Isolation of *soc-tuf* Gene Encoding Chloroplast Elongation Factor Tu (EF - Tu) Protein from Sugarcane (*Saccharum officinarum*)

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Abstract

Molecular chaperone is a molecule preventing the aggregation, denaturation, and inactivation of various proteins when the cells were exposed to extreme environmental conditions, such as high temperature or drought. One of such proteins is an Elongation factor Tu (EF - Tu). We have isolated an elongation factor Tu gene from sugarcane from cDNA which was heat-treated at 45°C for 16 h using reverse transcriptase PCR. The isolation was made in two steps. The first steps using internal primer developed using *tuf* gene sequence from maize available in the GenBank. Since partial cDNA sequence needs to be filled in, the sequence from sugarcane which is available in KEGG genome was added into a new primer designed to make overlapped PCR. This technique was used to clone the gene into bacterial expression vector pET-32b in order to test the *soc-tuf* gene function as molecular chaperone in bacterial system. The results showed that the gene had homology of 94% with maize *tuf* gene and 97% with sorghum amino acid sequence. The deduced amino acid sequence had homology of 92% with maize and 95% with sorghum, respectively. Gene analysis was conducted by generate phylogenetic molecular tree. Based on the tree, Sugarcane *tuf* sequence was actually clustered with *Sorghum bicolor* and *Zea mays* sequence. Thus, it had high possibility that *soc-tuf* gene from sugarcane having similar motif, protein structure, and can function as molecular chaperone as *tuf* gene from maize.

*Keywords: chloroplast protein elongation, environmental stress, heat tolerance, sugarcane (*Saccharum officinarum*)*

Introduction

Elongation factor Tu (EF - Tu) is a protein that involved in translational process. By binding to GTP and aminoacyl– tRNA, EF - Tu also translocate them into ribosome A-site (Brot, 1977; Riis, *et al.,* 1990). EF - Tu has three domains GTP-binding protein which also has similar characteristic with Ras proteins superfamily (Warren, *et al.,* 2001) belongs to the family of G proteins and is highly conserved in all organisms (Bhadula *et al.,* 2001).

EF – Tu in organelles (chloroplast and mitochondria) has the same function as EF - 1α in eukaryotic cytosol (Miller & Wissbach, 1977). Interestingly, the organellar EF – Tu shows more similarities to prokaryotic EF – Tu than to eukaryotic EF - 1α (Lee, *et al.,* 1999). Previous studies show that chloroplast EF – Tu has 80% similarity to prokaryotic EF - Tu and has 80 – 90% similarity to other EF – Tu from higher plants (Bhadula *et al.,* 2001; Riis, *et al.,* 1990). This is reflects their origin via ancient endosymbiosis (Gray, 1992). In most of lower photosynthetic eukaryotes, chloroplast elongation factor Tu (EF - Tu) is encoded by *tuf* gene in chloroplast genome (Baldauf & Palmer, 1990). While in higher plants, the genes encoding EF – Tu are located in the nucleus (Moriarty, *et al.,* 2002; Baldauf & Palmer, 1990) and after translation, the protein is transported into chloroplast.

In prokaryotes, EF - Tu is also involved in heat tolerance mechanism as molecular chaperones, that prevent the degradation of cellular proteins during heat – stress, increase the refolding of unfolded proteins, and increase the stability of protein (Moriarty, *et al.,* 2002; Momcilovic & Ristic, 2004; Rao, *et al.,* 2004 Ristic, *et al.,* 2008). During heat shock treatments, EF–Tu

molecules are accumulated in cytoplasm and near cytoplasmic membrane of *E. coli* (Caldas, *et al.,* 1998). Moreover, *E. coli* EF–Tu protein has ability to remain soluble and maintain its activity to 45°C (Caldas, *et al.,* 1998). It also binds other proteins and extends their stability up to 50°C (Caldas, *et al.,* 1998). Previous studies showed that plants also expressed chloroplast EF–Tu protein when it was exposed to high temperatures (Momcilovic & Ristic, 2004; 2007; Rao, *et al.,* 2004; Ristic, *et al.,* 2004; 2008). Exposure to a long term heat stress increased the level of chloroplast EF–Tu protein in mature wheat plants (Ristic *et al.,* 2008). The level of heat-induced EF–Tu protein in heat-tolerance wheat cultivars is greater than EF–Tu level in heat-sensitive cultivars (Ristic *et al.,* 2008). The same pattern was also observed in maize that heat sensitive maize line ZPL 389 did not accumulate EF-Tu under heat stress, while heat tolerant line ZPBL 1304 accumulate EF–Tu (Momcilovic & Ristic, 2004; 2007). Therefore, only plants, which show better tolerance to heat stress, express high amount of heat-induced EF–Tu protein. However, the evidence that chloroplast EF-Tu protein involved in heat tolerance, so far, reported only in maize (Bhadula *et al.,* 2001; Momcilovic & Ristic, 2004; Rao *et al.,* 2004; Ristic *et al.,* 2004).

In this study, EF-Tu from sugarcane (*Saccharum officinarum*) was used. Sugarcane is C4 plant commonly found in Southeast Asia region. Sugarcane has 3-4 times longer harvesting period than maize. It is assumed that sugarcane has been exposed to heat stress longer than maize and have higher temperature tolerance. The purposes of this research were to isolate *soc-tuf* gene encoding chloroplast elongation factor Tu (EF-Tu) from sugarcane and to investigate the gene similarities using phylogenetic tree. Therefore, this study was conducted based on the hypotheses, if *soc-tuf* gene is significantly homologous with *tuf* from maize or highly conserved, thus there is possibility that the protein may function to increase heat tolerance.

Materials and Methods

Samples preparation and treatment with high temperature

In this study, sugarcane plantlets with approximately of 4-6 cm of length were used. Before isolation, the plantlets were treated with different duration of incubation. Sugarcane plantlets were incubated at 45°C (Ristic, *et al.,* 2002) in incubator for 2, 4, 6, 14, and 16 h. The same treatment was also made to control plantlets exposed at 37ºC. Sugarcane from each treatment was taken and cleaned from the attached medium. Then, 600 mg of samples were used for RNA isolation total RNA from sugarcane was isolated by using Trizol® Solution Kit (Sigma, USA), followed by cDNA synthesis.

Primer design and tuf gene isolation from cDNA samples

There was no publication on sugarcane *tuf* gene so far, thus forward primer EF-TU-S for *tuf* gene amplification was designed by using maize tuf sequence. Meanwhile, maize reverse primer DO35690 (5'-TTACCACCCTCACGGATAGCAAACCTC-3') (Ristic, *et al*., 2004) was used to amplify sugarcane *tuf* gene. It was assumed that these primers will amplify about 1370 bp of sugarcane *tuf* gene. Amplification was generated using KAPA 2G™ Robust HotStart PCR system (KAPA Biosystems, USA) and sugarcane cDNA was used as a template.

Construction of full length tuf gene using overlap-extension PCR

Based on multiple sequence alignment, the amplified sugarcane *tuf* gene was not completed. To obtain full length sequence, sugarcane sequence was aligned with sugarcane EST from KEGG genome (http://www.genome.jp/kegg). Reverse primer was designed containing both sugarcane consensus sequence and expression vector sequence. While forward primer was designed containing both of *soc-tuf* partial sequence and expression vector sequence. Primers used to generate overlap-extension PCR were, forward primer EFTU-DA.F1 and reverse primer EFTU-DA.R1, respectively. Amplification was generated by using Phusion® Polymerase PCR

system (Finnzymes, USA) with touchdown PCR. Samples were visualized using DNA electrophoresis. Relevant DNA fragment was extracted from agarose gel and purified for further study.

Construction of tuf gene into expression vector pET-32b using PCR-cloning

In order to investigate the ability of sugarcane *tuf* gene, the gene was inserted into an expression vector pET-32b using PCR cloning (Bryksin & Matsumura, 2010). Instead of using two type of primer (forward and reverse primer), PCR cloning only used one DNA fragment derived from overlap–extension PCR product containing the gene of interest and two vector-homologous sequence at 3' and 5'- end of the sequence. This vector-homologous sequence acts as a primer and anneals to the plasmid vector to initiate the amplification.

Sequence analysis and construction of phylogenetic tree

Multiple sequence alignment was generated using CLUSTAL W in Bioedit and Geneious alignment in Geneious 4.8.5. Nucleotide and amino acid homology were analyzed using BLASTn and BLASTx from NCBI (http:// www.ncbi.nlm.nih.gov). Phylogenetic tree was constructed using MEGA 5.0 software.

Results and Discussion

Nucleotide sequence homology and similarity analysis

Based on BLASTn result (Table 1), sugarcane *tuf* sequence had 97% homology to *Sorghum bicolor* hypothetical protein sequence with 100% query coverage. *Sorghum bicolor* hypothetical protein has not been annotated yet. However, this protein has similar characteristics with EF-Tu proteins. In order to make the result more reliable, sugarcane *tuf* sequence was aligned with maize sequence and the result showed that sugarcane sequence had 94% similarity with maize *tuf* sequence encoding elongation factor Tu (EF–Tu) with 100% query coverage. E-value for these alignments are 0, thus the result was reliable.

Table 1. BLASTn result from sugarcane EF - Tu sequence using eucaryotes database

Accession Number	Species	Query Coverage	E-value	Homology
XM_002452345.1	*Sorghum bicolor* hypothetical protein, mRNA	100%	0	97%
NM_001156938.1	*Zea mays* elongation factor Tu (LOC100284040), mRNA >gb|EU966875.1| *Zea mays* clone 297889 elongation factor Tu mRNA, complete cds	100%	0	94%

In order to predict whether the nucleotide differences within sugarcane sequence has frame shift effect at amino acid sequence or not, sugarcane sequence must be analyzed by using BLASTx. Based on BLASTn result, sugarcane *tuf* sequence had 97% homology to *Sorghum bicolor* hypothetical protein sequence with 100% query coverage. The result (Table 2) showed that the differences within sugarcane nucleotide residues do not change amino acid sequence significantly. Sugarcane sequence still had 95% homology with *Sorghum bicolor* hypothetical protein and also 92% homology with *Zea mays* elongation factor Tu.

In order to investigate the homology between sugarcane and procaryotes *tuf* sequence, another BLASTx and BLASTn were conducted using procaryotes protein database. Based on BLASTn result, sugarcane *tuf* sequence had 67% homology to *Escherichia coli* (acc.

ZP_07125171.1) elongation factor Tu sequence with 90% query coverage. Moreover, The BLASTx result (Table 3) showed that sugarcane sequence still had 70% homology with *Thermus scotoductus* SA-01 and *Thermus Aquaticus* elongation factor Tu, also had 69% homology with *Thermus thermophilus* HB27 elongation factor Tu.

Table 2. BLASTx result from sugarcane EF - Tu sequence using eucaryotes database

Accession Number	Species	Query Coverage	E-value	Homology
XP_002452390.1	*Sorghum bicolor* hypothetical protein SORBIDRAFT_04g024850 >gb|EES05366.1|	99%	0	95%
NP_001149568.1	*Zea mays* LOC100283194 >gb|ACG35888.1| *Zea mays* elongation factor Tu	99%	0	92%

Table 3. BLASTx result from sugarcane EF - Tu sequence using procaryotes database

Accession Number	Species	Query Coverage	E-value	Homology
YP_004201365.1	Elongation factor Tu [*Thermus scotoductus* SA-01]	90%	3e-149	70%
1EFT_A	The crystal structure of elongation factor EF-Tu from *Thermus Aquaticus*	90%	3e-149	70%
YP_005299.1	Elongation factor Tu [*Thermus thermophilus* HB27]	90%	1e-148	69%

Based on BLAST results, it can be concluded that the sequence isolated from sugarcane was the *tuf* gene coding sequence. Characterization of sugarcane *tuf* gene was conducted by using various bioinformatics tools. FGENESH 2.6 from Softberry (www.softberry.com showed that *soc-tuf* cDNA sequence consisted of 1404 nucleotide residue which encodes 466 amino acid.

Construction of phylogenetic tree

Phylogenetic tree (Figure 1) of *tuf* sequences constructed based on sample sequence aligned with the various *tuf* sequences which were retrieved from GenBank and analyzed using Neighbor-Joining method with Kimura 2 parameter. The phylogenetic tree will confirm the BLASTn result. Tentative result of the BLASTn was used to collect other sequence related to the sample sequence, and construct the tree.

Sugarcane sequence is clustered with *Sorghum bicolor* with value 0.021 and *Zea mays* sequence with value 0.055 and also clustered at the same group as *Oryza sativa* with value 0.115 and *Triticum aestivum* with value 0.117 which belong to Poaceae family (the scale or branch length refers to the evolutionary distances used to infer the phylogenetic tree).

Based on sequence homology, several groups were formed with *Thermus thermophilus* as an outgroup. The homology of *tuf* sequence within plants (intra-clade) were higher than the inter-clade homology of *tuf* sequence. Thus, plant species formed a single cluster. While, bacterial species also formed a single cluster. Sugarcane sequence is actually clustered with *Sorghum bicolor* and *Zea mays* sequence at almost no nucleotide difference. It is also clustered at the same group as *Oryza sativa* and *Triticum aestivum* which are belong to Poaceae family. Due to *soc-tuf* gene has significant homology with maize *tuf*, thus there is high possibility that it also has similar motif, protein structure, and can function as molecular chaperone as *tuf* gene from maize in order to improve heat tolerance abillity in organisms.

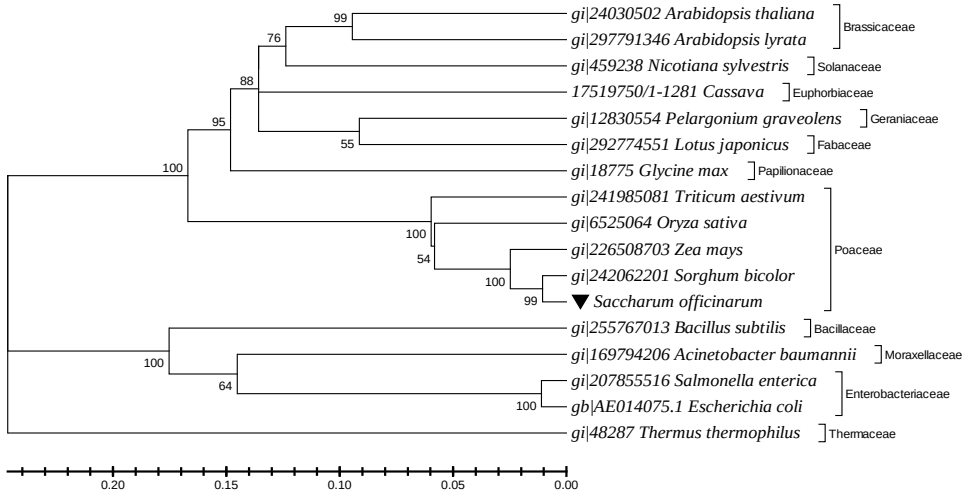

Figure 1. Neighbor-joining tree of *tuf* gene sequence using Kimura 2 parameter model with Gamma distribution.

References

Baldauf, S.L., J.D. Palmer. 1990. Evolutionary transfer of the chloroplast *tufA* gene to the nucleus. *Nature*. 344: 262 - 265.

Bhadula, S.K., T.E. Elthon, J.E. Habben, T.G. Helentjaris, S. Jiao, Z. Ristic. 2001. Heat stress induced synthesis of chloroplast protein synthesis elongation factor (EF-Tu) in a heat-tolerant maize line. *Planta*. 6:212-359.

Brot, N. 1977. *Molecular Mechanism of protein synthesis.* (H. Weissbach, & S. Pestka, Eds.). Academic Press, New York.

Bryksin, A.V., I. Matsumura. 2010. Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids. *BioTech*. 48: 463 - 465.

Caldas, T., A. Yaagoubi, G. Richarme. 1998. Chaperone properties of bacterial elongation factor EF-Tu. *Biol. Chem*. 273: 11478 - 11482.

Gray, M.W. 1992. The endosymbion hypothesis revisited. *Int. Rev, Cytol*. 141: 233 - 357.

Lee, J.H., J.W. Lee, Y-Y. Chung, K.H. Paek, J.S. Shin, C-H. Yun. 1999. Cloning and characterization of chloroplast elongation factor EF-Tu cDNA of *Oryza sativa* L. *Mol. Cells*. *9* (5): 484 - 490.

Momcilovic, I., Z. Ristic. 2007. Expression of chloroplast protein synthesis elongation factor, EF-Tu, in two lines of maize with contrasting tolerance to heat stress during early stages of plant develoment. *Plant Physiol*. 164: 90 - 99.

Momcilovic, I., Z. Ristic. 2004. Localization and abundance of chloroplast protein synthesis elongation factor (EF-Tu) and heat stability of chloroplast stromal protein in maize. *Plant Sci*. 81 - 88.

Moriarty, T., R. West, G. Small, D. Rao, Z. Ristic. 2002. Heterologous expression of maize chloroplast protein synthesis elongation factor (EF-Tu) enhances *Escherichia coli* viability under heat stress. *Plant Sci*. 163: 1075 – 1082.

Rao, D., I. Momcilovic, S. Kobayashi, E. Callegari, Z. Ristic. 2004. Chaperone activity of recombinant maize chloroplast protein synthesis elongation factor, EF-Tu. *Eur J. Biochem*. 271: 3684 - 3692.

Riis, B., S.I. Rattan, B.F. Clarck, W.C. Merrick. 1990. Eukaryotic Protein Elongation Factors. *Treds Biol. Sci*. 15: 420 - 424.

Ristic, Z., U. Bukovnik, I. Momcilovic, J. Fu, P.V. Prasad. 2008. Heat-induced accumulation of chloroplast protein synthesis elongation factor, EF-Tu, in winter wheat. Journal of plant physiology. *Plant Physiol*. 165: 192 – 202.

Ristic, Z., K. Wilson, C. Nelsen, I. Momcilovic, S. Kobayashi, R. Meeley. 2004. A maize mutant with decreased capacity of accumulate chloroplast protein synthesis elongation factor (EF-Tu) displays reduced tolerance to heat stress. *Plant Sci*. 167: 1367 – 1374.

Warren, C., I. Lathe, B. Peer. 2001. Evolution of *tuf* genes: ancient duplication, differential loss, and gene conversion. *FEBS Letters*. 502: 113 - 116.

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