Generation and Characterization of *oscsn1* Mutants Reveal That OsCSN1 Regulate ABI5 Degradation In Seedling Growth

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Abstract

In many developmental processes in plants, the COP9 signalosome (CSN) plays multiple effects. It is a complex composed of eight subunits CSN1 to CSN8, which is very conservative.The CSN1 acted in a network of signal transduction pathways critical for plant development. Although there are many studies on the CSN1 subunit in Arabidopsis, there are few studies on the CSN1 subunit in rice. We used CRISPR/Cas9 technology to edit the CSN1 subunit of Oryza sativa subsp. japonica (rice). We screened knockout mutants and then observed phenotypic changes of the mutants under different light conditions. Previous research demonstrated that atCSN1 promotes seed germination by regulating ABA effector ABI5. However, we found that this mechanism did not occur in rice. In the oscsn1 mutant, ABI5 protein was rapidly degraded at the seedling stage, and it did not show the displayed defects in degradation of ABI5. As a result, the mutants exhibited weak dormancy and the rapid growth phenotype of seedlings. Our observations demonstrate that osCSN1 plays a role in effecting growth and development by regulating protein turnover the ABA effector ABI5, but the direct the mechanism of their action and molecular targets are needed to explore.

1. Introduction

In many developmental processes in plants, the COP9 signalosome (CSN) plays multiple effects. It affects many repression of photomorphogenesis, DNA-damage response and protein subcellular localization in plants. The CSN complex is important in regulating gene expression, cell proliferation and the cell cycle, and it regulates the activity of E3 ubiquitin ligase complexes, which is a member of cullin-RING ligase (CRL) families. Studies on CSN in various organisms show that its function is very conservative, and the most studied plant is *Arabidopsis*. CSN is a greater than 300-kDa complex, it was first identified as a negative regulator of Constitutive Photomorphogenesis in plants [1, 2]. It is a complex composed of eight subunits CSN1 to CSN8, which is very conservative. Following the descending order of molecular weights, subunits named CSN1 to CSN8. All eight subunits share homologous sequences with “lid”, which components of the 26S proteasome regulatory particle and eIF3 (eukaryotic translation initiation factor 3) [3, 4]. In addition, the function of CSN and the lid subcomplex both act as metalloisopeptidases (Cope et al, 2002). Among these eight subunits, CSN1, CSN2, CSN3, CSN4, CSN7 and CSN8 have a PCI-domain (Proteasome, COP9 signalosome, Initiation factor eIF3), and the rest of the CSN subunits (CSN5 and CSN6) contain a conserved MPN domain (MOV34, Pad1N-terminal) [5]. The PCI-domain has two major domains, a globular winged-helix subdomain and an N-terminal helical bundle arrangement [7–9, 22]. Among the 8 subunits of COP9 signalosome complex (CSN), research on COP9 signalosome complex subunit 1(CSN1) began in 1994. The CSN1 proteins appear to act in a network of signal transduction pathways critical for plant development [10]. In the study of *Arabidopsis thaliana* CSN1 (atCSN1), this C-terminal domain (CTD) is necessary and sufficient for CSN1 to integrate into the CSN complex with other subunits, CTD harbors the binding sites for CSN2, 3, and 4 subunits [11]. In the light signaling pathway, Wang et al. (2009) found that COP1 can respond to light signals by the expression of CSN1. The N-terminal domain of CSN1 is necessary and sufficient for CSN1-induced COP1

nuclear localization [12]. In regulating plant photomorphogenesis, CSN also controls several CUL4-based E3 ligases by RUB/NEDD8 modification [13]. Although the most known function of CSN is to regulate the ubiquitin-proteasome pathway [14]. But CSN also plays a role in gene expression. Tsuge et al. (2011) found that CSN1 blocks ectopic expression of JNK1 by inhibiting c-Jun phosphorylation, without affecting c-Jun protein stability [15]. Li et al. (2011) demonstrated that TSK-associating protein 1 (TSA1) interacts with the NTD of COP9 signalosome complex subunit 1(CSN1), it is a reported Ca^{2+} binding protein. They also demonstrated a functional relationship between TSA1 and COP9 signalosome complex subunit 1(CSN1) in *Arabidopsis* seedling development in darkness [16].

Rice is an important food crop worldwide, and nearly half of the global population consumes rice as their staple food [17]. Although there are many studies on the CSN1 subunit in Arabidopsis, there are few studies on the CSN1 subunit in rice. Among all of the subunits of the complex, COP9 signalosome complex subunit 1(CSN1) plays a crucial role in complex integrity and it is the longest [11, 18]. In view of the high degree of conservation of the CSN complex, we used CRISPR/Cas9 technology to edit the CSN1 subunit of *Oryza sativa subsp. japonica* (rice). We screened knockout mutants and then observed phenotypic changes of the mutants under different light conditions.

2. Materials And Methods

2.1 Plant materials and culture conditions

O. sativa subsp. japonica (rice) was used as the wild type in this study. Two homozygous mutants (loss-of-function *oscsn1-580* and *reduce oscsn1-191*) were generated in the *O. sativa subsp. japonica* background using the CRISPR/Cas9 system. During the growing season, all materials were cultivated in a greenhouse at Jilin Agricultural University in Changchun, Jilin Province, China. *O. sativa subsp. japonica* (wild type) and *oscsn1* mutants were sterilized with 70% (v/v) ethanol for 2 min and with 40% (v/v) sodium hypochlorite (NaClO) for 40 min, then washed four times for 20 min with sterilized water. All seeds were germinated on 0.8% (w/v) agar solid medium in a glass tube for 10 d, and the glass tubes were incubated in a growth chamber at 28℃ with different light conditions. The 10-d-old (third leaf stage) seedlings were measured for radicle length, plant height, coleoptile length, length of the first leaf and the second leaf. The plants were frozen in liquid nitrogen for WB and qRT-PCR.

2.2 Construction of vectors and rice transformation

To generate *OsCSN1* (LOC_Os03g02540) knockout mutants, the CRISPR/Cas9-based genome editing method was used. Three target guide RNA (sgRNA) (sgRNA1, sgRNA 2 and sgRNA 3, Fig. 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 (Fig. 1A), and the others were chosen behind the beginning. These sgRNAs were assembled into the vector pP1C.3 (CRISPR/Cas9, Fig. 1B) using primers RG1, RG2, RG3 and U3p3-F (Table S1), following the method of CRISPR/Cas9 Plant Gene Knockout Vector (Genloci Biotechnologies Inc., China). The constructed vectors were confirmed by sequencing and transformed into *Agrobacterium tumefaciens* strain EHA105 [19, 20]. Calluses were

induced from mature seeds of *O. sativa subsp. Japonica* and transformed with the three constructed vectors (pP1C.3-sgRNA1, pP1C.3-sgRNA G2, and pP1C.3-sgRNA 3) through *Agrobacterium-mediated* genetic transformation of rice according with the method of Chen and Liu et al., (2018) [19, 20]. All primers that were used in this study are listed in Table S1.

2.3 Detection of mutations in T_0 plants

For detection of transgenes and mutations in regenerated T_0 plants, total genomic DNA of transgenes was extracted from rice leaves following a modified cetyltrimethylammonium bromide method [21]. To confirm that all T_0 plants were positive transgenic lines, the presence of the *hygromycin phospho-transferase* (HPT) gene was determined by PCR using a pair of primers, Hygjc2-F and Hygjc2-R, and plants were also tested by PCR using another pair of primers, RTCas9-F and RTCas9-R (Table S1).

2.4 Development of transgene-free mutant lines

To obtain homozygous transgene-free mutants, DNA and proteins of T_1 seedlings were extracted for testing. At least five T1 plants from each independent T_0 plant were selected for further analysis of site-specific mutations. To analyze the knock-out mutation genotypes of the T_1 plants, all transgenic lines were detected by PCR amplification using a pair of primers (JD-F3 and JD-R508) surrounding the target region of *OsCSN1*. The PCR products were generated using a 2×Taq PCR StarMix with Loading Dye (GenStar, China), and sequenced to determine the mutation sites of *OsCSN1*. Two transgene-free mutant lines *oscsn1-580* and *oscsn1-191*) were successfully identified (Fig. 2). The T_2 plants and advanced-generation seeds were used for the following experiments.

2.5 Protein extraction, western *blot analysis/antibodies and immunoblot analysis*

About 0.5 g of fresh seedling tissues were extracted with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 2.5 mM EDTA, 1 mM DTT, 0.1% NP-40, 1 mM PMSF, and 1× complete protease inhibitor cocktail (Roche, China). An equal amount of total proteins for each sample was added 5× SDS-PAGE Sample Buffer (Genstar, China), and then the mixture was vortexed. The mixed sample was boiled for 10 min, and then was centrifuged for 10 min. The supernatants were transferred to a new tube and loaded onto a 10% SDS-PAGE gel for western blotting. Antibodies used for this study include anti-osCSN1, anti-osSLR1, anti-osABI5, anti-plant actin (ABclonal, China) primary antibodies, and an HRP Goat Anti-Rabbit IgG secondary antibody (ABclonal, China). The signals were visualized with the Universal Hood III (731BR03292, BIO-RAD). All polyclonal Antibodies used for this study were made by Wuhan ABclonal Biotechnology Co., Ltd. (ABclonal, China), including rabbit polyclonal OsCSN1 antibody, rabbit polyclonal OsSLR1 antibody, and rabbit polyclonal OsABI5 antibody.

2.6 RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted from the seedling materials using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, Germany), and 0.5 mg of total RNA as a starting material was reverse transcribed with StarScript II First-strand cDNA Synthesis Mix With gDNA Remover (GenStar, China). A qRT-PCR was completed with 2× RealStar Green Fast Mixture with ROX (GenStar, China) on the StepOnePlus™ Real-Time PCR Instrument (Applied Biosystems by Thermo Fisher Scientific, China). The GAPDH gene of *O. sativa subsp. japonica* (JN848809) was used as an internal control to quantify relative mRNA levels that were calculated from the means of three replicates. The gene-specific primers of RT-PCR and qPCR are listed in Table S1.

2.7 Statistical analysis

At least three biological replicates were used for each treatment and control. Statistical analyses of the data were accomplished using SPSS 24.0 software. Mean values were compared using Fisher's least significant difference (LSD) test.

3. Results

3.1 CSN1 structure and function overview

The *Arabidopsis thaliana* CSN1 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) (Fig. 1C) [22]. The RPN7 domain of atCSN1 is composed of HR-I and HR-II. HR-I is two helix-turn-helix units, HR-II is three helix-turn-helix units, then HR-I and HR-II are tandem arrays through LH; LH is the longest single α-helix.

The PCID of *Arabidopsis thaliana* COP9 signalosome complex subunit 1(CSN1) consists of the helix bundle (HB) subdomain and the winged-helix (WH) subdomain (Fig. 1C). COP9 signalosome complex subunit 1(CSN1) (CSN1 or GPS1) is an important subunit for structural integrity and function of the CSN complex. The N-terminal region of CSN1 (CSN1-N) can control most of the repression functions of CSN1. The C-terminal region of subunit 1 (CSN1-C) regulates the combination of the COP9 signalosome complex subunits [22, 23].

In this study, we focused on the largest subunit of the COP9 signalosome, CSN1, for function analysis in rice. First, we compared the tertiary structures between the atCSN1 molecule and osCSN1 molecule to better understand osCSN1 (Fig. 1C). In the RPN7 domain, osCSN1 has LH and HR-II structure, which is longer than atCSN1. In the PCI domain, osCSN1 has HB and WH subdomains, but the HB subdomain of osCSN1 is shorter than atCSN1.

3.2 Mutations of OsCSN1 in T0 plants and development of homozygous transgene-free mutant lines

We designed a CRISPR/Cas9 construct to edit *OsCSN1* in the first exon (Fig. 1A), which was expected to cause mutation in the coding region and thus inactivate the OsCSN1 protein. In the T_0 generation, we

obtained 78, 67, and 53 independent transgenic plants, which were raised from transformation with the sgRNA1, sgRNA2 and sgRNA3 of CRISPR/Cas9 vectors. About 150 double positive HPT and Cas9 T_0 plants were confirmed (Fig. 2A). Seeds of the 87 mutated T_0 plants were harvested, raised from transformation with the sgRNA1 and sgRNA2 of CRISPR/Cas9 vectors, and grown as T_1 plant lines. Interestingly, the mutated T_0 plants raised from transformation with sgRNA3 of CRISPR/Cas9 vector had no seeds.

All T_1 plant lines were further tested for mutation affects. About 10 surviving plants of each T_1 line were sequenced for the target site. Ultimately, nine types of mutation were identified by PCR and sequencing, ranging from a single nucleotide (nt) deletion to deletion of short fragments up to 123 nt (Fig. 2C). Three mutants harbored a 1-nt change, and two mutants harbored changes of more than two nucleotides. However, the CSN1 protein of those mutants were not affected. 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; in particular, *oscsn1-580* is a null mutant of *osCSN1*, while *oscsn1-191* is a weak allele of *osCSN1* (Fig. 2C and 2D). Seeds of these verified transgene-free, homozygous mutant plants, designated as the mutant lines *oscsn1-580* and *oscsn1-191*, were harvested and used for further evaluation of osCSN1.

3.3 *Loss-of-function of OsCSN1 mutants displayed rice photomorphogenic growth under dark condition*

OsCSN1 null mutant have not been previously reported. To analyze the regulated function of osCSN1, 10-d-old seedlings of the wild type (*O. sativa spp.japonica*), *oscsn1-580* and *oscsn1-191* grown in the growth chamber were harvested. Light is one of the most important environmental factors in the normal life activities of plants. The process of plant development regulated by light is called photomorphogenesis. The seedlings of dicotyledons show short hypocotyls, no apical hooks, and large cotyledons with chloroplasts under light. The seedlings of dicotyledons growing in the dark are characterized by elongated hypocotyls, crooked apices, small and closed cotyledons, lack of chlorophyll, yellow or white color and abnormal chloroplast development. This is called dark morphogenesis or etiolation. The phenotype of rice seedlings growing in the dark includes coleoptile elongation and lack of chlorophyll with yellowing. However, when the etiolated seedlings are exposed to light, expression of light regulated genes is rapidly induced, elongation inhibition of coleoptiles and internodes is relieved and chlorophyll is synthesized for photosynthetic autotrophy. Loss-of-function mutants of atCSN1 have been described, and all of these mutants are phenotypically indistinguishable in that they display the pleiotropic *cop/det/fus* mutant phenotype, which is characterized by a short hypocotyl and open cotyledons in dark-grown seedlings, the accumulation of the anthocyanin pigment, and the expression of light-induced genes in the dark [24, 25, 27–29, 31].

Coleoptile growth in rice is an important feature of photomorphogenesis. So we examined radicle length, plant height, coleoptile length, the first leaf length and the second leaf length of 10-day-old seedlings *oscsn1-580*, *oscsn1-191* and wild type (10 seeds of every line were tested). We found that both *oscsn1*

mutants have identical phenotypes. They exhibit a short coleoptile phenotype in darkness, and even in light, the coleoptile length of the *oscsn1* mutant was significantly shorter than that of the wild type (Fig. 3A and B). These results indicated that the elongation of the mutant coleoptile was noticeable inhibited under dark conditions, showing a photomorphogenetic phenotype. Therefore, we believe that *oscsn1* plays an inhibitory role in rice photomorphogenesis. This is consistent with the role of CSN1 in Arabidopsis in darkness.

We also found that the plant height and root length of *oscsn1-580* and *oscsn1-191*mutants were significantly higher than that of the wild type in light, while in the dark, the root length of *oscsn1-580* and *oscsn1-191* mutants were significantly higher than that of the wild type. However in the dark, the plant height of *oscsn1-580* mutant was significantly higher than that of the wild type, the plant height of *oscsn1-191* mutant was the same as the wild type (Fig. 3A and B). In the light treatment, the length of the first leaf and the second leaf of *oscsn1-580* and *oscsn1-191* mutants increased compared to the wild type (Fig. 3A and B). Nevertheless, in the dark treatment, the length of the second leaf of *oscsn1-580* and *oscsn1-191* mutants increased significantly compared to the wild type, and the length of the first leaf of the mutant was the same as that of the wild type, or the increase was not significant (Fig. 3C).

We examined both of *oscsn1* mutants effect on seed germination in light or dark. Compared to wild type, *oscsn1-580* and *oscsn1-191* exhibited earlier germination (data not shown) and displayed more rapid growth (Fig. 3C). These results suggest that osCSN1 may have an additional function during rice germination. It may play an inhibitory role in rice photomorphogenesis, but it could also restrain the plant height of rice growth at the seedling stage.

3.4 The phenotype of OsCSN1 mutants under Red, Far-Red and blue condition

To evaluate the roles of osCSN1 in regulating rice seedling photomorphogenesis, we grew two mutants (*oscsn1-580 and oscsn1-191*) and wild type in growth chambers for 10 d with different light conditions, including blue light, red light, and far-red light. Then we measured the lengths of the first and second leaves, radicle length, plant height and coleoptile length. Under red conditions, the coleoptile length of the two mutants were shorter than those of the wild type; the plant height, radicle length and lengths of the first leaves and second leaves were similar among all examined plants (Fig. 4A and D). In far-red light conditions, all the data of *oscsn1-580* were lower than those of the wild type, radicle length of *oscsn1-580* was significantly shorter than those of the wild type, other data were not significantly different. In contrast, the radicle length, plant height and coleoptile length of *oscsn1-191* were much shorter than those of the wild type (Fig. 4B and D). In blue light conditions, the coleoptile lengths of two mutants were shorter than those of the wild type. In contrast, the radicle length and plant height of two mutants were much higher than those of the wild type. While lengths of the first leaves and second leaves were similar among all examined plants (Fig. 4C and D).

These results suggest that exposure to far-red light inhibited seedling elongation in *oscsn1-191* mutants. Under blue light, seedling elongation of all test materials were inhibited (Fig. 4C and D). However, under

blue light, the two mutant plants were much “stronger” than the wild-type plant. For example, the second leaves, the plant height and radicle length of two *oscsn1* mutants were longer than those of the wild type; in particular, the blade widths of the two mutants were much wider than that of the wild type. These observations implied that osCSN1 is an inhibitor during regulation of red, blue and far-red light-mediated responses, and the inhibition of osCSN1 is stronger in far-red and blue light conditions than in red light.

3.5 Characterization of ABA and GA biosynthesis in seedling period of null oscsn1 mutants

Gibberellins (GA) are plant hormones with important functions in modulating diverse processes in plant growth and development. The gibberellin response pathway is negatively regulated by DELLA proteins, which consist of five members in Arabidopsis: *RGA-LIKE3 (RGL3), GA-INSENSITIVE (GAI), RGA-LIKE1 (RGL1), RGA-LIKE2 (RGL2), and REPRESSOR OF ga1-3 (RGA)* [32, 33]. Rice has only one DELLA protein, *SLENDER RICE1 (SLR1*) [34, 35].

ABA levels become elevated during seed maturation to establish and maintain seed dormancy, and its levels drop sharply upon imbibition of seeds [33]. ABA induces several effectors, including the bZIP transcription factor ABA-insensitive5 (ABI5). ABI5 accumulates during seed maturation and in dry seeds [33, 36, 37]. ABI5 is suspected to be the final inhibitor of seed germination, acting downstream of GA repressor RGL2 [33, 38, 39]. Arabidopsis studies indicate that AtCSN1, or the atCSN complex, may play an important role in $SCF^{SLY1/2}$-mediated RGL2 ubiquitination in the GA pathway. RGL2 of Arabidopsis is a key inhibitor of germination and a substrate of $SCF^{SLY1/2}$, *csn1-10* of Arabidopsis exhibited hyperdormancy in seed germination and displayed clear defects in timely degradation of RGL2. The *atcsn1* mutants appear to be defective in ABI5 protein degradation during germination. In summary, the defects of the *atcsn1* mutant result in timely removal of RGL2 and ABI5 [34].

In the present study, the *oscsn1* mutant seedlings exhibited different responses to light compared to the *csn1-10* of Arabidopsis (Fig. 3). For example, the *atcsn1-10* mutant displayed hyperdormancy in seed germination, but *oscsn1* mutants had weak seed germination dormancy and sprouted earlier than wild type. The *oscsn1* mutants exhibited the rapid growth phenotype of rice seedlings and developed faster than the wild type in the seedling stage. Considering knowledge of the function of CSN, we hypothesized that osCSN1 has additional functions in the seedling stage in contrast to Arabidopsis. Also, *oscsn1* mutants could reduce the seed dormancy level through responses to GA or ABA and could promote growth and development through responses to GA in the seedling stage.

To test this hypothesis, we evaluated osSLR1 and osABI5 expression in *oscsn1* mutants and wild-type seedlings grown in light for 10 days. We examined osSLR1 protein levels in the *oscsn1* mutants and wild type by anti-SLR1 western blotting and examined osABI5 protein levels by anti-ABI5 western blotting. Both *oscsn1-580* and *oscsn1-191* (Fig. 5A) were similar to the wild type in timely degradation of SLR1. This result is not consistent with the abnormal accumulation of RGL2 in Arabidopsis. Examination of ABI5 showed that it was largely in the neddylated form in *oscsn1-580* and *oscsn1-191* mutants, while

ABI5 was primarily un-neddylated in the WT control (Fig. 5A). This result pointed to ABI5 as the factor potentially regulated by osCSN1. According to the results, *oscsn1* mutant embryos have the intrinsic capacity to initiate growth on an earlier time scale compared to the WT embryos. The rapid growth phenotype of *oscsn1* mutant seedlings could be caused by abnormal hormonal biosynthesis or metabolism.

Next, we examined ABI5 and SLR1 gene expression analyzed by qRT-PCR in *oscsn1-580 and oscsn1-191* mutants (Fig. 5B). Expression of SLR1 in the *oscsn1-580* mutant showed a trend similar to the wild type, while the *oscsn1-191* mutant displayed a comparatively higher level of SLR1. This result is inconsistent to the anti-SLR1 western blot and it may be caused by the presence of different amino acids. The characteristic over-accumulation of SLR1 protein in the *oscsn1-191* mutant might be responsible for their different phenotypes with the *oscsn1-580* mutant in far-red light conditions. However, the ABI5 expression level of two *oscsn1* mutants appears to be lower compared to the wild type. This result is consistent with the anti-ABI5 western blot results. Our observations demonstrate that osCSN1 plays a role in effecting growth and development by regulating protein turnover of the key inhibitor of rice seed germination, the ABA effector ABI5.

4. Discussion

The COP9 signalosome is involved in seed germination and seedling growth, and previous research demonstrated that CSN promotes seed germination by regulating DELLA protein RGL2 and ABA effector ABI5. However, we found that this mechanism did not occur in rice. The *atcsn1-10* mutant displayed clear defects in timely degradation of RGL2, but the *oscsn1* mutant was similar to the wild type in timely degradation of SLR1 and did not display defects in SLR1 degradation. In the *oscsn1* mutant, ABI5 protein was rapidly degraded at the seedling stage, and it did not show the displayed defects in degradation of ABI5. As a result, the mutants exhibited weak dormancy and the rapid growth phenotype of seedlings. Despite the apparently different phenotypes of the *oscsn1* mutants compared with the *atcsn1-10* mutant, the direct molecular targets and the mechanism of their action are unclear.

4.1 Functional difference of osCSN1 subunits and atCSN1 in the seedling stage

No functional subunits of the CSN1 have been reported in rice. We generated *osCSN1* knockout mutants and tested the photomorphogenic growth of mutants under darkness. In Arabidopsis, null mutants of the CSN1 subunits cause early mortality to seedlings, with a similar "*fusca*" phenotype [25, 33]. In *O. sativa spp. japonica*, null mutants of the CSN1 subunits exhibit a short coleoptile phenotype in darkness, and this is consistent with the role of CSN1 in Arabidopsis in darkness. However, in light, *oscsn1* mutants showed the rapid growth phenotype of wild rice seedlings, and this was different from the *atcsn1-10* mutants [25, 33]. These results indicate that the mechanisms used by osCSN1 and atCSN1 to regulate dormancy, germination and seedling growth are not identical. OsCSN1 might have an additional function during germination and growth of rice seedlings.

However, our analyses indicated that the *oscsn1* mutants differ in several aspects from wild type. First, the *oscsn1-580* and *oscsn1-191* mutants exhibit a short coleoptile phenotype in darkness; even in the light, the coleoptile of the *oscsn1* mutant was significantly shorter than that of the wild type (Fig. 3A and B). Second, the responses to red, far-red and blue light differ (Fig. 4). In red conditions, the coleoptile lengths of *oscsn1* mutants were shorter than those of the wild type, while the other measurements were similar among all examined plants (Fig. 4A and D). In far-red light conditions, the *oscsn1-191* mutant had a different phenotype than the *oscsn1-580* mutant. Radicle length, plant height and coleoptile length of *oscsn1-191* were shorter than the wild type, while *oscsn1-191* measurements were similar to the wild type (Fig. 4B and D). Blue light inhibited the seedling elongation of all test materials (Fig. 4C and D), which was much stronger in the *oscsn1* mutant than in the wild type. These different responses of *oscsn1-580* and *oscsn1-191* may be caused by different amino acid changes. The third difference is the protein level of ABI5 in the seedling stage. This was largely in the neddylated form in *oscsn1-580* and *oscsn1-191* mutants seedlings, while the protein level of SLR1 was similar to that of the wild type. It is possible that osCSN1 has a different function from atCSN1 that contributes to this phenotype. Alternatively, it is also possible that the *oscsn1* mutant only slightly enhances or reduces the level of SLR1 to an extent that was not detectable by western blotting. OsCSN1 might have an additional function that has different effects on rice at different developmental stages. OsCSN1 could play an inhibitory role in rice photomorphogenesis, but it could also restrain the height of seedling-stage rice.

4.2 OsCSN1 has functions in ABA signaling

Considering what is known about the function of the CSN1, we hypothesized that osCSN1 might have additional functions in the seedling stage in contrast to *Arabidopsis*. Additionally, *oscsn1* mutants could reduce the seed dormancy level by the responses to ABA and could promote growth and development through the responses to GA in the seedling stage. This study revealed that osCSN1 can directly or indirectly regulate the protein stability of b-ZIP transcription factor ABI5 (Fig. 5), a key transcription factor that mediates the response to ABA [40]. ABI5 protein stability is regulated by several factors and ubiquitin E3 ligases, including *ABI FIVE BINDING PROTEIN* (*AFP*) [41], *SALT- AND DROUGHT-INDUCIBLE RING FINGER 1* (*SDIR1*) [42], *KEEP ON GOING* (*KEG*) [43], CRL^{4ABD} and $CRL^{4DWA1/2}$ [44, 45]. However, in the *oscsn1* growth of the seeding stage, neddylated ABI5 is not associated with the above-mentioned genes. Our data suggest that osCSN1 may inhibit the protein degradation of ABI5 during the seedling stage, but it is unclear which of the known ABI5 E3 ligases osCSN1 works with. We also cannot preclude the possibility that a different E3 of rice exists that has not yet been identified that targets ABI5 specifically during the seedling stage. Our finding raises additional questions as to whether osCSN1 may affect other aspects of the ABI5 functions or other ABA responses. Nonetheless, the observation that ABI5 is specifically neddylated in the *oscsn1* mutant and not in the *atcsn1* mutant suggests that this function might represent the first osCSN1-specific activity in rice plants.

Table S1
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	GCTATTTCTAGCTCTAAAACGAAATACGCGCTCGACCAGGT–TGCCACGGATCATCTGC	
U3p3-F	CAGGAAACAGCTATGACCATATTCAAGGGATCTTTAAAC	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

Declarations

Declaration of Competing Interest

The authors report no declarations of interest.

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Author contributions

All authors contributed to the study conception and design. Material preparation, data collection by Shining Han, Yanxi Liu, Chen Shuhua and Xiaowei Gao. Data analysis and arrange were performed by Ming Wu, Miao Xu, Chunyu Zhang and Liquan Guo. The first draft of the manuscript was written by Ming Wu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

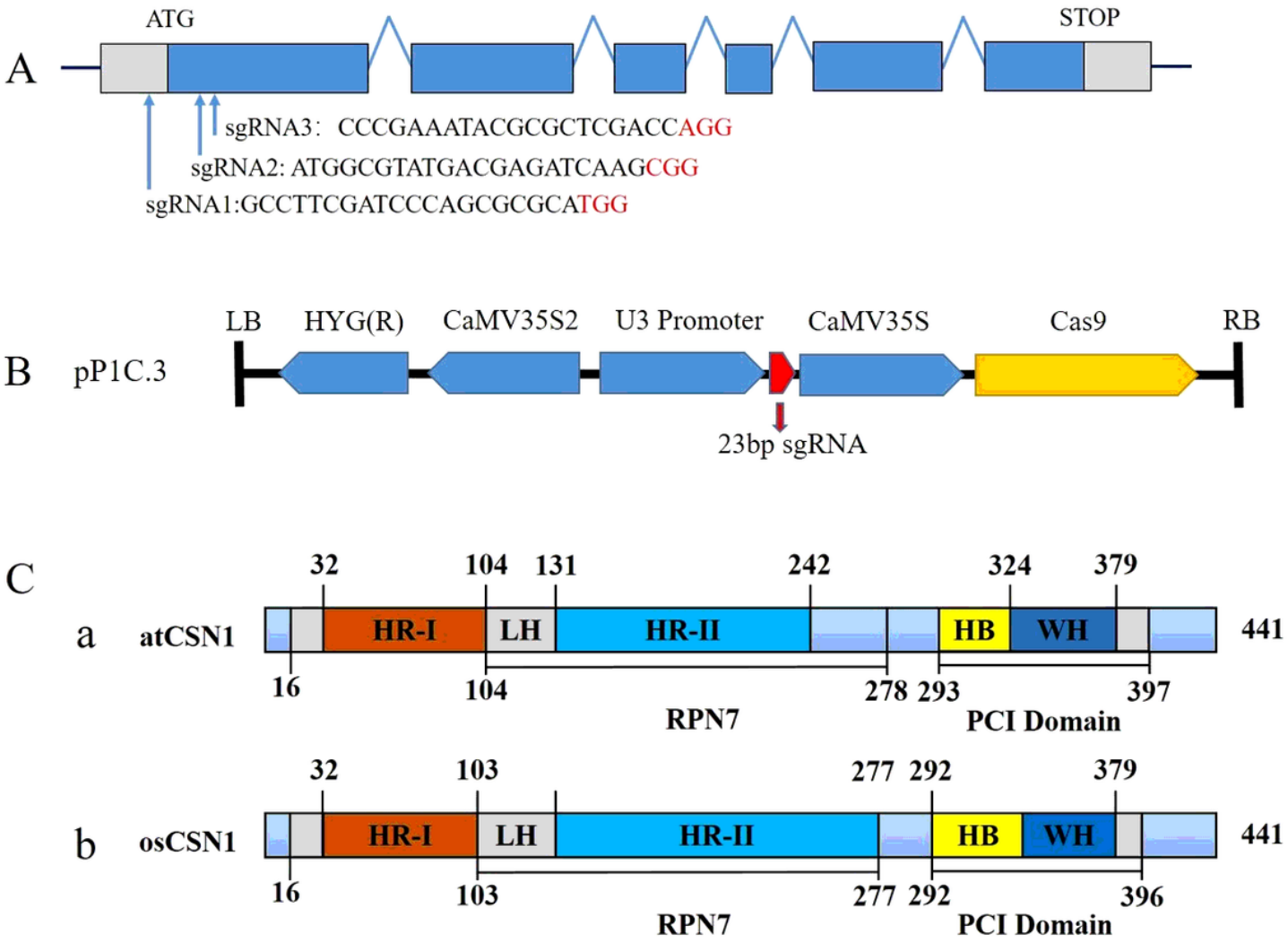


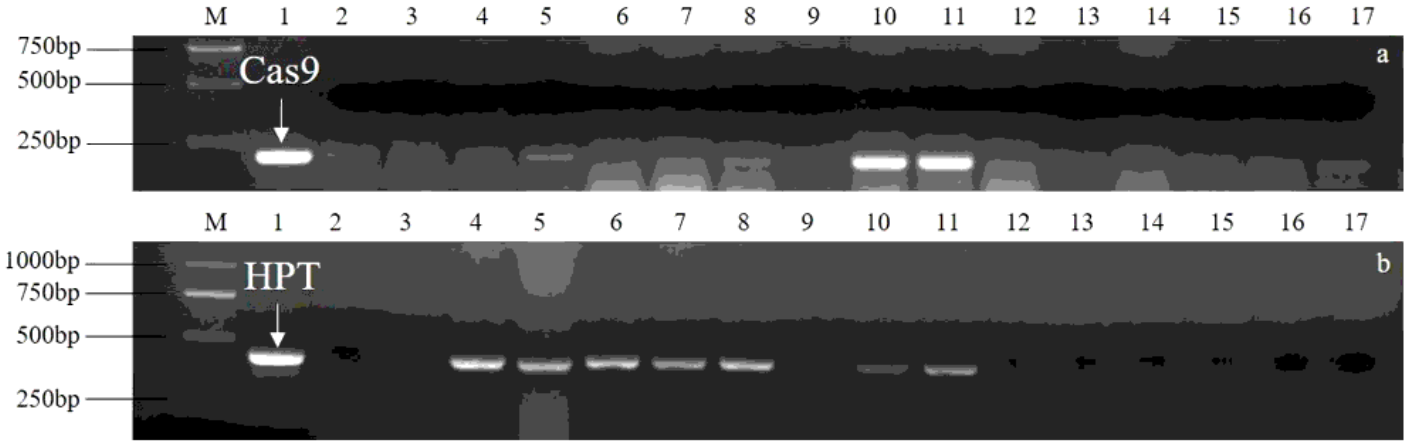
Figure 1

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 has four major domains, helical repeat-I (HR-I), linker helix (LH), helical repeat-II (HR-II), and PCI Domain (PCID).

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 PCR-positive transgenic plants for HPT and Cas9
pP1C.3-sgRNA1	78	78	45
pP1C.3-sgRNA2	67	67	42
pP1C.3-sgRNA3	53	53	63

B



C

Identification of the mutations in *oscsn1* mutant		
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 T0 rice plants for targeted mutagenesis of osCSN1. 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. (B) (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.

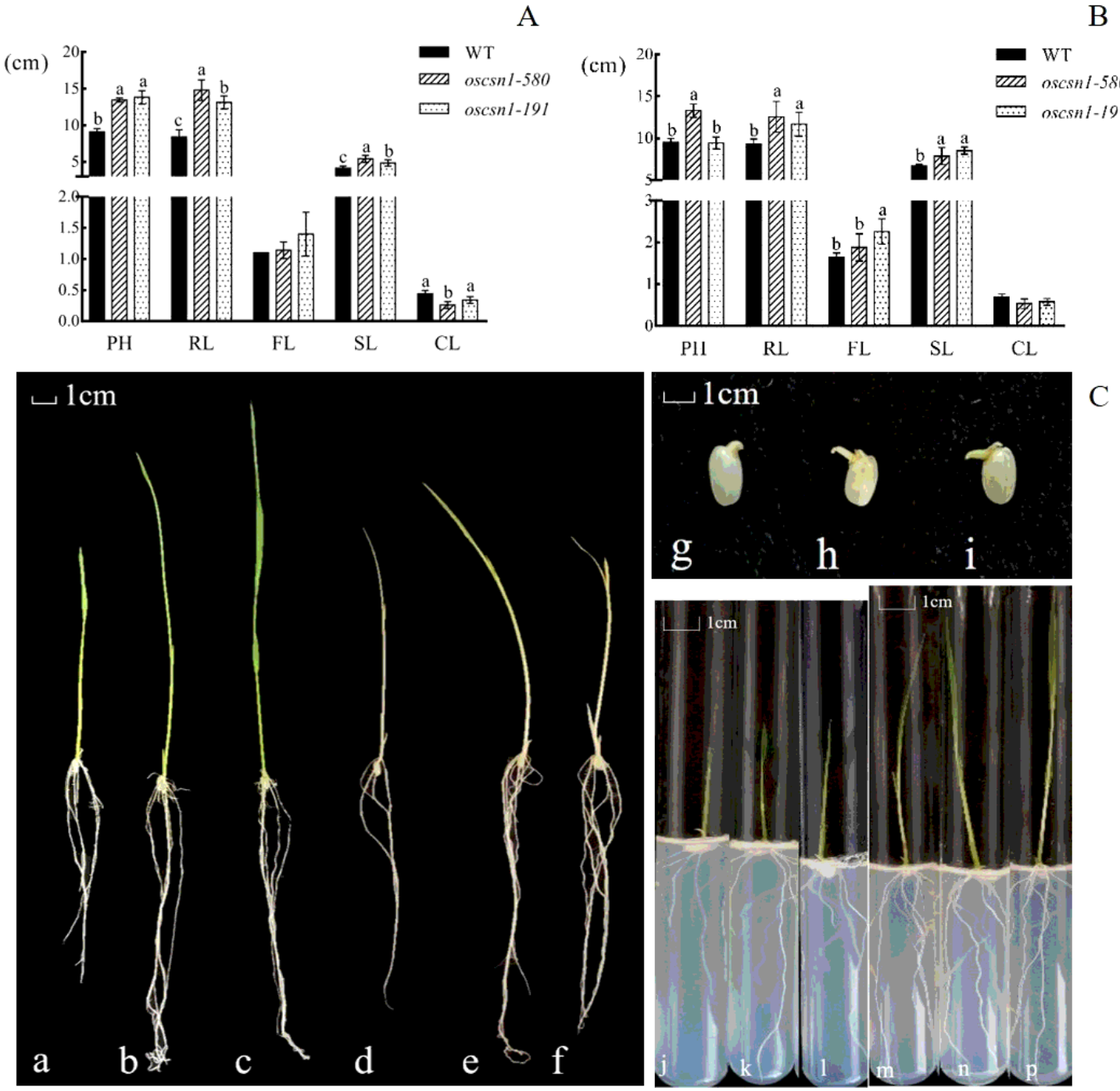


Figure 3

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. (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. 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 (b) and oscsn1-191 (c) grown in the light treatment for 10 days. Wild type (d), oscsn1-580 (e) and oscsn1-191 (f) grown in the dark treatment

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 (p) grown in light for 10 days.

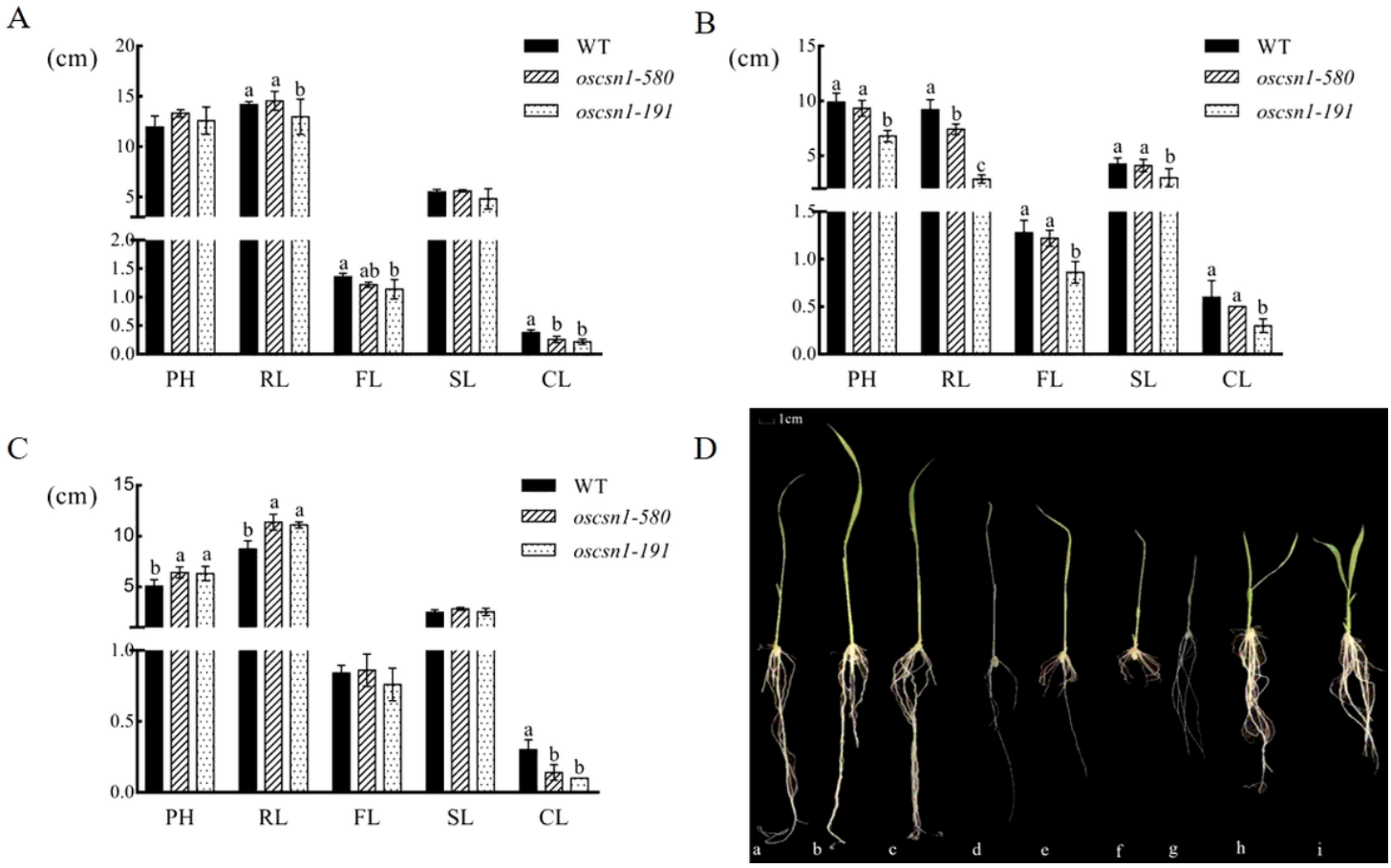


Figure 4

Phenotypic analysis of 10-day-old wild type and oscsn1 mutants grown under different conditions. (A) Phenotypic analysis of plants under the red light treatment. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. (B) Phenotypic analysis of plants under the far-red light treatment. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. (C) Phenotypic analysis of plants under the blue light treatment. PH, plant height; CL, coleoptile length; RL, radicle length; FL, the first leaf length; SL, the second leaf length. 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 the red light treatment. Wild type (d), oscsn1-580 (e) and oscsn1-191 (f) were grown under the far-red light treatment. Wild type (g), oscsn1-580 (h) and oscsn1-191 (i) were grown under the blue light treatment.

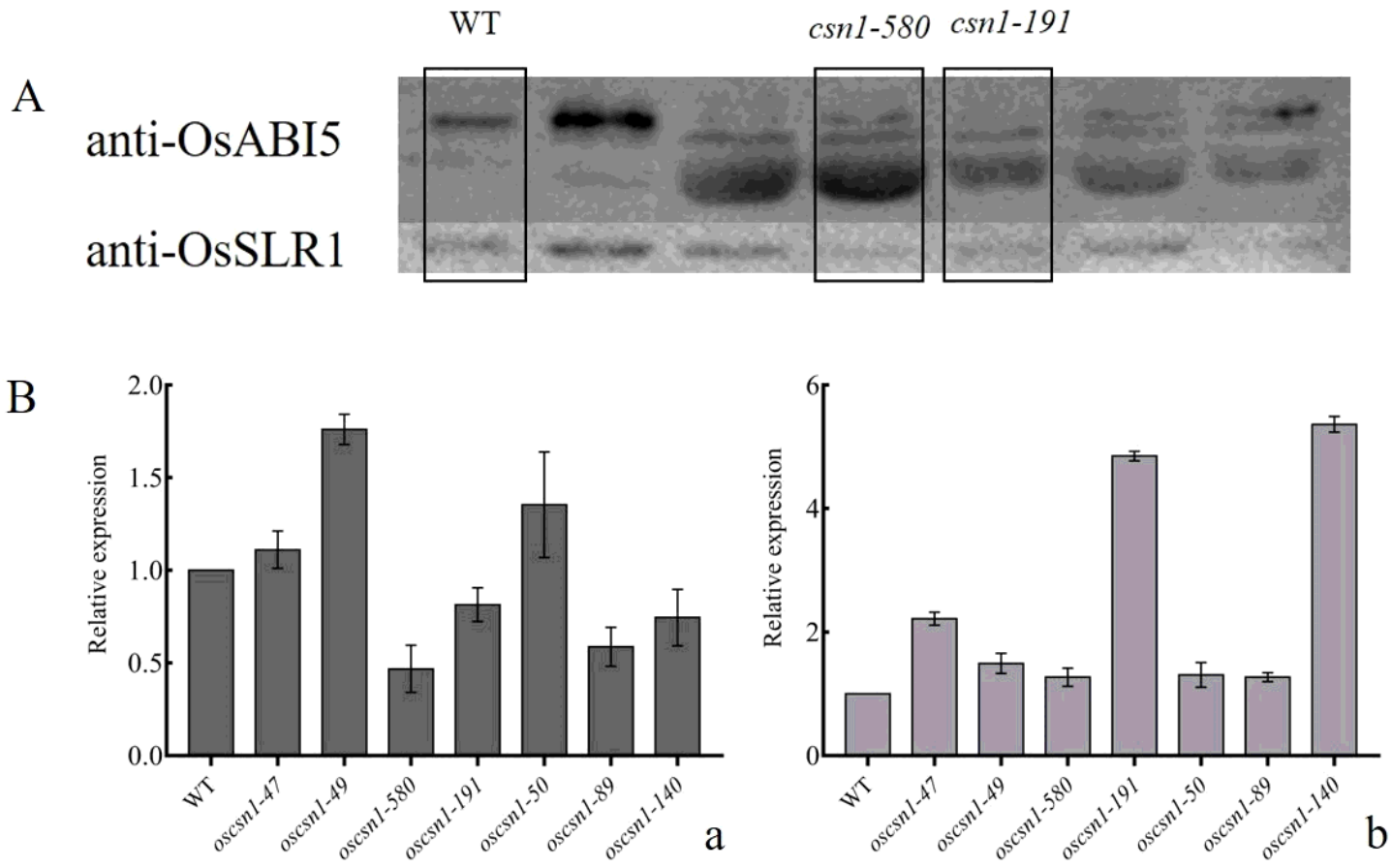


Figure 5

Expression ABI5 and SRL1 modification status in various oscsn1 mutants and the wild type. (A) Detection of ABI5 levels in the wild type and oscsn1 mutants lines using a western blot. OsSLR1 was probed as a loading control. (B) qRT-PCR analysis of the expression levels of ABI5 and SLR1 in the wild type and oscsn1 mutants lines. 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 $P< 0.05$.

Supplementary Files

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- TableS1.Alltheprimersthatwereusedinthisstudy..doc