J. Plant Biol. (2019) 62:419-428
DOI 10.1007/s12374-019-0380-z

RESEARCH ARTICLE

# Impaired Plastid Ribosomal Protein L3 Causes Albino Seedling Lethal Phenotype in Rice

**Jinwon Lee[1], Seonghoe Jang[1,5], Sanghoon Ryu[2], Seulbi Lee[1], Joonheum Park[3], Sichul Lee[3], Gynheung An[4] and Soon Ki Park[1,*]**

[1]*School of Applied Biosciences, Kyungpook National University, Daegu 41566, Korea*
[2]*Center for Scientific Instruments, Andong National University, Andong 36729, Korea*
[3]*Center for Plant Aging Research, Institute for Basic Science (IBS), Daegu 42988, Korea*
[4]*Crop Biotech Institute, Kyung Hee University, Yongin 17104, Korea*
[5]*Current address: World Vegetable Center Korea Office, Wanju-gun, Jeolabuk-do 55365, Korea*

Received: September 4, 2019 / Accepted: September 27, 2019


**Abstract** The chloroplast is a semiautonomous photosynthetic organelle that is essential for plant growth and development, particularly in crops via manipulation of its photosynthetic capacity and the biosynthesis of carbon skeletons. Plastid ribosomal proteins (PRPs) are crucial for the establishment of the transcription/translation apparatus during chloroplast differentiation. In this study, we isolated and characterized T-DNA-tagged rice mutants with defective chloroplasts, named *prpl3*, that exhibited a distinct albino seedling lethality. Transmission electronic microscopy (TEM) observations showed that the grana stacks in the mutant were not properly formed, with disrupted thylakoid structures in their chloroplasts. Chlorophyll content was also significantly reduced in the leaves of *prpl3* mutant seedlings. *PRPL3* contains nuclear genes encoding PRPs localized to the chloroplasts, and *prpl3* represents a novel mutant presentation of an impaired *PRPL3* gene. Our findings also demonstrated that *PRPL3* is responsible for phenotypic alterations by generating additional mutant alleles thereof using CRISPR/Cas9 systems. Expression levels of genes involved in photosynthesis and chloroplast development, including plastidial transcription and translation and photosynthesis, were altered in the *prpl3* mutant. These results collectively demonstrate that nuclear-encoded PRPL3 is indispensable for the proper development of chloroplasts in rice.

**Keywords:** Albino, Chloroplast development, *Oryza sativa*, Plastid ribosomal protein, PRPL3, Rice

*Corresponding author; Soon Ki Park
Tel : +82-53-958-6880
E-mail : psk@knu.ac.kr

## Introduction

Protein synthesis in plants occurs in the cytoplasm, plastids, and mitochondria. Plastid protein synthesis uses a bacterial-type 70S ribosome with two subunits, one small (30S) and one large (50S) (Schippers and Mueller-Roeber 2010; Tiller and Bock 2014). The 30S subunit contains 16S rRNA and 24 plastid ribosomal proteins (PRPs), including 12 proteins encoded by plastid genes and 12 encoded by nuclear genes (Yamaguchi and Subramanian 2003). The 50S subunit consists of three rRNAs (23S, 5S, and 4.5S) and 33 PRPs, including eight proteins encoded by plastid genes and 25 encoded by nuclear genes (Yamaguchi et al. 2003). Although the structure of the plastid ribosome is thought to be highly conserved relative to its cyanobacterial counterparts, some PRPs have diverged from their bacterial ancestry with respect to the formation of ribosomes in chloroplasts (Tiller and Bock 2014; Ahmed et al. 2017; Bieri et al. 2017). Impaired PRPs can affect plant growth and development, resulting in diverse phenotypes mediated by defective protein synthesis. While certain chloroplast ribosomal proteins are reported to be associated with plastid translation (Han et al. 1992; Barkan 1993), the nature of other relevant gene products and their modes of action remains elusive.

The Arabidopsis *ghs1* (*glucose hypersensitive1*) mutant has a T-DNA insertion in the *GHS1* gene encoding the plastid 30S ribosomal protein S21 and displays an increased sensitivity to glucose (Morita-Yamamuro et al. 2004). *PRPL33* knock-out tobacco plants are more sensitive to low temperatures despite normal viability and growth under standard growing conditions. Also, tobacco *PRPS18*, *PRPS2*, *PRPS4*, and *PRPL20* are indispensable for plastid ribosomal function, including plastidial translation during development (Rogalski et

al. 2006, 2008). The maize *prps17* mutant caused by a transposon insertion exhibits a seedling-lethal, high-chlorophyllic fluorescence phenotype (Schultes et al. 2000), and the *prps9* mutation induces early embryonic lethality (Ma and Dooner 2004). Similarly, many plastid ribosomal proteins are pivotal to embryonic development, as shown by mutant analyses of Arabidopsis, including *prps5*, *9*, *13*, *20* and *prpl1*, *4*, *6*, *10*, *13*, *18*, *19*, *21*, *22*, *27*, *28*, *31*, *32*, *35*, *36*) (Pesaresi et al. 2001; Morita-Yamamuro et al. 2004; Romani et al. 2012; Tiller et al. 2012; Yin et al. 2012; Zhang et al. 2016). Rice PRP mutants *albino seedling lethality 1* (*asl1*), *asl2*, and *albino lethal 1*(*al1*) exhibit albino and seedling-lethal phenotypes (Gong et al. 2013; Lin et al. 2015; Zhao et al. 2016). *ASL1* encodes the plastid 30S ribosomal protein S20 (PRPS20), *ASL2* encodes the chloroplast 50S ribosome protein L21 (PRPL21), and *AL1* encodes the PRPL12 protein. The rice *white leaf and panicles 1* (*wlp1*) and *thermo-sensitive chlorophyll-deficient mutant 11* (*tcd11*) mutants are impaired in *PRPL13* and *PRPS6*, respectively. These mutants display albino phenotypes at low temperatures, indicating both genes are required for normal chloroplast development, particularly in low temperature environments (Song et al. 2014; Wang et al. 2017). Even though the molecular functional characterizations of several rice PRPs have been reported, functional validation of many other rice PRPs has yet to be explored in detail.

Here we describe a rice T-DNA mutant showing albino seedling lethality, with impaired genes encoding the plastid ribosomal protein PRPL3. Additional mutant alleles for *PRPL3* were generated with CRISPR/Cas9 for phenotype verification. The ultrastructure of chloroplasts was defective in the *prpl3* mutant, and the expression levels of genes linked to chloroplast development and photosynthesis were also altered. We thereby concluded that *PRPL3* is critical to early development. During chloroplast development, nucleus-encoded RNA polymerase preferentially transcribes plastid housekeeping genes, such as those encoding the plastid-encoded RNA polymerase apparatus, rRNA, and tRNA, and the overall transcriptional and translational activities in the chloroplast dramatically increase. Thus, PRPs may be necessary for the early stages of chloroplast development in rice.

## Results

### Isolation of Albino Mutant in rice T-DNA Tagged Pool

Members of the rice T-DNA-inserted mutant pool were screened to isolate leaf senescence-related mutants (Jeon et al. 2000). One line presenting with an albino phenotype at the seedling stage was selected for further study. Analyses of flanking sequences (as determined by TAIL-PCR) in the albino mutant line demonstrated that the albino mutant had

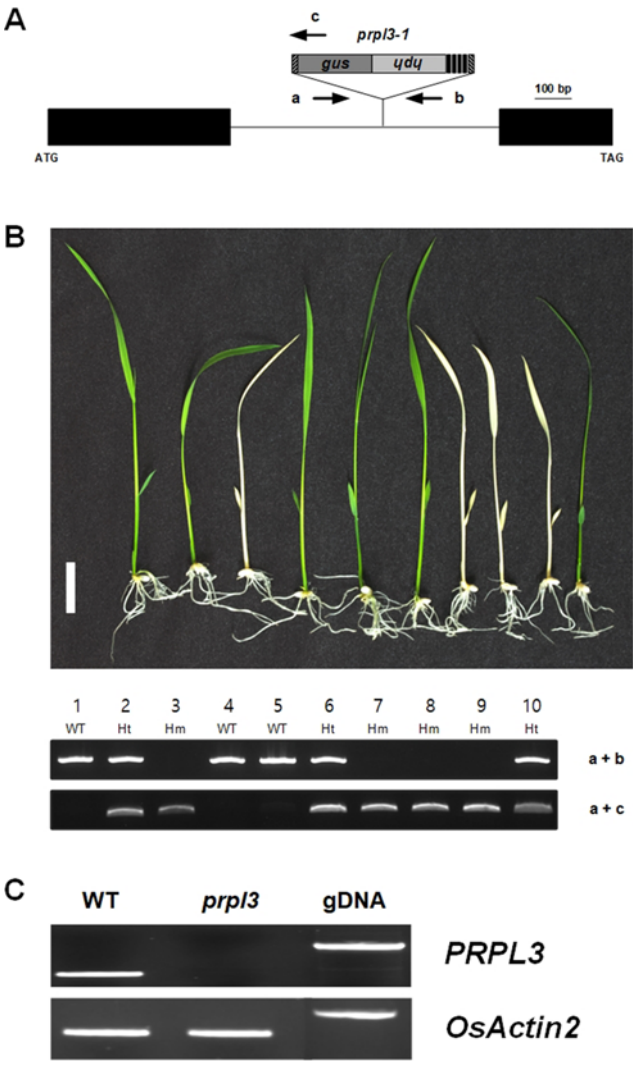


**Fig. 1.** Phenotype of *prpl3* mutant. (A) Genomic structure of *PRPL3* and T-DNA insertion position. Black boxes represent exons; intervening lines represent introns; a, b, and c primers were used for genotyping. (B) Phenotypes and genotypes of 7-day-old *PRPL3* plants. Albino phenotypes are co-segregated with T-DNA insertion. WT, Ht, and Hm denote wild type, heterozygote, and homozygote, respectively. Scale bar = 2 cm. (C) RT-PCR analysis of *PRPL3* transcripts in WT and *prpl3 (albino)* mutant. *OsActin2* was used as a control.

a T-DNA insertion in *PRPL3* (*LOC_Os02g04460*). The *PRPL3* mutant carrying a T-DNA insert in the middle of an intron was named *prpl3-1* (http://signal.salk.edu/cgi-bin/RiceGE; PFG_3A-02184) (Fig. 1A). The *PRPL3* gene encodes the 50S plastid ribosomal protein L3, consisting of 271 amino acids and a predicted chloroplast transit peptide region (cTP) at its N-terminus of 96 amino acids in length (Emanuelsson et al. 1999; Schein et al. 2001; http://www.cbs.dtu.dk/services/ChloroP/). Genotyping analysis showed that homozygous plants for each T-DNA tagged line displayed seedling-lethal albino phenotypes, indicating the mutations were recessive (Fig. 1B). RT-PCR analyses revealed that the *prpl3* mutant did not produce full-

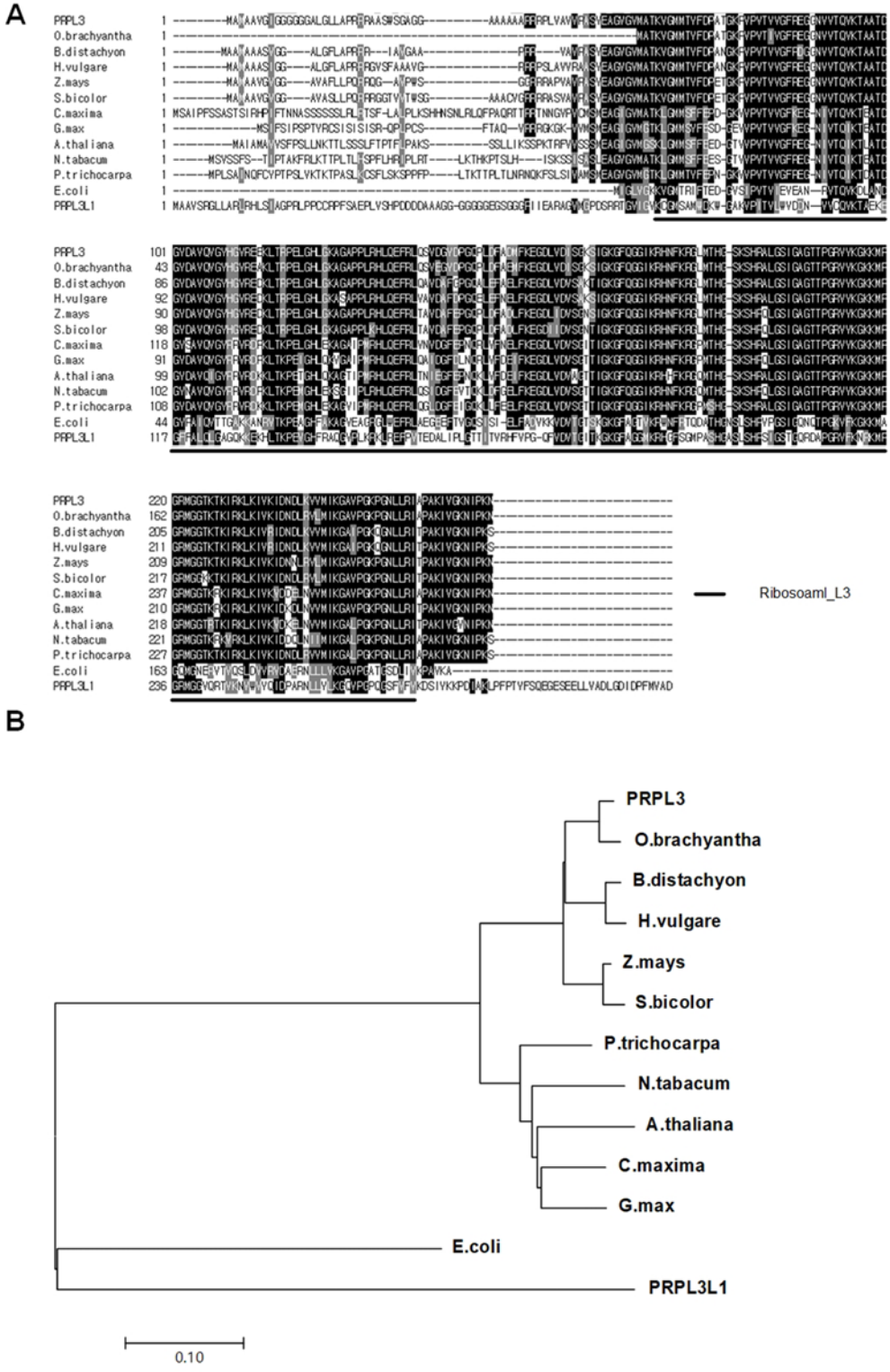


**Fig 2.** Multiple sequence alignment and phylogenetic tree of PRPL3 family. (A) Multiple sequence alignment of plant PRPL3 and *E. coli*. Black shading, identical residues; gray shading, similar residues. The sequence was multiple-aligned with *PRPL3* (XP_015625363), *PRPL3L1* (XP_015617381), *N. tabacum* (XP_016507364), *A. thaliana* (NP_181831), *B. distachyon* (XP_003574295), *H. vulgare* (BAJ95950), *Z. mays* (NP_001339224), *S. bicolor* (XP_002453273), *O. brachyantha* (XP_006646861), *P. trichocarpa* (XP_024439964), *C. maxima* (XP_022999904), *G. max* (XP_003522341), and *E. coli* (STI10773). (B) Phylogenetic tree constructed using the neighbor-joining method with the MEGA 7 program (Kumar et al., 2016). Bootstrap values indicated the number of amino acid substitutions per site.

length transcripts, demonstrating it was mRNA null (Fig. 1C). In the rice genome, another homologous gene, *PRPL3L1* (*LOC_ Os01g14830)*, was existed, with 47% homology to PRPL3 in the ORF sequence (Fig. 2A; Fig. S1). Sequence alignment indicated that the PRPL3 protein sequence in rice is homologous with that of other higher plants, including *Zea mays, Sorghum bicolor, Brachypodium distachyon, Hordeum vulgare, Arabidopsis thaliana*, and *Nicotiana tabacum*. Phylogenetic tree analysis suggested that PRPL3L1 was divided in to different clade, and PRPL3 was closely related to monocotyledonous plant species, a group that includes maize, sorghum, *Brachypodium*, and barley (Fig. 2A, B).

PRPL3 Mutant Present Impaired Chloroplast Development

We investigated the development of chloroplasts in the albino mutant by examining the ultrastructures of chloroplasts in the leaves of 10 day-old seedlings with transmission electron microscopy (TEM). Normal chloroplasts were observed in WT control plants; however, in the albino mutants, abnormal plastids were observed, and chloroplast development appeared to be arrested at the proplastid stage (Fig. 3A, B). The albino mutant showed reduced chlorophyll and carotenoid content compared to WT, likely due to disrupted chloroplast development (Fig. 3C). These observations imply that the *prpl3* mutation resulting in impaired plastid ribosomal proteins had a significant influence on chloroplast development.

Targeted Mutagenesis of PRPL3 by CRISPR/Cas9

We examined mutant alleles for *PRPL3* by designing a target site within the first exon of *PRPL3*; CRISPR/Cas9 on a Nippon bare background was then conducted to generate these mutants (Fig. 4A). This process produced 16 independent transgenic lines with albino phenotypes and 19 heterozygous plants (green phenotypes). Sequencing analysis of the flanking

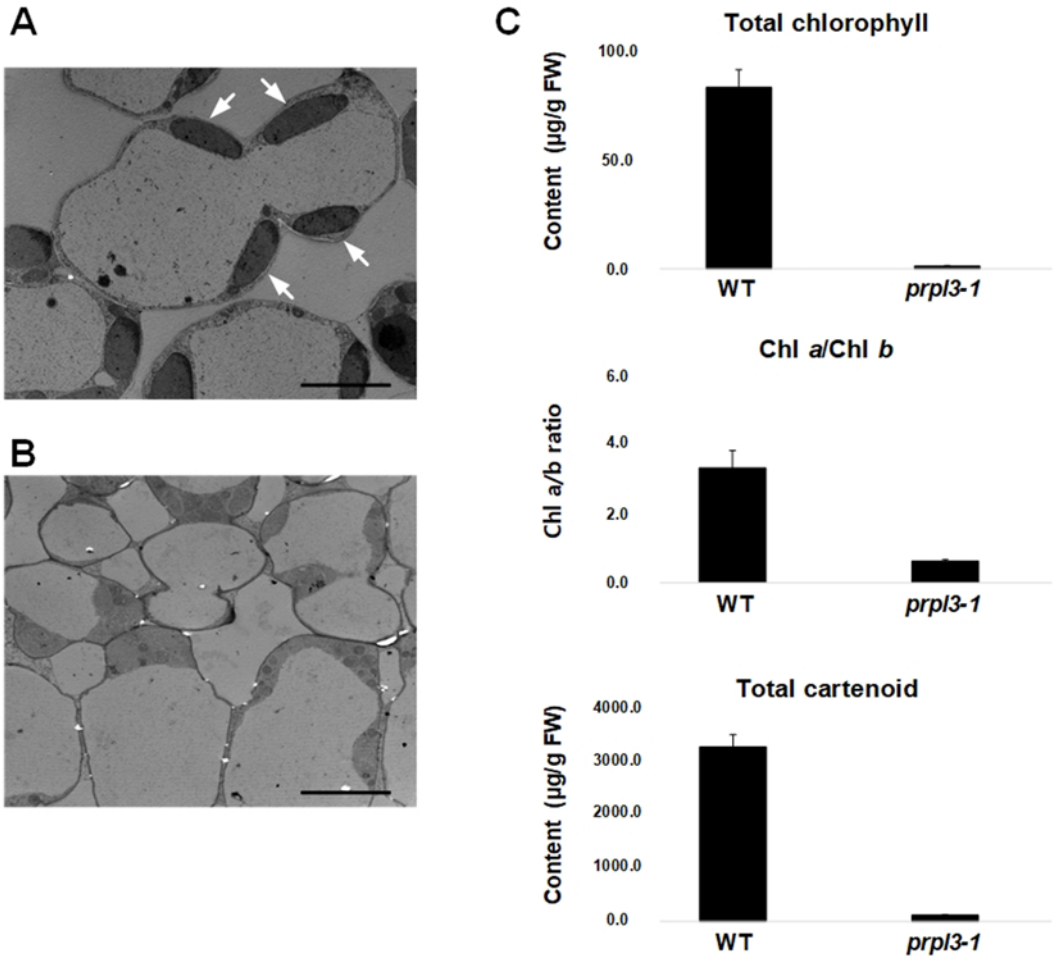


**Fig. 3.** TEM analysis and chlorophyll measurement of wild type and albino mutants. (A) Section of WT chloroplast. (B) Sections of *prpl3* chloroplast. Arrowhead indicates the chloroplast. Scale bar = 5 μm. (C) Pigment contents of WT and *prpl3* mutant. Total chlorophyll and carotenoid concentrations were obtained from 10-day-old plants. All values are means of three biological repeats. Error bars indicate standard deviation (SD).

region of target site showed that KO #1 had deletions in both chromosomes and KO #2 and KO #3 had a single base insertion (Fig. 4B; Fig. S2). Each homozygous mutant line for *PRPL3* phenocopied the albino *prpl3-1* mutant phenotype and was seedling lethal (Fig. 4C), implicating the *PRPL3* gene in the albino seedling lethal phenotype.

## Spatiotemporal Expression Patterns of PRPL3

We used qRT-PCR to examine the expression patterns of *PRPL3* in WT plants. *PRPL3* transcripts were detected in the young root, young shoot, mature flag leaf, and immature panicles. Expression of *PRPL3* was higher in the young shoot, mature flag leaf, and young root than in either the immature (~3 cm) or mature (~14 cm) panicles (Fig. 5A). We also examined the expression of *PRPL3* in the Rice Expression Profile Database and found that this expression pattern was relatively similar to those already documented (RicexPro: (http://ricexpro.dna.affrc.go.jp/) (Fig. S3). Binary vectors used for the generation of the PFG rice T-DNA insertional mutant population contained a promoterless reporter gene such as *GUS* or *GFP* for the purpose of gene trap (Jeong et al. 2002; Ryu et al. 2004; Kim et al. 2013). The expression of GUS driven by the *PRPL3* promoter and the first exon containing 532 bp in the T-DNA tagged line, PFG_3A-02184, was examined because the T-DNA was inserted in the middle of an intron, resulting in in-frame fusion to the *GUS* gene (Fig. 5B). In the homozygous seedling of PFG_3A-02184, GUS expression was detected in the root, leaf sheath, and leaf blade because plastids are also present in the root and chloroplasts. At mature stages in heterozygous plants, GUS expression was observed in the spikelet, internode, and in immature seeds (Fig. 5C). The GUS staining pattern mirrored the *PRPL3* mRNA expression pattern analyzed by quantitative RT-PCR, although the GUS staining appeared more intense in the green tissues (leaf and leaf sheaf) than in the root and young panicles. Taken together, our results indicates that *PRPL3* is constitutively expressed in the various tissues and may functions in the green tissues.

## Albino Mutations Caused by Impaired Ribosomal Proteins Affect Expression of Related Genes

We then investigated the expression levels of genes involved in chlorophyll biosynthesis, photosynthesis, and ribosome development in the albino mutant compared to WT plants.

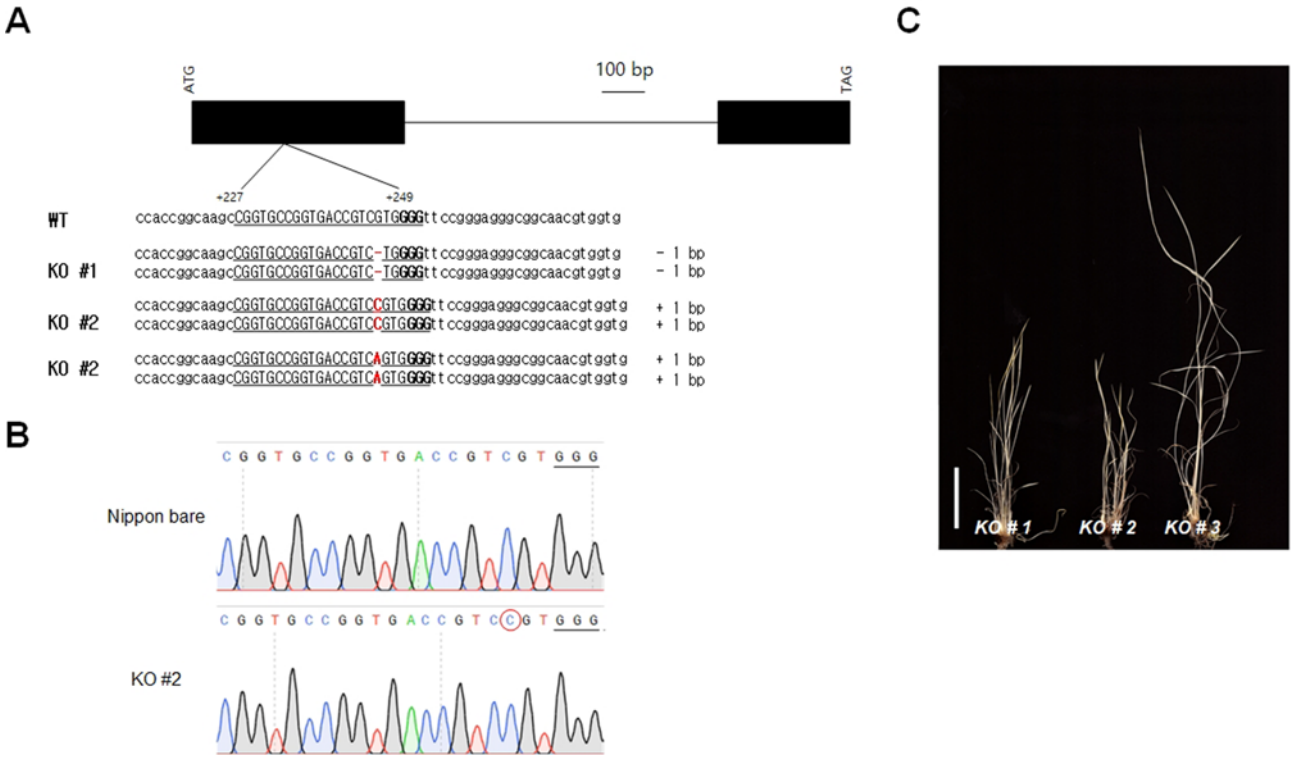


**Fig. 4.** Phenotypes of knock-out (*KO*) mutants of *PRPL3* in Nippon bare background. (A) Schematic diagram of sgRNA target site in *PRPL3*. Exons and introns are indicated by rectangles and lines, respectively. The target sequence and the protospacer adjacent motif (PAM) sequences of *PRPL3* sgRNA are underlined. The number of changed bases is shown on the right. "–" indicates deleted sequences; "+" indicates the inserted sequences. The indels are shown in red letters or dashes. (B) Chromatogram of sequence of the KO #2 mutant line. The cytosine inserted is marked by a red circle. The PAM sequences of the *PRPL3* sgRNA are underlined. (C) Phenotypes of *PRPL3* mutant alleles generated by CRISPR/Cas9 method. Scale bar = 2 cm.

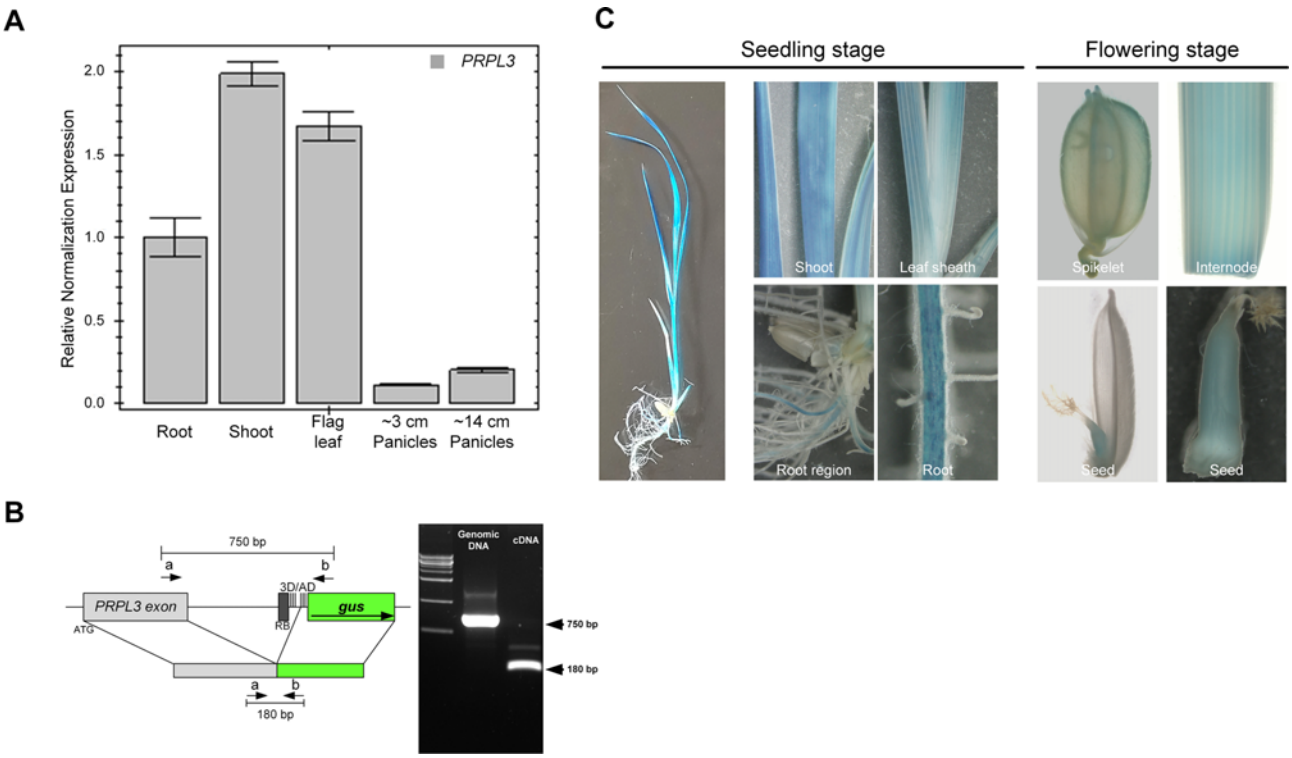


**Fig. 5.** Expression pattern analysis of *PRPL3*. (A) Relative expression levels of *PRPL3* in different organs. RNA was isolated from 10-day-old root, shoot, flag leaf, young panicle (~3 cm), and young panicles (~14 cm). Error bars indicate standard deviation (SD). (B) Schematic diagram of *PRPL3-GUS* fusion transcript. Fusion transcript was generated by splicing between the donor of the first intron and the first acceptor of the 3SD/AD (Jeon et al. 2000). RT-PCR analysis of *PRPL3-GUS* fusion transcript. PCR performed with the gene specific primer (a) and GUS-specific primer (b) using the genomic DNA and cDNA of *PRPL3* heterozygote plants. (C) PRPL3-GUS staining analysis of the root, leaf sheath, and leaf blades from 10-day-old plants in the homozygous plants and flowering stages including spikelet, internode, and early seed development in the heterozygous plants.

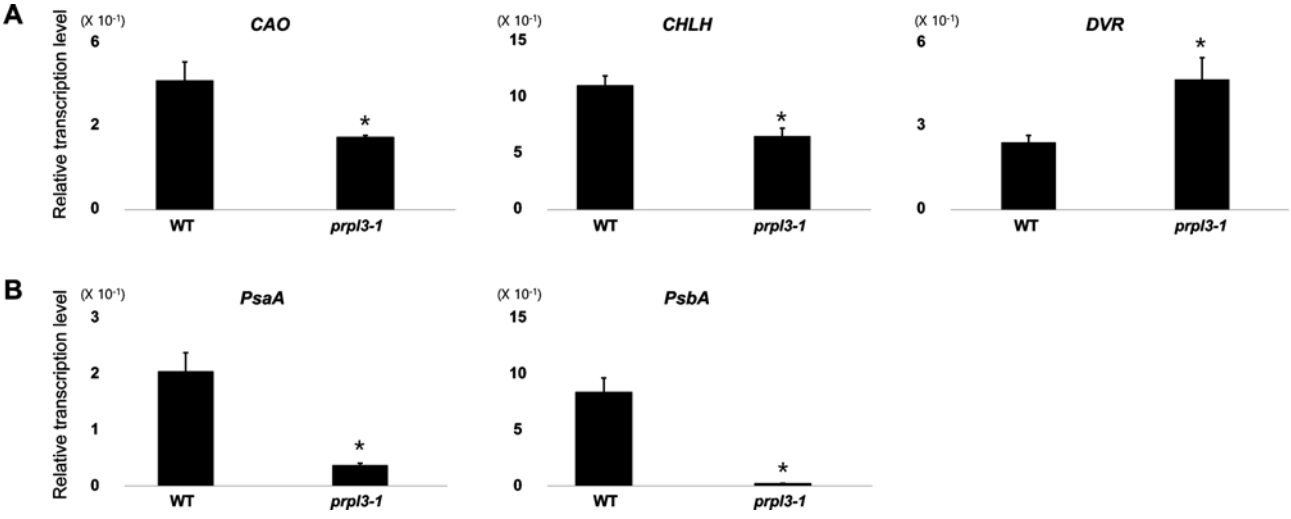


**Fig. 6.** Relative expression analysis of chloroplast-associated genes (A, B) Relative expression levels of chlorophyll biosynthesis-related genes and photosynthesis-related genes in WT and *prpl3* mutant. All values are means of three biological repeats; error bars indicate standard deviation (SD). Levels of significant difference are indicated by * ($P < 0.05$).

The expression levels of chlorophyll biosynthesis genes *chlorophyll a oxygenase1 (CAO1), Mg chelatase H subunit (CHLH),* and *divinyl reductase (DVR*) were examined. As shown in Fig. 6A, the expression of *CAO1* and *CHLH* genes was reduced in both mutants compared to WT plants. However, transcript levels of *DVR* were increased in the *prpl3* mutant. We also examined the expression levels of the photosynthesis genes *photosystem I P700 chlorophyll a*

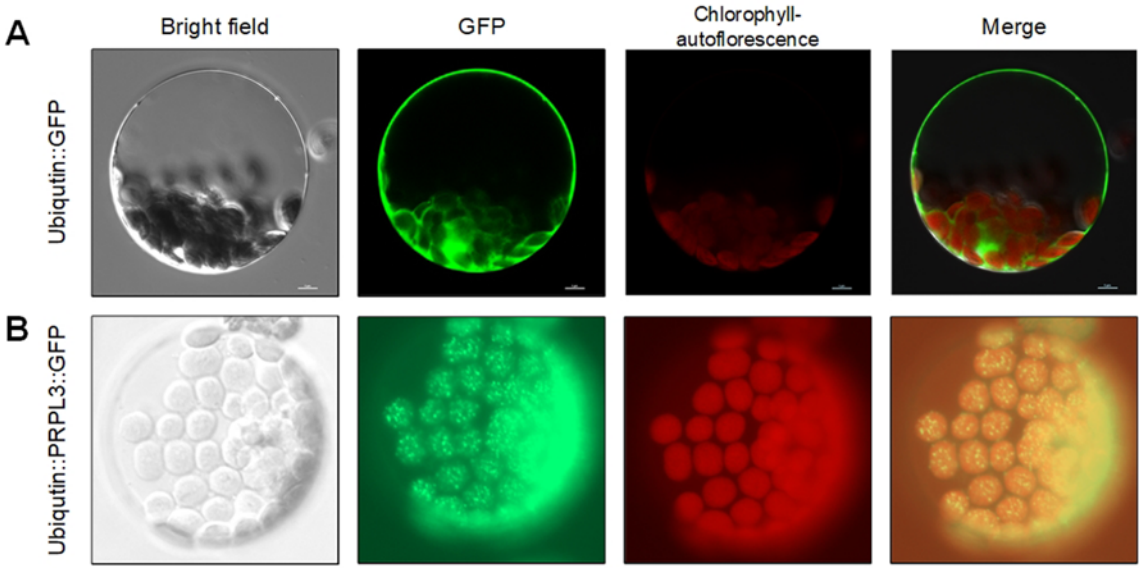


**Fig. 7.** Subcellular localization of PRPL3 protein. Transient expression of p*Ubi*::*GFP* in the Arabidopsis protoplast. (B) Transient expression of p*Ubi*::*PRPL3*::*GFP* in the Arabidopsis protoplast. p*Ubi*::*GFP* vector was used as a control. GFP pseudocolored - green, chloroplast autofluorescence pseudocolored – red. Scale bar = 5 μm.

*apoprotein A1 (psaA)* and *photosystem II protein D1 (psbA)* which encode photosystem components. Expressions of both the *psaA* and *psbA* genes were down-regulated in the *prpl3* mutant compared to WT plants (Fig. 6B).

### Subcellular Localization of PRPL3

Subcellular localization of PRPL13 was reported in the chloroplast, as predicted (Song et al. 2014). PRPL3 were predicted to localize to chloroplasts by ChloroP (Emanuelsson et al. 1999; Schein et al. 2001). To test actual subcellular localization of PRPL3 protein, p*Ubi*:*PRPL3*:*GFP* and p*Ubi*::*GFP* (empty vector) were transfected into Arabidopsis protoplasts, with the empty GFP vector serving as a negative control. Inverted fluorescent microscopy was applied to observe green florescence signal in the protoplasts 12 hours after transformation. The protoplasts transformed with the empty GFP vector displayed green fluorescent signals in the cytoplasm and the nucleus (Fig. 7A); however, green fluorescent signals from PRPL3-GFP fusion proteins were colocalized with chlorophyll autofluorescence in the chloroplasts, suggesting the PRPL3 protein localizes there (Fig. 7B).

## Discussion

Chloroplasts are cellular organelles that perform photosynthesis on behalf of plants. When chloroplast development or chlorophyll biosynthesis is disrupted, leaf color phenotypes are altered (Jung et al. 2003). The development of a functional chloroplast is controlled by multiple genetic factors, particularly nuclear-encoded factors. PRPs are crucial for ribosome biogenesis, plastidial protein biosynthesis, and early chloroplast development (Lin et al. 2015). Many PRP mutants have been identified in higher plants, with some shown to participate in many different biological processes in Arabidopsis (Romani et al. 2012). However, few studies have reported on the functions of PRPs in rice. The *asl1* mutant was the first plastid ribosomal protein isolated in rice and shows an albino lethal phenotype at the seedling stage. *ASL1* encodes PRPS20, and a mutation therein disrupts the expression of plastid and nuclear genes associated with chloroplast development (Gong et al. 2013). Mutation of the rice *ASL2* gene encoding PRPL21 causes chloroplast developmental defects and seedling death (Lin et al. 2015). The *AL1* gene encodes *PRPL12*, one of the genes involved in chloroplast biogenesis that participates in ribosomal assembly (Zhao et al. 2016). The rice *tcd11* mutant displayed an albino phenotype at low temperatures, similarly to the *wlp1* mutant. *TCD* encodes *PRPS6*, and *WLP1* encodes *PRPL13* (Wang et al. 2017; Song et al. 2014). *WGL2 (white green leaf 2)* encode plastid ribosomal protein S9, which mutant shows an albino phenotype at three leaf stage, and then gradually transitioned to green through the later developmental stages (Qiu et al. 2018).

Previous studies have shown that many genes participate in leaf color development using mutant analyses of rice. For example, *OsCHlH* (Jung et al. 2003), *OsCAO1*, and *OsCAO2* (Lee et al. 2005; Yang et al. 2016), *OsDVR* (Wang et al. 2010), *OsGluRs* (Liu et al. 2007), *YGL1* (Wu et al. 2007), *OsSGR1* (Park et al. 2007), *OsPPR1* (Gothandam et al. 2005), *YSA* (Su et al. 2012), *WGL2* (Qiu et al. 2018), and *VAL1* (Zhang et al. 2018) mutants all present with altered

leaf colors. Many PRP mutants have been identified in higher plants, as well, with some PRPs shown to contribute to a diverse array of biological processes including embryonic development, photosynthesis, and plant height in Arabidopsis (Romani et al. 2012).

In this study, we isolated rice mutant presenting with an albino seedling lethality phenotype, wherein *PRPL3* gene was disrupted by tagged T-DNA. The *prpl3* mutant exhibited altered pigment contents and defective chloroplast development. The phenotype of the *PRPL3* mutant was verified with additional mutant alleles generated by CRISPR/Cas9 systems, indicating that the phenotype was caused by impaired *PRPL3* gene function. Endogenous expression of *PRPL3* was observed through GUS staining wherein the promoter region plus 532 bp of the first exon of *PRPL3* was fused to the *GUS* gene from the T-DNA. PRPL3-GUS was found in the root, shoot, leaf sheath, and leaf blade at the seedling stage, and at the mature stage, was observed in the mature spikelet, internode, and immature seeds. Results of qRT-PCR analyses support this *GUS* expression data and indicated that PRPL3 is important for the general development of rice plants. PRPL3 was also found to localize to chloroplasts as evidenced by subcellular localization experiments using a construct for PRPL3-GFP fusion proteins, implying that PRPL3 protein is a component of the plastidial ribosome. Reduced expression of genes involved in chlorophyll biosynthesis (*CAO1*, *CHLH*) and photosynthesis (*psaA, psbA*) was observed in *prpl3* mutant. In the chloroplast and mitochondria, inter-organellar signaling (anterograde and retrograde signal) is important for controlling the organellar gene expression, metabolic and development of chloroplast and mitochondria. Retrograde signaling regulates the expression of nuclear-encoded chloroplast genes in response to the metabolic and/or developmental state of the plastid (Hess et al. 1993, 1994; Pesaresi et al. 2007; Kleine et al. 2009; Moller 2009). In *prpl3* mutant, the expression of chloroplast biosynthesis gene, *DVR*, was increased suggesting that a retrograde signaling may exist from chloroplast to nucleus. Also, lower chlorophyll and carotenoid contents were observed in the *prpl3* mutant, as well. Our findings suggest that PRPL3 is critical for chloroplast development, representing key components of chloroplast ribosomal proteins.

## Materials and Methods

### Plant Growth

Rice (*Oryza sativa* cv. Dongjin) lines used in this study were isolated from a T-DNA insertional mutant population (Jeon et al. 2000). Surface-sterilized T2 seeds of *prpl3-1* and wild type (WT) plants were germinated in half-strength Murashige and Skoog (MS) medium with 3% sucrose and 0.4% phytagel. Plants were grown in a greenhouse, and PCRs for genotyping were performed. PCR primers are listed in Supplementary Table 1.

### Generation of CRISR/Cas9 Plants

To investigate the function of *PRPL3* gene, we designed knockout target sequence (227-246$^{th}$ nucleotide from start codon) using the CRISPRdirect (Naito et al. 2015; https://crispr.dbcls.jp) and generated the U3p-gRNA construction in pRGEB32 vector (Addgene plasmid # 63142; Xie et al. 2015). After the plasmid was introduced into the *Agrobacterium tumefaciens* LBA4404, transgenic plants were generated using the *Agrobacterium*-mediated transformation in WT (Nippon bare), as previously reported (Lee et al. 1999; Lee et al. 2007).

### Subcellular Localization

Subcellular localization of PRPL3 was investigated by amplifying full length ORFs without a stop codon from WT plants using primers containing the BamHI and SpeI sites (Supplementary Table 1); these were introduced into vector pGA3651 (*pUbi::MSC::GFP)* which contains the maize (*Zea mays*) ubiquitin promoter and GFP protein (Kim et al. 2009). The GFP empty vector (pGA3651) and *pUbi:: PRPL3-GFP* vector were transfected into Arabidopsis protoplast cells (Yoo et al. 2007), and the transformed protoplasts were observed in the GFP and RFP (autofluorescence) channel and with a Nikon Eclipse Ti2 inverted fluorescent microscope (Nikon, JAPAN), respectively.

### RNA Isolation and RT-PCR and Quantitative RT-PCR Analyses

Total RNA was isolated from different plant organs, including seedling root, shoot, mature flag leaf, and immature panicles using QIAzol lysis reagent following the manufacturer's instructions. First, cDNA was synthesized with 2 μg of total RNA using reverse transcriptase (Solgent, KOREA) with 10 ng of the oligo (dT) primers. Synthesized cDNAs were used as templates for reverse transcription PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR). QRT-PCR was performed with a CFX Connect Real-Time System (Bio-Rad) using TOYOBO THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). Rice *actin 2* and *UBQ5* were used as internal controls for RT-PCR and qRT-PCR, respectively. All primers for PCR are listed in Supplementary Table 1. Data are expressed as the mean ± SD of three biological replicates. Relative expression levels were calculated by $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

### Histochemical GUS Staining Assay

PRPL3-GUS activity was evaluated in 10-day-old seedling using the homozygous plants and mature PFG_3A-02184 heterozygous plants at reproductive stage. GUS staining was performed according to methods described previously (Jefferson 1989).

### Chlorophyll Content Measurement

The total chlorophyll and carotenoid contents were spectrophotometrically evaluated in 10-day-old *prpl3-1* and WT plants according to methods described previously (Lichtenthaler 1987). Briefly, leaves (approximately 0.1 g fresh weight) were cut and soaked in 100% acetone before spectrophotometry.

### Phylogenetic Analysis

PRPL3 sequences were used as a query in a BLASTP search at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) for phylogenetic analysis. The full-length sequences were aligned with *ClustalW* (DDBJ, https://www.ddbj.nig.ac.jp/index-e.html) and a neighbor-joining tree was constructed using MEGA version 7.0 software with the Poisson correction method; data are shown in units of the number of amino acid substitutions per site (Kumar et al. 2016).

Transmission Electron Microscope (TEM) Analysis

Leaf samples were fixed with 2.5% glutaraldehyde in 0.02 M phosphate buffer, pH 7.2, for 2 h at 4°C. After several washes in phosphate buffer, samples were post-fixed with 1% osmium tetroxide overnight at 4? and then again washed in phosphate buffer. Samples were dehydrated with a graded series of ethanol substituted with propylene oxide and then embedded in Epon 812 for 48 h at 60°C. After embedding, 100 nm sections were cut on a Leica Ultracut UCT ultramicrotome using a diamond knife (Diatome). Ultrathin sections were transferred onto 200 mesh copper grids and stained with 2% uranyl acetate for 20 min, and then with lead citrate (Reynolds, 1963) for an additional 10 min. Sections were examined under TEM with a Hitachi H-7650 operated at 80 kV.

## Acknowledgements

The authors thank Junhyun Lim and Sang Dae Yun for taking care of the Arabidopsis plants. This work was supported by a grant from the Netx-Generation BioGreen 21 program (Project No. PJ01369001), Rural Development Administration, Republic of Korea.

## Author's Contributions

JL and SKP designed the research project; JL performed the research and wrote the manuscript; SR performed the TEM analysis; SL generated CRISPR/Cas9 plants; JP analyzed the chlorophyll contents; SJ, SL, and GA participated in the discussion; JL, SJ, and SKP wrote the manuscript. The authors declare no conflict of interest.

## Supporting Information

**Fig. S1.** Sequence alignment of PRPL3 and PRPL31 protein.
**Fig. S2.** Comparing the amino acid sequences of *PRPL3* between WT and *KO* mutants.
**Fig. S3.** Expression patterns of *PRPL3* in different organs and developmental stages.
**Table S1.** List of primers used for qRT-PCR, DNA constructs and genotyping in this study.

## References

Ahmed T, Shi J, Bhushan S (2017) Unique localization of the plastid-specific ribosomal proteins in the chloroplast ribosome small subunit provides mechanistic insights into the chloroplastic translation. Nucleic Acids Res 45:8581–8595

Barkan A (1993) Nuclear mutants of maize with defects in chloroplast polysome assembly have altered chloroplast RNA metabolism. Plant Cell 5:389–402

Bieri P, Leibundgut M, Saurer M, Boehringer D, Ban N (2017) The complete structure of the chloroplast 70S ribosome in complex with translation factor pY. EMBO J 36:475–486

Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural networkbased method for predicting chloroplast transit peptides and their cleavage sites. Protein Sci 8:978–984

Gong X, Jiang Q, Xu J, Zhang J, Teng S, Lin D, Dong Y (2013) Disruption of the rice plastid ribosomal protein s20 leads to chloroplast developmental defects and seedling lethality. G3 (Bethesda) 3:1769–1777

Gothandam KM, Kim ES, Cho H, Chung YY (2005) OsPPR1, a pentatricopeptide repeat protein of rice is essential for the chloroplast biogenesis. Plant Mol Biol 58:421–433

Han CD, Coe EH Jr, Martienssen RA (1992) Molecular cloning and characterization of iojap (ij), a pattern striping gene of maize. EMBO J 11:4037–4046

Hess WR, Prombona A, Fieder B, Subramanian AR, Borner T (1993) Chloroplast rps15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J. 12:563–571

Hess WR, Muller A, Nagy F, Borner T (1994) Ribosome-deficient plastids affect transcription of light-induced nuclear genes: genetic evidence for a plastid-derived signal. Mol Gen Genet 242:305–312

Jefferson RA (1989) The GUS reporter gene system. Nature 342: 837–838

Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G (2000) T-DNA insertional mutagenesis for functional genomics in rice. Plant J 22:561–570

Jeong DH, An S, Kang HG, Moon S, Han JJ, Park S, Lee HS, An K, An G (2002) T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol 130:1636–1644

Jung KH, Hur J, Ryu CH, Choi Y, Chung YY, Miyao A, Hirochika H, An G (2003) Characterization of a rice chlorophyll-deficient mutant using the T-DNA gene-trap system. Plant Cell Physiol 44:463–472

Kim SL, Choi M, Jung KH, An G (2013) Analysis of the early-flowering mechanisms and generation of T-DNA tagging lines in Kitaake, a model rice cultivar. J Exp Bot 64:4169–4182

Kim SR, Lee DY, Yang JI, Moon S, An G (2009) Cloning Vectors for Rice. J Plant Biol 52:73–78

Kleine T, Maier UG, Leister D (2009) DNA transfer from organelles to the nucleus: the idiosyncratic genetics of endosymbiosis. Annu Rev Plant Biol 0:115–138

Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874

Lee S, Kim JH, Yoo ES, Lee CH, Hirochika H, An G (2005) Differential regulation of chlorophyll a oxygenase genes in rice. Plant Mol Biol 57:805–818

Lee S, Jeon JS, Jung KH, An G (1999) Binary vectors for efficient transformation of rice. J Plant Biol 42:310–316

Lee J, Park JJ, Kim SL, Yim J, and An G (2007) Mutations in the rice liguleless gene result in a complete loss of the auricle, ligule, and laminar joint. Plant Mol Biol 65:487–499

Lin D, Jiang Q, Zheng K, Chen S, Zhou H, Gong X, Xu J, Teng S, Dong Y (2015) Mutation of the rice ASL2 gene encoding plastid ribosomal protein L21 causes chloroplast developmental defects and seedling death. Plant Biol (Stuttg) 17:599–607

Lichtenthaler HK (1987) Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol 148:350–382

Liu W, Fu Y, Hu G, Si H, Zhu L, Wu C, Sun Z (2007) Identification and fine mapping of a thermo-sensitive chlorophyll deficient mutant in rice (*Oryza sativa* L.). Planta 226:785–795

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408

Ma ZR, Dooner HK (2004) A mutation in the nuclear-encoded plastid ribosomal protein S9 leads to early embryo lethality in maize. Plant J 37:92–103

Moller (2009) Plastids Annual Plant Reviews, Volume 13, Wiley-Blackwell

Morita-Yamamuro C, Tsutsui T, Tanaka A, Yamaguchi J (2004)

Knock-out of the plastid ribosomal protein S21 causes impaired photosynthesis and sugar-response during germination and seedling development in *Arabidopsis thaliana*. Plant Cell Physiol 45:781–788

Naito Y, Hino K, Bono H, Ui-Tei K (2015) CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. Bioinformatics 31:1120–1123

Park SY, Yu JW, Park JS, Li J, Yoo SC, Lee NY, Lee SK, Jeong SW, Seo HS, Koh HJ, Jeon JS, Park YI, Paek NC (2007) The senescence-induced staygreen protein regulates chlorophyll degradation. Plant Cell 19:1649–1664

Pesaresi P, Schneider A, Kleine T, Leister D (2007) Interorganellar communication. Curr Opin Plant Biol 10:600–606

Pesaresi P, Varotto C, Meurer J, Jahns P, Salamini F, Leister D (2001) Knock-out of the plastid ribosomal protein L11 in Arabidopsis: effects on mRNA translation and photosynthesis. Plant J 27:179–189

Qiu Z, Chen D, He L, Zhang S, Yang Z, Zhang Y, Wang Z, Ren D, Qian Q, Guo L, Zhu L (2018) The rice white green leaf 2 gene causes defects in chloroplast development and affects the plastid ribosomal protein S9. Rice (N Y)11:39

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain for electron microscopy. J Cell Biol 17:208

Rogalski M, Ruf S, Bock R (2006) Tobacco plastid ribosomal protein S18 is essential for cell survival. Nucleic Acids Res 34:4537–4545

Rogalski M, Schottler MA, Thiele W, Schulze WX, Bock R (2008) Rpl33, a nonessential plastid-encoded ribosomal protein in tobacco, is required under cold stress conditions. Plant Cell 20:2221–2237

Romani I, Tadini L, Rossi F, Masiero S, Pribil M, Jahns P, Kater M, Leister D, Pesaresi P (2012) Versatile roles of Arabidopsis plastid ribosomal proteins in plant growth and development. Plant J 72:922–934

Ryu CH, You JH, Kang HG, Hur J, Kim YH, Han MJ, An K, Chung BC, Lee CH, An G (2004) Generation of T-DNA tagging lines with a bidirectional gene trap vector and the establishment of an insertion-site database. Plant Mol Biol 54:489–502

Schein AI, Kissinger JC, Ungar LH (2001) Chloroplast transit peptide prediction: a peek inside the black box. Nucleic Acids Res 29:E8

Schippers JH, Mueller-Roeber B (2010) Ribosomal composition and control of leaf development. Plant Sci 179:307–315

Schultes NP, Sawers RJH, Brutnell TP, Krueger RW (2000) Maize high chlorophyll fluorescent 60 mutation is caused by an Ac disruption of the gene encoding the chloroplast ribosomal small subunit protein 17. Plant J 21:317–327

Song J, Wei X, Shao G, Sheng Z, Chen D, Liu C, Jiao G, Xie L, Tang S, Hu P (2014) The rice nuclear gene WLP1 encoding a chloroplast ribosome L13 protein is needed for chloroplast development in rice grown under low temperature conditions. Plant Mol Biol 84:301–314

Su N, Hu ML, Wu DX, Wu FQ, Fei GL, Lan Y, Chen XL, Shu XL, Zhang X, Guo XP (2012) Disruption of a Rice Pentatricopeptide repeat protein causes a seedling-specific albino phenotype and its utilization to enhance seed purity in hybrid Rice production. Plant Physiol 159:227–238

Tiller N, Bock R (2014) The translational apparatus of plastids and its role in plant development. Mol Plant 7:1105–1120

Tiller N, Weingartner M, Thiele W, Maximova E, Schottler MA, Bock R (2012) The plastid-specific ribosomal proteins of Arabidopsis thaliana can be divided into non-essential proteins and genuine ribosomal proteins. Plant J 69:302–316

Wang P, Gao J, Wan C, Zhang F, Xu Z, Huang X, Sun X, Deng X (2010) Divinyl chlorophyll(ide) a can be converted to monovinyl chlorophyll(ide) a by a divinyl reductase in rice. Plant Physiol 153:994–1003

Wang WJ, Zheng KL, Gong XD, Xu JL, Huang JR, Lin DZ, Dong YJ (2017) The rice TCD11 encoding plastid ribosomal protein S6 is essential for chloroplast development at low temperature. Plant Sci 259:1–11

Wu Z, Zhang X, He B, Diao L, Sheng S, Wang J, Guo X, Su N, Wang L, Jiang L, Wang C, Zhai H, Wan J (2007) A chlorophyll-deficient rice mutant with impaired chlorophyllide sterification in chlorophyll biosynthesis. Plant Physiol 145:29–40

Xie K, Minkenberg B, Yang Y (2015) Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. Proc Natl Acad Sci USA 112:3570−3575

Yamaguchi K, Subramanian AR (2003) Proteomic identification of all plastidspecific ribosomal proteins in higher plant chloroplast 30S ribosomal subunit - PSRP-2 (U1A-type domains), PSRP-3 alpha/beta (ycf65 homologue) and PSRP-4 (thx homologue). Eur J Biochem 270:190–205

Yamaguchi K, Beligni MV, Prieto S, Haynes PA, Mcdonald WH, Mayfield SP (2003) Proteomic characterization of the Chlamydomonas reinhardtii chloroplast ribosome. Identification of proteins unique to the 70S ribosome. J Biol Chem 278:33774–33785

Yang Y, Xu J, Huang L, Leng Y, Dai L, Rao Y, Chen L, Wang Y, Tu Z, Hu J, Ren D, Zhang G, Zhu L, Guo L, Qian Q, Zeng D (2016) PGL, encoding chlorophyllide a oxygenase 1, impacts leaf senescence and indirectly affects grain yield and quality in rice. J Exp Bot 67:1297–1310

Yin T, Pan G, Liu H, Wu J, Li Y, Zhao Z, Fu T, Zhou Y (2012) The chloroplast ribosomal protein L21 gene is essential for plastid development and embryogenesis in Arabidopsis. Planta 235:907–921

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572

Zhao DS, Zhang CQ, Li QF, Yang QQ, Gu MH, Liu QQ (2016) A residue substitution in the plastid ribosomal protein L12/AL1 produces defective plastid ribosome and causes early seedling lethality in rice. Plant Mol Biol 91:161–177

Zhang J, Yuan H, Yang Y, Fish T, Lyi SM, Thannhauser TW, Zhang L, Li L (2016) Plastid ribosomal protein S5 is involved in photosynthesis, plant development, and cold stress tolerance in Arabidopsis. J Exp Bot 67:2731–2744

Zhang T, Feng P, Li Y, Yu P, Yu G, Sang X, Ling Y, Zeng X, Li Y, Huang J, Zhang T, Zhao F, Wang N, Zhang C, Yang Z, Wu R, He G (2018) VIRESCENT-ALBINO LEAF 1 regulates leaf colour development and cell division in rice. J Exp Bot 69:4791–4804