Biochemical and Biophysical Research Communications 397 (2010) 673–678

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of plant eukaryotic translation initiation factor 6 (eIF6) genes: The essential role in embryogenesis and their differential expression in *Arabidopsis* and rice

Yuki Kato [a], Mineko Konishi [a,1], Mikao Shigyo [a], Tadakatsu Yoneyama [a], Shuichi Yanagisawa [a,b,*]

[a] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan
[b] Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan

ARTICLE INFO

Article history:
Received 31 May 2010
Available online 11 June 2010

Keywords:
eIF6
Nitrogen response
Ammonia induction
Ribosome biogenesis
Arabidopsis
Rice

ABSTRACT

Eukaryotic translation initiation factor 6 (eIF6) is an essential component of ribosome biogenesis. In our present study, we characterize plant eIF6 genes for the first time. Although a single gene encodes eIF6 in yeast and animals, two genes were found to encode proteins homologous to animal and yeast eIF6 in *Arabidopsis* and rice, denoted At-*eIF6;1* and At-*eIF6;2*, and Os-*eIF6;1* and Os-*eIF6;2*, respectively. Analysis of the yeast *eif6* (*tif6*) mutant suggested that plant eIF6, at least in the case of At-eIF6;1, can complement the essential function of eIF6 in yeast. Evidence for the essential role of eIF6 in plants was also provided by the embryonic-lethal phenotype of the *at-eif6;1* mutant. In contrast, At-*eIF6;2* appears not to be essential due to its very low expression level and the normal growth phenotype of the *eif6;2* mutants. Consistent with the putative role of plant eIF6 in ribosome biogenesis, *At-eIF6;1* is predominately expressed in tissues where cell division actively proceeds under the control of intronic *cis*-regulatory elements. On the other hand, both Os-*eIF6;1* and Os-*eIF6;2* are probably active genes because they are expressed at significant expression levels. Interestingly, the supply of ammonium nitrate as a plant nutrient was found to induce specifically the expression of Os-*eIF6;2*. Our present findings indicate that the eIF6 genes have differently evolved in plant and animal kingdoms and also in distinct plant species.

1. Introduction

Eukaryotic translation initiation factor 6 (eIF6) was initially identified as a wheat protein interacting with the 60S ribosome [1]. Subsequent characterization of eIF6 in yeast and animals revealed that it is encoded by a single gene [2–5] and plays an essential role in growth. Disruption of yeast eIF6 gene (*TIF6*) has been shown to induce abnormal processing of the precursors for ribosomal RNA (rRNA) and a reduction in the abundance of 60S ribosome subunit. This results in a lethal phenotype and indicates that eIF6 is an essential component of ribosome biogenesis in yeast [3–6].

Since human eIF6 ($p27^{BBP}$) and murine eIF6 complement the function of eIF6 (Tif6) in yeast, this factor is likely to have an evolutionary conserved function [3–5]. Furthermore, recent studies have suggested that $p27^{BBP}$ might play additional roles in human cells, as it has been shown to interact with 60S subunits and then hamper the assembly of the 40S and 60S subunits in the cytosol [3,7]. Moreover, in conjunction with a multiprotein complex containing MOV10/Armitage that is involved in translational repression, and with proteins of the 60S ribosome subunit, $p27^{BBP}$ has been detected as a protein interacting with the RNA-induced silencing complex [8]. Gandin et al. [9] have also reported that mammalian eIF6 is the first of the eIF factors associated with the 60S subunit that regulates translation in response to extracellular signals and is required for efficient initiation of translation *in vivo*. In mouse, *eIF6* null embryos have a lethal phenotype at preimplantation, and the corresponding heterozygous mice are insensitive to insulin and have a decreased hepatic and adipose tissue mass and reduced level of protein synthesis [9]. Biffo et al. have also reported an interaction between $p27^{BBP}$ and β_4-integrin that mediates cell attachment to the surrounding tissue [10]. These recent results provide evidence that eIF6 plays versatile roles in animal cells.

Although eIF6 gene is ubiquitously expressed, its expression levels are modulated in animal cells. The $p27^{BBP}$ mRNA level has been reported to be 2- to 11-fold higher in cancer cells [11], and

Abbreviations: eIF6, eukaryotic translation initiation factor 6; MSX, methionine sulfoximine; RT, reverse transcription.

* Corresponding author. Address: Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81 3 5841 8032.
E-mail address: asyanagi@mail.ecc.u-tokyo.ac.jp (S. Yanagisawa).

[1] Research fellow of the Japan Society for the Promotion of Science.

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doi:10.1016/j.bbrc.2010.06.001

serum starvation of cultured human cells for 24 h downregulates the eIF6 gene expression levels [12]. On the other hand, the eIF6 mRNA level is reduced after several hours of nutrient starvation in *Dictyostelium discoideum* [13]. These cell type- or nutrient-dependent changes in the eIF6 mRNA level may be indicative of a linkage between ribosome biogenesis, protein synthesis and the regulation of eIF6 activity in response to environmental conditions.

In plants, amino acids are biosynthesized from inorganic nitrogen compounds in soils (nitrate and ammonia) and carbon skeletons (2-oxoglutarate) that are produced from the products of photosynthesis [14,15]. Recently, *Arabidopsis* transcriptome analyses have revealed a nitrate-responsive accumulation of ribosomal protein transcripts, suggesting that plant nitrogen response may involve the activation of ribosome biogenesis [16,17]. Our previous DNA microarray analyses in rice also suggested that a gene homologous to the yeast and animal eIF6 genes was a novel nitrogen-inducible gene (unpublished data), although eIF6 gene activation has not been reported in previous *Arabidopsis* transcriptome analyses [16,17].

An increasing number of studies on the yeast and animal eIF6 proteins have identified essential roles for eIF6 in growth control. However, although eIF6 was initially identified in wheat 30 years ago [1], the plant eIF6 genes have yet to be characterized. In our current study, we have done so for the *Arabidopsis* and rice eIF6 genes and have further investigated their expression in response to nitrogen, a critical macronutrient in plants.

2. Materials and methods

2.1. Identification of eIF6 genes in Arabidopsis and rice, and phylogenic analysis

Searches for putative eIF6 genes in *Arabidopsis* and rice were conducted in the NCBI, TAIR, and RAP databases. Two *Arabidopsis* genes showed similarity to the yeast and animal eIF6 genes were identified, and the corresponding expressed sequence tags (ESTs) were also found. These genes (*At3g55620* and *At2g39820*) were thus denoted At-*eIF6;1* and At-*eIF6;2*. Similarly, two rice genes were also found to be highly homologous to the animal and yeast eIF6 genes and are referred to as *Os-eIF6;1* and *Os-eIF6;2*. Three cDNAs (AK074012, AK104739 and AK104592) corresponding to *Os-eIF6;1* and a single cDNA (AK103139) for *Os-eIF6;2* were additionally found in the rice full-length cDNA collection. Although an additional cDNA clone (AK107378) was also identified that encodes a protein homolog of yeast and human eIF6, the sequence of this clone is not found in the complete rice genome sequence. We therefore, did not include this as a rice eIF6 cDNA clone. Phylogenic analysis was next performed via the alignment of amino acid sequences using Genetyx software (Genetyx Corp., Tokyo, Japan). Calculation of homologies among the sequences and the generation of the phylogenic tree were performed using the CLUSTAL W program [18]. The tree was displayed using the TREEVIEW program [19].

2.2. Plant materials

Seeds of *Arabidopsis* ecotype Colombia were used as wild-type. Seeds of two T-DNA insertion lines (SALK_139209 and SALK_017008) and the *emb1624* line [20] were provided by the Arabidopsis Biological Resource Center (http://abrc.osu.edu/).

2.3. PCR cloning, and semi-quantitative and quantitative real-time PCR

Total RNA extracts were prepared from *Arabidopsis* and rice seedlings using Trizol regent (Invitrogen, Carlsbad, CA) as described previously [21]. Reverse transcription (RT), and semi-quantitative and quantitative PCR assays were also performed as described previously [21,22]. The primers used are listed in Table S1. For PCR cloning experiments, KOD Fx DNA polymerase (Toyobo, Osaka, Japan) was used with specific primers, which are also listed in Table S1. All cloned DNA fragments were verified by sequencing.

2.4. Yeast transformation

To construct yeast expression constructs, the GAL4 (BD) sequence in the pGBT9 vector [23] was replaced with the respective coding sequences for the plant eIF6 genes. Yeast transformations were performed with these plasmids and the *Saccharomyces cerevisiae* strain y666 [4], as described previously [24]. The transformant phenotypes were examined using their growth profiles on media with or without glucose according to the method of Sanvito et al. [4].

2.5. Arabidopsis transformation

For the transformation of the *emb1624* line harboring the ammonium glufosinate-resistance gene, the binary vector pCB302-HYG-35SΩ-GUS was first constructed by replacing the ammonium glufosinate-resistance cassette in pCB302-35SΩ-GUS [25] with the hygromycin resistance cassette of pCAMBIA. A vector for complementation of the embryonic-lethal phenotype of the *emb1624* line, pAt-eIF6;1, was next generated by further replacing the 35S promoter and GUS gene in pCB302-HYG-35SΩ-GUS with the genomic DNA sequence for the *At-eIF6;1* locus (−707 to +1729, relative to the translation start codon). We attached a double Myc tag sequence to the At-eIF6;1 coding region in frame. The binary expression vector for the At-eIF6;1-GUS fusion protein was made by replacing the double Myc tag of pAt-eIF6;1 with the GUS gene. An additional binary vector for use in a GUS reporter assay was produced by replacement of the 35SΩ promoter in the pCB302-35SΩ-GUS vector [25] with *Arabidopsis* genomic DNA corresponding to the region from −707 to −1. GUS staining was performed as described previously [25].

3. Results

3.1. Identification of eIF6 genes in Arabidopsis and rice

To identify plant eIF6 genes, we surveyed putative candidates within the complete genomic DNA sequences of *Arabidopsis* and rice. Although it is known that a single eIF6 gene is present in yeast and animals [2–5], our screening of *Arabidopsis* and rice revealed two genes in each organism that are highly homologous to the yeast and animal eIF6 genes (Fig. S1). The presence of cDNA clones corresponding to these respective genes indicated actual transcription in each case (see Section 2.1). We therefore refer to the *Arabidopsis* genes as At-*eIF6;1* and At-*eIF6;2*, and the rice genes as *Os-eIF6;1* and *Os-eIF6;2*. The products of At-*eIF6;1*, At-*eIF6;2*, Os-*eIF6;1* and Os-*eIF6;2* show 74.6%, 60.6%, 70.5% and 66.8% identities, respectively, with yeast eIF6. However, despite these high homologies, subsequent phylogenic analysis suggested that our newly identified plant genes formed a new clade that was distinguishable from that containing the yeast and animal eIF6 genes (Fig. 1A).

3.2. The expression of At-eIF6;1 can complement the yeast tif6 mutant phenotype

It has been shown previously that human $p27^{BBP}$ and mouse eIF6 can complement the loss of TIF6 function in yeast [3–5]. To investigate whether products of the identified plant genes could do so, yeast expression vectors for *At-eIF6;1*, *At-eIF6;2*, *Os-eIF6;1*