Chlorosis seedling lethality 1 encoding a MAP3K protein is essential for chloroplast development in rice

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Abstract

Background: Mitogen-activated protein kinase (MAPK) cascades are conserved signaling modules in eukaryotic organisms and play essential roles in immunity and stress responses. However, the role of MAPKs in chloroplast development remains to be evidently established.

Results: In this study, a rice chlorosis seedling lethality 1 (csl1) mutant with a Zhonghua11 (ZH11, japonica) background was isolated. Seedlings of the mutant were characterized by chlorotic leaves and death after the trefoil stage, and chloroplasts were observed to contain accumulated starch granules. Molecular cloning revealed that OsCSL1 encoded a MAPK kinase kinase22 (MKKK22) targeted to the endoplasmic reticulum (ER), and functional complementation of OsCSL1 was found to restore the normal phenotype in csl1 plants. The CRISPR/Cas9 technology was used for targeted disruption of OsCSL1, and the OsCSL1-Cas9 lines obtained therein exhibited yellow seedlings which phenocopied the csl1 mutant. CSL1/MKKK22 was observed to establish direct interaction with MKK4, and altered expression of MKK1 and MKK4 was detected in the csl1 mutant. Additionally, disruption of OsCSL1 led to reduced expression of chloroplast-associated genes, including chlorophyll biosynthetic genes, plastid-encoded RNA polymerases, nuclear-encoded RNA polymerase, and nuclear-encoded chloroplast genes.

Conclusions: The findings of this study revealed that OsCSL1 played roles in regulating the expression of multiple chloroplast synthesis-related genes, thereby affecting their functions, and leading to wide-ranging defects, including chlorotic seedlings and severely disrupted chloroplasts containing accumulated starch granules.

Keywords: Rice, OsCSL1, Chloroplast development, MAP3K protein, Chloroplast-associated genes

Background

Chloroplasts are defined as organelles that play specific roles in the conversion of light energy to chemical energy via photosynthesis [1], and their functional and structural integrity are vital for normal plant growth and development [2, 3]. Abnormal chloroplast function is generally reflected in changes in leaf pigmentation [4, 5]. In rice, several pentatricopeptide repeat proteins (PPR) have been identified and are reportedly involved in chloroplast development, including Young Seedling Albino (YSA) [6], Albino Seedling Lethality3 (ALS3) [7], Thermo-sensitive Chlorophyll-Deficient 10 (TCD 10) [8], and OsPPR6 [9]. Additionally, genes from several other families have been implicated in chloroplast development. For example, Albino Leaf 1 (AL1) and AL2 encoding an octotricopeptide repeat protein and chloroplast group IIA intron splicing facilitator were identified, respectively [10, 11]; additionally, alterations in the number and structure of thylakoids resulted in the development of albino leaves.
and in the occurrence of seedling death at an early developmental stage in rice [10, 11]. Furthermore, rice Young Leaf Chlorosis 2 (YLC2) has been shown to encode the stroma-localized heme oxygenase 2, and seedlings of the ylc2 mutant have been observed to exhibit a chlorotic phenotype and defective chloroplast structures [12]. Recently, Glycinamide ribonucleotide synthetase (GARS), which catalyzes the second step in purine nucleotide biosynthesis, has been identified to be involved in chloroplast development in rice by affecting the expression of plastid-encoded genes [13].

Chloroplasts are semi-autonomous organelles containing a unique genome and gene expression system [14]. Precise chloroplast function is coordinately mediated by two types of RNA polymerase, namely the nuclear-encoded RNA polymerases (NEPs) and plastid-encoded RNA polymerases (PEPs) [15, 16], which are essential for the biogenesis of photosynthetically active chloroplasts in plants [16, 17]. The NEP complex is encoded by RPO1 and RPO1nmp, the knockout of which has been found to result in delayed chloroplast biogenesis [18, 19]. Apart from PEPs and NEPs, chlorophyll biosynthetic genes (CBGs) and nuclear-encoded chloroplast genes (NECGs) have also been established to be involved in chloroplast development.

Mitogen-activated protein kinase (MAPK) cascades are considered important mechanisms involved in the transmission of exogenous or developmental signals to target molecules, and are generally highly conserved in eukaryotes [20, 21]. These cascades are typically characterized by the sequential phosphorylation of MAPK kinase kinase (MKKK), MAPK kinase (MKK), and MAPK. In rice, MAPK signaling pathways have been shown to be involved in eliciting defense responses against blight disease [22], rice blast [23], and brown planthopper infestation [24, 25], and also in the responses to multiple abiotic stimuli [26–30]. OsMPK6 has been identified as a negative regulator involved in the resistance to bacterial pathogens, the reduced expression of which enhances resistance to different races of Xanthomonas oryzae pv. oryzae, mediated via salicylic acid and jasmonic acid signaling pathways [31, 32]. Recent studies have also revealed the involvement of MAPK cascades in plant development. For example, in rice, the OsMKK10–OsMKK4–OsMPK6 cascade has been established to positively control grain size and is negatively regulated by GRAIN SIZE AND NUMBER1 (GSN1) [33, 34]. Currently, however, much remains unknown regarding the role of MAPK cascades in chloroplast development.

In this study, characterization of a novel MAP3K gene involved in chloroplast development in rice was reported, designated chlorosis seedling lethality 1 (csl1), the mutation of which has been reported to result in energy deficiency and premature seedling death. OsCSL1 encodes a MAPK kinase kinase22 (MKKK22), which targets the endoplasmic reticulum (ER); furthermore, protein–protein interaction analyses revealed that OsCSL1/OsMKK22 interaction with MKK4. Expression of MKK4 was reduced in the csl1 mutant. Collectively, these observations indicate that the MKKK2–MKK4 signaling pathway potentially plays an important role in chloroplast development in rice.

**Results**

**The csl1 mutation results in the development of chlorotic seedlings and a lethal phenotype**

To investigate the mechanisms underlying MAPK involvement in chloroplast development, a mutant, chlorosis seedling lethality 1 (csl1), displaying defects in leaf pigmentation was identified among rice T-DNA insertion populations with a Zhonghua11 (japonica) background. Compared with the green leaves of wild-type (WT) plants, the csl1 mutant exhibited a chlorotic seedling phenotype that developed prior to the trefoil stage (Fig. 1A, B). Unlike other chlorotic or albino leaf mutants, which the leaf defects could be restored at later stages of development [19], csl1 seedlings gradually withered and eventually died. To determine whether the observed chlorosis was associated with altered photosynthetic efficiency, chlorophyll contents in leaf blades and sheaths at the trefoil stage were further evaluated. The contents of chlorophylls a and b and total chlorophyll content in both leaf blades and sheaths were significantly reduced in the csl1 mutant compared with WT seedlings (Fig. 1C), whereas no appreciable differences were detected between WT and heterozygous csl1 lines (Fig. S1).

Chloroplasts are indispensable for normal plant growth and development, not only as an essential site for photosynthesis but also for a wide range of biochemical processes, including the synthesis of pigments, lipids, and hormones. Considering that csl1 mutant seedlings were characterized by a yellow pigmentation and reduced chlorophyll contents, csl1 mutation exerted an influence on chloroplast development was investigated. Transmission electron microscopy (TEM) observations of the chloroplast structures in csl1 and WT seedlings at the two-leaf stage revealed that WT chloroplasts could be characterized by a distinct unidirectional thylakoid structure, whereas the intracellular structure of csl1 mutant chloroplasts seemed to be disordered with indistinct thylakoids oriented in different directions, which accordingly contributed to a marked disruption of chloroplasts in the csl1 mutant (Fig. 1D). Moreover, pronounced accumulation of starch granules in chloroplasts of the csl1 mutant was detected (Fig. 1D). Collectively, these
observations indicate that OsCSL1 is essential for normal chloroplast development in rice.

Molecular cloning of OsCSL1
Genetic analysis indicated that the csl1 phenotype could be subjected to control by a single recessive gene, as evidenced by the 3:1 segregation ratio between green and yellow seedlings (\( \chi^2 = 0.107 \), Table S1). To further characterize this gene, inverse polymerase chain reaction (IPCR) was performed to isolate the T-DNA genomic flanking regions in the csl1 mutant (Fig. S2). A BLAST search (https://rapdb.dna.affrc.go.jp/) revealed that the sequences of the flanking region were similar to those of a gene (Os03g0703400) predicted to encode a MAPK kinase kinase22 (MKKK22) located on rice chromosome 3, which has not been functionally characterized thus far. The coding region of Os03g0703400 contains eight exons and seven introns (Fig. 2A). Notably, the full-length OsCSL1 protein consists of 489 amino acids, as opposed to the predicted 654 residues found in databases, the difference between which is attributable to a 165-amino acid deletion at the beginning of the first exon in the amplified product (Fig. S3).

The site of the T-DNA insertion is located between bases +4214 and +4322 in the seventh intron, leading to a 109-bp segment deletion in Os03g0703400 (Fig. 2A). To eliminate interference from contiguous genes, expression levels of Os03g0703300, Os03g0703400 (MKKK22/OsCSL1), and Os03g0703600 were examined (Fig. 2B). RT-qPCR analysis revealed no appreciable differences between WT and csl1 mutant plants with respect to the levels of Os03g0703300 and Os03g0703600 transcripts (Fig. 2C); however, the level of Os3g0703400 (OsCSL1) was found to be significantly reduced in the csl1 mutant compared with the WT (Fig. 2C). Collectively, these findings indicate that Os3g0703400 is a strong candidate for the OsCSL1 gene.

Mutation of OsCSL1 is responsible for the development of a chlorotic seedling phenotype
To verify whether the chlorotic seedling phenotype was associated with a defective Os03g0703400 (OsCSL1), genetic complementation assay using heterozygous csl1/OsCSL1 plants was performed, owing to the lethality of the csl1 mutation in homozygous seedlings. A ~9-kb WT genomic DNA sequence, comprising a
~2.5-kb sequence upstream of the start codon, a ~5.5-kb open reading frame, and a ~1-kb sequence downstream of the stop codon, was cloned into a binary vector and transformed into heterozygous csl1/OsCSL1 plants. Positive transgenic plants derived from the csl1/csl1 background were obtained, and used for further analyses. As shown in Fig. 2D, csl1 seedlings characterized by a defective chlorotic phenotype were rescued by the OsCSL1 transgene (Fig. 2D). Moreover, the expression of OsCSL1 and chlorophyll contents in the transgenic seedlings recovered to normal levels (Fig. 2E, F). These observations thus revealed that the chlorotic phenotype of csl1 mutant seedlings was associated with a defective Os03g0703400, thereby indicating that OsCSL1 was the causal gene and it played an essential role in the regulation of rice leaf pigmentation.

To further examine the molecular function of OsCSL1 in rice, OsCSL1 knockout mutant lines in the Zhonghua11 background were generated using the CRISPR/Cas9 genome editing technique. Accordingly, a series of transgenic OsCSL1-Cas9 lines were generated using a target site located in the fifth exon (Fig. 3A). Subsequent phenotypic observations revealed that the seedlings of OsCSL1-Cas9 lines presented with a chlorotic phenotype prior to the trefoil stage (Fig. 3B), which was similar to that of the csl1 mutant. Consistently, similar to the findings documented with the csl1 mutant, chlorophyll contents (chlorophyll a, chlorophyll b, and total chlorophyll) in the OsCSL1-Cas9 line seedlings were reduced compared with those in the WT plants (Fig. 3C). Moreover, the findings revealed that deletion and/or substitution events occurred in the OsCSL1-Cas9 lines (Fig. 3D); for
example, OsCSL1-Cas9-1 and OsCSL1-Cas9-5, which contained a 1-bp deletion, and OsCSL1-Cas9-9, containing both deletions and substitutions (Fig. 3D). These findings indicated that OsCSL1 knockout plants phenocopied the T-DNA insertional mutant, and mutation of the OsCSL1 gene resulted in the development of the chlorotic seedling phenotype in rice.

OsCSL1 is targeted to the endoplasmic reticulum

To elucidate the temporal and spatial expression of OsCSL1, RT-qPCR to investigate the patterns of expression in different tissues was performed, including root, stem, leaf sheath, leaf blade, young panicle, and mature panicle. The results revealed that OsCSL1 was constitutively expressed in different tissues and organs, demonstrating markedly high levels in the leaf sheath and blade (Fig. 4A), and was weakly expressed in other tissues, such as the roots, stems, and young and mature panicles (Fig. 4A). These results thereby highlight the role of OsCSL1 in plant development, particularly, in the developmental stages in the leaves.

To examine the subcellular localization of OsCSL1, a CSL-GFP (green fluorescent protein) fusion protein was transiently expressed in rice protoplasts. An OsCSL1-GFP fusion protein under the control of the Zea mays ubiquitin promoter was assembled and subsequently transformed into rice protoplasts. Laser scanning confocal microscopy of the transformed protoplasts revealed an overlap of the green signals generated by OsCSL1-GFP, and the red signals derived from ER-mCherry [35], observed as an orange coloration (Fig. 4B), thus indicating that OsCSL1 was located in the ER.

Altered expression of chloroplast-related genes is observed in the csl1 mutant

Considering abnormal chlorophyll metabolism and plastid development in the csl1 mutant, the expression of chloroplast development- and photosynthesis-related genes were investigated, including chlorophyll biosynthetic genes (CBGs), plastid-encoded RNA polymerases (PEPs), nuclear-encoded plastid RNA polymerase (NEPs), and nuclear-encoded chloroplast genes (NECGs). In line with expectations, significant reductions in the expression levels of several CBGs in the csl1 mutant were detected, including those of OsHAP3, HemA, OsCAO, YGL1, and Cab1R, which were reduced by at least a 50% compared with those noted in the WT. Notable among these was the reduced expression of OsHAP3A and OsHAP3C, which were barely detectable in the csl1 mutant (Fig. 5A). Furthermore, examination of the expression of PEPs, NEPs, and NECGs revealed that, with the exception of psbA and psbP, the expression levels of all investigated genes were significantly impaired in the csl1 mutant (Fig. 5B-D). These findings accordingly provide evidence to indicate that chloroplast development,
Fig. 4 Expression pattern of OsCSL1. A Expression levels in different organs. B Subcellular localization of the OsCSL1-GFP protein in rice protoplasts.

Fig. 5 Expression of chloroplast-associated genes. Relative expression of chlorophyll biosynthetic genes (CBGs) (A), plastid-encoded RNA polymerases (PEPs) (B), nuclear-encoded RNA polymerases (NEPs) (C), and nuclear-encoded chloroplast genes (NECGs) (D). Data are presented as mean ± S.E., ** p-value < 0.01, two-tailed, two-sample Student’s t-test.
chlorophyll synthesis, and photosynthesis are potentially disrupted in the csl1 mutant.

**OsCSL1 establishes interaction with MKK4 and alters the expression of OsMKKs in the csl1 mutant**

In the MAPK cascade, MAP3K catalyzes the phosphorylation of MKK proteins, which subsequently leads to the phosphorylation of MAPKs and results in the stimulation of downstream target genes. Previously, yeast two-hybrid (Y2H) and protein microarray analyses have revealed the interactions established between MKK and MPK pairs and among a range of MAPKs and potential substrates [36, 37]. Thus, in the present study, Y2H assays were used to examine the interactions between OsCSL1/MKK22 and MKKs, using either full-length or truncated OsCSL1 as baits for assessments of protein interactions. The Y2H results revealed that OsCSL1 interactions with OsMKK4 (Fig. 6A), and that this interaction was dependent on the presence of a full-length OsCSL1 (Fig. 6A). Further analysis of the expression of OsMKK genes in csl1 mutant and WT plants revealed no significant differences in the expression of OsMKK3, OsMKK5, OsMKK6, or OsMKK10-2 between WT and the csl1 mutant (Fig. 6B); in contrast, a pronounced accumulation of OsMKK1 and OsMKK4 transcripts in the csl1 mutant was detected (Fig. 6B). Considering that it has been previously reported that OsMKK1 and OsMKK4 potentially establish interactions with OsMAPK3, OsMAPK4, and OsMAPK6 [33, 38], the expression of OsMAPK3, OsMAPK4, and OsMAPK6 were further investigated, and accordingly observed that the expression of OsMAPK3s was significantly higher in the csl1 mutant than that in the WT (Fig. 6C). Taken together, these findings indicate the potential role of the M KK22–M KK4 pathway in rice chloroplast development.

**Discussion**

In plants, events occurring during the early seedling phase are essential determinants of long-term growth and development [39]. In the present study, a homozygous chlorosis seedling lethality 1 (csl1) mutant was characterized using T-DNA insertion lines with a rice cultivar Zhonghua11 (O. japonica) background. csl1 seedlings were characterized by the development of yellow leaves (Fig. 1A) and occurrence of premature death after the trefoil stage. Inverse PCR analysis, used to identify DNA sequences flanking the T-DNA insertion region
in the csl1 mutant, indicated that Os03g0703400, encoding OsMKKK22, was a strong candidate for the csl1 gene (Fig. 2A-C).

MAP3Ks are vital components of MAPK cascades, a group of signal transduction pathways that tend to be highly conserved in eukaryotic organisms [38]. Generally, MAPK cascades consist of three types of functional protein kinases, namely MAP3Ks, MAP2Ks, and MAPKs. MAP3Ks activate downstream MAP2Ks, which subsequently activate MAPKs that in turn target different cytoplasmic and nuclear effector proteins. Numerous studies have revealed that MAP3Ks are extensively involved in the generation of responses of plants to biotic and abiotic stresses, as well as hormonal signal transduction. For example, GhMAP3K40 is reportedly associated with a reduced tolerance to biotic and abiotic stresses in *Nicotiana benthamiana* via negative regulation of plant development [40]. Additionally, *GhRaf19*, a Raf-like MAPKKK gene, has been demonstrated to negatively regulate the development of tolerance to drought and salt stress in cotton, and to positively regulate the development of resistance to cold stress [41]. Notably, mutation of *csl1*, encoding a MAP3K protein in rice, was associated with a lethal yellow seedling phenotype, and that chlorophyll contents in both the leaf blades and sheaths of csl1 mutant seedlings at the trefoil stage were significantly lower than those in the WT (Fig. 1). To verify whether the observed csl1 mutant phenotype was associated with MAP3K, complementation assays and knockout experiments were performed, the results of which indicated that the development of the lethal yellow seedling phenotype in rice csl1 seedlings was attributable to mutation of the Os03g0703400 gene (Figs. 2 and 3).

As mentioned previously, most studies reported in this field have highlighted the roles of MAP3Ks in plant adaptation to environmental conditions and as a regulator of signaling. For example, *CONSTITUTIVE TRIPLERE- SPONSE 1* (CTR1) and *ENHANCED DISEASE RESIST- ANCE 1* (EDR1), two of the best-studied MAP3Ks in plants, are known to be involved in the ethylene-mediated signaling response. Recent studies have revealed that MAP3Ks play important roles in plant growth and development. In this regard, it has been observed that compared with WT plants, transgenic *Arabidopsis* plants expressing MAP3K64 are characterized by earlier bolting and more vigorous growth [42]; furthermore, over-expression of *MAP3K18* has been found to be associated with a smaller phenotype in *Arabidopsis*, demonstrating the promotion of senescence of the rosette leaves in transgenic plants compared with those of WT plants [43]. Nevertheless, the molecular mechanisms underlying the MAP3K-mediated regulation of plant growth and development, particularly leaf pigmentation, remain to be determined.

MAPK pathways are triggered in response to a diverse range of external stimuli [44], with the activation of the MAPK cascade leading to the phosphorylation of specific targets, including transcription factors, cytoskeletal proteins, and phospholipases [45]. In this regard, Y2H assay results indicated that OsCSL1 established interactions with OsMKK4 (Fig. 6A), and the expression of OsMKK1 and OsMKK4 was significantly increased in the csl1 mutant compared with that in the WT (Fig. 6B). It has previously been established that *OsMKK1* is induced by salt stress, and that a high expression of this gene and its downstream target *OsMAPK4* contributes to enhanced seedling survival by increasing the transcript levels of *OsDREB2B* and *OsMYBS3* [38]. In rice, *OsMKK4/SMG1* plays multiple roles, among which the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the development of rice immunity [46]; additionally, the *OsMKK4–OsMPK1/OsMPK6* cascade and downstream transcription factor *OsWRKY53* are involved in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furthermore, the findings of a recent study have revealed that the *OsMKK4–OsMPK3* and *OsMKK4–OsMPK6* cascades are implicated in the rice wound response [47]. Furtherm...
of expression of chlorophyll biosynthetic, plastid, and nuclear genes, expression patterns of a series of chloroplast-related genes were analyzed, and accordingly found that the expression of genes involved in chlorophyll biosynthesis and chloroplast development, including those of CBGs, PEps, NEps and NECGs, was significantly suppressed in the csl1 mutant. Considering that the csl1 mutant exhibits a seedling lethal phenotype (Fig. 5), and that the chlorophyll content of mutant seedlings is lower than that in the WT plants (Fig. 1), suggesting that observed defects in pigmentation and chloroplast development are associated with the repression of chloroplast-associated genes [39].

Conclusions
In summary, the findings of this study provide evidence to indicate that OsCSL1 encodes a MAPK3 protein necessary for chloroplast development. Furthermore, OsCSL1 may demonstrate function by regulating the expression of multiple chloroplast synthesis-related genes, thereby affecting their functions. Thus, mutation of the OsCSL1 gene will result in wide-ranging defects, including severely disrupted chloroplasts containing accumulated starch granules and chlorotic seedlings observed in this study (Fig. 6D). Considering that most previous studies reported on MAPK cascades in plants have focused on their role in the development of immunity and elicitation of stress responses, characterization of OsCSL1 will contribute to enhancing currently limited understanding of the essential roles of MAPKs during chloroplast development.

Materials and methods

Plant materials and growth conditions
The rice (Oryza sativa L.) chlorosis seedling lethality 1 (csl1) mutant, initially identified in a T-DNA insertion population with a Zhonghua11 (japonica) background, was kindly provided by Dr. Jingliu Zhang (Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences). Over 3000 rice plants harboring T-DNA possessing a Ds element were generated via Agrobacterium tumefaciens-mediated transformation by Zhang and colleagues [49]. Wild-type (Zhonghua11) and csl1 mutant plants were grown in the paddy field of South China Agricultural University, Guangzhou, China (23.16° N, 113.23 °E; subtropical climate) during the early (from late February to early July) and late (from middle July to late October).

Insertion site analysis
Inverse polymerase chain reaction (IPCR) was performed to isolate the sequences flanking the aforementioned T-DNA region. The nested primers used for the right and left sites of the T-DNA were C1 and C2, and H1 and H2, respectively (Fig. S2). Genomic DNA digestion was initially performed using HindIII. The primers used for screening the T-DNA insertion locus were 48800+ and 5TF1 for the left site, and 49651 and 5TR2 for the right site, the sequences of which have been listed in Supplementary Table 1.

Yeast two-hybrid (Y2H) assays
To obtain yeast competent cells, single colonies of Y2HGld (qd2–3 mm) were dispersed in 1 mL of YPDA and then transferred to a 50-mL tube containing 10 mL YPDA. Cell suspensions were incubated at 30°C for 16-18 h under shaking conditions at 250 rpm, until an OD_{600} value >1.5 was obtained. Overnight cultures were subsequently centrifuged at 3,000 rpm for 5 min at room temperature, and the resulting supernatant was discarded. Following the addition of 10 mL of fresh YPDA, the tube containing the cell pellet was vortexed vigorously for 5 min to disperse cell clumps; subsequently, incubation was performed at 30°C for 3 to 4 h under shaking conditions at 250 rpm until an OD_{600} value ranging from 0.4–0.6 was achieved. After further centrifugation at 3,000 rpm for 5 min at room temperature, the supernatant was discarded and the cell pellet was resuspended with 1.5 mL of 1× TE. To this cell suspension, 0.1 μg of each plasmid DNA and 100 μg carrier DNA were in a fresh 1.5-mL tube, followed by addition of 0.1 mL of yeast competent cells and 0.6 mL of PEG/LiAc solution (PEG3350 40%, 0.01 M Tris-HCL, 1 mM EDTA, and 0.1 M LiAc, pH 7.5). This mixture was then incubated at 30°C for 30 min under shaking conditions at 200 rpm, followed by the addition of 70 μL of DMSO and thorough mixing. The cells were then subjected to heat shock by incubating in a 42°C water bath for 15 min, followed by incubation on ice for 2 min. Following centrifugation for 30 s at 13000 rpm and room temperature, the supernatant was discarded and the pellet cells were resuspended in 200 μL of 1× TE. Aliquots (100 μL) of this cell suspension were spread on SD/-Leu/-Trp and SD/-Leu/-Trp/-His/-Ade agar plates, respectively, followed by incubation at 30°C until the development of colonies was achieved.

Determination of chlorophyll contents
Chlorophyll content determination experiments were conducted in accordance with the methods described by Zhang et al. (2016) [10], with slight modifications. Fresh leaf samples were chopped using scissors, and approximately 0.1 g of the cut fresh leaf material was incubated with 2 mL of extraction buffer (ethanol: propanol: H2O, 4.5:4.5:1 v/v/v) in a 10-mL Eppendorf tube for 12 h in the dark at 4°C, until all leaf samples presented with white
coloration. Using the extraction buffer as a blank control, the maximum absorption of extracts was determined spectrophotometrically at 645 nm and 663 nm. Chlorophyll contents were calculated using the following equations: Chlorophyll $a = (12.72 \times 10^{-3} \times 2.59 \times 10^{-3}) \times V/W \times 1000$

$\text{Chlorophyll b} = (22.88 \times 10^{-3} - 4.67 \times 10^{-3}) \times V/W \times 1000$

Total chlorophyll $= (20.29 \times 10^{-3} + 8.05 \times 10^{-3}) \times V/W \times 1000$

Electron microscopy
The second leaves of the wild-type and $csl1$ the mutant seedlings were fixed using 2.5% glutaraldehyde for 24 h at 4°C. Thereafter, following three washes with 0.1 M PBS, the leaves were subjected to gradient dehydration using 30, 50, 70, 80, 90, and 100% acetone solution at room temperature, followed by the incubation of leaves in each concentration for 15 min. Resin penetration was then performed using an acetone: Epon812 mixture gradient (5:1, 3:1, 1:1, 1:3, and 1:5 v/v), after which the leaves were incubated in pure Epon812 for 12 h. The samples were subsequently embedded in paraffin and 60–80-nm thin sections were obtained using a microtome. The sections were subjected to staining with 2% uranyl acetate and observed using a transmission electron microscope (Leica EM UC6).

RNA extraction and reversion-transcription quantitative PCR(RT-qPCR)
Total RNA extraction was conducted using the TRIzol® Reagent (Invitrogen, USA) according to the manufacturer’s instructions. First-strand cDNA synthesis from 2 μg of the extracted RNA (pre-treated with DNase I) was performed using Rever’tra Ace® (TOYOBO, Japan) in 20-μL reaction systems. The reverse transcription products thus obtained were used as templates for quantitative real-time PCR (RT-qPCR) performed using the CFX ConnectTM real-time PCR system (Bio-Rad) with TB Green™ Premix Ex Taq™II (Ti RNaseH Plus) (Takara, Japan) according to the manufacturer’s instructions. Amplification conditions were 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 30 s. Standard curves were obtained from Ct values and log concentration, and amplification efficiencies (E) were calculated according to the equation: $E = 10^{(-1/\text{slope})} - 1$ [50]. The PCR efficiency of all primers was ≥90% (Table S2), and PCR products were sequenced to evaluate primer specificity. The reference gene was selected from rice ACTIN, β-TUB, GAPDH, 18S-rRNA, UBC, UBQ5 and eIF-4a. UBC gene was used as a reference after being identified using geNorm software [50]. The $2^{-\Delta\Delta Ct}$ analysis method was used to measure the relative expression levels. Each target gene was calibrated according to the WT sample. The primer sequences used in RT-qPCR, in accordance with the MIQE guidelines [51], are listed in Supplemental Table 2.

Plasmid construction and transformation
To generate transgenic OsCSL1 complementation lines, a ~9-kb DNA fragment (extending from ~2.5 kb upstream of ATG to ~1 kb downstream of TGA) was amplified from ZH11 and was then cloned into a binary vector comprising a pCAMBIA2300 backbone to yield a complementary vector. To produce OsCSL1 knockout transgenic lines, CRISPR/Cas9-induced genome editing technique was utilized. sgRNA expression cassette driven by the Zea mays U6α promoter was constructed, which was then assembled into a pYLCRISPR/Cas9Pubi-H vector as per methods previously described [52]. Upon verification via sequencing, the resultant vector was transformed into the Agrobacterium tumefaciens strain EHA105 for subsequent rice transformation. To examine the subcellular localization of the OsCSL1 protein, the pCAMBIA1300-Ubi-OsCSL1-GFP construct was generated, whereas for yeast two-hybrid (Y2H) analyses, different length segments of CSL1 and OsMKK4 were amplified using the corresponding primers. Segments of CSL1 were introduced into pGBKKT7 at EcoRI and BamHI sites, and OsMKK4 was inserted into pGADT7 subjected to digestion with NdeI and BamHI. The corresponding primers used for amplification are listed in Supplementary Table 2.

Subcellular localization
Rice protoplasts were isolated from 1–2-week-old rice seedlings grown on 1/2 MS medium, as per methods previously described by Zhang et al. [53] with minor modifications. The stems of seedlings were cut into 0.5-mm strips using a scalpel and were submerged in 0.6 M mannitol in the dark for 20 min. Subsequently, mannitol used herein was replaced with an enzyme solution (1.5% Cellulase RS, 0.75% Macerozyme R-10, 0.6 M mannitol, 10 mM MES at pH 5.7, 10 mM CaCl2, and 0.1% BSA), prepared immediately prior to use, and the stem tips were subject to digestion in the dark under shaking conditions (60 rpm) for 6 h. The protoplasts thus obtained were filtered through 40-μm nylon meshes into a 50-mL centrifuge tube and were centrifuged at 1,500 rpm to collect the protoplast pellet. After discarding the supernatant, the pellet was resuspended in 2 mL W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, and 2 mM MES, pH 5.7). The protoplast preparations were then transferred to several 2-mL centrifugal tubes, following which they were subjected to washing steps three times
with W5 and were centrifuged at 1,500 rpm. The resultant pellet was resuspended in MMG solution (0.4 M mannitol, 15 mM MgCl$_2$, and 4 mM MES, pH 5.7). For PEG-mediated transfection, 10 μg of total plasmid DNA was mixed with 100 μL of the protoplast preparation, and an equal volume of PEG solution [40% w/v PEG 4000 (Fluka), 0.2 M mannitol and 0.1 M CaCl$_2$]. The mixture was then incubated in the dark for 15 min at 25°C, after which, the protoplasts were collected via centrifugation at 1,500 rpm for 3 min and were then resuspended in 1 mL of the WI solution (0.5 M mannitol, 20 mM KCl, and 4 mM MES, pH 5.7). After incubation at 25°C for 16 h, the protoplasts were examined using a confocal laser scanning microscope (Leica TCS SP8).

**Supplementary Information**

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**Additional file 1:** Figure S1. Chlorophyll contents and phenotypes of the wild-type and heterozygous csl1 lines. Figure S2. Inverse polymerase chain reaction (IPCR) was performed to isolate sequences flanking the OsCSL1 T-DNA. Figure S3. Comparison of the predicted (upper) and amplified (lower) OsCSL1 protein products.

**Additional file 2:** Table S1. Segregation ratio of csl1 heterozygotes. Table S2. The primers used in this study.

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**Authors’ contributions**

JL, QZ, and ZZ designed the project; JL, QZ, and YL performed the experiments; JL, QZ, JZ, WW, and ZZ analyzed and interpreted the data; JL and QZ wrote the manuscript. All authors have read and approved the final version of the manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study will be available from the corresponding authors upon reasonable request.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

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