

Expression of Mangrove eEF1A Enhances Tolerance to Salt and Osmotic Stress in *Escherichia coli*

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Abstract

To analyze the mechanisms of salt tolerance in the mangrove plant, *Bruguiera sexangula*, functional screening for mangrove cDNAs encoding proteins essential for salt tolerance was performed using *Escherichia coli* as the host organism. A transformant expressing a eukaryotic elongation factor 1A homologue (Bs-eEF1A) displayed enhanced tolerance to salt and osmotic stress. This distinct function was not conferred by other plant eEF1A homologues isolated from two halophytes (*Suaeda japonica* and *Salsola komarovii*) or *Arabidopsis thaliana*. Levels of Bs-eEF1A transcripts in cultured *B. sexangula* cells were enhanced in response to salt stress. These data suggest that Bs-eEF1A plays an important role in the salt-tolerance mechanisms of *B. sexangula*, in addition to its original function in elongation during translation.

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Key words: *Bruguiera sexangula*, eEF1A, mangrove, *Salsola komarovii*, salt tolerance, *Suaeda japonica*.

Abbreviation

eEF1A, eukaryotic elongation factor 1A.

Although the importance of mangrove forests for the global ecosystem has been recognized, there are few molecular data available to explain why mangrove plants can grow in brackish habitats (Yamada *et al.*, 2002a, b, c; Hibino *et al.*, 2000). To isolate the genes essential for salt tolerance in mangrove plants, we constructed a mangrove cDNA library from suspension-cultured cells of the mangrove plant, *Bruguiera sexangula* (Mimura *et al.*, 1997a, b; Kura-Hotta *et al.*, 2001). We conducted functional screening of this library for genes essential to salt tolerance, using *Escherichia coli* as the host organism (Yamada *et al.*, 2002b, c). A transformant expressing a eukaryotic elongation factor 1A (eEF1A) homologue displayed enhanced salt tolerance.

Eukaryotic translation elongation factor 1A (eEF1A, formerly EF-1 α) is a highly abundant protein responsible for the delivery of aminoacyl-

tRNA to the acceptor site of ribosomes during peptide chain elongation (Browning, 1996). In addition to its role in protein synthesis, eEF1A appears to be involved in a number of other cellular processes, including microtubule severing (Shiina *et al.*, 1994), actin filament bundling (Edmonds *et al.*, 1995), and ubiquitin-dependent proteolysis of N-terminus-blocked proteins (Gonen *et al.*, 1994). Thus, eEF1A is a multifunctional protein. However, it has yet to be explained why enhanced salt tolerance is acquired by *E. coli* transformants expressing Bs-eEF1A.

In this study, we focused on *B. sexangula* eEF1A (Bs-eEF1A), and the *in vivo* effects of Bs-eEF1A and its homologues on salt tolerance were tested using *E. coli* as the host organism. The effects of salt stress on Bs-eEF1A transcription in cultured *B. sexangula* cells were also investigated. This research should extend our understanding of the salt-tolerance mechanisms of *B. sexangula*.

A *B. sexangula* cDNA library was constructed, as described previously (Yamada *et al.*, 2002a). The cDNA library was introduced into *E. coli* SOLR by

an *in vivo* excision system (Stratagene, La Jolla, CA, USA), and the transformants were selected on 2YT agar plates supplemented with 50 $\mu\text{g ml}^{-1}$ kanamycin, 50 $\mu\text{g ml}^{-1}$ ampicillin, 50 μM isopropyl- β -D-thiogalactopyranoside (IPTG), and 400 mM NaCl. From one million *E. coli* transformants, 29 transformants showed remarkable growth under conditions of salt stress. Analysis of their nucleotide sequences showed that two clones contained identical sequences encoding a full-length eukaryotic elongation factor 1A (eEF1A) homologue. Most of the other clones contained cDNAs encoding allene oxide cyclase homologue (Yamada *et al.*, 2002b), and α subunit of chaperonin containing TCP-1 (CCT α) (Yamada *et al.*, 2002c). The complete sequence of the eEF1A homologues was determined and deposited in the EMBL/GenBank/DBJ database under accession number AB073629. To ana-

lyze the activity of plant eEF1A homologues in enhancing salt tolerance in *E. coli*, cDNAs encoding eEF1A homologues were isolated from *Arabidopsis thaliana* and two halophytes, *Salsola komarovii* (Takeno and Yamaguchi, 1991) and *Suaeda japonica* (Tanimoto *et al.*, 1997), which belong to the family Chenopodiaceae. The *A. thaliana* eEF1A homologue (At-eEF1A; accession number P13905) cDNA was isolated by PCR using a cDNA library (constructed from 4.5-week-old Columbia leaves and stems) and the following primers: 5'-GAC-CAGTCTCTAACCATGGGAAAAG-3' (forward primer) 5'-TTTGAGTTCAGAGTTCACCTTGGCAC-3' (reverse primer). The amplified fragment was cloned into the *EcoRV* endonuclease site of pBluescript SK. The cDNAs encoding eEF1A homologues from the two halophytes were successfully isolated by the functional screening method, as described

Bs-eEF1A	MGKEKIHINIVVIGHVDSGKSTTTGELIYKLGIDKRVIERFEKEAAEMNKRSPKYAWVL	60
Sk-eEF1ASL.....	60
Sj-eEF1ASL.....	60
At-eEF1AF.....	60
	a	
Bs-eEF1A	DKLKAERERGITIDIALWKFETTKYCYTVIDAPGHRDFIKNMITGTSQADCAVLIIDSTT	120
Sk-eEF1AN.....I.....	120
Sj-eEF1AN.....I.....	120
At-eEF1AI.....	120
	b	
Bs-eEF1A	GGFEAGISKDGGQTRFHALLAFTLGVKQMICCCNKMDATTSKYSKARYDEIVKEVSSYLKK	180
Sk-eEF1AP.....S.F.....	180
Sj-eEF1AS.....R.....P.....	180
At-eEF1AP.....I.....	180
	c	
Bs-eEF1A	VGYNPEKIPFVPISGFEGDNMIERSTNLDWYKGPITLLEALDMIQEPKRPDKPLRLPLQD	240
Sk-eEF1AD..A.....N.....	240
Sj-eEF1AV.....N.....I.....	240
At-eEF1AD.....Q.N.....	240
	d	
Bs-eEF1A	VYKIGGIGTVPVGRVETGVLPKPGMVVTFGSPGLTTEVKSVEMHHEALQEQALPGDNVGFNV	300
Sk-eEF1AT.....S.P.....	300
Sj-eEF1AI.....N.....T.....S.P.....	300
At-eEF1AMI.....A.T.....S.L.....	300
	e	
Bs-eEF1A	KNVSVKDLKRGYVASNSKDDPAKEASSFTSQVIIMNHPGQIGNGYAPVLDCHTSHIAVKF	360
Sk-eEF1A	..A...I..F...D..N...AN..A.....	360
Sj-eEF1A	..I...D..N...G...A.....	360
At-eEF1A	..A...G..AN.....	360
	f	
Bs-eEF1A	SEILTKIDRRSGKELEKEPKFLKNGDAGFVKMIPTKPMVVETFSYPPVLRFAVRDMRQT	420
Sk-eEF1A	A.LV.....MI..V.....	420
Sj-eEF1A	A.L.....M.....A.S.....	420
At-eEF1AI.....M...T.....	420
	g	
Bs-eEF1A	VAVGVIRSVKKEPSSGAKVTKSAA-KKGGK	449
Sk-eEF1AN...D.T.....L.--.	447
Sj-eEF1AD...TS.....M.--.	447
At-eEF1AD..D.T.....V...A.	449

Fig. 1 Comparison of the Bs-eEF1A (AB073629) deduced amino acid sequence with the sequences of Sk-eEF1A (AB073631), Sj-eEF1A (AB073630), and At-eEF1A (P13905). Gaps are introduced to optimize the amino acid sequence alignment. Regions a, b, c, and d are involved in GTP binding and hydrolysis (Yang *et al.* 1990). Regions e, f and g constitute putative actin- and tRNA-binding domains (Yang *et al.* 1990), respectively. Arrows denote Bs-eEF1A-specific amino acid residues, relative to the three plant eEF1A homologues. Sequences were aligned using Genetyx software (Software Development, Tokyo, Japan).

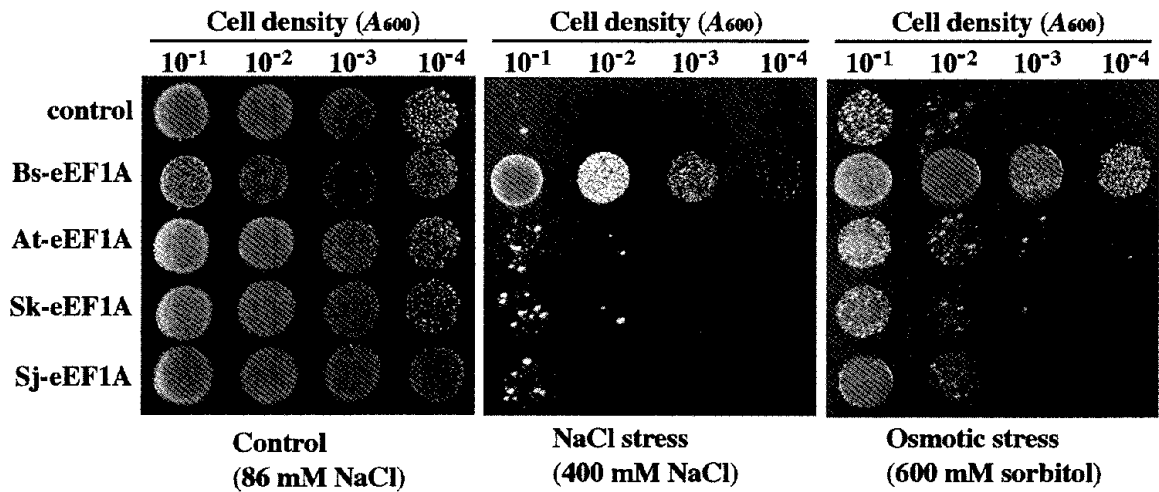


Fig. 2 Effects of the expression of Bs-eEF1A and homologous genes on salt and osmotic-stress tolerance in *E. coli*. Bs-eEF1A, Sk-eEF1A, Sj-eEF1A, or At-eEF1A cDNAs were introduced into *E. coli* (SOLR). Transformants were grown up to exponential phase in liquid 2YT medium containing 86 mM NaCl (normal NaCl concentration), 50 μ M IPTG, 50 μ g ml⁻¹ ampicillin, and 50 μ g ml⁻¹ kanamycin. The cell densities of *E. coli* transformants were adjusted to OD₆₀₀ = 0.1, and serial dilutions (1:10) were prepared. A 15 μ l aliquot of each dilution was spotted onto 2YT agar plates supplemented with 50 μ g ml⁻¹ kanamycin, 50 μ g ml⁻¹ ampicillin, 50 μ M IPTG, and 400 mM NaCl or 600 mM sorbitol. Empty vector, pBluescript SK, was used as a negative control. Plates were photographed after incubations of 12 h (normal condition) or 18 h (400 mM NaCl and 600 mM sorbitol conditions) at 37 °C.

above, using *S. japonica* and *S. komarovii* cDNA libraries constructed with a ZAP-cDNA Synthesis Kit (Stratagene). The nucleic acid sequences of the eEF1A homologues of *S. komarovii* (Sk-eEF1A) and *S. japonica* (Sj-eEF1A) were deposited in the EMBL/GenBank/DBJ database under accession numbers AB073630 and AB073631, respectively.

Plant eEF1As are highly conserved (Fig. 1). Homologies between the putative amino acid sequences of Bs-eEF1A and the other plant eEF1A homologues are 94.4% (At-eEF1A), 92.8% (Sk-eEF1A), and 93.3% (Sj-eEF1A). Fig. 2 shows the effects of the expression of plant eEF1A genes on salt and osmotic-stress tolerance in *E. coli*. All transformants grew on plates containing 86 mM NaCl (control). The growth of cells transformed with empty vector was strongly inhibited on plates containing over 400 mM NaCl or over 600 mM sorbitol. Slightly enhanced tolerance to salt and osmotic stress were observed in At-eEF1A, Sk-eEF1A, and Sj-eEF1A transformants. On the other hand, Bs-eEF1A transformants showed distinct growth under these stress conditions. Therefore, Bs-eEF1A might play an important role in the tolerance of salt and osmotic stress in transformed *E. coli*, whereas the other plant eEF1A homologues did not confer similar activity. Several amino acid residues specific to Bs-eEF1A were identified in

the eEF1A homologues examined (Fig. 1). The functional difference seen in Bs-eEF1A-transformed *E. coli* might be attributable to these residues.

To understand the relation between salt stress and the transcription of eEF1A mRNA in *B. sexangula*, northern blot analysis was performed using suspension-cultured cells cultivated under NaCl-free conditions for three days. Under these conditions, the effects of inoculation shock when cells are transferred into new medium are probably minimized (Yamada *et al.*, 2002b, c). Fig. 3 shows northern blot analysis of Bs-eEF1A mRNA expression after the addition of 100 mM NaCl to *B. sexangula* suspension cultures. Two bands are evident in each lane and the intensities of both bands in each lane are almost identical. The band intensities in both lanes were enhanced in response to salt stress. Maximum band intensities were detected 180–360 min after the addition of NaCl. Similar effect was also observed after addition of 300 mM sorbitol to *B. sexangula* suspension cultures (data not shown). Southern blot analysis indicated that there are at least four genes that are homologous to Bs-eEF1A (Fig. 4). To analyze the function of Bs-eEF1A in detail, isolation of cDNAs encoding Bs-eEF1A homologues should be required.

Bacterial elongation factor Tu (EF-Tu) shows

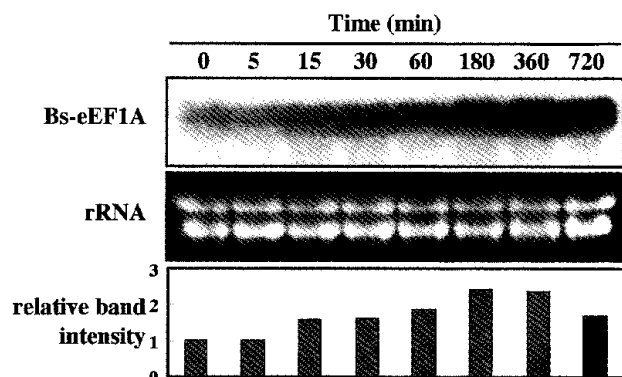


Fig. 3 Northern blot analysis of Bs-eEF1A mRNA expression after the addition of 100 mM NaCl to *B. sexangula* suspension cultures. Total RNA was extracted from the cultured *B. sexangula* cells by the guanidine thiocyanate/CsCl method (Kingston, 1991). Total RNAs (25 μ g) were separated by agarose gel electrophoresis and transferred to nylon Nytran SuPerCharge membrane (Schleicher & Schuell GmbH, Dassel, Germany). RNA blots were hybridized to complete Bs-eEF1A cDNA fragment labeled randomly with 32 P. The membranes were washed in 0.1 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 65 $^{\circ}$ C (high stringency). The band intensities corresponding to eEF1A mRNAs and ribosomal RNAs were determined using a Bioimage analyzer (BAS-1500, Fuji Film, Japan) and Typhoon 8600 (Amersham Bioscience Corp., USA), respectively. Relative band intensities indicate the intensities of the eEF1A mRNAs relative to rRNAs. Initial relative band intensities were adjusted to 1.0.

partial homology to eEF1A, and plays a role in elongation during translation, like eEF1A (Gaucher *et al.*, 2001). In addition to this principal function, EF-Tu has chaperon-like properties (Kudlicki *et al.*, 1997; Caldas *et al.*, 1998). Recently, it was reported that the expression of small heat shock proteins and CCT α , which show molecular chaperon properties, enhances the salt tolerance of *A. thaliana* (Sun *et al.*, 2001) and *E. coli* (Yamada *et al.*, 2002c, d). Therefore, we suggest that Bs-eEF1A has chaperon-like properties, like EF-Tu.

Further analysis of Bs-eEF1A will contribute to our understanding of the salt-tolerance mechanisms of *B. sexangula* at the molecular level.

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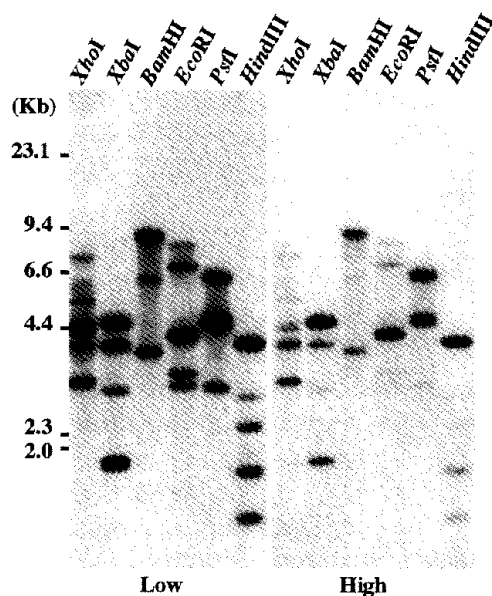


Fig. 4 Southern blot hybridization of *B. sexangula* genomic DNA fragments with Bs-eEF1A probe. *Bruguiera sexangula* genomic DNA was digested with *Xho*I, *Xba*I, *Bam*HI, *Eco*RI, *Pst*I, and *Hind*III. After hybridization, the membrane was washed in 1 x SSC and 0.5% SDS at 42 $^{\circ}$ C (low stringency; Low) or 0.1 x SSC and 0.1% SDS at 65 $^{\circ}$ C (high stringency; High).

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