OsCpn60 α 1*, Encoding the Plastid Chaperonin 60 α Subunit, Is Essential for Folding of *rbcl

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Chaperonins are involved in protein-folding. The rice genome encodes six plastid chaperonin subunits (Cpn60) - three α and three β . Our study showed that they were differentially expressed during normal plant development. Moreover, five were induced by heat stress (42°C) but not by cold (10°C). The *oscpn60 α 1* mutant had a pale-green phenotype at the seedling stage and development ceased after the fourth leaf appeared. Transiently expressed *OsCpn60 α 1:GFP* fusion protein was localized to the chloroplast stroma. Immuno-blot analysis indicated that the level of Rubisco large subunit (*rbcl*) was severely reduced in the mutant while levels were unchanged for some imported proteins, e.g., stromal heat shock protein 70 (Hsp70) and chlorophyll *a/b* binding protein 1 (Lhcb1). This demonstrated that *OsCpn60 α 1* is required for the folding of *rbcl* and that failure of that process is seedling-lethal.

INTRODUCTION

Chaperonins are oligomeric protein complexes that form a double-ring structure. They assist in the folding of newly synthesized or denatured polypeptides in their central cavity through the hydrolysis of ATP (Bukau and Horwich, 1998; Ditzel et al., 1998; Sigler et al., 1998). Two types of chaperonins have been identified. Type I chaperonins are found in bacteria (called GroEL), plastids (Cpn60), and mitochondria (Hsp60), while Type II chaperonins are present in archaea and the eukaryotic cytosol (CCT/TriC). Although both types have a markedly conserved crystal structure (Braig et al., 1994; Ditzel et al., 1998), they also are significantly different. Type I chaperonin is composed of seven subunits per ring while Type II chaperonin has eight or nine subunits. Those in Type I utilize co-chaperonin GroES/cpn10 for substrate encapsulation in the protein-folding process, whereas those in Type II have a built-in “cap” that performs the same function (Hartl, 1996; Horwich et al., 2007; Saibil, 2000). Both types have been identified in plants (Hill and Hemmingsen, 2001).

Type I chaperonins are evolutionarily conserved proteins that are encoded by multiple genes. Many bacteria have at least two *GroEL* genes. For example, *Bradyrhizobium japonicum* has seven *GroEL* homologues and *Rhizobium leguminosarum*

has three (Gould et al., 2007; Kaneko et al., 2002). Likewise, multiple mitochondria and plastid chaperonin homologues are encoded from the nuclear genome in plants (Hill and Hemmingsen, 2001; Peng et al., 2011). In contrast to the *Escherichia coli* GroEL and mitochondrial Hsp60, which have 14 identical subunits, the plastid Cpn60 has two distinct subunit types, α and β (Hemmingsen and Ellis, 1986; Martel et al., 1990; Nishio et al., 1999). The sequence identity between them is approximately 50%; a similar percentage is found between Cpn60 and GroEL (Hemmingsen et al., 1988; Martel et al., 1990). Although *Cpn60* evolved from *GroEL-1* in the cyanobacterial ancestor of plastids (Hill and Hemmingsen, 2001; Suzuki et al., 2009), the number of subunits in genomes differs among land plant species. For example, *Physcomitrella patens* has three α subunits and four β subunits (Suzuki et al., 2009), *Brassica napus* has three α but only one β (Cloney et al., 1994), while *Arabidopsis* has two α and four β (Hill and Hemmingsen, 2001).

Bacterial *GroEL* genes are differentially expressed and regulated by stresses such as high temperature (Glatz et al., 1997; Rodríguez-Quiriones et al., 2005). In *Arabidopsis*, *AtCpn60 α 1*, *AtCpn60 β 1*, and *AtCpn60 β 2* are strongly expressed while its remaining Cpn60s are expressed only weakly (Weiss et al., 2009). Other than that, expression patterns for the plastid Cpn60 gene family have not been well studied in most plants.

The structure and mechanisms of chaperonins have been intensively investigated in *E. coli* by using GroEL (Bukau and Horwich, 1998). At least one *GroEL* gene has been demonstrated to be essential for viability (Fayet et al., 1989). Approximately 250 of the ~2,400 cytosolic proteins interact with GroEL (Kerner et al., 2005). In plants, the chloroplast Cpn60 was initially identified as an abundant oligomeric protein that transiently binds nascent Rubisco large subunits (*rbcl*s) prior to their assembly into the Rubisco holoenzyme (Barraclough and Ellis, 1980). Transfer of newly synthesized *rbcl* subunits to the holoenzyme is inhibited by the addition of anti-chaperonin antibodies, support that Cpn60 plays a critical role in Rubisco assembly (Cannon et al., 1986; Milos and Roy, 1984). Functional analysis of plastid *Cpn60* genes has been conducted in *Arabidopsis*, where a T-DNA insertion mutant in *AtCpn60 α 1* shows an embryonic-lethal phenotype (Apuya et al., 2001). Deletion of *AtCpn60 β 1* leads to seedling death under short-day conditions

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(Ishikawa et al., 2003). A mutant lacking *AtCpn60 β 4* is defective in chloroplast NDH activity, indicating that *AtCpn60 β 4* is required for the folding of NdhH, a subunit of the chloroplast NADH dehydrogenase-like complex (Peng et al., 2011). However, involvement of Cpn60 in the folding of rbcL is unclear in these mutants.

Here, we describe how *OsCpn60 α 1* is essential for rice seedling development because it functions in such rbcL-folding.

MATERIALS AND METHODS

Plant materials, growing conditions, and genotyping

T-DNA-tagging lines were generated in *Oryza sativa* japonica cv. Dongjin and Hwayoung (An et al., 2003; Jeon et al., 2000; Jeong et al., 2006). Their T-DNA flanking sequences were identified via inverse PCR (Kim et al., 2011). In Line 3A-06578, T-DNA was inserted in *OsCpn60 α 1* (*Os12g17910*). As previously described by Kim et al. (2011), seeds were sterilized in a 0.025% (v/v) prochloraz solution, sown in soil, and placed in an incubator set at a constant 27°C with light from cool-white fluorescent lamps (30 μ mol photons m⁻²s⁻¹). Genotyping was performed with PCR, using two gene-specific primers (GT-F: 5'-AGCGCCTTGGTGCAGACAT-3' and GT-R: 5'-CTAGGACA GAGATAGTCTGAG-3') and two gene-specific/T-DNA primers (GT-R and LB1: 5'-TTGGGGATCCTCTAGAGTCGAG-3').

Quantitative real-time RT-PCR

Seedlings grown for 14 days at 27°C were then transferred to either heat (42°C) or cold (10°C) conditions. After these stress treatments, total RNAs were prepared from their third leaves, using RNeasy Plus (Takara, Japan). cDNAs were synthesized with M-MLV reverse transcriptase (Promega, USA) and oligo (dT)₁₅ as described previously (Lee et al., 2012). Using these cDNAs as template, we conducted quantitative real-time RT-PCR in a Rotor-Gene 6000 (Qiagen, Germany). The gene-specific primers were 5'-ACTCTCCGAAACTGATTCGAT-3' (a1-F) and 5'-TCAAGTTCTCATACTTGTCGTT-3' (a1-R) for *OsCpn60 α 1*, 5'-CTTTCTCAGACAGACTCAGC-3' and 5'-AAGTTCT CGTGTCTGTGTCAGC-3' for *OsCpn60 α 2*, 5'-GGATCTTG TAG ATGACTCTGA-3' and 5'-CCTTGACATCATGGACCTTTC-3' for *OsCpn60 α 3*, 5'-TGCTGCAACTGGACAGTAC-3' and 5'-TGAC AGCCGTTCCAGTTACA-3' for *OsCpn60 β 1*, 5'-CTGAAATCG TAAGGAAGTCCT-3' and 5'-CGTCTCAGATGCAACTGTGA-3' for *OsCpn60 β 2*, and 5'-AACGGCAGCTTCGTCATAGA-3' and 5'-TGAGTATCAGTGTGGGGAA-3' for *OsCpn60 β 3*. Cycling conditions included denaturing at 95°C for 10 s, annealing at 55°C for 15 s, and extension at 72°C for 25 s. Rice *UBQ5* was used to normalize the cDNA quantity (Jain et al., 2006), and transcript levels relative to that gene were calculated by the $\Delta\Delta Ct$ method. All experiments were conducted with three samples at each time point.

Chlorophyll quantification

The third leaves from seedlings were homogenized with liquid nitrogen and suspended in 95% ethanol. Residual plant debris was precipitated by centrifugation (13,000 $\times g$ for 10 min). The supernatant was used for measuring absorbance at 664.1 and 648.6 nm with a spectrophotometer (Shimadzu, Japan). Chlorophyll contents (*a* and *b*) were calculated by the equation of Lichtenthaler and Buschmann (2001).

Transmission electron microscopy

Leaf tissues were fixed in a solution containing 0.1 M sodium phosphate (pH 7.2), 3% glutaraldehyde, and 0.2% Brij35, then

post-fixed for 2 h in a solution containing 0.1 M sodium phosphate, 2% osmium tetroxide, and 0.2% Brij35. The samples were stained with 0.5% (w/v) uranyl acetate. Following dehydration, the specimens were embedded in Spurr's resin. After ultra-thin sectioning, the grids containing specimens were stained with 4% uranyl acetate and examined with a JEOL 1200 transmission electron microscope (JEOL Ltd, Japan).

Generation of *OsCpn60 α 1* overexpression transgenic plants

The full-length cDNA clone (AK120503) of *OsCpn60 α 1* was obtained from the Rice Genome Resource Center (RGR; <http://www.rgrc.dna.affrc.go.jp/index.html.en>). The plasmid was digested with *Sfi*I and the fragment encoding *OsCpn60 α 1* was ligated into pGA3593, a sub-cloning vector for rice full-length cDNA (Kim et al., 2009). The fragment isolated with *Bsr*GI/*Spe*I was inserted between the maize *Ubiquitin1* promoter and the *NOS* terminator in binary vector pGA3426 (Kim et al., 2009). Embryonic calli induced from 'Dongjin' wild-type (WT) seeds were co-cultured with *Agrobacterium tumefaciens* strain LBA4404 harboring the binary plasmid. Transgenic plants were generated as described previously (Lee et al., 1999).

Complementation of the *oscpn60 α 1* mutant

The *OsCpn60 α 1* overexpression (OX) construct was introduced into the *oscpn60 α 1* mutant by crossing the *OsCpn60 α 1* OX plant with *OsCpn60 α 1/oscpn60 α 1* heterozygote plants. The F1 offspring having both the OX construct and the T-DNA-inserted *OsCpn60 α 1* allele were selected and selfed to produce F2 seedlings that carried the OX construct in an *oscpn60 α 1* homozygous background.

Sub-cellular localization

The protein coding region of *OsCpn60 α 1* without the stop codon was amplified by PCR using a pair of primers (forward: 5'-GTTAACCTTATCCTCAGCTCTCAG-3' and reverse: 5'-AC TAGTGACGGTAAGGGTTCCTCT-3'). Restriction enzyme sites for cloning are underlined. The fragment was inserted into multiple cloning sites located between the CaMV 35S promoter and *green fluorescent protein (GFP)* coding region in the modified pGA3452 vector (Kim et al., 2009). We constructed the *p35S-AtTic21:red fluorescent protein (RFP)* vector based on the method of Teng et al. (2006). Particle bombardment was performed with onion epidermis cells, using a biolistic particle delivery system (PDS-1000; Bio-Rad, USA). To improve DNA adsorption into the gold particles, we used an alkaline buffer containing 50 mM Tris-HCl (pH 8.8) and 1 mM EDTA (Yoshimitsu et al., 2009). The other parameters were as described previously (Kim et al., 2009). Preparation of mesophyll protoplasts from rice seedling leaves and this transformation procedure were previously reported (Han et al., 2006). After the fusion proteins were transiently expressed, fluorescence signals were observed under a confocal laser scanning microscope (LSM 510 META; Zeiss, Germany).

Protein blot analysis

The third leaves were harvested from 7-d-old seedlings (*oscpn60 α 1* and WT) grown on half-strength MS media at a constant 27°C and under cool-white fluorescent light. Samples were ground under liquid nitrogen and suspended in a protein extraction buffer containing 250 mM sucrose, 25 mM KCl, 50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 0.5% (v/v) β -mercaptoethanol, 0.5% (v/v) Triton X-100, and a protease inhibitor cocktail (Roche, Switzerland), as described by Armstrong et al. (1995). After any

Fig. 1. Temporal-spatial expression patterns of plastid *Cpn60* genes using quantitative real-time RT-PCR. Roots and leaves were sampled from 14-d-old seedlings grown in soil at constant 27°C/ light. Flag leaves and spikelets at heading stage, young panicles (2-5 cm long), and developing seeds at 10 d after pollination were sampled from paddy field. *UBQ5* was internal control. Error bars indicate standard deviations; n = 3.

Fig. 2. Expression of plastid *Cpn60* genes in response to heat stress. Seedlings (14-d-old) first grown in soil at constant 27°C were subjected to 42°C. Their third leaves were sampled during treatment (15, 30, 60, and 120 min) and in recovery (2 h after end of exposure period, when returned to 27°C). *UBQ5* was internal control. Error bars indicate standard deviations; n = 3.

debris was precipitated by centrifugation (13,000 $\times$ g for 10 min at 3°C), the supernatant was analyzed. Protein concentrations were determined by Bradford assays. For blotting analysis, 15 μ g of total proteins was separated on 10% SDS-polyacrylamide gels. The blots were incubated with the primary anti-chlorophyll a/b binding protein 1 (Lhcb1), anti-stromal Heat shock protein 70 (Hsp70), or anti-rbcL antibodies (Agrisera, Sweden), then processed with an ECL kit (GE Healthcare, USA). To normalize quantities, we used the anti-Histone3 antibody (Santa Cruz Biotechnology, USA). Total proteins were visualized by Coomassie Brilliant Blue (CBB)-staining after SDS-PAGE. Images of the immunoblots were obtained with an LAS4000 image capture system (GE Healthcare).

RESULTS

Expression analysis of the rice *Cpn60* family

Rice has three α subunit genes and three β subunit genes of *Cpn60* (Peng et al., 2011). Here, we named them *OsCpn60 α 1* (*Os12g17910*), *OsCpn60 α 2* (*Os03g64210*), *OsCpn60 α 3* (*Os09g38980*), *OsCpn60 β 1* (*Os06g02380*), *OsCpn60 β 2* (*Os02g01280*), and *OsCpn60 β 3* (*ChrSy.fgenes.h.gene.28*). Sequencing of the cDNAs revealed that the public annotation of *OsCpn60 β 3* is incorrect because it misses 93 nucleotides (Supplementary Fig. S1). The primary structures share 60 to 86% identity among α subunits and 61 to 84% identity among β subunits (Supplementary Fig. S2A).

Expression patterns for these genes were studied in seedling roots and leaves, flag leaves, young panicles, spikelets, and developing seeds. Expression was strongest for *OsCpn60 β 1*,

Fig. 3. Phenotypes of *oscpn60α1* mutant. (A) Schematic diagram of *OsCpn60α1* genomic structure. Black box, exon; gray box, UTR region. T-DNA insertion, genotyping primers (GT-F, GT-R, and LB1), and RT-PCR primers (a1-F and a1-R) are depicted. (B) RT-PCR analysis of *OsCpn60α1* expression in segregant WT and *oscpn60α1* mutants. *OsAct1* was internal control. (C) Phenotypes of *oscpn60α1* mutant; 11-d-old seedlings were photographed. (D) Enlarged image of 3rd leaves in C. (E) Quantification of chlorophyll after being extracted from 3rd leaves of 10-d-old seedlings grown at constant 27°C. Error bars indicate standard deviations; n = 5. (F) TEM image of chloroplasts. Scale bar = 0.5 μm.

Fig. 4. Complementation of *oscpn60α1* mutant by introducing *OsCpn60α1* overexpression construct. (A) Relative expression level of overexpressing (OX) lines. For genetic complementation, OX-#3 line was used as female donor for crossing with *OsCpn60α1/oscpn60α1* line. (B) PCR analysis of complementation. Among 4 F1 progeny, plants 1, 3, and 4 carried OX construct in *OsCpn60α1/oscpn60α1* heterozygous background. Genotyping primers are described in Fig. 1A. (C) Phenotypes of F2 plants of *oscpn60α1* (left) and *oscpn60α1* carrying *OsCpn60α1*-OX construct.

moderate for *OsCpn60α1*, *OsCpn60α2*, *OsCpn60β2*, and *OsCpn60β3*, and low for *OsCpn60α3* (Fig. 1). Whereas *OsCpn60α2* and *OsCpn60β2* were dominantly expressed in developing seeds, transcript levels for the other genes were higher in the flag leaves. Our results are consistent with public microarray data from the Rice Oligonucleotide Array database (<http://www.ricearray.org/>) (Supplementary Fig. S3).

To examine whether these genes are responsive to heat or cold stress, we subjected seedlings first grown at 27°C to either 42°C or 10°C. Within 1 h of heat exposure, mRNA levels were significantly increased for $\alpha1$ and $\alpha2$ genes but not for the $\alpha3$

gene (Fig. 2). By comparison, transcription levels were elevated by approximately two-fold for all three β genes after such treatment. This indicated that the $\alpha1$ and $\alpha2$ proteins have dominant functions under heat stress. In contrast, cold conditions did not affect the expression of any of these six genes (Supplementary Fig. S4).

The *oscpn60α1* mutant showed pale-green and seedling-lethal phenotypes

We obtained a T-DNA insertion mutant in the eighth exon of *OsCpn60α1* (Fig. 3A). Its transcripts were barely detectable in

Fig. 5. Subcellular localizations of OsCpn60 α 1. (A) *p35S-OsCpn60 α 1::GFP* construct was co-transformed with plasmid marker *p35S-rbcS::RFP* into onion epidermis cells by particle bombardment. Scale bar = 100 μ m. (B) OsCpn60 α 1:GFP fusion protein was expressed alone in rice mesophyll protoplasts. GFP signal was merged with chlorophyll auto-fluorescence (Chl auto). (C) *p35S-OsCpn60 α 1::GFP* construct was co-electroporated with *p35S-AtTic21::RFP* plasmid (a chloroplast inner-membrane marker) into rice mesophyll protoplasts. BF, bright field; Scale bar = 10 μ m.

the T-DNA homozygous line (Fig. 3B), suggesting that *oscpn60 α 1* is a null allele. Homozygous mutant seedlings were pale green (Figs. 3C and 3D). In progeny of the *OsCpn60 α 1/oscpn60 α 1* heterozygote, that phenotype was segregated in an approximately 3:1 ratio (WT:*oscpn60 α 1*); all pale-green seedlings were *oscpn60 α 1* homozygous plants. This indicated that the mutant phenotype was recessive and was caused by a defect in a single genomic locus. When leaf chlorophyll contents were measured, we found that both chlorophyll *a* and *b* were severely decreased in the mutant, to approximately 37% of that recorded from the WT siblings (Fig. 3E). That is, after the fourth leaf emerged from an *oscpn60 α 1* plant, no further leaves were produced, resulting in a lethal character. TEM analyses showed that chloroplast development was impaired in the mutant (Fig. 3F).

To test their heat tolerance, we generated transgenic plants that over-express *OsCpn60 α 1*. Its protein coding region was laid between the maize *Ubiquitin1* promoter and the *NOS* transcription terminator. From 20 transgenics, we selected six that strongly over-expressed that gene (Fig. 4A). After they were exposed to a constant 40°C, we found that they developed chlorotic leaves to the same extent as similarly treated WT plants, thus indicating that *OsCpn60 α 1* overexpression did not confer heat tolerance.

To verify that the mutant phenotypes were due to a lack of *OsCpn60 α 1* functioning, we introduced the *OsCpn60 α 1* OX construct into the mutant. The *oscpn60 α 1* mutants carrying the *OsCpn60 α 1* transgene developed normal green leaves (Fig. 4C), thereby demonstrating that the mutant phenotype was due to an absence of functional *OsCpn60 α 1*.

Sub-cellular localization of OsCpn60 α 1

We performed transient expression analysis to examine the sub-cellular localization of OsCpn60 α 1. Full-length cDNA was fused to the *GFP* coding region in-frame and placed under the CaMV 35S promoter. The construct was co-introduced with the plastid marker plasmid *p35S-rubisco small subunit (rbcS)::RFP* into onion epidermal cells by particle bombardment. From this, we observed that the GFP signal was merged with RFP (Fig. 5A), indicating that OsCpn60 α 1 was localized to the plastids. To confirm this, we used protoplasts isolated from rice seedlings and noted that the OsCpn60 α 1:GFP signal was overlaid with chlorophyll autofluorescence (Fig. 5B).

Cpn60 is a major protein immuno-precipitated by Tic110, a component of the inner-membrane import apparatus (Kessler and Blobel, 1996). There, it is thought to assist in the re-folding of proteins imported into the chloroplasts (Li and Chiu, 2010). To test whether OsCpn60 α 1 is co-localized with the Tic complex, we used rice mesophyll protoplasts to co-electroporate the Cpn60 construct with the *p35S-AtTic21::RFP* vector, which encodes a subunit of the Tic complex (Teng et al., 2006). This experiment showed that the OsCpn60 α 1:GFP fusion protein was co-localized with AtTic21::RFP, which was located mainly in the envelope. However, the Cpn60 protein was also found in the stroma (Fig. 5C). These results indicated that OsCpn60 α 1 occurs predominantly in the stromal region but also has a minor association with the chloroplast inner membrane.

The *rbcl* protein level was dramatically reduced in the *oscpn60 α 1* mutant

We analyzed chloroplast proteins using immuno blot analysis. Total proteins were extracted from seedling leaves. Visualization by CBB-staining revealed two major bands that we de-

Fig. 6. Immuno-blot analysis of chloroplast proteins. Equal amounts of total protein extracted from 3rd leaves of segregant WT and *oscpn60α1* seedlings were separated by SDS-PAGE, visualized with CBB-staining (A), and immuno-detected by antibodies against chloroplast proteins Lhcb1, chloroplast Hsp70, and rbcL (B). Histone3 was loading control.

duced as rbcL encoded from the plastid genome and a Rubisco small subunit (rbcS) encoded from the nuclear genome. Both were severely diminished in the mutant (Fig. 6A). To confirm that one of those lost bands was rbcL, we conducted Western blotting with the anti-rbcL antibody and found that the rbcL level was dramatically reduced in the mutant (Fig. 6B).

Nuclear-encoded chloroplast proteins were also tested. Neither the chloroplast-localized Hsp70 (cpHsp70) nor the light-harvesting chlorophyll a/b binding protein 1 (Lhcb1), a subunit of the major light-harvesting antenna complex II (LHCII) in the thylakoid membrane, was reduced in the mutant (Fig. 6B). This suggested that folding of those proteins was not dependent on OsCpn60 α 1.

DISCUSSION

Rice plastid Cpn60 family

Subunit types for plant plastid Cpn60s are assumed to have arisen from an ancient gene duplication event (Archibald et al., 2000; Wastl et al., 1999). The numbers of subunits vary among land plant species (Cloney et al., 1994; Suzuki et al., 2009). Rice contains three α and three β subunits. Our study showed that expression of the rice Cpn60 genes is developmentally regulated. For example, *OsCpn60α1* was preferentially functional in vegetative organs, especially the flag leaves, whereas *OsCpn60α2* was expressed primarily in developing seeds. Among the β subunit genes, *OsCpn60β1* expression was much

higher than that of the other two, suggesting that it functions dominantly as a molecular chaperone.

In contrast to bacterial GroEL and mitochondrial Hsp60s that are composed of identical subunits, the plastid Cpn60 chaperonins from several plant species have roughly equal amounts of two distinct subunits, α and β (Bonk et al., 1996; Musgrove et al., 1987; Nishio et al., 1999; Viitanen et al., 1995). Through expression analysis we observed similar trends between the α and β subunit genes, with patterns for *OsCpn60α1* and *OsCpn60α3* resembling those for *OsCpn60β1* and *OsCpn60β3*. Expression was high in flag leaves and low in roots and young panicles. Similar patterns were found between *OsCpn60α2* and *OsCpn60β2*, which were preferentially expressed in the seeds. These results implied that the co-expressed α and β subunits form a functional hetero-oligomer. For example, $\alpha2$ and $\beta2$ act as a chaperone that preferentially functions in seeds.

In a reconstitution assay of Cpn60 oligomers that used pea α and β monomers expressed in *E. coli*, Dickson et al. (2000) showed that the β subunit monomers or α/β mixed monomers, but not the α subunit monomers, form functional tetradecamers in the presence of chaperonin 10 (Cpn10). Moreover, *Arabidopsis* β homo-oligomers function *in vitro* with authentic chloroplast Cpn10s (Vitlin et al., 2011). Among our six rice Cpn60 genes, *OsCpn60β1* expression was the strongest, presenting the possibility that it creates a functional $\beta1$ homo-oligomer *in planta*.

An *Arabidopsis* mutant lacking *AtCpn60β1* is more sensitive to heat stress when compared with WT plants (Ishikawa et al., 2003). Transcription of *OsCpn60α1* and *OsCpn60α2* was strongly induced by exposure to a high temperature while all of the β subunit genes were only slightly induced. This result is consistent with the heat transcriptome analyses (Jung and An, 2012; Jung et al., 2012). Therefore, these expression data suggest that the $\alpha1$ and $\alpha2$ subunits function dominantly under heat stress.

OsCpn60 α 1 is an essential subunit

In the early seedling stage, *oscpn60α1* null plants developed a pale-green phenotype and did not produce additional leaves after the fourth one emerged, resulting in lethality. This outcome demonstrated that OsCpn60 α 1 is essential for seedling growth. Similarly, an *Arabidopsis* mutant lacking *AtCpn60α1* has white cotyledons and fails to develop further (Apuya et al., 2001). However, the *oscpn60α1* phenotypes are milder than those of that *AtCpn60α1* mutant. Whereas the *Arabidopsis* mutants do not develop any leaves and their cotyledons do not accumulate chlorophyll, our rice mutants produced four leaves with reduced levels of chlorophyll. This was probably due to the presence of two genes in rice that are homologous to the *Arabidopsis* $\alpha1$ gene (Supplementary Fig. S2B). Those rice genes were expressed at similar levels in the seedling leaves. In *Arabidopsis*, a single mutant of *AtCpn60β1* or *AtCpn60β2* can complete its life cycle, but plants of the double mutant will be albino (Suzuki et al., 2009). Because our *oscpn60α1* mutant did not survive beyond the four-leaf stage, we could conclude that other Cpn60 subunits cannot fully complement the $\alpha1$ function and that subunits of OsCpn60 α 1 differ from those of other subunits.

OsCpn60 α 1 is a key factor for folding rbcL

The *Arabidopsis* chloroplast contains approximately 3000 nucleus-encoded proteins (Leister, 2003) and 88 plastid genome-encoded proteins (Li and Chiu, 2010). To gain functional activity these proteins should be folded correctly. In *E. coli*, approximately 250 different proteins interact with GroEL chaperonin;

however, obligate GroEL-dependence is limited to only ~85 substrates, including 13 essential proteins (Kerner et al., 2005).

In our *oscpn60 α 1* plants, only a trace amount of rbcL was observed but levels of Lhcb1 and stromal Hsp70 proteins were not altered. This indicated that OsCpn60 α 1 is a key factor for rbcL-folding in the rice chloroplast. Association of a newly synthesized rbcL with Cpn60 has been reported in higher plants (Barraclough and Ellis, 1980; Cannon et al., 1986; Gatenby and Ellis, 1990; Gutteridge and Gatenby, 1995; Roy, 1989; Roy and Cannon, 1988). The rbcL-Cpn60 binary complex is considered an obligatory intermediate before assembly into the Rubisco holoenzyme (Gutteridge and Gatenby, 1995; Liu et al., 2010). However, Cpn60 has not been shown to bind with newly synthesized rbcS subunits during or after their import from the cytosol (Ellis and Hemmingsen, 1989). The amount of rbcL subunit is unchanged in four mutants of *Arabidopsis*: β 1 (Ishikawa et al., 2003; Suzuki et al., 2009), β 2 (Suzuki et al., 2009), β 1/ β 2 double (Suzuki et al., 2009), and β 4 (Peng et al., 2011). Thus, our results provide the first functional identification of a Cpn60 subunit that is involved in the folding of rbcL.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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