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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*

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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.

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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.*

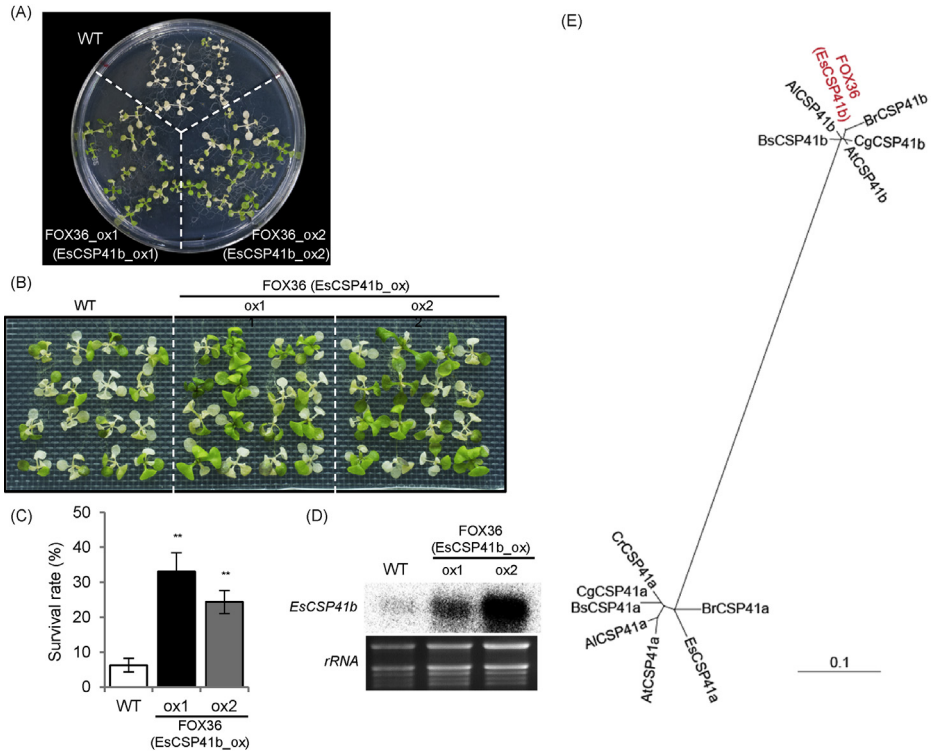

Fig. 1. Heat and salinity stress tolerance of wild-type (WT) and FOX36 plants. (A) Ten-day–old seedlings of WT, and FOX36_ox1 and _ox2 (two independent *A. thaliana* lines carrying the *EsCSP41b* gene), were exposed to 42 °C for 60 min and then moved to normal growth conditions (22 °C) for 5 d. (B) Ten-day–old seedlings of WT, FOX36_ox1, and FOX36_ox2 grown on nylon mesh (990 μm) on GM agar were transferred to GM agar supplemented with 200 mM NaCl and grown for a further 6 d. (C) Survival rate in the salinity tolerance assay (B). Survival rates were calculated from the results of five independent experiments (The number of plants n ≥ 12 for each experiment). Data are means ± SE for five individual experiments. $^{**}P < 0.01$ by Student's *t*-test. (D) The expression levels of the transgenes in lines FOX36_ox1 and _ox2 under normal growth conditions were confirmed by Northern blot analysis with a probe for *EsCSP41b*. (E) Phylogenetic tree based on 13 *CSP41* proteins in *Brassicaceae*, including proteins from *Arabidopsis lyrata* (AlCSP41), *Arabidopsis thaliana* (AtCSP41), *Boechera stricta* (BsCSP41), *Brassica rapa* (BrCSP41), *Capsella grandiflora* (CgCSP41), *Capsella rubella* (CrCSP41), and *Eutrema salsugineum* (EsCSP41).

lyrata, *A. thaliana*, *B. stricta*, *B. rapa*, *C. grandiflora*, *C. rubella*, and *E. salsugineum*. The predicted amino acid sequence of EsCSP41b showed the highest similarity to *B. rapa* CSP41b (BrCSP41b) (Fig. 1E).

3.3. *AtCSP41b*-overexpressing plants show heat and salinity stress tolerance

To determine whether the transgenic plants overexpressing *AtCSP41b* show greater heat tolerance than that of WT plants, as found for *EsCSP41b*-overexpressing plants, we generated *AtCSP41b*-overexpressing plants (Fig. 2A). Ten-day–old T2 seedlings of *AtCSP41b*-overexpressing and WT plants were incubated at 42 °C for 60 min and then at 22 °C for 10 d. Both independent *AtCSP41b*-overexpressing lines (AtCSP41b_ox1 and AtCSP41b_ox2) exhibited greater heat tolerance than that of WT plants, similar to that of *EsCSP41b*-overexpressing plants (Fig. 2B). In addition, the *AtCSP41b*-overexpressing plants showed significantly higher salinity stress tolerance than WT plants (Fig. 2C and D), as was shown above for *EsCSP41b*-overexpressing plants.

3.4. *EsCSP41b*-overexpressing plants have a high photosynthetic rate

The *atcsp41b* mutant is smaller and paler than wild-type plants, and has altered chloroplast morphology and photosynthetic performance [29]. Therefore, we examined the photosynthetic rate in 2-week–old *EsCSP41b*-overexpressing and WT seedlings under normal growth conditions or after heat stress at 42 °C for 20 min. The *EsCSP41b*-overexpressing seedlings showed a significantly higher photosynthetic rate under normal growth conditions or heat stress than WT seedlings (Fig. 3). These results suggest that the overexpression of *EsCSP41b* improves maintenance of photosynthesis even under heat stress.

3.5. Expression profiles of *EsCSP41b* and *AtCSP41b* under stress conditions

To compare the expression profiles of *EsCSP41b* and *AtCSP41b*, we performed RNA blot analysis. The expression levels of *EsCSP41b* and *AtCSP41b* in *E. salsugineum* WT and *A. thaliana* WT, respectively, were decreased under heat or salinity stress conditions (Fig. 4). The expression of *AtCSP41b* is known to be repressed by ABA and osmotic stress [30], and the expression levels of the photosynthesis-related genes, *rbcL* and *psbA*, are reduced in the *atcsp41b* knockout mutant [31]. Therefore, we examined the effect of *EsCSP41b* or *AtCSP41b* overexpression on the expression levels of heat-inducible genes (*Hsp70*, *Hsp17*, *MBF1c*, *ROF2* and *GolS1)* and photosynthesis-related genes (*rbcL* and *psbA)* in the transgenic plants under heat stress (37 °C for 0, 0.5, 1, or 2 h). There were no marked differences in the expression levels of these genes between transgenic and WT plants in this experiment (Fig. S1).

4. Discussion

In this study, we identified *EsCSP41b* as a gene that can confer heat and salinity stress tolerance on *A. thaliana*. We observed that

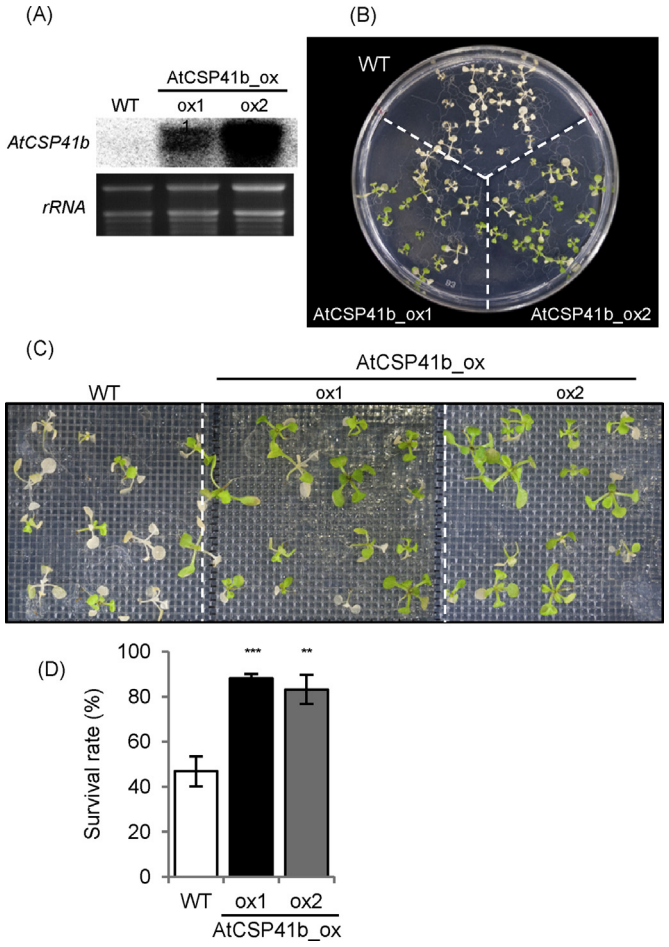

Fig. 2. Heat and salinity stress tolerance of *AtCSP41b*-overexpressing plants. (A) The expression levels of the transgenes in lines *AtCSP41b*-overexpressing-1 (AtCSP41b_ox1) and −2 (AtCSP41b_ox2) under normal growth conditions were confirmed by Northern blot analysis. (B) Ten-day−old seedlings of WT, AtCSP41b_ox1 and _ox2 were exposed to 42 °C for 60 min and then moved to normal growth conditions (22 °C) for 5 d. (C) Ten-day−old seedlings of WT, AtCSP41b_ox1 and _ox2 grown on nylon mesh (990 μm) on GM agar were transferred to GM agar supplemented with 200 mM NaCl and grown for 7 d. (D) Survival rate in the salinity tolerance assay (C). Survival rates were calculated from the results of five independent experiments ($n > 11$ for each experiment). Data are means ± SE for five individual experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

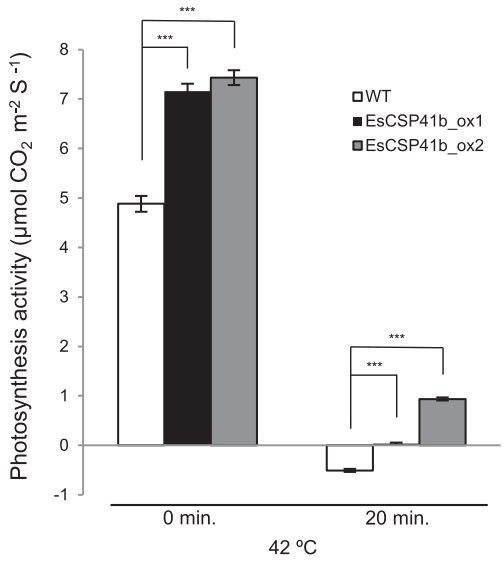

Fig. 3. Photosynthetic rate of WT and *EsCSP41b*-overexpressing seedlings after heat stress. CO_2 assimilation rates for 2-week−old seedlings of WT and *EsCSP41b*-overexpressing lines under normal growth conditions and after heat stress at 42 °C for 20 min were measured. White bars, WT plants; black bars, *EsCSP41b*-overexpressing lines. Data are means ± SE for three individual experiments ($n = 3$). $^{***}P < 0.001$, Student's *t*-test.

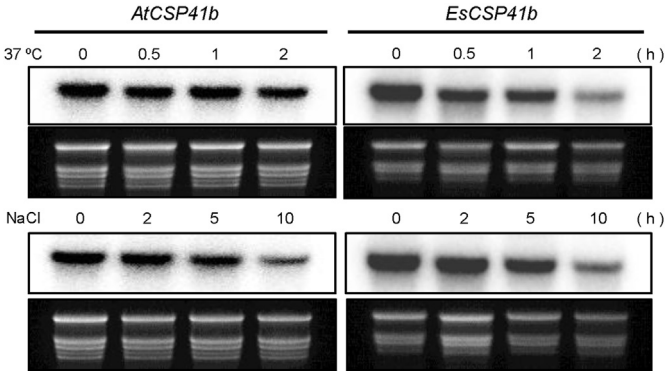

Fig. 4. Expression profiles of *EsCSP41b* and *AtCSP41b* in WT plants under stress conditions. Expression profiles of *EsCSP41b* and *AtCSP41b* during heat and salinity stress treatment of *E. salsugineum* and *A. thaliana* WT plants. Ten-day−old WT plants of *A. thaliana* and *E. salsugineum* were exposed to 37 °C for 0, 0.5, 1 or 2 h as a heat stress treatment or 250 mM NaCl for 0, 2, 5 or 10 h as a salinity stress treatment. For each gene, the corresponding full-length cDNA was used as the probe.

AtCSP41b-overexpressing plants exhibited heat and salinity stress tolerance, and *EsCSP41b*-overexpressing plants showed higher photosynthetic activity than WT plants under both normal growth and heat stress conditions.

Previous studies of *atcsp41b* knockout and *atcsp41ab* double mutants, described below, are consistent with our finding that *EsCSP41b* promotes photosynthesis. The *atcsp41b* knockout mutant shows a smaller and paler phenotype, reduced chloroplast RNA levels, and impaired photosynthesis compared with WT plants [29]. Although AtCSP41b interacts with its paralog, AtCSP41a in vivo [31], AtCSP41a and AtCSP41b do not have equivalent functions, and AtCSP41b appears to be functionally more important than AtCSP41a [32]. Loss of AtCSP41a has no obvious phenotypic effect and, accordingly, the *atcsp41ab* double mutant exhibits the same impaired photosynthesis phenotype as the *atcsp41b* mutant [33].

Environmental stress enhances photoinhibition, and reduces the expression level of *EsCSP41b* or *AtCSP41b*. Low transcript levels of *AtCSP41b* in response to stress might result in changes of chloroplast RNA metabolism and subsequently impair stress tolerance. In addition to being repressed by heat, salinity, ABA, and osmotic stresses, AtCSP41b is also repressed by senescence suggesting a role of AtCSP41b in senescence processes [30]. All these stresses are known to induce yellowing of leaves and thus affect chloroplast maintenance in *A. thaliana* through differential expression of chloroplast genes. Although the transcriptional regulation of *CSP41b* remains unclear, the repression of the *CSP41b* expression seems to be inversely correlated with the degree of photosynthetic activity (Figs. 3 and 4 and [30]). The maintenance of *CSP41b* expression under abiotic stresses by the constitutive 35S promoter in the transgenic plants may ameliorate the inhibition of photosynthetic activity under such stresses and result in improved survival.

We found no differences in the expression profiles of heat-inducible genes, such as *HSP*s, between *CSP41b*-overexpressing and WT plants under heat stress (Fig. S1), suggesting that the heat

tolerance of the transgenic plants was not caused by the higher expression of heat-inducible genes. In addition, there were no differences in expression levels of *rbcL* or *psbA* between *CSP41b*-overexpressing and WT plants under heat stress (Fig. S1); however, expression of these genes has previously been reported to be decreased in *atcsp41b* knockout mutants compared with WT plants [31]. The decrease of *rbcL* and *psbA* mRNAs in *atcsp41b* knockout mutants might be caused by secondary effects through dysfunction of chloroplasts. It is yet to be determined whether the overexpression of *CSP41b* affects the maintenance of *rbcL* and *psbA* expression levels under long-term heat stress.

EsCSP41b was selected as a target gene for the FOX hunting experiment because it was expressed highly in *E. salsugineum* compared with *A. thaliana* under normal growth conditions, even though it was not stress-inducible in the gene ontology analysis (Supplemental Table 1). We observed that the expression levels of *EsCSP41b* and *AtSCP41b* in WT plants were suppressed by heat or salinity stress. Although it remains unclear whether the decrease in *EsCSP41b* or *AtCSP41b* expression is actively controlled or occurs uncontrollably under various stress conditions, it seems uncontrollably for heat and salinity stress tolerance. A large number of stress-inducible genes have been used previously as target genes for generation of stress-tolerant plants. Although *CSP41b* is not a stress-inducible gene, our results suggest that it contributes to the maintenance of chloroplast function. Exposure to environmental stresses including heat, salinity, osmotic, cold, and oxidative stresses enhances photoinhibition and leads to chloroplast malfunction. Our results suggest that genes related to chloroplast function or photosynthetic performance may be good candidate genes for the production of stress-tolerant crops.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.151.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.151.

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