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Newly identified *CSP41b* gene localized in chloroplasts affects leaf color in rice

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ABSTRACT

A rice mutant with light-green leaves was discovered from a transgenic line of *Oryza sativa*. The mutant has reduced chlorophyll content and abnormal chloroplast morphology throughout its life cycle. Genetic analysis revealed that a single nuclear-encoded recessive gene is responsible for the mutation, here designated as *lgl1*. To isolate the *lgl1* gene, a high-resolution physical map of the chromosomal region around the *lgl1* gene was made using a mapping population consisting of 1984 mutant individuals. The *lgl1* gene was mapped in the 76.5 kb region between marker YG4 and marker YG5 on chromosome 12. Sequence analysis revealed that there was a 39 bp deletion within the fourth exon of the candidate gene *Os12g0420200* (TIGR locus Os12g23180) encoding a chloroplast stem-loop-binding protein of 41 kDa b (CSP41b). The *lgl1* mutation was rescued by transformation with the wild type *CSP41b* gene. Accordingly, the *CSP41b* gene is identified as the *LGL1* gene. *CSP41b* was transcribed in various tissues and was mainly expressed in leaves. Expression of CSP41b-GFP fusion protein indicated that CSP41b is localized in chloroplasts. The expression levels of some key genes involved in chlorophyll biosynthesis and photosynthesis, such as *ChlD*, *ChlI*, *Hema1*, *Ygl1*, *POR*, *Cab1R*, *Cab2R*, *PsaA*, and *rbcL*, was significantly changed in the *lgl1* mutant. Our results demonstrate that *CSP41b* is a novel gene required for normal leaf color and chloroplast morphology in rice.

1. Introduction

Chlorophyll molecules, crucial for photosynthesis, capture light energy from the sun and convert it to chemical energy, giving photosynthetic plants their green color. Chlorophyll is arranged in and around the thylakoid membranes of chloroplasts. The fine-tuned control of chlorophyll metabolism is required for chloroplast development and maintenance [1]. Mutant plants with altered leaf color have been found in many plant species and extensively used to explore chlorophyll metabolism and chloroplast development [2].

To date, more than 10 genes involved in yellow-green (or chlorina) mutations have been identified in rice. *OsCAO1*, encoding chlorophyll a oxygenase, plays a major role in chlorophyll *b* biosynthesis [3]. *Ygl1* encodes chlorophyll synthase, which catalyzes conversion of chlorophyllide *a* into Chlorophyll *a* to complete the last step of Chlorophyll *a* biosynthesis [4]. *Cde1(t)* encodes glutamyl-tRNA synthetase, which is required for chlorophyll synthesis [2]. *OsDVR* encodes a 8-vinyl reductase, which is involved in the conversion of divinyl chlorophyll(ide) a to monovinyl chlorophyll(ide) *a* [5]. *Vyl* encodes plastid caseinolytic protease P6 subunit [6]. *Ygl2* encodes heme oxygenase 1, which catalyzes heme degradation [7]. *YGL138(t)*, encoding a putative signal recognition particle 54 kDa protein, might be involved in the translocation of chloroplast proteins [8]. *Chl1/Ygl3/YGL7* and *Chl9* respectively encode the ChlD and ChlI subunits of Mg-protoporphyrin IX chelatase, which catalyzes the conversion of protoporphyrin IX to Mg-protoporphyrin IX [9–11]. *Ygl6* encodes a putative 3-β-hydroxysteroid dehydrogenase/isomerase family protein that might play a role in the synthesis of brassinosteroids [12]. *YGL8*, encoding a UMP kinase, catalyses the phosphorylation of UMP to UDP [13]. In addition, transgenic rice plants with RNAi construct of *OsHAP3A* or *OsNOA1* displayed the yellow-green leaf phenotype [14,15]. *OsHAP3A* encodes a HAP3 subunit of a CCAAT-box binding complex, which functions in regulating chloroplast development [14]. *OsNOA1* encodes a circularly permuted GTPase, which is implicated in chloroplast ribosome assembly [15]. Among

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these genes mentioned above, only *Ygl6* was located on chromosome 12.

In this study, we isolated a novel light-green leaf mutant, which is designated as *lgl1* (light-green leaf 1), from lines of transgenic rice (*Oryza sativa* L.) carrying a transfer DNA (T-DNA) insertion. By map-based cloning, the *lgl1* gene was bracketed in a 76.5 kb region between marker YG4 and marker YG5 on chromosome 12. Sequence and complementation analysis indicated that the *lgl1* phenotype is caused by the mutation of *CSP41b* (chloroplast stem-loop-binding protein of 41 kDa b).

2. Materials and methods

2.1. Plant materials

The *lgl1* mutant is in the 'Nipponbare' (*Oryza sativa* L. ssp. *Japonica*) background. The F_1 and F_2 generations of a cross between the *lgl1* mutant and 'Nipponbare' was used for genetic and phenotypic analysis. The *lgl1* individuals from the F_2 and F_3 generations of a cross between the *lgl1* mutant and 'Longtepu' (*Oryza sativa* L. ssp. *indica*) were used for fine mapping of the *lgl1* gene. For pigment analysis, the latest fully expanded leaves were harvested from the *lgl1* mutant and 'Nipponbare' at the seedling stage (2 weeks old), tillering stage (5 weeks old), and maturity stage (12 weeks old) with 6 replicates each. Plants used in this study were grown under natural conditions in the paddy field at China National Rice Research Institute (119°57′E, 30°03′N).

2.2. Measurement of pigment content

Pigments were extracted from fresh leaf tissues with 80% acetone. The extract was measured with a spectrophotometer at 470, 645 and 663 nm (DU 800 UV/Vis Spectrophotometer, Beckman Coulter, CA, USA). Total chlorophyll (Chl), Chl a, and Chl b contents were determined as described by Arnon [16]. The concentration of carotenoid was determined according to Wellburn's method [17].

2.3. Electron microscopy

Samples from fully expanded leaves of 2 weeks old seedlings were fixed with 2.5% glutaraldehyde, postfixed with 1% OsO_4, then dehydrated in a graded series of ethanol, and finally transferred from 100% ethanol to absolute acetone. Samples were embedded in Spurr resin and sectioned using an ultramicrotome. The specimens were post-stained by uranyl acetate and lead citrate and observed in a transmission electron microscope (JEM-1230, JEOL, Tokyo, Japan).

2.4. Map-based cloning

New insertion-deletion (InDel) markers were developed based on the sequence differences between 'Nipponbare' and '9311' (*Oryza sativa* L. ssp. *indica*). The primer sequences of these InDel markers used are listed in Supplemental Table S1. PCR reactions for mapping were set up according to previous procedure [2]. PCR products were analyzed by polyacrylamide gel electrophoresis [18].

The specific fragments within the 76.5 kb target region from the *lgl1* mutant were amplified with Roche FastStart High Fidelity PCR System. These fragments were cloned into the pMD18-T vector and then sequenced by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd (Sangon, Shanghai, China).

2.5. Construction of complementary vector and rice transformation

A 5.7 kb genomic DNA fragment containing the wild type *CSP41b* gene was amplified by PCR with primers containing *EcoR*I site (forward) and *Hind*III site (reverse): 5′-GAATTCGTTCTTGCCCATTTCGGAT-3′, 5′-AAGCTTACTCGTAGTGAAGCACACGC-3′ and introduced into the pMD18-T vector. After being verified, the fragment was cloned into the pCAMBIA1300 vector. The construct was transformed into the *lgl1* mutants by the *Agrobacterium tumefaciens*-mediated genetic transformation method. Transgenic plants were detected by PCR with primers (MU1: 5′-GCAGGGCTACTATGGTGGTT-3′, 5′-AAGTTCAGTGAGATGGCGTTAA-3′) flanking the mutation site.

2.6. Phylogenetic analysis

The homologous proteins of LGL1 were searched with BLASTP using the LGL1 protein as query. Full-length amino acid sequences were aligned using the DNAMAN program. The neighbor-joining phylogenetic tree was constructed using MEGA ver. 4 [19]. The values for nodes were obtained from 1000 bootstrap replicates.

2.7. Subcellular localization analysis

To determine the subcellular localization of CSP41b proteins, synthetic genes were constructed in which the fusion protein CSP41b-green fluorescent protein (GFP) was expressed under the control of the CaMV 35S promoter. The cDNA sequences corresponding to the CDS of the *LGL1* gene were amplified by PCR using specific primers (5′-GGATCCAGATGGCAGCAACAGCCTC-3′, 5′-TCTAGAGACGCTGACGAGCTTCTT-3′) to generate the *BamH*I site at the 5′ and the *Xba*I site at the 3′ end. The resulting fragment was cloned into the pMD18-T vector, and then sequenced. The wild type *CSP41b* gene was digested and fused in frame to the 5′-front of the *GFP* gene. The CSP41b-GFP vector was transformed into rice protoplast. Rice protoplasts were mounted on glass slides and observed with a Zeiss LSM700 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA).

2.8. Quantitative real time PCR analysis

Total RNA was extracted and purified from various tissues using Qiagen RNeasy Plant Mini Kit and RNase-free DNase Set (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Indianapolis, USA) with an oligo dT primer. The real time PCR was performed using the 2x SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) on the Applied Biosystems 7900HT Real Time PCR System. The relative expression levels of each transcript were normalized to the *OsACT1* gene using the comparative C_T method. PCR was carried out as follows: preheating at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. The primer sequences are listed in Supplemental Table S2.

3. Results

3.1. The lgl1 mutant showed a phenotype of light-green leaves

The *light-green leaves* (*lgl1*) mutant was first found among T_1 transgenic rice lines in the 'Nipponbare' background. The *lgl1* mutant could be clearly distinguished from wild type during the entire growth period (Fig. 1A–D). Chlorophyll and carotenoid contents of the latest full expanded leaves isolated from the mutants and wild type plants at different growth stages were measured.

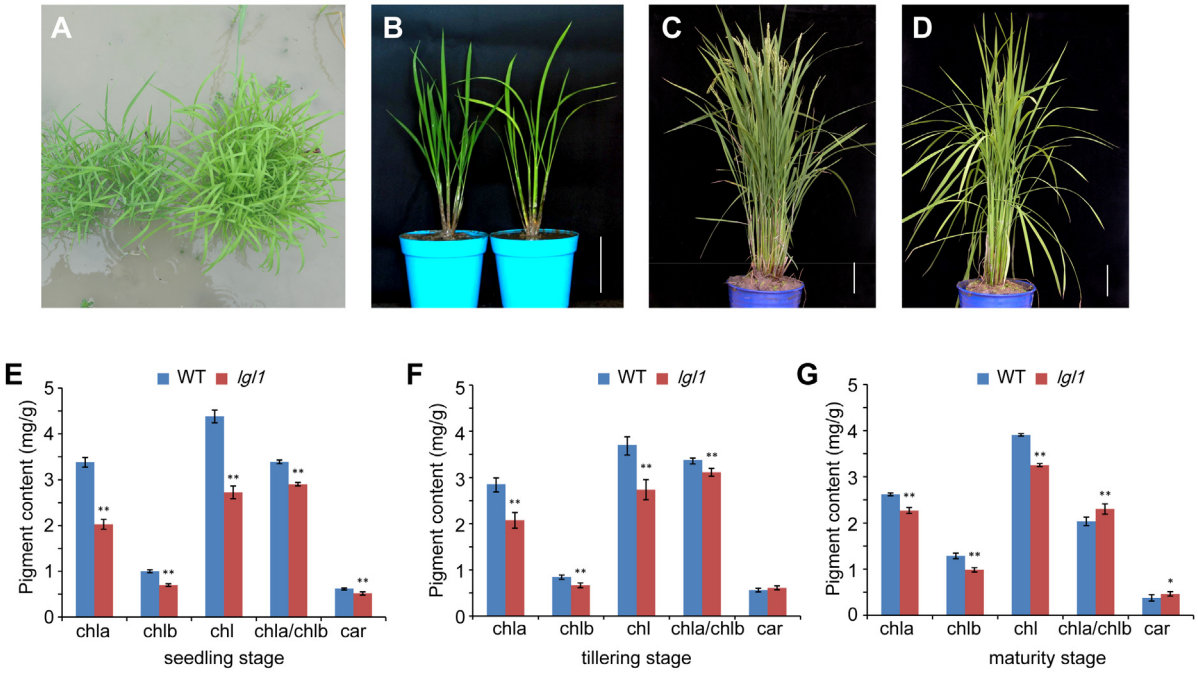

Fig. 1. Phenotypes and pigment contents of *lgl1* mutants.
(A) Morphology of wild type (WT) cultivar 'Nipponbare' (left) and the *lgl1* mutant (right) at the seedling stage. (B) Morphology of WT (left) and *lgl1* mutant (right) plants at the tillering stage. (C, D) Morphology of WT (C) and the *lgl1* mutant (D) at the maturity stage. (E) Pigment contents of WT and the *lgl1* mutant at the seedling stage. (F) Pigment contents of WT and the *lgl1* mutant at the tillering stage. (G) Pigment contents of WT and the *lgl1* mutant at the maturity stage. chl, chlorophyll; car, carotenoid. Values are means ± SD of 6 biological replicates. Scale bars = 10 cm.

The results indicated that leaves from the mutants contained less chlorophyll *a* and chlorophyll *b* than those from wild type plants at different growth stages (Fig. 1E–G). The *lgl1* mutants also had a lower chlorophyll *a* to chlorophyll *b* ratio and reduced carotenoid contents at the seedling stage (Fig. 1E). However, at the maturity stage, the *lgl1* mutants had a higher chlorophyll *a* to chlorophyll *b* ratio and higher carotenoid content (Fig. 1G).

The full expanded leaves from 30-day-old wild-type and *lgl1* mutant seedlings were analyzed by electron microscopy. No distinct differences in the size of chloroplasts were observed between the wild type and the mutant. However, the mutant had greater amounts of small starch grains compared with the wild type (Fig. 2A and B). Chloroplasts from *lgl1* mutant plants also contained a distorted thylakoid membrane system (Fig. 2C and D).

3.2. *Map-based cloning of the* lgl1 *gene*

The *lgl1* mutant was crossed with its parent cultivar 'Nipponbare'. The F_1 progenies exhibited the normal phenotype. In the F_2 generation, the ratio of wild-type to mutant individuals is 3:1 ($\chi^2 = 0.59 < \chi^2_{0.05} = 3.84$), which suggested that the *lgl1* phenotype was controlled by a single recessive nuclear gene.

To find the chromosomal location of *lgl1*, the genotypes of 21 F_2 *lgl1* individuals from a cross between the *lgl1* mutant and the *O. indica* cultivar 'Longtepu' were observed. The results revealed that the markers RM101 (at 48.2 cm) and RM1246 (at 65.3 cm) on chromosome 12 were linked to the *lgl1* gene. To map the *lgl1* gene with greater resolution, we developed six new InDel markers (Supplemental Table S1). The *lgl1* gene was further delimited to within 76.5 kb bracketed by L5 and L6 within the BAC clone OSJNBa0037L20 on chromosome 12 by using the six markers and 1984 F_2 and F_3 mutant individuals (Fig. 3). Sequence analysis of the 76.5 kb region found a deletion difference between wild type and the *lgl1* mutant. The *lgl1* allele carried a 39 bp deletion within the coding sequence of the fourth exon of the *CSP41b* gene (TIGR locus LOC_Os12g23180) (Fig. 3). *CSP41b*, encoding a chloroplast stem loop binding protein of 41 kDa, has been shown to be important for the proper functioning of the chloroplast using *Arabidopsis* [20,21]. Therefore, the *CSP41b* gene is most likely the candidate gene for *lgl1*.

3.3. *Transgenic complementation rescued the* lgl1 *phenotype*

To further confirm that the mutation in *CSP41b* is responsible for the *lgl1* phenotype, we constructed a recombinant binary vector containing the wild type *CSP41b* gene. The construct was introduced into the *lgl1* mutant using *Agrobacterium tumefaciens*-mediated genetic transformation. Transgenic plants were identified by PCR analysis with MU1 primer pairs. The PCR product in the *lgl1* mutant is 39 bp shorter than that of the wild type due to a deletion, whereas both of the products were observed in the CO1 complemented line, indicating the wild-type *CSP41b* gene has been successfully transformed into the *lgl1* mutant (Fig. 4B). Fifteen independent transgenic plants produced normal phenotype, like the wild type, indicating the *lgl1* phenotype was rescued (Fig. 4A). The results showed that the mutation of the *CSP41b* gene is responsible for the *lgl1* phenotype.

3.4. *Phylogenetic analysis of LGL1 and its homologous proteins*

The homologous proteins of the LGL1 protein were retrieved by a BLASTP search. CSP41b proteins were widely distributed among photosynthetic eukaryotes, including *Chlamydomonas reinhardtii* and *Physcomitrella patens*, which suggested an early origin of the CSP41b protein. There is only one copy of the CSP41b protein in all these organisms. Phylogenetic analysis indicated that the rice LGL1 has a closer phylogenetic relationship to CSP41b from grass family including maize and sorghum than it does with dicotyledonous species (Fig. 5). LGL1 shares considerable identity (68%, 221/324) with the CSP41b protein from *Chlamydomonas reinhardtii*,

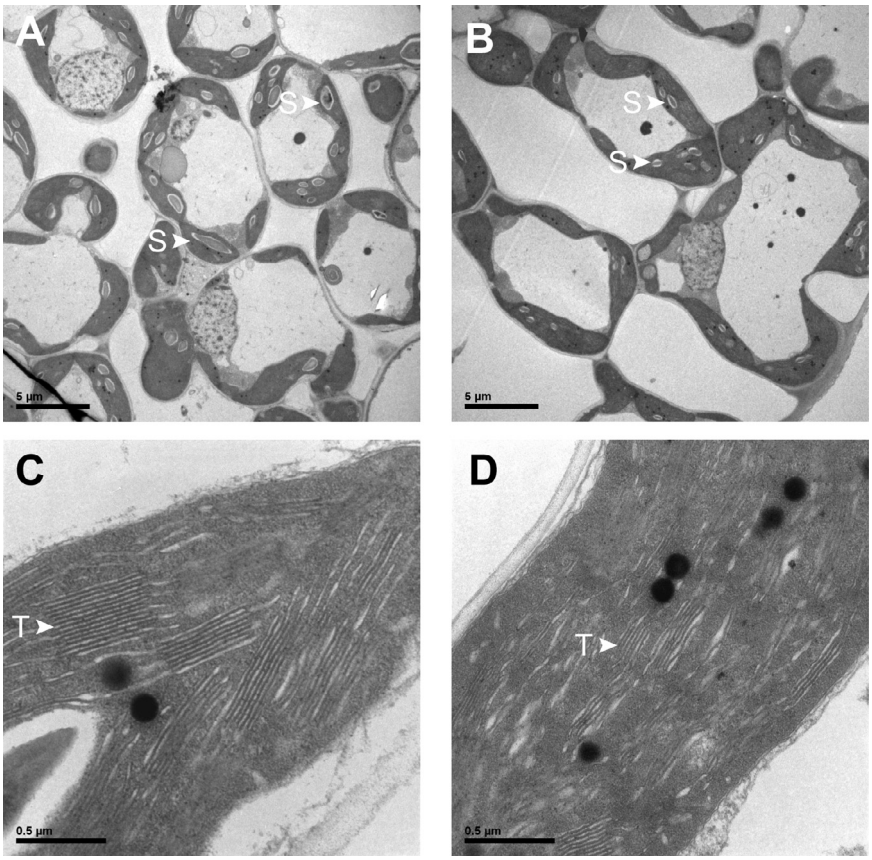

Fig. 2. Chloroplast ultrastructure of *lgl1* mutants at the seedling stage.
(A–D) Electron microscopic images of chloroplast of wild type (WT) cultivar 'Nipponbare' (A and C) and *lgl1* mutants (B and D). S, starch granule; T, thylakoid membrane system.

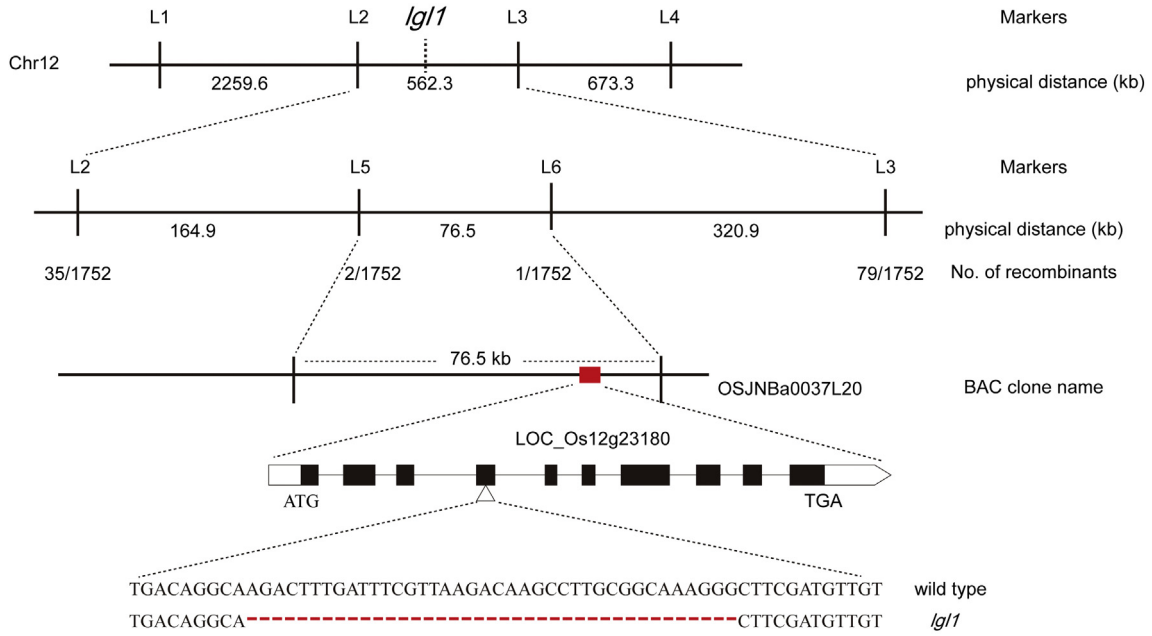

Fig. 3. Fine mapping and mutation site analysis of the *lgl1* gene.
The target gene was delimited to an interval of 76.5 kb between L5 and L6 within the BAC clone OSJNBa0037L20 on chromosome 12. The red rectangle indicates the *LGL1* gene (TIGR locus Os12g23180), which encodes a chloroplast stem-loop-binding protein of 41 kDa b (CSP41b). White and black boxes are exons; white boxes are untranslated regions; black boxes are the coding regions; the lines between two black boxes signify introns. The red dashed line indicates the 39 bp deletion in the *lgl1* mutant.

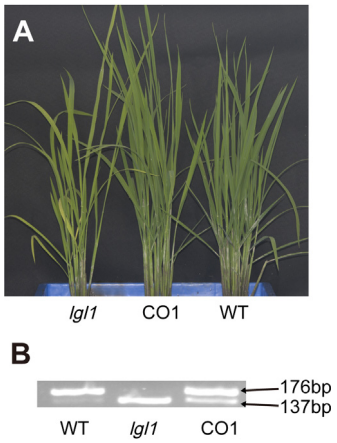

Fig. 4. Transgenic complementation experiment rescued the *lgl1* phenotype.
(A) Morphology of the *lgl1* mutant (left), transgenic *lgl1* plant with the *CSP41b* gene (CO1) (middle), and wild type (WT) cultivar 'Nipponbare' (right). (B) PCR detection of transgenic *lgl1* plants using the primer pair MU1 flanking the mutation site.

suggesting that this gene has been evolutionarily conserved among photosynthetic eukaryotes (Supplemental Fig. S1).

3.5. *The* CSP41b *gene encodes a chloroplast-targeted protein*

The TargetP program analysis showed that CSP41b is a chloroplast-targeted protein [22]. The subcellular localization of the protein was confirmed by fusing the *CSP41b* coding sequence with the GFP gene and introducing it into rice protoplast under the control of the cauliflower mosaic virus 35S promoter. GFP fluorescence of transgenic protoplast with CSP41b-GFP was co-localized with chlorophyll autofluorescence, whereas the control GFP proteins were localized to both the nucleus and the cytoplasm (Fig. 6A–D). This confirmed that LGL1 is a chloroplast-localized protein (Fig. 6E–H).

3.6. *Expression pattern of* LGL1

To determine the expression pattern of *LGL1*, we analyzed mRNA levels in various tissues, including root, panicle, leaf blade, leaf sheath and stem, from wild-type plants at the maturity stage (12 weeks old). The expression of *LGL1* was detected in all tissues with relatively high expression in leaves and relatively low expression in the other tissues (Fig. 7).

3.7. LGL1 *regulates the expression of chlorophyll biosynthesis and photosynthesis related genes*

Previous studies have shown that mutations that affect leaf color affect expression of chlorophyll biosynthesis and photosynthesis-related genes [11]. We also analyzed the expression transcripts of genes involved in chlorophyll biosynthesis, including *ChlD*, *ChlI*, and *ChlH* (Mg-chelatase D, I and H subunit), *Hema1* (glutamyl tRNA reductase), *Ygl1* (Chl synthetase) and *POR* (NADPH-dependent protochlorophyllide oxidoreductase) and genes associated with photosynthesis, including *Cab1R*, *Cab2R* (light-harvesting Chl a/b-binding proteins of PSII), *PsaA* (reaction center polypeptides of PSI), *PsbA* (reaction center polypeptides of PSII), and *rbcL* (the large subunit of RuBisCO) in wild type and the *lgl1* mutant at the maturity stage (12 weeks old) by quantitative real time PCR. Loss of function of *LGL1* resulted in significantly increased expression of *ChlD*, *ChlI*, *Hema1*, *Ygl1*, *POR*, *Cab1R*, *Cab2R*, and *PsaA* (Fig. 8). However, the transcript levels of *rbcL* were reduced in the *lgl1* mutant. There was no obvious difference in the expression of *ChlH* and *PsbA* (Fig. 8). The findings suggested that the *lgl1* mutation affects both chlorophyll biosynthesis and photosynthesis at a molecular level.

4. Discussion

In this study, we isolated a light-green leaf mutant *lgl1* from a transgenic rice line. Fine mapping showed that the *lgl1* gene was located within a 76.5 kb region between the INDEL markers L5 and L6 within BAC OSJNBa0037L20 on chromosome 12 (Fig. 3). Leaf color-related genes have not been reported previously in this region. Sequence and complementation analyses showed that the *LGL1* gene encoded a chloroplast stem-loop-binding protein of 41 kDa (CSP41b).

All photosynthetic eukaryotes encode two related proteins CSP41a and CSP41b that are of cyanobacterial origin [23–26]. *Arabidopsis* mutants that lack both CSP41 proteins are not viable, thus CSP41a and CSP41b may have evolved redundant functions [24]. However, CSP41b seems to be functionally more important than CSP41a. In *Arabidopsis*, the *csp41a* mutation does not result in obvious phenotype, whereas loss of *CSP41b* affects leaf coloration, chloroplast morphology, photosynthesis performance and circadian rhythms [21,24,27],. The CSP41b proteins are highly conserved between rice and *Arabidopsis* (Fig. 5; Supplemental Fig. S1). Not surprisingly, rice plants that have the *CSP41b* mutation have a phenotype of light-green leaves and altered chloroplast morphology (Figs. 1 and 2). The CSP41a proteins are less conserved among pho-

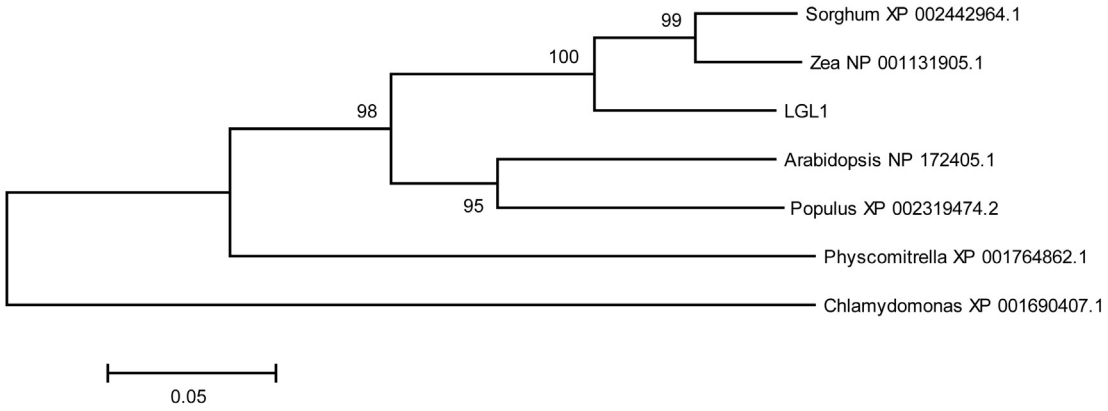

Fig. 5. Phylogenetic tree of LGL1 and its homologous proteins.
The tree was constructed using MEGA ver. 4. The full-length protein sequences used and the multiple alignments are shown in Supplemental Fig. S1. The values for nodes in the phylogenetic tree are from 1000 bootstrap replications.

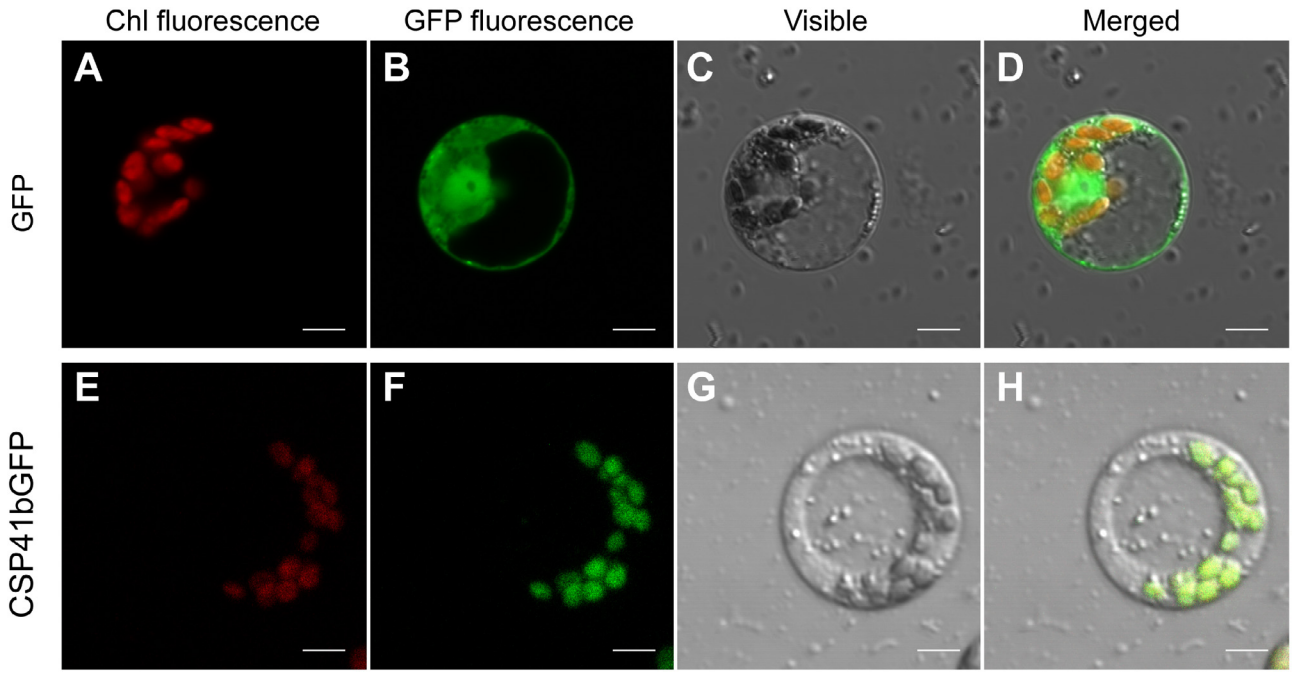

Fig. 6. Subcellular localization of the CSP41b-GFP protein in rice protoplasts.
(A–H) The 35S: GFP and 35S: CSP41b-GFP were transformed into rice protoplast. Chlorophyll (Chl) fluorescence (A and E), GFP fluorescence (B and F) and differential interference contrast images (C and G) in rice protoplasts were observed separately by confocal laser scanning microscopy and then the resulting images were merged (D and H). Scale bars = 5 μm.

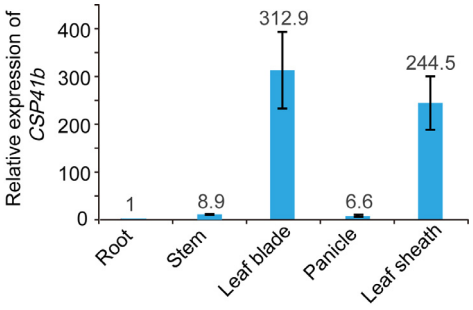

Fig. 7. Real time PCR analysis of *LGL1* transcript levels in different organs.
LGL1 transcript levels were normalized to the levels of *OsACT1* gene expression in respective samples. The levels of *LGL1* transcript in roots were arbitrarily set to 1. Values are the mean ± SD of 3 biological replicates.

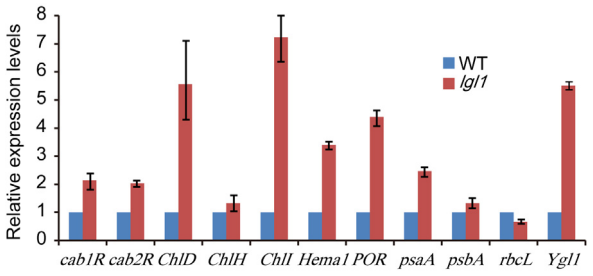

Fig. 8. The expression analysis of genes associated with chlorophyll biosynthesis and photosynthesis in the *lgl1* mutant and wild type.
The transcript levels of all tested genes were normalized to the levels of *OsACT1* gene expression. The transcript levels of all tested genes in wild type (WT) cultivar 'Nipponbare' were arbitrarily set to 1. Values are the mean ± SD of 3 biological replicates.

tosynthetic eukaryotes. It is unclear whether there is a phenotypic difference between the *CSP41a* mutants and wild type plants in rice.

CSP41 proteins are abundant in chloroplast [28]. CSP41a and CSP41b interact in vivo [20,27]. Multiple functions have been described for CSP41 proteins. CSP41a was originally found to be a member of a *petD* 3′ UTR binding complex and was thought to regulate pre-mRNA processing and stability [29,30]. Later, CSP41a was found to be a non-specific endoribonuclease and suggested to play a role in chloroplast mRNA decay [31,32]. CSP41b was originally discovered as part of the multi-subunit plastid-encoded RNA polymerase of mustard and it was proposed that it played a role in RNA stabilization and/or maturation [33]. CSP41 proteins participate in chloroplast ribosomal RNA (rRNA) metabolism, most likely acting in the final steps of 23S rRNA maturation [24]. In addition, complexes that contain CSP41b assemble in the absence of light and disassemble in the presence of light. These complexes bind and stabilize mRNAs that code for photosynthetic proteins and some rRNAs [27]. Although a large number of studies on CSP41a and CSP41b have been done, knowledge of rice CSP41 proteins is lacking. Our studies show that a majority of genes for chlorophyll biosynthesis and photosynthesis were upregulated in rice *csp41b* mutant (Fig. 8). These findings suggest that CSP41b might bind and stabilize only a small number of mRNAs for chlorophyll biosynthesis and photosynthesis in rice.

The *lgl1* mutants have a lower chlorophyll *a* to chlorophyll *b* ratio and reduced carotenoid contents at the seedling stage (Fig. 1E). However, in mature plants, the *lgl1* mutants have a higher proportion of chlorophyll *a* to chlorophyll *b* and increased carotenoid contents (Fig. 1G). Chlorophyll *a* is essential for most photosynthetic organisms, including rice, to release chemical energy, whereas chlorophyll *b* and carotenoid are accessory pigments. We speculate that the content of chlorophyll *b* and carotenoids is more variable than chlorophyll *a* content and more likely to differ under altered growth conditions.

Author contributions

M.J. and L.W. conceived and designed research. L.X., L.F., and L.W. carried out fine mapping and sequence analysis. M.J. and L.W. per-

formed subcellular location, electron microscopy, and expression analysis. F.Y. and H.G. conducted rice transformation. M.J., L.F., and L.W. analyzed data. L.F. and L.W. wrote and revised the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2016.12.005.

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