Previously, we characterized a mitochondrial co-chaperonin (Cpn10) and a chloroplast co-chaperonin (Cpn20) from Arabidopsis thaliana (Koumoto, Y., Tsugeki, R., Shimada, T., Mori, H., Kondo, M., Hara-Nishimura, I., and Nishimura, M. (1996) Plant J. 10, 1119–1125; Koumoto, Y., Shimada, T., Kondo, M., Takao, T., Shimonishi, Y., Hara-Nishimura, I., and Nishimura, M. (1999) Plant J. 17, 467–477). Here, we report a third co-chaperonin. The cDNA was 603 base pairs long, encoding a protein of 139 amino acids. From a sequence analysis, the protein was predicted to have one Cpn10 domain with an amino-terminal extension that might work as a chloroplast transit peptide. This novel Cpn10 was confirmed to be localized in chloroplasts, and we refer to it as chloroplast Cpn10 (chl-Cpn10). The phylogenetic tree that was generated with amino acid sequences of other co-chaperonins indicates that chl-Cpn10 is highly divergent from the others. In the GroEL-assisted protein folding assay, about 30% of the substrates were refolded with chl-Cpn10, indicating that chl-Cpn10 works as a co-chaperonin. A Northern blot analysis revealed that mRNA for chl-Cpn10 is accumulated in the leaves and stems, but not in the roots. In germinating cotyledons, the accumulation of chl-Cpn10 was similar to that of chloroplastic proteins and accelerated by light. It was proposed that two kinds of co-chaperonins, Cpn20 and chl-Cpn10, work independently in the chloroplast.

Chaperonins are proteins that play a vital role in protein folding in eukaryotic and prokaryotic cells. They are generally divided into two groups (1). Group I chaperonins are localized in the stroma of chloroplasts, the matrix of mitochondria, and euas, and group II chaperonins are found in the eukaryotic cytosol and archaeabacteria. One of the differences between the two groups is the requirement for co-chaperonins for their function. Group I chaperonins work together with co-chaperonins, whereas group II chaperonins work alone.

The chaperonin (GroEL) and co-chaperonin (GroES) of Escherichia coli have been characterized in detail. They are heat-shock proteins that are also required for viability under normal conditions (2). GroEL is composed of two heptameric rings, each consisting of seven 57-kDa subunits. Two stacked rings of the GroEL heptamer form a central cavity that captures incompletely folded proteins. The co-chaperonin GroES, a dome-shaped ring consisting of seven 10-kDa subunits, binds to GroEL. GroES has been shown to increase the cooperativity of the ATPase activity of GroEL (3). The crystal structures of the GroEL tetradecamer (4), the GroES heptamer (5), the GroEL-GroES complex (6), and the GroEL-peptide complex (7) have been characterized.

Whereas the structures and basic mechanism of reaction have been well defined, little is known about the roles of the chaperonins in vivo. From the analysis of a temperature-sensitive GroEL mutant strain of E. coli, it has been shown that about 30% of newly translated poly peptides fold via GroEL (8). Moreover, it has been shown that about 10–15% of cytoplasmic proteins of E. coli cells interact with GroEL (9). These results suggested that the majority of proteins fold without the help of chaperonins in vivo. However, it is a fact that the folding of some proteins is extremely dependent on chaperonins (10).

From import experiments of temperature-sensitive mutants of yeast mitochondrial chaperonin (Cpn60) and co-chaperonin (Cpn10), it was shown that some proteins form aggregates in the absence of either Cpn60 or Cpn10. In particular, the folding of a newly imported Cpn60 itself is extremely dependent on Cpn10. The chaperonin system in the chloroplast stroma of higher plants is unique. The stromal chaperonin oligomer consists of two isoforms, Cpn60α and Cpn60β (11), and stromal co-chaperonin (Cpn20) is comprised of two GroES-like tandem domains (12). Schlicher and Soll (13) showed that the thylakoid lumen contains a Cpn10 homologue that is recognized by a peptide-specific antiserum raised against a peptide of stromal Cpn20. The reported size of the luminal homologue on SDS-PAGE is about 10–12 kDa. On the other hand, in a recently performed proteomics study on the chloroplast of pea, Pelletier et al. (14) identified Hsp70, Cpn60α, and Cpn20 on the two-dimensional electrophoresis map with thylakoid luminal proteins. The explanation for the presence of stromal chaperonins in the luminal fraction was that they are bound to the stromal side of the thylakoid membrane. The authors supposed that the 10–12-kDa protein recognized by the antiserum against Cpn20 was a degradative product of Cpn20 and not a Cpn10 homologue. Thus, we performed studies to examine whether a Cpn10 homologue exists in the chloroplast or not. In this study, we report the existence of a novel Cpn10 homologue in the chloroplast. Sequence analysis showed that chloroplast Cpn10 was divergent from the other co-chaperonins, suggesting that it might have evolved to have a special function.
EXPERIMENTAL PROCEDURES

Expression and Purification of the His-tagged Cpn10 Homologue in E. coli—The cDNA fragment for the deduced mature region of chl-Cpn10, residues 50–139, was inserted into expression vector pQE30 (Qiagen, Chatsworth, CA). Six histidine residues (His-tagged) located at the amino-terminal of chl-Cpn10 were derived from this vector. E. coli was transformed with the construct, and the expression of proteins was induced with isopropyl-β-D-thiogalactopyranoside.

Chaperonin-assisted Protein Folding Assay—The chaperonin-assisted protein folding assay was performed as described by Schmidt et al. (15). Citrate synthase (CS) was denatured at a concentration of 15 mM in a solution containing 6 M guanidine hydrochloride, 100 mM Tris-Cl (pH 7.7), and 20 mM dithiothreitol at least 1 h at room temperature. Denatured CS was rapidly diluted to a concentration of 150 μM in a solution containing 50 mM Tris-Cl (pH 7.7), 10 mM MgCl₂, 10 mM KCl, and 225 nM GroEL tetradecamer at 0 °C. The temperature was adjusted to 35 °C, and 2 mM ATP and 300 nM co-chaperonin oligomer were added. After various time periods of incubation, aliquots were removed and assayed for CS activity. The assay for CS was essentially as described previously (16). GroEL and GroES were purchased from Takara (Tokyo, Japan). The content of co-chaperonin was determined by a protein assay (Bio-Rad Laboratories, Hercules, CA).

Immunogold Localization—Transgenic Arabidopsis cotyledons were fixed, dehydrated, and embedded in LR White resin (London Resin Co., Basingstoke, United Kingdom) as described previously (17). Immunogold procedures were essentially the same as those described by Kinnoshita et al. (18), except for the use of antiserum against the His-tagged chl-Cpn10 diluted 50-fold. 15 nM protein A-gold (Amersham...
Pharmacia Biotech) was diluted 100-fold prior to use. An antiserum was raised in a rabbit against the His-tagged chl-Cpn10 expressed in E. coli.

The sections were examined with a transmission electron microscope (1200EX; JEOL, Tokyo, Japan) at 80 kV.

Preparation of Chloroplasts—Intact chloroplasts were prepared from homogenates of mature tobacco leaves as described in a previous study (19). The chloroplast pellets (equivalent to 130 /H9262 g of chlorophyll) were lysed in 150 /H9262 lo f1 0m M Hepes-KOH (pH 6.0) and 10 mM MgCl2. After 5 min on ice, the membrane and the soluble fractions were separated by centrifugation at 4000 /H11003 g for 10 min.

Northern Blotting—Total RNA was purified by LiCl precipitation after the extraction of RNA with ISOGEN (Nippongene, Tokyo, Japan). 20 μg of total RNA was electrophoresed on a 1% agarose/formaldehyde gel and transferred to a nylon membrane under a vacuum with 1 N ammonium acetate. Hybridization was performed as described by Church and Gilbert (20). After hybridization, the membrane was sequentially washed twice in 2× SSC and 0.1% (w/v) SDS at room temperature for 30 min and twice in 0.1× SSC and 0.1% (w/v) SDS at 60 °C for 30 min. The washed membrane was exposed to an imaging plate, and the radioactivity of signals was detected with an analyzer system (Fuji Film, Tokyo, Japan).

Electrophoresis and Immunoblotting—SDS-PAGE (15% acrylamide) was performed by following the method of Laemmli (21). The separated proteins on the gels were electrophoretically blotted onto polyvinylidene difluoride membranes (Nihon Millipore Ltd., Tokyo, Japan). Immunochemical detection was performed by the ECL detection system (Amer- sham Pharmacia Biotech). After detection, the blotted membrane was rinsed once in water and stained in 0.25% Coomassie Brilliant Blue.
solution for 1 min. The stained membrane was washed once in 50% methanol until the protein bands appeared and were dried.

Plant Transformation—To generate overproducing transgenic plants, the ρ-glucuronidase gene of binary vector pBI121Hm was replaced by a SalI-XbaI cDNA fragment derived from an Arabidopsis EST clone (GenBank accession number T44192). pBI121Hm is a derivative of pBI121 and contains two drug resistance genes, the neomycin phosphotransferase II gene and the hygromycin phosphotransferase gene. The resulting construct was introduced into Agrobacterium tumefaciens (strain EHA101) and used to transform Arabidopsis thaliana ecotype Columbia plants by the in planta method (22).

Quantitative Reverse Transcription-PCR—First-strand cDNA was synthesized using a Superscript™preamplification system for the first-strand cDNA synthesis kit (Life Technologies, Inc.) from total RNA. The oligo(dT) primer was used for reverse transcription. Quantitative PCR assays were performed by an ABI Prism 7700 sequence detection system (PE Biosystems Japan, Tokyo, Japan). This system is capable of detecting PCR products as they accumulate during PCR. For detection, it is necessary to prepare the fluorescent DNA probe that is specific to the PCR target sequence flanked by PCR primers. To select the PCR primers and the fluorescent probes, we used the Prime Express™ program (PE Biosystems Japan). All PCR procedures were carried out with 40 cycles of denaturation at 95 °C for 15 s with annealing and extension at 60 °C for 1 min according to the supplier’s recommendations.

RESULTS AND DISCUSSION

Sequence Analysis—In A. thaliana, cDNAs for mitochondrial Cpn10 and chloroplast Cpn20 have already been cloned (19, 23, 24). Using the amino acid sequences of full-length mitochondrial Cpn10 and the mature region of chloroplast Cpn20, we searched the EST data base for another homologous cDNA. As a result, some EST clones were found to have the amino-terminal conserved motif of Cpn10, PXXD/K(R) (Fig. 1a) but showed low similarity to the two clones mentioned above as a whole. The sequence of the longest EST clone (GenBank accession number T44192) was determined. The cDNA was 603 bp long, encoding a protein of 139 amino acids (Fig. 1a). The protein was predicted to be a homologue of Cpn10 containing an amino-terminal extension from sequence analysis. This extension was predicted to be a chloroplast transit peptide using the PSORT program. Therefore, the Cpn10 homologue is referred to as chl-Cpn10. Moreover, we found tomato and soybean EST clones that exhibit high sequence homology with chl-Cpn10. Moreover, we found tomato and soybean EST clones that exhibit high sequence homology with chl-Cpn10. Furthermore, these EST clones showed 72% identity.

The phylogenetic tree was generated with amino acid sequences of the two Arabidopsis clones showed 72% identity. The phylogenetic tree was generated with amino acid sequences of other co-chaperonins including bacterial GroES (Fig. 2). The amino-terminal conserved motif of GroES starts from the fifth proline. Because it is expected that sequences with similar length align more accurately, the amino acid sequence from Thr-50 to Glu-139 of chl-Cpn10, in which the conserved motif also starts from the fifth proline (as in GroES), was used for alignment. As shown in Fig. 2, chl-Cpn10 is highly divergent from other co-chaperonins. For a more detailed analysis, chl-Cpn10 was compared with GroES (Fig. 1c). The crystal
Activity of chl-Cpn10 as a Co-chaperonin—The chaperonin-assisted protein folding assay was performed to study whether chl-Cpn10 works as a co-chaperonin. The polypeptide containing residues 50–139 of chl-Cpn10 was expressed in E. coli. The segment containing the 6 histidine residues (His tag) was fused to the amino terminus. Because the expressed His-tagged chl-Cpn10 was insoluble, it was immobilized on a Ni-coupling column after being solubilized with 8 M urea and renatured by a gradual decrease of urea from 6 M to 1 M and then eluted by imidazole. The purified His-tagged chl-Cpn10, as judged from the Coomassie Brilliant Blue staining pattern (Fig. 3b), was used for assays. CS was used as a substrate. At 35 °C, denatured CS is not permissive for spontaneous folding, and the recovery of CS activity depends on chaperonins (15). GroEL was used as a chaperonin. As shown in Fig. 3b, about 30% of the CS activity was recovered with His-tagged chl-Cpn10. By doubling the amounts of chl-Cpn10, the recovery of CS activity was increased slightly. These results confirmed that the His-tagged Cpn10 homologue could interact with GroEL and work as a co-chaperonin.

In this assay, the authentic co-chaperonin, GroES, recovered about 70% activity. The previous study showed that the activity of mitochondrial Cpn10 containing the His tag at its amino terminus was the same as that of GroES (19). This indicates that the amino-terminal His tag has no effect on His-tagged mitochondrial Cpn10 in this assay, and the His tag did not seem to prevent co-chaperonins from binding to GroEL. It was shown that about 55% of the CS activity was recovered with His-tagged Cpn20 (Fig. 3). The activity of Cpn20 was higher than that of chl-Cpn10, although chl-Cpn10 is the same single-type co-chaperonin as GroES. It was suggested that the low activity of chl-Cpn10 was caused by sequence specificity. Because the length of the mobile loop of chl-Cpn10 is expected to be longer than that of GroES, it seems that chl-Cpn10 is not able to bind GroEL efficiently.

Localization of chl-Cpn10—To confirm the localization of chl-Cpn10, immunogold labeling was performed. We have previously revealed by immunogold labeling that mitochondrial Cpn10, which was expressed in a transgenic plant, is exclusively localized in mitochondria (23). Because endogenous chl-Cpn10 was hardly detectable by immunogold labeling, the transgenic Arabidopsis harboring chl-Cpn10 cDNA was prepared. chl-Cpn10 was expressed under regulation of the 35S promoter from cauliflower mosaic virus. An immunoblot analysis of wild-type and transgenic Arabidopsis using rosette leaves is shown in Fig. 4a. Because the immunoreactive bands in both lanes were almost the same size, it is expected that the overexpressed chl-Cpn10 was imported into chloroplasts and processed correctly.

Seedlings of transgenic Arabidopsis, which were grown in the dark for 4 days, were transferred and kept in continuous light for 1 day. Cotyledons became green within 1 day of illumination, and their chloroplasts had stacked thylakoid membranes. The gold particles for chl-Cpn10 were restricted exclusively to a chloroplast (Fig. 4b), and most of them were in contact with thylakoid membranes (Fig. 4c). Fractionation analysis was performed to examine whether chl-Cpn10 was associated with thylakoid membrane. Because it was difficult to isolate chloroplasts from Arabidopsis, intact chloroplasts were isolated from mature tobacco leaves. After disruption by osmotic shock, the insoluble precipitates containing thylakoid membrane and the soluble fraction were separated. Immunoblot analysis of these two fractions is shown in Fig. 5. The immunoreactive band for chl-Cpn10 was detected in the soluble fraction. Cpn20, which was used as a stromal control, was concentrated in the soluble fraction. These results indicated...
that chl-Cpn10 was not associated with thylakoid membrane.

**Analysis of Expression Patterns**—The expression pattern of the chl-Cpn10 gene was also examined. Fig. 6c shows the Northern blotting of RNA extracted from the roots, stems, and leaves of mature Arabidopsis plants. Because of the weak chl-Cpn10 signal, 20 µg of total RNA was used. The amount of ribosomal RNA in each lane was almost the same. mRNA for chl-Cpn10 was present in the leaves and stems, but the signal was not detected in the roots. Cpn20 was observed in these three organs (19). These results suggested that chl-Cpn10 and Cpn20 work independently.

Next, the effect of heat treatment on the accumulation of chl-Cpn10 mRNA was examined (Fig. 6b). For detailed study, we performed quantitative reverse transcription-PCR with a fluorescent probe, which is specific to the target PCR product. First, the total RNA was prepared from Arabidopsis plants incubated at 35 °C for various lengths of time. Five µg of RNA was used for reverse transcription with an oligo(dT) primer. Because almost the same results were observed with duplicates, it seems to be unnecessary to take the efficiency of reverse transcription into consideration. One gene, APETALA 2 (AP2; Ref. 27) was used as a control because the amount of AP2 mRNA was constant during heat treatment. Fig. 6c shows the amount of chl-Cpn10 mRNA that was normalized by the amount of AP2 mRNA. Heat treatment did not change the amount of chl-Cpn10 mRNA drastically. This result was distinct from those of both mitochondrial Cpn10 and chloroplast Cpn20, whose mRNAs showed nearly 10-fold and 5-fold increases by heat treatment, respectively. This also indicated that chl-Cpn10 belongs to a divergent class of co-chaperonin.

**Chaperones in Chloroplasts**—To investigate the differences between chl-Cpn10 and Cpn20, the accumulation pattern of chl-Cpn10 protein during germination and greening was examined. Arabidopsis seeds were germinated in the dark. Cotyledons were yellow during the incubation in the dark and became fully green within 1 day of exposure to light. In cells exposed to light, proteins involved in photosynthetic electron transfer, such as light harvesting complex II, were rapidly accumulated (Fig. 7, bottom). One of the major stromal proteins involved in photosynthetic carbon metabolism, RuBisCO, was accumulated gradually in the dark, especially after a 5-day incubation. A comparison of the amounts of RuBisCO in lane 5 and in lane 4D1L indicated that the accumulation was also accelerated by light. Accumulation of chl-Cpn10 was observed after a 5-day incubation in the dark and accelerated by light, similar to that of RuBisCO (Fig. 7, top panel). The amount of chl-Cpn10 in cotyledon grown for 7 days in the dark (7D cotyledon) was comparable with that in a light-exposed cotyledon. Transgenic Arabidopsis that overexpresses chl-Cpn10 was also germinated and grown for 7 days in the dark. Immunogold labeling of transgenic Arabidopsis showed that chl-Cpn10 was localized in the etioplasts of a 7D cotyledon (Fig. 4d).

In contrast to chl-Cpn10, accumulation of Cpn20 was observed at an early stage of germination. This also suggested that chl-Cpn10 and Cpn20 work independently. Cpn60α and Cpn60β had been shown to bind to immobilized Cpn20 (28). Because these Cpn60s were originally referred to as RuBisCO-binding proteins, it is known that one of their substrates is RuBisCO. Therefore, it is thought that Cpn20 facilitates the folding of RuBisCO with Cpn60α and Cpn60β. It might be reasonable that Cpn20 protein seemed to accumulate slightly faster than RuBisCO. By analogy, chl-Cpn10 might mediate the folding of the protein that accumulates later.

From a search of the Arabidopsis genomic sequence, we found two Cpn60α genes and four Cpn60β genes. One of the two Cpn60α genes and three of the four Cpn60β genes may
actually be expressed, judging from the presence of EST clones. Because chl-Cpn10 and Cpn20 are thought to work independently, there is a possibility that they regulate a specific partner, Cpn60. As reported previously (29), Cpn60a and Cpn60β are expressed in E. coli and have been characterized. This report indicated that Cpn60β could form oligomer by itself, although Cpn60α could not. Moreover, it also indicated that a homo-oligomer of Cpn60β works only with mitochondrial Cpn10, in contrast to a hetero-oligomer that is compatible with various co-chaperonins. These results suggested the possibility that one Cpn60β homologue could form its homo-oligomer and function with its specific co-chaperonin. It is possible that a chaperonin system containing chl-Cpn10 and a Cpn60β homologue exists in chloroplasts, in addition to one containing Cpn20, Cpn60α, and Cpn60β.

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