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Expression of Plant CCTα Genes Enhance Salt and Osmotic Stress Tolerance in *Escherichia coli*

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Abstract

To analyze the mechanisms of salt-tolerance in a mangrove plant, *Bruguiera sexangula*, functional screening of cDNAs encoding proteins essential for the salt-tolerance was performed using *Escherichia coli* as a host organism. A transformant expressing the α subunit of cytosolic chaperonin containing TCP-1 (CCTα) homologue was found to have enhanced salt-tolerance. A similar function was also observed in CCTα from *Arabidopsis thaliana*. The amount of CCTα transcript in the mangrove-cultured cells did not change in response to salt-stress and the transcript was continuously produced in the presence of NaCl or not. The role of plant CCTα in salt-tolerance was discussed.

Keywords: *Bruguiera*, CCTα, mangrove, salt tolerance

Abbreviations

CCTα, α subunit of cytosolic chaperonin containing TCP-1; TCP-1, t-complex peptide-1.

To analyze the mechanism of salt-tolerance in higher plants, numerous key genes have been cloned; e.g., late-embryogenesis abundant proteins (Xu *et al.*, 1996), P5CS (Kishor *et al.*, 1995), DREB1A (Kasuga *et al.*, 1999) and AtNHX1 (Apse *et al.*, 1999). In contrast, there are few reports (Hibino *et al.*, 2001) dealing with genes for salt-stress tolerance in mangrove plants. To find key genes essential for salt-tolerance in mangrove plants, we have constructed a mangrove cDNA library from suspension-cultured cells of a mangrove plant, *Bruguiera sexangula* (Mimura *et al.*, 1997a; Mimura *et al.*, 1997b; Kura-Hotta *et al.*, 2001) and have conducted functional screening of key genes essential for the salt-tolerance mechanisms using *Escherichia coli* as a host organism. In this screening, transformants expressing the α subunit of cytosolic chaperonin containing TCP-1 (CCTα) homologue were found to have enhanced salt-tolerance.

Chaperonins are a class of molecular chaperones that mediate the folding of non-native polypeptides by ATP hydrolysis (Frydman, 2001), and have been assigned to either type I or type II (Kubota *et al.*, 1995). Type I includes GroEL from eubacteria (Georgopoulos *et al.*, 1973), HSP60 from mitochondria (Cheng *et al.*, 1989), and the Rubisco-subunit binding proteins from chloroplast (Hemmingsen *et al.*, 1988). Type II includes TF55 (Trent *et al.*, 1991) and thermosomes (Phipps *et al.*, 1991) from archaebacteria, and the CCT complex also called TRiC (TCP-1 ring complex) from the cytosol of eukaryotes (Kubota *et al.*, 1995). Similar to other chaperonins, the CCT complex is a high molecular protein whose subunits are arranged in two stacked multimeric rings with a central cavity. Whereas type I chaperonins such as GroEL are promiscuous, assisting in the folding of many other proteins, only a few proteins, mainly actin and tubulin, have been described as natural substrates for the CCT complex (Ursic *et al.*, 1994). At present, information concerning the role of the CCT complex in the salt-tolerance of higher plants is lacking. In this study, we focused on the α subunit of the CCT complex, CCTα, and the *in vivo* function of plant CCTα in salt and osmotic tolerance was investigated using *Escherichia coli* as a host organism.

The *B. sexangula* cDNA library containing over one million independent clones was constructed using ZAP cDNA synthesis kit (Stratagene, La

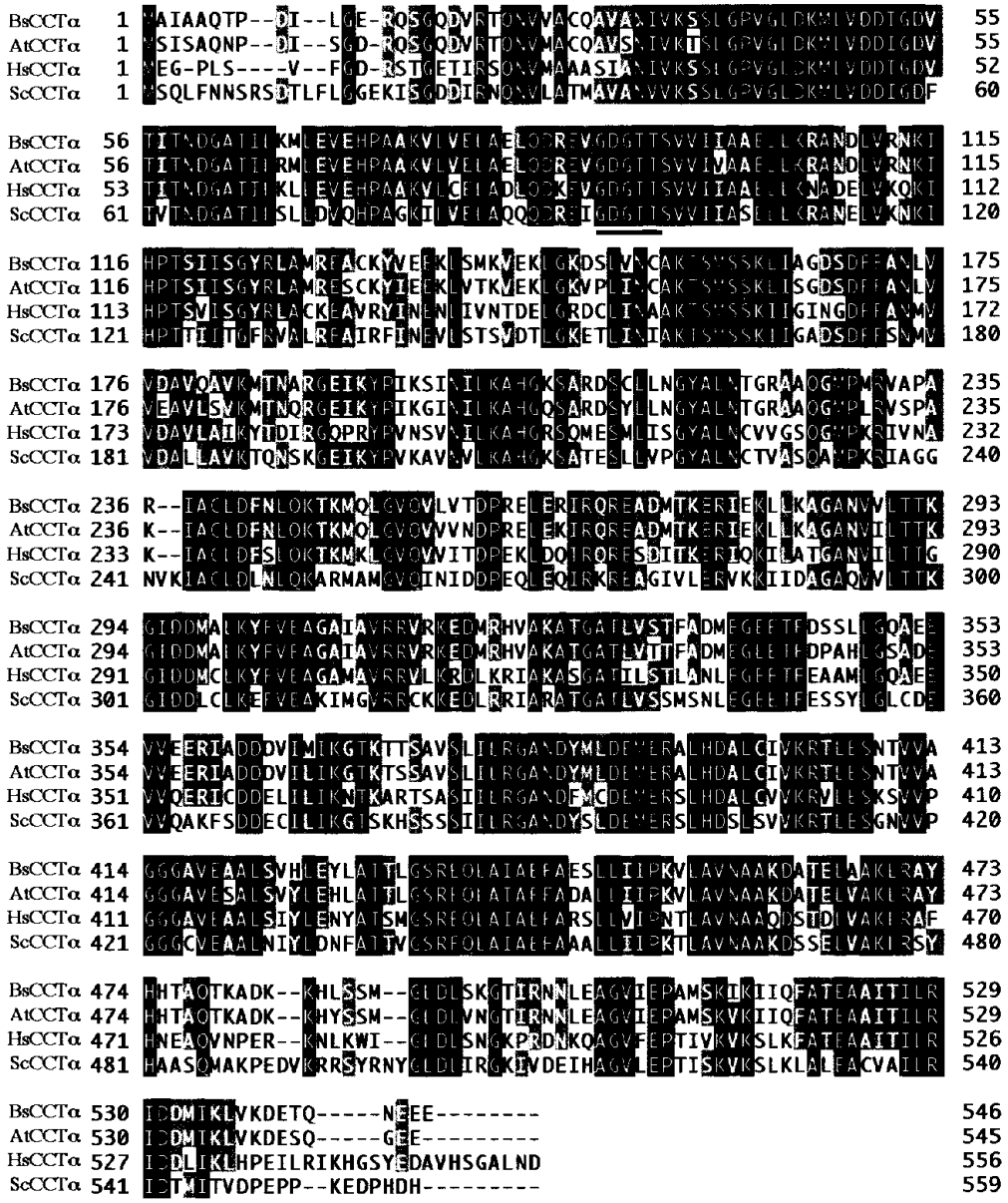

Fig. 1 Comparison of CCTα amino acid sequence of *B. sexangula* with the homologues of *A. thaliana* (AtCCTα, accession no. P28769), *Homo sapiens* (HsCCTα, accession no. X52882), and *S. cerevisiae* (ScCCTα, accession no. M21160). Gaps were introduced to optimize the amino acid sequence alignment. Underlining indicates ATP binding domains (GDGTT), which were completely conserved in each CCTα sequence. The alignment was done using GENETYX software (Software Development, Tokyo, Japan).

Jolla, CA USA) and transformed into *E. coli* SOLR (Stratagene) using the *in vivo* excision system. The transformants were plated on 2YT agar plates containing 400 mM NaCl. The growth of the host strain was strongly inhibited at this condition. From one million *E. coli* transformants, twenty-nine transformants showed remarkable growth under salt stress conditions. Analysis of their partial nucleotide sequences showed that two clones had an identical sequence encoding full length of CCTα homologue. The whole sequence was determined and deposited in the EMBL/GenBank/DDBJ database under accession No. AB073552. The length of the cDNA was 2060 bp and it encoded a putative protein containing 546 amino acids. Database searches with the BLAST program revealed that this amino acid sequence shows homology with other CCTα sequences from higher plants, mammals, and yeast (**Fig. 1**). The homologies between the putative amino acid sequences of the *B. sexangula* CCTα homologue (BsCCTα) and the other CCTα sequences from *Arabidopsis thaliana* (AtCCTα), *Homo sapiens* (HsCCTα), and *Saccharomyces cerevisiae* (ScCCTα) were 90%, 67% and 62%, respectively. At the moment, there are no entries about plant CCTα sequences in EMBL/GenBank/DDBJ

database without AtCCTα. To confirm weather BsCCTα have the specific function in *E. coli* or not, AtCCTα was also tested as a control. Nested PCR was performed to amplify the AtCCTα cDNA. As a template, an *A. thaliana* cDNA library (4.5-week-old Columbia leaves and stem) was used. Primers were designed based on previously determined sequences (Mori *et al.*, 1992) as follows:

1st atCCTaF:
5'-ACTGCACTTTATCTCGAGAGCTCAGATCTC-3'
1st atCCTaR:
5'-CAGCTCTTTCAGAGGCTACATTGTTACAGC-3'
2nd atCCTaF:
5'-CTGAATAATGTCGATCTCCGCCCAA-3'
2nd atCCTaR:
5'-GAGGTTTGCTTATTCTTCGCCTTGG-3'.

After the first PCR, the PCR products containing several fragments were purified by QIAquick PCR Purification kit (QIAGEN, Valencia, CA USA) to remove first primers. The second PCR was done using the first PCR products as a template. Approximately 2.0 Kb of a single band was detected in the second PCR. The band was cloned into the *Eco*RV endonuclease site of pBluescript SK.

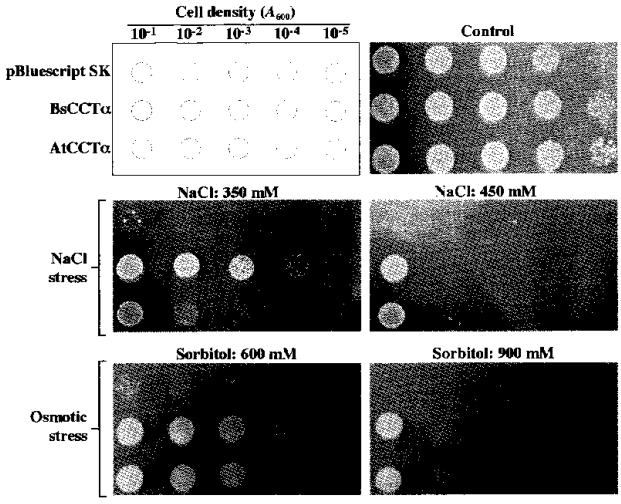

Fig. 2 Effect of BsCCTα and AtCCTα expression on salt and osmotic stress tolerance in *E. coli*. Plant CCTα cDNAs (in pBluescript SK) were introduced into *E. coli* (SOLR). The transformants were grown up to exponential phase in liquid 2YT medium containing 86 mM NaCl (normal NaCl concentration), 0.05 mM IPTG, 50 μg ml^{-1} ampicillin, and 50 μg ml^{-1} kanamycin. Serial dilutions, 1:10, were made from these *E. coli* transformants, whose cell density was adjusted to OD_{600} at 0.1. A 15 μl aliquot of each dilution was spotted on 2YT agar plates supplemented with 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} ampicillin, 0.05 mM IPTG, and various concentrations of NaCl or sorbitol. Empty vector, pBluescript SK, was used as a control. Plates were photographed after 12 h of incubation at 37 ℃.

Fig. 2 shows the effects of plant CCTα genes expression on salt and osmotic stress tolerance in *E. coli* (SOLR). All transformants could grow on the plate containing 86 mM NaCl (control). The empty vector transformant could not grow on the plate containing over 350 mM NaCl or over 600 mM sorbitol. On the other hand, BsCCTα and AtCCTα transformants grew in the presence of 450 mM NaCl or 900 mM sorbitol. Therefore, it can be said that BsCCTα and AtCCTα have similar function in *E. coli*.

The structures of type I and type II chaperonins have similar organization in their three domains (apical, intermediate, and equatorial) within the monomer (Llorca *et al.*, 1998). Chatellier *et al.* (1998) showed that the fragment encompassing the apical domain of GroEL, called minichaperones, facilitated the refolding of several proteins *in vivo* and *in vitro* without requiring GroES, ATP, or the cage-like structure of multimeric GroEL. Plant CCTα could not compose cage-like structure, because other CCT subunits are absent in *E. coli*, It can be postulated that plant CCTα also has chaperone activity like that of minichaperones.

Recently other group of a molecular chaperon, small heat shock protein (At-HSP17.6A) was characterized. Over production of At-HSP17.6A could increase salt and drought tolerance in *A. thaliana* (Sun *et al.*, 2001) and suggested that At-HSP17.6A play an important role in protecting cellular components under the stress conditions. Plant CCTα may have similar function that the molecular chaperone activity could give rise to enhanced salt and osmotic stress tolerance in *E. coli*.

In order to analyze the effect of salt-stress on CCTα mRNA expression in the *B. sexangula* suspension-cultured cells was investigated. Culture condition of the suspension culture was described previously (Kura-Hotta *et al.*, 2001). **Fig. 3A** shows the growth curves of the suspension-cultured cells in the presence of 0 and 100 mM NaCl. Growth was evaluated by measuring fresh weight and cell number. A lag phase was detected in the culture containing 100 mM NaCl, but no significant difference was found in the maximum growth rates and final concentrations of both cultures. The amounts of BsCCTα mRNA in both cultures were investigated by northern blot analysis (**Fig. 3B**). Approximately one or two days after inoculation, BsCCTα transcript was strongly expressed in both cultures. This effect may have been caused by inoculation shock. After such a shock, the expression of BsCCTα

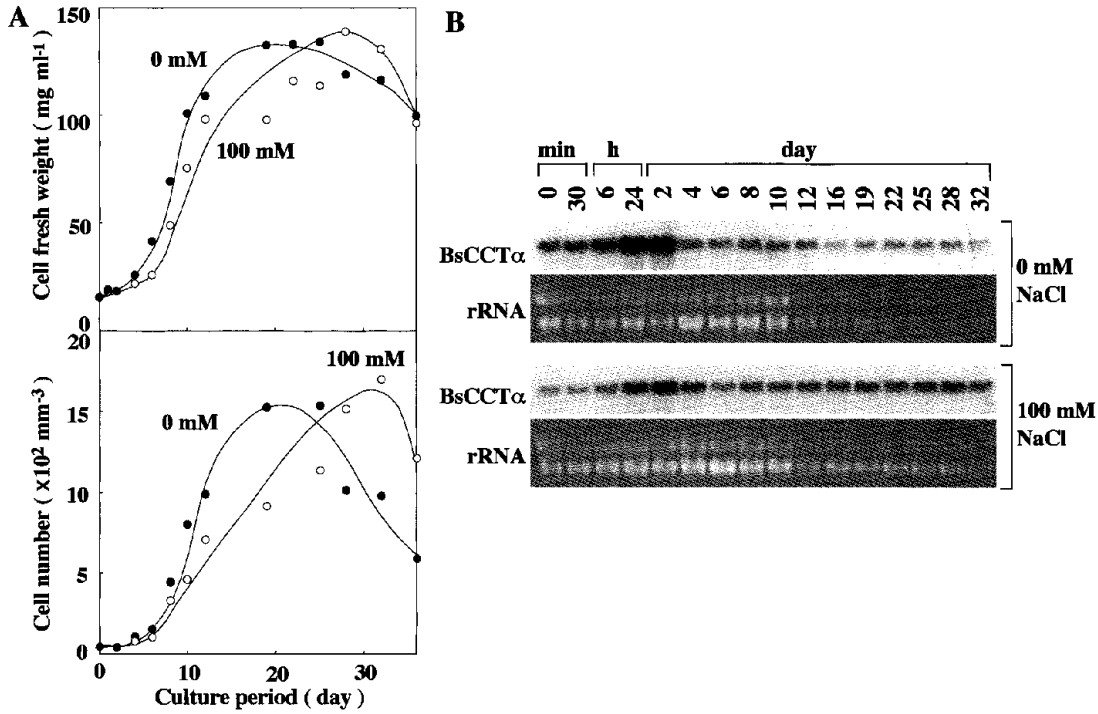

Fig. 3 Growth curves of *B. sexangula* cultured cells in the presence of 0 or 100 mM NaCl (A), and detection of CCTα mRNAs in these cells (B). Open and closed circles indicate growth curves under 100 and 0 mM NaCl conditions, respectively. Total RNA was extracted from the *B. sexangula* cultured-cells by the guanidine thiocyanate/CsCl method (Kingston, 1991). 25 μg of total RNAs were separated by agarose gel electrophoresis and transferred to nylon transfer membrane, Nytran supercharge (Schleicher & Schuell GmbH, Dassel Germany). The RNA blots were hybridized to BsCCTα cDNA fragments labeled randomly with ^{32}P. The membranes were washed in 0.1 x SSC and 0.1% SDS at 65 ℃ (high stringency conditions).

transcript in both cultures was continuous, irrespective of the presence or absence of NaCl.

To understand the relationship between salt-stress and transcription level of CCTα mRNA in *B. sexangula* in more detail, northern blot analysis was performed using the suspension-cultured cells that were cultivated under NaCl-free conditions for three days. Under these conditions, the effect of the inoculation shock in the new medium was probably reduced and BsCCTα mRNA was stably expressed. Therefore, the effect of NaCl addition on expression of BsCCTα mRNA could be detected easily. **Fig. 4** shows the effect of BsCCTα mRNA expression on various concentrations of NaCl (A) and the time course of BsCCTα mRNA expression after the addition of 100 mM NaCl (B) in *B. sexangula* cells. In both treatments, the transcription level of BsCCT α mRNA remained unchanged after the addition of NaCl. In general, stress responsive proteins, including HSP17.6A are synthesized after salt-stress was started (Sun *et al.*, 2001). On the other hand, BsCCT α is probably produced constantly. Therefore, it can be postulate that BsCCTα may play an important role to protect cells from salt stress, immediately. To confirm this hypothesis, the activity of BsCCTα should be revealed in further analyses.

To isolate genes essential for salt-stress tolerance in higher plants, mRNAs that were up regulated by salt-stress were detected and the corresponding genes were cloned by several differential screening methods (Rippmann *et al.*, 1997; Posas *et al.*, 2000; Kawasaki *et al.*, 2001). However, this strategy may not be suitable for mangrove plants because the plants grow in brackish habitats and may produce the key proteins for the salt-tolerance irrespective of NaCl concentration. We therefore established a "functional screening method", which successfully isolated BsCCTα cDNA. We also cloned cDNAs encoding elongation factor 1A homologue, allene oxide cyclase homologue, and unknown proteins in this screening. At the moment, function of these proteins in salt-tolerance is still enigma. However, this functional screening method may be useful in cloning genes that are "functionally important" in salt-tolerance. Further analyses of plant CCTα and other screened proteins will contribute to our under-

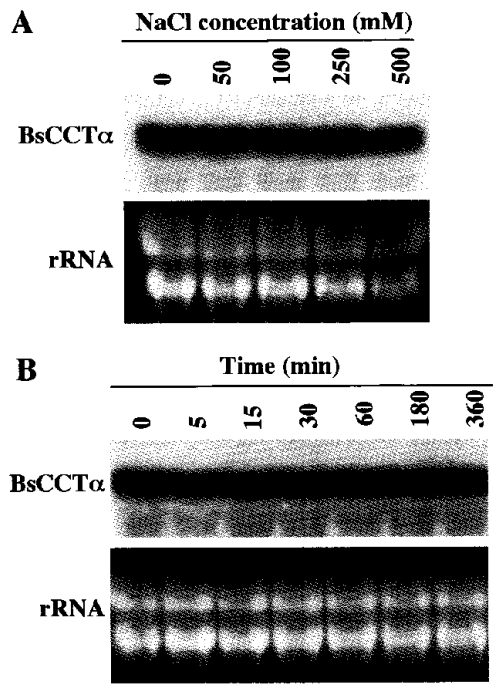

Fig. 4 Effect of BsCCTα mRNA expression on various concentrations of NaCl (A) and the time course of BsCCTα mRNA expression after addition of 100 mM NaCl (B) in *B. sexangula* culture. After the addition of NaCl, cultures were incubated for 3 h and then cells were collected and total RNA extraction was conducted (A). The conditions of the northern blot analyses were indicated in **Fig. 3** legend.

standing of the molecular level of salt-tolerance mechanisms in higher plants, including mangrove plants.

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