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OsCSN1 Regulates the Growth and Development of Rice Seedlings through the Degradation of SLR1 in the GA Signaling Pathway

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Abstract: The constitutive photomorphogenesis 9 (COP9) signalosome (CSN) is involved in various regulations during plant development. The CSN is a highly conserved protein complex with nine subunits, and CSN1 acts in a network of signaling pathways critical for plant development. Although CSN1 has been widely studied in *Arabidopsis thaliana*, there have been few investigations on CSN1 in rice. In this paper, using the CRISPR/Cas9 system, CSN1 was edited from *Oryza sativa subsp. japonica* (rice). After screening out the *OsCSN1* knockout mutant and *OsCSN1* reduce mutant, the phenotype and protein expression were identified under different light conditions. Experiments showed that in *OsCSN1* knockout mutant and *OsCSN1* reduce mutant, the SLR1 protein was rapidly degraded at the rice seedling. In this study, the OsCSN1 acted as a negative regulator to affect seedling growth and development through CUL4-based E3 ligase, which is involved in the degradation of SLR1 in the GA signaling pathway. However, its direct target and mechanism of action are not clear.

Keywords: COP9 signalosome; CSN1; rice; SLR1

Citation: Musazade, E.; Liu, Y.; Ren, Y.; Wu, M.; Zeng, H.; Han, S.; Gao, X.; Chen, S.; Guo, L. OsCSN1 Regulates the Growth and Development of Rice Seedlings through the Degradation of SLR1 in the GA Signaling Pathway. *Agronomy* **2022**, *12*, 2946. https://doi.org/10.3390/agronomy12122946

Academic Editors: Mirosława Chwil and Renata Matraszek-Gawron

Received: 11 October 2022
Accepted: 21 November 2022
Published: 24 November 2022

1. Introduction

Light is one of the most needed environmental signals during plant development, participating in and regulating most plant developmental processes [1]. Plants rely on light to control cell differentiation, structural and functional changes, and eventually to assemble into tissues and organs [2]. The process of light-controlling plant development is called photomorphogenesis [2]. In studying the photomorphogenesis of *Arabidopsis thaliana*, a series of *Arabidopsis* photomorphogenesis mutants were obtained through genetic screening. The photomorphogenesis of these mutants was characterized by open apical hooks, short hypocotyls, expanded cotyledons, cell differentiation, chloroplast development, and anthocyanin accumulation, referred to as constitutive photomorphogenesis (COP) [3].

In higher eukaryotes (animals, plants, etc.), the COP9 signalosome (CSN) is a highly conserved protein complex [4]. CSN is acidic, with a molecular weight of about 560 kDa, and is a uniform spherical particle with a diameter of about 12 nm, mainly located in the nucleus [5]. The CSN regulates the plant response to light signals through in the protein degradation pathway, enabling plants to complete the transition from dark growth to light morphogenesis. CSN is considered to be an inhibitor of light regulation and development [6]. The CSN has two functional domains: the PCI domain and MPN domain, in which the PCI domain exists in CSN1-4, CSN7, and CSN8, and the MPN domain exists in CSN5 and CSN6 [4]. It was shown that the N-terminal helical repeat domains of PCI proteins (CSN1-4 and CSN7-8) radiate from the PCI ring at the bottom of the complex, where the most enormous helical bundle (CSN1-4) located on the PCI ring forms a prominent arm-like protrusion. Additionally, the heterodimers formed by the MPN domains of CSN5 and CSN6 are situated on the helical bundle [7]. The MPN dimer, helical pile, and PCI loop include a complex three-layer assembly. Since eukaryotic translation initiation factor 3

(eIF3), the 19S proteasome "lid" subcomplex, and CSN all contain two domains, PCI and MPN, and share this overall structure, it can be assumed that these three complexes are functionally similar and conserved [8].

The CSN can interact directly with the 26S proteasome, a proteasomal degradation pathway, by regulating the activity of ubiquitin E3 ligase [9]. The CSN is involved in and affects the aspects of the plant, regulating the nucleocytoplasmic distribution of COP1, regulating optical signal transduction, temperature signal transduction (cold response and heat stress), and hormone signal transduction in organic plants, etc. [10–13]. The CSN plays a vital role in cell division from the G_0 phase to the whole cell cycle. The knockout mutants of *Arabidopsis* CSN exhibit abnormal cell division, and individual subunits regulate cell cycle progression by controlling the activity of different cell cycle factors [14,15].

The CSN1 is the largest molecular weight of all subunits, and plays a vital part in the structural integrity of CSN [16]. The PCI domain of CSN1 is located at the C- terminal of the amino acid sequence. The CSN1 interacts with other subunits to promote the complex formation and maintain stability [16]. The N- terminal of CSN1 is not involved in the composition of CSN, but has an essential physiological function in plant development and is required for the nuclear localization of COP1 [17]. The CSN1 can interact with TSK-associating protein 1 (TSA1). Expression of TSA1 is significantly reduced in *csn1* null mutants and regulates *Arabidopsis* hypocotyl development under dark conditions [18]. When a specific upstream signal is detected and the N-terminal end of the CSN is unable to bind to the complex, its cellular localization will translocate to the nucleus [19]. In the studies of *Arabidopsis*, it was found that CSN cannot be assembled, and the stability of each subunit would be destroyed when CSN1 was lacking. Thus, it can be seen the accumulation of CSN1 requires the assembly of a complete complex [20]. At the same time, the *csn1* null mutant showed defective skotomorphogenesis and seedling growth stagnation, the mutant lacked Rub1/Nedd8 deconjugate activity on CULIINs. The phenotype of the mutant mainly depended on the function of the N-terminal of CSN1. Loss of CSN1 function can also lead to abnormal floral organ development, low fecundity, defects in the product of shoots, and inflorescences in *Arabidopsis* [21].

In plants, gibberellin (GA) and abscisic acid (ABA) in seed germination and dormancy are involved in the regulation, and these two hormones have antagonistic effects on seed germination. Seed germination was delayed in *Arabidopsis csn5a-1* and *csn1-10* mutants, and the two germination inhibitors expression RGL2 and ABI5 was increased, but the functional deletion of the CSN1 subunit in the *csn1-10* mutant affected the RGL2 degradation [13]. The RGL2 excessive accumulation promoted the synthesis of ABA and activated the expression of ABI5, while CSN5A regulates the proliferation of ABI5 through another mechanism unrelated to CSN1 [13,22]. RT-qPCR was used to analyze the expression of eight *OsCSN* genes in 14-day-old rice seedlings. The results showed that the expression of these eight *OsCSN* genes was significantly reduced after GA treatment, and the transcript level of *OsCSN1* was slightly increased after ABA treatment [23]. These results suggest that *OsCSN* genes may respond to plant hormones and perform different functions [23]. The length of the CSN1 homologous gene in rice is the same as that in *Arabidopsis*, with a total 441 amino acids of CSN1, and the amino acid sequence homology with AtCSN1 is 67% [24]. A previous study showed that rice homologous genes can ultimately rescue the defects of the *AtCSN1* mutant, and can replace AtCSN1 to assemble CSN [25]. *OsCSN1* overexpression mutants can make seedlings have longer hypocotyls and less anthocyanin pigmentation, and lead to enhanced CSN function. The abovementioned studies show that CSN1 plays a significant role in the germination process of plants.

The CSN1 is critical for structural integrity and functionality, and it is the largest-molecular-weight subunit in the CSN complex [19,26]. In rice, CSN1 research is still limited. Therefore, there is an urgent need to conduct studies on phenotypic changes in *OsCSN1* mutants under different light conditions to elucidate the mechanism of action further. Because of the CSN complex and its highly conserved nature, this study used CRISPR/Cas9 technology to edit the CSN1 subunit of *Oryza sativa subsp. japonica* (rice). In addition,

OsCSN1 knockout mutant and *OsCSN1* reduce mutant were screened. Under various light conditions, phenotypic changes in the mutants were observed, and the OsCSN1 function was investigated. Therefore, it is necessary to further elucidate the growth and development mechanism of the *OsCSN1* mutant in the seedling stage.

2. Materials and Methods

2.1. Cultivation Conditions of Plant Materials

O. sativa subsp. japonica (rice) was utilized as the no-treatment control in this paper. Use the CRISPR/Cas9 system to edit and screen out two homozygous mutants. During the reproductive cycle, it was sterilized with ethanol and sodium hypochlorite at *O. sativa subsp. japonica* (wild type) and *oscsn1* mutants, washed with sterile distilled water, and soaked. Seeds on 0.8% (*w*/*v*) solid agar medium were germinated, and grown in glass tubes at 28 °C under various light conditions for 10 days. That the 10 days seedling mutants measured with the height of the plant, the length of the radicle, the length of the coleoptile, and the length of the first and second leaves. Plant samples were subjected to quantitative real-time PCR (qRT-PCR) and western blot (WB). All experimental materials were grown in a greenhouse at Jilin Agricultural University in Changchun, Jilin Province, China.

2.2. Vector Construction and Transformation of Rice

The knockout mutants of *OsCSN1* (LOC_Os03g02540) by the CRISPR/Cas9-based genome editing method were constructed. Three targets guide RNA (sgRNA, Figure 1A) (http://cbi.hzau.edu.cn/cgi-bin/CRISPR, accessed on 4 December 2018) were selected according to the method of CRISPR/Cas9 Plant Gene Knockout Vector (Genloci Biotechnologies Inc., Nanjing, China) (Figure 1B). These sgRNAs using primers in Table 1 were assembled into the vector pP1C.3 (RG1, RG2, RG3, and U3p3-F) (Table 1). Transformation of *Agrobacterium tumefaciens* strain EHA105 with the correctly sequenced vector [27]. Calluses were used to induce wild type mature seeds. Rice was transformed by *Agrobacterium-mediated* transformation using three constructed vectors (pP1C.3-sgRNA1, pP1C.3-sgRNA2, and pP1C.3-sgRNA3). Table 1 shows all the primers used in this study.

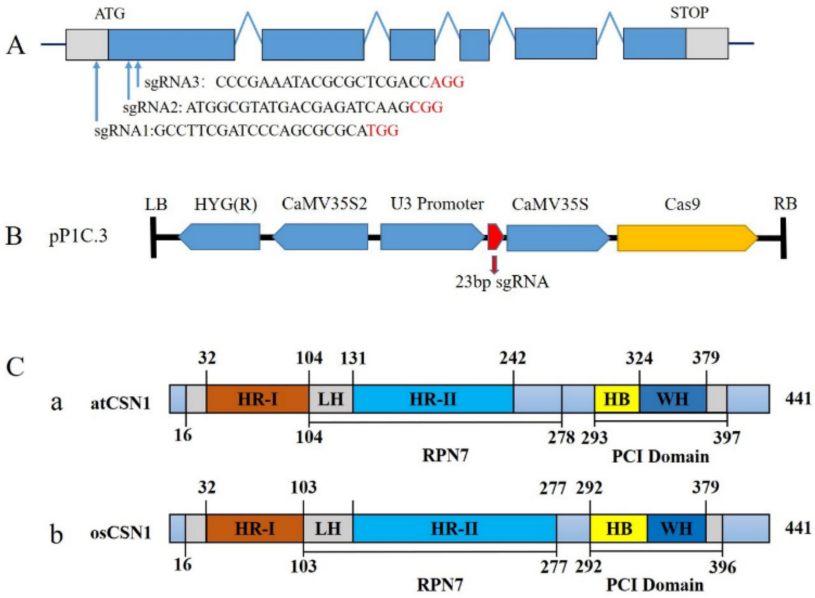


Figure 1. Schematic map of the genomic region of OsCSN1 and structure of OsCSN1. (**A**) The 20 bp sequences for sgRNA and PAM motif (NGG) are highlighted in black and red, respectively. Three target guide RNA sequences were designed in the exon1 of the *OsCSN1* gene. (**B**) The structure of pP1C.3 plasmid. (**C**) Domain structure of OsCSN1 (**b**) and AtCSN1 (**a**). They have four major domains, helical repeat-I (HR-I), linker helix (LH), helical repeat-II (HR-II), and PCI Domain (PCID).

Table 1. All the primers that were used in this study.

Primer	Sequence (5′–3′)	Purpose
RG1	GCTATTTCTAGCTCTAAAAC-TGCGCGCTGGGATCGAAGGCT–TGCCACGGATCATCTGC	Plasmid construction
RG2	GCTATTTCTAGCTCTAAAAC-CTTGATCTCGTCATACGCCAT–TGCCACGGATCATCTGC	Plasmid construction
RG3	GCTATTTCTAGCTCTAAAACGAAAT ACGCGCTCGACCAGGT–TGCCACGGATCATCTGC	
U3p3-F	CAGGAAACAGCTATGACCATATTCAAGG GATCTTTAAAC	Plasmid construction
JD-F3	CGTCTCGTCTCGCACTCTCGCATCG	Mutant detection
JD-R508	CCTGTAGCCATTGAGCTCGCTCTCG	Mutant detection
Hygjc2-F	GTCCGTCAGGACATTGTTGGAGCC	Mutant detection
Hygjc2-R	GTCTCCGACCTGATGCAGCTCTCGG	Mutant detection
RTCas9-F	AAGCCCATCAGAGAGCAGG	Mutant detection
RTCas9-R	TGTCGCCTCCCAGCTGAG	Mutant detection
G1	TGCGCGCTGGGATCGAAGGCT	sgRNA
G2	CTTGATCTCGTCATACGCCA	sgRNA
G3	GAAATACGCGCTCGACCAGG	sgRNA
GAPDHF	AAGCCAGCATCCTATGATCAGATT	q RT-PCR
GAPDHR	CGTAACCCAGAATACCCTTGAGTTT	q RT-PCR
Dye-ABI5F	TGGGATCTGGCATGGTCAAC	q RT-PCR
Dye-ABI5R	TACATGGCGTTTACCGGTCC	q RT-PCR
Dye-SLR1F	CATGCTTTCCGAGCTCAACG	q RT-PCR
Dye-SLR1R	TGACAGTGGACGAGGTGGAA	q RT-PCR
Dye-CUL4F	AGGACAGACAGTATCAGGTGGATGC	q RT-PCR
Dye-CUL4R	TCCGATGGCTTGATTGGGAACTTG	q RT-PCR
Dye-CSN2F	GAGCAGCTCTTGGTCTCACTCATTC	q RT-PCR
Dye-CSN2R	CGACCTGTCACCACGTTCTAGTAAC	q RT-PCR

2.3. Detection of Mutations in T_0 Plants

The modified cetyltrimethylammonium bromide method was used to extract genome-wide transgenic DNA from leaves. The use of the Hygjc2-F and Hygjc2-R paired primers PCR confirmed the *hygromycin phosphotransferase* (HPT) gene presence, and the plants received another pair of primers, RTCas9-F and RTCas9-R (Table 1). This is a PCR method to identify the introduction and mutation, ensurung that all T_0 plants are positively transgenic.

2.4. Transgene-Free Mutant Lines Development

To obtain homozygous transgene-free mutants, DNA and proteins of T_1 seedlings were extracted for testing. At least five T_1 plants from each independent T_0 plant were selected for further analysis of site-specific mutations. We used a pair of primers to analyze T_1 genotype knockout mutants around the OsCSN1 target region by PCR amplification. Sequencing using PCR products generated by 2× Taq StarMix PCR using a loading die (GenStar, China) was performed to detect the *Os*CSN1 mutation sites. Two transgene-free mutant strains were successfully identified: *oscsn1-580* and *oscsn1-191* (Figure 2). The T_2 generation plants and their harvested seeds were used in subsequent experiments.

2.5. Protein Extraction and Western Blot/Antibody Analysis

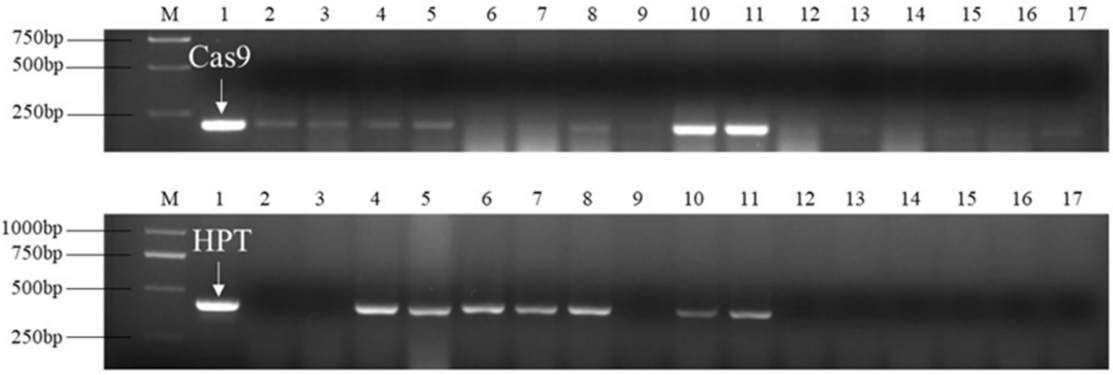
Approximately fresh seedling tissue 0.5 g, 2.5 mM EDTA, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, and 1× complete protease inhibitor cocktail (Roche, China) were used. We transferred the supernatant to a new tube after centrifugation for 10 min, added the same amount of total protein to each sample, and added sample buffer 5× SDS-PAGE (Genstar, China) for mixing. For western blot, boil the sample was mixed for 10 min and loaded onto 10% SDS-PAGE gel. The

antibodies employed in this study were all purchased from Wuhan ABclonal Biotechnology Co., Ltd. These include primary rabbit polyclonal antibody OsCSN2, rabbit polyclonal antibody OsABI5, rabbit polyclonal antibody OsCUL4, and rabbit polyclonal antibody OsSLR1, anti-plant actin, and secondary antibody IgG HRP goat anti-rabbit antibody. Universal Hood III (731BR03292, BIO-RAD) was used to visualize the signal.

A

Development and analysis of T_0 rice plants for targeted mutagenesis of *OsCSN1*			
Plasmid	No. of transgenic plants	No. of plants tested for mutation	No. of double positive of HPT and Cas9 mutated plants
pP1C.3-sgRNA1	78	78	45
pP1C.3-sgRNA2	67	67	42
pP1C.3-sgRNA3	53	53	63

B



C

Mutant allele	Nucleotide (nt) change	Amino acid (aa) change
oscsn1-191	N-terminal delete 20nt	Delete ATG
oscsn1-89	N-terminal delete 50nt	Delete ATG
oscsn1-200	Delete exon1	Delete ATG
oscsn1-580	N-terminal delete 123nt	Delete ATG

D

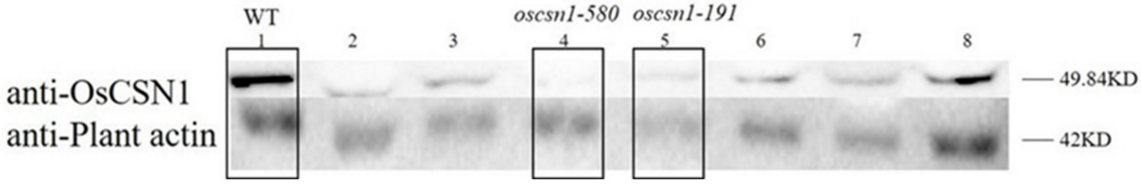


Figure 2. CRISPR/Cas9-induced mutations in OsCSN1. (**A**)Development and analysis of T_0 rice plants for targeted mutagenesis of OsCSN1. (**B**) The upstream and downstream primers were designed using the *hygromycin phosphotransferase* gene (HPT) and *Cas9* gene as templates, and the extracted template DNA of *oscsn1* mutants was double screened to identify positive plants containing knockout plasmids. Identification of transgenic plants by PCR. M, DL-2000marker; 1, pP1C.3 plasmid (PCR-positive control), 4, 5, 8, 10, and 11, double PCR-positive transgenic plants. (**a**) The gene of *Cas9*. (**b**) The gene of *HPT*. (**C**) Nucleotide mutations and the corresponding changes in amino acid sequences are summarized. (**D**) Detection of OsCSN1 in *oscsn1* mutants and wild type by western blot. Actin was probed and served as a loading control. 1, wild type; 2–8, *oscsn1* mutants.

2.6. Real-Time RNA Separation and Quantitative Polymerase Chain Reaction

We used the Spectrum Plant Total RNA kit (Sigma-Aldrich, Darmstadt, Germany) to extract total RNA from seedlings. Reverse transcription was performed using StarScript II First Strand cDNA Synthetic Mix and gDNA Remover (GenStar, Beijing, China) using total

RNA 0.5 mg as a template. We used 2× RealStar Green Fast Mixture with ROX (GenStar, China) to complete a rapid quantitative polymerase chain reaction on the StepOnePlusTM real-time PCR device (Applied Biosystems by Thermo Fisher Scientific, Shanghai, China). We used the blank control (*O. sativa subsp. Japonica*) GAPDH gene (JN848809) to quantify relative mRNA levels calculated by averaging three replicates. The details of the qPCR and RT-PCR gene-specific primers are shown in Table 1.

2.7. Analysis of Statistical

We used at least three biological replicates in each control and treatment. Fisher's least significant difference (LSD) test was employed to compare the average value and SPSS 24.0 software for statistical data analysis.

3. Results

3.1. Overview of the Structure and Function of CSN1

The AtCSN1 included the RPN7 domain and PCI domain, and its crystal structure consisted of a PCI domain (PCID), a linker helix (LH), and two helical repeat domains (HR-I and HR-II) (Figure 1C) [16]. The PCI domain of AtCSN1 can be divided into two subdomains, the winged-helix (WH) and the helix bundle (HB) subdomain in the PCID. Additionally, the HR-I and HR-II are composed in the AtCSN1 RPN7 domain (Figure 1C). The LH, HR-I, and HR-II are respectively tandem arrays of two and three helix-turn-helix units. AtCSN1 and OsCSN1 molecules are compared to the tertiary structure to understand OsCSN1 in this study better. OsCSN1 has subdomains WH and HB in the PCI domain, but the AtCSN1 is longer than the OsCSN1 HB subdomain. However, in the RPN7 domain, the AtCSN1 is shorter than OsCSN1 because of has HR-II and LH structures in OsCSN1. In addition, it allows the protein to integrate into the COP9 signalosome in the C-terminal part of subunit 1 (CSN1-C) (Figure 1C). Therefore, in the present study, we focused on the functional analysis of CSN1 in the rice COP9 signalosome.

3.2. OsCSN1 Mutation Homozygous Transgene-Free Mutant Lines Development in T_0 Plants

In this study, three target guide RNA (sgRNA) (sgRNA1, sgRNA 2 and sgRNA 3, Figure 1A) sequences were designed in the exon1 of the OsCSN1 gene using the CRISPR/Cas9 system. One of them was chosen at the beginning of the coding region of the OsCSN1 gene (Figure 1A), and the others were chosen behind the beginning. This was predicted to raise mutations in the coding region, thereby inactivating the OsCSN1 protein. At T_0 generation, the transformation of the CRISPR/Cas9 vector with sgRNA1, sgRNA2, and sgRNA3 yielded 78, 67, and 53 independent transgenic plants, respectively. However, Cas9 and HPT implants identified approximately 150 double positives in T_0 plants (Figure 2A). Seeds from 87 mutated T_0 plants were collected and bred from CRISPR/Cas9 vector sgRNA1 and sgRNA2 transformations and grown as T_1 plant lines. Interestingly, mutant T_0 plants grown and transformed from the CRISPR/Cas9 vector sgRNA3 had no seeds.

In addition, the mutational effects of all T_1 plant lines were investigated, and each T_1 line sequenced approximately 10 surviving plants for the target site. At last, nine mutations were identified by PCR and sequencing, from single nucleotide (nt) deletion to short fragments, maximum length of 123 nt (Figure 2C). However, these mutants have a change in the size of the OsCSN1 protein. The other mutants displayed many nucleotide deletions from the N-terminal, which deleted a start codon in the respective genes and displayed the most noticeable reductions in the steady-state level of CSN1. Especially, *oscsn1-191*, a *OsCSN1* reduced mutant, had diminished expression of the OsCSN1 protein. Additionally, *oscsn1-580* is a *OsCSN1* knockout mutant, and the OsCSN1 protein was knocked-out in the *oscsn1-580* mutant (Figure 2C,D). Seeds of the stably inherited *oscsn1-580* and *oscsn1-191* mutants were obtained from transgene-free plants and used in subsequent experiments.

3.3. Under Dark Conditions, Oscsn1 Mutants Exhibit Photomorphogenic Growth of Rice

Light is one of the most critical environmental influences in plant growth and development. Plants depend on light to control cell differentiation and structural and functional changes, which eventually converge into tissue and organ building. The process of controlling plant development by light is called photomorphogenesis. The CSN is a negative regulator of photomorphogenesis. The growth state of rice coleoptile is an essential feature of photomorphogenesis. The experiment measured phenotypic data of 10-day-old wild type seedlings to analyze the regulated function of OsCSN1 by measuring coleoptile length, radicle length, plant height, and the incomplete leaf length of each line in *oscsn1-191* and *oscsn1-580* mutants. All *oscsn1* mutants exhibited short coleoptile phenotypes in darkness. Additionally, under the light, the coleoptile length was significantly longer in the wild type than in the oscsn1 mutants (Figure 3A,B). These results suggest that mutant coleoptile elongation was significantly inhibited under dark conditions and exhibited a photomorphogenesis phenotype. Thus, *oscsn1* and *atcsn1* mutants play an inhibiting role in photomorphogenesis.

Moreover, the analyzed data revealed that both *oscn1-191* and *oscsn1-580* mutants had radically longer radicle length and plant height than the wild type under light and dark conditions. The *oscsn1-580* mutant was significantly higher in the dark. The wild type plant height was shorter than that of the *oscsn1-191* mutant (Figure 3A,B). Under light, the wild type first and second leaves lengths were shorter than those of the mutants (Figure 3A,B). The wild type second leaf length was appreciably shorter than that of mutants under dark, and the first leaf length of the mutants was appreciably different from that of the wild type (Figure 3C).

This paper also investigated the effect of two *oscsn1* mutants on seed germination under light and dark conditions. The *oscsn1-191* and *oscsn1-580* mutants grew faster compared with the wild type (Figure 3C). These results suggest that *Os*CSN1 has supplementary functions in rice germination that may inhibit rice photomorphogenesis, and may suppress rice height at the seedling stage.

3.4. Oscsn1 Mutants Phenotype Comparison under Different Light Conditions

To analyze the regulatory effects of OsCSN1 on the photomorphogenesis of rice seedlings. The *oscsn1* mutants and wild type were incubated under different light conditions of red, far-red, and blue light for 10 days, and the plant height, radicle length, incomplete leaves length, and coleoptile length were measured. Under red light, the wild type coleoptile was longer than the two *oscsn1* mutants. The radicle length, plant height, and incomplete leaves lengths among the mutants were similar to the wild type (Figure 4A,D). Under far-red light, the wild type plant height, radicle length, and coleoptile length were much longer than *oscsn1-580* mutant, but all wild type phenotypic data were longer than *oscsn1-191* mutant. There were no significant differences between wild type data and other data (Figure 4B,D). Under blue light, the wild type radicle length was longer than the *oscsn1* mutant. Both plant height and coleoptile length were much shorter in the wild type than in the *oscsn1* mutant. However, the incomplete leaf lengths of the wild type and mutants did not differ much (Figure 4C,D).

These results indicated that red light inhibited seedling elongation of the *oscsn1-191* mutants. Under blue light, the growth of wild type and mutants were inhibited, but that of the *oscsn1* mutants were significantly suppressed (Figure 4C,D). These observations suggest that OsCSN1 is an inhibitor of red, far-red, and blue light-mediated regulatory processes and that the inhibitory effect of OsCSN1 is stronger under far-red light and blue light conditions than under red light conditions.

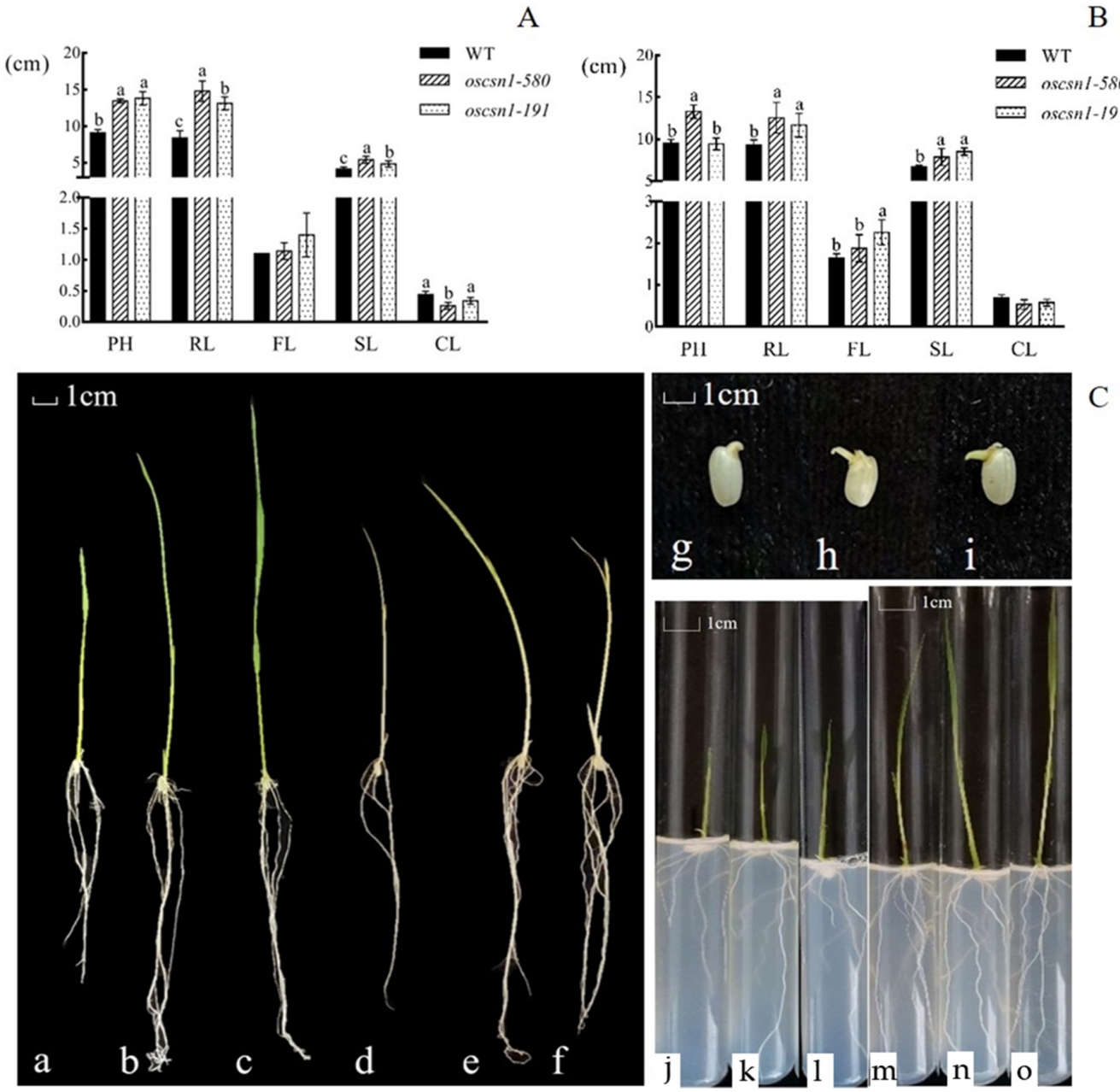


Figure 3. Phenotypic characteristics of wild type and *oscsn1* mutants grown under different light conditions. The seeds were incubated in an artificial climate chamber set at 28 °C for 10 days. (**A**) Plants were grown in the light. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. The small letters a, b, c represent the within-group difference significance analysis. Data are mean ± SD (n = 5). Means with different letters are significantly different ($p < 0.05$). (**B**) Plants were grown in the dark. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. The small letters a, b represent the within-group difference significance analysis. Data are mean ± SD (n = 5). Means with different letters are significantly different ($p < 0.05$). (**C**) Image of wild type and oscsn1 mutant seedlings grown under different light conditions. Scale bar = 1 cm. Wild type (**a**), *oscsn1-580* mutant (**b**) and *oscsn1-191* mutant (**c**) grown in the light for 10 days. Wild type (**d**), *oscsn1-580* mutant (**e**) and *oscsn1-191* mutant (**f**) grown in the dark for 10 days. Wild type (**g**), oscsn1-580 (**h**) and oscsn1-191 (**i**) seed germinated in light for 2 days. Wild type (**j**), oscsn1-580 (**k**) and oscsn1-191 (**l**) grown in light for 6 days. Wild type (**m**), oscsn1-580 (**n**) and oscsn1-191 (**o**) grown in light for 10 days.

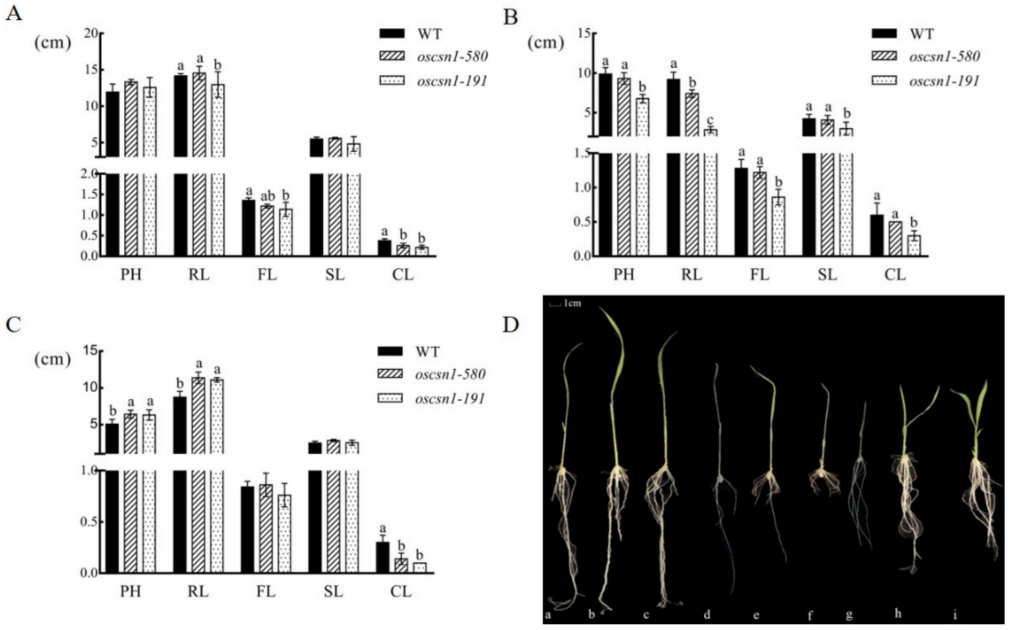


Figure 4. Phenotypic analysis of 10-day-old wild type and *oscsn1* mutants grown under different conditions. (**A**) Phenotypic analysis of plants under red light. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. The small letters a, b represent the within-group difference significance analysis. (**B**) Phenotypic analysis of plants under the far-red light. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. The small letters a, b, c represent the within-group difference significance analysis. (**C**) Phenotypic analysis of plants under the blue light. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. The small letters a, b represent the within-group difference significance analysis. Different letters within a column indicate significant differences at $p < 0.05$ according to Duncan's multiple range test. (**D**) Image of 10-day-old wild type and *oscsn1* mutant seedlings grown under different light conditions. Scale bar = 1 cm. Wild type (**a**), *oscsn1-580* (**b**) and *oscsn1-191* (**c**) were grown under red light. Wild type (**d**), *oscsn1-580* (**e**) and *oscsn1-191* (**f**) were grown under the far-red light. Wild type (**g**), *oscsn1-580* (**h**), and *oscsn1-191* (**i**) were grown under the blue light.

3.5. GA Biosynthesis Characterization of Oscsn1 Null Mutants at the Seedling Stage

Gibberellin (GA) is a growth-regulating phytohormone that affects various developmental processes in plants. In *Arabidopsis*, the gibberellin response pathway is negatively regulated by five DELLA proteins: RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2), RGA-LIKE3 (RGL3) [12,13], REPRESSOR OF ga1-3 (RGA), and GA-INSENSITIVE (GAI). The SLENDER RICE1 (SLR1) of DELLA protein is only one protein in rice [28,29].

In seedling growth and development, the wild type develops more slowly than the *oscsn1* mutants. In addition, the *oscsn1* mutants may regulate the level of seed dormancy by responding to the GA signaling pathway and promote plant growth and development at the seedling stage by responding to GA. It is hypothesized that in rice, OsCSN1 may have a function in the seedling stage that is not present in AtCSN1.

To test this hypothesis, OsSLR1 and OsABI5 protein levels were examined by western blot in wildtype and *oscsn1* mutants grown in the light for 10 days. The SLR1 protein in *oscsn1-580* and *oscsn1-191* mutants exhibited a decrease compared with the wild type, inconsistent with the abnormal accumulation of SLR1 protein in *Arabidopsis*. The amount of ABI5 protein was significantly reduced in *oscsn1-580* and *oscsn1-191* mutants compared with the wild type (Figure 5A). Under red, far-red, and blue light, SLR1 protein expression was significantly up-regulated in *oscsn1* mutants compared with light (Figure 5B–D). The ABI5 expression was significantly down-regulated in wild type and mutants compared with light (Figure 5B–D). The SLR1 protein showed up-regulation in mutants under blue

light, but the ABI5 protein showed down-regulation (Figure 5B–D). The resulting analysis showed that knockout or reduce of OsCSN1 in response to different light caused abnormal synthesis or degradation of SLR1 and ABI5 in the *oscsn1* mutant, thus affecting the *oscsn1* mutant seedlings to exhibit a fast growth phenotype. Based on the analysis of the results, it can be concluded that the rapid-growth phenotype of the *oscsn1* mutants in seedling may be due to abnormal hormone metabolism and biosynthesis.

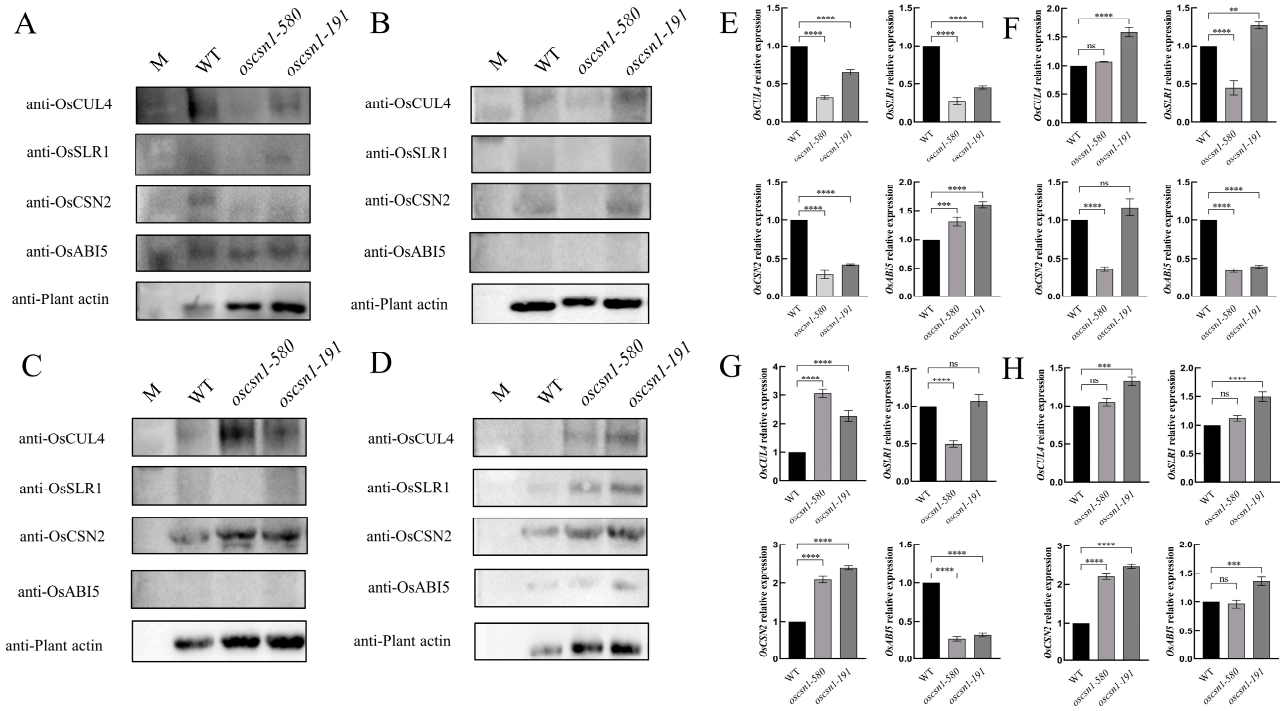


Figure 5. Expression protein and gene modification status in the *oscsn1* mutants and the wild type under different light. Detection of proteins level in the wild type and *oscsn1* mutants using a western blot. (**A**) Under light. (**B**) Under red light. (**C**) Under far-red light. (**D**) Under blue light. qRT-PCR analysis of the expression levels of gene in the wild type and *oscsn1* mutants. (**E**) Under light. (**F**) Under red light. (**G**) Under far-red light. (**H**) Under blue light. The average values (± SD) from three biological repeats are shown. For each gene, different letters indicate significant differences in expression according to Duncan's multiple range test, ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns stands for no significant difference.

In this study, qRT-PCR was used to analyze the gene expression of *ABI5* and *SLR1* in *OsCSN1* knockout mutant and *OsCSN1* reduce mutant (Figure 5B). Under light, the expression of the *SLR1* gene in *oscsn1-580* and *oscsn1-191* mutants showed significant down-regulation compared with the wild type (Figure 5E). Additionally, the expression of the *ABI5* gene in mutants showed up-regulation compared with the wild type (Figure 5E). The *SLR1* gene expression in the *oscsn1-580* mutant showed significant down-regulation under red and far-red light, and the *SLR1* gene expression in the *oscsn1-191* mutant showed no significant change compared to the wild type (Figure 5F,G). The *ABI5* gene showed considerable down-regulation in mutants under red light and far-red light (Figure 5F,G). Under blue light, the *SLR1* gene showed considerable up-regulation in *oscsn1* mutants, and the *ABI5* gene expression was unchanged (Figure 5H). The results suggest that OsCSN1 may be degraded and play a role in rice seedling growth and development through the DELLA protein SLR1 in the GA signaling pathway.

3.6. OsCSN1 Affects Seedling Growth via CUL4-Based E3 Ligase

The CSN2 is a component of CSN and is stable only when present in the form of a complex. It was found that the CSN2 protein interacts strongly with the CSN1 protein

and with the CSN complex in *Arabidopsis* [30]. The CSN accumulates steadily only when all subunits are present. CULLIN-4 (CUL4) is a member of the CULLIN family of related ubiquitin ligases that primarily mediates the ubiquitination and subsequent proteasomal degradation of target proteins. The CUL4 can exist as a ubiquitin E3 ligase complex, and CSN regulates plant photomorphogenesis by controlling the RUB/NEDD8 modification of CUL4-based E3 ligases. It can also interact with the CSN complex in response to DNA damage [4,31,32].

In the study, the protein expression of CSN2 was significantly lower in *oscsn1-580* and *ocsn1-191* mutants under light than in the wild type (Figure 5A). Under red light, it resulted in a significant up-regulation of CSN2 protein expression in wild type and *oscsn1-191* mutant compared with light (Figure 5B). Additionally, the CSN2 protein expression of wild type and mutant under far-red and blue light also showed some degree of up-regulation (Figure 5C,D). Under light, the *CSN2* gene expression in *oscsn1* mutants showed down-regulation compared with the wild type (Figure 5E). The *OsCSN1* knockout mutant and *OsCSN1* reduce mutant showed significant up-regulation of *CSN2* gene expression under red, far-red, and blue light compared with light (Figure 5F–H). According to the analysis of the results, when *OsCSN1* was knockout or had reduced expression, it resulted in a significant downregulation of CSN2 expression. Under light, the protein expression of CUL4 in the *oscsn1-580* mutant showed significant down-regulation compared with the wild type. The protein expression of CUL4 in the *oscsn1-191* mutant showed down-regulation compared with the wild type but to a lesser extent than that of *oscsn1-580* mutant (Figure 5A). Under red light, the protein expression of CUL4 in wild type and mutants showed significant up-regulation compared with that under light (Figure 5B). The CUL4 protein expression in the wild type appeared up-regulated under both far-red and blue light compared to light (Figure 5C,D). The expression of CUL4 protein in the mutants under far-red light appeared up-regulated compared with light conditions. No significant changes were observed in the expression under blue light (Figure 5C,D). Under far-red light, *OsCSN1* mutants showed significant up-regulation of CUL4 protein expression compared with the wild type. Among them, the CUL4 protein expression was significantly up-regulated in the *oscsn1-580* mutant (Figure 5C). Under light, the *CUL4* gene expression in the *oscsn1-580* and *oscsn1-191* mutants exhibited significant down-regulation compared with the wild type (Figure 5E). The expression of *CUL4* gene showed significant up-regulation in mutants under light, far-red light and blue light (Figure 5F–H). Analysis of the results indicated that rice seedling growth may be regulated by the CSN complex controlling the CUL4 protein in the CULLIN-Ring family, which governs seedling growth or photomorphogenesis establishment in rice. Deletion of the OsCSN1 protein leads to some degree of abnormal expression of CUL4 protein.

4. Discussion

Previous papers have shown that the CSN is involved in seed germination and seedling growth, and promotes germination by regulating the degradation of the DELLA protein RGL2 and the ABA effector ABI5 in *Arabidopsis*. That showed apparent defects in the timely degradation of RGL2 in the *atcsn1-10* mutant, and this mechanism does not exist in rice. The ABI5 protein is rapidly degraded during the seedling stage in the *oscsn1* mutant. Furthermore, the *oscsn1* mutant phenotype is significantly different from that of the *atcsn1-10* mutant, showing a weak resting phenotype and a fast-growing phenotype. Yet, its direct molecular target and mechanism of action are still indistinct.

4.1. The Functional Difference between OsCSN1 and AtCSN1 Subunits in the Seedling Period

Since the function of CSN1 has been less studied in rice, *OsCSN1* knockout mutant exhibit photomorphogenesis in the dark. In contrast, null mutants of CSN1 in *Arabidopsis* cause premature seedling death with a typical "*fusca*" phenotype [13,33]. Ineffective mutants of the OsCSN1 subunit exhibited a short germinal sheath phenotype in the dark, consistent with the role of AtCSN1. However, the *ocsn1* mutant exhibited a fast-growth

phenotype, which differed from the *atcsn1-10* mutant [13,33]. These causes suggest that OsCSN1 and AtCSN1 have different regulatory mechanisms in germination and seedling growth and development, which they have additional functions in OsCSN1 seedling germination and growth.

However, the analyses showed that the phenotype of the *oscsn1* mutant was different from the wild type in the following aspects. First, the *oscsn1* mutant had a significantly shorter radicle sheath than the wild type under light and darkness (Figure 3A,B). Second, the phenotypes of the mutants differed under different lights (Figure 4). The wild type had a longer coleoptile length than the *oscsn1* mutant under red conditions (Figure 4A,D). The *oscsn1-191* and the *oscsn1-580* mutant had different phenotypes: plant height, coleoptile length, and radicle length were suppressed in *oscsn1-191* under far-red light conditions (Figure 4B,D). Seedling elongation was inhibited under blue light conditions in the *oscsn1* mutant, to a much stronger extent than in the wild type (Figure 4C,D). The OsCSN1 may have an auxiliary function that acts differently, inhibiting photomorphogenesis and rice seedling height.

4.2. OsCSN1 Plays a Role in GA Signaling

By understanding the comparison, the OsCSN1 may have a different function than AtCSN1. It can decrease seed dormancy levels by responding to ABA in *oscsn1* mutants, followed by seedling growth and development by responding to GA. This study showed that SLR1 and ABI5 proteins exhibited significant down-regulation in *oscsn1* mutants (Figure 5A). The SLR1 protein expression in OsCSN1 knockout and reduce mutants showed significant up-regulation compared with light under red, far-red, and blue light. In contrast, ABI5 protein expression in wild type and mutants showed significant down-regulation compared with light (Figure 5B–D). At the mRNA level, the expression of the *SLR1* gene showed considerable down-regulation in the two mutants under light. The expression of the *ABI5* gene did not differ from the wild type (Figure 5E). Under red, far-red and blue light, the *SLR1* gene expression in *OsCSN1* knockout and reduced mutants showed significant up-regulation compared with light (Figure 5F–H). However, the *ABI5* gene expression was up-regulated in *OsCSN1* knockout and reduced mutants under red and far-red light (Figure 5F,G). There was no significant change in *ABI5* gene expression under blue light (Figure 5G). The results indicated that OsCSN1 can directly or indirectly regulate the expression of the DELLA protein SLR1 in the GA signaling pathway and inhibit rice seedling development. The OsCSN1 may also inhibit ABI5 protein degradation at the seedling stage and cause ABI5 to be specifically modified in the *OsCSN1* mutants.

The *OsCSN1* knockout mutant and *OsCSN1* reduce mutant showed significant down-regulation of CSN2 protein under light (Figure 5A). The CSN2 protein expression was upregulated in *oscsn1* mutants under different light compared with light (Figure 5B–D). The changes in the *CSN2* gene expression at the mRNA level in the two *oscsn1* mutants under different light were consistent with the trend of protein expression. Combining the protein functions of CSN1 and CSN2, the analysis revealed that the presence of CSN1 affects the expression of CSN2 protein and even the assembly of the CSN complex. Red light can up-regulate the expression of CSN2. When CSN1 completely lost its function, there was no significant change in CSN2 expression. When in the *OsCSN1* reduced mutant, the CSN2 protein expression could be restored to match that of the wild type. It is hypothesized that OsCSN1 can promote the expression of CSN2 in response to red light, and it will even promote the assembly of the CSN complex.

CULLIN-RING ligases (CRL) are the most prominent family of ubiquitin E3s regulated by the COP9 signalosome (CSN) [34]. The CUL4 protein is a member of the CULLIN-Ring family of proteins. CSN can control the RUB/NEDD8 modification of several CUL4-based E3 ligases, thereby regulating plant photomorphogenesis. The results showed that the *OsCSN1* knockout resulted in a significant downregulation of CUL4 protein and *CUL4* gene expression due to the complete loss of function of CSN1. In contrast, OsCSN1 reduction also resulted in down-regulation of CUL4 protein and *CUL4* gene expression, but to a

lesser extent than in the *OsCSN1* knockout mutant. There was no significant change in the expression of the *CUL4* gene at the mRNA level under red and blue light compared to that under light. Far-red light resulted in significant up-regulation of *CUL4* gene expression in mutants. The changes in CUL4 protein expression in the two mutants under different light were consistent with the trend of gene expression. It is thus suggested that CSN1 may have some effect on rice seedling growth by regulating the expression of CUL4 protein. It may also have an impact on the photomorphogenesis of rice. We hypothesize that OsCSN1 may affect the degradation of SLR1 in the GA signaling pathway through CUL4-based E3 ligase, which affects rice seedling growth and development.

Based on the above results and analysis, the OsCSN1 may respond to different light and affect the photomorphogenesis of rice, which has some effects on the growth and development of rice seedlings. The OsCSN1 senses different light signals and exerts a crucial role in the seedling growth and development process through the GA signaling pathway. In this study, the OsCSN1 acted as a negative regulator to affect seedling growth and development through CUL4-based E3 ligase, which is involved in the degradation of SLR1 in the GA signaling pathway. However, the exact molecular mechanism of its action is still unknown, which will be the direction of future research. Studies on the function of OsCSN1 gene revealed that loss of OsCSN1 function resulted in rapid growth occurring in rice seedlings. The *oscsn1* mutants exhibited significant fertility shortening. Therefore, it could be an important breakthrough affecting the shortening of fertility in rice. Based on the phenotype and related protein expression, the molecular regulatory mechanism of CSN1 affecting seedling growth was explored to improve grain production to meet the food demand of the growing population.

Author Contributions: Data curation, E.M., Y.L., Y.R., H.Z., S.H., X.G. and S.C.; formal analysis, E.M., Y.L., Y.R., H.Z., S.H. and X.G.; funding acquisition, S.C.; investigation, Y.L., Y.R., H.Z., S.H., X.G. and S.C.; methodology, E.M., M.W. and L.G.; resources, M.W. and L.G.; supervision, M.W. and L.G.; writing—original draft, E.M., Y.L. and S.H.; writing—review and editing, M.W. and L.G. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Department of Jilin Province Science & Technology [grant numbers 20210509019RQ, 20210203011SF].

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the article.

Acknowledgments: We thank the Department of Jilin Province Science & Technology. We thank editors and reviewers for critical and helpful comments for the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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