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OsNAC300 Positively Regulates Cadmium Stress Responses and Tolerance in Rice Roots

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Abstract: Transcriptional regulation is involved in responding to cadmium (Cd) stress in plants. However, the molecular mechanisms of Cd stress responses regulated by transcription factors remain largely unknown in plants. In this study, a rice (*Oryza sativa*) NAC (no apical meristem [NAM]; Arabidopsis transcription activation factor [ATAF]; cup-shaped cotyledon [CUC]-related) family transcription factor, OsNAC300, was isolated and functionally characterized for its involvement in Cd stress responses and tolerance. OsNAC300 was localized to the nucleus. *OsNAC300* was mainly expressed in roots and significantly induced by Cd treatment. Knockout of *OsNAC300* resulted in increased sensitivity to Cd stress, while its overexpression lines enhanced tolerance to Cd stress. RNA-Seq analysis revealed that the mutant is impaired in regulating some important genes that were responsive to Cd stress in wild-type rice, such as the pathogenesis-related genes 10a (*OsPR10a*), *OsPR10b*, chalcone synthase 1 (*OsCHS1*), and several others, which was validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. Moreover, chromatin immunoprecipitation-qPCR assays and luciferase reporter assays demonstrated that OsNAC300 directly binds to the promoters of *OsPR10a*, *OsPR10b*, and *OsCHS1* and activates their transcription. Overall, OsNAC300 is an important regulatory factor in Cd stress responses and tolerance in rice.

Keywords: cadmium; OsNAC300; rice (*Oryza sativa*); stress; RNA-Seq; OsPR10a; OsCHS1

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1. Introduction

Cadmium (Cd) is a highly toxic metal element to almost all organisms, including humans and plants [1,2]. Anthropogenic activities such as mining and smelting, and modern agricultural practices including overusing fertilizers and herbicides/pesticides have led to increased Cd contamination in the environment, which poses a serious threat to human health [3]. Rice (*Oryza sativa* L.) is the most important staple cereal crop for most Asian countries and can accumulate a high Cd level in its grains [4]. Excess Cd can also affect plant growth and development via the disruption of nutrient homeostasis, dysfunction of proteins, and the generation of toxic reactive oxygen species (ROS) [5]. Several defense mechanisms against Cd stress have been identified in plants and other organisms, including chelation, extrusion, sequestration of Cd, and the removal of Cd-triggered ROS, for example, cys-rich proteins or peptides such as metallothionein, phytochelatin (PC), and defensin-like protein, are often used to chelate heavy metal Cd and are involved in Cd stress tolerance in plants [6–8].

The content of ROS in plant cells rapidly increases upon heavy metal Cd stress [9]. In addition, stress-responsive flavonoids have substantial potential to inhibit the generation

of ROS and reduce ROS levels, i.e., flavonoids act as antioxidants. Chalcone synthase (CHS) is the rate-limiting enzyme in the flavonoid synthesis pathway in *Petunia hybrida* [10]. The expression of *CHS* has been induced in various plant species under different forms of stress, such as UV, wounding, herbivory, and microbial infections (reviewed in Dao et al. [11]). These findings implied the potential role of flavonoids as antioxidants to remove ROS generated under Cd stress in plants.

Pathogenesis-related (PR) proteins are highly conserved proteins that have been well defined; these can quickly respond to biotic and abiotic stresses, including pathogen infection, wounding, drought, high salinity, and heavy metal toxicity [12]. The major families of PRs have been divided into 17 classes (PR1 to PR17), primarily based on their amino acid sequence identity and biological activities [13]. Among these, PR10 proteins are typically intracellular, small (16–19 kDa), and acidic, with similar three-dimensional structures [14]. PR10 proteins have been reported to have various functions, such as antimicrobial, ribonuclease, and norcoclaurine synthase activities, and the ability to bind ligands [14–16]. Some studies also revealed that the *PR10* genes are responsive to various environmental stresses, such as drought, high salinity, low and high temperatures, wounding, and UV exposure [17]. There are four *PR10* genes, *OsPR10a*, *OsPR10b*, *RSOsPR10* (root-specific PR 10), and *JIOsPR10* (jasmonic acid-inducible PR 10) in the rice genome [18–20]. *OsPR10a* and *OsPR10b* encode predicted proteins of 158 and 160 amino acids, respectively, and share 71% amino acid identity [18]. *OsPR10a* (encodes PBZ1) was first characterized as a probenazole-inducible protein in rice [21]. OsWRKY6 directly regulates the expression of *OsPR10a*, since WRKY transcription factors are known to be transcriptional activators of *PR* genes [22]. Rice and bentgrass (*Agrostis* spp.) that overexpressed *RSOsPR10* displayed improved tolerance to drought (rice and bentgrass) and salt (bentgrass) [17]. Overexpression of *JIOsPR10* enhances the tolerance to biotic and abiotic stress in rice [12]. The expression of pathogenesis-related class 5 (*OsPR5*) was induced in leaves of rice that were wounded and upregulated by salicylic acid (SA), jasmonic acid (JA), and kinetin and protein phosphatase (PP) 2A inhibitors [23]. However, whether PR proteins respond to Cd stress and/or are involved in plants' tolerance to Cd stress have not been reported.

Transcriptional regulation is extensively involved in the response of plants to signal molecules in the external environment, such as biotic and abiotic stresses. The expression of many genes responds to biotic and abiotic stress in plants, including genes encoding transcription factors (TFs) and defense-related proteins [24,25]. The NAC (NAM, no apical meristem; ATAF, Arabidopsis transcription activation factor; CUC, cup-shaped cotyledon) domain TFs include a plant-specific gene family with few members that has been studied for its roles in plant growth and biotic/abiotic stress tolerance across various plant species [26]. More than 151 rice NAC (ONAC) TFs have been identified, but only a few of these have been functionally defined [27,28]. Yuan et al. [29] specified that ten *ONAC* genes exhibit pivotal functions in rice response to abiotic stress tolerance. The ten genes are *ONAC002*, *ONAC003*, *ONAC009*, *ONAC022*, *ONAC045*, *ONAC048*, *ONAC058*, *ONAC066*, *ONAC095*, and *ONAC122*.

A large number of TFs cooperate to complete the specific regulation of downstream functional genes, thereby improving the ability of plants to adapt to fluctuations in the external environment [30]. It is difficult for plant roots to avoid detrimental substances in the soil, such as Cd and aluminum (Al), and plants must have a set of transcriptional regulatory mechanisms to manage such toxicities. In plant, for the regulatory responses to Cd stress, studies of a few TFs, such as OsMYB45, AtZAT6, TaHSFA4a, and CaPF1, have been reported, since these TFs facilitate the tolerance of plants to Cd stress by improving their antioxidant capacity, metallothionein expression, and content of glutathione (GSH) [31–34]. Comparative transcriptome analysis under Cd stress has shown that there are still many unreported genes that are strongly induced by Cd stress [35–37]. This indicates that many unknown TFs are involved in the process of regulating plant cells in response to Cd stress.

In our previous study, several TFs that increased in expression after Cd stress were identified in rice [35]. To characterize these regulators that function in the response to Cd

stress, we performed phenotypic screening based on the mutants of these TFs and found that OsNAC300 (also designated ONAC300/OsNAC077); (Os12g0123800/LOC_Os12g03050) may play an important role in response to Cd stress in rice. Kusano et al. [38] had shown that high levels of *OsNAC300* mRNA were detected by in situ hybridization in the developing shoot apical meristem (SAM) and in young leaves, however, the biological function of OsNAC300 is unknown. Here, we show that OsNAC300 plays an important regulatory role in Cd stress responses and Cd tolerance in rice.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Three *osnac300* knockout lines were generated by editing *OsNAC300* in the "Nipponbare" background. One target sequence (gRNA1, 257–276 bp; Supplemental Table S1) for CRISPR/Cas9 was used from the genomic sequence of *OsNAC300* and inserted into the CRISPR/Cas9 vector pRGEB31 (Addgene, Watertown, MA, USA) [39]. The risk of off-target mutations was minimized by the selection of highly specific target sequences via a thorough genome search (www.genome.arizona.edu/crispr/). *Agrobacterium*-mediated transformation was performed using vigorously growing calluses derived from mature rice embryos [40]. Homozygous lines were confirmed by sequencing PCR-amplified products from potential mutant plants.

To generate the *OsNAC300* overexpression (OE) lines, the full-length *OsNAC300* cDNA sequence was amplified using the primer pairs (Supplemental Table S1). The amplified cDNA fragment was digested with *Kpn* I and *Bam*H I and cloned into the pCAMBIA1300 vector, followed by rice transformation as described above [40]. The relative expression levels of *OsNAC300* were determined in the transgenic OE lines with reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Seeds of both wild-type rice (cv. Nipponbare) and *OsNAC300* mutants were soaked in water for 2 days (d) before being transferred to a solution of 0.5 mM $CaCl_2$. After 4 d, seedlings were transferred into $\frac{1}{2}$ Kimura B solution (pH 5.5) and cultivated in a greenhouse maintained at 25 to 30 °C. The composition of the nutrient solution was as follows: 0.18 mM $(NH_4)_2SO_4$, 0.27 mM $MgSO_4{\cdot}7H_2O$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.09 mM KH_2PO_4, 0.5 μM $MnCl_2{\cdot}4H_2O$, 3 μM H_3BO_3, 1 μM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.4 μM $ZnSO_4{\cdot}7H_2O$, 0.2 μM $CuSO_4{\cdot}5H_2O$, and 20 μM Fe(III)-EDTA. This solution was renewed every 2 d. All the experiments were repeated at least three times.

2.2. Subcellular Localization

An *OsNAC300* cDNA fragment was amplified from the rice cDNA library using a pair of *OsNAC300*-specific primers (Supplemental Table S1) and cloned into a pBluescript SK vector that harbored the 35S cauliflower mosaic virus promoter [31]. An OsNAC300-synthetic Green Fluorescent Protein (sGFP) fusion protein expression construct was transformed into onion epidermis cells using a gene gun (Bio-Rad Laboratories Inc., Hercules, CA, USA). After bombardment, the onion layers were incubated in the dark for 12–18 h at 25 °C. Photographs were taken using laser confocal microscopy (UltraVIEW VOX, PerkinElmer, Waltham, MA, USA).

2.3. Gene Expression Analysis

Rice (cv. Nipponbare) was grown in a paddy field from mid-June to the end of September. To examine the expression pattern of *OsNAC300*, different tissues, including the roots, basal stems, old leaf blades, old leaf sheaths, new leaf blades, new leaf sheaths, flag leaf blades, flag leaf sheaths, node-Is, and seeds, were sampled at different stages of growth. To examine the *OsNAC300* expression in response to Cd stress, 2-week-old wild-type rice seedlings were exposed to Cd (0–100 μM) for different time intervals. The roots were sampled for RNA extraction, with treatments conducted in triplicates. Total RNA (1 μg) was used for first-strand cDNA synthesis using a HiScript II Q RT SuperMix Kit (Vazyme, Nanjing, China). RT-qPCR was performed using ChanQTM SYBR Color

qPCR Master Mix (Vazyme). *Actin* (LOC_Os03g50885) was used as an internal standard. The primers for analysis of the gene expression are listed in Supplemental Table S1.

2.4. β-glucuronidase (GUS) Staining

The construction of *OsNAC300* promoter-GUS was performed as previously described [31]. Primers listed in Supplemental Table S1 were used for the amplification of the promoter region. proOsNAC300::GUS was introduced into the *Agrobacterium tumefaciens* strain *EHA105* and transformed into rice callus (Nipponbare) [40]. GUS staining of seedlings used 50 mM sodium phosphate buffer (pH 7.0) that contained 0.1% Triton X-100 (*v*/*v*) and 0.5 mM X-Gluc (Sangon, Shanghai, China), which was incubated at 37 °C for 6 h. The stained tissues were observed using a Carl Zeiss laser scanning system (Zeiss, Oberkochen, Germany) and a Leica MZ95 stereomicroscope (Leica Instruments, Wetzlar, Germany).

2.5. Phenotypic Analysis

To compare the tolerance of *OsNAC300* knockout lines and the wild-type rice to Cd stress, 2-week-old seedlings of the wild-type rice and three *OsNAC300* knockout lines were exposed to a solution that contained 200 μM $CdCl_2$ for 2 d, and then $CdCl_2$-free conditions were restored for 5 days. To observe the sensitivity of root elongation to Cd stress, 3-day-old rice seedlings were transferred to a solution that contained 0.5 mM $CaCl_2$ (as control) or 5 μM $CdCl_2$ + 0.5 mM $CaCl_2$ (as +Cd) for 3 days. Daily elongation of the root was measured until day 3 of the Cd stress.

2.6. Elemental Concentration Measurement

The plants were grown on 1/2 strength Kimura solution with 0.5, 5, or 50 μM $CdCl_2$ for 3 days, harvested, dried at 80 °C for 3 d, and then subjected to acid digestion. At least 0.2 g dry weight of tissue was used for each replicate; samples were digested with HNO_3/$HClO_4$ (87:13 [*v*/*v*]) at 100 °C for 1 h, 120 °C for 1 h, 140 °C for 1 h, 160 °C for 1 h, and 180 °C for 1 h. After liquefying the processed samples in 2% HNO_3, the concentrations of Cd were determined using inductively coupled plasma mass spectrometry (ICP-MS, Perkin-Elmer NexION 300X, Waltham, MA, USA).

2.7. RNA-Seq Analysis

The seedlings of the wild type rice and the *OsNAC300* knockout line (*osnac300-1*) were germinated and grown in culture solution for 2-weeks. The seedlings were treated with or without (as the controls) 100 μM $CdCl_2$ for 12 h. The seedling roots were sampled and rapidly frozen in liquid nitrogen for RNA extraction. There were 3 replicates in each group, 12 samples in total. Total RNA was extracted using an RNA extraction kit (TaKaRa, Dalian, China). The RNA quantity was determined using an ND-8000 spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA), 1% agarose gel electrophoresis, and a 2100-Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples, in the final analysis, had been subjected to electrophoresis with no visible smears on agarose gels, with 260/280 ratios above 2.0, and RNA integrity numbers greater than 8.0 [35]. Total RNA samples of the roots were then sent to Genewiz Biotechnology Corporation (http://www.genewiz.com.cn; Genewiz, Suzhou, China) for sequencing. The libraries were sequenced as 101-bp paired-end reads using an Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequence reads were mapped to the rice genome with combined gene annotation of RAPDB (http://rapdb.dna.affrc.go.jp/download/archive/irgsp1/IRGSP-1.0_representative_2013-04-24.tar.gz, IRGSP-1.0_predicted_2013-03-19.tar.gz) and MSU 7.0 (ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/all.gff3) databases. The relative transcript abundance of each gene was measured in terms of the fragments per kilobase of transcript per million mapped reads (FPKM). The differential expression genes (DEGs) between the

two sets of samples were identified using DESeq. The detailed bioinformatics analysis procedures can be found in our previous paper, He et al. [41].

To confirm the RNA-Seq results, we performed an RT-qPCR analysis of root RNA samples treated exactly the same way as described above. We examined the expression of the following genes: *OsNAC300*, *OsPR10a*, *OsPR10b*, *RSOsPR10*, *JIOsPR10*, *OsPR5*, *OsPR1-71,* and *OsCHS1*. *Actin* was used as an internal standard. The specific primers are listed in Supplemental Table S1. RNA-Seq data: Illumina reads of all samples were deposited in the Sequence Read Archive at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA622262.

2.8. Transient Luciferase (LUC) Reporter Assay

The 1184-bp, 1898-bp, and 1688-bp promoter regions of *OsPR10a*, *OsPR10b,* and *OsCHS1* were amplified with their specific primer pairs, respectively (Supplemental Table S1). Three fragments were then cloned into the binary vector p1381Z-LUC to generate p1381Z-proPR10a::LUC, p1381Z-proPR10b::LUC, and p1381Z-proCHS1 using the single *Bam*H I restriction site, and the three constructs, including pCAMBIA1300-35S::OsNAC300, were subsequently mobilized into *A. tumefaciens* (strain EHA105). Transient luciferase reporter assays on 4-week-old *Nicotiana benthamiana* leaves were performed and checked. The relative expression level of LUC was quantified following transfection with different vectors. The hygromycin B resistance gene (*HPTII* redesignated as *Hyg*) was used for an internal standard based on its high and stable expression level under the same 35S promoter. For this analysis, the relative expression level of LUC was measured by RT-qPCR from three biological replicates as previously described [42].

2.9. ChIP-qPCR Assay

A Chromatin Immuno-Precipitation Kit (Merck Millipore, Burlington, MA, USA) was used to perform ChIP-qPCR assays as previously described [43]. Briefly, approximately 2.5 g samples of 2-week-old 35S::NAC300-GFP transgenic rice seedlings were fixed with 50 mL of 1.0% formaldehyde under vacuum for 10 min. Chromatin was extracted and sheared to 200- to 1000-bp fragments using ultrasonication. Then, 60 µL of sheared DNA was immuno-precipitated with 4 mg of anti-GFP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 60 rpm and 4 °C. DNA fragments that were specifically associated with the OsNAC300 protein were released, purified, and used as templates for ChIP-qPCR. Genomic fragments from nonbinding sites were used as negative controls. Normalized relative expression was calculated by the $^{\Delta\Delta}C_t$ (cycle threshold) method, and *Actin* in the "noAB" samples was used as the internal standard.

2.10. Statistical Analysis

Statistical analysis was performed using SPSS ver. 20.0 (IBM, Inc., Armonk, NY, USA) for all the data obtained. To compare the wild-type plants and *osnac300* mutants, the data were analyzed using a one-way analysis of variance (ANOVA) followed by a Student's *t*-test or Tukey's test where appropriate. Data are shown as the means ± SD as error bars.

3. Results

3.1. Sequence and Subcellular Localization Analysis of OsNAC300

The full-length open reading frame of *OsNAC300* cloned from rice root cDNA was identical to the sequence in a rice sequence database (http://rapdb.dna.affrc.go.jp). *OsNAC300* consists of two exons and one intron and encodes a protein of 396 amino acids with a calculated molecular mass of 43.86 kDa. Phylogenetic analysis showed that OsNAC300 is a member of the NAM subfamily of the rice NAC TF family (Supplemental Figure S1A). Among rice members, the closest homologs of OsNAC300 are OsNAC77L and OsNAC45, which share 70.82% and 66.75% identity, respectively, and belong to a subgroup (Supplemental Figure S1A). OsNAC45 plays complex roles by mediating the expression of development-related genes under various abiotic stresses in rice

roots [44]. The OsNAC300 protein contains one conserved NAM domain for DNA binding (Supplemental Figure S1B). Using the ProtComp 9.0 program (http://www.softberry.com/), we predicted that OsNAC300 is a nuclear protein with nuclear localization signal peptide (NLS).

To confirm the nuclear localization of OsNAC300, an OsNAC300-sGFP fusion construct was transiently expressed in the onion epidermal cells. Using confocal laser scanning microscopy, we found that the sGFP-tagged OsNAC300 signal was co-localized with the nuclear localization marker, red fluorescent protein-Histone 2A (RFP–H2A) [31], whereas, in cells that expressed solely sGFP, green fluorescence was observed throughout the cell (Figure 1). This result indicated that OsNAC300 is a nuclear-localized protein.

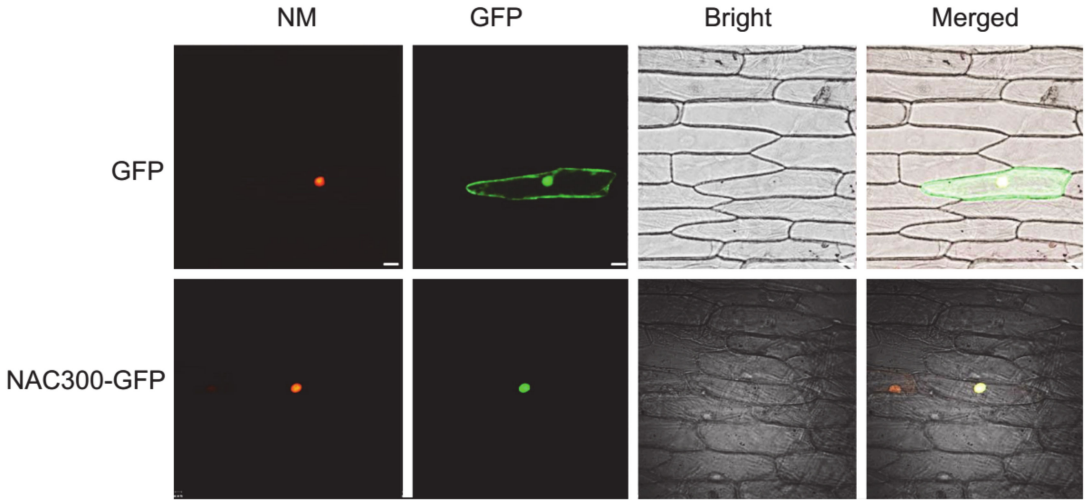

Figure 1. Subcellular localization of OsNAC300. Confocal images of onion epidermal cells expressing *GFP*, *OsNAC300-GFP*. *RFP-H2A* (red fluorescent protein Histone 2A); (nuclear marker, NM) is a nuclear localization tag. (Scale bars = 70 μm.)

3.2. Expression Analysis of OsNAC300

The expression pattern of *OsNAC300* was investigated in rice plants grown in either soil or nutrient solution by using RT-qPCR. *OsNAC300* was mainly expressed in the rice roots at all growth stages grown in a paddy field (Figure 2A). GUS staining analysis on the *OsNAC300* promoter-GUS transgenic lines showed that *OsNAC300* was primarily expressed in the primary and lateral roots (Figure 2B–G), and was strongly induced by Cd stress (Figure 2H). Furthermore, RT-qPCR experiments showed that Cd stress induced the expression of *OsNAC300* in the roots in a dose-dependent manner (Figure 2I). An analysis of the time-course assay of Cd-treated roots showed that the expression of *OsNAC300* was rapidly upregulated in response to Cd stress from 6 h and reached its maximum at 24 h (Figure 2J). These results indicate that *OsNAC300* was specifically expressed in roots and was induced by Cd stress.

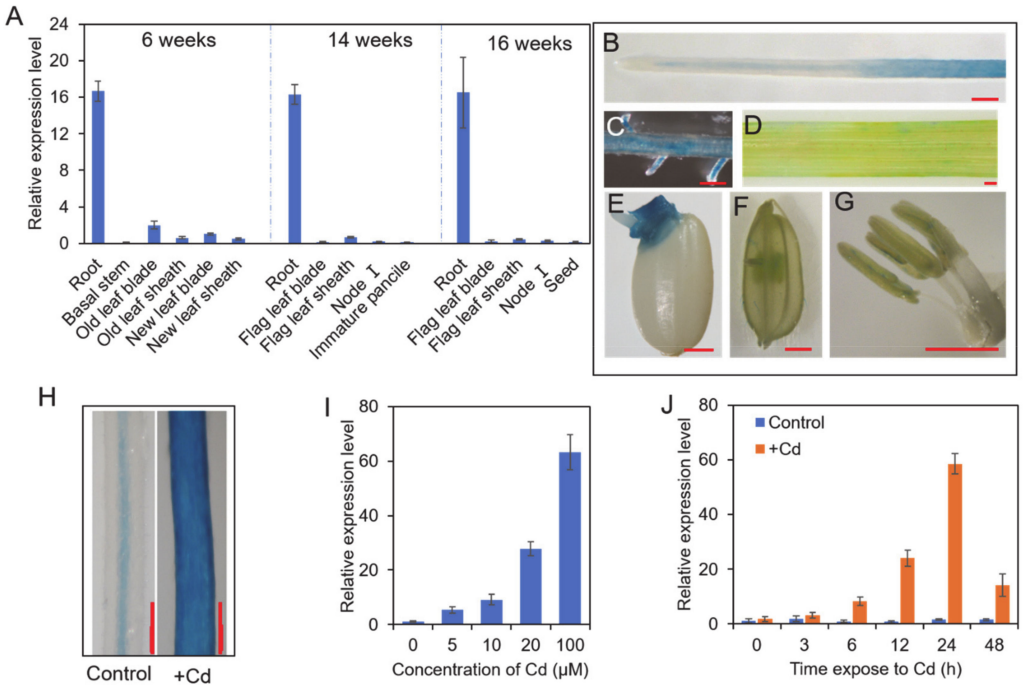

Figure 2. Expression pattern of *OsNAC300*. (**A**) Relative expression of *OsNAC300* in various tissues at different growth stages. Samples of various organs were taken from rice grown in a paddy field. (**B**–**H**) β-glucuronidase (GUS) staining, bar = 1 mm. (**B**) Region of primary roots (PR). (**C**) Region of lateral roots (LR). (**D**) Leaf. (**E**) Germinating seed. (**F**) Floret. (**G**) Stamen. (**H**) Region of PR under –Cd and +Cd (100 μM) for 24 h. (**I**) Dose-dependent expression of *OsNAC300* in the roots. Rice seedlings were exposed to a solution containing different concentrations of $CdCl_2$ for 24 h. (**J**) Time-dependent expression of *OsNAC300* in the roots. Rice seedlings were exposed to a solution containing 100 μM $CdCl_2$ for different times. The expression of *OsNAC300* was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR). *Actin* was used as an internal standard. Expression relative to seedlings without addition of Cd is shown. Data are means ± SD of four biological replicates.

3.3. Knock Out of OsNAC300 Enhances Sensitivity to Cd Stress

To investigate the physiological role of OsNAC300 in Cd stress response and tolerance in rice, *osnac300* knockout mutants were generated via CRISPR/Cas9. Three independent mutant lines (*osnac300-1*, *osnac300-2*, and *osnac300-3*) with nucleic acid deletions in the gRNA region were identified by sequencing (Supplemental Figure S2). Under control growth conditions without Cd, no visible differences were observed between the wild-type plants and *osnac300* knockout lines (Figure 3A). However, when grown in a solution containing 200 μM Cd^{2+} for 2 days and then transferred into a solution without Cd for 5 days' recovery, all of the three *osnac300* mutant plants withered and died, whereas the wild-type plants were still alive (Figure 3B). We observed necrosis and browning on the leaves of *osnac300* mutants after 2 days of Cd treatment, which was not observed in the wild-type leaves (Figure 3C). The survival ratios of the three mutant lines were only 10, 12, and 13% of that of the wild-type plants (Figure 3D). The daily elongation of the roots of the three *osnac300* mutants was also significantly less than that of the wild-type plants under Cd stress (Figure 3D). These results indicated that mutation of *OsNAC300* enhances the sensitivity to Cd stress in rice, suggesting that OsNAC300 is required for Cd tolerance in rice.

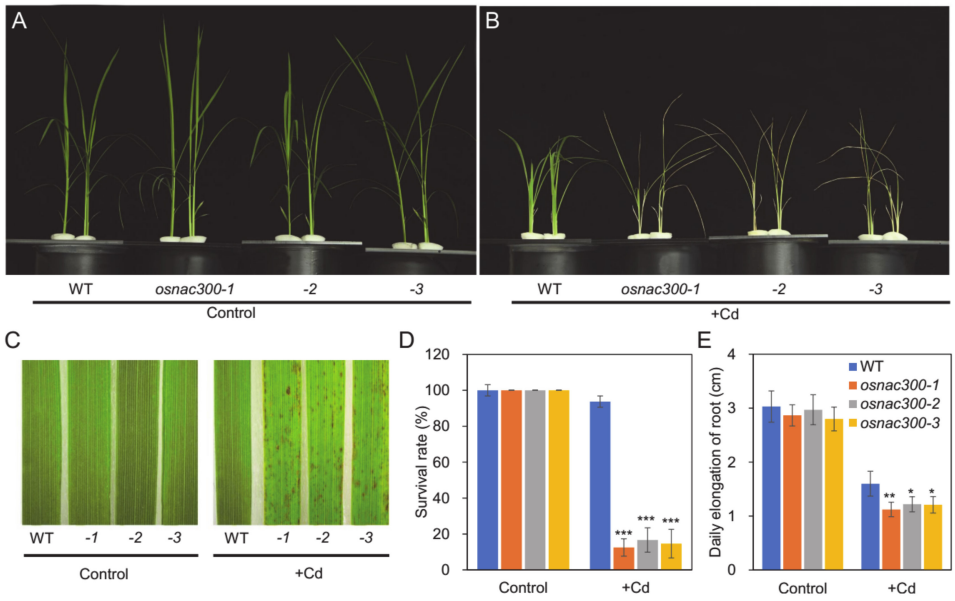

Figure 3. *osnac300* mutants are hypersensitive to Cd stress. (**A**,**B**) Phenotype of *osnac300* knockout lines subjected to Cd treatment. 2-week-old rice seedlings were grown on either control (1/2 Kimura solution) (**A**) or +Cd (+200 μM $CdCl_2$ in 1/2 Kimura solution) (**B**) for 2 days, and subsequently restored in Cd-free solution for 5 days. (**C**) The images of leaves were taken when 2-week-old rice seedlings were grown under 200 μM $CdCl_2$ for 2 days. (**D**) Survival rate of rice seedlings subjected to Cd treatments as described in (**A**). (**E**) Daily elongation of roots on the 3rd day of Cd stress. 3-d-old rice seedlings were grown on either control (0.5 mM $CaCl_2$) or +Cd (5 μM $CdCl_2$ +0.5 mM $CaCl_2$) for 3 days. Mean values ± SD are shown. The values of the indicated genotypes were compared to that of the wild-type (WT) plants (Student's *t*-tests, *** p <0.001, ** p <0.01, * p < 0.05, n = 12).

To further investigate the function of OsNAC300, *OsNAC300* overexpression lines were generated. The three lines OE1, OE2, and OE3 showed significantly higher (18.2, 15.8, and 14.3 times) expression levels of *OsNAC300* compared to the wild-type plants (Figure 4A). The daily root elongation of OsNAC300-OE lines (OE1, OE2, and OE3) was significantly higher than that of the wild-type plants in the presence of Cd (Figure 4B), whereas there was no significant difference between the wild-type plants and the over-expressing lines under control conditions. These results suggest that overexpression of *OsNAC300* enhanced the tolerance to Cd in rice.

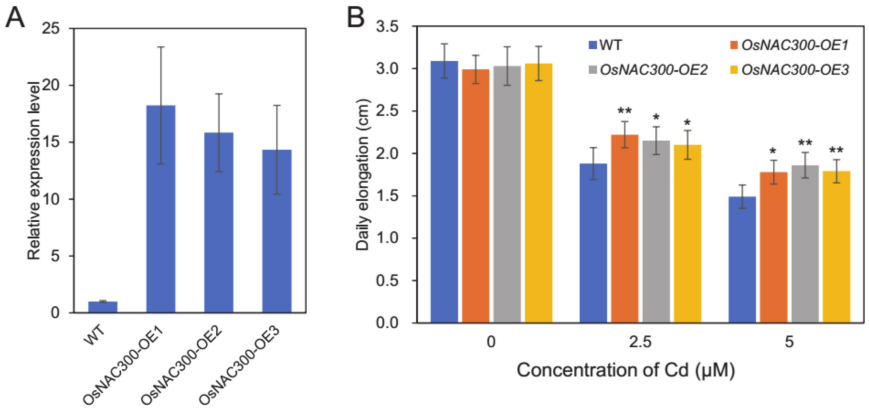

Figure 4. Overexpression of *OsNAC300* enhanced the tolerance of rice roots to Cd. (**A**) RT-qPCR analysis of the relative transcription level of *OsNAC300* in the roots of the wild-type (WT) and three *OsNAC300* overexpression lines. (**B**) Daily elongation of roots on the 3rd day of Cd stress. 3-d-old rice seedlings were grown on 0, 2.5 or 5 μM $CdCl_2$ for 3 days. Mean values ± SD are shown. The values of the indicated genotypes were compared to that of the WT (Student's *t*-tests, ** p <0.01, * p < 0.05, n = 12).

3.4. Role of OsNAC300 in the Accumulation of Cd in Rice

To examine whether OsNAC300-mediated Cd tolerance is associated with the uptake and transport of Cd in rice, we grew *osnac300* knockout lines and the wild-type plants in a hydroponic solution with three different Cd concentrations for 3 days. There were no differences between the wild-type plants and *osnac300* knockout lines in the concentration of Cd in both the roots and shoots under all three growth conditions (Figure 5A,B). Furthermore, the Cd accumulation in the roots and shoots of *OsNAC300*-overexpressing lines were also compared with the wild-type plants under the same conditions as above. Similarly, no differences were observed in the concentration of Cd in both roots and shoots between the wild-type plants and *OsNAC300* overexpression lines under all conditions (Figure 5C,D). These results indicate that Cd accumulation or exclusion in rice is not significantly regulated by *OsNAC300*.

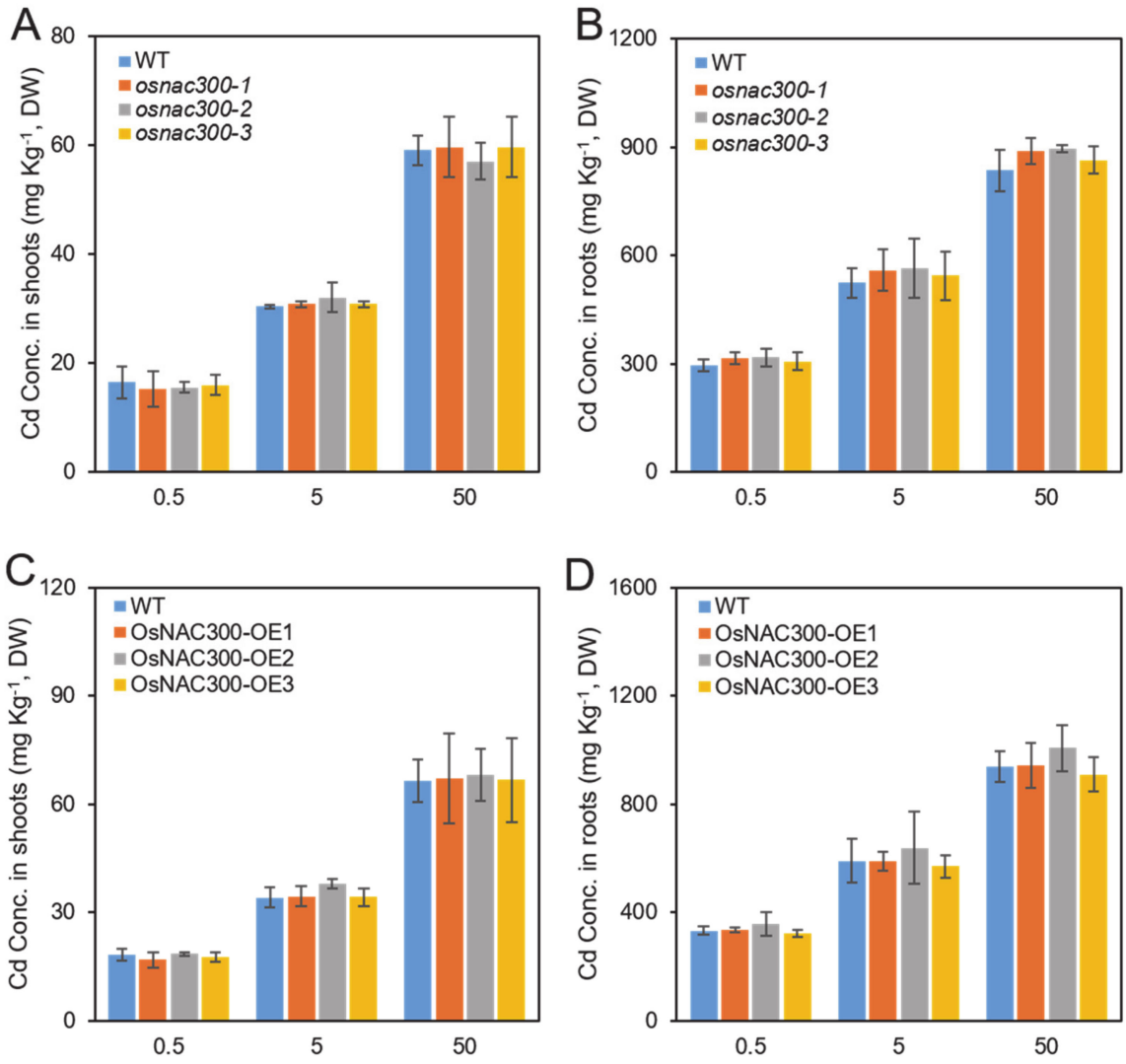

Figure 5. Cd concentrations of *osnac300* mutants and overexpression lines. (**A**,**B**) Concentration of Cd in the shoots (**A**) and the roots (**B**) of *osnac300* knockout lines. (**C**,**D**) Concentration of Cd in the shoots (**C**) and the roots (**D**) of *osnac300* overexpression lines. 2-week-old rice seedlings were grown on 0.5, 5 or 50 μM $CdCl_2$ for 3 days. The Cd concentration in the roots and shoots was determined by ICP-MS. Data are means ± SD of four biological replicates.

3.5. OsNAC300 Plays an Important Role in Changes in Global Gene Expression in Response to Cd Stress

To identify genes regulated by OsNAC300, we performed RNA-seq analysis using the wild-type plants and *osnac300-1* mutant treated with or without Cd stress. A large number of differentially expressed genes (DEGs) were identified from the wild-type plants under Cd stress. Using $p \leq 0.01$ and fold change (FC) ≥ 1.5 as a cutoff, 4443 and 3671 genes (Supplemental Tables S2 and S3) were identified as induced and repressed by Cd stress. The genes induced by Cd stress include many encoding TFs (*OsNAC300*, *OsERF102*, and *OsERF104*), defense-related proteins (*OsPR10a*, *OsPR10b*, and *RSOsPR10*), and many transporters (*OsABCG1*, *OsABCG3*, and *OsABCG5*). The genes repressed by Cd stress include many genes related to nutrient homeostasis, such as macronutrient metabolism (*OsNAR2.1*, *OsNRT2.1*, *OsNRT2.2*, and *OsPT2*), and micronutrient metabolism (*OsNAS1/2/3*, *OsNAAT1*, *OsTOM1*, *OsZIP5*, *OsHMA2*, and *OsNramp5*). Meanwhile, a total of 393 genes (256 upregulated and 137 downregulated genes) in the *osnac300-1* mutant plants were identified as differentially expressed compared with the wild-type plants under normal conditions (Supplemental Tables S4 and S5).

Then, the Cd stress-induced genes that were affected by the loss of function of OsNAC300 were studied in more detail. 4227 of the 4443 Cd stress-induced genes (e.g., *OsDREB1B* and *OsDREB1C*) were regulated normally in the *osnac300-1* mutants (Supplemental Table S6). A total of 158 genes, such as *OsERF4* and *OsERF103*, were hyper-induced in *osnac300-1* mutants under +Cd conditions (Supplemental Table S7), suggesting that OsNAC300 could either directly or indirectly function as a repressor of these genes. Moreover, 58 genes were not induced (or induced to a lesser extent) in the *osnac300-1* mutants compared with the wild-type plants under +Cd conditions (Supplemental Table S8). "Defense-related protein" was the most enriched biological process in the 58 genes, including *OsCHS1*, *OsPR10a*, and *OsPR10b*. These genes have been proposed to be involved in environmental stresses [14] and were deregulated in *osnac300-1* mutants. These results indicated that OsNAC300 is either directly or indirectly involved in the activation of genes induced by Cd stress, including *OsPR10a*, *OsPR10b*, and *OsCHS1*.

3.6. The Response of PR Genes and OsCHS1 to Cd Stress Are Dependent on OsNAC300

To verify the OsNAC300-dependent Cd stress-induced expression of the *PR* and *OsCHS1* genes identified by RNA-seq, we performed RT-qPCR experiments. After Cd treatment, the expression levels of *OsNAC300* in the roots of both the wild-type plants and mutants were upregulated approximately 20-fold (Figure 6A). This is consistent with those of the other RNA-Seq experiments [35], indicating that the effects of two Cd stress experiments are similar, and the expression of *OsNAC300* that responds to Cd stress does not depend on its protein function. The expression of *OsPR10a*, *OsPR10b*, *RSOsPR10*, and *JIOsPR10* was induced up to 470-, 25-, 60- and 4.3-fold by Cd stress in the roots of the wild-type, respectively (Figure 6B–E). However, in the roots of the *osnac300-1* mutant, the expression levels of *OsPR10a*, *OsPR10b*, and *RSOsPR10* were severely weakened (Figure 6B–E). The other two *PR* genes, *OsPR5* and *OsPR1-17*, were also induced by Cd stress, dependent on OsNAC300 (Figure 6F,G). This showed that except for *JIOsPR10*, the expression of *OsPR10a*, *OsPR10b*, *RSOsPR10*, *OsPR5*, and *OsPR1-71* that respond to Cd stress are dependent on OsNAC300. Chalcone synthase (CHS) is the rate-limiting enzyme in the flavonoid synthesis pathway. *OsCHS1* in the wild-type plant roots was also induced by Cd stress. However, in the *nac300-1* mutant roots, the expression of *OsCHS1* was not affected by Cd stress (Figure 6H).

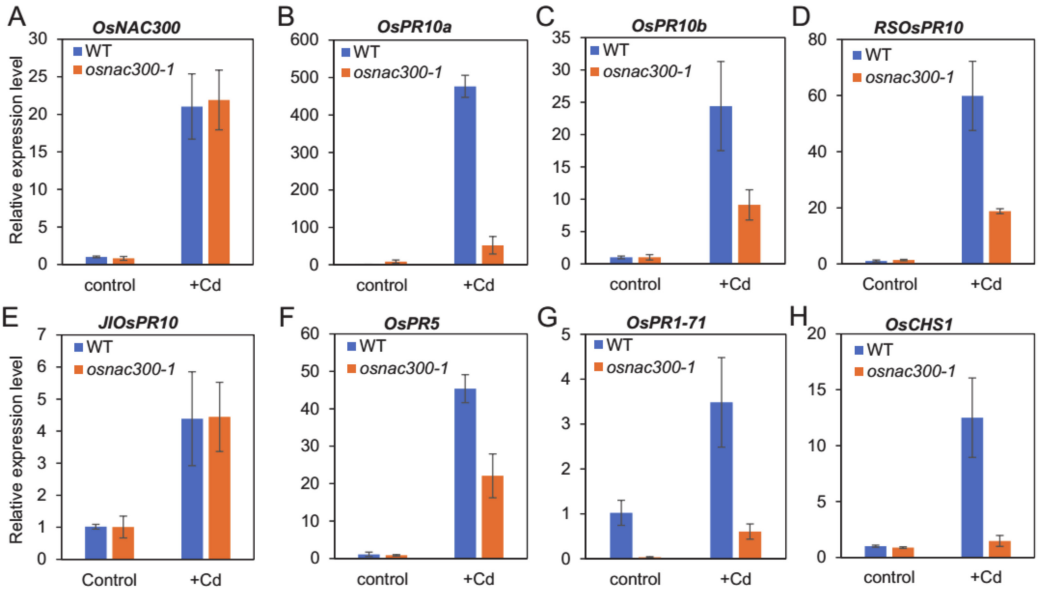

Figure 6. RT-qPCR analysis of potential downstream genes of OsNAC300 under Cd stress. The expression level of *OsNAC300* (**A**), *OsPR10a* (**B**), *OsPR10b* (**C**), *RSOsPR10* (**D**), *JIOsPR10* (**E**), *OsPR1-71* (**F**), *OsPR5* (**G**), and *OsCHS1* (**H**) in 2-week-old wild-type and *osnac300-1* seedlings treated without or with 100 μM $CdCl_2$ for 12 h assayed by RT-qPCR. The relative expression level was obtained by normalization to the expression level in the WT without Cd treatment. *Actin* was used as the internal standard. Data represent the mean ± SD (n = 4).

3.7. OsNAC300 Directly Binds to the Promoters of OsCHS1, OsPR10a, and OsPR10b and Enhances Their Transcription

Eleven genes that showed significantly lower levels of expression in the os*nac300-1* line than in the wild-type plants under Cd stress and were highly induced by Cd stress (FC ≥ 3) in the wild-type plants were selected for promoter analysis (Table 1) to verify their potential direct regulation relationship with OsNAC300. Bu et al. [45] reported that ANAC019 shows high affinity with the CATGTG motif. In our study, most of these genes (11 out of 14) contained one or more CATGTG motifs in their promoter (Supplemental Table S9). *OsCHS1*, *OsPR10a*, and *OsPR10b* contained one, two, and one CATGTG motifs in their promoters, respectively (Figure 7, Supplemental Table S9). This suggests that CATGTG motifs may play an important role in OsNAC300 response to Cd stress.

Table 1. Differential expression (in roots) of genes was affected by OsNAC300.

	WT			***osnac300-1***				
Gene ID	**Control**	**+Cd**	**FC**	**Control**	**+Cd**	**FC**	**Symbol**	**Description**
pathogenesis-related protein								
Os12g0555500	1.3	229.2	173.8	8.4	62.2	7.4	*OsPR10A*	Probenazole-inducible protein PBZ1.
Os12g0555200	19.3	253.9	13.2	16.2	78.0	4.8	*OsPR10B*	Similar to Probenazole-inducible protein PBZ1.
Os12g0555000	39.3	956.6	24.3	48.6	479.8	9.9	—	Similar to Probenazole-inducible protein PBZ1.
Os03g0663600	4.1	155.3	38.1	4.6	87.6	19.2	—	Similar to Pathogenesis-related thaumatin-like protein.
Os12g0628600	1.7	69.8	41.6	1.6	29.7	18.1	*OsPR5*	Similar to Thaumatin-like pathogenesis-related protein 3 precursor.
Metabolism								
Os11g0530600	0.4	3.9	9.2	0.4	0.5	1.3	*OsCHS1*	Similar to Chalcone synthase C2 (EC 2.3.1.74).

Table 1. *Cont.*

	WT			*osnac300-1*				
Gene ID	**Control**	**+Cd**	**FC**	**Control**	**+Cd**	**FC**	**Symbol**	**Description**
Other biology process								
Os11g0514500	7.6	51.5	6.8	9.3	29.1	3.1	—	leucine-rich repeat-containing extracellular glycoprotein precursor.
Os03g0661600	19.7	74.7	3.8	24.6	46.9	1.9	—	Similar to Alpha-amylase/trypsin inhibitor (Antifungal protein).
Unknown								
Os09g0272600	75.8	754.7	10.0	86.4	482.2	5.6	—	Conserved hypothetical protein.
Os10g0452100	11.1	50.5	4.5	15.0	32.3	2.2	—	Conserved hypothetical protein.
Os04g0653700	2.3	9.4	4.1	3.3	5.6	1.7	—	Conserved hypothetical protein.

The locus identifiers are given for genes that are at least 3-fold upregulated in response to Cd in WT, are at least 1.5-fold downregulated in *osnac300-1* Cd stress roots compared to WT Cd stress roots, and are 1.5-fold downregulated in fold change (FC) (*osnac300-1* in +Cd) compared to FC (WT in +Cd). 11 genes met these three requirements and were found to be statistically significant. WT and *osnac300-1* signal intensities and fold changes are presented for the average of three biological replicates. Genes are grouped based on their regulation by OsNAC300 and secondly by predicted function.

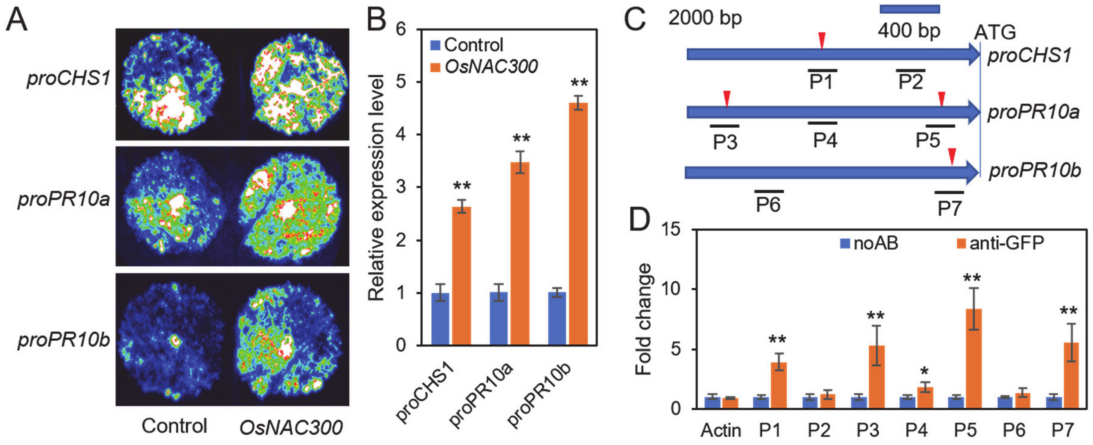

Figure 7. OsNAC300 directly binds to the promoters of *OsCHS1*, *OsPR10a* and *OsPR10b*, and enhances their transcription in tobacco leaves. (**A**) Luciferase signals were detected in leaf cells for 48 h following co-infiltration with empty vector and pCAMBIA1300-NAC300 and with p1381-proCHS1::LUC, p1381-proPR10a::LUC, and p1381-proPR10b::LUC. (**B**) The relative expression levels of luciferase were quantified by RT-qPCR following transfection with different vectors. *Hyg* was used for an internal standard (Student's *t*-test, ** $p < 0.01$). Transient luciferase reporter assays show the activation of *OsCHS1*, *OsPR10a*, and *OsPR10b* expression by OsNAC300. Tobacco leaf transient expression assays using 1688-, 1184-, and 1898-bp promoter fragments of *OsCHS1*, *OsPR10a*, and *OsPR10b*, respectively. (**C**) Diagram of *OsCHS1*, *OsPR10a*, and *OsPR10b* promoters. Red wedge represents CATGTG motifs. P1–7 indicates genomic DNA fragments around the three promoters for ChIP-qPCR. (**D**) ChIP-qPCR assay. Binding of OsNAC300 to specific regions of the *OsCHS1*, *OsPR10a*, and *OsPR10b* promoters was examined with 4-week-old 35S::GFP-NAC300 transgenic seedlings. The noAB (no antibody) and a fragment of *Actin* were used as negative controls. Data are means ± SD ($n = 4$). Statistical comparison was performed by Tukey's multiple comparison test. Asterisks indicate significant differences from the control (* $p < 0.05$ and ** $p < 0.01$).

To elucidate whether OsNAC300 could directly activate the promoter of these candidate genes, a luciferase (LUC) transient transcriptional activity assay was performed in tobacco leaves using the firefly LUC gene as a reporter, while OsNAC300, driven by the CaMV 35S promoter, served as an effector. Thus, 35S::OsNAC300 co-expression with the promoters of three genes (p1381-proCHS1::LUC, p1381-proPR10a::LUC, and p1381-proPR10b::LUC) was observed to enhance the bioluminescence signal (Figure 7A), and the transcript abundance of LUC relative to that of the control was calculated (Figure 7B). This result indicated that OsNAC300 could directly enhance the transcriptional activ-

ity of the promoters of the three genes and provided additional confirmation that the upregulation of these genes under Cd stress is dependent on OsNAC300.

The binding of TFs to *cis*-regulatory elements in the promoter region of their target genes is a key step to control their expression, and they can act as activators or repressors. To determine whether OsNAC300 binds to the native promoter that contains CATGTG motifs, ChIP-qPCR was used with the *35S::OsNAC300-GFP* line in vivo. Seven fragments, spanning different regions of the *OsCHS1*, *OsPR10a*, and *OsPR10b* promoters (P1 to P7), were selected for qPCR analysis (Figure 7C). As indicated in Figure 7D, the fragments that contain CATGTG motifs (P1 for *OsCHS1,* P3 and P5 for *OsPR10a*, and P7 for *OsPR10b*) were considerably enriched compared with the negative controls. These results demonstrated that OsNAC300 binds to regions that contain the CATGTG motifs, such as the *OsCHS1*, *OsPR10a*, and *OsPR10b* promoters, thus controlling their levels of expression in response to Cd stress.

4. Discussion

Kusano et al. [38] reported that *OsNAC300* was expressed in the root, shoot and flower, as well as in the mature phloem of vascular tissues at very early developmental stages, while via GUS staining and RT-qPCR we find that *OsNAC300* was mainly expressed in roots but was only found at low levels in leaves (Figure 2A–G). Some previous studies showed that plant NAC TFs have roles in growth and development processes, particularly under biotic or abiotic stress conditions [29,46,47]. To our knowledge, this is the first report that NAC TF (OsNAC300) is very highly expressed in roots and strongly induced under Cd stress (Figure 2A,H–J). Its loss-of-function mutants are hypersensitive to Cd stress (Figure 3). In the *OsNAC300*-OE lines, the levels of NAC300 expression were higher than in the wild-type and roots exhibited an enhancement of tolerance to Cd toxicity (Figure 4A,B).

However, the mutation or overexpression of *OsNAC300* did not affect the uptake and transport of Cd in rice (Figure 5C,D). This implies that under normal conditions or Cd stress, Cd uptake or efflux genes such as *OsNramp5* [48], *OsNramp1* [49], or *OsABCG36* [50] are not downstream of OsNAC300. Besides, increasing the content of sulfhydryl-rich compounds, such as GSH and phytochelatin-PC, to enhance the tolerance of plant cells to Cd stress is typically accompanied by an increase in the content of Cd [6,51]. This also results in a hypothesis that OsNAC300 is not likely to regulate the PC-GSH pathway. The transcriptome data also revealed that the PC-GSH pathway is not regulated by OsNAC300 (Supplemental Tables S4 and S5).

Via the RNA-Seq analysis of rice roots, we identified the differential expression of genes that were affected by Cd stress in the *osnac300* mutant compared with those in the wild-type plants (data are shown in Table 1). Although the expression of nutrition-related genes was inhibited by Cd stress, this process has nothing to do with the function of OsNAC300 (Supplemental Table S6, and Figure 8). This implies that OsNAC300 may improve the tolerance of rice to Cd stress by specifically regulating the expression of genes that encode resistance to stress. In this study, we found that four *OsPR10* genes were induced by Cd stress in the rice genome, namely *OsPR10a*, *OsPR10b*, *RSOsPR10,* and *JSOsPR10* (Table 1). Among these, *OsPR10a* was induced 500-fold, and *OsPR10b* was induced 25-fold (Figure 6B–E). Combined with the previous results, this study adds more detail to our knowledge that the *OsPR10* gene family responds largely to stress conditions, i.e., both abiotic and biotic stresses [12]. *PR10* genes might be used as a group of marker genes to show the degree of stress on the rice roots; this seems particularly likely for *OsPR10a*. Through gene knockout and RNA-Seq, we found that *OsPR10a*, *OsPR10b*, *RSOsPR10*, *OsPR5*, and *OsPR1-71* responded to Cd stress, via dependence on OsNAC300.

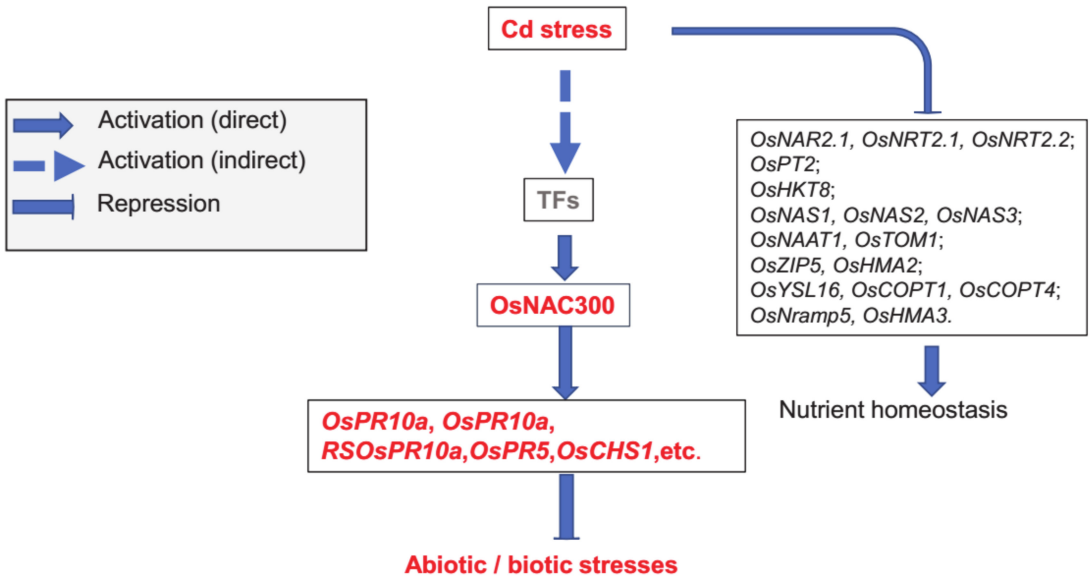

Figure 8. The OsNAC300-dependent Cd stress response regulatory pathway in rice roots. In rice, the Cd stress signal activates downstream gene expression through OsNAC300-dependent and OsNAC300-independent regulatory pathways. In the OsNAC300-dependent regulatory pathway, OsNAC300 directly binds to promoter regions containing CATGT of Cd stress response genes, including *OsPR10a*, *OsPR10b*, and *OsCHS1*, which respond to abiotic and biotic stress. On the other hand, the Cd stress signal also regulates other nutrient homeostasis (e.g., *OsNAR2.1*, *OsNRT2.1* and *OsNRT2.2*) that are not dependent on OsNAC300.

Additionally, confirmation of these findings was elaborated by performing a luciferase reporter assay and ChIP-qPCR experiments (as presented in Figure 7A–D). The ChIP-qPCR experiments demonstrated that OsNAC300 binds directly to the promoters of *OsPR10a* and *OsPR10b* (Figure 7D). This evidence indicates that *OsPR10a* and *OsPR10b* receive stress signals through OsNAC300. The loss of the *OsNAC300* function does not affect the response of its own promoter to Cd stress (Figure 6A). This implies that there are other transcription factors upstream of *OsNAC300* that regulate the expression of *OsNAC300* in response to stress signals. The OsNAC300 homolog in Arabidopsis is a TF, i.e., *AtNAC087*, which was found to be expressed downstream of the master regulator AtORE1 during leaf senescence [52], while AtORE1(ANAC092) is itself an NAC domain TF factor [53]. Future studies on such interacting NAC TFs or others (yet to be known) in rice will precisely explain the stress signaling and regulatory mechanisms of OsNAC300.

OsCHS1 is a rate-limiting enzyme in the process of isoflavone synthesis [54]. Isoflavones play an important role in the antioxidant processes of plant cells [55]. In RNA-Seq, we found that *OsCHS1* in the rice roots was significantly upregulated by Cd stress (Table 1 and Figure 6H). We hypothesize that the rice root cells may synthesize more isoflavones by increasing the expression of *OsCHS1*, thereby enhancing their antioxidant capacity. In Arabidopsis, it was hypothesized that NAC TF-ANAC078 was involved in inducing the genes of flavonoid biosynthesis under conditions of high light stress [56]. The results of RNA-Seq and RT-qPCR showed that *OsCHS1* is dependent on OsNAC300 in response to Cd stress. The ChIP-qPCR experiments enabled an observation that OsNAC300 binds directly to the *OsCHS1* promoters. This evidence indicates that *OsCHS1* receives stress signals through OsNAC300.

5. Conclusions

Taken together, we determined that OsNAC300 is a nuclear-localized TF that is highly expressed in the roots of rice. OsNAC300 plays an important role in the process of Cd stress tolerance in rice, and it directly regulates the levels of expression of *OsPR10a*, *OsPR10b*, and *OsCHS1* in response to Cd stress.

Overall, as summarized in Figure 8, we conclude that OsNAC300 is an important regulator under stress. OsNAC300 may transmit stress signals in the roots, which provides new insights into the link between Cd stress and other stress response genes. However, further study will reveal new molecular insights for NAC TF and enhance our understanding to develop stress-tolerant crops.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/1/95/s1. Figure S1. Phylogenetic analysis and multiple alignments of amino acid sequences of NAC family proteins in rice, *Arabidopsis*, tomato, maize. Supplemental Figure S2. Identification of *osnac300* mutants generated by CRISPR/Cas9. Supplemental Table S1. Primers used in this study. Supplemental Table S2. Genes in WT induced by Cd stress (4443 genes). Supplemental Table S3. Genes in WT repressed by Cd stress (3671 genes). Supplemental Table S4. Gene expressed lower in *osnac300-1* mutant than WT under control (137 genes). Supplemental Table S5. Genes expressed higher in *osnac300-1* mutant than WT under control (256 genes). Supplemental Table S6. Genes normally expressed in *osnac300-1* mutant under +Cd condition among Cd stress-induced genes (4227 genes). Supplemental Table S7. Genes expressed higher in *osnac300-1* mutant than WT under +Cd among Cd stress-induced genes (158 genes). Supplemental Table S8. Gene expressed lower in *osnac300-1* mutant than WT under +Cd among Cd stress-induced genes (58 genes). Supplemental Table S9. Motifs in 6 possible downstream genes of OsNAC300.

Author Contributions: L.Z., L.L., and S.H. conceived and designed the experiments; S.H., Y.S., K.I.S., H.X., and B.D. performed the experiments; collected, documented, and analyzed the data; S.H., and K.I.S. wrote the early draft; L.Z., J.X., and S.H. revised the manuscript, and all authors read and acknowledged the final form of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Illumina reads of all samples were deposited in the Sequence Read Archive at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra) under accession number PRJNA622262.

Conflicts of Interest: The authors affirm no conflicts of interests or any other concerns.

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