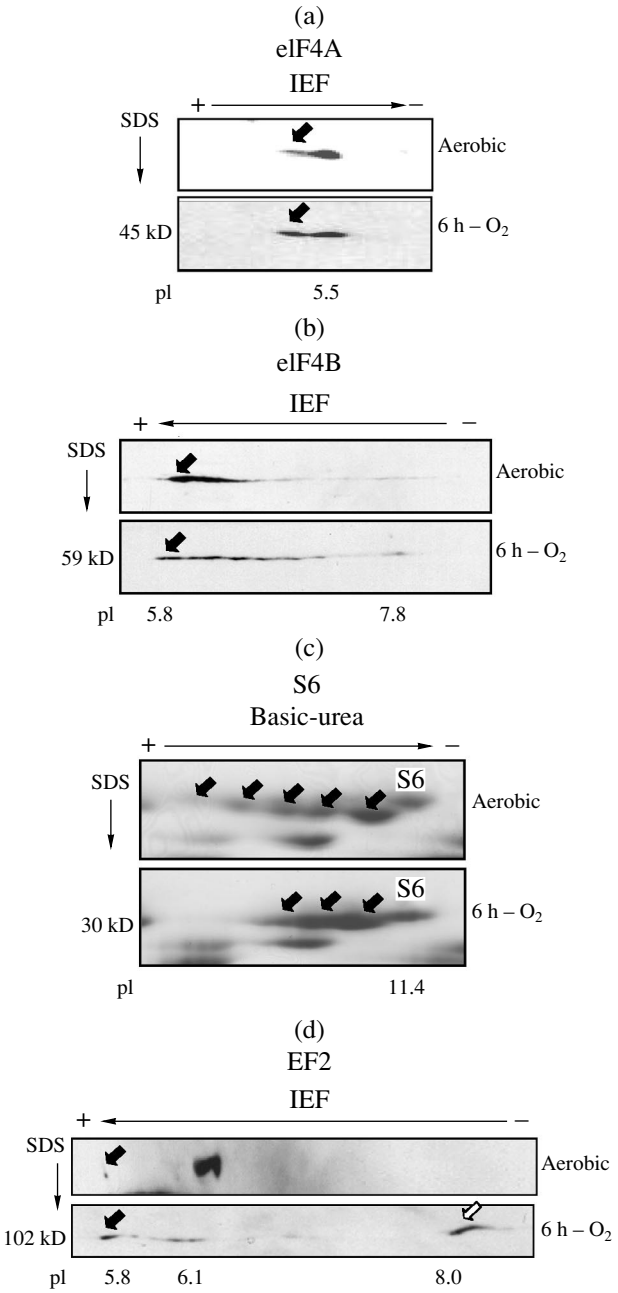

Fig. 2. Posttranslational modification of eIF4A, eIF4B, ribosomal protein S6, and eEF2 in response to oxygen deprivation.

Five-day-old seedlings were untreated (aerobic) or deprived of oxygen for 6 h (6 h $-O_2$).

(a, b, and d) For analysis of posttranslational modification of eIF4A, eIF4B, and eEF2, total cellular protein was precipitated with TCA and fractionated by isoelectric focusing (IEF) gel electrophoresis in the first dimension and Laemmli 12% SDS-PAGE in the second dimension. Proteins were blotted to nitrocellulose membranes and incubated with antisera prepared against recombinant maize eIF4A, native wheat eIF4B or native wheat eEF2. Filled black arrows indicate phosphorylated eIF4A and eEF2 and the most highly phosphorylated isoform of eIF4B. An open arrow indicates an eEF2 isoform of unknown modification. (c) For analysis of ribosomal protein S6 (RPS6), ribosomes were isolated, rRNA was extracted with acetic acid, proteins were fractionated using a basic-urea gel in the first dimension and a Laemmli 12% SDS-PAGE in the second dimension, and stained with Coomassie blue. RPS6 was identified by western blot analysis using antiserum prepared against maize RPS6 (data not shown). The protein labeled S6 is nonphosphorylated. Filled black arrows indicate the mono-phosphorylated to penta-phosphorylated isoforms of the RPS6. The region of the gels with the proteins of interest is shown. The direction of electrophoresis is indicated with large arrows. Determination of apparent pI and mol wt was based on determination of gel pH in the IEF dimension and mol wt standards in the SDS-PAGE dimension.

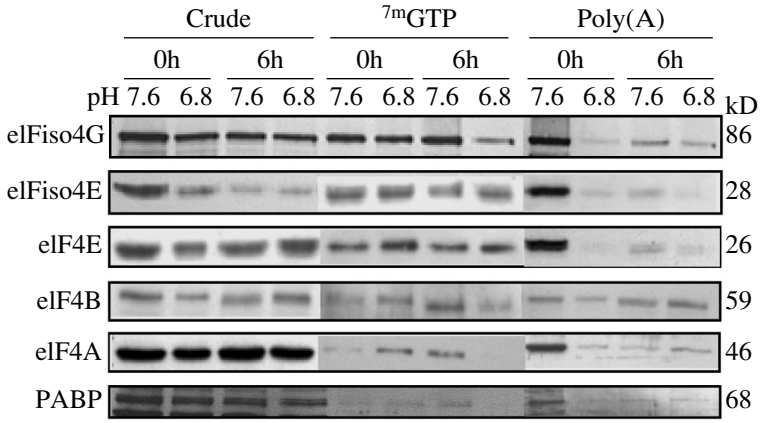

Fig. 3. Affinity purification of the mRNA cap-binding complex.

Crude cell lysates were prepared under low-salt conditions from aerobic (0 h) and 6-h oxygen-deprived root tips of 5-day-old seedlings and used for affinity purification of protein complexes with ^{7m}GTP-sepharose or poly(A)-agarose at pH 7.6 and 6.8. Proteins were fractionated by Laemmli 12% SDS-PAGE, blotted to nitrocellulose, and incubated with specific antisera prepared against native wheat eIFiso4F (for detection of eIFiso4G and eIFiso4E), recombinant maize eIF4E, recombinant maize eIF4A, native wheat eIFiso4B or native wheat PABP. The region of the gel with the proteins of interest is shown. Apparent mol wt was determined based on migration of mol wt standards.

translated in hypoxic protoplasts [38], raises the possibility that maintenance of 5'-cap and 3'-tail interactions via sequences in *ADH1* mRNA allows for circumvention of translational repression under oxygen deprivation.

Anoxia affects phosphorylation of subunits of the mRNA 5'-cap-binding complex. Previously, we reported that oxygen deprivation resulted in the accumulation of additional acidic eIF4E isoforms [17]. Both the aerobic and acidic eIF4E isoforms that accumulated under anoxia were phosphorylated, based on *in vivo* labeling with orthophosphate. We concluded that eIF4E is hyperphosphorylated in response to oxygen deprivation. By contrast, we observed no isoelectric variation in eIFiso4E, under aerobic or oxygen deprivation conditions [17]. To further elucidate the mechanisms that underlie translational regulation in response to oxygen deprivation we investigated whether additional components of the 5'-cap and 3'-tail complex are post-translationally modified. Proteins isolated from maize root tips were fractionated by two-dimensional IEF/SDS-PAGE and initiation factors were identified by western immunoblot analysis.

An antiserum was prepared against recombinant maize eIF4A and used to detect this protein in crude extracts from root tips. The level of nonphosphorylated eIF4A (pI 5.5) was higher than that of the phosphorylated isoform in aerobic root tips, and the level of phosphorylated eIF4A increased to about 50% following 6 h of oxygen deprivation (Fig. 2a). These results indicate that phosphorylation of eIF4A, documented in the first 1 h of anoxia in maize root tips [39], was sustained in response to this stress. In addition, immunological detection of eIF4B with an antiserum prepared against native wheat eIF4B revealed that this factor was multiply phosphorylated in aerobic roots and partially to fully dephosphorylated in response to oxygen deprivation (Fig. 2b) In summary, these results indicate that several factors of the cap-binding complex are differentially post-translationally modified in response to low oxygen stress.

Anoxia affects mRNA 5'-cap and 3'-tail-binding complexes. The functional significance of the alterations in the 5'-cap-binding factors was further studied by purification of the 5'-cap and 3'-tail binding complexes from root tips using ^{7m}GTP-sepharose or poly(A)-agarose. In this assay, binding of eIF4E or eIFiso4E to ^{7m}GTP can result in copurification of eIF4G and eIFiso4G, respectively, as well as eIF4B, eIF4A and PABP associated with either scaffold factor. The affinity purification was done at pH 7.6 or 6.8, to mimic the cytosolic pH of in root tips following under aerobic and anoxic conditions, respectively [40, 41]. pH was considered of relevance since the *in vitro* binding of the wheat germ eIFiso4G–eIFiso4E complex to oligoribonucleotides displayed a narrow pH optimum around pH 7.6 [42], due to pH-sensitive conformational changes in eIFiso4G [43, 44]. We observed similar affinity purification of eIFiso4E with ^{7m}GTP at pH 7.6 and 6.8 using either aerobic or 6-h oxygen-deprived roots (Fig. 3), suggesting that pH did not influence the ability of eIFiso4E to bind ^{7m}GTP. However, the purification of eIFiso4G was slightly lower at pH 6.8, as evident in the sample from the oxygen deprived roots. The affinity purification of eIF4E with ^{7m}GTP-sepharose did not appear to be affected by pH or hyperphosphorylation caused by oxygen deprivation. The dephosphorylation of eIF4B in response to the stress was evident in the one-dimensional gel system (Fig. 2b). The binding of the dephosphorylated eIF4B was lower at pH 6.8 than pH 7.6. This could be due to a pH effect on the

binding of eIF4B to either scaffold factor or to reduced binding of eIFiso4G to eIFiso4E. The affinity purification of both eIF4A and PABP was greatly reduced in anoxic roots at pH 6.8, suggesting that pH may significantly alter the interaction of these proteins to the cap-binding complex. Additional studies are needed to address whether the phosphorylation status of eIF4E, eIF4B or eIF4A or pH sensitivity of eIFiso4G affect the ATP-dependent α-helicase activity of the cap-binding complex and/or maintenance of interactions between the 5'-cap and 3'-poly(A) tail.

Poly(A)-agarose was used as an affinity substrate to provide additional insight into the alterations in the 5'-cap and 3'-tail interactions that occur in response to oxygen deprivation. Both PABP and eIF4B of plants bind to poly(A) oligoribonucleotides [33, 45]. eIF4B was efficiently purified from aerobic and 6 h oxygen deprived roots when incubated with poly(A) agarose at pH 7.6 and 6.8. There was no evidence that the phosphorylation status of eIF4B affected the binding of the factor to poly(A). However, a reduction in purification of eIFiso4G, eIFiso4E, eIF4E and eIF4A was evident at pH 6.8 with aerobic root extracts and at pH 7.6 and 6.8 with extracts from oxygen-deprived roots, suggesting that association of these factors with the 3'-end of the mRNA may be affected by alterations in cytosolic pH. The decrease in cap-binding protein association occurred concomitantly with a reduction in PABP binding, indicating that pH-sensitive binding of this factor to poly(A) may be the primary cause of reduced binding of the eIF4F subunits. However, it remains to be clarified whether reduced co-purification of eIFiso4G, eIFiso4E, eIF4E and eIF4A is due to disrupted PABP–poly(A), PABP–eIF4G (or eIFiso4G) and/or PABP–eIF4B interactions, since the association of eIF4B with PABP was reduced in response to heat shock as a result of eIF4B dephosphorylation in wheat [45]. These results provide evidence that physical interaction between factors that unite the 5'-cap and 3'-poly(A) tail of the mRNA are disrupted in response to oxygen deprivation.

Anoxia reduces ribosomal protein S6 phosphorylation. Our studies have also considered a potential role of the ribosome in selective mRNA translation. Ribosomal protein S6 (RPS6) is a component of the small subunit that is located in the mRNA binding region. In animals, RPS6 is sequentially phosphorylated at multiple Ser residues at its carboxyl terminus by S6 kinase ($p70^{s6k}$) [46]. Insulin and other growth regulators stimulate RPS6 phosphorylation via a phosphatidylinositide-3-OH kinase and target of rapamycin-dependent pathway. When RPS6 is phosphorylated mRNAs that possess a 5' terminal polypyrimidine tract (5'TOP) associate efficiently with polysomes. This subpopulation includes mRNAs that encode ribosomal proteins and translation factors that are required for cell growth. When starved cells are provided with serum, this signaling pathway is stimulated leading to the activation of $p70^{s6k}$. It is thought that a 5'TOP-binding protein interacts with phosphorylated RPS6 in the mRNA binding region of the small ribosomal subunit to stimulate translation of 5'TOP mRNAs. We considered that RPS6 could be involved in the regulation of mRNA translation in response to oxygen deprivation. In aerobic roots RPS6 accumulated in six isoforms from nonphosphorylated (pI 11.4) to penta-phosphorylated (Fig. 2c) (Williams *et al.*, [61]). The level of RPS6 phosphorylation was reduced after 6 h of oxygen deprivation, as evidenced by the increase in pI of the abundant isoforms. Additional studies are necessary to address if mRNAs with a 5'-TOP or other *cis*-acting element directs translational regulation in response to changes in RPS6 phosphorylation in plant cells.

Translational Regulation at Elongation

Anoxia reduces ribosomal 12-kD P-protein phosphorylation. The universally conserved lateral stalk structure on the large ribosomal subunit has been demonstrated to facilitate the translocation phase of protein synthesis [47]. In animals, the structure is formed by a complex of acidic phosphoproteins (P-proteins), including P0 (~35 kD), P1 and P2 (~12 kD) [48]. In higher plants, the stalk is composed of P0, P1, two types of P2 (P2a and P2b), and a plant specific 12-kD P-protein, P3 [49, 50]. Several studies suggest the phosphorylation of the 12-kD P-proteins is involved in ribosome-mediated translational regulation in yeast and animals [51–53]. Our previous investigations indicated that the phosphorylation status of the 12-kD P-proteins is altered in response to anoxia [49, 54]. Immunoblot analyses demonstrated that, when intact maize seedlings were deprived of oxygen for up to 24 h, an increase was observed in the amount of the faster migrating, dephosphorylated forms of P1, P2a, and P3 (P2b was not detected in maize root tips) [54]. In mammals phosphorylated P2 functions as a GTPase activating protein, stimulating GTP hydrolysis by eEF2 and promoting translocation. We propose that the reduced phosphorylation of the 12-kD P-proteins in response to low oxygen stress may serve to impair eEF2 activity.

Anoxia affects eukaryotic elongation factor-2 post-translational modification. Eukaryotic elongation factor 2 (eEF2) is a GTPase that catalyzes the translocation of the peptidyl tRNA from the A site to the P site of the ribosome during translation. eEF2 activity requires the hydrolysis of GTP which promotes the forward movement of the mRNA by three bases to expose the next codon and ejection of the deacylated tRNA from the E site of the ribosome [55]. eEF2 binds to the pretranslocation state ribosome and is released from the structurally distinct posttranslocation state ribosome. eEF2 is posttranslationally modified in two ways. A conserved His residue of eEF2 is modified to dipthamide, a modification that is only known to occur on eEF2 and provides the target for ADP ribosylation by diptheria toxin [56]. Two conserved Thr residues within the GTP-binding domain of eEF2 are sites of phosphorylation. The

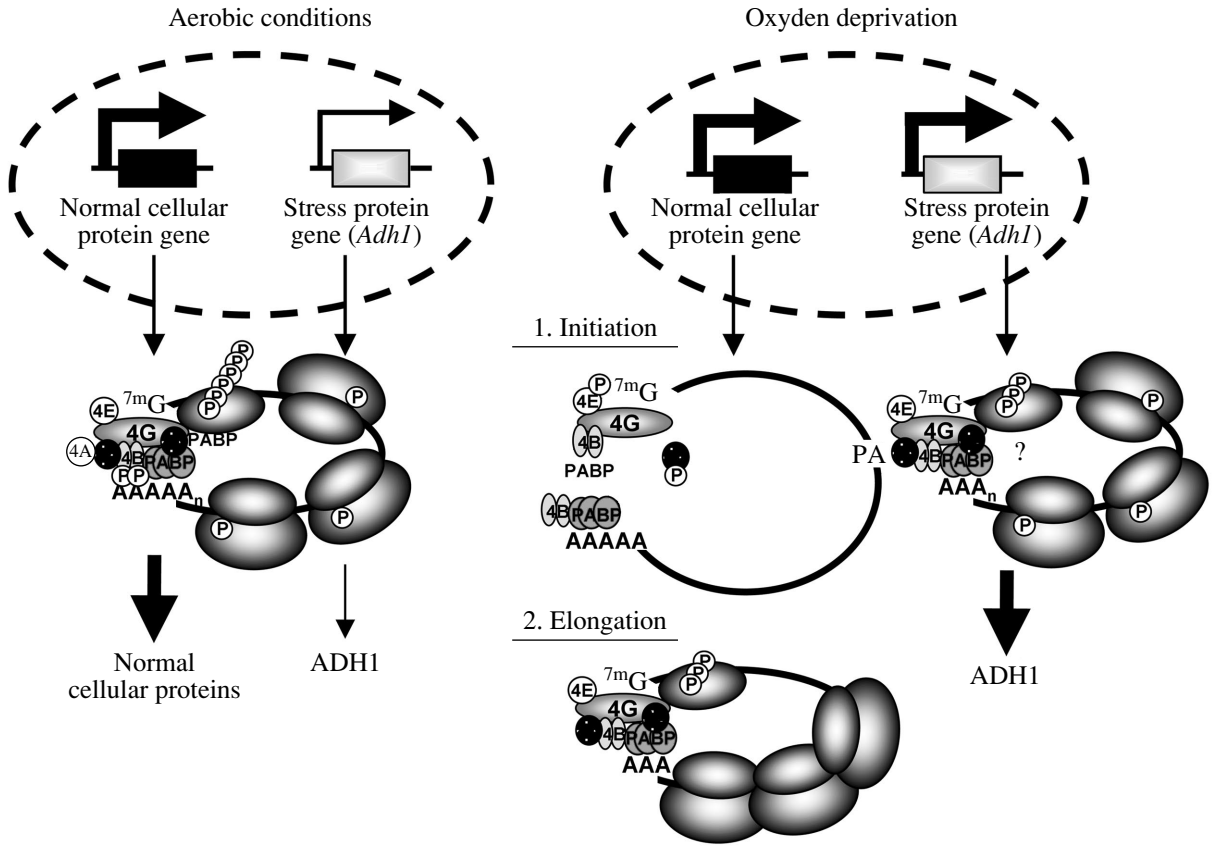

Fig. 4. Model of transcriptional and translational regulation in response to oxygen deprivation in maize.

Transcription and translation of normal cellular protein genes and stress protein genes (e.g., *Adh1*) is depicted under aerobic conditions and in response to oxygen deprivation. Under aerobic conditions, both gene types are transcribed and both mRNA types are efficiently translated. Under oxygen deprivation, transcription of normal cellular protein genes is maintained and that of stress protein genes is increased. The stress protein mRNAs are preferentially translated, as indicated by the presence of ribosomes on the ADH1 mRNA. Features of the 5' and 3' regions of the ADH1 mRNA or additional translational components, indicated by the "?", may allow for maintained initiation. Translation of the normal cellular mRNAs may be regulated at the level of (1) initiation or (2) elongation. Alterations in phosphorylation of initiation factors and/or the reduction in cytosolic pH from 7.6 to 6.8 affect the interactions between proteins at the 5'-cap and 3'-poly(A) tail that facilitate circularization of the mRNA, resulting in the regulation of initiation (1), whereas alterations in phosphorylation of the 12-kD P-proteins and eEF2 could provide additional control through an effect on eEF2 GTPase activity, resulting in the regulation of elongation (2), represented by the message containing stalled ribosomes. The 5'-cap of the mRNA is designated ^{7m}G, the 3-poly(A) tail of the mRNA is designated with multiple "A"s. Phosphorylation of translation factors and ribosomal proteins is represented with a "P" (as summarized in Table 2). Phosphorylation of ribosomal protein S6 is indicated on the 40S ribosomal subunit; phosphorylation of the 12-kD ribosomal P-proteins is indicated on the 60S ribosomal subunit of the 80S ribosome.

mammalian eEF2 kinase is a Ca^{2+}/calmodulin-dependent enzyme that is regulated via the phosphatidylinositide-3-OH kinase pathway that also controls phosphorylation of RPS6 [57]. In mammals, eEF2 GTPase activity is inhibited by both eEF2 ADP-ribosylation and phosphorylation [58]. eEF2 phosphorylation is reversible and is thought to modulate global levels of ribosome translocation [56].

Wheat eEF2 has an apparent molecular mass of 102 kD [59]. The deduced sequences of plant eEF2s are highly conserved relative to animal eEF2; the conservation includes the GTPase domain and the post-translationally modified His and Thr residues (Bailey-Serres, unpublished data). Although a plant eEF2 kinase has not been identified, phosphorylation of wheat eEF2 by the rabbit enzyme inhibits translocation [60]. An antiserum prepared against wheat eEF2 detected multiple eEF2 isoforms in maize root tips (Fig. 2d). The pI of these proteins was consistent with the nonphosphorylated (pI 6.1) and phosphorylated (pI 5.8) isoforms of wheat eEF2 [60]. The level of phosphorylated eEF2 appeared to increase in response to 6 h of oxygen deprivation, as judged by the decrease in pI (Fig. 2d). An eEF2 isoform of pI 8.0 was also observed, but the nature of this alteration in pI is unclear. The apparent increase in eEF2 phosphorylation in response to oxygen deprivation may reduce translational elongation at a global level.

Table 2. Effect of oxygen deprivation on the phosphorylation of the translational machinery

Component of translation	Aerobic		Oxygen deprivation	
	Phosphorylation status			
	phosphorylated	dephosphorylated	phosphorylated	dephosphorylated
Initiation factors				
eIF4A		×	×	
eIF4B	×			×
eIF4E[a]		×	×	
eIFiso4E[a]	No change			
Ribosomal proteins				
RPS6	×			×
P-proteins[b]	×			×
Elongation factor				
eEF2		×	×	

Note: [a] [17]; [b] [54].

CONCLUSIONS

A Model for the Regulation of mRNA Translation in Response to Oxygen Deprivation

Changes in both gene transcription and mRNA translation underlie alterations in protein synthesis in response to oxygen deprivation in maize roots (summarized in Fig. 4). In aerobic roots both normal cellular protein and stress protein genes (i.e., *Adh1*) are transcribed [12, 13]. Under aerobic conditions both classes of transcripts appear to be translated. The transcription of stress protein genes is induced in response to oxygen deprivation, whereas there is little to no change in the transcription of the normal cellular protein genes [12, 13]. The initiation of protein synthesis is significantly reduced; however, stress protein mRNAs, such as *ADH1* mRNA, escape translational repression [9, 12, 13]. The initiation of translation is a complex mechanism. Most mRNAs initiate via a 5'-cap and 3'-poly(A) dependent mechanism that involves competition for limiting initiation factors and ATP-dependent removal of mRNA secondary structure. The ability of maize *ADH1* mRNA to maintain efficient translation under low-oxygen stress suggests that it may be translated by a mode that is distinct from that of most cellular mRNAs. The finding that both 5' and 3' sequences of *ADH1* mRNA are necessary and sufficient to maintain translation of a reporter gene transcript in hypoxic cells provides evidence that this mRNA has evolved an alternative method to maintain 5'-cap and 3'-tail interactions [38]. How might translational regulation be mediated in response to low oxygen stress? The reduction in nucleotide triphosphate levels is most likely the primary cause of the global reduction in protein synthesis. However, global and message-specific regulation of translation may be mediated by the decrease in cytosolic pH and modifications in phosphorylation of initiation factors, elongation factors, and ribosomal proteins (Table 2). Affinity purification of cap-binding complex factors with ^{7m}GTP-sepharose revealed that oxygen deprivation affected the interaction of eIF4A and PABP via eIF4G/eIFiso4G. Consideration of protein interactions with poly(A)-agarose showed that eIF4B binding was unaffected by pH or reduced phosphorylation caused by oxygen deprivation. However, low pH appeared to markedly reduced the binding of PABP to poly(A) agarose. Together, these observations indicate that mRNA 5'-cap and 3'-tail interactions necessary for efficient scanning of the 43S pre-initiation complex may be disrupted by initiation factor modifications and/or reduced cytosolic pH during oxygen deprivation. Based on findings in animal cells, an additional component of message-specific regulation of initiation may be the reduced phosphorylation of RPS6 in response to the stress. We hypothesize that translation of most cellular mRNAs is impacted by the depletion of nucleotide triphosphates, reduced cytosolic pH, and alterations of translation factors that disrupt the physical interactions between the 5'-cap and 3'-tail of the mRNA (Fig. 4). Sequences in the 5' and 3' regions of the ADH mRNA appear to compensate for the alterations in the translational machinery, allowing this mRNA to escape translational repression.

Oxygen deprivation also effects elongation of translation [12–16]. The down regulation of elongation may be due to energetic reasons, however, the alterations in phosphorylation of the 12-kD P-proteins and eEF2 could provide additional control through an effect on eEF2 GTPase activity. Hence, regulation of initiation and elongation of translation may not be mediated solely by nucleotide triphosphate availability but may have evolved to maintain the initiation of translation of select mRNAs that are crucial for survival of transient low oxygen stress. The observation that certain mRNAs

are associated with large polysomes under low oxygen conditions, but their gene products appear not to be synthesized, suggests that regulation of elongation may also be a selective process.

In conclusion, our studies have shown that alterations in gene regulation occur at both transcriptional and translational levels in response to oxygen deprivation. Stress protein mRNAs are selectively translated despite a dramatic reduction in overall levels of protein synthesis. By contrast, mRNAs that are required for normal growth and metabolism continue to be synthesized, accumulate, but are poorly translated. The selective translation of stress protein mRNAs and impaired translation of normal cellular mRNAs may allow for expedient recovery once oxygen becomes available and mitochondrial oxidative phosphorylation is restored.

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