Arabidopsis Stromal 70-kD Heat Shock Proteins Are Essential for Plant Development and Important for Thermotolerance of Germinating Seeds\textsuperscript{1[C][W][OA]}

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The 70-kD heat shock proteins (Hsp70s) are molecular chaperones involved in a variety of cellular processes including protein folding, protein translocation, and stress responses in almost all organisms and in almost all subcellular compartments. However, the function of plastid stromal Hsp70s has been shown to be important for protein folding, protein translocation, and stress responses in almost all organisms and in almost all subcellular compartments. However, the function of plastid stromal Hsp70s in higher plants is still uncertain. Genomic surveys have revealed that there are two putative stromal Hsp70s in Arabidopsis thaliana, denoted cpHsc70-1 (At4g24280) and cpHsc70-2 (At5g49910). In this study, we show that cpHsc70-1 and cpHsc70-2 could indeed be imported into the chloroplast stroma. Their corresponding T-DNA insertion knockout mutants were isolated and designated as ΔcpHsc70-1 and ΔcpHsc70-2. No visible phenotype was observed in the ΔcpHsc70-2 mutant under normal growth conditions. In contrast, ΔcpHsc70-1 mutant plants exhibited variegated cotyledons, malformed leaves, growth retardation, and impaired root growth, even though the protein level of cpHsc70-2 was up-regulated in the ΔcpHsc70-1 mutant. After heat shock treatment of germinating seeds, root growth from ΔcpHsc70-1 seeds was further impaired, indicating that cpHsc70-1 is important for thermotolerance of germinating seeds. No ΔcpHsc70-1 ΔcpHsc70-2 double mutant could be obtained, suggesting that the ΔcpHsc70 double knockout was lethal. Genotype analyses of F\textsubscript{1} seedlings from various crosses indicated that double-knockout mutation was lethal to the female gametes and reduced the transmission efficiency of the male gametes. These results indicate that cpHsc70s are essential for plant development and the two cpHsc70s most likely have redundant but also distinct functions.

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Therefore, whether stromal Hsp70s are involved in chloroplast protein import requires further studies. In the green algae Chlamydomonas and Dunaliella, chloroplast stromal Hsp70B has been shown to function in PSII protection and repair during and after photoinhibition (Schroda et al., 1999; Yokthongwattana et al., 2001). Chlamydomonas stromal Hsp70B regulates the assembly state of VIPP1 (vesicle inducing protein in plastid 1), which may be important for thylakoid biogenesis or maintenance (Liu et al., 2005, 2007). It is not known whether higher plant stromal Hsp70 has any similar function.

Genomic surveys revealed that there are 14 genes encoding Hsp70s in Arabidopsis (Sung et al., 2001a). Among them, two genes encode putative plastid stromal Hsp70s, cpHsc70-1 (At4g24280), and cpHsc70-2 (At5g49910). They harbor a predicted chloroplast-targeting transit peptide and a C-terminal motif (PEGD-VIDADFTDSK) conserved among plastid Hsp70s (Guy and Li, 1998). Their polypeptide sequences share a 90.7% identity. Although reverse transcription (RT)-PCR analyses indicated that the amplified signal for cpHsc70-1 was very low and the transcripts of cpHsc70-2 was much higher than cpHsc70-1 in all organs (Sung et al., 2001b), according to the public microarray and MPSS databases (Brenner et al., 2000 [MPSS, http://)].

![Figure 1. Arabidopsis cpHsc70s were imported into the stroma of isolated chloroplasts.](image1)

![Figure 2. Confirmation of the T-DNA insertion lines for cpHsc70s. A, Schematic representation for the genomic fragments of cpHsc70-1 (At4g24280) and cpHsc70-2 (At5g49910). Black boxes and lines represent exons and introns, respectively. ATG represents the translation initiation sites. The locations of the T-DNA insertions in Δcphsc70-1 and Δcphsc70-2 mutants are illustrated. Positions and directions of primers used in B are also indicated. B, Δcphsc70-1 and Δcphsc70-2 are null mutants. Primer sets for cpHsc70-1 are: 1E2-S + 1E8-AS for wild-type copy of cpHsc70-1 DNA and RT-PCR of RNA, and SALK-Lba1 + 1E8-AS for Δcphsc70-1 T-DNA insertion. Primer sets for cpHsc70-2 are: 2E1-S + 2E8-AS for wild-type copy of cpHsc70-2 DNA and RT-PCR of RNA, and SALK-Lba1 + 2E8-AS for Δcphsc70-2 T-DNA insertion.](image2)
mpss.udel.edu/at/]; Zimmermann et al., 2004 [Genevestigator, http://www.genevestigator.ethz.ch/]; Toufighi et al., 2005 [Botany Array Resource, http://bbc.botany.utoronto.ca/]), both transcripts are quite abundant in almost all tissues, with the level of \( cpHsc70-1 \) slightly higher than \( cpHsc70-2 \) in most tissues.

In this report, we show that the two putative plastid Hsp70s were indeed imported into the chloroplast stroma. T-DNA insertion knockout mutants of the two genes were analyzed. Our data indicate that the stromal Hsp70s are important for plant development under both normal and heat-stress conditions.

**RESULTS**

**Arabidopsis cpHsc70-1 and cpHsc70-2 Could Be Imported into the Stroma of Chloroplasts**

To verify the plastid localization of Arabidopsis \( cpHsc70-1 \) and \( cpHsc70-2 \), \(^{35}\)S-labeled \( cpHsc70-1 \) and \( cpHsc70-2 \) were synthesized by in vitro translation and incubated with isolated pea chloroplasts. As shown in Figure 1, the precursor form of both \( cpHsc70-1 \) and \( cpHsc70-2 \) was about 80 kD. After import, a mature protein of approximately 71 kD was produced, suggesting the cleavage of a transit peptide. The imported mature \( cpHsc70 \)s were present in the soluble fraction of chloroplasts (lane 4) and were resistant to thermolysin (data not shown) and trypsin digestion (lanes 2 and 3). Trypsin can penetrate the outer but not the inner envelope membrane (Jackson et al., 1998), and resulted in complete digestion of the outer membrane protein Toc75 but not the thylakoid membrane protein chlorophyll \( a/b \)-binding protein of PSII (CAB; Fig. 1). These data suggested that the two \( cpHsc70 \)s were located in the chloroplast stroma. It is less likely that they were located in the thylakoid lumen because their transit peptides do not resemble the typical bipartite luminal-targeting sequence (Gutensohn et al., 2006).
Identification of T-DNA Insertion Mutants of cpHsc70s

To study the function of cpHsc70s, we obtained their putative T-DNA insertion mutants, SALK_140810 and SALK_095715, from the SALK T-DNA collection (Alonso et al., 2003). The T-DNA insertion sites were confirmed by genomic PCR and DNA sequencing. As shown in Figure 2A, both SALK_140810 and SALK_095715 have a T-DNA insertion in the intron II of cpHsc70-1 and cpHsc70-2, respectively. RT-PCR analyses indicated that both T-DNA insertion lines were null mutants (Fig. 2B). We designated these two mutants as \( \Delta cphsc70-1 \) (SALK_140810) and \( \Delta cphsc70-2 \) (SALK_095715). Genomic DNA analyses further indicated that both \( \Delta cphsc70-1 \) and \( \Delta cphsc70-2 \) contained T-DNA insertion in a single locus and the variegated-cotyledon phenotype \( \Delta cphsc70-1 \) (see below) was linked to the T-DNA insertion (Supplemental Fig. S1; Supplemental Table S1).

\( \Delta cphsc70-1 \) Plants Exhibited Variegated Cotyledons, Malformed Leaves, and Growth Retardation

The cotyledons of \( \Delta cphsc70-1 \) plants were variegated (Fig. 3A). Furthermore, in the early vegetative stage, the true leaves of \( \Delta cphsc70-1 \) seedlings often had irregular leaf margins and small lesions (Fig. 3B). The mutant was also smaller than the wild type. At 15 d after germination, the fresh weight of \( \Delta cphsc70-1 \) plants was about 50% of wild-type plants (Fig. 3D). We also measured root growth on vertical plates. Root length of \( \Delta cphsc70-1 \) plants was also about 50% of wild type (Fig. 3, C and E). In contrast, there was no visible phenotype in the \( \Delta cphsc70-2 \) mutant. These results suggest that cpHsc70-1 is important for both shoot and root growth. Because there is only one mutant allele available for \( \Delta cphsc70-1 \), we tried to complement \( \Delta cphsc70-1 \) with a cpHsc70-1 genomic fragment to confirm that the mutant phenotypes were caused by the loss of cpHsc70-1. As shown in Figure 3, \( \Delta cphsc70-1 \) transformed with a 5-kb cpHsc70-1 genomic fragment (transformants designated as cpHsc70-1g) fully recovered the wild-type phenotype.

We further analyzed the cpHsc70 protein level in the mutants and wild type using an antibody specifically recognizing the pea stromal Hsp70, S78 (Akita et al., 1997). This antibody was made against the C-terminal region of S78. This region of S78 shares a 75% sequence identity to the two Arabidopsis cpHsc70s, but only a 22% to 32% identity to other Arabidopsis Hsp70s. The antibody recognized a single band in Arabidopsis total leaf proteins on SDS-PAGE and immunoblots.

Table I. Progeny genotypes of the cross \( \Delta cphsc70-2 \) ♀ × \( \Delta cphsc70-1 \) ♂

<table>
<thead>
<tr>
<th>Generation</th>
<th>Gene</th>
<th>Genotypea</th>
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<tbody>
<tr>
<td></td>
<td>cpHsc70-1</td>
<td>cpHsc70-2</td>
</tr>
<tr>
<td>F2</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Observed no.</td>
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<td>71</td>
</tr>
<tr>
<td>Observed ratio</td>
<td>1.97</td>
<td>0.97</td>
</tr>
<tr>
<td>Theoretical ratio</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F3</td>
<td>+/+</td>
<td>+/+</td>
</tr>
<tr>
<td>Observed no.</td>
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<td>14</td>
</tr>
<tr>
<td>Observed ratio</td>
<td>0.34</td>
<td>0</td>
</tr>
<tr>
<td>Theoretical ratio</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a+, Wild-type copy of the gene; \( \Delta \), gene with the T-DNA insertion. bThe parental F1 genotype is +/Δcphsc70-1 +/Δcphsc70-2. The F2 plants were from self-pollination of the F1 parents. cThe parental F2 genotype is +/Δcphsc70-1 Δcphsc70-2/Δcphsc70-2. The F3 plants were from self-pollination of the F2 parents.
(Supplemental Fig. S2). However, the two Arabidopsis cpHsc70s could be resolved on isoelectric focusing (IEF) gels and each mutant was indeed missing the corresponding cpHsc70 (Fig. 3F). Interestingly, the level of cpHsc70-2 was up-regulated in the ΔcpHsc70-1 mutant. This result suggested that in the ΔcpHsc70-1 mutant, even though cpHsc70-2 was up-regulated, the ΔcpHsc70-1 phenotype could not be rescued. In comparison, the ΔcpHsc70-2 mutant had a lower amount of total cpHsc70 (presumably cpHsc70-1) than the wild type and this amount of cpHsc70-1 was sufficient for normal plant development.

**cpHsc70s Are Essential for Plant Development**

As a first step toward investigating whether cpHsc70-1 and cpHsc70-2 had different or redundant functions, we tried to generate a ΔcpHsc70-1 ΔcpHsc70-2 double mutant. As shown in Table I, in the F2 progeny of ΔcpHsc70-2 flowers crossed with ΔcpHsc70-1 pollen, no double-knockout seedling was identified. In the meantime, reduced seed sets in the siliques of F1 plants were observed (Fig. 4A). Siliques of F1 plants from a cross of the reverse direction had the same reduced seed sets (Fig. 4A). In addition, plants with the genotype +/ΔcpHsc70-1 ΔcpHsc70-2/ΔcpHsc70-2 were extremely small (Fig. 4B). No plants with the genotypes ΔcpHsc70-1/ΔcpHsc70-1 ΔcpHsc70-2+/ΔcpHsc70-2 were identified (Table I). To confirm the absence of plants with this genotype, we crossed the ΔcpHsc70-1 single mutant flowers with pollen from plants with the genotype +/ΔcpHsc70-1 ΔcpHsc70-2/ΔcpHsc70-2 (Table II, third cross). Half of F1 progeny were expected to have the genotype ΔcpHsc70-1/ΔcpHsc70-1 ΔcpHsc70-2/. ΔcpHsc70-2. However, after analyzing 104 F1 plants, all of them had the genotype heterozygous for both ΔcpHsc70s. This result indicated that plants with only one copy of cpHsp70-2 are not viable. This may suggest that a threshold level of cpHsc70 is critical for plant development, although the cause of this lethality is currently unknown.

The lethality of the double mutation was further confirmed by the absence of double mutants in the self-pollinated progeny of plants with the genotype +/ΔcpHsc70-1 ΔcpHsc70-2/ΔcpHsc70-2 (Table I, bottom half); no double mutant was observed even though one-quarter of the progeny was expected to be double mutants. To further analyze if the absence of the double mutant was caused by gametophytic defects, we performed reciprocal crosses of the +/ΔcpHsc70-1 ΔcpHsc70-2/ΔcpHsc70-2 mutant with wild type (Table II, first two crosses). Genotype analyses of F1 seedlings showed that when the +/+ΔcpHsc70-1 ΔcpHsc70-2/ΔcpHsc70-2 mutant flowers were crossed with wild-type pollen, no plant containing the ΔcpHsc70-1 T-DNA insertion was obtained. This result indicated that there was no viable ovule with the double-knockout mutation. Genotype analysis of F2 seedlings from crosses of the reverse direction was expected to show 50% of the seedlings with the genotype +/+ΔcpHsc70-1 ΔcpHsc70-2 and 50% with the genotype +/+ΔcpHsc70-1 +/+ΔcpHsc70-2. However, we found 56 plants with the former genotype and only 17 plants with the latter genotype. This result suggested that pollen with the double-knockout mutation had reduced transmission efficiency. These data indicated that cpHsc70s were essential for ovule development and the two cpHsc70s had at least partially redundant functions.

### Effect of Individual ΔcpHsc70 Mutations on Chloroplast Protein Import

We next tested whether the ΔcpHsc70 knockout mutants had reduced protein import efficiency. As shown in Figure 5, A and B, chloroplasts isolated from adult mutant plants did not show impaired prRBCS import compared to the wild type. Because ΔcpHsc70-1 had the most apparent phenotype in cotyledons, we further compared protein import efficiency of chloroplasts isolated from 7-d-old seedlings, in which cotyledons represent the majority of green tissue. As shown in Figure 5C, ΔcpHsc70-1 chloroplasts had a greatly reduced protein import efficiency compared to the wild type, confirming that ΔcpHsc70-1 was the most important gene for chloroplast development in Arabidopsis. This result suggested that the ΔcpHsc70-1 mutant may have impaired protein import efficiency in cotyledons, which lead to the smaller size of ΔcpHsc70-1 mutant plants.

### Table II. Genotypes of F1 plants from various crosses

<table>
<thead>
<tr>
<th>Cross</th>
<th>Gene</th>
<th>F1 Genotypea</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>cpHsc70-1</td>
<td>Δ70-1/Δ70-2</td>
</tr>
<tr>
<td>Δ70-2 × Δ70-2</td>
<td>+/Δ70-2</td>
<td>+/Δ70-2</td>
</tr>
<tr>
<td>Δ70-1 × Δ70-2</td>
<td>Δ70-1/Δ70-2</td>
<td>Δ70-1/Δ70-2</td>
</tr>
<tr>
<td>Δ70-2 × Δ70-2</td>
<td>+/Δ70-2</td>
<td>+/Δ70-2</td>
</tr>
<tr>
<td>Δ70-1 × Δ70-2</td>
<td>Δ70-1/Δ70-2</td>
<td>Δ70-1/Δ70-2</td>
</tr>
<tr>
<td></td>
<td>Observed no.</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Δ70-1/Δ70-2</td>
<td>0b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Observed no.</td>
<td>17b</td>
</tr>
<tr>
<td></td>
<td>Δ70-1/Δ70-2</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Δ70-1/Δ70-2</td>
<td>0</td>
</tr>
</tbody>
</table>

a, Wild-type copy of the gene; Δ, gene with the T-DNA insertion.

bNo plant with the genotype +/+ΔcpHsc70-1 ΔcpHsc70-2 was observed, indicating that no viable ovule with the genotype ΔcpHsc70-1 ΔcpHsc70-2 was produced.

The transmission efficiency of the double-knockout pollen was reduced compared to pollen with the ΔcpHsc70-2 single mutation.
reduced amount of imported mature RBCS. However, noticeably the amount of envelope-associated prRBCS was also greatly reduced. We, therefore, further analyzed association of prRBCS with the chloroplast surface when no or little ATP was present in the import system. As shown in Figure 5D, fewer prRBCS associated with the Δcphsc70-1 mutant chloroplast surface in the absence of ATP. This result suggested that the Δcphsc70-1 mutation might have some secondary effects that caused damage to the chloroplast surface when isolated, which resulted in less efficient precursor association with the chloroplast. However, the amount of major translocon components like Toc159, Toc75, and Tic110 was not reduced in Δcphsc70-1 compared to the wild type (Supplemental Fig. S2). Therefore, the reason for the reduced precursor association of Δcphsc70-1 chloroplasts is not clear.

**cpHsc70-1 Is Important for Thermotolerance of Germinating Seeds**

Many Hsp70s have been shown to be important for protection of organisms against heat stress. Expression of Arabidopsis cpHsc70-1 and pea stromal Hsp70 S78 were 5 and 9 times higher, respectively, after heat shock (Marshall and Keegstra, 1992; Busch et al., 2005). We, therefore, investigated if cpHsc70s were involved in plant thermotolerance. In acquired thermotolerance tests (Fig. 6A), in which plants were acclimated at 37.5°C before they underwent heat shock at 44.5°C, the survival rate of the two Δcphsc70 mutants was similar to the wild type. In comparison, the hot1 mutant (SALK_099583c), which has a T-DNA insertion in the gene-encoding cytosolic ClpB (At1g74310), was totally killed. Interestingly, both Δcphsc70-1 and Δcphsc70-2 exhibited pale-green leaves after recovery. When the chlorophyll content was determined, both mutants had a reduced amount of total chlorophylls compared to the wild-type and cpHsc70-1g plants (Fig. 6B). For basal thermotolerance tests, seedlings were directly heated at 44.5°C for 30 or 40 min, and then allowed to recover for 7 d. Almost all seedlings, mutants and wild type alike, were killed by the 40-min heat shock, but all survived the 30-min heat shock (Fig. 6C). Again the Δcphsc70 mutants had a reduced chlorophyll content after the recovery (Fig. 6D).

The cpHsc70-1 gene has its highest expression level in seeds (Genevestigator; Zimmermann et al., 2004). We therefore tested seed basal thermotolerance by heating imbibed seeds at 44.5°C for 150 min. We did not observe a consistent difference in the germination rate between the mutants and the wild type. However, the root growth of the Δcphsc70-1 was further impaired.
by the heat treatment (Fig. 7). After growing on vertical plates for 7 d, for both wild type and ΔcpHsc70-2, heated seeds exhibited a root growth approximately 90% of the unheated wild type and ΔcpHsc70-2. In contrast, root length of plants from heat-treated ΔcpHsc70-1 seeds was only approximately 30% of the root length of plants from unheated ΔcpHsc70-1 seeds (Fig. 7B). This result indicates that cpHsc70-1 is important for root growth from heat-stressed seeds.

**DISCUSSION**

Mutant phenotype analyses revealed that ΔcpHsc70-1 plants had the most apparent phenotypes in altered growth of cotyledons and roots, and also in basal seed thermotolerance. Interestingly, expression of cpHsc70-1 also happens to be significantly higher than cpHsc70-2 only in cotyledons, root tips and seeds (Genevestigator; Zimmermann et al., 2004). This correlation may suggest that the differences observed between the ΔcpHsc70-1 and ΔcpHsc70-2 mutants result from the different promoter activities of the two genes, rather than different functions of the two proteins. Furthermore, plants that were homozygous for ΔcpHsc70-1 and heterozygous for ΔcpHsc70-2 were not found (Tables I and II), suggesting that in the absence of cpHsc70-1, the level of cpHsc70-2 became critical for plant growth. ΔcpHsc70-1 ΔcpHsc70-2 double-knockout mutation was lethal for the development of ovules and reduced the transmission efficiency of pollen. These results support that the two cpHsc70s have overlapping essential functions. It has been suggested that this redundancy may serve as a safety net against mutations that would otherwise be lethal (Sung et al., 2001a).

However, in the ΔcpHsc70-1 mutant, cpHsp70-2 was up-regulated to a level higher than the two cpHsc70s combined in the wild type (Fig. 3F; Supplemental Fig. S2) and ΔcpHsc70-1 still showed clear growth defects. This result suggests that cpHsc70-1 may have some specific functions that cannot be substituted by cpHsc70-2. Therefore it is most likely that the two cpHsc70s have overlapping but distinct functions. The two cpHsc70s share more than a 95% identity in both the ATPase domain and the substrate-binding domain, but only a 63% identity in the C-terminal 5-kD subdomain. The C terminus of Hsc70s has been shown to be important for interacting with cochaperones like Hop (Hsp70/Hsp90 organizing protein). It is possible that the two cpHsc70s may interact with different cochaperones, which lead them to different substrates.

Figure 6. Acquired and basal thermotolerance analyses. A, Seedlings were heated to 37.5°C for 1 h, returned to 22°C for 2 d, heated to...
It is interesting that there are also two putative stromal Hsp70s in the fully sequenced genomes of other land plants, such as moss (*Physcomitrella* spp.), poplar (*Populus* spp.), rice (*Oryza sativa*; Supplemental Table S2), and sorghum (*Sorghum bicolor*; Supplemental Table S2), but green algae only harbor a single cpHsp70. A second copy of cpHsc70 may be beneficial to cope with the more stressful and variable environment on land. However, phylogenetic analyses (Fig. 8) suggested that land-plant cpHsc70s were duplicated recently during evolution because the two copies of cpHsc70s could not be grouped into two protein subfamilies among different plant families. In most cases, the two copies from the same family are more similar to each other than to cpHsc70s from other families, although the two monocot cpHsc70s have a more divergent relationship. Experiments are required to first define the elements for specific expression of the two genes and then to perform promoter swap experiments to determine the functional specificity/redundancy of the two proteins.

The isolation of the *cpHsc70*-knockout mutants enabled us to directly test if plastid Hsp70s was involved in chloroplast protein import. Mature chloroplasts isolated from either single *Δcphsc70*-knockout mutant showed no import defect. Under normal import conditions, both the amount of precursor bound and the amount of mature protein imported were reduced in chloroplasts isolated from cotyledons of *Δcphsc70-1* (Fig. 5C). For isolated Arabidopsis chloroplasts, most of the precursor proteins that were still bound to the envelope after a 20- to 30-min import could not be imported even after prolonged incubation and were thought to represent nonspecific sticking of precursors on the chloroplast surface (Fitzpatrick and Keegstra, 2001; data not shown). Indeed, in all translocon mutants reported so far, only the amount of imported mature proteins, but not the amount of envelope-bound precursor proteins, was reduced (Jarvis et al., 1998; Sun et al., 2001; Kubis et al., 2003; Constan et al., 2004; Hung et al., 2004; Kovacheva et al., 2007). Hence, there seem to be some differences between the surface of isolated *Δcphsc70-1* cotyledon chloroplasts and that of other mutant chloroplasts. Indeed, when analyzed under conditions in which almost no ATP was present, precursor association with the mutant chloroplasts was reduced. Therefore, it is likely that defects in chloroplast physiology had resulted in damage to the surface of *Δcphsc70-1* cotyledon chloroplasts when isolated. More informative results on the function of cpHsp70 in chloroplast protein import await conditional double knockout or extreme double knockdown of the cpHsc70s. Another chaperone, Hsp100 (ClpC), has been shown to stably associate with the translocon complex (Akita et al., 1997; Nielsen et al., 1997). In addition, Cpn60 also has been found in isolated protein-import complexes (Kessler and Blobel, 1996). Therefore, it would be interesting to test whether multiple chaperones are involved in chloroplast protein transport across the inner membrane, with each chaperone assisting the import of certain precursors or under certain conditions.

Heat shock proteins have been shown to play important roles in helping cells to cope with environmental stress. The cytosolic Hsp100/ClpB plays major roles in acquired thermotolerance (Hong and Vierling, 2000, 2001; Nieto-Sotelo et al., 2002; Hong et al., 2003;
Katiyar-Agarwal et al., 2003). Cytosolic Hsp70 and plastid ClpB and small heat shock proteins have also been implicated in heat sensitivity of plants (Lee and Schofl, 1996; Sung and Guy, 2003; Wang and Luthe, 2003; Miroshnichenko et al., 2005; Myouga et al., 2006; Yang et al., 2006; Lee et al., 2007). However, no such role has been proposed for plastid Hsp70s. Here we have provided genetic evidence to show that Hsp70s in the plastid stroma are important for thermotolerance of germinating seeds, indicating that plastid physiology is important for seeds to endure heat stress. Plastids are involved in the assimilation of nitrogen and in the syntheses of lipids and many hormones, which are important for seed germination and root growth. It is therefore reasonable to have chaperones protecting the biosynthesis enzymes during heat stress. Accumulation of toxic intermediates due to heat inactivation of the enzymes may also have contributed to the inhibition of root growth observed. For acquired and basal thermotolerance in seedlings, we could not detect a difference in the survival rate between the mutants and the wild type. However, both δphsc70 mutants had reduced chlorophyll contents during heat shock recovery (Fig. 6, B and D). These results suggest that cpHsc70s may play a role in protection of the photosystems during heat shock and/or recovery. In green algae, the chloroplast Hsp70B has been shown to participate in the protection and repair of the PSII during and after photoinhibition (Schroda et al., 1999; Yokthongwattana et al., 2001). Therefore the involvement of stromal Hsp70s in the maintenance of chloroplast photosystems during stress conditions may be a general mechanism in plants. The mild phenotypes in seedling thermotolerance of the Arabidopsis δphsc70 mutants could be due to the presence of two homologous genes. It would be interesting to reduce the total level of cpHsc70s by antisense or RNAi techniques, coupled with inducible promoters to circumvent the essential nature of the proteins to further analyze the function of stromal Hsp70s in stress protection, chloroplast protein import, thylakoid formation, or even other novel functions.

MATERIALS AND METHODS

Plant Growth Conditions

All Arabidopsis (Arabidopsis thaliana) plants used in this study were of the ecotype Columbia. Sterilized Arabidopsis seeds were plated on 0.3% Gelrite-solidified 1× Murashige and Skoog (MS) medium containing Gamborg's B5 vitamin and 0.5% Suc. After a 3-d cold stratification, seeds were grown in growth chambers under a 16-h photoperiod with a light intensity approximately 80 μmol m⁻² s⁻¹ at 22°C. Soil-grown Arabidopsis plants were grown on a 9:1 mixture of peat, vermiculite, and perlite at 22°C under a constant light with an intensity approximately 120 μmol m⁻² s⁻¹, except all plants used for cross-experiments were grown on soil to maturity under a 16-h photoperiod with a light intensity approximately 120 μmol m⁻² s⁻¹ at 22°C. For growing pea seedlings (Pisum sativum 'Little Marvel'), imbibed seeds were
grown on vermiculite for 9 to 12 d under a 12-h photoperiod at 20°C with a light intensity of approximately 150 μmol m⁻² s⁻¹.

**Identification of Δcphsc70-1 and Δcphsc70-2 T-DNA Insertion Mutant Lines**

Mutant lines SALK_140810 (Δcphsc70-1), SALK_095715 (Δcphsc70-2), and SALK_099583c (hot1) were obtained from the SALK T-DNA collection (Alonso et al., 2003) via the Arabidopsis Biological Resource Center; they were screened by PCR amplification of genomic DNA because Δcphsc70-1 had lost kanamycin resistance and Δcphsc70-2 showed heterogenous growth on kanamycin plates. Primers used for amplifying the wild-type copy of cpHsc70-1 were 1E2-S (5′-gattcaagggagccacatggcatcttcagccgcccaa-3′) and 1E8-AS (5′-ttctctattggctgtctgtgaagtcag-3′). For amplifying the wild-type copy of cpHsc70-2, the primers used were 2E1-S (5′-gcttttcgtaaaggcttgtaagc-3′) and 2E8-AS (5′-ctgaggtagttgtagttgac-3′). To identify the T-DNAs inserted in cpHsc70-1 and cpHsc70-2, the SALK Lba1 primer (5′-tgcttttcgtaaaggcttgtaagc-3′) was combined with 1E8-AS and 2E8-AS, respectively. The T-DNA insertion sites were verified by sequencing the PCR products. Primer pairs used for checking cpHsc70-1 and cpHsc70-2 transcripts by RT-PCR were 1E2-S + 1E8-AS and 2E1-S + 2E8-AS, respectively. To test the expression of the cpHsc70 protein in each mutant, total stomatal proteins were analyzed in IEF gels (pI 3–7) and immunoblots decorated with the anti-S78 antibody.

**Vector Construction and Plant Transformation**

Using genomic DNA extracted from wild-type Arabidopsis seedlings as the template, a 5-kb genomic fragment containing the promoter and coding regions of cpHsc70-1 was amplified by PCR with primers H70A-PS (5′-aagctttccttgaagttgaagc-3′) and H70At-A5 (5′-gaagctttccctgagctgaagc-3′). The PCR product was first cloned into the pGEM-T vector (Promega), and then subcloned into the binary vector pCAMb1390. The resulting plasmid was named pCAMb1390/cpHsc70-1g and was transformed into Agrobacterium GV3101. The Δcphsc70-1 mutant plants were infected by the floral spray method (Chung et al., 2000). Transgenic plants harboring the introduced cpHsc70-1 genomic fragments were screened on MS media containing 50 mg L⁻¹ hygromycin.

**In Vitro Translation and Protein Import Assays**

T3 promoter fused cpHsc70-1 and cpHsc70-2 linear DNAs were amplified by PCR with pBluescript (Stratagene) using plasmids containing full-length cDNA as templates and primers containing the T3 promoter sequence. Primers used for amplifying T3cpHsc70-1 were 5′-caattaccaccttaaaaggaggaagttcctcgattttgacctcccccacccaa-3′ and 5′-gctctctattggctgtctgtgaagtcag-3′. For amplifying T3cpHsc70-2, the primers used were 5′-caattaccaccttaaaaggaggaagttcctcgattttgacctcccccacccaa-3′ and 5′-ttctcttttctggcttgagttgagttgac-3′. [35S]Met-labeled precursors of cpHsc70-1 and cpHsc70-2 were in vitro translated in a TNT reticulocyte lysate system (Promega), with the addition of PCR-generated T3cpHsc70-1 and T3cpHsc70-2 DNA templates. [35S]Met-pRBCC synthesis, pea, and Arabidopsis chloroplast isolation, and import assays were conducted as described (Perry et al., 1991), except the grinding buffer for Arabidopsis chloroplast isolation was modified to 50 mM HEPES-KOH (pH 8.0), 330 mM sorbitol, 2 mM EDTA, and 0.5% bovine serum albumin. Trypsin digestion of chloroplasts and separation of chloroplasts after digestion into soluble and membrane fractions were performed as described (Jackson et al., 1998; Hung et al., 2004). For import in the absence of ATP (Fig. 5D), ATP was removed from the in vitro-translated precursors by gel filtration (Perry et al., 1991) and chloroplasts were depleted of internal ATP by incubation in the dark at 4°C for 1 h. Import was performed under a green-safe light at room temperature for 15 min. Quantification of gel bands was performed using the Fuji FLA5000 phosphomager (Fujiﬁlm). Antibodies against Tic and Toc proteins were prepared as described (Chou et al., 2003; Tu et al., 2004).

**Heat-Stress Treatments and Measurement of Chlorophylls**

Thermotolerance assays of seeds and seedlings on plates were performed according to Charrng et al. (2006) with minor modifications. To make the heat treatment more uniform, heat shock was conducted by heating tape-sealed agar plate in water bath in the dark. Plates were then cooled down to room temperature by floating on tap water, and kept in the dark at room temperature for 2 h to avoid possible additional effects of light stress. Seedlings were allowed to recover for an additional 7 d in the original growth conditions prior to calculating the survival rate. Plants that were still green and producing new leaves were scored as survived. For the acquired thermotolerance test, 5- to 7-d-old seedlings were initially acclimated at 37.5°C for 1 h and returned to the original growth condition for 2 d, then challenged at the lethal temperature 44.5°C for 1 h. Total chlorophyll was determined by the method of Lichtenthaler (1987). Root length was measured using pictures of seedlings and the software ImageJ (National Institutes of Health image).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AL161561 (cpHsc70-1) and AB024032 (cpHsc70-2).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Genomic Southern analyses of the T-DNA in Δcphsc70 mutants.

**Supplemental Figure S2.** Δcphsc70-1 had a higher amount of cpHsc70-2 but a normal amount of chloroplast translocon proteins.

**Supplemental Figure S3.** Alignment of the amino acid sequences of plastid Hsp70s used for constructing the phylogenetic tree.

**Supplemental Table S1.** Progeny segregation of the heterozygous Δcphsc70-1 mutant.

**Supplemental Table S2.** Plastid Hsp70s used for the phylogenetic analysis.

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**LITERATURE CITED**


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