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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.

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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.

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