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CSP41b, a protein identified via FOX hunting using *Eutrema salsugineum* cDNAs, improves heat and salinity stress tolerance in transgenic *Arabidopsis thaliana*

Hirotaka Ariga, Tomoko Tanaka, Hirokazu Ono, Yoichi Sakata, Takahisa Hayashi, Teruaki Taji*

Department of Bio-Science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya, Tokyo 156-8502, Japan

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ABSTRACT

Eutrema salsugineum (also known as *Thellungiella salsuginea* and formerly *Thellungiella halophila*), a species closely related to *Arabidopsis thaliana*, shows tolerance not only to salt stress, but also to chilling, freezing, and high temperatures. To identify genes responsible for stress tolerance, we conducted Full-length cDNA Over-eXpressing gene (FOX) hunting among a collection of *E. salsugineum* cDNAs that were stress-induced according to gene ontology analysis or over-expressed in *E. salsugineum* compared with *A. thaliana*. We identified *E. salsugineum CSP41b* (chloroplast stem-loop-binding protein of 41 kDa; also known as *CRB*, chloroplast RNA binding; named here as *EsCSP41b*) as a gene that can confer heat and salinity stress tolerance on *A. thaliana*. *A. thaliana* CSP41b is reported to play an important role in the proper functioning of the chloroplast: the *atcsp41b* mutant is smaller and paler than wild-type plants and shows altered chloroplast morphology and photosynthetic performance. We observed that *AtCSP41b*-overexpressing transgenic *A. thaliana* lines also exhibited marked heat tolerance and significant salinity stress tolerance. The *EsCSP41b*-overexpressing transgenic *A. thaliana* lines showed significantly higher photosynthesis activity than wild-type plants not only under normal growth conditions but also under heat stress. In wild-type plants, the expression levels of both *EsCSP41b* and *AtCSP41b* were significantly reduced under heat or salinity stress. We conclude that maintenance of *CSP41b* expression under abiotic stresses may alleviate photoinhibition and improve survival under such stresses.

1. Introduction

High temperature is a major abiotic stress that greatly affects plant growth and crop production [1,2]. A 10–15 °C rise above the optimum temperature for growth causes heat stress responses in higher plants and leads to inhibition of photosynthesis [3]. Environmental stress enhances photoinhibition, a process that is determined by the balance between the rate of photodamage to photosystem II (PSII) and the rate of its repair [4]. Recent investigations suggest that exposure to environmental stresses, such as heat, cold, and salinity, do not affect photodamage but inhibit the repair of PSII through suppression of the synthesis of PSII proteins [5–8].

Eutrema salsugineum (also known as *Thellungiella salsuginea*) is closely related to a genetic model plant *Arabidopsis thaliana* [9], and its genes show 90% sequence identity to those of *A. thaliana*. *E. salsugineum* is tolerant not only to extreme salinity, but also to chilling, freezing, and ozone, suggesting that it is a good genomic resource to study tolerance to these abiotic stresses [10–16]. In addition, we previously reported that *E. salsugineum* showed greater heat tolerance than *A. thaliana* [17]. A number of studies of *E. salsugineum* have provided novel insights into the mechanisms of salt tolerance [18–20], and many candidates for genes conferring salt tolerance have been isolated from an *E. salsugineum* cDNA expression library [21].

A full-length cDNA library of *E. salsugineum* derived from various tissues and whole seedlings subjected to environmental stress treatments (high salinity, chilling, or freezing) or abscisic acid (ABA) treatment was constructed [19,22]. To identify *E. salsugineum* genes

Abbreviations: FOX, full-length cDNA overexpressing gene; CSP, chloroplast stem-loop-binding protein; CRB, chloroplast RNA binding; GM, germination medium; EST, expressed sequence tag; OX, overexpressing.

* Corresponding author.
E-mail address: t3teruak@nodai.ac.jp (T. Taji).

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involved in stress tolerance, we performed Full-length cDNA Over-eXpressing (FOX) gene hunting, a high-throughput strategy to analyze the physiological functions of genes [23], and identified *HsfA1d* as a gene that can confer marked heat tolerance on *A. thaliana* [17]. Here, we performed FOX hunting under heat or salinity stress independently from among a collection of *E. salsugineum* cDNAs that were stress-induced, according to gene ontology analysis, or over-expressed in *E. salsugineum* compared with *A. thaliana*.

2. Materials and methods

2.1. Plant materials and growth conditions

E. salsugineum ecotype Shandong and *A. thaliana* ecotype Columbia (Col-0) were used in this study. Each seedling was grown on germination medium (GM [24]) agar plates. The seeds were stratified at 4 °C for 7 d and then transferred to 80 μmol m^{-2} s^{-1} white light and an 8 h:16 h day/night cycle at 22 °C for germination and growth.

2.2. Construction of binary vectors containing full-length cDNAs from E. salsugineum and production of FOX A. thaliana lines

We selected 433 genes that were stress-induced according to gene ontology analysis or over-expressed in *E. salsugineum* compared with *A. thaliana* from a collection of 9569 *E. salsugineum* cDNAs [22] to use for the FOX hunting experiment. *AtCSP41b* cDNAs were obtained from RAFL clones (RIKEN Arabidopsis full-length cDNA clones; RIKEN BRC, Tsukuba, Japan). Each cDNA was digested individually with *Sfi*I and cloned into the *Sfi*I site of a *Rhizobium radiobacter* binary vector, pBIG2113SF, by using T4 ligase. The pBIG2113SF vector was derived from pBIG2113N [25] by insertion of two *Sfi*I sites into the *Xba*I site of pBIG2113N so that full-length cDNAs could be inserted in the sense orientation relative to the 35S promoter [23]. *R. radiobacter* strain GV3101 was transformed by electroporation with each plasmid. *A. thaliana* (Col-0) plants were transformed with each FOX plasmid independently by the floral dipping method. T2 seeds were collected separately from each transgenic plant and used for subsequent experiments.

2.3. Heat-shock treatment on agar plates

Seeds were sown in Petri dishes (90 mm × 20 mm) containing 20 ml GM agar. Ten-day–old seedlings were placed in a water-bath at 42 °C for 60 or 70 min. After the heat-shock treatment, the seedlings were transferred to normal growth conditions at 22 °C.

2.4. Salinity stress treatment on agar plates

Seeds were sown on nylon mesh–layered (mesh size 990 μm) GM agar plates. Ten-day–old seedlings were mesh-transferred to plates supplemented with 200 mM NaCl.

2.5. Phylogenetic analysis

BLASTX searches of various genomes were performed with FOX36 (EsCSP41b) coding sequence by using Phytozome 10.2 (http://phytozome.jgi.doe.gov/pz/portal.html#). We obtained orthologous protein sequences [locus name in Phytozome] in *Arabidopsis lyrata* (AlCSP41a [486816], AlCSP41b [471025]), *A. thaliana* (AtCSP41a [At3g63140], AtCSP41b [At1g09340]), *Boechera stricta* (BsCSP41a [Bostr.13158s0317], BsCSP41b [Bostr.25219s0010]), *Brassica rapa* (BrCSP41a [Brara.D00026], BrCSP41b [Brara.F00592]), *Capsella grandiflora* (CgCSP41a [Cagra.0664s0051], CgCSP41b [Cagra.4395s0120]), *Capsella rubella* (CrCSP41a [Carubv10017370m]) and *E. salsugineum* (EsCSP41a [Thhalv10006498m]). Phylogenetic analysis by the neighbor-joining method was performed with ClustalW software and the phylogenetic tree was built by TreeView software.

2.6. Measurement of photosynthetic activity

Photosynthetic activity was measured by using an open gas-exchange system (LI-6400XT; LI-COR Inc.) with a custom-made chamber for the Petri dish. The parameters in the chamber were set as follows: TempR (relative temperature) = 25 °C, CO_2R (relative CO_2 concentration) = 400 μmol m^{-2} s^{-1}, flow = 500 μmol s^{-1} and PQntm (light intensity and quality) = 200 μmol photon m^{-2} s^{-1}.

2.7. RNA blot analysis

RNA blot analysis was performed as described in Ref. [17].

3. Results

3.1. Identification of a heat- and salinity-stress tolerance gene, EsCSP41b, by FOX hunting

Several groups have performed transcriptome analyses using *E. salsugineum* [11,12,26,27]. In addition, EST libraries have been constructed from *E. salsugineum* plants subjected to drought, salinity, and cold stresses [28]. For the FOX hunting in the current study, we selected 433 genes from 9569 *E. salsugineum* full-length cDNAs [22]; these target genes included stress-inducible genes and genes expressed highly in *E. salsugineum* compared with *A. thaliana* under normal growth conditions (Supplemental Tables 1 and 2). We generated *E. salsugineum* FOX plasmids by introducing each cDNA into a binary vector downstream of the CaMV 35S promoter. *A. thaliana* (Col-0) plants were transformed independently with each FOX plasmid.

To evaluate the heat-stress tolerance of the transgenic plants, we incubated 10-day–old T2 seedlings from eight independent transformants per cDNA at 42 °C for 60 or 70 min and then at 22 °C for 5 d. In addition, to screen the transgenic plants for salinity-stress tolerance, 10-day–old seedlings grown on a nylon mesh on GM agar plates were mesh-transferred to plates supplemented with 200 mM NaCl for 6 d. We considered the genes as candidates if two or more independent transgenic lines were clearly more tolerant than WT plants. *FOX36* gene was independently identified from both stress assays as a heat- and salinity-stress tolerance gene (Fig. 1A–C). *FOX36* encodes a chloroplast stem-loop-binding protein of 41 kDa, CSP41 (also known as chloroplast RNA binding, CRB). Two independent lines, FOX36-ox1 and -ox2, showed high expression of the transgene compared with WT plants (Fig. 1D). There were no significant differences in growth under normal growth conditions between the two FOX36-transgenic lines and WT plants (data not shown).

3.2. Phylogenetic analysis of EsCSP41b in Brassicaceae

We obtained the complete sequence of *FOX36* full-length cDNA and compared the predicted amino acid sequence with that of *A. thaliana* orthologs, AtCSP41a and AtCSP41b. FOX36 showed higher similarity to AtCSP41b than to AtCSP41a (Fig. 1E). Thus, we named the *FOX36* gene as *E. salsugineum CSP41b* or *EsCSP41b*. We identified a *CSP41a* ortholog (*EsCSP41a*) in the *E. salsugineum* genome. To verify that the *FOX36* gene, *EsCSP41b*, encodes CSP41b, not CSP41a, we constructed a phylogenetic tree of the deduced amino acid sequences of 13 *CSP41* genes in *Brassicaceae* including those from *A.*

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