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Research Article

Differential expression of soybean *SLTI100* gene encoding translation elongation factor 1A by abiotic stresses

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Abstract The translation elongation factor 1A, eEF1A, catalyzes the binding of aminoacyl-tRNA to the A-site of the ribosome by a GTP-dependent mechanism. By subtractive suppression hybridization technique, we have isolated a soybean low-temperature inducible gene, *SLTI100* encoding translation elongation factor 1A. Multiple sequence alignments and phylogenic analysis showed that *SLTI100* and other eEF1As originated from diverse organisms are highly conserved. RNA expression of *SLTI100* was specifically induced by low temperature, high salt, ABA, or drought stress. Based on the subcellular localization of the corresponding gene product fused to GFP, we were able to confirm that SLTI100-GFP was restricted to the nucleus and cytoplasm. We propose that soybean eEF1A may play an important role in translational regulation during abiotic stress responses in plants.

Introduction

In the translational elongation process, eukaryote translational elongation factor 1 (eEF1) is a protein synthesis factor composed of four subunits: A, Bα, Bβ and Bγ (Browing et al. 1990). Subunit eEF1A, also known as EF1α, binds aminoacyl-tRNAs to the acceptor (A) site of the ribosome during the peptide chain elongation phase of protein synthesis. eEF1A (49,174 kDa) is an abundant protein representing upto 5% of the soluble protein in wheat germ extracts (Browing et al. 1990) and is highly conserved in evolution (77% conservation of wheat EF1A with human EF1A) (Metz et al. 1992). In higher eukaryotes, eEF1A is typically encoded by a multigene family: in man there are 18 genes (Lund et al. 1996) while in *Arabidopsis* there are 4 genes (Axelos et al. 1989).

In plants, eEF1A gene expression seems constitutive (Axelos et al. 1989), but its expression was shown to vary with organ type and developmental stage such as cotton fiber elongation (Xu et al. 2007), maize endosperm (Carneiro et al. 1999), fully expanded sugarcane leaves (Vijaykumar et al. 2002), or germinating rice seeds (Kidou and Ejiri 1998). There are many evidences that plant eEF1A gene was induced by abiotic stresses such as wounding (Morelli et al. 1994), low oxygen (Vayada et al. 1995) or low temperature stress (Dunn et al. 1993; Berberich et al. 1995). It is speculated that translational efficiency by increased eEF1A level may enhance stress tolerance in plants.

At the subcellular level, maize eEF1A was shown to be closely associated with F-actin surrounding the rough endoplasmic reticulum of the endosperm cells (Clore et al. 1996; Lopez et al. 2003). Furthermore, Lopez-Valenzuela et al. (2004) proved that high level of maize eEF1A protein increased the amount of cytoskeleton surrounding ER and also Lys content in maize endosperm. It supports that eEFlA acts as a 'bridge' linking protein synthesis with the cytoskeleton network (Condeelis et al. 1995).

In the present study, we isolated the full-length cDNA of *SLTI100* encoding eEF1A regulated by abiotic stresses. It was shown that there are 4 copies of *SLTI100* in soybean genome. SLTI100-GFP was targeted to the nucleus and cy-

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toplasm. We propose that *SLTI100* gene encoding eEF1A be implicated in the protein synthesis in plants during abiotic stresses.

Materials and methods

Plant materials and treatments

Soybean plants (*Glycine max* cv. 'Sinpaldal 2') were grown in a growth chamber with 16 h/day light supply (22,000 lux) at 28°C for 3-4 weeks. As a non-stress control, leaves were sampled from plants in a growth chamber. For the low temperature stress, plants were placed in a refrigerator at 4°C upto 24 h as previously described (Cho et al. 2007). To induce salt stress, plants were soaked in a solution of NaCl (250 mM) at 28°C for 24 h. For the ABA treatment, whole plants were sprayed with ABA stock solution (100 μM) and the leaves were sampled at the indicated time. For drought stress, whole plants were removed from the soil and dehydrated at 28°C upto 24 h. Plant samples were immediately frozen in liquid nitrogen, and stored at -75°C.

Subtractive suppression hybridization (SSH)

SSH was performed using a PCR-Select cDNA Subtraction kit (Clontech, USA), as described by the manufacturer. Total RNA from 5 time-points (3, 6, 12, 24 and 48 h) (150 μg/time-point) was pooled from low temperature-treated leaves (4°C) (tester) or untreated leaves (driver), and mRNA isolated using an Oligotex mRNA Isolation kit (Qiagen, USA). Each mRNA sample (2 mg) was subjected to subtractive hybridization. Subtracted cDNAs were cloned into pCR2.1 vector (Invitrogen, USA), and transformed in TOP10F *E. coli* (Invitrogen, USA). Bacterial colonies obtained from SSH were arrayed in duplicate on nylon membranes. Specifically, each bacterial suspension (1 ml) was dotted on nylon membranes, which were incubated overnight on LB plates for bacterial growth. The duplicated membrane was hybridized with radiolabeled tester cDNA or driver cDNA. Positively selected clones were sequenced (Macrogen, Korea), and analyzed by comparing with the GenBank/EMBL database using BLAST analysis.

Cloning of *SLTI100* gene using 5' and 3' rapid amplification cDNA ends (5' RACE)

cDNA synthesis was carried out using reverse transcription kit (Promega, USA). The full-length *SLTI100* cDNA was obtained using SMART RACE cDNA amplification kit (Clontech, USA) according to the manufacturer's instruction. Gene-specific primers including 5' primer FSP1-100 and 3' primer RSP1-100 were synthesized for 5' and 3' RACE (Bioneer, Korea). The full-length cDNA of *SLTI100* was PCR-amplified with the F-100 and the R-100 primer. The resulting PCR product was cloned to pGEMT-Easy (Promega, USA) for the sequencing.

Northern blot analysis

Total RNA sequences were isolated using RNA extraction Kit (Ambion, USA). Total RNA (20 μg) was fractionated on a 1% formaldehyde agarose gel, and transferred to nylon membrane. The *SLTI100* probe was PCR-amplified using the F-100 primer and the R-100 primer covering the entire cDNA region of *SLTI100* gene (1,344 bp). The probe was labeled by a random priming method using the Ladderman labeling kit (Takara, Japan). The membrane was hybridized to a ^{32}P-dCTP labeled probe using fragments encompassing *SLTI100* cDNA and washed, as described by Church and Gilbert (Church et al. 1984). The membrane was exposed to X-ray film (Kodak, Japan) for detection of signals.

Phylogenetic analysis of eEF1As

Deduced amino acids sequences of 14 eEF1As and *SLTI100* in this study were subjected to protein phylogenetic analysis. A total of 15 sequences were aligned with the CLUSTALW program (Thompson et al. 1994) and visually examined with the GENEDOC program as previously described. Phylogenetic trees were constructed using PRODIST with the Jones-Taylor-Thornton matrix model and NEIGHBOR with the neighbor-joining method (Saitou et al. 1987) in the PHYLIP (phylogeny inference package) programs, version 3.62. We generated 1,000 bootstrapped replicate resampling data sets with SEQBOOT (PHYLIP, version 3.62). We followed the standard protocol for the default settings of the computer programs used in this procedure.

Construction of SLTI100-GFP

To construct a translational fusion of GFP and SLTI100, SLTI100 open reading frame region (447 aa; 1,344 bp) was amplified by PCR using Ex Taq (Takara, Japan) with the F-GFP100 primer containing a unique *Xba* I and an ATG initiation site and the R-GFP100 primer. PCR-amplified DNA fragment was cloned into pGEM-T Easy vector yielding and the nucleotide sequences of the inserted fragment were confirmed by DNA sequencing. The *Xba*I DNA fragments from pGEMT-SLTI100 (*Xba*I) were introduced into the

corresponding sites of pSMGFP plasmids (Genbank accession no; U70495) (Davis and Vierstra. 1996) resulting in pSLTI100-GFP. Thus, the start codon of GFP is translationally fused to the C-terminal region of *SLTI100* (Davis and Vierstra. 1996).

Subcellular localization of GFP and SLTI100-GFP

pSMGFP and pSLTI100-GFP constructs were transformed into *Agrobacterium* sp. strain C58c1, respectively and the transformed *Agrobacterium* cells with pSMGFP or pSLTI100 -GFP were expressed in the leaves of 4- to 5-week-old *Nicotiana benthamiana* plants as previously described (Kim et al. 2009). Expression of the fusion protein and vector control was monitored 36 h after infiltration. The fluorescence photographs of epidermis and protoplasts were taken using a Carl Zeiss LSM510 laser confocal microscope as previously described (Kim et al. 2009). Excitation wavelength of 488 nm was used and GFP signals were detected with the filter set for fluorescein isothiocyanate (FITC; BP 505-530 nm). Excitation wavelength of 543 nm was used and RFP was detected with the filter set of LP 560.

Table 1 Lists of primers used in this study

Primer	Sequences
FSP1-100	5'-CAATGTGAGAAGTGTGGCAG-3'
RSP1-100	5'-CTGCCACACTTCTCACATTG-3'
F-100	5'-ATGGGTAAGGAAAAGGTTCACATCAGT-3'
R-100	5'-TCACTTCTTCTTCTGGGCAGCC-3'
FS-100	5'-GAGGGAGACAACATG ATTGAGAGG-3'
F-GFP100	5'-TCTAGAATGGGTAAGGAAAAGGTTCA-3'
R-GFP100	5'-TCTAGACCCTTCTTCTTCTGGGCA-3'

Results and discussion

A subset of genes differentially expressed during low temperature stress in soybean was identified by SSH (Cho et al. 2007). Out of low-temperature induced genes, there were several genes encoding ribosomal protein associated with translational regulation (Kim et al. 2009a, 2009b). In this study, a 600-bp cDNA clone (*SLTI100*) displayed strong homology to eEF1A was characterized in relation to abiotic stress. The full-length cDNA of *SLTI100* was obtained by 5' and 3' RACE using cDNA of soybean leaf with a pair of specific primer as described in materials and methods. The full-length cDNA sequence of *SLTI100* is composed of an open reading frame (1,344 bp) (Genbank number EU790565). Deduced amino acid sequences of *SLTI100* cDNA are composed of 447 amino acid residues (M.W. 49.4 kDa; pI 9.03).

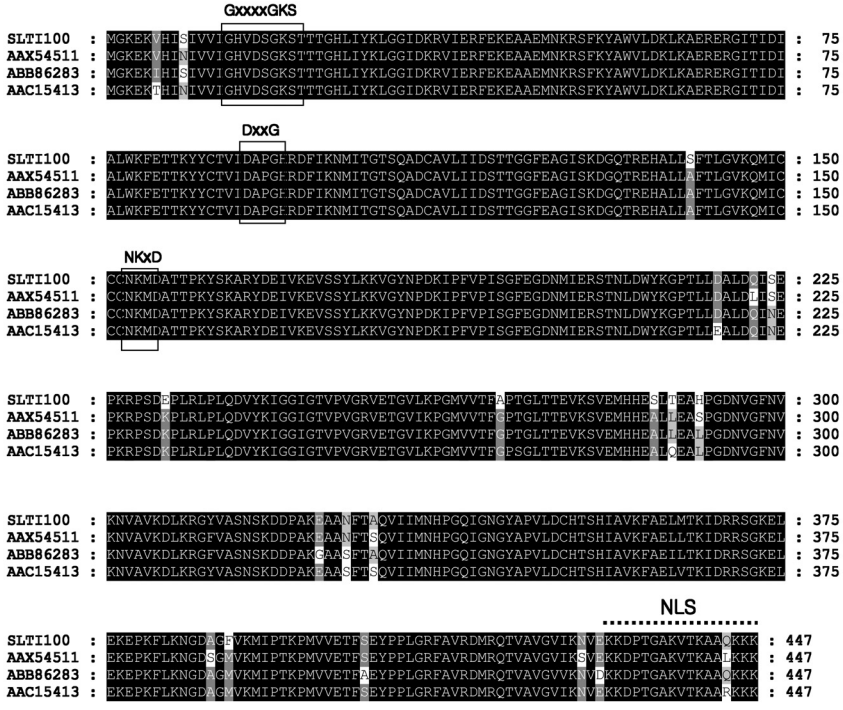

Fig. 1 Alignment of deduced amino-acid sequences of eEF1A cDNA sequences from other plant species; SLTI100 (this work), *O. sativa* (AAC15413), *S. tuberosum* (ABB86283), and *A. deliciosa* (AAX54511). Alignment of eEF1A sequences was performed using the T-Coffee program (Thompson et al. 1994). The regions corresponding to the GDP/GTP binding or GTPase activity domains of GTP-binding proteins are boxed; the consensus amino acid sequences are shown above. Nucleus localization signal (NLS) mainly composed of lysine (K) residues is indicated in the C terminus

SLTI100 cDNA sequences show 99% identity with tefS1, which was cloned and characterized before (Aguilar et al. 1991). Aguilar et al. showed that tefS1 transcript sharply increased 3 h after illumination (Aguilar et al. 1991). However, it was tested its RNA expression during abiotic stresses. A multiple sequence alignment of the deduced protein sequences of *SLTI100* gene with other eEF1A protein sequences is shown in Fig. 1. Multiple alignments of *SLTI100* and other plants eEF1A proteins showed that amino acid sequences are highly conserved each other (Fig. 1). There are three representative regions in the N-terminus of eEF1A known to be GTP binding domain important for the hydrolysis of GTP to GDP (Kidou et al. 1998) (Fig. 1). Based on the prediction of localization of plant eEF1A by Expasy (http://expasy.org/), it was shown that the C-terminal region contains a nucleus localization signal (NLS) (Fig. 1).

An extensive phylogenetic analysis using the deduced amino acid sequences of SLTI100 and fourteen eEF1A genes was inferred on the basis of the multiple sequence alignment (Fig. 2). The analysis indicated that SLTI100 was clustered with plant eEF1As rather than animal and microbe eEF1As (Fig. 2). In fact, SLTI100 showed protein sequence identity from 93% to 96% to eEF1As originated from plants. Homology between soybean eEF1A and non-plant species eEF1As is about between 73% and 76%. This shows that eEF1As are highly conserved in eukaryotes.

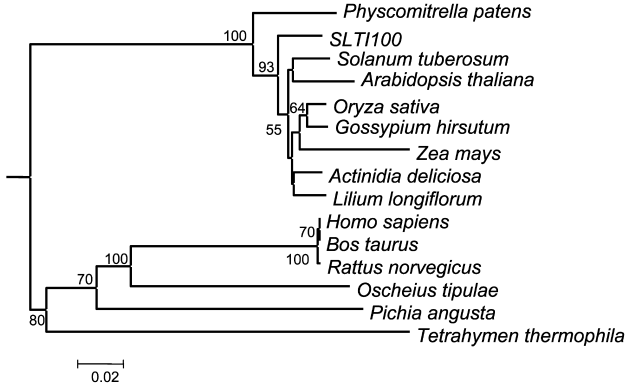

Fig. 2 Phylogenetic tree for the aligned amino acid sequences of eEF1A from diverse organisms; SLTI100 (this work), *O. sativa* (AAC15413), *L. longiflorum* (AAD56020), *Z. mays* (AAF42980), *A. deliciosa* (AAX54511), *G. hirsutum* (ABA12218), *S. tuberosum* (ABB86283), *B. taurus* (AJ238405), *P. angusta* (AY179868), *H. sapiens* (NM_001402), *R. norvegicus* (NM_175838), *O. tipulae* (AY928342), *T. thermophila* (XM_001032213), *P. patens* (XP_001753167) and *A. thaliana* (BAF02151). Phylogenetic trees were constructed using PRODIST with the Jones-Taylor-Thornton matrix model and NEIGHBOR with the neighbor-joining method (Saitou et al. 1987) to analyze phylogenetic relationships. Bootstrap values are shown for each node that had >50% support in a bootstrap analysis of 1,000 replicates. The scale bar indicates 0.1 change per amino acid

We investigated SLTI100 RNA expression level in soybean plants treated with abiotic stresses by northern blot analysis (Fig. 3). SLTI100 RNA level showed maximum after 6 h by low temperature stress and its RNA level was reduced after that. SLTI100 RNA expression was monitored by other abiotic stress such as high salt (NaCl), ABA or drought stress. SLTI100 RNA was strongly induced by high salt, ABA, or drought stress. After 24 h of drought stress, it seems that RNA got degraded to smaller sizes, which resulted in stronger staining with ethidium bromide (Fig. 3). Taken together, RNA expression of SLTI100 gene is regulated by low temperature, salt, ABA or drought stress in plants.

There are evidences proving that eEF1A gene expression is induced by environmental stresses in plants (Dunn et al. 1993; Morelli et al. 1994; Berberich et al. 1995; Vayada et al. 1995). Cold-inducible eEF1A genes were isolated from barley (Dunn et al. 1993) and maize (Berberich et al. 1995). It was reported that increases in protein synthesis are accompanied by an accumulation of eEF1A protein and transcript and that the expression of eEF1A may serve as an indicator of translational stimulation (Morelli et al. 1994). Increased RNA level of SLTI100 may contribute to tolerance to osmotic stress by stimulated translation efficiency in plants.

To examine the cellular localization of the gene product, pSLTI100-GFP was constructed and transiently expressed in the *N. benthamiana* via *Agrobacterium*-mediated transformation. Control GFP was abundant in the nucleus and cytoplasm (Fig. 4). Fluorescence signal of SLTI100-GFP was also found in the nucleus and the cytoplasm (Fig. 4). According to the prediction of *SLTI100* protein by PSORT (http://psort.ims.u-tokyo.ac.jp/form.html), the probability of the nucleus localization by *SLTI100* was 0.18 and ER targeting probability of *SLTI100* was 0.65. It is possible that *SLTI100* protein is associated with ER of outer layer of nuclear envelope and cytoplasm in cells. There are many reports demonstrated that eEF1A protein is associated with ER, and cytoskeleton, (Clore et al. 1996) and even virus pro-

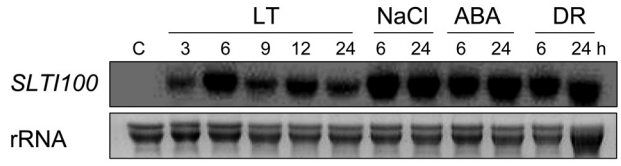

Fig. 3 Expression of *SLTI100* gene during low temperature, salt, ABA, or drought stresses. Soybean plants were incubated in a refrigerator (4ºC) upto 24 h. As a salt stress, soybean plants were fed with salt solution (NaCl, 100 mM) for 6 and 24 h. ABA (100 μM) was exogenously applied to soybean plants for 6 and 24 h. For drought stress, soybean plants were air-dried for 6 and 24 h. RNA blot analysis was carried out at least 3 times showing the same result

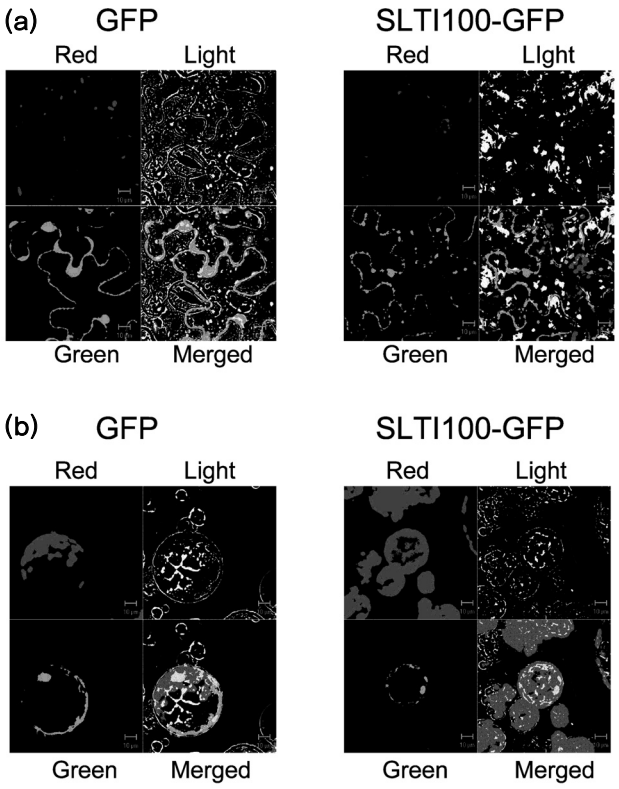

Fig. 4 Confocal images of GFP and SLTI100-GFP protein in N. benthamiana epidermal cells (a) and protoplasts (b). (a) Leaf epidermis was used for GFP localization analysis after 24 h of Agro -infiltration. (b) Protoplasts were isolated from tobacco leaves transiently expressing GFP proteins. The images of GFP were visualized at 800 times magnifications using a confocal microscope. For each panel, DIC shows the actual image, red channel shows chloroplast fluorescence and green channel shows GFP fluorescence. Merged image shows red and green channel at once

tein in cytoplasmic membrane vesicles derived from ER (Thivierge et al. 2008). In the C terminus (431-447 aa) of deduced amino acid sequences of *SLTI100* contain the signal sequence for the nucleus targeting composed of Lys rich domain (Fig. 1). However, it is not clear if fluorescence signal of SLTI100-GFP in nucleus is present in the outer layer of nuclear envelope or inside nucleus (Fig. 4). It is most likely that *SLTI100* is associated with translational process in plants.

In conclusion, we demonstrated that *SLTI100* RNA expression is induced by abiotic stresses such as low temperature, salt, ABA and drought stresses. It was shown that SLTI100 -GFP fusion protein was localized to the nucleus and cytoplasm. We propose that *SLTI100* may play an important role in protein translation process especially during abiotic stresses in plants.

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