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Molecules and Cells

The Calmodulin-Binding Transcription Factor OsCBT Suppresses Defense Responses to Pathogens in Rice

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We previously isolated the *OsCBT* gene, which encodes a calmodulin (CaM)-binding protein, from a rice expression library constructed from fungal elicitor-treated rice suspension cells. In order to understand the function of OsCBT in rice, we isolated and characterized a T-DNA insertion mutant allele named *oscbt-1*. The *oscbt-1* mutant exhibits reduced levels of *OsCBT* transcripts and no significant morphological changes compared to wild-type plant although the growth of the mutant is stunted. However, *oscbt-1* mutants showed significant resistance to two major rice pathogens. The growth of the rice blast fungus *Magnaporthe grisea*, as well as the bacterial pathogen *Xanthomonas oryzae pv. oryzae* was significantly suppressed in *oscbt-1* plants. Histochemical analysis indicated that the hypersensitive-response was induced in the *oscbt-1* mutant in response to compatible strains of fungal pathogens. *OsCBT* expression was induced upon challenge with fungal elicitor. We also observed significant increase in the level of pathogenesis-related genes in the *oscbt-1* mutant even under pathogen-free condition. Taken together, the results support an idea that OsCBT might act as a negative regulator on plant defense.

INTRODUCTION

Plants have evolved defense response systems to protect themselves from pathogen attack. The successful resistance of plants to pathogens depends upon timely recognition of the invading pathogens and rapid activation of defense responses *via* a number of signal transduction pathways. To successfully resist invading pathogens, plants employ efficient pathogen sensing systems and effective defense response mechanisms. Plant resistance (R) protein-mediated signal perception is a sophisticated pathogen recognition system in which the R proteins directly or indirectly recognize the specific effectors of pathogens and trigger defense reactions at the infected sites of the plant (Chisholm et al., 2006; Jones and Dangl, 2006). Plant defense responses are often accompanied by programmed cell death (hypersensitive response, HR) at the site of pathogen invasion and trigger generation of reactive oxygen species, pathogenesis-related (*PR*) gene expression, and accumulation of antimicrobial compounds (Dangl and Jones, 2001; Greenberg and Yao, 2004; Veronese et al., 2003). Transcriptional reprogramming is also a key step in plant defense signaling cascades in response to pathogen invasion (Eulgem, 2005). In combination with genetic and biochemical approaches, large-scale gene expression profiling approaches have been recently used to elucidate the gene regulation networks during plant immune responses (Kottapalli et al., 2007; Maleck et al., 2000; Tao et al., 2003). Microarray analysis has revealed that in addition to the classical *PR* genes, numerous other genes exhibit differential expression after activation of the defense program. Moreover, several types of transcription factors, such as WRKY, ERF, TGA-bZIP, Whirly, NPR1, and Myb, have been implicated in disease resistance (Eulgem, 2005). Despite extensive advances in the field of plant disease resistance, the precise molecular mechanisms of the interactions between plant and pathogen and the detailed elucidation of complex signaling networks remain elusive.

The Ca^{2+}/CaM complex plays vital roles in plants in sensing various environmental biotic and abiotic signals and triggering appropriate cellular responses by modulating the activities or functions of a wide range of CaM-binding proteins including metabolic enzymes and transcription factors, as well as ion channels and pumps (Bouché et al., 2005; White and Broadley, 2003; Yang and Poovaiah, 2003). Ca^{2+}/CaM-mediated modulation of cellular responses occurs *via* two distinct pathways. First, Ca^{2+}/CaM triggers rapid responses by direct binding to cytosolic target proteins, which modulates their activity. In addition, Ca^{2+}/CaM triggers indirect and relatively slow cellular responses by

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regulating gene expression through interactions with transcription factors (Snedden and Fromm, 1998). To date, many CaM-binding transcription factors, such as TGA3, WRKY, and AtMyb2, as well as CaM-binding transcriptional activators containing the CG-1 DNA-binding domain (AtSRs/AtCAMTAs and OsCBT), have been identified (Bouché et al., 2002; Choi et al., 2005; Park et al., 2005; Szymanski et al., 1996; Yang and Poovaiah, 2002; Yoo et al., 2005). CaM directly binds to these transcription factors and regulates the expression of their target genes by modulating the activity of the factors. Genes encoding CaM-binding transcription factors containing the CG-1 DNA-binding domain have been identified in various plant species, including parsley, tobacco, tomato, *Arabidopsis*, and rice, and the gene expression patterns of these proteins have been analyzed (Choi et al., 2005; da Costa e Silva, 1994; Reddy et al., 2000; Yang and Poovaiah, 2000; 2002; Zegzouti et al., 1999). The results from the analyses of plant CG-1 family members suggest that CG-1 family transcriptional activators might play important roles in Ca^{2+}/CaM-mediated plant responses to environmental stresses including biotic and abiotic stresses, as well as to developmental stimuli. However, the physiological functions of CG-1 transcription factor family proteins remain to be elucidated. Recently the first genetic evidence for the biological function of CG-1 transcriptional activator family member was indicated. The *camta3* mutants, loss-of-function alleles of one of the *Arabidopsis* CAMTA family (CAMTA3) show enhanced spontaneous lesion formation, expression of defense-related genes, and resistance to pathogens, indicating that CAMTA3 functions as a negative regulator in plant defense responses to pathogen (Galon et al., 2008).

In this report, we examined the functions of *OsCBT*, which encodes a CaM-binding CG-1 family transcription factor, using a reverse genetic approach in rice. Gene expression of *OsCBT* was upregulated in response to fungal pathogen invasion. A transfer (T)-DNA insertion allele mutant of *OsCBT*, *oscbt-1*, exhibited reduced *OsCBT* transcript levels and strong resistant to both fungal and bacterial pathogens. Down-regulation of *OsCBT* expression triggers constitutive expression of *PR* genes even under pathogen-free condition and induces HR in response to compatible pathogens. Our results provide valuable cues to understand not only the biological functions of OsCBT, but also the molecular mechanisms underlying plant defense responses mediated by transcriptional re-programming.

MATERIALS AND METHODS

Rice suspension cell culture and treatments

Suspension cell lines of rice (*Oryza sativa* L. Dongjin) were cultured and maintained as previously described (Cheong et al., 2003). Treatment with a fungal elicitor or Mock was performed in the dark. The fungal elicitor was prepared from the rice blast fungus *M. grisea* as described (Kim et al., 2003), and was added to the cell cultures at a final concentration of 50 μg/ml total reducing sugar. Subsequently, 3 ml of the cells (0.2-0.3 g fresh weight) were harvested by filtration at various times after treatment, quickly frozen in liquid nitrogen, and stored at -70°C until analyzed.

Plant materials and pathogen inoculations

Five- or six-leaf stage rice seedlings grown under natural light in a green house (25-30°C) were used for pathogen inoculation. For fungal inoculation of rice leaves, a conidial suspension (1×10^5 conidia/ml) of *Magnaporthe grisea* (*M. grisea*, strains KJ401 and KI313, which are compatible to the Dongjin cultivar and incompatible to the Jinheung cultivar) was sprayed onto the leaves using an atomizer. Inoculated plants were kept in a humidified chamber at 28°C and harvested at various times after inoculation. After 3 days, the leaves were observed for the appearance of disease symptoms.

For bacterial pathogen inoculation, *Xanthomonas oryzae pv. oryzae* (*X. oryzae pv. oryzae*) strain K1 or K2 were clip inoculated on leaves of approximately 6-week-old plants. Lesion lengths were measured at 16 days after inoculation. To quantify bacterial growth, rice leaves were collected at 14 days after inoculation, ground in water, and plated on agar plates after serial dilution and colonies were counted.

Screening T-DNA insertion mutants in the *OsCBT* gene from mutant pools

An *oscbt-1* mutant line was isolated by PCR screening of T-DNA insertion mutants with *OsCBT* gene-specific primers (F1 and R1) and the T-DNA right border primer (RB) using DNA pools (Table 1) (Lee et al., 2003). The exact position of the T-DNA insertion was determined by sequencing the amplified DNA fragment. All procedures were performed as described previously (Lee et al., 2003).

Reverse transcriptase (RT)-PCR and quantitative real-time PCR

RT-PCR was carried out using total RNA extracted from rice plants. First-strand cDNA was synthesized in a 20 μl reaction mixture using 1 μg of total RNA with Superscript II RNase H-reverse transcriptase and oligo-dT primers, as recommended by the manufacturer (Invitrogen). PCR reactions were carried out in a 20 μl volume with gene specific primer pairs (Table 1). PCR products were resolved by 1.2% agarose gel electrophoresis.

For quantitative real-time PCR, total RNA was extracted from suspension cell cultures that were treated with fungal elicitor or water as a control. Real-time PCR was conducted using the MyiQ Real-Time PCR Detection system (Bio-Rad) and iQ SYBRGreen Supermix (Bio-Rad) according to the manufacturer's instructions. At least three independent experiments were performed. The data were normalized by the value of *OsACTIN*, and fold change in expression was calculated by the MyiQ software.

Genomic Southern blot analysis

Rice genomic DNA (10 μg) was digested with *Hin*dIII or *Cla*I, fractionated on a 0.8% agarose gel, and transferred onto a nylon membrane (Hybond-N^+, Amersham). The membrane was hybridized with ^{32}P-labeled hygromycin phosphotransferase (*HPT*) and glucuronidase (*GUS*) gene probes, respectively. Hybridization was carried out at 65°C for 18 h in Church buffer (1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS). The membrane was finally washed in 1× SSC, 0.1% SDS at 50°C and exposed to X-ray film (Kodak).

Histochemical analysis

For aniline blue staining, rice leaves harvested at 3 days after fungal inoculation were excised and rinsed in deionized water. To observe mycelia and callose formation by fluorescence microscopy, leaves were preserved in 1 N KOH. The KOH-preserved specimens were autoclaved at 121°C for 15 min, rinsed with 0.067 M K_2HPO_4, pH 9.0, and stained with 0.05% aniline blue dye in 0.067 M K_2HPO_4, pH 9.0, as previously described (Diez-Navajas et al., 2007).

To detect cell death in rice leaves, trypan blue staining was performed as described (Belenghi et al., 2003). Detached leaves were immersed in a boiling solution of 10 ml lactic acid,

Table 1. Primers used for genotyping and RT-PCR analysis

Gene name	Forward primer	Reverse primer
Primers for genotyping		
OsCBT	5′-GAT GCC AGC AAT TCT GTA CTG ATT GAG T-3′	5′-ACA AAA CGA GAG GAA ATA GAA AAG GCC G-3′
Right Border	5′-TTG GGG TTT CTA CAG GAC GTA ACA TAA G-3′	
Primers for RT-PCR		
OsCBT	5′-TCA TTT CCG TAC ATG GAA GAT GAG-3′	5′-CTG CAG GTT GTT CTA TCA TTT CCA-3′
OsACTIN	5′-GTA AGC AAC TGG GAT GAC ATG GAG AA-3′	5′-CCT CCA ATC CAG ACA CTG TAC TTC CTC-3′
PR1a	5′-GCA AAT TGT GTA GTA GTG TTG CAT-3′	5′-AAC TTA TTA AGC AGA GAG AGT GAG-3′
PR1b	5′-TAG CTT CAA TTA ATG GCG AGT TCG-3′	5′-CAC GTA GCA TAG CAT ACG ATA TGA-3′
PR4	5′-CAT CTA TAA ATC GTA GTA ACC ACC-3′	5′-TTA CTC GTC TGT CCG AAA TGT GTA-3′
PR10a	5′-TCA TCT CTG CAT TTG CAT TTG CAG-3′	5′-GCA TAG GCA TGA TGA CGA TTT AGT-3′
PR10b	5′-TCA GGT GGA GAC TAG CTT AGA TA-3′	5′-C CTT AAA CAC AGA ATA ATT CTT CTC-3′
PBZ1	5′-AGA TTA TAT CTT CAG TGA TGG CTC-3′	5′-TAG AGG CAG TAT TCC TCT TCA TCT-3′

20 ml 50% (v/v) glycerol, 0.02 g trypan blue and 10 ml phenol for 2 min. The trypan blue solution was decanted and the leaves were destained with 10 ml of 70% (w/v) chloral hydrate.

RESULTS

Isolation of the *oscbt-1* mutant

Previously, we isolated the *OsCBT* gene, which encodes a CaM-binding transcriptional activator, by screening a rice cDNA expression library prepared from fungal elicitor-treated rice suspension cell cultures (Choi et al., 2005). To investigate the biological role of *OsCBT* in rice, we isolated an *OsCBT* T-DNA insertion mutant from rice activation-tagged T-DNA insertion mutant pools using a PCR-based screening strategy (Jeong et al., 2002; Lee et al., 2003). To confirm the candidate *OsCBT* mutant allele, the genomic regions flanking both sides of the T-DNA insertion were amplified by PCR and sequenced. Database analysis (http://www.tigr.org) of the T-DNA flanking sequences indicated that the T-DNA was inserted 219 bp upstream from the ATG translation initiation site of *OsCBT* (Fig. 1A). This T-DNA insertion mutant was named *oscbt-1*. To isolate a homozygous T-DNA insertion mutant we genotyped ten T_2 plants by PCR using the T-DNA right border primer and two gene specific primers (Table 1) and were able to identify two homozygous T-DNA insertion lines five heterozygous T-DNA insertion lines (Fig. 1B). The segregation ratios determined by PCR-genotyping of self-fertilized heterozygous plants in T_3 generation suggested that a single T-DNA was inserted in the *oscbt-1* mutant (data not shown). A single T-DNA insertion in *oscbt-1* mutant was also confirmed by genomic Southern blot analysis. Genomic DNA was isolated from wild-type and *oscbt-1* plants, digested with *Hin*dIII or *Cla*I and hybridized with either the *GUS* or *HPT* region of the T-DNA (Fig. 1C). As shown in Fig. 1D, we detected a single hybridizing band in the *oscbt-1* mutant for both probes, indicating that the *oscbt-1* mutant carries only a single copy of T-DNA in the genome. To examine the effect of the T-DNA insertion on *OsCBT* gene expression, we performed an RT-PCR analysis using total RNA isolated from wild-type and *oscbt-1* mutant plants. The T-DNA insertion into the promoter region of the *OsCBT* gene partially suppressed *OsCBT* expression (Fig. 1E) and quantitative analysis showed that the transcript level of the mutant allele was approximately 45% of that in wild-type plants (Fig. 1F). Moreover we also tested whether insertion of activation-tagged T-DNA triggered ectopic upregulation of genes flanking the activation-tagged T-DNA insertion site. To confirm this, we analyzed expression patterns of two down-stream genes (Os07g30750, Os07g30760) and four up-stream genes (Os07g30790, Os07g 30800, Os07g30810, Os07g30820) of *OsCBT* (Os07g30774) in *oscbt-1* mutant compared to those in wild-type by using RT-PCR, but we did not observe any obvious changes in mRNA levels of those genes between wild-type and *oscbt-1* mutant plants, indicating that the activation-tagged T-DNA insertion do not trigger the activation of flanking genes (data not shown). Since *oscbt-1* is not a complete loss-of-function allele, we have tried to identify the second independent mutant allele from searching other rice mutant populations available so far, however no other RNA-null or loss-of-function *OsCBT* mutant alleles are available suggesting that the complete loss of OsCBT function may be lethal. Thus, subsequent experiments were conducted using the *oscbt-1* mutant allele. The homozygous *oscbt-1* mutant plants exhibited a dwarf phenotype compared to wild-type plants (Fig. 1G). Additionally, *oscbt-1* mutants displayed reduced fertility and have lower grain yields compared to wild-type plants (data not shown). These results indicate that down-regulation of *OsCBT* expression may affect normal development of the plant.

The *oscbt-1* mutant exhibits enhanced resistance to fungal and bacterial pathogens

Isolation of *OsCBT* from the screening of a cDNA expression library prepared from fungal elicitor treated rice suspension cells prompted us to test whether OsCBT participates in plant defense signaling against pathogen invasion. We inoculated 5 or 6 leaf stage wild-type and *oscbt-1* mutant plants with compatible strains of the rice blast fungus *M. grisea* (strains KJ401 and KI313). While wild-type rice plants (Dongjin cultivar) showed typical leaf blast symptoms, such as development of grey lesions on the leaves, *oscbt-1* mutant plants showed almost no disease symptoms (Fig. 2A). On the leaves of wild-type plants, lesions started to appear 2 to 3 days after inoculation (DAI) and the average lesion diameter reached up to around 30 mm by 13 DAI. In contrast, *oscbt-1* mutant plants exhibited a highly resistant phenotype for two compatible blast strains, KJ401 and KI313, with the average lesion diameters ranging from 3 to 8.7 mm (Fig. 2B). To analyze the resistance

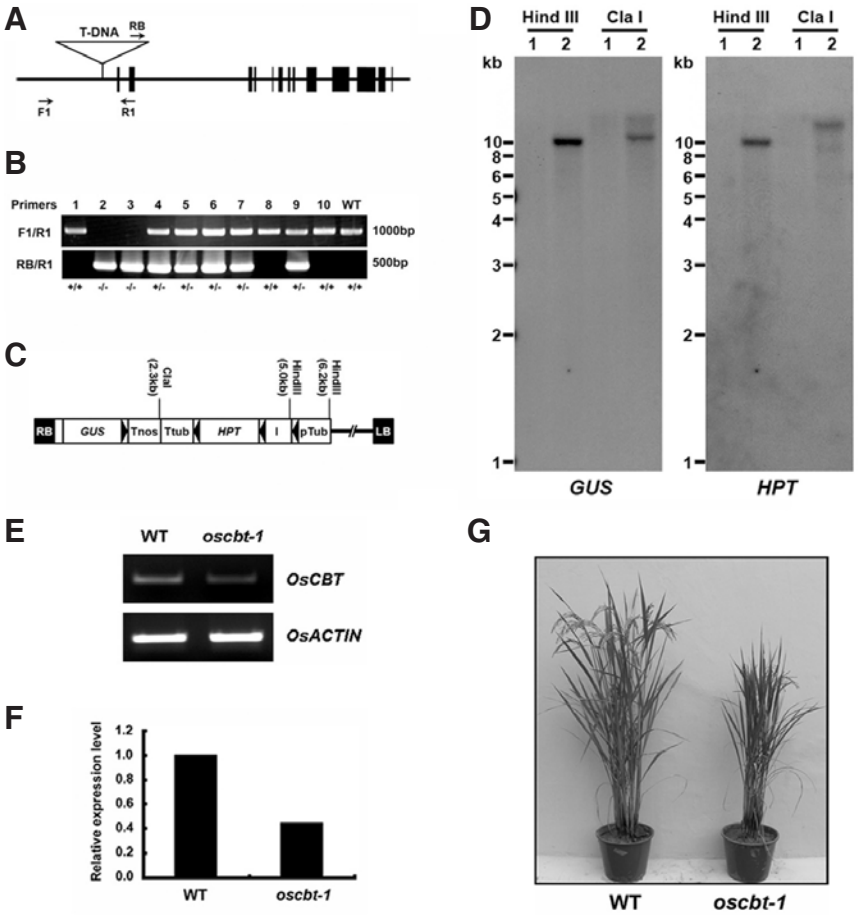

Fig. 1. Characterization of the *oscbt-1* mutant. (A) Genomic structure of the *OsCBT* gene and insertion position of the T-DNA. Exons are indicated by the filled boxes. T-DNA insertion is indicated by the open inverted-triangle. The T-DNA was inserted into the promoter region of the *OsCBT* gene. Arrows indicate the primers (F1, R1 and RB) used for genotyping. (B) Genotyping of *oscbt-1* progeny. Genomic DNA from wild-type and ten independent T2 *oscbt-1* plants was analyzed by PCR. Lanes 1, 8, and 10 are wild-type (+/+); lanes 2 and 3 are homozygotes (-/-); lanes 4, 5, 6, 7 and 9 are heterozygotes (+/-). (C) Map of the T-DNA region including positions of probes used for Southern blotting (GUS and HPT). (D) Southern blot analysis of wild-type and *oscbt-1* mutant plants. Genomic DNA extracted from wild-type and *oscbt-1* mutant plants was digested with *Hin*dIII and *Cla*I. The blot was hybridized to a *GUS* probe. After the blot was stripped, the blot was rehybridized to a *HPT* probe. Lanes 1 and 2 indicate the wild-type and *oscbt-1* mutant samples, respectively. (E) Comparison of *OsCBT* transcript levels in wild-type and *oscbt-1* mutant plants by RT-PCR. *OsACTIN* was used as a control (lower panel). (F) Relative expression level of the *OsCBT* transcript. Densitometric analysis of RT-PCR results, normalized to *OsACTIN*. (G) Phenotype of the *oscbt-1* mutant plant.

phenotype of *oscbt-1* in more detail, fungus inoculated leaves were harvested at 4 days after infection from two wild-type cultivars, Dongjin and Jinheung, which is compatible and incompatible to KJ401 strains used, respectively (Kim et al., 2004), and *oscbt-1* plants (Dongjin background) and stained with trypan blue, a histochemical indicator of cell death. In compatible wild-type plants, deep blue staining was observed in cells at sites of necrosis as well as in yellowing cells flanking the necrotic lesions (Fig. 2C). In contrast, staining was localized to small cell clusters in *oscbt-1* mutant plants as well as in incompatible wild-type plants. Since the localized staining pattern of trypan blue in *oscbt-1* mutant plants was similar to that seen during HR, we checked whether the lesions on *oscbt-1* mutant leaves exhibited an HR-like phenotype or a necrosis-like phenotype using aniline blue staining (Fig. 2D). During blast fungus infection, no hyphal development was observed in *oscbt-1* mutant leaves. Furthermore, callose deposition and accumulation of autofluorescent material in the cells surrounding fungal penetration sites, which is typical of HR, were detected in *oscbt-1* (Fig. 2D, bottom panel). These HR-like phenotypes of *oscbt-1* plants were identical to those of incompatible wild-type control plants (Fig. 2D, middle panel). In compatible wild-type plants, however, invading hyphae surrounded leaf epidermal cells and no HR-like phenotype was observed (Fig. 2D, top panel). Thus, down-regulation of *OsCBT* gene expression triggered HR-associated resistance to fungal pathogens which do not normally trigger HR in the compatible cultivar.

We next examined whether this resistance is specific only to the fungal pathogen or if *oscbt-1* mutant plants also exhibited resistance to other rice pathogens. Wild-type and *oscbt-1* mutant plants were inoculated with the bacterial pathogen *X. oryzae pv. oryzae*, the causal agent of rice bacterial blight disease. Rice leaves were inoculated with the K1 or K2 strains of *X. oryzae pv. oryzae*. After inoculation, wild-type plants showed typical disease symptoms, such as leaf blight, wilting, and pale yellow leaves. However, the development of these disease symptoms progressed only slightly beyond the inoculation sites in the *oscbt-1* mutant plants (Fig. 3A). The average of lesion lengths at 16 DAI were 50.7 and 79.9 mm in wild-type plants for the K1 and K2 bacterial blight strains, respectively, whereas the lesions on *oscbt-1* plants were restricted to within 2.2 to 3.3 mm from the inoculation sites (Fig. 3B). The strong resistance of *oscbt-1* plants to bacterial blight was confirmed by a significant reduction in bacterial growth of more than 40-fold in *oscbt-1* plants compared to wild-type plants (Fig. 3C). These results demonstrate that down-regulation of *OsCBT* expression confers significant resistance to two major rice pathogens.

Induction of OsCBT expression in response to pathogenic signal and constitutive expression of defense-related genes in *oscbt-1* mutant plants

The enhanced resistance to both fungal and bacterial pathogens conferred by the down-regulation of *OsCBT* expression suggests that OsCBT may be involved in plant defense signaling against pathogen attack. To test this hypothesis, we examined *OsCBT* gene expression in response to fungal pathogen. We treated rice suspension cell cultures with fungal elicitor prepared from *M. grisea* and analyzed the levels of *OsCBT*

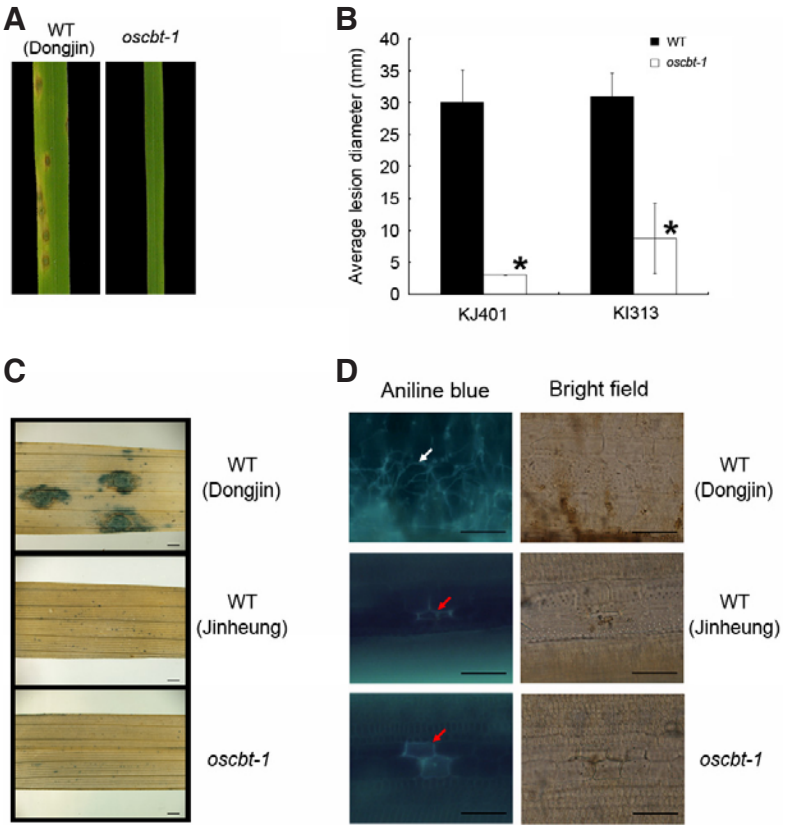

Fig. 2. Resistance phenotype of *oscbt-1* mutants to fungal pathogen. Five to six leaf stage (three- to four-week-old) rice seedlings were inoculated with spores of *M. grisea* strains, which are compatible and incompatible to Dongjin and Jinheung cultivars, respectively. (A) Disease symptoms of compatible wild-type (WT) and *oscbt-1* mutant plants spray-inoculated with *M. grisea* spores (race KJ401). Cell death lesions were visible on rice leaves at 4 days after inoculation. The *oscbt-1* mutant plants showed resistance to the fungal pathogen. (B) Average lesion diameter developed on leaves of compatible WT and *oscbt-1* mutant plants after spot inoculation with *M. grisea* spores (strains KJ401 and KI313). Lesion diameters were measured at 13 days after inoculation. The *oscbt-1* mutant showed a significant reduction in lesion size. The Data are means and standard deviations obtained from at least three independent experiments. Asterisks indicate significant difference to wild-type control ($P < 0.001$). (C) Trypan blue staining of leaves spray inoculated with *M. grisea* spores (KJ401). Trypan blue staining shows the cell death phenotype in rice leaves 4 days after inoculation with *M. grisea* spores. Scale bars = 1 mm. (D) Aniline blue staining of leaves spray-inoculated with *M. grisea* spores (KJ401). Aniline blue staining shows the hyphae of *M. grisea* in the leaves of compatible WT plants (indicated by white arrow). Callose deposited HR cell death was observed in *oscbt-1* mutant and incompatible WT plants (indicated by red arrows). Scale bars = 50 μm.

transcript using quantitative real-time PCR. Upon fungal elicitor treatment, *OsCBT* expression gradually increased in a time dependent manner and peaked by 24 h after treatment, while the expression of *OsCBT* was not affected by a mock treatment (Fig. 4A).

Mutant plants exhibiting broad-spectrum pathogen resistance, including barley *mlo*, rice *spl* and *blm*, and *Arabidopsis camta3* often also exhibit spontaneous HR-like lesion formation and elevated *PR* gene expression, even in pathogen free conditions (Büschges et al., 1997; Galon et al., 2008; Jung et al., 2005; Yin et al., 2000). To examine whether the enhanced resistance of *oscbt-1* is due to the presence of spontaneous micro-HR in the leaves, we conducted both trypan blue and aniline blue staining with the leaves of axenically grown *oscbt-1* mutant plants. Neither trypan blue nor aniline blue staining revealed development of spontaneous HR-like cell death in the leaves of *oscbt-1* mutant plants in pathogen-free conditions (data not shown). We then examined *PR* gene expression in *oscbt-1* mutant plants. *PR1s* (*PR1a* and *PR1b*), *PR4*, and *PR10s* (*PBZ1*, *PR10a*, *PR10b*) function in the responses to various biotic and abiotic stresses in rice (Agrawal et al., 2000a; 2000b; Guimil et al., 2005; Kim et al., 2004; McGee et al., 2001). In *oscbt-1* mutant plants, the expression of the *PR* genes tested, with the exception of *PR1a*, was upregulated compared to wild-type plants, in pathogen free conditions (Fig. 4B). These results indicate that the significant resistance to two major rice pathogens of the *oscbt-1* mutant is at least partly attributable to constitutive expression of *PR* genes.

DISCUSSION

CaM-binding transcriptional activators (CAMTAs) containing a CG-1 DNA binding domain have been identified from various organisms including flies, mammals, and plants (Finkler et al., 2007). In plants, several CAMTA homologs have been isolated from various species and analysis of their gene expression patterns has revealed that plant CAMTAs modulate gene expression in response to various biotic and abiotic environmental stimuli. In the rice genome, there are six CAMTA homologs containing a CG-1 DNA binding domain at the N-terminus (Os01g69910, Os03g09100, Os04g31900, Os07g30774 (OsCBT), Os07g43030, Os10g22950). In our previous report, we showed that OsCBT functions as a transcriptional activator and that its activity is negatively regulated by CaM-binding (Choi et al., 2005). Here, we examined the function of OsCBT in rice using a reverse genetic approach. The *OsCBT* T-DNA insertion mutant, *oscbt-1*, exhibits significant resistance to both fungal and bacterial pathogens. The resistance of *oscbt-1* plants to compatible fungal pathogens is accompanied by HR (Fig. 2). Moreover, several rice *PR* genes are constitutively expressed in *oscbt-1* plants and *OsCBT* gene expression itself is up-regulated by fungal elicitor treatment. These results suggest that OsCBT functions in plant defense responses against fungal and bacterial pathogens and that it appears to normally suppress plant defense responses. Consistent with our observations, a recent study reported that a loss-of-function mutant of *CAMTA3,* one of the *Arabidopsis* CG-1 family members, exhibits enhanced resistance to virulent bacterial and fungal pathogens in association with spontaneous lesion formation, elevated defense-related gene expression, and high levels of reactive oxygen species (Galon et al., 2008). These results suggest that this member of the plant CG-1 family plays a role in defense signaling by suppressing normal defense responses. Moreover, our results suggest not only that OsCBT might be a rice functional ortholog of

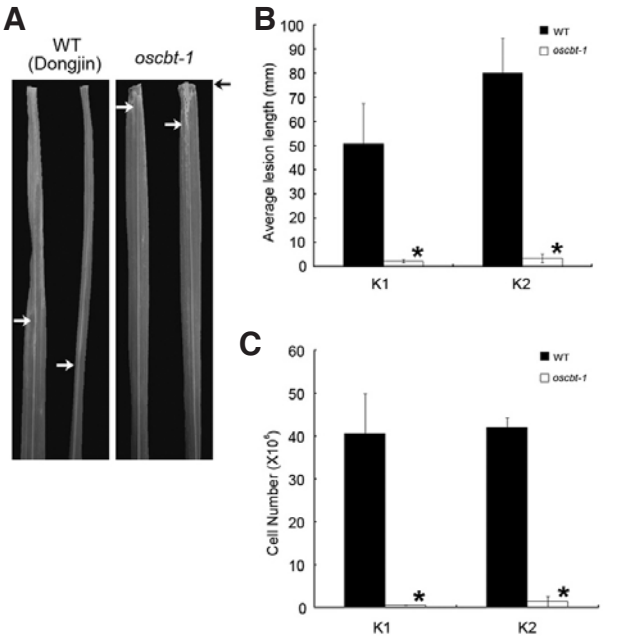

Fig. 3. Enhanced resistance of *oscbt-1* mutant plants to a bacterial pathogen. Six-week-old rice seedlings were inoculated with the virulent K1 and K2 strains of *X. oryzae pv. oryzae*. (A) Disease symptoms on the leaves of WT (Dongjin cultivar) and *oscbt-1* mutant plants at 14 days after inoculation with *X. oryzae pv. Oryzae.* The white arrows indicate the points where disease symptoms have progressed. The black arrow indicates the sites of pathogen inoculation. (B) Average lesion length on the leaves of WT and *oscbt-1* mutant plants measured at 16 days after inoculation with *X. oryzae pv. oryzae*. (C) Growth of the virulent strains of *X. oryzae pv. oryzae* 14 days after inoculation of the leaves of WT and *oscbt-1* mutant plants. The Data are means and standard deviations obtained from at least three independent experiments. Asterisks indicate significant difference to wild-type control ($P < 0.001$).

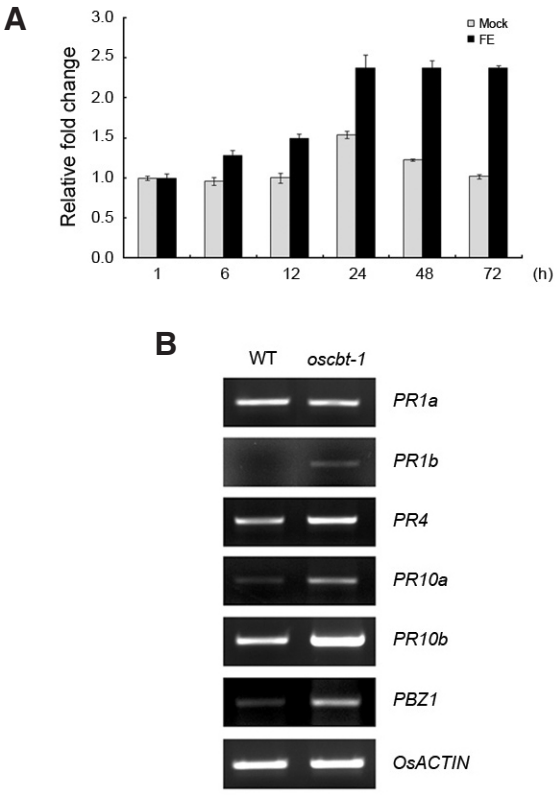

Fig. 4. Expression analysis of *OsCBT* and *PR* genes. (A) Expression of *OsCBT* in response to treatment with a fungal elicitor (FE) in suspension cultured rice cells. Suspension cells were treated with FE or Mock as a control. Samples were harvested at the indicated times. Transcript levels of *OsCBT* were determined by quantitative real-time PCR. (B) Comparison of *PR* gene expression in WT (Dongjin cultivar) and *oscbt-1* plants. Total RNA was extracted from WT and *oscbt-1* mutant plants. Transcript levels of *PR* genes were analyzed by RT-PCR. *OsACTIN* was used as a loading control. We obtained the similar results from the two independent RT-PCR experiments with technical replicates.

Arabidopsis CAMTA3, but also that the defense signaling pathway transduced by Ca^{2+}/CaM-mediated transcriptional regulation is well conserved between di- and monocotyledous plant species.

Mutant plants that show broad-spectrum resistance, such as barley *mlo*, rice *spl* and *blm*, and *Arabidopsis camta3*, often also exhibit development of HR and constitutive expression of defense-related genes under pathogen-free conditions (Büschges et al., 1997; Galon et al., 2008; Jung et al., 2005; Yin et al., 2000). Although the expression of several rice *PR* genes is elevated in *oscbt-1* plants, any HR-like lesions were not formed on the leaves of *oscbt-1* plants, even though *oscbt-1* plants exhibited HR to virulent pathogens. These results suggest that OsCBT may not directly participate in modulation of HR cell death, but may participate in the regulation of defense-related gene expression during normal growth. Disease resistance is not always correlated with HR (Lorrain et al., 2003). For example, the *Arabidopsis dnd1* mutant exhibits broad-spectrum resistance against virulent viral, fungal, and bacterial pathogens despite the virtual absence of HR cell death (Yu et al., 1998). Moreover, the *Arabidopsis lsd1* mutant showed resistance to virulent pathogens under conditions in which no spontaneous cell death lesions were observed (Dietrich et al., 1997). In addition, constitutive expression of defense-related genes in the *oscbt-1* mutant may affect the normal development of this mutant plant, resulting in the dwarf phenotype and reduced fertility. Constitutive activation of plant defense systems has been shown to cause defects in normal vegetative growth, flowering, and seed production (Heil, 2002), consistent with the developmental defects of the mutant *oscbt-1* (Fig. 1G).

Cytosolic Ca^{2+} elevation switches on a network of signaling events during plant innate immune responses to pathogen attack (Ma and Berkowitz, 2007). One of the downstream events from cytosolic Ca^{2+} elevation in this signaling cascade is formation of the Ca^{2+}/CaM complex, which transmits the perceived signal to downstream effectors. Moreover, there is a growing body of evidence indicating that CaM plays a crucial role in plant defense signaling (Ali et al., 2003; Bouché et al., 2005; Heo et al., 1999; Kim et al., 2002a; 2002b; Takabatake et al., 2007). Our previous results showed that OsCBT functions as a transcriptional activator and that the binding of Ca^{2+}/CaM to the C-terminal region of OsCBT suppresses OsCBT-mediated transcriptional activation (Choi et al., 2005). Our observations in the current study suggest that OsCBT plays a role as a negative regulator in plant disease resistance as well as in regulation of defense related gene expression. Taken together, our observations indicate that Ca^{2+}/CaM may trigger plant defense responses, at least in part, by suppressing OsCBT func-

tion. However, the precise regulation of OsCBT by Ca^{2+}/CaM binding remains to be elucidated.

In this report, we examined the biological functions of OsCBT using a reverse genetic approach in rice. The T-DNA insertion allele mutant of *OsCBT*, *oscbt-1*, exhibits strong resistant to both fungal and bacterial pathogens with the constitutive expression of defense-related genes and induces hypersensitive-response (HR) in response to compatible pathogens, indicating that OsCBT functions as a negative regulator of defense response. To our knowledge, this is the first report to show the biological function of a CG-1 DNA-binding domain containing family member in rice. Further studies on downstream targets of OsCBT will help understand how OsCBT modulates Ca^{2+}/CaM signaling in plant defense response.

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