Research article

Functional analysis of the Chloroplast GrpE (CGE) proteins from Arabidopsis thaliana

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ABSTRACT

The function of proteins depends on specific partners that regulate protein folding, degradation and protein-protein interactions, such partners are the chaperones and cochaperones. In chloroplasts, proteins belonging to several families of chaperones have been identified: chaperonins (Cpn60α, Cpn60β), Hsp90s (Hsp90-5/Hsp90C), Hsp100s (Hsp93, ClpC) and Hsp70s (cpHsc70α). Several lines of evidence have demonstrated that cpHsc70 chaperones are involved in molecular processes like protein import, protein folding and oligomer formation that impact important physiological aspects in plants such as thermotolerance and thylakoid biogenesis. Despite the vast amount of data existing around the function of cpHsc70 chaperones, very little attention has been paid to the roles of DnaJ and GrpE cochaperones in the chloroplast. In this study, we performed a phylogenetic analysis of the chloroplastic GrpE (CGE) proteins from 71 species. Based on their phylogenetic relationships and on a motif enrichment analysis, we propose a classification system for land plants’ CGEs, which include two independent groups with specific primary structure traits. Furthermore, using in vivo assays we determined that the two CGEs from A. thaliana (ATCGEs) complement the mutant phenotype displayed by a knockout E. coli strain defective in the bacterial grpE gene. Moreover, we determined in planta that the two ATCGEs are bona fide chloroplastic proteins, which form the essential homodimers needed to establish direct physical interactions with the cpHsc70-1 chaperone. Finally, we found evidence suggesting that ATCGE1 is involved in specific physiological phenomena in A. thaliana, such as the chloroplastic response to heat stress, and the correct oligomerization of the photosynthesis-related LHCII complex.

1. Introduction

Chloroplasts are photosynthetic organelles of endosymbiotic origin that contain their own genome (Martin et al., 1998), which encodes 130 genes in average (RefSeq: https://www.ncbi.nlm.nih.gov/genome/organelle/). Estimations indicate that functional chloroplasts require 2000 to 3500 proteins (van Wijk and Baginsky, 2011), however the chloroplast genome codes for an average of only 83 proteins (RefSeq: https://www.ncbi.nlm.nih.gov/genome/organelle/). The rest of the chloroplastic proteome is encoded in the nuclear genome; using the cellular transcription and translation apparatus such proteins are synthesized in the cytoplasm and post-translationally imported into the organelle (Villarejo et al., 2005; Jarvis and Kessler, 2014; Paila et al., 2015). From their synthesis, chloroplast proteins are subjected to regulatory events that impact the performance of their functions; targeting, folding, post-translational stabilization, and degradation are processes that affect the homeostasis of chloroplast proteins (Trösch et al., 2015); these processes are regulated by molecular chaperones. In the cytoplasm, precursor proteins interact with the Hsp70, 14-3-3 and Hsp90 chaperones that keep them in a proper folding state and facilitate their interaction with the translocon receptor proteins Toc159 and Toc34 (Rial et al., 2000, May and Soll, 2000; Qbadou et al., 2006; Flores-Pérez and Jarvis, 2013).

In the chloroplast stroma, several chaperone systems have been identified. The chaperonins (Cpn60α and Cpn60β) are involved in the folding of RbcL (Ribulose Bisphosphate Carboxylase/Oxygenase large chain) and NdhH (NAD(P)H-quinone oxidoreductase subunit H) (Barrera and Ellis, 1980; Peng et al., 2011), Hsp90s mediate protein import, protein maturation and thylakoid formation (Pratt and Toft, 2003; Heide et al., 2009; Inoue et al., 2013), Hsp100s prevent

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Arabidopsis thaliana impaired growth (Apuya et al., 2001), and point mutations in the defective phenotypes and contain aberrant chloroplasts embryo-lethal (Cao et al., 2003; Inoue et al., 2013). The knockout of the Hsp90-5 gene also generate albino seedlings while null mutants are 

photosystem assembly (Yalovsky et al., 1992; Schroda et al., 1999; Liu et al., 2005, 2007; Clarke, 2012; Sjogren et al., 2014), and Hsp70s (cpHsc70-1 and cpHsc70-2) participate in thylakoid formation, protein import and protein aggregation and facilitate protein import and degradation (Kovacheva et al., 2007; Doyle and Wickner, 2009; Olinares et al., 2011; Clarke, 2012; Sjogren et al., 2014), and Hsp70s (cpHsc70-1 and cpHsc70-2) participate in thylakoid formation, protein import and photosystem assembly (Yalovsky et al., 1992; Schröda et al., 1999; Liu et al., 2007; Su and Li, 2008; Shi and Theg, 2010; Su and Li, 2010).

Mutant plants defective in chloroplast chaperones display pigment-defective phenotypes and contain aberrant chloroplasts. For example, Arabidopsis thaliana mutants in cpn60α display albino phenotypes with impaired growth (Apuya et al., 2001), and point mutations in the Hsp90-5 gene also generate albino seedlings while null mutants are embryo-lethal (Cao et al., 2003; Inoue et al., 2013). The knockout of the major isoform of Hsp93/ClpC causes chloroplast development and protein import defects (Constan et al., 2004; Kovacheva et al., 2005, 2007). Finally, mutation of cpHsc70-1 gene causes cotyledon and leaf variegation with chloroplast protein import defects, and double mutants for the two plastidic Hsc70s are embryo lethal (Su and Li, 2008).

To function efficiently, chaperones interact with other proteins known as cochaperones (Fink, 1999; Mayer, 2010). In chloroplasts, the cochaperonins Cpn10 and Cpn20 form hetero-oligomeric complexes to form the lid of the Cpn60 barrel-shaped oligomer (Tsai et al., 2012). Additionally, the C-terminus of Tic40 has homology to the proteins Hip (Hsp70 interacting protein) and Hop (Hsp70-Hsp90-organizing protein), which are known cochaperones that bind and organize Hsp70 and Hsp90 in the cytoplasm (Stahl et al., 1999; Bédard et al., 2007). In accordance, Tic40 has been found to directly interact with Hsp93/ClpC to enhance ATP hydrolysis (Chou et al., 2003). Additionally, other cochaperones have been identified in chloroplasts, including DnaJ and GrpE domain-containing proteins. In Escherichia coli, such cochaperones directly interact with DnaK (Hsp70) to mediate protein folding. DnaJ is known to recruit target proteins and enhance the ATP hydrolysis of DnaK, while GrpE is a nucleotide exchange factor that regulates the exchange of ADP to ATP in the nucleotide-binding site of DnaK, thus regulating the affinity of the chaperone for the unfolded polypeptides (Fink, 1999; Mayer, 2010). In plastids, 19 DnaJ domain-containing proteins have been identified (Chiu et al., 2013), recent studies have found that mutant plants defective in individual DnaJ proteins (AtJ8, AtJ11, and AtJ20) display defects in PSII (Photosystem II) homeostasis in A. thaliana (Chen et al., 2010). Also, AtJ20 was found involved in the turnover of DXS (1-deoxy-D-xylulose-5-phosphate synthase) enzyme in A. thaliana (Pulido et al., 2013), whereas the DnaJ-containing protein CDJ2 from Chlamydomonas reinhardtii is required for the formation of the essential VIPP1 (Vesicle-inducing Protein in Plastids 1) oligomers during thylakoid biogenesis (Liu et al., 2005, 2007).

In contrast, the information regarding the function of the chloroplast GrpE proteins (CGEs) is fragmentary, with a comprehensive description available only for the C. reinhardtii CGE1. It is known that the CGE1 from C. reinhardtii is localized in the chloroplast stroma, where it interacts with HSP70B (the chloroplastic Hsp70 from C. reinhardtii), forms homodimers and participates in both protein import and VIPP1 oligomerization (Schröda et al., 2001; Liu et al., 2007; Willmund et al., 2007). Additionally, in Physcomitrella patens CGE proteins are localized in the chloroplast, where they interact with the stromal Hsp70-2

**Nomenclature**

| A. thaliana CGEs AtCGEs | Hsp70-Hsp90-organizing protein |
| BiFC | NDH-H NAD(P)H-quinone oxidoreductase subunit H |
| RbcL | nYFP Yellow Fluorescent Protein N-terminus |
| CGEs | pCE pSPYCE vector |
| CrCGE1 | pD14 pDEST14 vector |
| cYFP | pD22 pDEST22 vector |
| DXX | pD32 pDEST32 vector |
| Hip | pEG pEarleyGate103 vector |
| | pNE pSPYNE vector |
| | TCA Trichloroacetic acid |
| | VIPP1 Vesicle-inducing Protein in Plastids 1 |

**Fig. 1. Conserved motifs in CGE proteins.** Sequence motifs found enriched in Type A (A) and Type B (B) CGEs based on the analysis with DREME tool (http://meme-suite.org/doc/dreme.html?man_type=web). The input sequences were randomized to generate a control set, the E-value threshold for motif discovery was set to 0.05. Alignment between the *E. coli* GrpE and the consensus sequences of Type A and Type B CGEs is shown (C). The numbers, arrows and boxes over positions 73, 74, 82, 122, 183, and 192 point to the amino acids that have been shown to be important for GrpE activity in *E. coli*. 

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**Table 1.** Nomenclature for proteins involved in chloroplast protein import and degradation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BiFC</td>
<td>Biluminescent Fluorescence Complementation</td>
</tr>
<tr>
<td>RbcL</td>
<td>Ribulose Bisphosphate Carboxylase/Oxygenase large chain</td>
</tr>
<tr>
<td>CGEs</td>
<td>Chloroplast GrpE proteins</td>
</tr>
<tr>
<td>CrCGE1</td>
<td>C. reinhardtii CGE1</td>
</tr>
<tr>
<td>cYFP</td>
<td>Yellow Fluorescent Protein C-terminus</td>
</tr>
<tr>
<td>DXX</td>
<td>1-deoxy-D-xylulose-5-phosphate synthase)</td>
</tr>
<tr>
<td>Hip</td>
<td>Hsp70 interacting protein</td>
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protein to regulate protein import into the organelle (Shi and Theg, 2010). However, no experimental evidence exists that analyzes CGE function from land plants. This study presents data that gives insight into the functional traits of the A. thaliana CGEs, it is focused on the in vivo determination of several CGE protein traits that are relevant to its proposed function as a cochaperones for cpHsc70-1 protein, such as activity as nucleotide exchange factor, subcellular localization, physical interaction with cpHsc70 and dimer formation, it also presents information regarding the biological processes mediated by CGEs in A. thaliana.

2. Results

2.1. CGE proteins share functionally-relevant amino acid residues

To gain understanding of the evolution of CGEs, we performed a phylogenetic analysis of 136 CGE protein sequences from 71 species (61 embryophytes: 60 tracheophytes and 1 bryophyte, 7 chlorophytes, 1 charophyte, and 1 cryptophyte) (Fig. S1). A full alignment between all the analyzed CGEs and the E. coli GrpE is shown in the supplemental file CGEs full alignment. This analysis showed that the CGE protein sequences from embryophytes form a monophyletic group that further divides into two subgroups, named Type A and Type B. Analysis of the phylogenetic tree showed that 57% of the plants contain both Type A and Type B CGEs, while 18% contain only Type A and 24% only Type B CGEs. The two subgroups of CGEs can be distinguished from each other by variations in short motifs conserved in the analyzed CGE protein sequences. The SYQGI and VKVS motifs are present in Type A CGEs, while the (N/D)SYQSI and MVKVS are characteristic of Type B sequences. The SYQGI and VKVS motifs are present in Type A CGEs, while the (N/D)SYQSI and MVKVS are characteristic of Type B sequences. Furthermore, sequence comparison with the CGEs, it is focused on the phylogenetic tree showed that 57% of the plants contain both Type A and Type B CGEs, while 18% contain only Type A and 24% only Type B CGEs. The two subgroups of CGEs can be distinguished from each other by variations in short motifs conserved in the analyzed CGE protein sequences. The SYQGI and VKVS motifs are present in Type A CGEs, while the (N/D)SYQSI and MVKVS are characteristic of Type B sequences (Fig. 1A and B). Furthermore, sequence comparison with the E. coli GrpE showed that two of the six amino acid positions essential for GrpE activity (Harrison et al., 1997; Gelinas et al., 2003, 2004) have been modified in the plant sequences. In embryophytes, amino acid position R74 was substituted by a K residue, and position K82 by an S residue (Fig. 1C and Table S2). Moreover, the G residue at position 122 of the E. coli GrpE is only conserved in Type A CGEs, while it has been substituted by S in 86% of the Type B CGEs (Fig. 1C and Table S2). The remaining functionally-relevant amino acids (R73, R183, and V192) from the bacterial GrpE are fully conserved among all the CGEs analyzed (Fig. 1C and Table S2). Finally, we observed that the two CGE genes present in the genome of A. thaliana belong to each of the two subgroups; CGE2 to Type A and CGE1/EMB1241 to Type B.

2.2. A. thaliana type A and type B CGEs are functional nucleotide exchange factors

To assess the functionality of the A. thaliana CGEs (AtCGEs), AtCGE1 and AtCGE2 genes were used to complement an E. coli knockout line (OD212) that is defective in the grpE gene. This mutant carries an additional mutation the Hsp70 gene (dnaK332) that suppresses the lethal phenotype conferred by the knockout of the endogenous grpE. Thus, OD212 line can grow normally at 25 °C but has growth defects when cultured at higher temperatures (Deloche et al., 1997). The predicted transit peptides were deleted from the CDSS of AtCGE1 and AtCGE2 genes, and the Shine-Dalgarno sequence was added to the genetic constructs to facilitate bacterial translation; the chimeric genes were cloned into the pDEST14 bacterial expression vector, generating the constructs pD14::ΔCGE1 and pD14::ΔCGE2 (Fig. S2). These constructs were transformed into OD212 cells and their phenotype was evaluated under permissive (25 °C), mild (37 °C), and restrictive (43 °C) temperature conditions (Fig. 2). The empty pD14::ΔccdB vector was used as negative control and the full length CDS of the E. coli grpE gene was cloned into pDEST14 and used as positive control (pD14::EcgrpE). Under permissive conditions, no detectable differences in growth were observed between the OD212 strain transformed with the empty vector and those carrying pD14::ΔCGE1, pD14::ΔCGE2 and pD14::EcgrpE plasmids (Fig. 2). However, under mild temperature conditions (37 °C) improved growth was observed in the cells containing pD14::ΔCGE1, pD14::ΔCGE2 and pD14::EcgrpE plasmids compared with the empty vector (Fig. 2). At the restrictive conditions (43 °C), the growth defects displayed by all the strains were more severe; however, cells transformed with pD14::ΔCGE1, pD14::ΔCGE2 and pD14::EcgrpE plasmids performed better than the empty vector-transformed cells (Fig. 2). Furthermore, pD14::CGE2-carrying cells displayed improved growth compared with those containing pD14::CGE1 (Fig. 2), indicating a better complementation capacity for AtCGE2 compared to AtCGE1. These results demonstrate that the AtCGEs alleviate the defective phenotype of the OD212 mutant; supporting the idea that both AtCGEs retain key molecular traits that allow interaction with DnaK, to promote the essential nucleotide exchange needed by this chaperone.

2.3. A. thaliana CGEs are chloroplast localized

To investigate the role of CGEs as cochaperones for chloroplastic Hsp70s, their subcellular localization was determined in vivo and contrasted to the localization of the chloroplastic Hsp70, the cpHsc70-1 protein. Translational fusions of AtCGE1, AtCGE2, and cpHsc70-1 to

![Fig. 2. Functional complementation assay in bacteria.](Image) The growth of the thermo-sensitive E. coli OD212 strain, transformed with the pD14::ΔccdB (empty vector) or the expression vectors pD14::ΔCGE1 (ΔCGE1), pD14::ΔCGE2 (ΔCGE2) or pD14::EcgrpE (EcgrpE), was analyzed at 25 °C (A), 37 °C (B), and 43 °C (C). Five μL drops from the indicated bacterial samples were cultured for 20 h in LB-agar plates at the corresponding dilutions (1, OD600nm = 0.1; 10⁻¹, OD600nm = 0.01; 10⁻², OD600nm = 0.001; 10⁻³, OD600nm = 0.0001) and temperature conditions.
GFP and c-Myc tag were generated (Fig. S2) and used to transform *N. benthamiana* leaves. Protoplasts from transformed leaves were analyzed 96 h post-transformation under the confocal microscope. The protoplasts expressing AtCGE1-GFP (Fig. 3B) or AtCGE2-GFP (Fig. 3C) fusions displayed GFP fluorescence distributed inside the chloroplast stroma and in discrete cumuli at the periphery of plastids. The formation of peripheral fluorescence cumuli has been described as chloroplast envelope deformations caused by overaccumulation of membrane-associated proteins (Breuers et al., 2012). Interestingly, protoplasts expressing cpHsc70-1-GFP fusion displayed a distribution pattern characteristic of stromal proteins (Fig. 3D), with intense fluorescence in a few foci inside the organelle (Farmaki et al., 2006; Perello et al., 2016). Additionally, we addressed the possibility of AtCGEs being transported to mitochondria by colocalization analysis of AtCGE1-GFP and AtCGE2-GFP and a translational fusion between mCherry and the yeast mitochondrial protein COX4 (Cytochrome c oxidase subunit 4) (Nelson et al., 2007), but no co-localization was found (Fig. S5). Altogether, these data indicate that both AtCGEs are *in vivo* imported into the chloroplast, where they coexist with cpHsc70-1 chaperone.

To confirm our data and to determine the suborganellar localization of the AtCGEs and cpHsc70-1 proteins, chloroplasts from *N. benthamiana* leaves transformed with *pNE::CGE1*, *pNE::CGE2* or *pNE::CPHSC70-1* constructs were isolated and fractionated into stromal and envelope fractions. Western blot analysis of purified suborganellar fractions was carried out using the c-Myc epitope tag present in the *pNE* expression vectors (Fig. S2). Fig. 4 shows that AtCGE1 and AtCGE2 proteins accumulate in both the chloroplast envelope and in the stromal fractions (Fig. 4: lanes 3 and 5), while cpHsc70-1 protein was only detected in the stromal fractions (Fig. 4 stromal fractions: lanes 2 and 4). Western blots against RbcS (stromal protein marker) and Tic40

![Image](https://example.com/image.png)

**Fig. 3. Subcellular localization of AtCGE1, AtCGE2 and cpHsc70-1 proteins.** Images of mesophyll protoplasts from *N. benthamiana* leaves expressing GFP (A) and the translational fusions AtCGE1-GFP (B), AtCGE2-GFP (C), and cpHsc70-1-GFP (D) are shown. Images corresponding to the bright field, chlorophyll fluorescence (Chlorophyll), GFP fluorescence, and the merge between the two fluorescence channels are shown in the indicated columns. Protoplasts were prepared 96 h after leaf agroinfiltration. Scale bars correspond to 10 μm.
confirmed that no cross-contamination between the suborganellar fractions existed in the samples. This information corroborates the confocal microscopy data (Fig. 3) and demonstrates the co-localization between AtCGEs and cpHsc70-1 in the chloroplast stroma, supporting the hypothesis of a common function for these proteins.

2.4. *A. thaliana* CGEs interact with cpHsc70-1 in vivo

To analyze the existence of a physical interaction between the AtCGEs and the cpHsc70-1 chaperone in vivo, BiFC (Bimolecular Fluorescence Complementation) assays were performed. *N. benthamiana* leaves were transformed by agroinfiltration with the plasmids containing cpHsc70-1, AtCGE1 and AtCGE2 proteins fused to the N- (nYFP) and C-terminal (cYFP) regions of YFP protein (Fig. S2). Reciprocal co-transformation assays (i.e. cpHsc70-1-nYFP/AtCGE1-cYFP and cpHsc70-1-cYFP/AtCGE1-nYFP) were performed to corroborate the complementation of fluorescence. Fig. 5 shows that fused to nYFP or to cYFP, AtCGE1 directly interacts with cpHsc70-1 chaperone, as demonstrated by the presence of YFP fluorescence in the chloroplasts of transformed plant cells (Fig. 5A and B). Similar results were found for AtCGE2 and cpHsc70-1, the cells co-transformed with the nYFP and cYFP protein fusions displayed YFP fluorescence complementation in the chloroplast stroma (Fig. 5A and B). Furthermore, *N. benthamiana* protoplasts transformed with the individual translational fusions (i.e. only AtCGE1-nYFP or AtCGE2-cYFP) were used as negative control for these experiments. Alternatively, using c-Myc and GFP-tagged proteins, we detected co-immunoprecipitation of AtCGE1 and AtCGE2 with cpHsc70-1 (Fig. S6), corroborating the interactions detected in the BiFC assays. Altogether, these observations demonstrate that the two AtCGEs form stable physical interactions with the cpHsc70-1 chaperone inside the chloroplasts in vivo.

2.5. *A. thaliana* CGEs form homo and heterodimers

It is known that bacterial GrpE interacts with DnaK as a homodimer, and this conformation is key for the regulation of the DnaK reaction cycle (Harrison et al., 1997). Dimerization has been reported for *C. reinhardtii* CGE1 (CrCGE1) (Schroda et al., 2001). To test the capacity of AtCGEs to form dimers, BiFC experiments were performed in *N. benthamiana* leaves transformed with the expression vectors containing AtCGE1 and AtCGE2 proteins fused to the cYFP and nYFP moieties of YFP protein (Fig. S2). Complementation of YFP fluorescence was detected in the chloroplasts of the cells transformed with the plasmids containing the fusions AtCGE1-cYFP and AtCGE1-nYFP (Fig. 6A), indicating that AtCGE1 protein forms homodimers in vivo. Similarly, YFP fluorescence was detected in the chloroplasts of the cells expressing the fusions AtCGE2-cYFP and AtCGE2-nYFP (Fig. 6B), demonstrating that AtCGE2 is capable of homodimerization in vivo too. Given that AtCGE1 and AtCGE2 belong to different clades of the CGE phylogenetic tree (Fig. S1), we were also interested in analyzing their ability to form heterodimers. YFP fluorescence was detected in the chloroplasts of the cells expressing the protein fusions AtCGE1-cYFP and AtCGE1-nYFP (Fig. 6A), indicating that AtCGE1 protein forms homodimers in vivo. Similarly, YFP fluorescence was detected in the chloroplasts of the cells expressing the fusions AtCGE2-cYFP and AtCGE2-nYFP (Fig. 6B), demonstrating that AtCGE2 is capable of homodimerization in vivo too. Additionally, using c-Myc and GFP-tagged proteins, we detected co-immunoprecipitation of AtCGE1 and AtCGE2 with cpHsc70-1 (Fig. S6), corroborating the interactions detected in the BiFC assays. In conclusion, these experiments support that the AtCGE1 and AtCGE2 proteins can form homodimers and heterodimers as well. Finally, as no data suggests the formation of cpHsc70-1 homodimers, protoplasts from *N. benthamiana* leaves transformed using the plasmids.
containing the fusions cpHsc70-1-cYFP and cpHsc70-1-nYFP were used as a control, no fluorescence was detected in protoplasts from such leaves (Fig. 6E), indicating that the overexpression of proteins targeted to the chloroplast stroma does not result in unspecific interactions between the two moieties of YFP, validating the BiFC observations presented in Figs. 5 and 6.

2.6. CGE1 and CGE2 genes are differentially regulated in response to heat stress

In E. coli, grpE and dnaK genes are transcriptionally activated upon heat stress (Yura et al., 1993), a regulation that is conserved in the CGE1 and HSP708 genes of C. reinhardtii but not in mitochondrial GrpEs from yeast or rat (Ang et al., 1986; Ikeda et al., 1994; Naylor et al., 1996; Schroda et al., 2001). To determine the transcriptional response of AtCGEs and CPSC70-1 genes to heat stress in seedlings, we first determined by Northern-blot the mRNA accumulation levels of AtCGE1, AtCGE2 and CPSC70-1 under normal growth conditions in several developmental stages of wild-type seedlings (Fig. 7A and B). We found that AtCGE1, AtCGE2 and CPSC70-1 genes are co-expressed at 8, 10, 12, and 14 days after germination (Fig. 7A and B). We also observed that the mRNA levels of AtCGE2 and CPSC70-1 increase after 8 days of development and remain the same for the rest of the evaluated time points; furthermore, we found that the mRNA levels of AtCGE1 are consistently downregulated 12 days after germination, but the initial levels are recovered later during development (Fig. 7A and B). Considering the downregulation of AtCGE1 in 12-day-old seedlings, we decided to perform the heat stress experiments in 14-day-old seedlings exposed to 40 °C for 30, 60 and 90 min, and the mRNA levels of AtCGE1, AtCGE2 and CPSC70-1 were analyzed by Northern blot. As shown in Fig. 7, AtCGE1 transcript levels do not significantly change in response to heat treatment (Fig. 7C and D). In contrast, AtCGE2 mRNA levels decrease after 30 min of heat treatment (Fig. 7C and D). This response was like the observed in DXS1, a transcript that encodes a chloroplast-
localized protein with no chaperone function that was used as a control of the treatment (Fig. 7C and D). Finally, the abundance of CPHSC70-1 mRNA shows downregulation after 30 and 60 min of heat stress but increases 90 min after the onset of the heat treatment (Fig. 7C and D); this response agrees with previous reports (Schroda et al., 2001). To our understanding, the fact that AtCGE1 transcript levels do not change in response to heat stress, suggests that the AtCGE1 protein levels remain unchanged during the development of the stress response; at the same time, the downregulation of AtCGE2 mRNA accumulation suggests that AtCGE2 protein levels decrease in response to heat stress. This hypothesis suggests that there are specific molecular mechanisms that differentially regulate the transcription or mRNA accumulation of the two AtCGEs; specifically, during the heat stress response, these mechanisms increase the AtCGE1 to AtCGE2 ratio, favoring the interactions between AtCGE1 and cpHsc70-1. These data suggest that AtCGE1 is the major AtCGE involved in the chloroplast’s response to heat stress.

Fig. 6. In vivo determination of AtCGE1 and AtCGE2 dimer formation. Mesophyll protoplasts from N. benthamiana leaves co-expressing the translational fusions AtCGE1-nYFP and AtCGE1-cYFP (A), AtCGE2-nYFP and AtCGE2-cYFP (B), AtCGE2-nYFP and AtCGE1-cYFP (C), AtCGE1-nYFP and AtCGE2-cYFP (D), and cpHsc70-1-nYFP and cpHsc70-1-cYFP (E). Images corresponding to the bright field, chlorophyll fluorescence (Chlorophyll), reconstituted YFP fluorescence (YFP fluorescence), and the merge between the two fluorescence channels are shown in the indicated columns. Protoplasts were prepared 96 h after the agroinfiltration of the N. benthamiana leaves. Scale bars correspond to 10 μm.
While AtCGE2 might be involved in a different biological process. However, the post-transcriptional regulation that controls the abundance of AtCGE proteins under heat stress remains to be analyzed.

2.7. cge1 mutant plants have distinctive phenotypes

To further explore the physiological roles of AtCGEs, we performed a phenotypic characterization of mutant plants for AtCGE1, and CPBEC70-1 genes. As previously described, homozygous T-DNA insertion mutants in the AtCGE1 gene (emb1241-1 and emb1241-2 mutants) are embryo-lethal (Meinke et al., 2008), preventing the phenotypic characterization of such plants. Thus, we analyzed the available heterozygote mutant lines emb1241-1(±), emb1241-2(±), and cge2-1(±), and the homozygous mutant Dcphsc70-1 (Fig. S3). The pigment content analysis in these mutants showed a significant decrease in the content of chlorophyll b (chl b) in Dcphsc70-1 and emb1241-2(±) mutant lines (Fig. 8A and Table S3). In contrast, no significant differences in pigment content were found in the emb1241-1(±) plants (Fig. 8A and Table S3). These results indicate the presence of alterations in the photosynthetic apparatus of emb1241-2(±) and Dcphsc70-1 plants.

To support these findings, the accumulation of the photosystem I psaD1 and photosystem II D1 reaction center proteins was analyzed by Western blot in emb1241-2(±) and Dcphsc70-1 mutants. In Fig. 8B, we observed a higher accumulation of psaD1 and D1 in emb1241-2(±) and Dcphsc70-1 mutants than in the wild-type plants (Fig. 8B), suggesting that the stoichiometry of the reaction centers or other parts of the photosynthetic apparatus might be altered in these plants. Using BN-PAGE, the accumulation of photosynthetic complexes was analyzed in emb1241-2(±) and Dcphsc70-1 mutants. It was observed that both emb1241-2(±) and Dcphsc70-1 accumulate lower levels of the functional oligomers of LHCII than the wild-type plants (Fig. 8C). Accordingly, the levels of LHCII monomer were higher in the emb1241-2(±) and Dcphsc70-1 than in the wild-type plants (Fig. 8C). No significant differences in the abundance of the other major complexes (PSI and PSII) resolved in the gel were detected. These results suggest that cpBEC70-1 and AtCGE1 protein have a role in the proper oligomerization of the LHCII complex.

3. Discussion

Protein function largely depends on the protein’s ability to interact...
with specific molecular partners to regulate aspects like folding, localization, and degradation; the most well-known protein partners are the chaperones. Chloroplastic chaperones of the Hsp70 family are known to be involved in protein import into the chloroplast and the formation of protein oligomers, and to have roles in cellular and physiological traits such as thylakoid biogenesis and thermotolerance (Schroda et al., 2001; Liu et al., 2007; Su and Li, 2008; Shi and Theg, 2010; Su and Li, 2010). It is known that Hsp70-family proteins work together with DnaJ and GrpE proteins (Mayer, 2010). In the plastids of land plants, it has been demonstrated that chloroplastic DnaJ proteins have roles in the homeostasis of proteins and PSII function (Pulido et al., 2013; Kong et al., 2013). However, the role that GrpE proteins play in the chloroplasts of higher plants has not been analyzed in detail.

Through a phylogenetic analysis, we identified the chloroplastic protein homologues of the bacterial GrpE protein in 61 land plant species, in accordance to previous reports we found two CGEs in A. thaliana that were named AtCGE1 and AtCGE2 (Schroda et al., 2001; Shi and Theg, 2010). Using a genetic complementation assay, we established the activity of the CGEs from A. thaliana in vivo. Due to the knockout of the grpE gene, the E. coli mutant strain OD212 has growth defects under heat stress conditions. Here we found that transformation with the coding regions of AtCGE1 and AtCGE2 genes can rescue the defective-growth phenotype of the E. coli strain OD212 at high temperatures. However, the complementation shown by the cells transformed with the AtCGEs is partial, since the heat-resistant phenotype conferred by the expression of the AtCGEs is weaker than the conferred by EcGrpE at 43 °C. This result might be due to differences in the amino acid positions important for the establishment of interactions with DnaK protein of AtCGE1 and AtCGE2, in comparison to the bacterial GrpE. It is known that mutation of six amino acid positions in the bacterial GrpE protein negatively affects the interaction with DnaK (Harrison et al., 1997; Gelinas et al., 2003, 2004); three of the six mentioned positions are conserved between GrpE and CGEs (R73, R183, and V192). However, positions R74 and K82 in the GrpE sequence are substituted by K and S residues respectively in CGEs. It is possible that these amino acid substitutions generate interactions between AtCGEs and DnaK, that are not as stable at 43 °C as the interactions between DnaK and EcGrpE, suggesting that these substitutions are responsible for the partial complementation displayed by the AtCGEs. Furthermore, the amino acid residue corresponding to position 122 of GrpE is the only functionally-relevant position that is different between Type A and Type B CGEs; Type A CGEs (such as AtCGE2) conserve the G residue present in the bacterial GrpE, while Type B CGEs (such as AtCGE1) present an S residue substitution at the corresponding position. The conservation of G122 in Type A CGEs explains the improved complementation displayed by AtCGE2 at 43 °C, apparently this position makes AtCGE2 more similar to the bacterial GrpE and likely results in better DnaK binding in comparison to AtCGE1. These observations suggest that AtCGEs form stable interactions with DnaK to supplement for the lack of endogenous GrpE in E. coli. Similar

Fig. 8. Biochemical phenotype of emb1241 (±) and Dphsc70-1 mutants. The Chlorophyll b accumulation in wild-type, emb1241-1 (±), emb1241-2 (±), and Dphsc70-1 plants is shown (A). The error bars represent the standard error, the symbol (*) over the bars represent statistically significant differences calculated in a one-way analysis of variance with a significance level of p < 0.05. The Immunodetection of psaD1 and D1 proteins (B) in total protein extracted from wild-type (1), emb1241-2 (±) (2), and Dphsc70-1 (3) adult plants is shown. The Ponceau S staining of the protein-containing membranes is shown as a loading control, molecular weight marker (in kDa) is shown at the left. Blue native-PAGE of total leaf protein (C) from wild-type (1), emb1241-2 (±) (2), and Dphsc70-1 (3) plants. Arrows point to the bands corresponding to the supramolecular complexes visible in the samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
complementation has been shown for other eukaryotic GrpE functional orthologues, such as the C. reinhardtii CGE1 (CrCGE1) and the yeast mitochondrial protein Mge1p (Deloche and Georgopoulos, 1996; Schroda et al., 2001), indicating that the two AtCGEs are functional nucleotide exchange factors that can be considered functional orthologues of the bacterial GrpEs.

In addition to the activity and the amino acid conservation, several aspects of the AtCGEs biology were investigated to find a correlation between CGEs and chloroplastic Hsp70s in higher plants, given the fact that there are not available specific anti CGE antibodies against the CGEs from higher plants, our main experimental strategies are based on the recombination of recombinant proteins in N. benthamiana leaves. Using GFP-tagged proteins, colocalization of the chloroplastic homolog of DnaK (cpHsc70-1) and the two AtCGEs was detected, indicating that the two AtCGEs are effectively targeted to the chloroplast. Additionally, our mRNA accumulation experiments showed that AtCGE1, AtCGE2 and CPHC70-1 genes are co-expressed during seedling development in A. thaliana, indicating a strong correlation between the AtCGEs and CPHC70-1 at the transcriptional and posttranscriptional level. Accordingly, CrCGE1 and the chloroplastic Hsp70 HSP70B, have been shown to accumulate mainly in the chloroplast stroma, indicating that their activities are spatially coordinated in algae (Schroda et al., 2001).

In contrast, the present work showed that c-Myc tagged AtCGE1 and AtCGE2 accumulate not only in the stroma but also at the envelope of chloroplasts. In these experiments, c-Myc tagged cpHsc70-1 was only detected in the stromal fraction of the plastids. However, AtCGEs do not display features of integral membrane proteins, thus their localization at the chloroplast envelope must be mediated by interactions with unknown membrane proteins with functions not related to chaperone activity, or that might serve as scaffold to mediate interaction with the cpHsc70-1. In chloroplasts, the stromal side of Tic40 protein has sequence homology to the Hip and Hop chaperones that are known to bind and regulate the activities of Hsp70 and Hsp90 chaperones (Stahl et al., 1999; Chou et al., 2003; Bédard et al., 2007); this observation indicates that similar interacting proteins might work to regulate the interaction between AtCGEs and cpHsc70-1 at the chloroplast envelope. Additionally, the immunodetection of AtCGEs at the chloroplast envelope corroborates the fluorescence distribution displayed by the translational fusions AtCGEs-GFP, which (in addition to the stromal localization) show the formation of cumuli that resemble envelope deformations caused by the overaccumulation of envelope proteins (Breuer et al., 2012). Altogether, these data demonstrate that the AtCGEs have a dual suborganellar distribution that might be due to interactions with unknown membrane proteins.

BiFC and co-immunoprecipitation assays were performed to demonstrate the existence of direct interactions between CGEs and chloroplastic Hsp70s. These experiments demonstrate that AtCGE1 and AtCGE2 form stable physical interactions with cpHsc70-1 in vivo inside the chloroplasts, corroborating the information obtained in the GFP-tagging experiments. Previous work has demonstrated that dimerization of GrpE proteins is fundamental for the establishment of interactions with Hsp70 proteins in E. coli and S. cerevisiae (Deloche and Georgopoulos, 1996; Wu et al., 1996; Azem et al., 1997). Taking advantage of our genetic constructs for BiFC and GFP-tagged proteins, we showed that the two CGEs from A. thaliana can form homodimers in vivo, indicating that the interaction mechanism between AtCGEs and cpHsc70-1 might be like the described for E. coli and S. cerevisiae (Deloche and Georgopoulos, 1996; Wu et al., 1996; Azem et al., 1997). In addition, we found that AtCGE1 and AtCGE2 form heterodimers in vivo; however, the relevance to chaperone activity of the formation of such heterodimers remains to be addressed. Together, these results indicate that the chloroplastic GrpE proteins of A. thaliana have the specific traits needed to physically interact with cpHsc70-1 and that their biological roles might be functionally linked to the activity of cpHsc70-1.

The activity of DnaK and GrpE proteins has been linked to heat stress responses (Yura et al., 1993). In C. reinhardtii, increased accumulation of HSP70B transcript has been detected upon incubation at 40 °C (Schroda et al., 2001). However, the transcriptional response of A. thaliana CPHC70-1 to heat stress is not fully consistent, with some studies detecting upregulation (Suzuki et al., 2013) and others detecting downregulation (Sung et al., 2001) in response to heat stress. In this regard, the heat stress experiments performed in this study showed overaccumulation of the CPHC70-1 mRNA 90 min after the onset of the heat treatment, supporting the physiological data of Su and Li (2008) that suggested the involvement of cpHsc70-1 protein in the thermotolerance of A. thaliana seedlings. In C. reinhardtii, increased accumulation of CrCGE1 transcript was found in response to heat stress (Schroda et al., 2001). In our experiments, no significant changes in the accumulation of AtCGE1 mRNA were detected after the heat treatment, whereas downregulation of the AtCGE2 transcript was found after the onset of the treatment. The transcriptional response of AtCGE2 is commonly observed in genes not related to heat stress responses (Rizhsky et al., 2002; Echevarría-Zomeño et al., 2016; Jiang et al., 2017). Based on this transcriptional data, we hypothesized that AtCGE1 protein has a higher likelihood to have a function that is coordinated with cpHsc70-1 in the chloroplastic heat stress responses. Also, the data presented suggest that the two AtCGE proteins might have independent roles in chloroplast biology.

In the past, cpHsc70-1 protein activity has been linked to several biological roles, including thermotolerance, thylakoid biogenesis, protein import and photosystem II assembly (Yalovisky et al., 1992; Schroda et al., 1999; Liu et al., 2007; Sun and Li, 2008; Shi and Theg, 2010; Su and Li, 2010). As stated before, Hsp70 proteins work together with DnaJ cochaperones. Accordingly, different DnaJ proteins have been found to have biological roles like those of cpHsc70-1; such as the essential oligomerization of VIPP1 protein during thylakoid biogenesis, and the formation of PSI dimers and PSII-LHCII supercomplexes (Liu et al., 2005, 2007; Chen et al., 2010). To investigate the existence of a shared role for AtCGEs and cpHsc70-1, the accumulation of photosynthetic pigments was determined in leaves of the plant lines Dcphsc70-1, and the two alleles of AtCGE1 emb1241-1( ± ) and emb1241-2( ± ). Such analysis showed that a decrease in the content of chlorophyll b in the Dcphsc70-1, emb1241-1( ± ), and emb1241-2( ± ) plant lines. As the biochemical phenotype is stronger in emb1241-1( ± ), only emb1241-2( ± ) plants were used for further analysis. In photosynthetic organisms, chlorophyll b is mainly associated with the LHCCI complex of PSI (Green and Durnford, 1996; Kitajima and Hogan, 2003; Mascia et al., 2017). In agreement, the BN-PAGE analysis showed that the intensity of the protein bands corresponding to the LHCCI trimer and the LHCCI assembly from the emb1241-2( ± ) and Dcphsc70-1 mutants, is reduced in comparison to the intensity of the corresponding bands in wild-type plants. In contrast, the abundance of the band corresponding to the monomeric state of the LHCCI is increased in both mutants compared to the wild-type. This observation indicates that similar to what is observed for cpHsc70-1, the AtCGE1 protein is necessary for the correct assembling of the supramolecular organization of the LHCCI complex, further supporting that the activity of AtCGE1 is functionally linked to that of cpHsc70-1 in A. thaliana.

As reported before, mutation of CGE1 causes defects that result in the stunning of embryo development at the preglobular stage (Meinke et al., 2008). The fact that homozygous mutants for AtCGE1 are embryo-lethal indicates that AtCGE2 protein is not able to supplement the absence of AtCGE1 protein. Furthermore, the phylogenetic analysis performed here shows that 35 plant species contain both Type A and Type B CGEs, while 26 species contain only one type of CGE protein (11 species have Type A and 15 species have Type B). These observations indicate that either type of CGE can perform all the functions of the two types of CGEs in the species that have only one type of CGE protein, a phenomenon that does not happen in the plant species that have the two types of CGEs, such as A. thaliana. This hypothesis suggests that
CGEs from species containing only one type of CGE must have different biochemical or molecular properties compared to CGEs from two type-containing species, that enables them to carry out the entire set of biological functions associated to CGE activity. These fundamental regulatory aspects of CGE biology will be investigated in detail in future research. Finally, the fact that the two AtCGEs are functional nucleotide-exchange factors that establish direct physical interactions with cpHsc70-1, suggests that despite their different biological roles, the two AtCGEs exert their functions through the enhancement of the activity of Hsp70 chaperones.

In conclusion, AtCGE1 and AtCGE2 proteins are the chloroplastic functional orthologues of the bacterial GrpE protein, they physically interact with cpHsc70-1 and mediate independent biological processes. We have evidence that suggests the involvement of AtCGE1, but not AtCGE2, in the homeostasis of LHCII functional oligomers.

4. Materials and methods

4.1. Plant material and growth conditions

*Nicotiana benthamiana* plants were cultured for 6 weeks in MetroMix 300 (Sun Gro Horticulture, USA) substrate supplemented with a controlled-release fertilizer (Osmocote Smart-release, The Scotts Miracle-Gro Company, USA) at 26 °C in a 16h light:8h dark photoperiod. For the experiments involving aseptic culture of plants, seeds were surface sterilized by incubation in a solution of 1% (v/v) NaClO, Miracle-Gro Company, USA) at 26 °C in a 16h light:8h dark photoperiod. Mix 300 (Sun Gro Horticulture, USA) substrate supplemented with a 4% sucrose and 0.8% (w/v) phytoagar was used to inoculate fresh medium and allowed to grow to an OD600nm of 0.1. Serial dilutions were prepared and 5 μL drops were plated on solid LB medium. The inoculated plates were incubated for 20 h at either 25 °C, 37 °C or 43 °C. All the LB media was supplemented with ampicillin to a final concentration of 100 μg/mL. The complementation experiments were replicated at least three times.

4.2. In silico analysis

Chloroplast GrpE protein sequences were identified and retrieved using the UniProtKB (http://www.uniprot.org/help/uniprotkb) BLAST tool, using the *E. coli* GrpE protein sequence as a query against the Plants database. In total, 136 CGE protein sequences were sorted by the Arabidopsis Biological Resource Center (www.arabidopsis.org). Populations of 30 adult plants (30 days old) of the mutant lines, were PCR-screened (Table S1) and only T-DNA-carrying plants were used for further analysis.

4.3. Gene cloning and plasmid construction

The *A. thaliana* CGE1 (At5g17710), CGE2 (At1g36390) and CPHSC70-1 (At4g24280), and the bacterial EgrpE (NC_000913.3) coding regions (CDS) were amplified by PCR using specific oligonucleotides to obtain the full CDS or to delete the transit peptides (Table S1). Entry vectors for Gateway cloning were generated by either recombination into pDONR/Zeo donor vector or by directional cloning into pENTR/D-TOPO entry vector. The expression plasmids (Fig. S2) were generated by LR clonase II-mediated (Invitrogen, USA) recombination of the entry vectors containing the coding regions of interest and destination vectors pDST14 (pD14), pDST22 (pD22), pDST32 (pD32), pEarleyGate103 (pEG), pSPYCE (pCE), and pSPYNE (pNE). The expression plasmids constructed using pEG, pCE, and pNE were transferred to *Agrobacterium tumefaciens* C58S1 cells, individual clones were used for plant agroinfiltration.

4.4. *E. coli* complementation assay

The *E. coli* strain OD212 (dnaK332 ΔgrpE:::12-camR) (Deloche et al., 1997) was transformed with pD14::ΔCGE1, pD14::ΔCGE2, pD14::EgrpE and pD14::Δcdbi plasmids (Fig. S2), the cells were cultured overnight at 25 °C in solid lysogeny broth (LB) media. Individual colonies were cultured overnight in liquid LB medium at 25 °C, 100 μL of these cultures were used to inoculate fresh medium and allowed to grow to an OD600nm of 0.1. Serial dilutions were prepared and 5 μL drops were plated on solid LB medium. The inoculated plates were incubated for 20 h at either 25 °C, 37 °C or 43 °C. All the LB media was supplemented with ampicillin to a final concentration of 100 μg/mL. The complementation experiments were replicated at least three times.

4.5. Subcellular and suborganellar localization, and Bimolecular Fluorescence Complementation (BiFC)

The subcellular localization and the BiFC experiments were performed using agroinfiltrated *Nicotiana benthamiana* leaves. Subcellular localization was determined using translational fusions between the proteins of interest and GFP, while BiFC experiments were carried out using the split YFP system (Walter et al., 2004). The agroinfiltration of *N. benthamiana* leaves was performed using *A. tumefaciens* C58C1 cells, individual T-DNA insertion lines (Bailey, 2011) fed with Type A or Type B sequences. Motif discovery was performed using the DREME tool from the MEME-suite (Bailey, 2011) fed with Type A or Type B sequences.

4.6. Confocal microscopy

Confocal microscopy images of *N. benthamiana* leaf protoplasts were obtained with an Olympus FV1000 microscope (Olympus, USA) using excitation lasers of 488 nm for GFP and 515 nm for YFP, chlorophyll fluorescence was captured using a 515 nm laser and a barrier filter BA655-755. Z projections were rendered using the Fiji software maximum intensity projection type (Schindelin et al., 2012).
4.7. Plant heat stress experiments and northern blot

A. thaliana (Col-0) seedlings were cultured for 8, 10, 12 and 14 days under normal growth as described before. Fourteen days old A. thaliana (Col-0) seedlings were exposed to heat stress by incubation at 40 °C for 30, 60 or 90 min. Total RNA was prepared from the seedlings using TRIzol (Ambion, Life Technologies, USA) reagent, following the manufacturer’s protocol. For northern blot analyses, 10 μg of total RNA were fractionated in 1.5% (w/v) agarose gels under denaturing conditions [2% (v/v) formaldehyde] and transferred to Hybond-N+ nylon membrane (GE Healthcare Bio-Sciences, UK). Specific DNA probes for each of the genes were isolated from the CDSs and encompass the regions of the genes.

Hybridization and washing of the RNA-containing membranes were performed according to the manufacturer’s protocol. For northern blot analyses, 10 μg of total RNA were fractionated in 1.5% (w/v) agarose gels under denaturing conditions [2% (v/v) formaldehyde] and transferred to Hybond-N+ nylon membrane (GE Healthcare Bio-Sciences, UK). The specific DNA probes were radiolabeled with α-dCT32P using the Megaprime DNA labeling system (GE Healthcare Bio-Sciences, UK), following the protocol provided by the manufacturer. Hybridization and washing of the RNA-containing membranes were performed under stringent conditions (55 °C and 0.8825 M Na+). All the experiments described in this section were performed by triplicate.

4.8. Pigment quantitation

Photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) of adult plants were extracted by overnight incubation of leaves in 80% acetone solution. The absorbance of the samples was measured at 663 nm, 646 nm, and 470 nm. Using the absorbance data, the concentration of pigments was calculated with the following equations (Lichtenthaler and Wellburn, 1983):

Chlorophyll a (Ca) = (12.21*A663)-(2.81* A646)
Chlorophyll b (Cb) = (20.13* A663)-(5.03* A663)
Carotenes = ((1000* A470)-(3.27*Ca)-(104*Cb))/229

The statistical significance of the data was determined using a One-Way ANOVA calculator (http://www.socscistatistics.com/tests/anova/Default2.aspx), with a significance level of p < 0.05.

4.9. Protein analyses

The protein isolated from suborganellar fractions was precipitated by addition of Tris-EDTA [100 mM Tris, 10 mM EDTA, pH 8], 0.3% sodium deoxycholate and 72% Trichloroacetic acid (TCA) in a reagent at sample ratio of 1:5 (v/v), the samples were incubated on ice for 1 h. The protein was collected by centrifugation at 25, 000 x g, resuspended in 90% acetone and incubated overnight at 4 °C. The samples were centrifuged at 25, 000 x g, and the pellets were allowed to air-dry before resuspension in a minimal volume of 2X Laemmli buffer [65 mM Tris-HCl pH 6.8, 30% (v/v) glyceral, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 355 mM 2-mercaptoethanol] (Laemmli, 1970).

The protein samples (10–20 μL) were fractionated in denaturing 12% polyacrylamide gels, transferred to nitrocellulose blotting membrane (GE Healthcare Bio-Sciences, UK), and incubated in PBS-T buffer [137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.1% (v/v) Triton X-100] supplemented with 0.5% (w/v) nonfat dry milk. For Western blot analysis, monoclonal primary antibodies α-Myc (Sigma-Aldrich, Mexico) and αHA (Santa Cruz Biotechnology Inc, USA), and secondary antibodies αMouse-HRP (Thermo Fisher Scientific, USA) and αRabbit-HRP (Thermo Fisher Scientific, USA) were used. Detection of the recombinant proteins was performed using the Amersham ECL Prime Western blotting detection reagent kit (GE Healthcare Bio-Sciences, UK), following the manufacturer’s instructions.

For the BN-PAGE analysis, total protein from wild-type, emb1241-2, and DcpHsc70-1 mutants was extracted by incubation of frozen-pulverized leaf tissue with BN extraction buffer [70 mM Tris-HCl pH 7.5, 1 mM MgCl2, 25 mM KCl, 5 mM EDTA pH 8, 0.25 mM Sucrose, 39.1 mM n-Dodecyl β-D-maltoside], supplemented with the complete protease inhibitor cocktail as indicated by the manufacturer (Sigma-Aldrich, Mexico). Protein samples were subjected to BN-PAGE in a 4%–14% acrylamide gradient, according to the protocol of Heinemeyer et al., (2007). Additionally, the BN-PAGE gels were destained by 10 consecutive washes of boiling-hot distilled water. All the experiments described in this section were performed at least three times.

Contribution

L. A. de Luna-Valdez: performed experiments, analyzed the results and wrote the manuscript.

C. I. Villaseñor-Salmerón: performed experiments.

A. A. Guevara-García: designed and supervised experiments, edited the manuscript and funded the research.

P. León- Mejía: edited the manuscript funded the research.

E. Cordoba: designed experiments and edited the manuscript.

R. Vera-Estrella: designed experiments and edited the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.027.

References


