OsCpn60β1 is Essential for Chloroplast Development in Rice (Oryza sativa L.)

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Abstract: The chaperonin 60 (Cpn60) protein is of great importance to plants due to its involvement in modulating the folding of numerous chloroplast protein polypeptides. In chloroplasts, Cpn60 is differentiated into two subunit types—Cpn60α and Cpn60β and the rice genome encodes three α and three β plastid chaperonin subunits. However, the functions of Cpn60 family members in rice were poorly understood. In order to investigate the molecular mechanism of OsCpn60β1, we attempted to disrupt the OsCpn60β1 gene by CRISPR/Cas9-mediated targeted mutagenesis in this study. We succeeded in the production of homozygous OsCpn60β1 knockout rice plants. The OsCpn60β1 mutant displayed a striking albino leaf phenotype and was seedling lethal. Electron microscopy observation demonstrated that chloroplasts were severely disrupted in the OsCpn60β1 mutant. In addition, OsCpn60β1 was located in the chloroplast and OsCpn60β1 is constitutively expressed in various tissues particularly in the green tissues. The label-free qualitative proteomics showed that photosynthesis-related pathways and ribosomal pathways were significantly inhibited in OsCpn60β1 mutants. These results indicate that OsCpn60β1 is essential for chloroplast development in rice.

Keywords: rice; OsCpn60β1; chloroplast; proteomics

1. Introduction

Most proteins must fold into their native states to attain functional activity. But in the constantly changed cellular environment and under unpredictable environmental stress conditions, newly synthesized proteins are susceptible to misfolding and aggregation, potentially bring dangers to cells. To maintain cellular protein homeostasis, molecular chaperones play an irreplaceable role in promoting protein efficient folding and preventing protein aggregation [1–3]. Among them, chaperonins are a critical group of ATP-driven molecular chaperones that widely exist in prokaryotes and eukaryotes [4–6]. They form large double-ring complexes consisting of 14–16 subunits and each ring encloses a central cavity that assists in the folding of encapsulating substrate proteins [7–9]. Depending on structure and weather co-chaperonin dependence, chaperonins are classified into two distantly related subgroups, group I and group II [10]. Group I chaperonins were found in eubacteria (GroEL), chloroplast (Cpn60) and mitochondria (Hsp60), while group II chaperonins present in archaea (thermosomes) and the eukaryotic cytosol (CCT or TRiC).

The structure and functional mechanism of Group I chaperonins have been extensively investigated in GroEL/GroES complex [11–13]. GroEL forms a homo-oligomer protein composed of two stacked heptameric rings, each of which contains seven identical ~57KD subunits. The co-chaperonin, GroES forms a ring of seven ~10KD subunits which functionally interacts with GroEL in
the presence of ATP to assist protein folding. And the functional mechanism of GroEL/GroES complex has been elucidated by two models—An asymmetric “bullet” model and a symmetric “football” model [13,14]. As the homolog of GroEL and Hsp60, chloroplast chaperonin Cpn60 was initially found as the Rubisco binding protein that could combine with Rubisco large subunit (rbcL) and participate in the assembly process of Rubisco holoenzyme [15–17]. In contrast to bacteria and mitochondria, Cpn60 contains two distinct subunit types, termed Cpn60α and Cpn60β [16,18,19]. Notably, the amino acid sequences of these two subunit types are only about 50% identical, which also share the equal similarity with GroEL [17,18]. Besides, the structure and regulatory mechanism of Cpn60 are much more complicated than GroEL and Hsp60, due to the existence of multiple copies in photosynthetic eukaryotes genomes [20,21]. In vitro, Cpn60αβ hetero-oligomer and Cpn60β homo-oligomer have been reconstructed in E.coli cells. In the presence of MgATP, both of these two types of oligomers could display refolding activity when assisted by co-chaperonins in vitro [22]. However, Cpn60β subunits preferentially form Cpn60αβ hetero-oligomers in the presence of Cpn60α. Besides, only hetero-oligomeric Cpn60αβ1β2 other than homo-oligomeric Cpn60β can cooperate functionally with GroES in refolding activity [23]. And in Chlamydomonas reinhardtii, both Cpn60 monomers and homo-oligomers possessed ATPase activity; but only protein complexes containing all three subunits, the Cpn60αβ1β2 oligomeric complex, have functional cooperation with GroES in refolding a model substrate [24,25]. These results suggest that in vivo Cpn60αβ hetero-oligomers play a much more important role than homo-oligomers. It has been proposed that Cpn60 hetero-oligomers contain seven α subunits and seven β subunits (Cpn60α7β7) [16,18,26]. However, a recent study found that in Chlamydomonas reinhardtii, Cpn60α:Cpn60β1:Cpn60β2 was determined in a 6:2:6 ratio [27], which indicated that the structures of Cpn60 vary significantly among species and need to be further studied.

The molecular mechanism of Cpn60 proteins has been well investigated in Arabidopsis. There are two AtCpn60α genes (AtCpn60α1: At1g28000, AtCpn60α2: At5g18820) and four AtCpn60β genes (AtCpn60β1: At1g55490, AtCpn60β2: At3g13470, AtCpn60β3: At5g56500, AtCpn60β4: At1g26320) in Arabidopsis genome. Among them, the expression level of AtCpn60α1, AtCpn60β1 and AtCpn60β2 is much higher than three others. A T-DNA insertion mutant of AtCpn60α1, termed schlepperless (slp), was the firstly identified mutant of Cpn60 in Arabidopsis [28]. The slp mutant exhibited an embryonic lethality phenotype due to a defect in plastid development which affected embryo development before the heart stage. Besides, a missense allele (caused by a single nucleotide mutation) of AtCpn60α1, arc2, showed a dwarf phenotype with reduced chlorophyll contents and chloroplast numbers, which suggested that AtCpn60α1 was critical to the development of chloroplast and embryo [29]. AtCpn60α2 mutants showed abnormal embryos arrested at the globular stage, which was possibly caused by the reduction in the KASI (β-ketoacyl-[acyl carrier protein] synthase I) protein level. In contrast to AtCpn60α1, AtCpn60α2 could form functional hetero-oligomers with AtCpn60β2 and AtCpn60β3, which is specifically required for the folding of KASI [30]. A mutant of AtCpn60β1, br04, showed a growth retardation phenotype with enlarged chloroplasts, while the mutant of AtCpn60β2 did not exhibit embryo development defects or chloroplast division defects [29]. The atcnp60β1atcnp60β2 double mutant exhibited albino and seedling lethal phenotypes, suggesting AtCpn60β1 and AtCpn60β2 have redundant functions in chloroplast division. AtCpn60β4 can form a hetero-oligomeric complex with AtCpn60α1 and three other AtCpn60β subunits and is essential for the folding of NdhH [31]. Besides, a recent report showed that AtCpn60β4 also regulates growth, development and flowering in Arabidopsis [32].

In rice, there are three OsCpn60α genes (OsCpn60α1: LOC_Os12g17910, OsCpn60α2: LOC_Os03g64210, OsCpn60α3: LOC_Os09g38980) and three OsCpn60β genes (OsCpn60β1: LOC_Os06g02380, OsCpn60β2: LOC_Os02g01280, OsCpn60β3: Chr8Sy.fgenesh.gene.28). However, few experiments have focused on OsCpn60 and only two of the six family members have been studied in detail. A T-DNA insertion mutant of OsCpn60α1 exhibited pale-green and seedling lethal phenotypes, whose protein level of rbcL was severely reduced, suggesting that OsCpn60α1 is an essential factor for rbcL folding [33]. In addition, the rice gene TCD9 encodes a subunit of chaperonin protein (OsCpn60α2), which is important for chloroplast development during the early leaf stage.
In this study, in order to investigate the molecular mechanism of OsCpn60β1, we constructed OsCpn60β1 knockout mutants by CRISPR/Cas9 technology which exhibited albino leaf phenotype. By combining a phenotypic and comparative proteomics analysis, we found that OsCpn60β1 plays an important role in the chloroplast development.

2. Results

2.1. OsCpn60β1 Deficiency in Rice Results in Albino Leaf Phenotype

In order to clarify the physiological function of OsCpn60β1, we attempted to disrupt the OsCpn60β1 gene by CRISPR/Cas9 genome editing system (Figure 1A). We designed two specific guide RNA sequences in the first exon of OsCpn60β1 as the editing targets (Figure 1B). And in the T2 generation, two transgene-free homozygous knockout lines were recovered after sequencing, which were named β1-1 and β1-2 (Figure 1C,D). Both of these tow mutants display albino leaf and growth retardation phenotypes (Figure 1D). Consistent with their phenotypes, the accumulation of chlorophyll was much lower in OsCpn60β1 mutants than that of wild type (Figure 2A). The seedling fresh weight and plant height of OsCpn60β1 mutants were much lower compared with wild type (Figure 2B,C), while there were no obvious difference in root length (Figure 2D). Unlike some other albino or chlorotic leaf mutants, who can turn to be green and normal during the later developmental stage [35–38], the OsCpn60β1 mutant could not survive past three leaves stage. To examine whether there were other off targets genes in OsCpn60β1 mutants, we produced the ProCpn60β1::Cpn60β1-GFP (green fluorescent protein) vector construct and transformed into OsCpn60β1 heterozygous plants. Finally, we obtained 8 independent transgenic lines in the OsCpn60β1 homozygote background and both of them can rescue the albino leaf phenotype (Figure S1). These results suggest that the phenotypes of the mutant are resulted from OsCpn60β1 deficiency and OsCpn60β1 mutants are photosynthesis-deficient mutants in rice.

Figure 1. Production of OsCpn60β1 knockout mutants via the CRISPR/Cas9 system. (A) Diagram of CRISPR/Cas9 system for editing OsCpn60β1. (B) Schematic diagram of targets sites in OsCpn60β1.
Black boxes show exons, black lines show introns and white boxes show untranslated regions (UTR). (C) Mutation sites of OsCpn60β1 knockout lines. β1-1 mutant has a 38-bp deletion and a 1-bp insertion, which has a 37-bp deletion in total; β1-2 mutant has a 5-bp deletion and a 1-bp insertion, which has a 4-bp deletion in total. (D) Phenotypes of OsCpn60β1 mutants, 7-day-old seedlings were photographed. Scale bar, 1 cm.

Figure 2. Characteristics of OsCpn60β1 mutants at 7-day-old seedling stage. (A) Pigment content of wild type (WT) and OsCpn60β1 mutant. Chlorophyll a (Chla), chlorophyll b (Chlb), total chlorophyll (Chl) and carotenoid (Car). (B) Fresh weight of WT and OsCpn60β1 mutants. (C) Plant height of wild type and OsCpn60β1 mutant. (D) Root length of WT and OsCpn60β1 mutants. The data are mean ± SD (n = 3) and ** indicates statistical significance at p < 0.01.

2.2. Chlorophyll Fluorescence Analysis of OsCpn60β1 Mutants

Chlorophyll fluorescence is a non-invasive signature of photosynthesis, which has been widely used to monitor changes in the physiological state of the photosynthetic apparatus [39]. Therefore, we measured the fluorescence parameters in wild type and the OsCpn60β1 mutant by a Dual-PAM 100 chlorophyll fluorescence analyzer (Heinz Walz, Effeltrich, Germany) to monitor whether or not the OsCpn60β1 mutant lost the physiological function. The Fv/Fm ratio reflects the maximum quantum efficiency of photosystem II (PSII) photochemistry and the Fv/Fm was about 0.79 in the wide type plants, whereas the Fv/Fm was just 0.32 in OsCpn60β1 mutants (Figure 3A). The actual photochemical efficiency (ΦPSII and ΦPSI) was also reduced dramatically compared with that of wild type, suggesting that the light energy harvest and transfer were severely affected in OsCpn60β1 mutants (Figure 3B,C).
Figure 3. Parameters measurement of WT and OsCpn60β1 mutants. (A) Maximum photochemical efficiency of PSII, Fv/Fm. (B) The actual photochemical efficiency of PSII, ФPSII. (C) The actual photochemical efficiency of PSI, ФPSI. The data are mean ± SD (n = 3) and ** indicates statistical significance at \( p < 0.01 \).

2.3. Chloroplast Development is Disturbed in OsCpn60β1 Mutants

Abnormal chloroplast development should result in lower total chlorophyll contents in plants [40]. To investigate chloroplast development in OsCpn60β1 mutants further, we observed the ultrastructure of chloroplasts at the three leaves stage of wild type plants and OsCpn60β1 mutants using transmission electron microscopy (Hitachi H-7650). We observed that the chloroplasts in wild type plants were well developed with dense and well-structured grana stacks (Figure 4A,D). Conversely, the OsCpn60β1 mutant had abnormal chloroplast architecture without observable grana lamellae stacks, which just displaying oval-shaped vesicles (Figure 4B,C,E,F). These results demonstrated that OsCpn60β1 plays a key role in early chloroplast development.

Figure 4. Transmission electron microscopic images of chloroplasts in WT and OsCpn60β1 mutants. (A) The cell of WT; (B) the cell of β1-1 mutant; (C) the cell of β1-2 mutant; (D) a normal chloroplast in WT; (E) an abnormal chloroplast in β1-1 mutant; (F) an abnormal chloroplast in β1-2 mutant.
2.4. Expression Pattern and Subcellular Localization of OsCpn60β1

To investigate the expression pattern of OsCpn60β1, we examined the OsCpn60β1 expression level in various tissues of wild type rice (Donjin) during different growth stages by qRT-PCR. As shown in Figure S2, OsCpn60β1 was expressed in root, stem, leaf, root-stem transition region, node, flower, panicle and filling seed. And the expression level in the leaves was higher than that of other tissues, especially in the flag leaf expression was the highest and expression in node and filling seed rarely, indicating that OsCpn60β1 mainly functions in leaves (Figure S2). To determine the spatiotemporal expression profile of OsCpn60β1 in detail, we also developed transgenic plants with OsCpn60β1 promoter driven β-glucuronidase (GUS). Consistent with the results of qRT-PCR, GUS staining was observed in different tissues including axial root, lateral root, leaf, leaf sheath and anther (Figure 5). Taken together, our results indicated that OsCpn60β1 was constitutively expressed in various tissues particularly in the green tissues.

SignalP (http://www.cbs.dtu.dk/services/SignalP/) [41] analysis of the OsCpn60β1 polypeptide predicted it contains a chloroplast transit peptide. To explore the precise subcellular localization of OsCpn60β1, we produced the 35S::OsCpn60β1-GFP transgene and transiently expressed in rice protoplasts. The green fluorescent signals of OsCpn60β1-GFP were overlaid with chloroplast autofluorescence in transformed rice protoplasts (Figure 6). These data demonstrated that OsCpn60β1 was localized in the rice chloroplast.

![Figure 5. Organic expression pattern of OsCpn60β1. Gus staining in axial root (A), lateral root (B), node (C), leaf (D), cross section of leaf sheath (E), anther (F).](image)

![Figure 6. Subcellular localization of the OsCpn60β1-GFP (green fluorescent protein) in rice protoplasts. GFP signals show that the OsCpn60β1-GFP fusion protein produced from the 35S::OsCpn60β1-GFP construct localized to the chloroplast. Green fluorescence shows GFP, red fluorescence shows chloroplast auto-fluorescence and yellow fluorescence shows the merged fluorescence. Scale bar, 5μm.](image)
2.5. Analysis of Differentially Accumulated Proteins (DAPs) in OsCpn60β1 Mutants

In order to gain an extensively understanding of the function of OsCpn60β1, total proteins in leaves were extracted from the wild type plants and OsCpn60β1-1 mutants and the proteins expression profiles were detected by using relative liquid chromatography electrospray ionization tandem mass spectrometry (LC-MS/MS)-based label-free protein quantification technique. A total of 3534 proteins were identified using the two biological replicates, with 3168 proteins in wild type plants and 3379 proteins in OsCpn60β1 mutants. The proteins whose fold change was greater than 2 (up-regulation greater than 2 or down-regulation less than 0.5) and the p-value lower than 0.05 were considered as being differentially expressed. Consequently, there were 558 differentially accumulated proteins (DAPs) between wild type and OsCpn60β1 mutants, among which 277 proteins were up-regulated and 281 were down-regulated in OsCpn60β1 mutants (Table S1).

The overall identified proteins were then classified into three sets of ontologies including biological process (BP), molecular function (MF), cellular component (CC) based on GO analysis. The identified proteins were mainly involved in such as metabolic process, cellular process, biological regulation, catalytic activity, protein binding, cell and cell parts (Figure S3). Furthermore, using Fisher’s exact test method, the GO functional enrichment analysis of DAPs was performed to understand the changed cellular metabolism in OsCpn60β1 mutants. For the BP category, photosynthesis related terms such as light reaction, light harvesting and photosynthetic electron transport chain were the most important terms. For the MF category, chlorophyll binding, RNA binding and disulfide oxidoreductase activity were enriched. For the CC category, thylakoid proteins were significantly enriched. And these results suggest that DAPs are mainly primarily involved in light reaction, light harvesting, photosynthetic electron transport chain, rRNA binding, disulfide oxidoreductase activity (Figure 7).

Figure 7. GO functional enrichment analysis of differentially accumulated proteins (DAPs) in OsCpn60β1 mutants compared with WT. The label at the top of the bar chart shows the enrichment factor (rich Factor ≤ 1), which represents the proportion of the number of DAPs annotated into a GO function category to the number of all identified proteins annotated into the GO function category.
The color of the bar chart represents the significance of enriched GO functional classification, which is based on Fisher’s exact test to calculate the P value. BP, biological process; MF, molecular function; CC, cellular components.

The DAPs were further analyzed for kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment. Using the KEGG database, 558 DAPs were classified into 78 metabolic pathways. According to the numbers of DAPs, the top 20 pathways mainly composed ribosome, photosynthesis, glyoxylate and dicarboxylate metabolism, carbon fixation in photosynthetic organisms and protein processing in endoplasmic reticulum (Figure S4). With Fisher’s exact test, KEGG pathway enrichment analysis of DAPs was performed. And as shown in Figure 8, three pathways were significantly enriched (p-value < 0.05), including glyoxylate and dicarboxylate metabolism (17 proteins), photosynthesis (24 proteins) and photosynthesis-antenna proteins (10 proteins).

Figure 8. Pathway enrichment analysis of differentially accumulated proteins (DAPs) in OsCpn60β1 mutants compared with WT. For the bar graph, color represents the significance of enriched kyoto encyclopedia of genes and genomes (KEGG) pathways. Fisher’s exact test is used to calculate the p-value. The label at the top of the bar chart shows enrichment factor (rich Factor ≤ 1), which represents the proportion of the number of DAPs involved in a KEGG pathway to the number of proteins involved in this pathway among all identified proteins.

2.6. Photosynthesis Metabolism Proteins Were Greatly Decreased in OsCpn60β1 Mutants

As a chloroplast located chaperone, Cpn60 plays an important role in in modulating the folding of numerous chloroplast protein polypeptides, such as rbcl and NdhH [16,31]. The abundance of photosynthesis related proteins contain photosystem I, photosystem II, light harvesting antenna and cytochrome b6/f complex proteins were greatly decreased in OsCpn60β1 mutants. In Figure 9 is shown the photosynthesis pathway, which had the higher number of associated proteins of all the significantly enriched pathways found in the Fisher’s exact test shown in Figure 8. And 24 proteins were down-regulated significantly (proteins levels were shown in Table S2), including some photosystem II (PSII) complex subunits (such as Psb27, Psb28, PsbE, PsbH, PsbO, PsbQ, PsbR, PsbS), five proteins in photosystem I (PSI) complex (such as PsaD, PsaE, PsaG, PsaH, PsaK), cytochrome b6/f complex (Petc), photosynthetic electron transport proteins (PetE, PetF, PetH) and F-type ATPase (alpha, gamma, delta, b). Light harvesting antenna is one of the most abundant chloroplast proteins in plants, which plays an important role in the absorption, transmission and regulation of excitation energy distribution between photosynthetic reaction centers, as well as in maintaining the stack of the thylakoid membrane [42]. In OsCpn60β1 mutants, several light harvesting antenna proteins were also down-regulated (Table S2). And these results suggested that light reaction was inhibited seriously in the OsCpn60β1 mutant. Besides, some proteins participated in the pathway of carbon fixation in photosynthetic organisms were down-regulated seriously (Table S2), especially the abundance of rbcl and Rbcs, which indicated that OsCpn60β1 is also critical to Calvin cycle regulation.
Figure 9. Differentially accumulated proteins (DAPs) involved in photosynthesis pathway. The known pathways were obtained from KEGG database. Red borders indicate differentially accumulated proteins in OsCpn60β1 mutants. Green background borders indicate proteins which were unidentified identified in WT but not in OsCpn60β1 mutants. White background borders indicate proteins which were not identified in both WT and OsCpn60β1 mutants.

2.7. Ribosomal Pathway Was Inhibited in OsCpn60β1 Mutants

Ribosomes are the cell’s protein-synthesizing machinery, which comprise a large (60S or 50S) and small (40S or 30S) subunit containing rRNAs and various ribosomal proteins. In eukaryotic cells, ribosomes are found in chloroplast, mitochondria and cytoplasm [43,44]. In the present study, a total of 27 ribosomal proteins were altered significantly in the OsCpn60β1 mutant compared with wild type, among which except for two 40S ribosomal proteins were up-regulated and the others including most chloroplast ribosomal proteins were down-regulated seriously (Table S3). The chloroplast ribosome includes four rRNA (23S, 16S, 5S and 4.5S) and other ribosomal proteins [45]. We hypothesized that the chloroplast ribosome biosynthesis in the OsCpn60β1 mutant might be impaired. To prove this hypothesis, we further analyzed the composition and amount of rRNAs using the Agilent 2100 bioanalyzer. In OsCpn60β1 mutants seedlings, 16S rRNA and 23S rRNA were marginally detected (Figure 10). Overall, the chloroplast ribosome biosynthesis is severely impaired in OsCpn60β1 mutants.
Figure 10. OsCpn60β1 affects the development of chloroplast ribosome. (A) rRNA analysis using an Agilent 2100 bioanalyzer. The rRNAs isolated from 7-day-old WT and OsCpn60β1 mutants seedlings. (B) qRT-PCR analysis of rRNA accumulation in WT and OsCpn60β1 mutants seedlings. The data are mean ± SD (n = 3) and ** indicates statistical significance at p < 0.01.

2.8. Validation of Proteomics Data by Parallel Reaction Monitoring (PRM) Method

To confirm the reliability of the proteomics data, 8 DAPs were selected to verify the protein expression levels determined by the PRM method. And the expression trends of these selected proteins were basically consistent with our label-free qualitative proteomics data (Table 1), endorsing that our proteomics data was reliable.

Table 1. Confirmation of Daps in Proteomic Analysis Using parallel reaction monitoring (PRM) Analysis.

| Protein ID | Description                                         | Fold Changes in Proteomics (Mutant/WT) | Fold Changes in PRM (Mutant/WT) |
|------------|-----------------------------------------------------|----------------------------------------|---------------------------------|
| Q7XDY9     | Rubisco accumulation factor 1                       | 0.452                                  | 0.473                           |
| Q9ZST1     | 30S ribosomal protein S17                            | 0.234                                  | 0.212                           |
| Q6Z7V2     | 24.1 kDa heat shock protein, mitochondrial          | 15.022                                 | 14.833                          |
| Q6H4L2     | Elongation factor 2                                  | 2.907                                  | 2.722                           |
| Q7F8S5     | Peroxiredoxin-2E-2, chloroplastic                   | 0.329                                  | 0.313                           |
| Q2QQ99     | Protein SPIRAL1-like 3                              | 2.256                                  | 2.051                           |
| B7E5F1     | Carbonic anhydrase                                  | 0.061                                  | 0.064                           |
| Q7F2L7     | PsbE                                                | 0.016                                  | 0.012                           |
3. Discussion

3.1. OsCpn60β1 is an Essential Subunit of Cpn60 Complex

In this study, we succeeded in generating OsCpn60β1 knockout mutants using CRISPR/Cas9 technology, which exhibited albino leaves and eventually died at the seedling stage (Figure 1). However, in Arabidopsis, neither a single mutation of AtCpn60β1 nor AtCpn60β2 could cause a visible phenotype, only the atcpn60β1atcpn60β2 double mutant displayed albino and seedling lethal phenotypes, suggesting functions of AtCpn60β1 and AtCpn60β2 are redundant in chloroplast division [29]. However, there are three OsCpn60β genes in rice, although the phenotypes of OsCpn60β2 and OsCpn60β3 are unclear and OsCpn60β1 plays an irreplaceable role in chloroplast development and plant growth. Besides, the oscp60β1 mutation had a pale-green phenotype and development ceased at the seedling stage [33]; OsCpn60α2 mutation caused albino phenotype at low temperature before three leaves seedling stage [34]. Because the different phenotypes of Cpn60 family members and the seedling lethal trait of OsCpn60β1 mutants (Figure 1), we could conclude that OsCpn60β1 is an essential subunit of Cpn60 complex, whose functions differ from those of other subunits.

3.2. OsCpn60β1 is Critical to Rubisco Folding and Carbon Fixation

In the 1980s, when John Ellis studied light driven-protein synthesis in isolated chloroplasts, he found that before combined with RbcS to form a whole enzyme, rbcL first combined with other proteins to form a complex, which was later widely known as Cpn60 [15-17]. Later more studies showed that Cpn60 and rbcL were correlated, especially Cpn60α subunit types played an important role in the folding of rbcL. In maize, rbcL was closely bound to two specific Cpn60 subunits, namely ZmCpn60α1 (cps2, encoded by AC215201.3) and ZmCpn60β1 (GRMZM2G083716) [46]. Compared with the wild type, the cps2 mutant showed pale-green phenotype and the protein abundance of Rubisco was down-regulated by 95%, while the expression level of some other chloroplast proteins did not change significantly, indicating that ZmCpn60α1 is specific to rubisco folding [47]. In the same way, when the ZmCpn60α1 homologous gene in rice (OsCpn60α1) was mutated and it also showed a yellowish and Rubisco protein specific down-regulation phenotype [33].

It is interesting to note that the abundance of rbcL is unchanged in four mutants of Arabidopsis: β1, β2, β1/β2 double and β4 [29,31]. However, the reconstruction of Arabidopsis thaliana Rubisco in E. coli had been achieved with co-expression of Cpn60 hetero-oligomers and other co-chaperonins. Meanwhile, AtCpn60β, which forms tetradeameter complexes by itself, can also mediate production of Rubisco with low efficiency [23]. Here, we found that the protein abundance of rbcL and Rbcs was marginally detected in our OsCpn60β1 mutants according to our proteomic data (Table S2) and we also found some proteins participated in Calvin cycle regulation such as fructose-bisphosphate aldolase, malate dehydrogenase are also down-regulated significantly (Table S2), which demonstrates that OsCpn60β1 is critical to Rubisco folding and plays an important role in carbon fixation.

3.3. OsCpn60β1 is Crucial in Ribosome Biogenesis

Chloroplasts contain 70S ribosomes, consisting of a 30S small subunits and a 50S large subunit, similar to prokaryote-type ribosomes and distinct from the cytosolic ribosomes, which are 80S ribosomes [43]. The 30S subunit contains a 16S rRNA and 24 different proteins. The 50S subunit contains 4 rRNAs (23S, 16S, 5S and 4.5S) and 33 different proteins. In addition, plastid ribosomal proteins (PRPs) play important roles in ribosome biogenesis, plastid protein biosynthesis and chloroplast differentiation [48]. Mutation of PRPs caused diverse phenotypic effects in plants, including lethality, reduced plant height and decreased photosynthetic capacity [49]. The maize photosynthetic mutant hcf60 exhibited a pale green seedling lethal and high-chlorophyll fluorescence phenotypes, which was caused by the deletion of ribosomal small subunit protein 17 (RPS17) and was the first reported PRP mutant in higher plants [48]. In recent years, numerous PRP large subunits and small subunits have been reported to be essential for embryogenesis in Arabidopsis [49–52]. In
addition, when some subunits such as RPS1, RPS17 and RPL24 were disturbed, the plastid protein synthesis and photosynthesis of the mutant were impaired in these mutants but they still could survive normally, which indicates that these subunits may not be essential for basal ribosome activity [49].

In rice, albino seedling lethality 1 (asl1) was the first identified PRP mutant and the mutated ASL1 gene encodes the chloroplast 30S ribosomal protein S20 (RPS20) [53]. Similarly, the rice mutants asl2 and all were reported to show albino phenotypes at the seedling stage and could not survive past the three leaves stage, due to lacking of the chloroplast 50S ribosomal protein L21 (PRPL21) and 50S ribosome protein L12 (PRPL12) [54,55]. More recently, PRPL13 and RPS6 were found to be required for normal chloroplast development under low temperature conditions [56]. In addition, PRS9 was essential for early chloroplast development in rice, as the wgl2 mutant displayed an albino phenotype from germination through the three leaves stage and then gradually turned from albino to green through the later developmental stage [56]. In our study, we found that a total of 27 ribosomal proteins were altered significantly in the OsCpn60β1 mutant compared with wild type, among which 25 ribosome proteins including many chloroplast ribosomal proteins were down-regulated seriously (Table S3). Ribosomes are the cell’s protein-synthesizing machinery and these down-regulated ribosomal proteins would cause a block in the mutant proteins synthesis especially chloroplast proteins. It is worth to note that two cytosolic ribosomal proteins (40S ribosomal protein S3a and 40S ribosomal protein S29) were up-regulated in mutants (Table S3). And cytosolic ribosomal proteins play important roles in many biological processes such as shoot meristematic function, lateral root initiation and leaf variegation [57–60], we speculated that the increased abundance of these two proteins were essential for plant growth in OsCpn60β1 mutants at early leaf stage. And the detailed relationships between OsCpn60β1 and these two cytosolic ribosomal proteins need to be further investigated in the future. Besides, the chloroplast ribosome consists of 4 rRNAs (23S,16S, 5S and 4.5S) and these rRNAs represent essential components of the chloroplast translational apparatus [45].

We detected the composition and content of rRNAs (23S,16S, 5S and 4.5S) and these rRNAs represent essential components of the chloroplast translational apparatus [45]. And the detailed relationships between OsCpn60β1 and these two cytosolic ribosomal proteins need to be further investigated in the future. Besides, the chloroplast ribosome consists of 4 rRNAs (23S,16S, 5S and 4.5S) and these rRNAs represent essential components of the chloroplast translational apparatus [45]. We detected the composition and content of rRNAs in OsCpn60β1 mutants and wild type. And we found that 23S and 16S rRNAs were significantly decreased in mutants (Figure 10). These results suggested that OsCpn60β1 was crucial in chloroplast ribosome biogenesis and defects in chloroplast ribosomes would result in abnormal synthesis of chloroplast proteins, which seemed to be a main cause of albino leaf phenotype.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Cultivar ‘Donjin’ was used in the study. Germinated rice seeds were grown in hydroponic solution recommended by the International Rice Research Institute. Rice seedlings were grown in a greenhouse under a 12-h-light (30 °C)/12-h-dark (22 °C) photoperiod and a photosynthetic photon flux density (PPFD) of 500 μmol photons m⁻²s⁻¹ as previously described [61].

4.2. Construction of Vectors and the Generation of Transgenic Plants

We employed the CRISPR/Cas9 system to establish OsCpn60β1 mutants according to the protocol described previously [62]. Briefly, the first coding exon of OsCpn60β1 was selected for guide RNA design based on the CRISPR-PLANT database (www.genome.arizona.edu/crispr/) [63]. Two Polycistronic tRNA-gRNA (PTG) genes were inserted in the pRGEB32 vector to create the OsCpn60β1-pRGEB32 vector by Golden Gate Assembly. To determine the OsCpn60β1 expression pattern, a 1800-bp sequence upstream of the ATG codon in the OsCpn60β1 gene was amplified from genomic DNA of wild type, which was cloned into the pBII101.3 vector to drive expression of the GUS reporter gene. For complementation of the OsCpn60β1 mutant, the CDS of OsCpn60β1 driven by its 1800-bp native promoter was inserted into the modified pCambia1300-GFP vector to generate the ProCpn60β1::Cpn60β1-GFP vector. Transgenic rice plants were generated by the Agrobacterium tumefaciens strain EHA105–mediated transformation using rice mature seeds derived callus according to a conventional protocol [64]. All of the primers are listed in Table S4.
4.3. Chlorophyll Quantification and Chlorophyll Fluorescence Measurements

Leaf chlorophyll content was determined according to the previously described method [65] with slight modifications. Leaves (approximately 0.2 g fresh weight) at 7-day-old seedling stage were cut and immersed in 5 mL of 80% acetone for 12 h in the dark. Residual debris was removed by centrifugation. Absorbance of the supernatants was measured by spectrophotometric scanning (DU800, Beckman, Fullerton, CA, USA) at 663 nm, 645 nm and 470 nm. Three biological replicates were analyzed for each sample.

The chlorophyll fluorescence parameters of wild type plants and OsCpn60β1 mutants were measured using a Dual-PAM 100 chlorophyll fluorescence analyzer (Heinz Walz, Effeltrich, Germany). Prior to measurements, all plants were first dark adapted for 40 min. The photochemical efficiency (Fv/Fm) and electron transfer quantum efficiency (ΦPSII and ΦPSI) were recorded and calculated using Dual-PAM 100 software according to the manufacturer’s instructions. The measurement was repeated three times and averaged.

4.4. Transmission Electron Microscopy

The transmission electron microscopy analysis was carried out as described previously [66]. Concisely, leaf samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C, then further fixed in 1% OsO4 overnight at 4 °C. Then, tissues were further dehydrated in a gradient of ethanol solutions and finally embedded in resin. Ultrathin sections (50 nm) were cut on a Leica EM UC7 ultra-microtome and stained with uranyl acetate. Samples examined with a Hitachi H-7650 (Hitachi, Tokyo, Japan) transmission electron microscope.

4.5. Subcellular Localization of OsCpn60β1

To investigate the subcellular localization, we cloned the full-length CDS sequence of OsCpn60β1 without the termination codon and the fragment was fused into the modified pCambia1300-GFP vector under the CaMV35S promoter. Then the 35S::OsCpn60β1-GFP fusion construct was transformed into rice protoplasts according to a previous study [67]. And images were captured by a florescence microscope (Zeiss LSM710, Zeiss, Jena, Germany). The PCR amplification primers are listed in Table S4.

4.6. Histological β-glucuronidase (GUS) Assay

GUS staining was performed according to a standard protocol [68]. Transgenic rice tissues were incubated overnight at 37 °C in GUS staining buffer (0.1 M K2HPO4 (pH 7.0), 0.1 mM KH2PO4 (pH 7.0), 5 mM KFe(CN)6, 5 mM K4Fe(CN)6·3H2O, 0.1% Triton X-100, 20% methanol, 1 mg mL−1 X-Gluc). After staining, the tissues were soaked in 70% ethanol to remove chlorophyll and surface dyes. Images were captured under a stereomicroscope (Nikon AZ100 microscope, Nikon, Kyoto, Japan).

4.7. Protein Extraction and Digestion

The shoots of wild type and OsCpn60β1 mutants at 7-day-old seedling stage were used for protein extraction. In total, there were 6 samples including 2 independent biological replicates and three technical repetitions. Approximately 500 mg fresh tissue of each sample was ground into powder in liquid nitrogen and then incubated in extraction solution (50 mM DTT, 6 M urea, 1% Protease Inhibitor Cocktail and 0.5 M Tris-HCl, pH 8.0). The suspension was cracked by ultrasonication at 4 °C for 5 min and then incubated for 30 min on ice. Samples were subsequently centrifuged at 13,000 g for 30 min at 4 °C and the supernatant was transferred to a new clean tube and stored at −80 °C. The protein concentration was measured with BCA kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions.

Protein solution was digested using the FASP procedure as previously described [69]. After being reduced with 10 mM DTT at 37 °C for 1 h, the protein solution was alkylated with 30 mM iodoacetamide at 37 °C for 30 min in darkness. Then, 50 mM NH4HCO3 was added to dilute the urea agent less than 2M. Finally, the sample was further digested using trypsin (Promega, Madison,
Wisconsin, USA) in a 1:50 trypsin-to-protein mass ratio at 37 °C overnight. Reactions were stopped by adding formic acid to a final concentration of 1% and the mixture was desalted by a Zorbax column C18 (Phenomenex, Torrance, CA, US). Then, the peptides were vacuum-dried in a SpeedVac Concentrator (Savant, Thermo Fisher Scientific, Waltham, MA, USA) and redissolved in 0.1% formic acid. The concentration of peptides was measured by spectrophotometric scanning at 280 nm.

4.8. Label-free Qualitative Proteomics Analysis

The isolation and analysis of tryptic peptides were performed using a quadrupole time-of-flight mass spectrometer (Agilent model 6500, Wilmington, DE, USA) based on the operation manual of MALDI-TOF MS. The resulting MS/MS data were searched against the UniProt plant protein database (http://www.uniprot.org) [70] and the phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) [71] using the MaxQuant 1.5.3.30 (Computational Systems Biochemistry, Max-Planck Institute for Biochemistry, Martinsried, Germany) software (http://www.coxdocs.org) [72]. The following conditions were used—the enzyme digestion mode was set to Trypsin/P, allowing for up to 2 missing cleavages; the mass tolerance for precursor ions was set at 20 ppm in the first search and at 5 ppm in the main search and the mass tolerance for fragment ions was set at 0.02 Da; carbamidomethyl-modified cysteine residues were specified as a fixed modification and oxidation of methionine was specified as a variable modification. Protein quantitation was calculated using intensity-based absolute quantification (iBAQ) method in MaxQuant (Computational Systems Biochemistry, Max-Planck Institute for Biochemistry, Martinsried, Germany) software, with the p-values ≤ 0.05 and the global false discovery rate (FDR) ≤ 0.05. The iBAQ data was weighted and normalized by the median ratio in Mascot. Proteins with a fold change ≥ 2 coupled with p-values < 0.05 were determined as differentially accumulated proteins (DAPs).

The bioinformatics analysis was performed according to the method reported in our earlier study [73]. The Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/) [74]. Firstly, identified protein IDs were converted to UniProt ID and then mapped to GO IDs by the protein ID. If some identified proteins are not annotated by UniProt-GOA database, the InterProScan software (http://www.ebi.ac.uk/interpro) [75] was used to annotate protein’s GO functional by protein sequence alignment method. GO items can be classified into three categories including cellular component (CC), biological process (BP) and molecular function (MF). For each category, a two-tailed Fisher’s exact test was carried out to test the significance of the enrichment of each differentially accumulated protein (DAPs) against all identified proteins. A GO term with a corrected p-value < 0.05 was considered to be significant. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/) [76] was used to annotate the biological pathway. In brief, we got the protein’s KEGG database descriptions by KEGG online service tools KAAS firstly and then mapped the DAPs to the KEGG pathway database by using the KEGG mapper tool.

4.9. Parallel Reaction Monitoring (PRM) Verification

In order to confirm the reliability of label-free quantitative proteomics analysis, some DAPs were analyzed by using parallel reaction monitoring (PRM) method. The remaining samples of precious proteomics were used for direct tryptic digestion. The PRM analyses were performed on a mass spectrometer and the peptide fragments monitored for each protein were selected depended on the ion signal intensities in the spectral library. The MS acquisition mode was a combination of two scan events—a full scan and a time-scheduled scan. The full MS scan was carried out with a resolution of 70,000 (at 200 m/z), an AGC target value of 3.0×10^6 and a maximum ion injection time of 250 ms. The time-scheduled scan was taken at a resolution of 35,000 resolution (at 200 m/z), an AGC target value of 3.0 × 10^6 and a maximum ion injection time of 200 ms. And a 2 Th (Thomson) window was used for target precursor ions isolation. Precursor ions were fragmented by HCD (higher-energy collisional dissociation) with normalized collision energy of 27. After each precursor ion (light and heavy masses) was selected by the fragmented quadrupole, all fragment ions were quantified in the
to demonstrate that pathways critical to chloroplast development in rice were altered in OsCpn60β1 mutants compared with wild type plant. These results demonstrate that OsCpn60β1 is critical to chloroplast development in rice.

**Supplementary Materials:** The following are available online at www.mdpi.com/1422-0067/21/11/4023/s1:

- Figure S1. Characteristics of complementation lines at 7-day-old seedling stage. (A) Phenotypes of complementation lines. (B) qRT-PCR analysis of OsCpn60β1 expression in complementation lines. (C) Pigment content of WT and complementation lines. COM1 represents complementation line 1 of the OsCpn60β1 mutant, COM2 represents complementation line 2 of the OsCpn60β1 mutant. Figure S2. Organic expression analysis of OsCpn60β1 by qRT-PCR. Relative expression level of OsCpn60β1 was calculated in each tissue of Donjin (DJ) at different growth stages. DJ was cultivated in normal culture solution for 3 weeks and transferred to the field. Figure S3. The GO annotation results of identified proteins in WT and OsCpn60β1 mutants. Figure S4. The first 20 KEGG pathways with the most DAPs in OsCpn60β1 mutants compared with WT. Table S1 All DAPs in mutants were listed. Table S2 DAPs in photosynthesis metabolism pathway. Table S3 DAPs in ribosomal pathway. Table S4 Primers sequence in this study were listed.

**Author Contributions:** DJ conceived and designed the research. Q.W. and C.Z. performed the experiments; Y.C., K.Z. and Y.Z. analyzed the data; DJ and Q.W. contributed to writing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| WT           | Wild type   |
| GFP          | Green fluorescent protein |
| UTR          | Untranslated regions |
| LC-MS/MS     | Liquid chromatography electrospray ionization tandem mass spectrometry |
| DAPs         | Differentially accumulated proteins |
| GO           | Gene ontology |
| CC           | Cellular component |
| BP           | Biological process |
| MF           | Molecular function |
| KEGG         | Kyoto encyclopedia of genes and genomes |
| TEM          | Transmission electron microscopy |
| PRM          | Parallel reaction monitoring |
| DTT          | Dithiothreitol |
| PSII         | Photosystem II |
| PSI          | Photosystem I |
| RbcL         | Ribulose bisphosphate carboxylase large chain |
| Rbcs         | Ribulose bisphosphate carboxylase small chain |

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