Plastid caseinolytic protease OsClpR1 regulates chloroplast development and chloroplast RNA editing in rice

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Abstract

Background: Plant plastidic caseinolytic protease (Clp) is a central part of the plastid protease network and consists of multiple subunits. The molecular functions of many Clps in plants, especially in crops, are not well known.

Results: In this study, we identified an albino lethal mutant al3 in rice, which produces albino leaves and dies at the seedling stage. Molecular cloning revealed that AL3 encodes a plastid caseinolytic protease, OsClpR1, homologous to Arabidopsis ClpR1 and is targeted to the chloroplast. Compared with the wild type, chloroplast structure in the al3 mutant was poorly developed. OsClpR1 was constitutively expressed in all rice tissues, especially in young leaves. The OsClpR1 mutation affected the transcript levels of chlorophyll biosynthesis and chloroplast development-related genes. The RNA editing efficiency of three chloroplast genes (rpl2, ndhB, ndhA) was remarkably reduced in al3. Using a yeast two-hybrid screen, we found that OsClpR1 interacted with OsClpP4, OsClpP5, OsClpP2, and OsClpS1.

Conclusions: Collectively, our results provide novel insights into the function of Clps in rice.

Keywords: Oryza sativa, OsClpR1, Chloroplast development, Chloroplast RNA editing

Background

Chloroplasts are a semi-autonomous organelle responsible for photosynthesis, and the biosynthesis and storage of multiple metabolites (Moreira et al. 2000; Sugimoto et al. 2004). The functional chloroplast is derived from the proplastid, and is synergistically regulated by the plastid and nuclear genomes (López-Juez 2007; Sakamoto et al. 2008). Accumulating evidence indicates that protein degradation plays an important role in chloroplast biogenesis (Clarke et al. 2005; Kato and Sakamoto 2010).

Plastids contain several proteases, such as the stromal Ser Clps, the thylakoid-bound FtsH metalloproteases, and EGY1 proteases (Adam et al. 2006; Zheng et al. 2006). The ATP-dependent Clp peptidase has been studied, and the plastid Clp proteolytic system in plants consists of five ClpP proteins, four ClpR proteins, and three Clp chaperones (ClpC1, ClpC2, and ClpD; Constan et al. 2004; Sakamoto 2006; Sjögren and Clarke 2011). Additionally, plants have two specific accessory ClpTs and an adaptor ClpS (Sjögren and Clarke 2011). Recent studies have reported that plant chloroplast Clps are involved in controlling chloroplast development and plant growth. In Arabidopsis thaliana, the loss of function mutants clpp4 and clpp5 are embryonic lethal, while the loss of function mutant clpp3 is seedling lethal (Kovacheva et al. 2007; Kim et al. 2013). The ClpC1 knockout mutant exhibits growth retardation and leaf chlorosis, while there was no obvious phenotype in the clpC2 mutant (Zhang et al. 2018). However, the clpC1/clpC2 double mutant exhibits defects in embryogenesis. In rice, the leaves of the homozygous mutant osclpP5 were light yellow, and died at the three-leaf stage (Tsugane et al. 2006).
A rice yellow leaf mutant vyl showed a yellowing phenotype during the whole growth period, and then gradually turned green from the top to the bottom (Dong et al. 2013). VYL encodes a subunit of the plastid caseinolytic protease homologous to the Arabidopsis ClpP6 subunit. In addition, the OsClpP6 mutation affected plant height, panicle length, and leaf morphology (Li et al. 2013). However, little is known about the structure and function of chloroplast Clps in rice.

In this study, we isolated a rice albino lethal mutant al3 with decreased chlorophyll contents and impaired chloroplasts. AL3 encodes a subunit of the plastid caseinolytic protease, OsClpR1, and influenced the transcription of chlorophyll biosynthesis and chloroplast development-related genes. Notably, OsClpR1 affected the chloroplast RNA editing of rpl2, ndhB, and ndhA. Furthermore, using the yeast two-hybrid analysis, we found that OsClpR1 interacted with OsClpP4, OsClpP5, OsClpP2, and OsClpS1.

Results
Characterization of the al3 Mutant in Rice
We isolated a leaf color mutant albino lethal 3 (al3) by screening the T-DNA insertion library in rice (Dongjin background). The al3 mutant exhibited the albino phenotype and could not be recovered at a later developmental stage compared with wild-type (Fig. 1a, b). Eventually, the al3 plants died. Consistent with the albino leaf phenotype of al3, the chlorophyll a and b contents were reduced significantly in al3 (Fig. 1c).

To further investigate the leaf color phenotype between wild-type and al3, we performed TEM to observe and compare the chloroplast ultrastructure of the al3 mutant and wild-type. The chloroplasts of al3 leaves were poorly developed and lacked organized thylakoids (Fig. 1d, e), whereas the development of chloroplasts in wild-type leaves were well developed and had well-structured thylakoids (Fig. 1f, g). Taken together, the AL3 gene is essential for rice chloroplast development.

Molecular Cloning of the AL3 Gene
To clone the gene for al3, we used tail-PCR to obtain the flanking sequence in al3. Only one fragment sequence was aligned with the rice gene LOC_Os05g51450, suggesting that one T-DNA was located in al3. Furthermore, we identified a genomic flanking sequence by searching the T-DNA insertion database (http://orgyenesdb.cirad.fr/). Subsequently, we designed three PCR primers (P1, P2, and P3) and identified the T-DNA insertion site in al3 (Fig. 2a, b). The result suggested that
the T-DNA was located in the first exon of LOC_Os05g51450 (Fig. 2a, b), which consists of nine exons and eight introns (Fig. 2a). To explore the influence of the T-DNA insertion on LOC_Os05g51450, we analyzed the expression level of LOC_Os05g51450. The transcript level of LOC_Os05g51450 was significantly reduced by the T-DNA insertion in al3 (Fig. 2c). Therefore, we speculated that LOC_Os05g51450 was the candidate AL3 gene. To confirm that LOC_Os05g51450 was the candidate AL3 gene, we used CRISPR/Cas9 to knock out LOC_Os05g51450 in the Nipponbare background. We sequenced LOC_Os05g51450 in the 25 T0 transgenic plants and obtained six heterozygous mutants which were similar to the leaf color of wild-type plants. Then, three T1 transgenic homozygous plants were obtained, which exhibited albino leaves (Fig. 3a, b). In addition, we detected the potential off-target sites and did not find any mutations in any of the potential off-target sites (Additional file 1: Table S1). These results revealed that LOC_Os05g51450 is the AL3 gene.

**AL3 Is a Plastid-Localized and Conserved Clp Protease**

Sequence and bioinformatic analysis indicated that the AL3 contains nine exons and eight introns (Fig. 2a). AL3 is predicted to encode a Clp protease of 386 amino acids. A BLAST search of the sequence suggested that the protein encoded by AL3 shares 52.5% amino acid sequence identity with the plastid ClpPR protease ClpR1 in Arabidopsis thaliana (Kim et al. 2009; Fig. 4). Therefore, we named AL3 as OsClpR1. The ClpR1 mutant developed slower than the wild type with pale green leaves and affected chloroplast development (Koussevitzky et al. 2007).

Amino acid comparison indicated that proteins homologous to OsClpR1 are highly conserved among Zea mays, Hordeum vulgare, Sorghum bicolor, Setaria italica, Brachypodium distachyon (Fig. 4).

ChloroP analysis revealed that OsClpR1 has a chloroplast transit peptide at the N-terminus with 40 amino acids (www.cbs.dtu.dk/services/ChloroP/). To verify this prediction, we constructed two transformation vectors expressing the OsClpR1-GFP and OsClpR141–386-GFP fusion protein and introduced them into rice protoplasts. The results showed that the green fluorescence of OsClpR1-GFP fully co-localized with the Chl fluorescence of the chloroplasts (Fig. 5b). However, OsClpR141–386-GFP fluorescence was observed in the cytosol (Additional file 1: Figure S2). These results indicated that the N-terminal 40 amino acids are essential for OsClpR1 to target the chloroplasts.

![Fig. 2 Molecular cloning of AL3.](image-url)

**a** Schematic representation of the AL3 gene. Black boxes represent exons, and the lines represent introns. T-DNA is inserted into the first exon of LOC_Os05g51450. **b** PCR verification of the T-DNA insertion site. **c** Expression level of AL3 in the wild-type and al3.
Expression Pattern of AL3

To analyze the expression pattern of AL3, we first searched the AL3 gene in the Rice eFP Browser (http://bar.utoronto.ca/eFP-browser/cgi-bin/efpWeb.cgi), and found that OsClpR1 was expressed in various tissues, and especially in leaves (Additional file 1: Figure S2). To test the result of the prediction, we carried out qRT-PCR to analyze the OsClpR1 expression in roots, young leaves, seedlings, and panicles. The result indicated that OsClpR1 was constitutively expressed in various tissues (Fig. 5a).
Altered Expression of Chlorophyll Biosynthesis and Chloroplast-Associated Genes in al3

We observed that chloroplasts were impaired in al3 and hypothesized that the loss of function of OsClpR1 may influence the expression of chlorophyll biosynthesis and chloroplast-associated genes. To test this hypothesis, we carried out qRT-PCR to analyze the expression of these genes. Our results indicated that the expression levels of three tetrapyrrole biosynthesis genes (HEMA, HEMC, and HEME), three subunits of Mg-chelatase (CHLH, CHLI, and CHLM), magnesium-protoporphyrin IX monomethyl ester cyclase CRD, and divinyl reductase DVR were remarkably reduced in al3, compared with wild-type (Additional file 1: Figure S3A). In addition, the expression levels of chloroplast-associated genes, e.g. psaA, atpB, and rps2, were significantly down-regulated (Additional file 1: Figure S3B). Taken together, our results revealed that the OsClpR1 may coordinate the chlorophyll biosynthesis and chloroplast-associated genes to regulate rice chloroplast development.

Altered RNA Editing of rpl2–1, ndhA-1019, ndhB-611 and ndhB-737 in al3

Previous studies have shown that plastid RNA editing plays an important role in regulating chloroplast development in plants (Xiao et al. 2018; Huang et al. 2020; Wang et al. 2020). To verify whether the OsClpR1 mutation affects plastid RNA editing, we examined the RNA editing efficiency of 21 editing sites in wild-type and al3. We observed that the RNA editing of rpl2–1, ndhA-1019, ndhB-611 and ndhB-737 were significantly reduced compared to wild-type (Fig. 6a), whereas the other 17 editing sites were not affected (Additional file 1: Figure S4).

In addition, we examined the editing efficiency of rpl2–1, ndhA-1019, ndhB-611 and ndhB-737 in the CRISPR/Cas9 knock-out lines. RNA editing at these four editing sites was also reduced in the CRISPR/Cas9 knock-out lines (Fig. 6b). These results suggest that the OsClpR1 mutation affects chloroplast RNA editing in rice.

Interactions between OsClpR1, OsClpP2, OsClpP4, OsClpP5, and OsClpS1

To investigate the role of OsClpR1 in the assembly of the rice Clp complex assembly, we carried out a yeast two-hybrid screen to identify the proteins that interact directly with OsClpR1. We cloned the rice homologs of the Arabidopsis protease complex and named them as OsClpR2 (Os06g0136800), OsClp5 (Os03g0344900), OsClpS1 (Os08g0432500), OsClpP2 (Os04g0525600), OsClpP3 (Os01g0507900), OsClpP4 (Os10g0508000), OsClpP5 (Os03g0308100), and OsClp-T (Os03g0247000). As shown in Fig. 7, OsClpR1 interacted with OsClpS1, OsClpP2, OsClpP4, OsClpP5, but not with OsClpR2, OsClp5, OsClpP3, VYL and OsClp-T (Additional file 1: Figure S5A-B). This result indicated that OsClpR1 likely serves as a core component in the assembly of the rice Clp complex. OsClpP4 and OsClp-T are reported to interact with themselves and form
homodimers (Dong et al. 2013). Therefore, we used yeast two hybrid analysis to verify whether OsClpR1 can form a homodimer. However, OsClpR1 could not interact with itself to homodimerize (Additional file 1: Figure S5C).

Discussion

Plastid caseinolytic protease has been extensively studied in dicotyledonous plants, but it has been scarcely studied in monocotyledonous plants, especially in rice (Zhang et al. 2018). In this study, we isolated and identified a rice plastid caseinolytic protease protein OsClpR1. The OsClpR1 gene was highly expressed in young leaves, and lowly expressed in roots and panicles. Knockout of OsClpR1 caused a significant decrease of chlorophyll contents and functional chloroplasts. OsClpR1 was located in chloroplasts and is conserved among Zea mays, Hordeum vulgare, Sorghum bicolor, and Arabidopsis thaliana. In summary, our results provide novel insights for the regulation of chloroplast development by Clps in rice. Leaf color is one of the important factors affecting photosynthesis, which directly affects rice yield. To date, more than 120 genes have been reported to regulate chloroplast development in rice, such as YGL8, TSC1, and Lhca4 (Kong et al. 2016; Shi et al. 2018; Yamatani et al. 2018). The expression levels of chlorophyll biosynthesis and plastid-encoded genes in the al3 mutant were remarkably reduced compared with the wild type (Additional file 1: Figure S3). Our study was helpful to improve the molecular mechanism of Clps regulating chloroplast development in rice.

In addition, we observed defects in RNA editing of rpl2–1, ndhA-1019, ndhB-611 and ndhB-737 in the al3 mutant (Fig. 7a), whereas all other chloroplast editing sites were similar to the wild-type (Additional file 1: Figure S3). Additionally, the RNA editing of rpl2–1, ndhA-
1019, *ndhB*-611 and *ndhB*-737 in CRISPR/Cas9 knock-out plants were also reduced (Fig. 7b). RNA editing, as a posttranscriptional modification, is essential for generating mature transcripts of plastid genes (Yan et al. 2018). However, not all albino mutants in rice exhibit chloroplast RNA editing defects in rice. For example, the rice albino mutant *wsl3* has almost no impairment in chloroplast RNA editing (Wang et al. 2020). Previous studies have shown that multiple organellar RNA editing factor (MORF) proteins are essential for plastid and mitochondrion RNA editing in *Arabidopsis* and rice (Bentolila et al. 2012; Zhang et al. 2017). The MORF protein WSP1 is involved in the editing of six plastid RNA editing sites in rice (Zhang et al. 2017). To investigate whether OsClpR1 affects RNA editing by interacting with MORFs, we used the yeast two-hybrid screen to examine the interaction between OsClpR1 and the 7 MORF proteins in the rice genome. Unfortunately, OsClpR1 did not interact with any of the seven MORF proteins in the screen (data not shown), suggesting that OsClpR1 influences chloroplast RNA editing independent of an interaction with MORFs. Our future studies will be focus on identifying the interacting proteins of OsClpR1 to elucidate the mechanism of its regulation of chloroplast RNA editing.

**Conclusions**

OsClpR1 regulates chloroplast development in rice and influences chloroplast RNA editing. Our findings contribute to understanding the mechanisms of chloroplast development and the functional characterization of Clps in monocots.

**Methods**

**Plant Materials**

The *al3* mutant (1A-08939) was isolated from a T-DNA insertion library with the cv Dongjin background (Jeon et al. 2000; Jeong et al. 2006). The mutant and wild-type plants were planted in paddy fields under natural conditions in Huaian, China.

**Chl Content Determination and Transmission Electron Microscopy (TEM)**

Leaves from wild-type and *al3* plants were collected at the seedling stage. Approximately 200 mg of powdered leaves were soaked in 20 mL of 95% ethanol for 48 h in the dark. Chl contents for each sample were measured in triplicate as previously described (Liu et al. 2020).

For TEM, leaves from wild-type and *al3* plants were collected and fixed in 2.5% (v/v) glutaraldehyde. The sample preparation was performed as previously described (Liu et al. 2020). The chloroplast structure of leaf cells was observed under a JEOL 1200EX transmission electron microscope.

**Identification of T-DNA Insertion Locus and CRISPR/Cas9 Knock-out of *AL3***

To identify the T-DNA insertion locus in *al3*, we searched the flanking sequence database (Jeong et al. 2006; http://orygenesdb.cirad.fr/). The T-DNA locus was detected by PCR with the primers P1, P2, and P3 (Additional file 1: Table S2).

To knock out *AL3*, one CRISPR/Cas9 vector was constructed as previously described (Lu et al. 2017). The recombinant vector was transformed into rice cv Nipponbare by Agrobacterium. The genotype of CRISPR/Cas9 plants was analyzed by PCR and the amplification products were sequence-verified. Genome target sequences and PCR primers are listed in Additional file 1: Table S2.

**RNA Extraction and Quantitative RT-PCR**

Total RNA from roots, young leaves, seedlings at the two-leaf stage, and panicles were isolated by the RNAprep Pure Plant Kit (CW/BIO, Jiangsu, China). First-strand cDNA was generated from 1 μg of total RNA using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa). Quantitative RT-PCR was performed as previously described and the primers used to measure the expression level of chlorophyll biosynthesis and chloroplast-associated genes were obtained from the study by Liu et al. 2020. The primers used for the expression level of OsClpR1 are listed in Additional file 1: Table S2.

**Subcellular Localization of OsClpR1**

To confirm the subcellular localization of OsClpR1, the coding region of *OsClpR1* (without the stop codon) was introduced into the pAN580-GFP vector and transformed into rice protoplasts. To detect the chloroplast signal of the OsClpR1 protein, and one truncated OsClpR1 segment were inserted into the pAN580-GFP vector, termed OsClpR1<sup>141–386</sup>-GFP. Fluorescence was observed by a Zeiss LSM700 confocal laser-scanning microscope. The primers used for constructing the vector are listed in Additional file 1: Table S2.

**Analysis of RNA Editing**

Specific cDNA fragments containing editing sites were amplified and sequenced. The cDNA sequences were compared to identify C to T changes resulting from RNA editing as previously described (Tan et al. 2014; Cui et al. 2019; Huang et al. 2020).

**Yeast Two-Hybrid Analysis**

The full-length cDNA of *OsClpR1* and nine Clp genes were cloned into pGBKKT7 and pGADT7, respectively. The vectors were transformed into yeast strain AH109 according to the manufacturer’s instructions (Clontech). The primers used for constructing the vectors are listed in Additional file 1: Table S2.
Abbreviations

Chl: Chlorophyll; Chl a: Chlorophyll a; Chl b: Chlorophyll b; qRT-PCR: Quantitative real-time polymerase chain reaction; MORF: Multiple organellar RNA editing factor; Clp: Plastidic casenoetic protease; TEM: Transmission electron microscopy; WT: Wild type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-021-00489-6.

Additional file 1: Figure S1. Subcellular localization of OsClpR1.585. GFP. Figure S2: Expression pattern of OsClpR1 at various growth periods. Data was collected from the Rice eFP Browser. Figure S3: Expression analysis of chlorophyll biosynthesis and chloroplast development related-genes in wild-type and ω3. Figure S4. The rest 17 RNA editing sites in wild-type and ω3. Figure S5. A yeast two-hybrid interaction assay between OsClpR1 and five Clp proteins in rice. Table S2. Off-target effect detection. Table S2. Primers used in this study.

Acknowledgements

The authors thank AiMi Academic Services (www.aimieditor.com) for English language editing and review services.

Authors’ Contributions

LX, XZY, and CPH designed the research. LX, YYR, and CH performed the yeast two-hybrid assays. All authors read and approved the final manuscript.

Funding

This work was supported by the Program for Huaihangh Tails, Suzhou Agricultural Science and Technology Innovation Project (SN20200048), Suzhou Academy of Agricultural Sciences Foundation (202019009X), andEffects on chloroplast development during early leaf development in rice. New Phytol doi 228(4):1401–1416. https://doi.org/10.1111/nph.16769

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