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The 5′ untranslated region of the maize alcohol dehydrogenase gene contains an internal ribosome entry site

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ABSTRACT

Adh1, the maize gene encoding alcohol dehydrogenase ADH1, mRNA is efficiently translated in O_2-deprived roots of maize, whereas many normal cellular mRNAs are poorly translated. It has been shown that *adh*, the 5′ untranslated region of *adh1* mRNA, provides effective translation of mRNA under hypoxia and heat shock conditions in *Nicotiana benthamiana* plants. We found that *adh* contains the internal ribosome entry site (IRES) active both *in vivo*, in *N. benthamiana* cells, and *in vitro*, in rabbit reticulocyte lysate translation system. It is widely supposed that cap-independent internal initiation may maintain efficient translation of particular cellular mRNAs under a variety of stresses and other special conditions when cap-dependent protein synthesis is impaired. We evaluated the level of IRES activity of *adh* and found that its contribution to the overall translation of *adh*-containing mRNA in plant cells is less than 1% both under normal conditions and under heat shock. The low efficiency of this IRES is inconsistent with its possible role as a main factor ensuring efficient translation of *adh1* mRNA under stress conditions.

1. Introduction

Roots of maize (*Zea mays* L.) seedlings respond to O_2 deprivation by alternation of gene expression at transcriptional and post-transcriptional levels. In O_2-deprived roots many genes encoding normal cellular proteins are constitutively transcribed but their mRNAs are poorly loaded with ribosomes (Fennoy and Bailey-Serres, 1995). Genes encoding anaerobic polypeptides, including certain enzymes required for glycolysis and ethanolic fermentation, are transcribed at elevated levels and efficiently translated in O_2-deprived roots (Sachs et al., 1980; Sachs, 1994; Fennoy and Bailey-Serres, 1995).

Adh1, the maize gene encoding alcohol dehydrogenase-1, has been extensively characterized as a model anaerobic polypeptide gene. The effect of ADH1 mRNA sequences on message stability and translation had been studied in maize protoplasts (Bailey-Serres and Dawe, 1996). 5′ capped and 3′ polyadenylated mRNA constructs containing different 5′ untranslated regions and reporter gene were synthesized *in vitro* and electroporated into protoplasts that were cultured at 40 or 5% O_2. The mRNA with a 17-nucleotide polylinker 5′ untranslated region (UTR) was expressed 10-fold lower under hypoxic conditions than under aerobic conditions. The level of expression of the mRNA containing the 5′-UTR of *adh1* (termed *adh*) upstream the coding region of the reporter gene was not reduced under hypoxia (Bailey-Serres and Dawe, 1996). Previously we found that, constituting the 5′ UTR, *adh* provides efficient translation of mRNA not only in maize protoplasts but also in tobacco (*Nicotiana benthamiana*) leaves under hypoxia and heat shock (Mardanova et al., 2007).

Translation of most eukaryotic mRNAs involves interaction of the mRNA 5′ m^7GpppN cap with the eIF4E subunit of the eIF4F translation initiation complex. Once the eIF4F complex has assembled on the mRNA the small ribosomal subunit is recruited to the mRNA and scanning for a favourable AUG initiation codon commences. The amount of eIF4E can be limiting for translation and its availability is dependent upon the phosphorylation state of its inhibitory binding partners, 4EBP1 and 4EBP2 (Pause et al., 1994). Hypoxia has been shown to reduce the availability of eIF4E by increasing its association with 4EBP1, although it is suspected that other mechanisms contribute to the suppression of protein synthesis (Tinton and Buc-Calderon, 1999).

An alternative mode of translation initiation, that does not require eIF4E and the 5′ cap, involves recruitment of the translation initiation complex by an internal ribosome entry site (IRES). Translation by internal ribosome entry was first identified in picornaviruses, but a number of cellular mRNAs have subsequently been found to contain an IRES (Macejak and Sarnow, 1991; Komar and Hatzoglou, 2005;

Abbreviations: adh, the 5′-untranslated region of maize alcohol dehydrogenase *adh1* gene; IRES, internal ribosome entry site; GFP, green fluorescent protein; GUS, β-glucuronidase; DNAse, deoxyribonuclease; PVX, potato virus X; RLU, relative light units; RNAse, ribonuclease; RRL, rabbit reticulocyte lysate; UTR, untranslated region; WGE, wheat germ extract.

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Baird et al., 2006). It is widely supposed that cap-independent internal initiation may maintain efficient translation of particular cellular mRNAs under a variety of stresses and other special conditions when cap-dependent protein synthesis is impaired (reviewed by Holcik and Sonenberg, 2005).

However, the debates over the existence and biological significance of cellular IRESs continue (Kozak 2001, 2003). Some candidate IRES sites were subsequently dismissed because the sequences were shown to harbor cryptic transcriptional promoters or splice sites (Holcik et al., 2005; Kozak, 2005). The low efficiency of some putative IRESs may be inconsistent with their postulated biological roles (Kozak, 2005).

Because adh1 5′UTR (*adh*) provides effective translation of mRNA in plant cells under hypoxia and heat shock, we hypothesized that *adh* contains an IRES site efficient under stress conditions. Using bicistronic assays we demonstrated that *adh* in fact exhibit IRES activity in experiments on a transient expression *in vivo*, in *N. benthamiana* leaves, and *in vitro*, in rabbit reticulocyte lysate cell-free translation system. However, we found that internal initiation of translation makes only minor contribution to the overall translation of mRNA.

2. Materials and methods

2.1. Adh sequence

Adh sequence was amplified by PCR form maize genome DNA using primers adhNco (CAT CCA TGG CCC CCC TCC GCA AAT CTT C) and adhBK (CT GGA TCC GGT ACC ATT TTC TCG CTC CTC ACA G).

2.2. Viral vectors carrying bicistronic gfp–uidA reporter cassettes

Plasmids pA8151 and pA8175 were constructed on the basis of plasmid PVXdt:GFP–RFP (Schwartz et al., 2006) by substitution of RFP gene by *uidA* gene fused with IREScp148 (in case of pA8151) and by *uidA* gene fused with polylinker sequence (in case pA8175). IREScp148 — *uidA* and polylinker — *uidA* fragments were excised from p35S-GFP–IRES–GUS and p35S-PL–GUS constructs described in (Dorokhov et al., 2002).

To construct the similar vector carrying *adh* between *gfp* and *uidA*, we excised the IREScp148 sequence from pA8151 using KpnI/NcoI digestion and replace it by *adh* sequence. The resulting vector was named pA7248adh.

Plasmid pA7248adh_del was constructed from pA7248adh following deletion of the most parts of coding sequences of *gfp* (deletion of XbaI–SmaI fragment) and *uidA* (deletion of NcoI–BamHI fragment) genes.

2.3. Plasmid vectors carrying bicistronic gfp–uidA reporter cassettes downstream of T7 promoter

The T7 promoter vectors contained the *gfp* gene as the first and the *uidA* gene as the second cistron, separated by spacer sequences. Plasmids pT7hGFPIcpGUS and pT7hGFPGT×GUS containing, respectively, IREScp148 of cruciform-infecting Tobacco mosaic virus and polylinker sequence between *gfp* and *uidA*, were described previously (Dorokhov et al., 2002). To obtain the similar vector pT7hGFPadhGUS carrying adh between the two cistrons, we replaced the IREScp148 fragment in pT7hGFPIcpGUS by *adh* sequence using HindIII and MunI.

2.4. Viral vectors carrying monocistronic uidA reporter cassettes

Vector pAGUS carrying only *uidA* downstream of the promoter of PVX subgenomic RNA was obtained from pA7248adh by deletion of NcoI fragment comprising *gfp* together with *adh* sequence. pAadhGUS carry *adh* and *uidA*, it was constructed by deletion of XbaI–SmaI fragment comprising *gfp* in pA7248adh. Two additional plasmids, pAhGUS and pAhadhGUS, carrying the inverted repeat sequence upstream of *uidA* or *adh–uidA*, respectively, were constructed. To construct pAhadhGUS we replaced the *gfp* gene in pA7248adh by the inverted repeat excised from pT7hGFPGTxGUS. To construct the second plasmid, pAhGUS, we deleted the *adh* sequence from pAhadhGUS.

2.5. Binary vectors carrying reporter gfp gene

Binary vector pNRadhGFP contained the *adh* sequence between the cauliflower mosaic virus 35S RNA promoter and the reporter gene, *gfp*. The similar vector pNRGFP lacks the *adh* (Mardanova et al., 2007). Vectors pNRhadhGFP and phGFP differs from pNRadhGFP and pNRGFP, respectively, in that they contains additional inverted repeat sequence immediately downstream of the 35S promoter.

2.6. In vitro transcription and translation

RNA was transcribed from 0.5 μg linearized plasmid DNA templates with T7 RNA polymerase according to the manufacturer protocols for the RiboMax kit (Promega). Transcripts were purified by phenol-chloroform extraction, their integrity was verified by agarose electrophoresis. *In vitro* translation was performed according to manufacturer protocols for the wheat germ extract and rabbit reticulocyte lysate kits (Promega). Translation of *adh*-containing mRNAs in WGE system was performed with different concentration of magnesium acetate and potassium acetate according to manufacturer protocol (Promega). The mRNA concentration in translation reactions was 0.06 μg/μl.

2.7. Agroinfiltration of leaves

Agrobacterium tumefaciens (strain GV3101) cells carrying binary vectors were grown overnight at 28 °C in LB broth supplemented with kanamycin (50 μg/ml), rifampicin (50 μg/ml) and gentamycin (25 μg/ml). The cells were collected by centrifugation at 2500 g for 10 min, and the pellet was resuspended in solution containing 10 mM $MgSO_4$ and 10 mM MES (pH 5.6) to OD_{600}=0.2. Leaves of greenhouse grown *N. benthamiana* plants were injected with this suspension using a syringe without needle.

2.8. Protein extraction from N. benthamiana cells and Western analysis

Proteins from *A. tumefaciens*-infiltrated plant leaves were extracted from frozen samples by homogenization in extraction buffer (10 mM KCl, 5 mM $MgCl_2$, 0.4 M sucrose, 50 mM Tris pH 8.0, 10% glycerol, 0.08% β-mercaptoethanol) using a Kontes pestle (Fisher). Samples were suspended with buffer and centrifuged at 14,000 ×*g* for 15 min to obtain the supernatant containing soluble proteins. Protein concentrations in the extracts were determined using Bio-Rad protein assay kit.

Proteins separated by 10% SDS/PAGE in 10% gel were immunoblotted with polyclonal antibodies raised against GFP. The GFP protein was detected by chemiluminescence by using the ECL system from Amersham.

2.9. Determination of GUS activity

GUS activity was determined according to (Jefferson, 1987) and measured in relative light units (RLU). Protein concentrations were determined using Bio-Rad protein assay kit. The mean values for three independent experiments are given.

2.10. RNA isolation and analysis

Total RNA was isolated from *N. benthamiana* leaves with RNeasy Plant Mini Kit (Qiagen). After treatment with RQ1 RNase-Free DNase