THESIS FOR DEGREE OF MASTER OF SCIENCE

Functional Analysis of *Cryptochrome-Interacting Basic-helix-loop-helix1* (*OsCIB1*) in Controlling Leaf Angle and Grain Size in Rice (*Oryza sativa*)

벼의 잎 각도와 종자 크기에 관여하는 *OsCIB1*의 기능 연구

BY

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MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Cryptochrome-Interacting basic-helix-loop-helix (CIB), an critical transcription factor in plant, plays important roles associated several development processes, including hypocotyl elongation, flowering, and plastid development. It has been reported that a soybean CIB orthologue gene *Cryptochrome-interacting bHLH1* (*GmCIB1*) promotes leaf senescence by activating transcription of senescence-associated genes such as *WRKY DNA BINDING PROTEIN53b* (*WRKY53b*). However, any functions of *CIB1* have not been studied or reported yet in rice. In this study, we screened two T-DNA mutants to identify the function of *OsCIB1*. Especially, a rice gain of function mutant, *oscib1-D,* displayed wide leaf angles and slender grains, similar to plants with increased brassinosteroid (BR) levels or enhanced BR signaling. qRT-PCR analysis showed that gene in brassinosteroid signaling pathway are upregulated in *oscib1-D*, but there was no significant difference of the expression level of BR biosynthesis-related genes between WT and *oscib1-*

D. In addition, *osicb1-D* showed more sensitive phenotype than WT to BR. Histological analysis revealed that increased cell length in adaxial surface of lamina joint is responsible for larger angles. Moreover, expression level of genes involved in cell elongation such as expansins and xyloglucan endotransglycosylase/hydrolase(XTH), two major cell wall-loosening enzymes, was significantly increased in *oscib1-D*. Thus, these results strongly suggest that *OsCIB1* is involved in the BR signaling pathway and determines not only leaf inclination but also grain shape by regulating cell-elongation-related genes.

Keywords: rice, *Cryptochrome-interacting bHLH1* (*OsCIB1*), transcription factor, lamina joint, leaf angle, brassinosteroid, cell elongation

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ABBREVIATION

CIB	Cryptochrome-interacting bHLH
BR	Brassinosteroid
LJ	Lamina joint
DAS	Days after sowing
Ad	Adaxial
Ab	Abaxial
SEM	Scanning electron microscopy
LD	Long day
NLD	Natural long-day
XTH	Xyloglucan endotransglycosylase/hydrolase
DAF	Day after flowering

INTRODUCTION

Cryptochrome(CRY), one of the photolyase-related blue light receptors, plays important role in regulating photomorphogenic response and circadian rhythm in plants, including various organisms[1-4]. In case of Arabidopsis, three cryptochromes, cryptochrome1 (CRY1) and cryptochorme2 (CRY2), and cryptochrome3 (CRY3) are encoded. CRY1 controls blue light inhibition of hypocotyl elongation [5] and CRY2 mediates photoperiodic response of flowering time [6]. CRY3 is a CRY-DASH type CRY and is localized in mitochondrion or chloroplasts. It plays important role to repair damage caused by UV on single-stranded-DNA [7, 8].

It has been reported that not only a few proteins but also the basic helix-loop-helix (bHLH) transcription factor CIB1 interacts with CRY2 [9, 10]. This protein complex affects various phenotypes such as hypocotyl, flowering, stomata opening, bending of hypocotyl, calcium concentration in cytoplasmic, programmed cell death, plastid development, and fruit(silique) elongation [11-18]. CIB1 is one of the signaling partner proteins of CRY2 to regulate gene expression and they interact with each other when photoexcited CRY2 are phosphorylated to make an open conformation in response to blue light. In soybean, CIB1 plays a transcription activator to regulate downstream genes involved in leaf senescence interacting with the E-box (CANNTG) DNA

sequences [19]. However, it has not been fully understood that which functions rice orthologue gene of Arabidopsis *CIB1* (*OsCIB1*) have.

The inclination between the leaf blade and leaf sheath, leaf angle, is a key factor determining the plant shape and architecture [20, 21]. CIB1 is the basic helix-loop-helix (bHLH) transcription factor to affects various phenotypes. Similar to control of hypocotyl bending, several bHLH proteins such as OsBU1 and OsIBH1 regulate leaf angle in rice [22, 23]. In addition to these two regulators, various genes have been reported to have a role in controlling leaf angle, including *Ta1*, *OsDWARF4*, *D2*, *OsBRI1*, *ILI1*, *LC2*, and *ILA1* [24-28]. It is considered that lamina joints connecting the leaf blade and leaf sheath is most important organ controlling leaf inclination. The degree of the leaf inclination largely depends on cell proliferation and cell expansion as well as cell wall composition at the lamina joint [29]. Nevertheless, it is well known that BR is a critical hormone to affect lamina joint and stimulate leaf angle in rice [30].

Brassinosteroids (BRs) are a group of steroidal phytohormones that are widely distributed in plants. Thus far, more than 69 types of BRs have been isolated from diverse plants. BRs play a number of roles in biological, physiological and developmental processes, including cell expansion, cell division, vascular bundle differentiation, male fertility, senescence, seed germination, grain filling, photomorphogenesis, skotomorphogenesis, flowering, senescence, abiotic, and biotic stresses [31-38]. In rice, it has been reported that BR plays important roles in the in the regulation of grains size,

leaf angle, and yield potential. For instance, loss-of-function mutants with low BR contents or weak BR signaling such as *d2*, *d11*, and *d61* exhibit short grains, erect leaves, and dwarf phenotypes [39-41]. More importantly, it has been proved that modulating the expression level of BR-related genes such as *OsDWARF4* and *OsBRI1* improves rice grain yield due to higher planting densities [26, 42].

Grain size is mainly determined by grain length, grain width, and grain thickness and it not only contributes to grain yield but also affects preference for rice [43]. The cell number and cell size largely determines the organ size during organogenesis [44, 45]. In recent years, it has been reported that several genes and quantitative trait loci (QTLs) including *GS3*, *GW2*, *GW5*, *GS5*, *GW8*, *qGL3*, *TGW6*, *GW6a*, and *BG1* affect grain size by regulating cell number in rice. In addition, *PGL1*, *GL7*, and *GS2*/*GL2* regulate grain size by influencing cell size [46-58].

Here we demonstrate that *OsCIB1* acts as a key regulator in leaf angle and grain size. T-DNA insertion *oscib1* and *oscib1-D* mutants exhibited exaggerated leaf inclination with slender grains. Histological analysis showed that the length of cells in adaxial surface was significantly increased in *oscib1-D*, whereas there was no obvious alteration of the length of cells in adaxial surface. Since leaf angle is closely related to BR, we investigated expression level of genes involved in BR biosynthesis and signaling. Lamina joint inclination assay showed that *oscib1-D* was hypersensitive to BR. Because organ size is determined by cell proliferation or cell expansion, we identified

expression levels of genes such as *OsEXPA*s and *OsXTH*s. As a results, six *OsEXPA*s and two *OsXTH*s were upregulated in *oscib1-D*. This study provides new insights into the roles of *OsCIB1* in leaf angle and grain size.

MATERIALS AND METHODS

Plant materials and growth conditions

The T-DNA insertion knockdown mutant of *OsCIB1* (LOC_Os02g47660;PFG_3C-00039.L; *oscib1-D*) and T-DNA insertion overexpression mutant of *OsCIB1*(LOC_02g47660; PFG_3A-01275.L;*oscib1*) in rice was isolated in the Korean *japonica* cultivar 'Dongjin' (hereafter termed wild type; WT) and was obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/cgi-bin/RiceGE) [59]. Rice plants were grown in the paddy field of the Seoul National University Experiment Farm under natural long day (NLD) conditions (37°N latitude, Suwon, Korea). Seeds were sown on the seed beds in the greenhouse on 29 April and transplanted to the paddy field on 3 June. Seeds were sown on the seed beds in the greenhouse on 29 April and transplanted to the paddy field on 3 June. Rice cultivation followed normal agricultural practices for Korean rice varieties. Rice was also grown in growth chamber under long day (LD) (14.5 h light, 30°C/9.5 h dark, 24°C) conditions with 60% relative humidity. The light source was light-emitting diodes producing mixed red, blue and white light, and photon flux density was around 500μmol $m^{-2}s^{-1}$.

Identification of the T-DNA insertion *oscib1-D and oscib1* mutant

To identify the homozygous *oscib1-D* mutant, we extracted genomic DNAs

from the *oscib1*-segregating population using a cetyl trimethyl ammonium bromide (CTAB) method [60] and performed PCR analysis. PCR was conducted with a T-DNA plasmid pGA2715 left border primer (pGA2715-LB) in combination with an *OsCIB1* left genomic primer (*OsCIB1*-LP) and right genomic primer (*OsCIB1*-RP) (Table 1.). PCR was performed with 32 cycles of 95 °C for 30s, 60 °C for 30 s and 72 °C for 1 min.

RNA extraction, reverse transcription (RT) and quantitative real-time PCR (qPCR) analysis

Total RNA was extracted from Lamina joints using the MG Total RNA Extraction kit (Macrogen, Seoul, Korea) according to the manufacturer's instructions. First-strand cDNAs for RT were synthesized from 3μg of total RNA using oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA) and diluted with water to 100μL. Relative expression levels of *OsCIB1* and flowering-related genes were measured by RT-qPCR using gene-specific primers, and *Ubiquitin5* (*UBQ5*) was used for an internal control (Table 1) [62]. GoTaq qPCR Master Mix (Promega) was used in a 20 μL total reaction volume. Expression levels of each gene were measured by the relative quantification method using a LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland), and qPCR conditions were 95°C for 2min, and then 45cycles of 95°C for 10s and 60°C for 1min.

Histological analysis of lamina joints

Samples were fixed in 3.7% formaldehyde, 5% acetic acid and 50% ethanol overnight at 4°C, and dehydrated in a gradient series of ethanol, cleared through a xylene series, then infiltrated through a series of Paraplast (Sigma) and finally embedded in 100% Paraplast at 55-60°C for thin sections. Then 8-12μm-thick microtome sections were mounted on glass slides. The sections were deparaffinized in 100% xylene and were dried staining with Toluidine blue O (Sigma) before observation on an Axioscope2 microscope(Zeiss).

Lamina joint inclination assay

Sterilized Seeds were grown on Murashige and Skoog (MS) medium in a growth chamber at 30°C under dark condition for 10days. ~2-cm segments including the second-leaf lamina joint were sampled under dim light. These were floated on distilled water containing various concentrations of 24-epibrassinolide (BL; an active form of BRs, sigma, http://www.sigmaaldrich.com/) for 48h in the dark. After incubation, the angle between the lamina joint and leaf sheath was measured using IMAGEJ software [31].

Subcellular localization of OsCIB1

To identify the subcellular localization of OsCIB1, green fluorescent protein(GFP) was fused in front of the *OsCIB1* cDNA in the pMDC43 vector

through LR recombination (Lambda integrase/excisionase;Elpis-Biotech), resulting in the 35S:GFP-OsCIB1 plasmid. The fusion constructs, as well as the control (empty pMDC43 vector; *35S:GFP*), were transformed into rice protoplast. The transformed rice protoplast were examined with a confocal laser scanning microscope (Carl Zeiss LSM710, Oberkochen, Germany).

Scanning electron microscopy

In order to examine the adaxial surface of lamina joints, 1cm transverse sections of the middle region of a fully expanded third lamina joints were sampled. Sections were fixed in primary fixative (2% paraformaldehyde, 2% glutaraldehyde), post-fixed with 1% osmium tetroxide, and dehydrated in a series of ethanol and propylene oxide, then finally embedded in Spurr's resin. After polymerization, sections were observed with a scanning electron microscope (JSM 5410LV; JEOL, Tokyo, Japan).

Table1. Information of primers used in this study.

Marker	Forward primer (5'→3')	Reverse primer(5'→3')
A. Primers for verification of transgenic plants		
PFG 3A-01275.L	CTAGAGTCGAGAATTCAGTACA	ACCAACAGAAGCACTCCAGG
PFG 3C-00039.L	CTAGAGTCGAGAATTCAGTACA	ACGAACAGAAGCACTCCAGG
B. Primers used for gene cloning		
OsCIB1	ATGGCGCAATGCGGCGGCGGCGACGTTTCACGACA	TTACATTTCCATTTTGAGATTGTCTAGC
C. Primers used for qRT-PCR		
OsCIB1	CTGGAGATGGTCGTCAGTAACCAG	ACGGTGCATCAAATCCACTGAAC
BR6ox	GTCGACATCCAGGCCAAGAC	TCTGCCTTGAGAGCGTCAGA
OsCPD	GAAAAATAGTTCTCGCATAGGTAGTG	GGTCATTTTACAAGCAGCACAAC
OsD2	AGCTGCCTGGCACTAGGCTCTACAGATCAC	ATGTTGTCGGAGATGAGCTCGTCGGTGAGC
OsBRI1	AAATGCCCGTAGAAACAACAGTC	TGTAGCACCATCAGAAGCGA
OsBZR1	CGTCGCCCACCTACAACCTC	TCGCCCAAATCGCAGCAT
OsBAK1	GAGTTGATCTTGGGAATGCTGC	CACTAGGTATCGTTCCGCTTATGTT
OsILI1	TTCTCTCCAAGCTTCAGGCCCT	TGCACGTCTCCTGCAAAACCCT
OsBU1	TCCAAGCTCCAGTCCCTCCT	CGGTGCAGGCTCTTGATGTA
OsGSK1	CATCGCAGAGCGTGCTGTTGG	CATCGTTTGCAGCTCCCGGTT
OsCDC6	GTTGAGTGGGCATAGGGTAGG	TCCGACTTGAAATACGAGGCAAT
OsMCM3	GGTACACTTCCGATCACAGCG	CCACATCCATTGGGTCGTTTC
E2F2	GGGTGACGCTGATGGTGATAC	TGCTTTCTTTGGGATTGTTGA
OsCYCA3;1	CAAATCCATCAACCGCCAGAA	TCAACTCAGATGCCCTGTAGCC
OsEXPA1	CCTGCTTTTTTCAATGCGAAT	AAAGCATGCCGATCATCGA
OsEXPA3	CAGGGGTGTGGGTGTTTGAG	GCCTCTCCAGAACCTCGTAGC
OsEXPA4	GGCTATGCTGAGGCTGCTAATT	CTCCCCTTTACTACTACCACTACACT
OsEXPA5	GTGTCACTGCTAGTGGTAGTTTGTT	GCCTTACCACCACTTCCAATCC
OsEXPA6	GCAAGAGAAGGCAAGGCATTTC	ACTAAGAGCAGAGCAGCAAACTG
OsEXPA7	TGAAGCAAAACTGCAATATACCCTCTT	CCGAAGAAGAACCCAAACTTAACCA
OsXTH2	AGCCGTACATCCTGCAGACGA	GCCCAGGTCCTTGCTGTTCT
OsXTH28	GCGTACCACCACGGGTTCTTCA	CGTTGGTCTGCACCCTCCACTC
PGL1	GAA ATTGAAGCAGTGCAGGGA	TCACGATGCTGGCTGGTCTC

PGL2	ATGTCGAGCAGAAGGTCGTC	TCAGGAGCGGAGGATGCTGC
GW7	CCCAAGCAAGAAGTCCAG	TGAAGCAAGAACTGAAGGA
GLW7	AACCCGCCGTTCCAGATCAG	AAGAAGGGACGTAGGTGGTG
GS2	TGCGTCCCTTCTTTGATGAGT	ACAGTTGGGTGCCTGAGAATG
SRS5	ATGAGGGAGTGCATCTCGAT	CAAGATCGACGAAGACAGCA

RESULTS

Phenotypic characterization of the *oscib1-D*, a T-DNA insertion mutant of rice

O.sativa CIB1 (*OsCIB1*; LOC_Os02g47660) is the rice orthologue of *Arabidopsis thaliana CIB1* (*AtCIB1*; At4g68050) [12]. To study key factors controlling rice leaf angle, we screened a collection of activation-tagging T-DNA insertion rice lines. As a result, we identified a mutant with enlarged leaf inclination and slender grain shape (Figure 1A, D). The leaf angles of upper four lamina joints in *oscib1-D* were larger than those of the WT, especially for the flag leaves (Figure 1B, C). The grain length of *oscib1-D* was significantly longer than that of WT but, in case of grain width, *oscib1-D* had narrower shape than WT (Figure 1E, F). In addition, the grain thickness of *oscib1-D* was considerably decreased in comparison with WT and also the 500-grain weight of *oscib1-D* was slightly reduced (Figure1G, H).

In order to screen T-DNA mutant lines, we searched database and were provided with two different T-DNA mutant alleles. One is *OsCIB1*-knockdown(hereafter reffered as *oscib1*) and the other is *OsCIB1*-overexpressing rice lines(hereafter reffered as *oscib1-D*). *oscib1* showed reduced plant height and narrower leaf angle than that of WT. These phenotypes are similar to those of BR-deficient or BR-insensitive mutants, such as *d61* and *d11* (Figure 2A, B) [40, 41]. Genotyping showed that T-DNA

of both *oscib1* and *osicb1-D* was inserted in the 1000-bp upstream region from the ATG start codon (Figure 2C). *oscib1* has no detectable accumulation of *OsCIB1* transcripts (Figure 2D) and *oscib1-D* has more *OsCIB1* transcripts that that of WT(Figure 2E). These results indicate that expression of *OsCIB1* affects the leaf angle, grain size, and height as BR controls above traits.

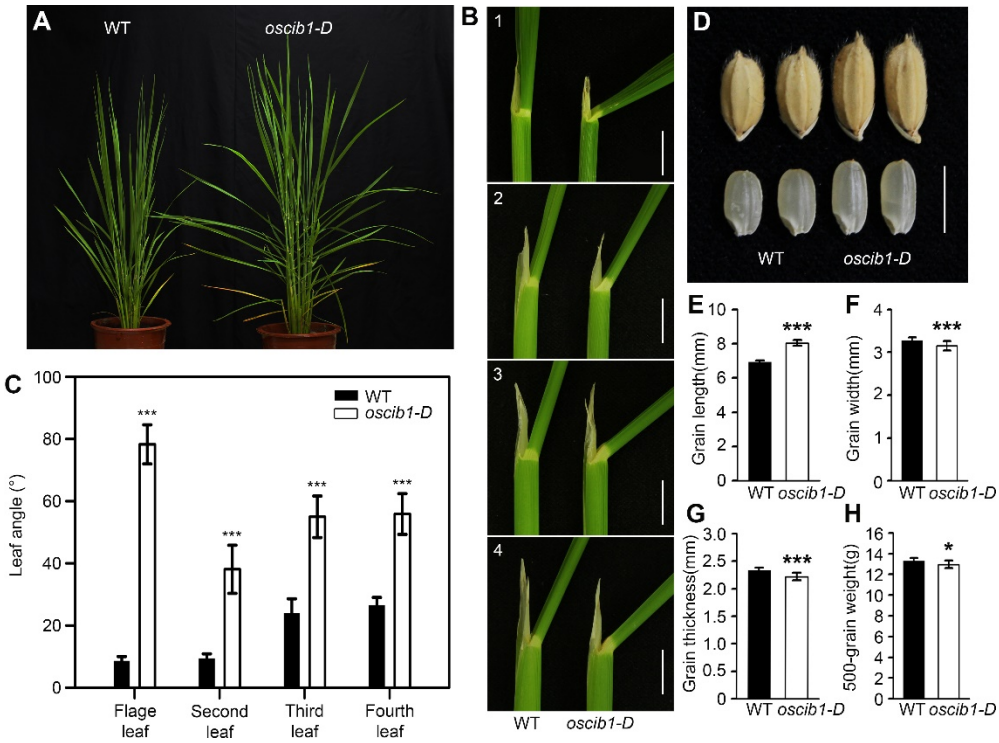

Figure 1. Phenotypic characterization of *oscib1-D* mutant

(A) Phenotypes of 15-week-old plants. (B) Lamina joints of the flag (1), second(2), third(3), and fourth (4) leaves between WT and *oscib1-D* (counted from the flag leaf downwards). (C) Comparison of the leaf angles at upper four leaves of WT and *oscib1-D*. (D) grain comparisons of WT and *oscib1-D*. (E-H) Measurements of grain traits with longer, narrower, and thinner shape (E and F), and reduced grain weight (H) in *oscib1-D*. Mean and SD values were obtained from more than ten biological replicates (n=10). Statistical analysis using Student′s *t*-test identified significant differences (**P*<0.05, ***P*<0.01). Scale bars = 1cm (B), 0.5cm (D).

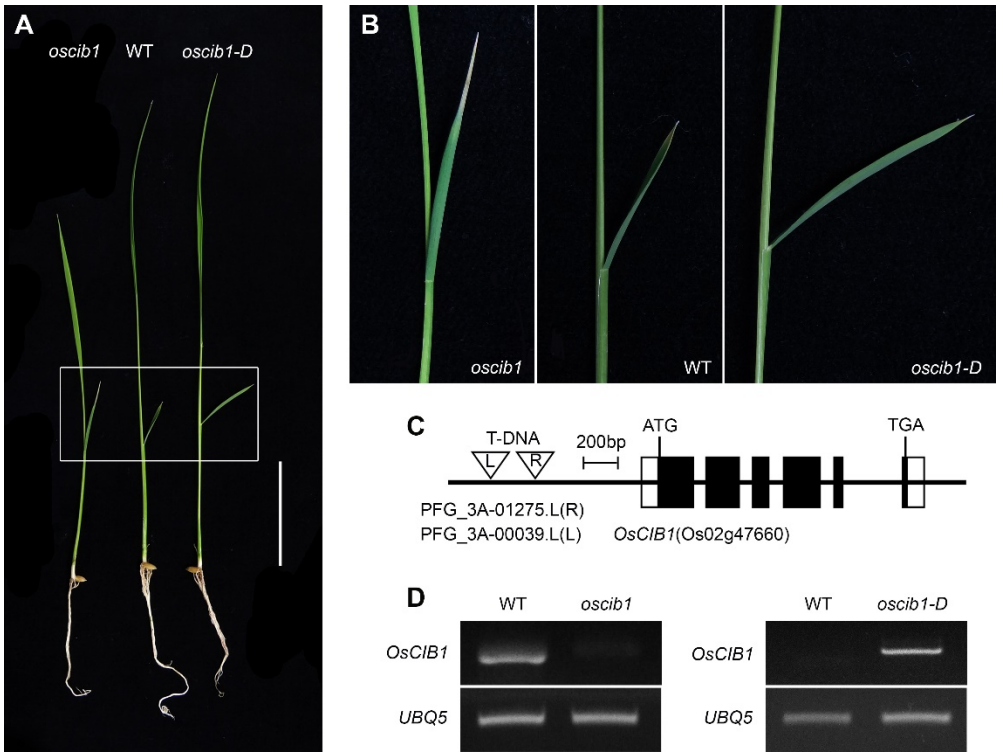

Figure 2. Enhanced or decreased expression of *oscib1-D* leads to the mutant phenotype.

(A) Phenotypes of the 10-day-old WT, *oscib1*, and *oscib1-D* seedlings grown in growth chambers under long day (LD) condition. (B) The boxed regions in (A) were enlarged to highlight the differences. (C) Gene structure and two T-DNA insertion site (horizontally inverted triangle) in 1000bp-upstream region of *OsCIB1* (PFG_3A-01275.L, PFG_3C-00039.L). (D) The low and high amount of *OsCIB1* transcripts in *oscib1* and *oscib1-D* mutants respectively was confirmed by RT-PCR. *UBQ5* was used for internal control. Scale bar = 5cm.

Increased cell length in adaxial surface of lamina joint is responsible for wide leaf angle.

The degree of the leaf inclination is strongly controlled by cell proliferation and expansion as well as cell wall composition at the lamina joint. Phenotypic observation showed that *oscib1-D* has exaggerated leaf angle and extended adaxial side of lamina joint (Figure 3A). To investigate morphological differences between WT and *osicb1-D*, we performed scanning electron microscopy analysis (Figure 3B, C). However, there was no significant morphological differences between WT and *oscib1-D*. To identify whether the exaggerated leaf angle was caused by alterations in cell number or cell size, we performed histological analysis by using paraffin embedding and sectioning. The histological analysis on the third leaf lamina joints showed that there was no significant alteration of cell length in the abaxial surface between WT and *oscib1-D*. In contrast, in adaxial surface, the cell length of *osicb1-D* was increased (Figure 3D-I). Therefore, elongated cell not cell division in adaxial surface of lamina joints in *oscib1-D* causes a wider leaf bending. These results indicate that increased cell length in adaxial side of lamina joint is responsible for larger leaf angle in *oscib1-D*.

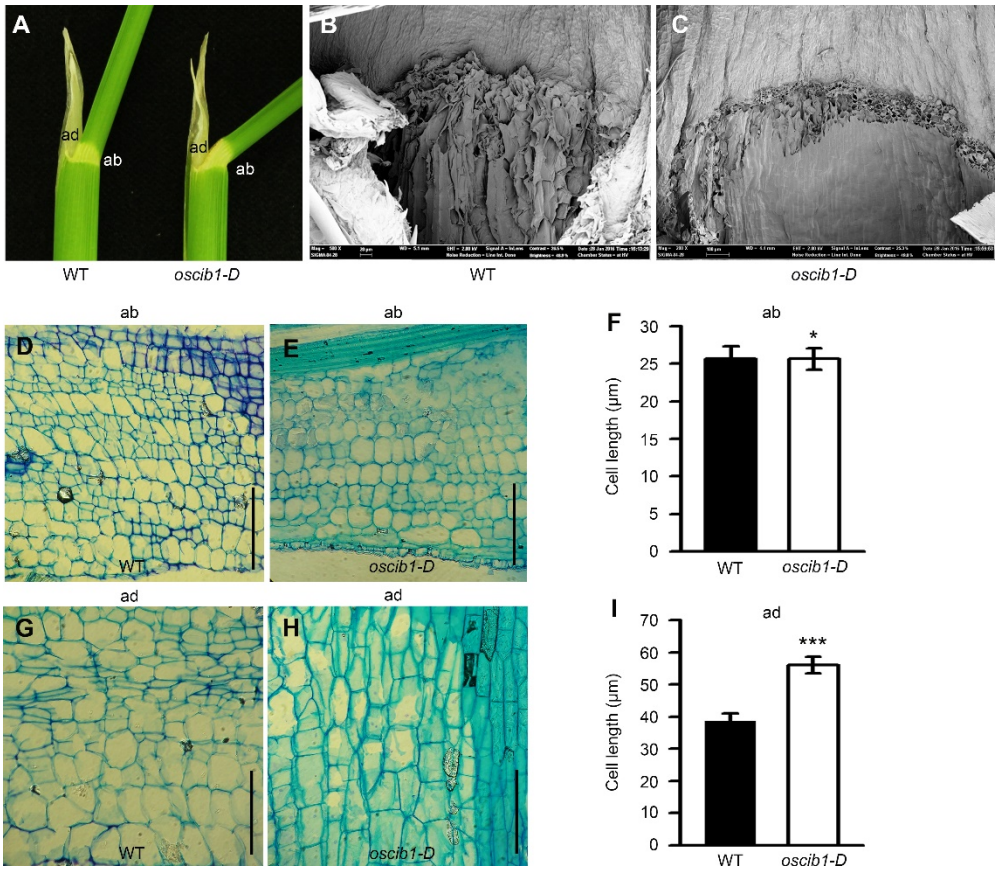

Figure 3. Morphological comparison between WT and *oscib1-D*.

(A) Comparison of the lamina joint of WT and *oscib1-D* at the heading stage. ad, adaxial; ab, abaxial. (B,C) SEM images of adaxial surface of WT (B) and *oscib1-D* (C) at the heading stage. (D,E) Longitudinal sections of the abaxial sides of WT(D) and *oscib1-D*(E) of the third leaf lamina joint shown in (A). (F) Measurement of average lengths of cells shown in (D) and (E). (G,H) Longitudinal sections of adaxial sides of WT(G) and *oscib1-D* (H) third lamina joints shown in (A). (I) Measurement of average lengths of cells shown in (G) and (H). Mean and SD values were obtained from more than ten biological replicates (n>10). Statistical analysis using Student's *t*-test identified significant differences (**P*<0.05, ****P*<0.005). Scale bar = 100μm

Spatial and temporal accumulation of *OsCIB1* transcripts.

In previous studies, it has been reported that as bHLH transcription factor, CIB1 regulated genes involved in flowering and leaf senescence in Arabidopsis and Soybean respectively [12, 19]. In rice, the function of OsCIB1 remains unclear. Therefore, to examine the subcellular localization of OsCIB1, GFP was fused in front of the *OsCIB1* cDNA in the pMDC43 vector. We constructed an OsCIB1-GFP fusion and deliver the *35S:GFP-OsCIB1* construct into rice protoplast. GFP-OsCIB1 proteins only accumulated in nucleus (Figure 4A).

As many of the genes regulating leaf angle are expressed at high levels in the lamina joint or leaf tissue [23,66-68], we examined the mRNA levels of *OsCIB1* in various organs at heading stage in NLD. However, Quantitative RT-PCR analysis revealed that *OsCIB1* mRNAs are most abundant in the node (Figure 4B). These results suggested that *OsCIB1* is mainly expressed in node and might function in nucleus as transcription factor.

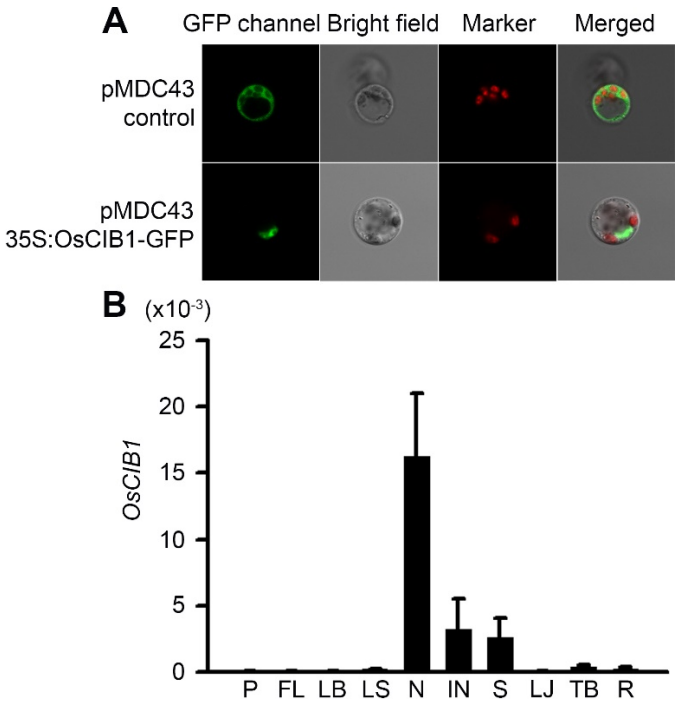

Figure 4. Subcellular localization and expression pattern of *OsCIB1*.

(A) Transient expression of GFP-OsCIB1 fusion protein in rice protoplast analysed by confocal laser scanning microscopy. (B) Quantitative RT-PCR analysis of *OsCIB1* expression in various rice tissues. P, panicle; FL, flag leaf; LB, leaf blade; LS, leaf sheath; N, node; IN, internode; S, stem; LJ, lamina joint; TB, tiller base; R, root. Mean and SD values were obtained from more than three biological replicates.

oscib1-D has more sensitive phenotype to BR than WT and increased expression of genes involved in BR signaling pathway.

It has been reported that the steroid phytohormone BR plays important roles in controlling the leaf inclination of rice [26, 61]. In Arabidopsis, almost all major BR signaling components have been isolated by extensive genetic and biochemical studies. Most components of the BR signaling pathway are conserved between Arabidopsis and rice. However, in Arabidopsis, there are some BR signaling components that have not been identified in rice. The phenotypes of *oscib1-D* is similar to plants with elevated BR levels or enhanced BR signaling [63, 64], which means that *OsCIB1* may be involved in BR biosynthesis or BR signaling. To identify whether the response of *oscib1-D* to BR is more sensitive than that of WT, we performed lamina joint inclination assay using excised leaf segments (Figure 5A) [30]. Lamina joint inclination is one of the most sensitive BR responses in rice [23]. When leaf segments of WT and *oscib1-D* including lamina joint were treated with various concentration of BR, the leaf angle of both WT and *oscib1-D* was increased in a dose-dependent manner. However, in contrast to WT, the degree of lamina joint bending of *oscib1-D* was higher under all BR concentration treatments than that of WT. These results indicate that BR signaling is enhanced in *oscib1-D*.

To further investigate whether wide leaf angle of *oscib1-D* is caused by BR signaling independent of endogenous BR contents, we identified

expression level of genes known to involved in BR biosynthesis or BR signaling. As shown in Figure 6A, the expression of the four BR-biosynthesis-related genes was not obviously altered in *oscib1-D* (Figure 6A-D). On the other hand, in accordance with the above BR sensitivity test, the expression of six genes in BR signaling pathway is increased in *oscib1-D* (Figure 7A-F). Taken together, these results indicate that *oscib1-D* had higher sensitivity to BR than WT, suggesting that OsCIB1 could regulate genes in BR signaling pathway.

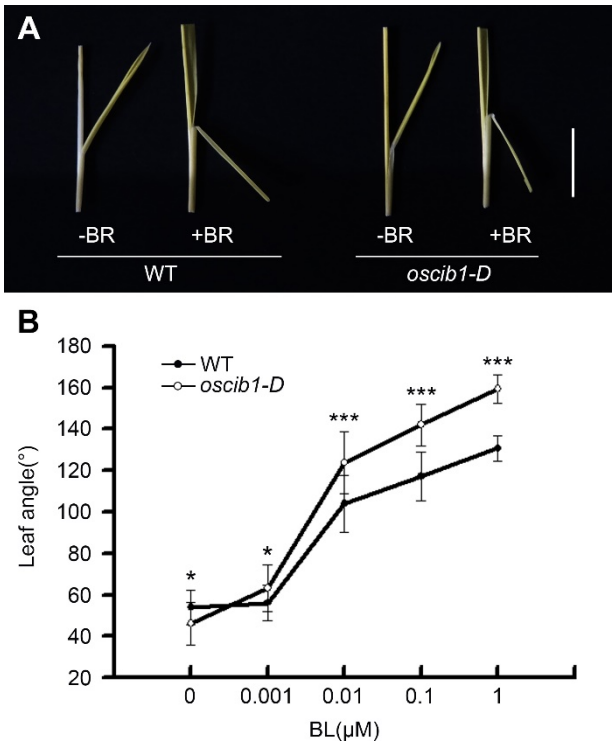

Figure 5. Response of wild type and *oscib1-D* to 24-epibrassinolide(24-eBL). (A) Morphological Response of the second leaf lamina joint to 1μM BL treatment by the excised leaf segment method. (B) Dose response of the lamina joints angle to various concentrations of BL in WT and *oscib1-D*. Mean and SD values were obtained from more than biological replicates (n>10). Statistical analysis using Student's *t*-test identified significant differences (**P*<0.05, ****P*<0.005). Scale bar = 1cm.

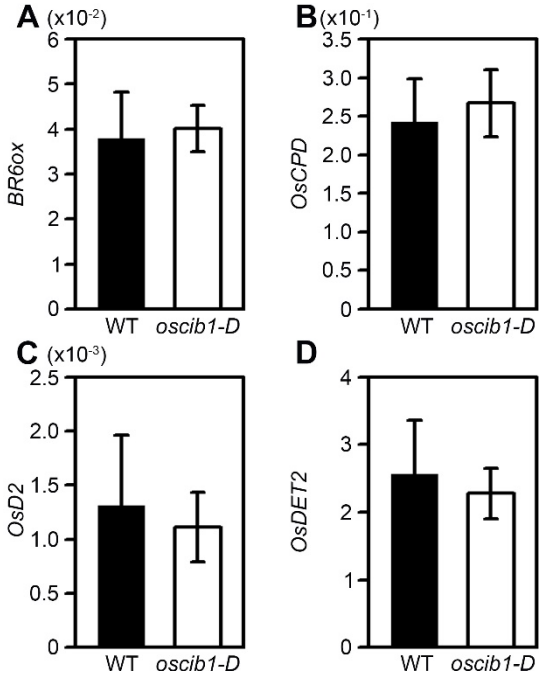

Figure 6. Expression analysis of BR-biosynthesis-related genes.

(A-D) Total RNA was extracted from ~2-cm lamina joint segments including leaf blade and leaf sheath (2-week-old) of WT and *oscib1-D*. Relative expression levels of *BR6ox* (A), *OsCPD* (B), *OsD2* (C),and *OsDET2* (D) were obtained by normalizing to the transcript level of *UBQ5*. Mean and SD values were obtained from more than three biological replicates. Statistical analysis using Student's *t*-test identified significant differences

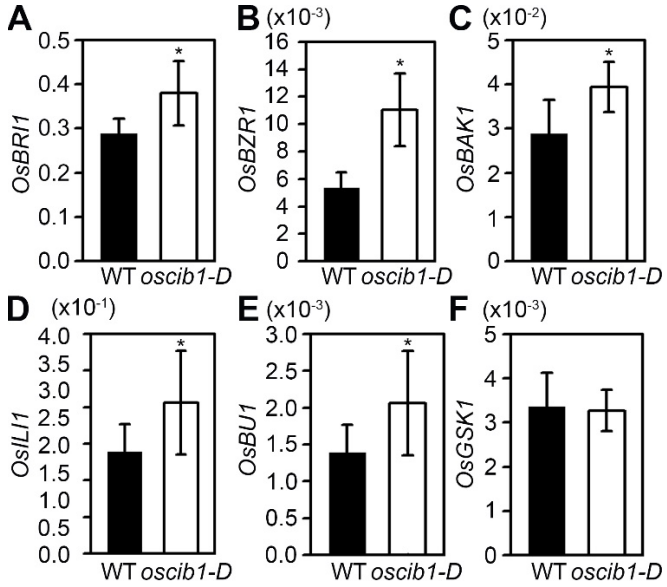

Figure 7. Expression analysis of BR signaling-related genes.

(A-F) Total RNA was extracted from ~2-cm lamina joint segments including leaf blade and leaf sheath (2-week-old) of WT and *oscib1-D*. Relative expression levels of *OsBRI1* (A), *OsBZR1* (B), *OsBAK1* (C), *OsILI1* (D), *OsBU1* (E),and *OsGSK1* (F) were obtained by normalizing to the transcript level of *UBQ5*. Mean and SD values were obtained from more than three biological replicates. Statistical analysis using Student′s *t*-test identified significant differences (**P*<0.05).

OsCIB1 might directly regulate genes involved in cell elongation.

Like Arabidopsis CIB1, rice OsCIB1 is a transcription factor which can directly regulate the expression of target genes [12]. To investigate downstream genes regulated by the OsCIB1 transcription factor, we performed quantitative real-time PCR analysis using the lamina joints of WT and *oscib1-D*. Because *oscib1-D* showed increased leaf angle (Figure 1) and had elongated cells in adaxial surface of lamina joint (Figure 3), there can be a possible explanations for the effect of genes involved in cell-elongation. However, we could not exclude the possibility that cell division affects the phenotype of *oscib1-D*. Therefore, to further investigate the functional mechanism of *OsCIB1* in regulation of cell division at the lamina joint, we firstly identified expression levels of genes known to be involved in cell division. We identified the expression of cell division-related genes such as *OsCDC6*, *OsMCM3*, *E2F2*, and *OsCYCA3;1*. As shown in Figure 8, there was no significant difference between WT and *oscib1-D*. Subsequently, we investigated the expression levels of *OsEXPAs* (members of the α-expansin gene family critical for cell elongation in rice) and *OsXTHs* (encoding xyloglucan endotransglycosylase, the cell-wall loosening enzyme necessary for cell elongation), the two regulators loosening cell walls during cell elongation or expansion. The expression level of these two genes in lamina joints of *oscib1-D* was significantly increased (Figure 9). Collectively, these results indicate that *OsCIB1* mainly regulates cell expansion through

activating the expression of cell elongation-related genes.

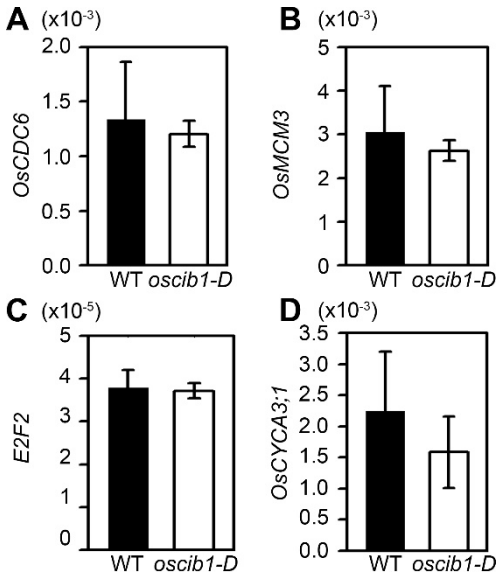

Figure 8. Expression analysis of cell cycle-related genes.

(A-D) Total RNA was extracted from ~2-cm lamina joint segments including leaf blade and leaf sheath (2-week-old) of WT and *oscib1-D*. Relative expression levels of *OsCDC6* (A), *OsMCM3* (B), *E2F2* (C),and *OsCYCA3;1* (D) were obtained by normalizing to the transcript level of *UBQ5*. Mean and SD values were obtained from more than three biological replicates.

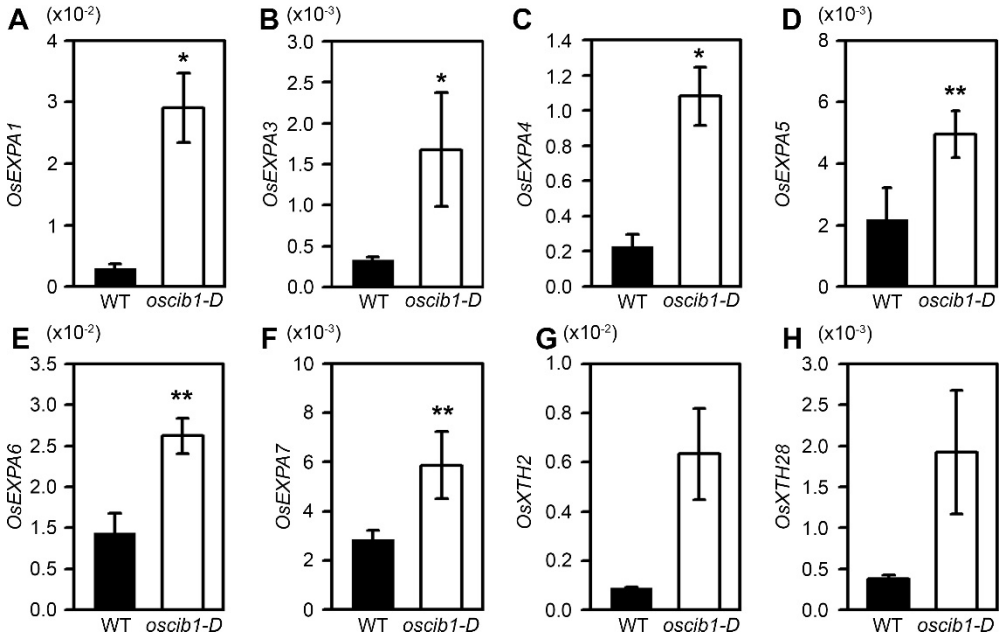

Figure 9. Expression analysis of *OsEXPA* and *OsXTH* family genes.

(A-D) Total RNA was extracted from ~2-cm lamina joint segments including leaf blade and leaf sheath (2-week-old) of WT and *oscib1-D*. Relative expression levels of *OsEXPA1* (A), *OsEXPA3* (B), *OsEXPA4* (C), *OsEXPA5* (D), *OsEXPA6* (E), *OsEXPA7* (F), *OsXTH2* (G),and *OsXTH28* (H) were obtained by normalizing to the transcript level of *UBQ5*. Mean and SD values were obtained from more than three biological replicates. Statistical analysis using Student′s *t*-test identified significant differences (**P*<0.05, ***P*<0.01, ****P*<0.005).

OsCIB1 controls grain size independent of grain-specific regulators.

To verify whether OsCIB1 is specifically expressed or functions in grains, we identified the expression level of *OsCIB1* in panicle tissues at various stages (Figure 10). We sampled panicles according to the developmental stage such as length and day after flowering. As shown in Figure 10, there was no obvious difference of expression level at respective stages.

Grain size is mainly determined by grain length, grain width, and grain thickness and it not only contributes to grain yield but also affects preference for rice [43]. The cell number and cell size largely determines the organ size during organogenesis [44, 45]. In recent years, it has been reported that several genes and quantitative trait loci (QTLs) including *GS3*, *GW2*, *GW5*, *GS5*, *GW8*, *qGL3*, *TGW6*, *GW6a*, and *BG1* affect grain size by regulating cell number in rice. In addition, *PGL1*, *GL7*, and *GS2*/*GL2* regulate grain size by influencing cell size [46-58]. To investigate the possible regulatory relationship between *OsCIB1* and other previously genes that affect grain size by regulating cell size, such as *PGL1*, *PGL2*, *GL7*, *GLW7*, *GS2*, and *SRS5*, we examined the transcript level of these genes and confirmed there was no significant difference between WT and *oscib1-D*, but rather decreased expression in *oscib1-D* (Figure 11). Taken together, these results indicate that *OsCIB1* not affects developmental stage of panicle and may regulate grain size in a pathway independent of *PGL1*, *PGL2*, *GL7*, *GLW7*, *GS2*, and *SRS5*.

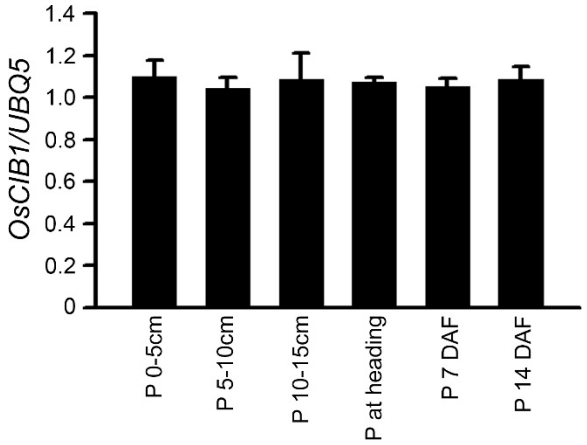

Figure 10. *OsCIB1* expression in panicle tissues at various stages.

Total RNA was extracted from panicles of WT. Relative expression level of *OsCIB1* were obtained by normalizing to the transcripts level of *UBQ5*. Mean and SD values were obtained from more than three biological replicates. P, Panicle; DAF, day after flowering.

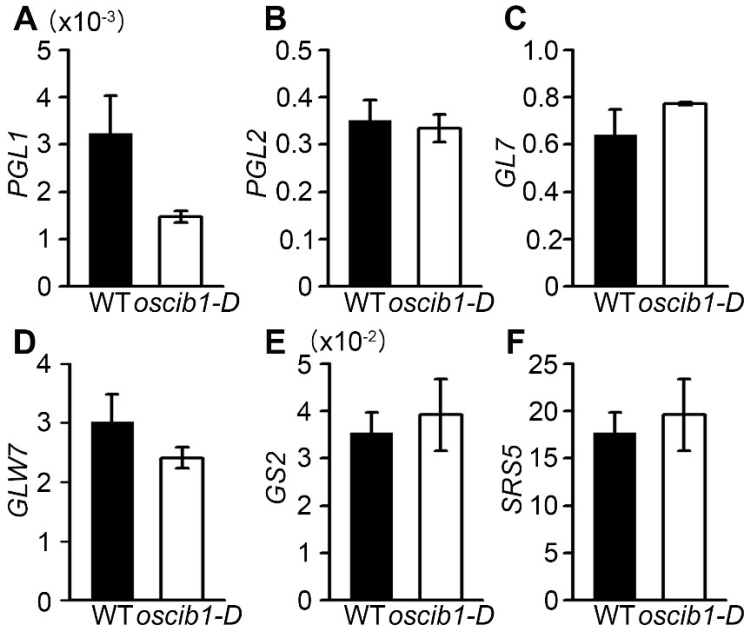

Figure 11. Quantitative RT-PCR analysis of several genes that control grain size by regulating cell expansion.

Total RNA was extracted from ~10-cm panicle segments of WT and *oscib1-D* at heading stage. Relative expression levels of *PGL1* (A), *PGL2* (B), *GL7* (C), *GLW7* (D), *GS2* (E), and *SRS5* (F) were obtained by normalizing to the transcript level of *UBQ5*. Mean and SD values were obtained from more than three biological replicates.

DISCUSSION

CIB1 in Arabidopsis are involved in transcriptional regulation of *FT* genes. In previous study, in soybean (*Glycine max*), CRY2a interact with the soybean basic helix-loop-helix transcription activator CIB1 (for cryptochrome-interacting bHLH1) and it activates senescence-associated genes, such as *WRKY DNA BINDING PROTEIN53b* (*WRKY53b*) [12, 19]. However, the function of CIB1 in rice remains unclear and little is known about BR signaling components in rice.

In this study, our data provide evidence that *OsCIB1* involved in BR signaling and functions as a critical regulator of genes related to the cell elongation to make enlarged lamina joint in rice. Several results in this study support this conclusion: (ⅰ) *oscib1-D* showed exaggerated leaf angle via increased cell size in adaxial surface of lamina joints, whereas *oscib1* had narrower leaf inclination than that of WT (Figure 2); (ⅱ) *oscib1-D* plants are sensitive to exogenous BR and alter expression of genes involved in BR signaling independent of BR biosynthesis (Figure 5-7); and (ⅲ) OsCIB1 might directly regulates cell-elongation-related genes such as Os*EXPA*s and Os*XTH*s(xyloglucan endotransglycosylase/hydrolase) (Figure 9). The size of an organ is determined by cell proliferation and cell expansion [46-48]. Our data showed that *OsCIB1* is required for cell expansion in lamina joints.

It has been reported that phytochrome-interacting-factor-like 1 (OsPIL1) functions as a key regulator of internode elongation [65]. Overexpression of *OsPIL1* in transgenic rice plants activated internode elongation. In addition, the abnormal internode elongation was caused by larger cells in plants overexpressing *OsPIL1* (OsPIL1-OXs). Transcriptome analysis identified that cell wall-related genes were upregulated in OsPIL1-OXs, which means that OsPIL1 can directly regulates downstream genes such as expansins and cellulose synthases. Moreover, the results of transient expression assay suggested that OsPIL1 could activate expression of the *OsEXPA4* and 1-ACC oxidase genes via the G-box element. Therefore, in this scenario, it is strongly possible that OsCIB1 is also involved in regulating cell-elongation-related genes. However, how OsCIB1 regulates the cell elongation and exact relationship between OsCIB1 and BR signaling pathway remain unclear. Therefore, additional studies are needed to further elucidate the mechanism underlying BR-induced cell elongation of lamina joint in rice.

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초 록

본 연구는 식물체에서 blue light 를 인지하는 cryptochrome 에 binding 하여 전사조절인자로써 역할을 하는 OsCIB1 의 기능을 T-DNA 삽입 돌연변이체를 이용하여 *oscib1-D* 돌연변이체에서 나타나는 잎의 각도와 종자크기의 차이의 매커니즘을 밝힌 연구이다. 잎의 각도 차이는 식물생장상(growth chamber), field 조건에서 모두 일주일 정도가 지나면서 나타나기 시작하고 종자 길이의 경우, heading 하기 전 즉, 등숙 전 상태를 비교하여도 과다발현체의 경우가 더 폭이 좁고 긴 형태를 보이는 것을 관찰 할 수 있었다. 이러한 표현형의 차이가 *OsCIB1* 유전자에 의한 것인지를 확인하기 위하여 knockout 돌연변이체 *oscib1*, Wild type, *oscib1-D* 각 개체들을 seedling stage(10-day-old)에서 관찰한 결과, *OsCIB1* 의 expression 이 감소한 *oscib1* 의 경우는 좁아진 잎의 각도를 보였고 반대로, *OsCIB1* 의 expression 이 증가한 *oscib1-D* 의 경우 Wild type 보다 더 넓어진 잎의 각도를 보였다. 따라서 *OsCIB1* 의 expression 의 양에 따라서 잎의 각도가 조절 됨을 확인 할 수 있었다. 잎의 각도를 조절하는 lamina joint 를 관찰한 결과 adaxial side 가 신장되어 있음을 확인하였고 구조적으로 어떠한 변화에 의한 것인지를 확인하기 위하여 lamina joint 의 SEM(scanning electron microscopy)촬영을 실시 하였지만 큰 차이를 발견하지 못했다. 따라서 세포수준에서의 차이를 확인하기 위하여 paraffin embedding 과 sectioning 을 하여 현미경으로 관찰한 결과, Wild type 과 *oscib1-D* 의 adaxial side 에서의 세포의 길이는 큰 차이가 없었지만, 반대쪽인 adaxial surface 의 세포의 길이가 *oscib1-D* 에서 더 길어져 있음을 확인하였다. 잎의 각도는 식물 호르몬 중 하나인 brassinosteroid(BR)와 밀접한 관련이 있다는 선행 연구결과를 토대로 두 가지 가능성을 제시하였다. 첫 번째는 OsCIB1 이 전사조절인자로서 brassinosteroid 합성 pathway 에 있는 유전자들을 조절 할 수 있다는 가능성을 확인 하기 위해서 Quantitative RT-PCR 을 통해서 expression level 을 확인한 결과 BR 합성에 관여하는 유전자인 *BR6ox*, *OsCPD*, *OsD2*, *OsDET2* 는 WT 과 *oscib1-*

D 에서 큰 차이가 없었고, 두번째 가능성인 BR-signaling 을 조절 할 수 있다는 가능성을 확인하기 위해서 *OsBRI1*, *OsBZR1*, *OsBAK1*, *OsILI1*, *OsBU1*, *OsGSK1* 의 expression 을 체크한 결과 모두 *oscib1-D* 에서 증가하는 것을 확인하였다. 세포의 길이에 의한 것임을 확인한 것과 같은 맥락으로 세포분열에 관여하는 유전자들의 expression 은 큰 변화가 없었던 반면, *OsEXPA* 와 *OsXTH* 같이 세포신장에 관여하는 대표적인 두 유전자의 경우는 *oscib1-D* 에서 증가함을 확인하였다. 따라서 *OsCIB1* 이 잎의 각도와 종자의 길이를 조절하는데 있어 BR signaling 에 관여하는 유전자들뿐만 아니라 *OsEXPA* family 에 속하는 유전자들을 조절하여 세포신장을 일으키고 이것이 잎의 각도를 조절함을 추측할 수 있었다.