Title

The Transcription Factor OsNAC5 Mediates Cadmium Stress Response and Accumulation in Rice

Shubao Hu[a,1], Jinfen Chen[a,1], Hui Wang[c], E Ji[b], Xinxin Su[a], Muyao Zhu[d], Xiaoyan Xiang[a], Li Gong[a], Qiang Zhou[e], Xin Xiao[c], Ganlin Wu[a]

[a]Provincial Key Laboratory of the Biodiversity Study and Ecology Conservation in Southwest Anhui Province, Anqing Normal University, Anqing, China

[b]College of Life Sciences, Nanjing Agricultural University, Nanjing, China

[c]College of Resources and Environment, Anqing Normal University, Anqing, China

[d]College of Resources and Environment, Anhui Science And Technology University, Bengbu, China

[e]College of Biology and Environmental Sciences, Jishou University, Jishou, China

Corresponding author：Ganlin Wu

E-mail address: wugl@sina.cn

[1] Authors are equally contributed.

Running title: OsNAC5, a key regulator of Cd stress response in rice.

Abstract: Cadmium (Cd) is a toxic heavy metal that endangers human health via contaminated rice consumption. Reduction Cd uptake in rice is essential for minimizing grain Cd accumulation. However, the transcriptional regulatory of Cd uptake in rices remains largely elusive. This study reveals that the transcription factor OsNAC5 in *Oryza sativa* positively regulates the Cd transporter gene *OsNRAMP1*, influencing Cd uptake. OsNAC5 is primarily expressed in roots, localized to the nucleus and is upregulated by Cd induced H_2O_2. *OsNAC5* knockout reduces Cd levels in both shoots and roots and increases Cd sensitivity. *OsNRAMP1* expression, enhanced by Cd stress, dependents on OsNAC5, which binds to CATGTG motifs in the *OsNRAMP1* promoters activating its expression. Loss of *OsNAC5* function decreases Cd accumulation in rice grains. Our findings elucidate the transcriptional regulation of Cd stress response in rice and suggest biotechnological approaches to reduce Cd uptake in crops.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Keywords: Cadmium; Oryza sativa; OsNAC5; OsNRAMP1; Cd uptake

1. **Introduction**

Cadmium (Cd) is a highly toxic heavy metal that poses a threat to human health, as it can cause kidney damage and increase the risk of cancer (Nordberg, 2009). Environmental contamination with Cd arises from various anthropogenic activities, including mining, the use of Cd-containing fertilizers, and the application of sewage sludge (Zhao et al., 2015). Rice, a staple crop in many Asian countries, exhibits higher Cd uptake and accumulation compared to other cereals such as wheat and maize (Sui et al., 2018). The current levels of Cd exposure in most populations exceed the threshold for adverse health effects, underscoring the urgent need for strategies to mitigate Cd transfer from soil to edible plant parts and protect human health (Chen et al., 2018).

Recent advances have been made in identifying key transporters involved in Cd uptake and translocation in plants (Clemens and Ma, 2016). The natural resistance-associated macrophage protein (NRAMP) family, particularly OsNRAMP5, has been identified as a major influx transporter for manganese (Mn), Cd and Lead (Pb) in rice (Chang et al., 2022; Ishikawa et al., 2012; Ishimaru et al., 2012; Sasaki et al., 2012; Yang et al., 2014). OsNRAMP5 is localized at the plasma membrane of the exodermis and endodermis in the rice roots, facilitating the uptake of these metals (Chang et al., 2022; Sasaki et al., 2012). However, mutations in OsNRAMP5, while effective in restricting Cd uptake, result in compromised growth and grain yield under Mn-limiting conditions (Sasaki et al., 2012; Yang et al., 2014). Conversely, overexpression of *OsNRAMP5* has been shown to decrease Cd accumulation in the shoots and grains by disrupting radial Cd transport into the stele (Chang et al., 2020). OsNRAMP1, another member of the NRAMP family, shares high amino acid sequence similarity with OsNRAMP5 and has been implicated in Mn and Cd uptake in rice (Chang et al., 2020; Takahashi et al., 2011). Both OsNRAMP1 and OsNRAMP5 contribute to metal uptake, although their functions are not completely redundant (Chang et al., 2020; Takahashi et al., 2011). Once internalized, Cd is transported to vacuoles by OsHMA3, a P-type heavy metal ATPase, or translocated to shoots via OsHMA2 (Ueno et al., 2010). Despite the understanding of these transporters' roles, the transcriptional regulatory mechanisms controlling their expression are not well characterized.

Transcription factors (TFs) are pivotal in modulating gene expression in response to Cd stress (Lin et al., 2017). Several TFs, including AtWRKY12 (Han et al., 2019), AtWRKY13 (Sheng et al., 2019; Zhang et al., 2020), AtWRKY45 (Li et al., 2023), AtZAT6 (Chen et al., 2016), and AtMYB4

(Agarwal et al., 2020), AtMYB49 (Zhang et al., 2019), and AtNAC102 (Han et al., 2023) in *Arabidopsis*, as well as OsHsfA4a (Shim et al., 2009), OsMYB45(Hu et al., 2017), and OsNAC300(Hu et al., 2021) in rice have been identified as important regulators of Cd tolerance by controlling the expression of Cd-detoxification genes(Lin et al., 2017). However, the transcriptional regulatory mechanisms controlling Cd accumulation in crops remain poorly understood.

Previous studies have emphasized the critical role of *OsNAC5* (*ONAC009/ONAC071*) in mediating stress responses in rice (Takasaki et al., 2010; Jeong et al., 2013; Li et al., 2023; Song et al., 2011). *OsNAC5* is known to be induced by a variety of abiotic stresses, including drought, cold, high salinity, and exposure to abscisic acid (ABA) and methyl jasmonic acid, and it is localizes to the nucleus. Overexpression of *OsNAC5* in transgenic plants has been demonstrated to confer enhanced tolerance to high salinity (Takasaki et al., 2010). As a transcriptional activator, *OsNAC5* bolsters stress tolerance by upregulating the expression of stress-responsive genes in rice, such as late embryogenesis abundant 3 (*OsLEA3*) (Takasaki et al., 2010). Song et al. (2011) reported that *OsNAC5* plays a significant roles in enhancing tolerance to abiotic stress in rice by modulating downstream targets associated with the accumulation of compatible solutes, sodium ions (Na^+), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA). Jeong et al. (2013) showed that root-specific overexpression of *OsNAC5* leads to substantial root enlargement, which in turn improves drought tolerance and increases grain yield under field conditions. Additionally, a recent study has elucidated the role of OsNAC5 in coordinating with OsABI5 to finetuning cold tolerance in rice (Li et al., 2023). Furthermore, overexpression of *OsNAC5* has shown to result in increased iron (Fe) concentrations in seeds compared to wild-type plants (Wairich et al., 2023). However, the roles of OsNAC5 in Cd tolerance and accumulation has yet to be fully explored.

In this study, we aimed to investigate the role of OsNAC5 in the regulation Cd tolerance and uptake in rice. Through analysis of its expression pattern, subcellular localization, phenotypic response, Cd concentration, we provide compelling evidence that OsNAC5 significantly influences Cd tolerance and accumulation in rice. Moreover, we demonstrate that OsNAC5 directly interacts with "CATGTG" motifs in the promoters of *OsNRAMP1*, promoting its expression under Cd stress conditions.

2. Materials and Methods

2.1. Generation of rice genetic materials and plant growth conditions

Generation of The *osnac5* Knockout Line: The *osnac5* knockout line was generated using the CRISPR/Cas9 genome editing system in the 'ZH11' rice cultivar background. A specific guide RNA (gRNA) sequence (297–319 bp; see Supplementary Tab. S1) targeting the *OsNAC5* gene was designed and cloned into the pRGEB31 CRISPR/Cas9 vector (Addgene, Watertown, MA, USA) (Xie and Yang, 2013). To minimize off-target effects, target sequences with highly specificity were identified through a comprehensive genome-wide search (www.genome.arizona.edu/crispr/) (Xie et al., 2014). Agrobacterium tumefaciens-mediated transformation was performed on actively dividing calli derived from mature rice embryos (Nishimura et al., 2006). Homozygous mutant lines were confirmed by sequencing PCR-amplified genomic fragments from putative mutants.

Construction of Genetic Complementation Lines: For the genetic complementation of the *osnac5* mutant, a genomic fragment encompassing the *OsNAC5* promoter (1011 bp upstream of the start codon) and codon sequence (990 bp starting from ‘ATG’) was PCR-amplified from rice root DNA. This fragment was subsequently cloned into the binary vector pCAMBIA1300□HA. The primers used for amplification are listed in Supplementary Tab. S1. The resulting construct, Pro*OsNAC5*::*OsNAC5*□HA, was introduced into the *osnac5* mutant plants (free of selection marker) via Agrobacterium□mediated transformation. Two independent complementation lines (ProOsNAC5::*OsNAC5*□HA, T3 generation) were selected based on immunoblotting analysis using anti-HA antibody. These lines used for further phenotypic characterization and chromatin immunoprecipitation (ChIP) assays.

Growth Conditions and Nutrient Solution: Seeds of both wild-type 'ZH11' and *osnac5* mutant rice were initially soaked in water for 48 hours, followed by germination in a 0.5 mM $CaCl_2$ solution. After five days, seedlings were transferred to a half-strength Kimura B solution (pH 5.5) and grown in a greenhouse with controled conditions at 25—30□. The nutrient solution was as composed of 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 100 μM Fe(III)-EDTA. This solution was refreshed every two days to ensure optimal nutrient availability.

2.2. *Subcellular localization*

A cDNA fragment encoding OsNAC5 was amplified from a rice cDNA library using *OsNAC5*-specific primers (listed in Supplementary Tab. S1.). The PCR product was cloned into the pBluescript SK(+) vector, which harbors the 35S cauliflower mosaic virus (CaMV) promoter, to drive constitutive expression. For subcellular localization studies, the *OsNAC5* coding sequence was fused in-frame with the coding sequence of Yellow Fluorescent Protein (YFP) fusion protein to generate an OsNAC5-YFP fusion construct. This expression cassette was then introduced into rice protoplasts using the polyethylene glycol (PEG) 3350/Ca^{2+} transfection method (Shi et al., 2014). Following transfection, the rice protoplasts were incubated in the dark at 25°C for 12–18 hours to allow for expression of the fusion protein. After the incubation period, the rice protoplasts expressing the OsNAC5-YFP fusion protein were examined for fluorescence localization. Images were captured using a laser confocal microscope (UltraVIEW VOX, PerkinElmer, Waltham, MA, USA).

2.3. Gene expression analysis

To elucidate the tissue-specific expression pattern of *OsNAC5* in wild-type (WT) rice, samples were harvested from various tissues, including roots, basal stems, mature leaf blades, mature leaf sheaths, young leaf blades, young leaf sheaths, of two-week-old seedlings. To evaluate the expression of *OsNAC5* under Cadmium Nitrate ($Cd(NO_3)_2$) stress, WT rice seedlings at the same developmental stage were subjected to a gradient of $Cd(NO_3)_2$ concentration (0 to 100 μM) and to hydrogen peroxide (H_2O_2, 10 mM) for predetermined time periods. Furthermore, to investigate the expression profiles of Cd transporter genes (*OsNRAMP1*, *OsNRAMP5*, *OsHMA2* and *OsHMA3*), as well as other potential downstream functional genes (*OsLEA3*, *OsNAS3* and *OsYSL2*) of *OsNAC5*, 2-week-old WT rice seedlings were exposed to 10 μM $Cd(NO_3)_2$ for a duration of 12 hours.

For RNA extraction, total RNA was isolated from the treated rice tissues utilizing a Fastpure Plant Total RNA Isolation Mini Kit (Product code: RC401, Vazyme, Nanjing, China). First-strand cDNA synthesis was carried out employing the HiScript II 1st Strand cDNA Synthesis Kit (Product code: R211-01, Vazyme) with 1 μg of the extracted total RNA as a template. RT-qPCR analysis was performed using the ChamQ SYBR Color qPCR Master Mix (Product code: Q311-02, Vazyme), with *Actin* being utilized as the internal control for normalization. Primers sequences for gene expression analysis are detailed in Supplementary Tab. S1.

2.4. Phenotypic analysis

To evaluate the impact of Cd stress on root elongation, seeds from the WT rice, the *osnac5* mutant line, and two independent *osnac5* complementation lines were germinated. Post-germination, seedlings transferred to nets floating on a solution in 4 L containers. The control solution containing 0.5 mM $CaCl_2$ (-Cd group), while the treatment solution comprised 0.5 mM $CaCl_2$ supplemented with 5 μM $Cd(NO_3)_2$ (+Cd group). The containers were incubated at 30 ℃ for a period of three days. Root length measurements were taken at 0, 24, 48 and 72 hours subsequent to the initiation of Cd exposure. For each rice line and Cd treatment condition, eight biological replicates were conducted.

2.5. Soil pot experiment

A soil sample contaminated with Cd was collected from a paddy field located in Xiangtan, Hunan Province, China, exhibiting a total Cd content of 1.13 mg/kg. The collected soil was air-dried, ground, and passed through a 50-mesh sieve to ensure homogeneity. Subsequently, the sieved soil was uniformly blended with fertilizers at a ratio of 0.3 g KH_2PO_4 and 0.5 g $(NH_4)_2SO_4$ per kilogram of soil in a single mixing step. Plastic buckets, each with a 22-liter capacity, were filled with 17 kg of the prepared soil-fertilizer mixture. Tap water was added to each bucket to submerge the soil, which was then allowed to soak for a period of 7 days. The pH of the soil was determined to be approximately 5.7. One seedling from each of the following rice lines: WT plants, the *osnac5* mutant and two *osnac5* complementation lines, were transplanted into individual plastic buckets. Throughout the vegetative growth phase, the plants were irrigated twice daily to maintain a water level of 1—3 cm above the soil surface. During the rice grain-filling stage, the water was drained from the buckets. Upon reaching grain maturity, the plants were harvested. The biomass and metal concentrations in the grains were determined.

2.6. Elemental Concentration Measurement

To evaluate the translocation efficiency of Cd from roots to shoots, 14-day-old plants of WT, the *osnac5* mutant line and two *osnac5* complemental lines were subjected to 0.1, 1, or 5 μM $Cd(NO_3)_2$ for a duration of 7 days. Post-exposure, the roots were meticulously washed twice with 5 mM $CaCl_2$ to remove surface-bound Cd, followed by a thorough rinse with deionized water. The roots and shoots were then separated, harvested and oven-dried at 75 □ for 5 days to achieve a constant dry weight. For

the determination of metal element content, the dried plant tissues were digested using a mixed acid protocol. Each sample, consisted of a minimum of 0.1 g of dry tissue, was digested in a heating block with a mixture of perchloric acid ($HClO_4$) and nitric acid (HNO_3) in a 13:87 (v/v) ratio. The digestion process involved a temperature gradient: the samples were heated at 110 □ for 2 hours, following by 140 □ for 1.5 hours, then 160 □ for 0.5 hours and finally maintained at 180 □ until complete dryness was achieved. After digestion, the residue was dissolved in 2.5% HNO_3 for analysis. The concentration of metal element in the digested samples was quantified using inductively coupled plasma mass spectrometry (ICP-MS, Perkin-Elmer NexION 300x, Waltham, MA, USA).

2.7. Chromatin Immunoprecipitation Quantitative PCR assay (ChIP-qPCR)

ChIP□qPCR assays were performed using a commercial chromatin immuno□precipitation Kit (Merck Millipore) in accordance with the method described by Hu et al. (2021). In brief, 5 g of fresh roots harvested from OsNAC5Pro::NAC5-HA transgenic seedlings were subjected to cross-linking with 1.2% formaldehyde under vacuum for 8 minutes. Subsequent to fixation, chromatin was extracted and sonicated to generate fragments ranging from 200 to 1000 base pairs (bp) in length, as per the manufacturer's instructions.

A total of 100 μL of the sonicated chromatin solution was then immunoprecipitated with 5 μL of anti□HA antibody (Santa Cruz Biotechnology) under gentle agitation at 50 rpm and 4 °C overnight. The immunocomplexes were processed to release and purify the DNA fragments associated with the OsNAC5 protein, which were then utilized as templates for qPCR analysis. Genomic fragments from regions not expected to bind OsNAC5 served as negative controls. Quantification of the relative abundance of target DNA sequences was performed using the $^{\Delta\Delta}C_t$ (cycle threshold) method. *Actin* was employed as the internal reference gene, with its expression in the 'no antibody' (noAB) samples providing the baseline for normalization.

2.8. Electrophoretic mobility shift assay (EMSA)

The *OsNAC5* gene was PCR-amplified from ‘ZH11’ cDNA using Phanta Super-Fidelity DNA Polymerase (Vazyme Biotech Co., Ltd) . The resulting PCR product was cloned into the pGEX-4T-1 vector utilizing ClonExpress II One Step Cloning Kit (Vazyme) according to the manufacturer's instructions. The recombinant GST-OsNAC5 fusion protein was subsequently purified with the

MagneGST™ Protein Purification System (Promega), following the provided protocol.

For EMSA, a DNA fragment encompassing the "CATGTG" motif within the *OsNramp1* promoter was synthesized and labeled at 5′ end with biotin. Additionally, an unlabeled version of the same DNA fragment was synthesized to function as a competitor in the binding assay. EMSA assay was conducted in accordance with the procedure outlined by (Hu et al., 2024). The sequences of the probes used for EMSA are detailed in Supplementary Tab. S1.

2.9. Gene numbers

OsNAC5 (LOC_Os11g08210), *Actin* (LOC_Os03g50885), *OsNRAMP1* (LOC_Os07g15460), *OsNRAMP5* (LOC_Os07g15370), *OsHMA2* (LOC_Os06g48720), *OsHMA3* (LOC_Os07g12900) , *OsLEA3* (LOC_Os05g46480), *OsYSL2* (LOC_Os02g43370), and *OsNAS3* (LOC_Os07g48980).

3. Results

3.1. Sequence and subcellular localization of Analysis of OsNAC5

The full-length open reading frame (ORF) of *OsNAC5* was successfully cloned from rice root cDNA and found to be consistent with the sequence reported in the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp). Structural analysis of the *OsNAC5* gene revealed that comprises three exons and two introns. The gene encodes a protein of 329 amino acids with a predicated molecular mass of 35.53 kDa. Phylogenetic analysis positioned OsNAC5 within the rice NAC TF family, featuring a conserved NAM domain implicated in DNA binding (Supplementary Fig. S1A). Within this family, OsNAC6 emerged as the closest relative to OsNAC5, exhibiting 65.23% sequence identity (Supplementary Fig. S1B). Previous studies have documented that *OsNAC6* is responsive to various abiotic and biotic stressors, including drought, abscisic acid (ABA) and jasmonic acid (JA) (Nakashima et al., 2007). Moreover, overexpression of *OsNAC6* has been demonstrated to confer enhanced tolerance to drought and high salinity in rice (Lee et al., 2017; Rachmat et al., 2014).

Bioinformatic analysis using the ProtComp 9.0 program (http://www.softberry.com/) predicted that OsNAC5 is a nuclear protein, possessing a nuclear localization signal (NLS) peptide. To experimentally confirm the nuclear localization of OsNAC5, a transient expression assay was performed in rice protoplasts using an OsNAC5-YFP (yellow fluorescent protein) fusion construct.

Confocal laser scanning microscopy revealed that the fluorescence emitted by the YFP-tagged OsNAC5 overlapped with the nuclear localization marker, red fluorescent protein-tagged Histone 2A (RFP-H2A),as reported by (Hu et al., 2017). In contrast, protoplasts expressing YFP alone displayed yellow fluorescence throughout the cell (Fig. 1). These observation are in agreement with a previous study by Takasaki et al., (2010), which showed nuclear localization of OsNAC5-GFP (green fluorescent protein) fusion protein in rice roots harboring OsNAC5pro::OsNAC5-GFP construct. Taken together, these results robustly support the conclusion that OsNAC5 is localized to the nucleus.

3.2. Expression pattern of OsNAC5

The expression profile of *OsNAC5* was assessed by RT-qPCR across various tissues of rice plants (cv. ZH11) cultivated in a hydroponic nutrient solution. The analysis revealed that *OsNAC5* expression was predominantly detected in the roots (Fig. 2A). This finding contrasts with a report by Song et al. (2011), which indicated that *OsNAC5* was mainly expressed in the leaves of cv. Zhonghua10, suggesting cultivar-specific expression patterns for OsNAC5.

To elucidate the response of *OsNAC5* to Cd or hydrogen peroxide (H_2O_2) stress, hydroponic experiments were performed. Exposure to Cd resulted in a dose-dependent induction of *OsNAC5* expression in rice roots (Fig. 2B). A time-course analysis of Cd-treated roots showed a rapid increase in *OsNAC5* transcript levels, peaking at 24 hours post-treatment (Fig. 2C). Previous studies have demonstrated that *OsNAC5* is inducible by various abiotic stresses, including drought, high salinity, and cold (Takasaki et al., 2010; Jeong et al., 2013; Song et al., 2011). Given the role of H_2O_2 as a key signaling molecule (Mittler et al., 2011; Ni et al., 2019), we further examined its effect on *OsNAC5* expression. The results indicated a swift up-regulation of *OsNAC5* following H_2O_2 treatment, with a peak expression level observed at 12 hours (Fig. 2D), suggesting a more rapid response to H_2O_2 than to Cd stress.

To investigate the potential involvement of H_2O_2 in the signaling pathway of Cd-induced *OsNAC5* expression, rice plants were pre-treated with two reactive oxygen species (ROS) modulators, dimethylthiourea (DMTU), a scavenger for H_2O_2, and diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, prior to Cd exposure (Zhang et al., 2014). The pre-treatments with these two ROS modulators significantly attenuated the Cd-induced up-regulation of *OsNAC5* expression

(Supplementary Fig. S2), indicating that H_2O_2 produced in cell is a essential mediator for the Cd-evoked increase in *OsNAC5* transcript levels.

3.3. Knockout of OsNAC5 results in a Cd-sensitive phenotype in rice

To elucidate the physiological role of *OsNAC5* in Cd stress response and tolerance in rice, we utilized the CRISPR/Cas9 system to generate an *osnac5* mutant line in the cv. ZH11 background. Sanger sequencing confirmed the homozygous *osnac5* mutant line, revealing a frameshift mutation due to a 1 bp (thymine base) insertion at the target gRNA site (Supplementary Fig. S3). To validate the function role of OsNAC5, we performed a genetic complementation assay by introducing the genomic sequence of *OsNAC5* (Pro*NAC5*::*NAC5*□HA) into the *osnac5* mutant. This resulted in the creation of two complementation lines (C1 and C2), which were verified by immunoblotting analysis using anti-HA antibody, setting the stage for subsequent phenotypic analysis and chromatin immunoprecipitation (ChIP) assay (Supplementary Fig. S4).

Under control growth conditions, the root length of the *osnac5* mutant seedlings was comparable to that of WT seedlings (Fig. 3A). However, when upon exposure to 5 μM Cd for 3 days, the root length of the mutant seedlings was significantly reduced relative to the WT seedlings (Fig. 3B). Notably, both complementation lines exhibited root lengths similar to that of the WT under both control conditions and Cd stress (Fig. 3B). The daily root elongation rate for all four rice lines was approximately 25 mm under control conditions. Under Cd stress, the daily root elongation was markedly inhibited on the first day across all lines (Fig. 3C). On the second and third days of Cd exposure, the *osnac5* mutant line showed a more pronounced inhibition of root elongation compared to the WT and complementation lines (Fig. 3C). Following 3 days of Cd treatment, the WT rice showed a relative inhibition rate of root elongation of 36.5% compared to the non-Cd-treated control, while the *osnac5* mutant line exhibited a higher inhibition rate of 57.1% (Fig. 3D). These results provide compelling evidence for the critical role of OsNAC5 in mediating the response of rice roots to Cd stress, with its disruption leading to increased sensitivity to Cd stress in rice.

3.4 Knockout of OsNAC5 decreased Cd uptake in rice

To explore the role of OsNAC5 in mediating Cd accumulation in rice, two-week-old plants of WT, the *osnac5* mutant, two *osnac5* complementation lines C1 and C2 were subjected to 0.1, 1 or 5 μM

$Cd(NO_3)_2$ for 7 days. At Cd concentration of 0.1 μM, the *osnac5* mutant plants showed a marginal decrease in Cd accumulation in both the roots and shoots compared to the WT and complementation lines, however, this reduction was not statistically significant (P>0.05; Fig. 4A, B). In contrast, at 1 μM Cd exposure, the *osnac5* mutant plants exhibited a significant reduction in Cd concentration by 15.6% in roots and 15.2% in shoots relative to the WT and two complementation lines (both $P < 0.05$; Fig. 4A, B). This trend persisted at the higher concentration of 5 μM Cd, with the *osnac5* mutants displaying a significant decrease of 15.2% in root Cd concentration and 15.1% in shoot Cd concentration compared to the WT and complementation lines (both $P < 0.05$; Fig. 4A, B).

Conversely, the Cd concentrations in the shoot and root tissues of the complementation lines did not differ significantly from those of the WT under any of the tested Cd treatments (Fig. 4A, B). Additionally, the efficiency of Cd translocation from roots to shoots did not show significant variation among the WT, the *osnac5* mutant, and complementation lines under three Cd treatment conditions (Fig. 4C). These results indicate that the *osnac5* knockout may lead to reduced Cd uptake in rice without significantly altering the translocation of Cd from roots to shoots.

3.5 OsNRAMP1 is induced by Cd stress dependent on OsNAC5.

To further examine the potential link between OsNAC5 knockout and Cd accumulation in rice, we performed real-time qPCR analysis of these Cd transporter genes. After 12 hours of exposure to 10 μM Cd, the expression of *OsNRAMP1* in WT rice roots was significantly upregulated, showing an approximate 5.6-fold increase compared to the untreated control (Fig. 5A). In contrast, the *osnac5* mutant did not exhibit any change in *OsNRAMP1* expression levels upon Cd treatment (Fig. 5A). The complementation lines C1 and C2, however, showed a strong induction of *OsNRAMP1* expression, mirroring the response seen in WT rice (Fig. 5A).

Under the same Cd exposure, the expression levels of *OsNRAMP5* and *OsHMA3* in WT rice roots were significantly downregulated to about 0.5-fold of the control, whereas *OsHMA2* expression remained unchanged (Fig. 5B–D). Notably, there were no significant differences in the expression levels of *OsNRAMP5*, *OsHMA2*, and *OsHMA3* between the WT, the *osnac5* mutant, and complementation lines (Fig. 5B–D). Additionally, under control conditions, the transcript levels of these four Cd transport-related genes were similar across the roots of WT, the *osnac5* mutant, and

complementation lines (Fig. 5A–D). These observations suggest that of the four Cd transporters analyzed, only *OsNRAMP1* expression is induced by Cd stress in a manner dependent on the functional presence of OsNAC5.

We also assessed whether the *OsNAC5* knockout would alter the gene's own expression response to Cd stress. Real-time qPCR analysis indicated that under Cd stress, the expression level of *OsNAC5* in the *osnac5* mutant was similar to that in WT rice (Supplementary Fig. S5A). Given that the reduced Cd accumulation in the *osnac5* mutant does not account for its increased sensitivity to Cd stress, it is postulated that *OsNAC5* may regulate the expression of other stress-related genes. The "late embryogenesis abundant" gene *OsLEA3*, known to be directly upregulated by OsNAC5 and involved in various stress responses (Takasaki et al., 2010), was found to be strongly induced by Cd stress in an OsNAC5-dependent manner (Supplementary Fig. S5B). This may partly explain the *osnac5* mutant's heightened sensitivity to Cd stress. Furthermore, Jeong et al. (2013) showed that overexpression of *OsNAC5* enhances the expression of genes involved in the homeostasis of divalent metals, such as *OsNAS3* and *OsYSL2*. In our study, *OsNAS3* expression was suppressed by Cd stress but was unaffected by the *OsNAC5* knockout (Supplementary Fig. S5C), while *OsYSL2* expression was not influenced by either Cd stress or OsNAC5 status (Supplementary Fig. S5C). These findings suggest that *OsNAC5*'s biological function is multifaceted, encompassing the regulation of Cd transport as well as broader stress response mechanisms.

3.6 OsNAC5 binds directly to the promoter of OsNramp1

The interaction of transcription factors (TFs) with cis-regulatory elements in gene promoters is essential for the regulation gene expression. OsNAC300 has been shown to have a strong affinity for the NAC transcription factor binding element motif (CATGTG) (Hu et al., 2021). Analysis of the approximately 1.0 kb promoter region of *OsNRAMP1* revealed the presence of two "CATGTG" motifs located at positions -846 to -841 bp and -131 to -126 bp upstream of the transcription start site (Fig. 6A). To determine whether OsNAC5 directly interacts with these motifs in the *OsNRAMP1* promoter *in vivo*, ChIP-qPCR assays were performed using the ProOsNAC5::OsNAC5-HA transgenic rice line and an anti-HA monoclonal antibody. Enrichment of specific amplicons (S1, S2, and S3) within the promoter region was observed. Upon Cd exposure, the fragments (S1 and S3) containing the

"CATGTG" motif exhibited significant enrichment compared to the negative controls, whereas no notable enrichment was detected under control conditions without Cd treatment (Fig. 6B).

To further confirm the binding affinity of OsNAC5 to the "CATGTG" motif, EMSA were conducted using bacterially expressed, recombinant purified GST-OsNAC5 fusion protein and synthetic oligonucleotides corresponding to the "CATGTG" motif from the *OsNRAMP1* promoter. The EMSA results demonstrated a strong DNA binding complex with oligonucleotides from the *OsNRAMP1* promoter, confirming the specific *in vitro* interaction of OsNAC5 with the "CATGTG" motif (Fig. 6C). Collectively, these findings indicate that OsNAC5 directly binds to regions containing the "CATGTG" motifs within the *OsNRAMP1* promoter, modulating its expression in response to Cd stress.

3.7 Knockout of OsNAC5 reduced Cd accumulation in rice grain

To assess the impact of *OsNAC5* knockout on Cd accumulation in rice grains (unpolished brown rice), a pot experiment was conducted with the WT, the *osnac5* mutant, and two complementation lines (C1 and C2) grown in Cd-contaminated paddy soil (total Cd concentration of 1.13 mg/kg, pH 5.77). Upon reaching maturity, no significant differences were observed among the four lines in terms of plant height, effective panicle number per plant, seed-setting rate, or grain yield per plant (Supplementary Tab. S2). However, the *osnac5* mutant displayed a marked reduction in Cd concentrations, with a 39.4% decrease in grains and a 41.8% decrease in husks compared to the WT (Fig. 7). In contrast, the Cd concentrations in the grains and husks of the two complementation lines were similar to those observed in the WT (Fig. 7).

Given that OsNRAMP1 is also known to transport Mn (Chang et al., 2020), we measured Mn concentrations in rice grains and detected no significant differences among the four rice lines (Supplementary Fig. S6A). Recent studies have indicated that overexpression of the *OsNAC5* gene enhances the translocation of Fe and Zn from vegetative tissues to seeds (Wairich et al., 2023). Consequently, we measured the concentrations of Fe and Zn in rice grains and found no significant differences among the four lines (Supplementary Fig. S6B, C). These results are consistent with the notion that Fe, Zn, and Mn are essential micronutrients, and plants have developed intricate regulatory mechanisms to maintain their homeostasis, avoiding both deficiency and toxicity.

4.Discussion：

Previous research has established that *OsNAC5* expression is induced by a variety of adverse environmental stressors, including drought, cold, high salinity, abscisic acid (ABA), and methyl jasmonate (Takasaki et al. 2010; Song et al., 2011). In line with these observations, our study demonstrates that *OsNAC5* is also responsive to heavy metal Cd and H_2O_2. Intriguingly, the response to H_2O_2 was observed as early as three hours post-treatment, whereas the response to Cd stress was significantly delayed in comparison to H_2O_2 (Fig. 2B-D). Moreover, we have shown that the induction of *OsNAC5* by Cd stress is contingent upon intracellular H_2O_2 production (Supplementary Fig. S2). These results imply that OsNAC5 may be implicated in a broad spectrum of biotic and abiotic stress responses, although its specific role in biotic stress remains to be elucidated.

Previous work has shown that overexpression of *OsNAC5* under the maize ubiquitin promoter significantly enhances salt tolerance in rice (Takasaki et al., 2010). Conversely, rice lines with reduced *OsNAC5* expression through RNA interference (RNAi) display decreased tolerance to cold, drought, and high salinity, while overexpression of *OsNAC5* in rice or *Arabidopsis* confers increased resistance to these stressors (Song et al., 2010). Additionally, driving *OsNAC5* expression in rice roots with the root-specific promoter RCc3 leads to increased root diameter, improved drought tolerance, and higher yield (Jeong et al., 2013). In our study, we utilized CRISPR-Cas9 technology to generate an *osnac5* mutant and found that this mutant exhibited increased sensitivity to Cd stress compared to WT plants (Fig. 3B, D). However, no phenotypic differences were detected between the *osnac5* mutant and WT plants under normal growth conditions (Fig. 3A, D).

OsNRAMP1 and OsNRAMP5 are two critical transporters involved in Cd uptake, with OsNRAMP5 playing a dominant role and OsNRAMP1 a subsidiary role, and their functions are non-redundant (Chang et al., 2020; Sasaki et al., 2012). The transcriptional regulation of OsNRAMP5 and OsNRAMP1 also differs, with *OsNRAMP1* expression being strongly induced by Cd stress, while *OsNRAMP5* expression is suppressed (Fig. 5A, B). Both *OsNAC5* and *OsNRAMP1* are induced by Cd stress (Fig. 2B, 5A). Notably, in the *osnac5* mutant, *OsNRAMP1* does not respond to Cd stress induction (Fig. 5A). However, in the complementation lines, *OsNRAMP1* expression is restored to WT levels (Fig. 5A). Interestingly, the loss of *OsNAC5* function does not influence the extent of Cd

stress-induced suppression of *OsNRAMP5* expression (Fig. 5B). Considering the established transcriptional activation function of the OsNAC5 protein (Takasaki et al., 2010), our findings indicate that OsNAC5 exerts a distinct positive transcriptional regulatory influence on *OsNRAMP1* in response to Cd stress, while it does not appear to regulate *OsNRAMP5* in the same manner. This specificity underscores the nuanced role of OsNAC5 in the plant's adaptive response to heavy metal stress, selectively modulating the expression of key transporters involved in Cd uptake.

Moreover, our observations revealed that under non-stress conditions (0.1 μM Cd, serving as the control), the loss of OsNAC5 function does not affect the Cd concentration in the roots and shoots of rice plants (Fig. 4A, B). Correspondingly, the expression level of *OsNRAMP1* is not impacted by the loss of OsNAC5 function under these conditions (Fig. 5A). These findings lend further credence to the hypothesis that OsNAC5 specifically upregulates *OsNRAMP1* expression under conditions of Cd stress. Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) experiments substantiated that OsNAC5 interacts with the NAC binding elements in the *OsNRAMP1* promoter exclusively in the presence of Cd stress *in vivo* (Fig. 6B). Consequently, we propose that OsNAC5 directly associates with the *OsNRAMP1* promoter to positively influence its expression during Cd stress, highlighting a targeted regulatory mechanism activated by heavy metal exposure (Fig. 8).

OsHMA2, a plasma membrane transporter for Cd, is localized in the pericycle of roots and is constitutively expressed during the vegetative stage (Yamaji et al., 2013). Loss-of-function mutations in OsHMA2 lead to diminished translocation of Cd from roots to shoots, as previously reported (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013). Our study indicates that the expression of *OsHMA2* is minimally influenced by Cd stress and does not appear to be associated with the function of *OsNAC5* (Fig. 4C). OsHMA3, located in the tonoplast of all root cells, has been shown to have its expression modestly reduced by Cd in a cultivar-dependent manner (Ueno et al., 2010; Yan et al., 2016). In line with these findings, we observed that Cd stress suppresses the expression of *OsHMA3*, but this suppression is independent of *OsNAC5* function (Fig. 4D).

Recent molecular biology strategies have aimed to mitigate Cd accumulation in rice grains. CRISPR/Cas9-mediated knockout of *OsNRAMP5* in both japonica and indica rice varieties has been demonstrated to significantly lower Cd accumulation in seeds (Tang et al., 2017; Yang et al., 2019).

Overexpression of *OsNRAMP5* has been shown to disrupt radial Cd transport into the stele, thereby reducing Cd accumulation in rice grains (Chang et al., 2020). Similarly, CRISPR/Cas9-mediated knockout of *OsNRAMP1* in japonica rice (ZH11) has been effective in significantly reducing Cd content in both grains and husks (Chang et al., 2020). Alterations in *OsHMA2* expression have also been reported to significantly decrease Cd levels in rice seeds (Takahashi et al., 2012). Moreover, overexpression of *OsHMA3a* from japonica rice can reduce grain Cd content by over 90% without compromising essential nutrient levels such as Zn and Fe (Ueno et al., 2010). In our research, we found that knockout of the transcription factor *OsNAC5* similarly results in reduced Cd content in japonica rice grains (ZH11) (Fig. 7). Unlike the transporters previously mentioned, transcription factors such as OsNAC5 do not directly mediate Cd uptake or transport. This discovery provides an alternative molecular biology strategy to regulate Cd content in rice grains, expanding the toolkit for food safety.

4. Conclusion

In addition to its established roles in mediating responses to drought, cold, high salinity, and ABA, our research has unveiled a novel function of the rice transcription factor OsNAC5 in responding to Cd stress. We have demonstrated that OsNAC5 is pivotal in modulating rice's response to Cd stress and its subsequent accumulation in plant tissues. The expression of the *OsNAC5* gene is upregulated in the presence of Cd stress, a response that is contingent upon the production of H_2O_2. Notably, OsNAC5 specifically interacts with the "CATGTG" motifs within the promoter region of *OsNRAMP1*, thereby orchestrating its expression in response to Cd exposure (Fig. 8). Crucially, the knockout of *OsNAC5* leads to a reduction in Cd accumulation in rice grains, underscoring its potential utility in mitigating heavy metal content in crops. These insights not only enhance our comprehension of the molecular mechanisms governing the response to Cd stress in rice but also offer promising avenues for the development of biotechnological strategies to minimize Cd uptake and accumulation in agricultural produce.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (41907145); Key project support from Anhui Provincial Department of Education (2022AH051048, 2023AH050484); Research Project of Anqing Normal University (100001195); Open Project Program of Provincial Key

Laboratory of the Biodiversity Study and Ecology Conservation in Southwest Anhui Province(Wsz202205,Wxn202303,Wxn202309).

Environmental Implication

Cadmium (Cd) is readily taken up by plants and can accumulate to toxic levels in edible tissues, posing a significant risk to human health upon consumption. The transporter OsNRAMP1 has been identified as a critical gateway for Cd uptake in plants, yet the intricacies of its regulation have remained largely unexplored. Our research uncovers the pivotal role of the transcription factor OsNAC5 in directly upregulating *OsNRAMP1* expression, thereby modulating Cd uptake and enhancing the plant's tolerance to Cd stress.

Supplementary Data

Supplementary Fig. S1. Amino acid sequence multiple alignment and phylogenetic tree analysis of NAC family proteins in rice, Arabidopsis, maize, and wheat.

Supplementary Fig. S2. Cd stress induces the expression of OsNAC5 in rice roots depending on H_2O_2 production.

Supplementary Fig. S3. Identification of the osnac5 mutant generated by CRISPR/Cas9.

Supplementary Fig. S4.Two complementary lines (ProOsNAC5::OsNAC5□HA) were determined by immunoblotting analysis with anti-HA antibody.

Supplementary Fig. S5. Transcript levels of Cd transporter genes OsNAC5 (A), OsLEA (B), OsNAS3 (C) and OsYSL2 (D) in roots of Cd-treated rice plants.

Supplementary Fig. S6. The concentrations of Mn (A) , Zn (B) and Fe (C) in rice grain (unpolished brown rice) of wild type (cv. ZH11) , osnac5 mutant and two complementation lines (C1 and C2).

Supplementary Tab. S2. Primers sequence used in this study.

Supplementary Tab. S2. Analysis of agronomic traits in WT plants (ZH11), the osnac5 mutants and two osnac5 complementation lines (C1 and C2).

References:

Takasaki H., Maruyama K., Kidokoro S., Ito Y., Fujita Y., Shinozaki K., et al., 2010. The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. Mol Genet and Genomics 284,173-183. https://doi.org/ 10.1007/s00438-010-0557-0.

Agarwal, P., Mitra, M., Banerjee, S., Roy, S., 2020. MYB4 transcription factor, a member of R2R3-subfamily of MYB domain protein, regulates cadmium tolerance via enhanced protection against oxidative damage and increases expression of PCS1 and MT1C in Arabidopsis. Plant Sci 297, 110501. https://doi.org/10.1016/j.plantsci.2020.110501.

Chang, J., Gao, W., Wang, P., Zhao, F., 2022. OsNRAMP5 Is a Major Transporter for Lead Uptake in Rice. Environ Sci Technol 56, 17481-17490. https://doi.org/10.1021/ acs.est.2c06384.

Chang, J., Huang, S., Konishi, N., Wang, P., Chen, J., Huang, X., et al., 2020. Overexpression of the manganese/cadmium transporter OsNRAMP5 reduces cadmium accumulation in rice grain. J Exp Bot 71, 5705-5715. https://doi.org/10.1093/jxb/eraa287.

Chang, J., Huang, S., Yamaji, N., Zhang, W., Ma, J., Zhao, F., 2020. OsNRAMP1 transporter contributes to cadmium and manganese uptake in rice. Plant Cell Environ 43, 2476-2491. https://doi.org/10.1111/pce.13843.

Chen, H., Yang, X., Wang, P., Wang, Z., Li, M., Zhao, F., 2018. Dietary cadmium intake from rice and vegetables and potential health risk: A case study in Xiangtan, southern China. Sci Total Environ 639, 271-277. https://doi.org/10.1016/j.scitotenv.2018.05.050.

Chen, J., Yang, L., Yan, X., Liu, Y., Wang, R., Fan, T., et al., 2016. Zinc-Finger Transcription Factor ZAT6 Positively Regulates Cadmium Tolerance through the Glutathione-Dependent Pathway in Arabidopsis1. Plant physiol 171, 707-719. https://doi.org/10.1104/pp.15.01882.

Clemens, S., Ma, J., 2016. Toxic Heavy Metal and Metalloid Accumulation in Crop Plants and Foods. Annu rev plant biol 67, 489-512. https://doi.org/10.1146/annurev-arplant-043015- 112301.

Han, G., Huang, R., Hong, L., Xu, J., Hong, Y., Wu, Y., et al., 2023. The transcription factor NAC102 confers cadmium tolerance by regulating WAKL11 expression and cell wall pectin metabolism in Arabidopsis. J Integr Plant Biol 65, 2262-2278. https://doi.org/10.1111/jipb.13557.

Han, Y., Fan, T., Zhu, X., Wu, X., Ouyang, J., Jiang, L., et al., 2019. WRKY12 represses GSH1 expression to negatively regulate cadmium tolerance in Arabidopsis. Plant Mol Biol 99, 149-159. https://doi.org/10.1007/s11103-018-0809-7.

Hu, S., Du B, Mu, G., Jiang, Z., Li, H., Song, Y., et al., 2024. The transcription factor OsbZIP48 governs rice responses to zinc deficiency. Plant Cell Environ https://doi.org/10.1111/pce.14825.

Hu, S., Shinwari, K., Song, Y., Xia, J., Xu, H., Du, B., et al., 2021. OsNAC300 Positively Regulates Cadmium Stress Responses and Tolerance in Rice Roots. Agronomy 11, 95. https://doi.org/10.3390/agronomy11010095.

Hu, S., Yu, Y., Chen, Q., Mu, G., Shen, Z., Zheng, L., 2017. OsMYB45 plays an important role in rice resistance to cadmium stress. Plant Sci 264, 1-8. https://doi.org/10.1016/j.plantsci.2017.08.002.

Ishikawa, S., Ishimaru, Y., Igura, M., Kuramata, M., Abe, T., Senoura, T., et al., 2012. Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. Proc Natl Acad Sci 109, 19166-19171. https://doi.org/10.1073/pnas.1211132109.

Ishimaru, Y., Takahashi, R., Bashir, K., Shimo, H., Senoura, T., Sugimoto, K., et al., 2012. Characterizing the role of rice NRAMP5 in Manganese, Iron and Cadmium Transport. Sci Rep 2:286. https://doi.org/ 10.1038/srep00286.

Jeong, J., Kim, Y., Redillas, M., Jang, G., Jung, H., Bang, S., et al., 2013. OsNAC5 overexpression enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain yield in the field. Plant Biotechnol J 11, 101-114. https://doi.org/10.1111/pbi.12011.

Lee, D., Chung, P., Jeong, J., Jang, G., Bang, S., Jung, H., et al., 2017. The rice OsNAC6 transcription factor orchestrates multiple molecular mechanisms involving root structural adaptions and nicotianamine biosynthesis for drought tolerance. Plant Biotechnol J 15, 754-764. https://doi.org/10.1111/pbi.12673.

Li, F., Deng, Y., Liu, Y., Mai, C., Xu, Y., Wu, J., et al., 2023. Arabidopsis transcription factor WRKY45 confers cadmium tolerance via activating PCS1 and PCS2 expression. J Hazard Mater 460, 132496.

Li, R., Song, Y., Wang, X., Zheng, C., Liu, B., Zhang, H., et al., 2023. OsNAC5 orchestrates OsABI5 to fine-tune cold tolerance in rice. J Integr Plant Biol. https://doi.org/10.1111/jipb.13585.

Lin, T., Yang, W., Lu, W., Wang, Y., Qi, X., 2017. Transcription Factors PvERF15 and PvMTF-1 Form a Cadmium Stress Transcriptional Pathway. Plant Physiol 173, 1565-1573. https://doi.org/ 10.1104/pp.16.01729.

Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V., Vandepoele, K., et al., 2011. ROS signaling: the new wave? Trends Plant Sci 16, 300-309. https://doi.org/10.1016/j.tplants.2011.03.007.

Nakashima, K., Tran, L., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., et al., 2007. Functional analysis of a NAC□type transcription factor OsNAC6 involved in abiotic and biotic stress□responsive gene expression in rice. Plant J 51, 617-630. https://doi.org/10.1111/j.1365-313X.2007.03168.x.

Ni, L., Fu, X., Zhang, H., Li, X., Cai, X., Zhang, P., et al., 2019. Abscisic Acid Inhibits Rice Protein Phosphatase PP45 via H_2O_2 and Relieves Repression of the Ca^{2+}/CaM-Dependent Protein Kinase DMI3. Plant Cell 31, 128-152. https://doi.org/10.1105/tpc.18.00506.

Nishimura, A., Aichi, I., Matsuoka, M., 2006. A protocol for Agrobacterium-mediated transformation in rice. Nat Protoc 1, 2796-2802. https://doi.org/10.1038/nprot.2006.469.

Nordberg, G., 2009. Historical perspectives on cadmium toxicology. Toxicol Appl Pharm 238, 192-200. https://doi.org/10.1016/j.taap.2009.03.015.

Rachmat, A., Nugroho, S., Sukma, D., Aswidinnoor, H., Sudarsono, 2014. Overexpression of OsNAC6 transcription factor from Indonesia rice cultivar enhances drought and salt tolerance. Emir J Food Agr 26, 519. https://doi.org/10.9755/ejfa.v26i6.17672.

Sasaki, A., Yamaji, N., Yokosho, K., Ma, J., 2012. Nramp5 Is a Major Transporter Responsible for Manganese and Cadmium Uptake in Rice. Plant Cell 24, 2155-2167. https://doi.org/10.1105/ tpc.112.096925.

Sheng, Y., Yan, X., Huang, Y., Han, Y., Zhang, C., Ren, Y., et al., 2019. The WRKY transcription factor, WRKY13, activates PDR8 expression to positively regulate cadmium tolerance in Arabidopsis. Plant Cell Environ 42, 891-903. https://doi.org/10.1111/ pce.13457.

Shi, B., Ni, L., Liu, Y., Zhang, A., Tan, M., Jiang, M., 2014. OsDMI3□mediated activation of OsMPK1 regulates the activities of antioxidant enzymes in abscisic acid signalling in rice. Plant Cell Environ 37, 341-352. https://doi.org/10.1111/pce.12154.

Shim, D., Hwang, J., Lee, J., Lee, S., Choi, Y., An, G., et al., 2009. Orthologs of the Class A4 Heat Shock Transcription Factor HsfA4a Confer Cadmium Tolerance in Wheat and Rice. Plant Cell 21,

4031-4043. https://doi.org/10.1105/tpc.109.066902.

Song, S., Chen, Y., Chen, J., Dai, X., Zhang, W., 2011. Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234, 331-345. https://doi.org/ 10.1007/s00425-011-1403-2.

Sui, F., Chang, J., Tang, Z., Liu, W., Huang, X., Zhao, F., 2018. Nramp5 expression and functionality likely explain higher cadmium uptake in rice than in wheat and maize. Plant Soil 433, 377-389. https://doi.org/10.1007/s11104-018-3849-5.

Takahashi, R., Ishimaru, Y., Senoura, T., Shimo, H., Ishikawa, S., Arao, T., et al., 2011. The OsNRAMP1 iron transporter is involved in Cd accumulation in rice. J Exp Bot 62, 4843-4850. https://doi.org/10.1093/jxb/err136.

Tang, L., Mao, B., Li, Y., Lv, Q., Zhang, L., Chen, C., et al., 2017. Knockout of OsNramp5 using the CRISPR/Cas9 system produces low Cd-accumulating indica rice without compromising yield. Sci Rep 7:14438. https://doi.org/10.1038/s41598-017-14832-9.

Ueno, D., Yamaji, N., Kono, I., Huang, C., Ando, T., Yano, M., et al., 2010. Gene limiting cadmium accumulation in rice. Proc Natl Acad Sci 107, 16500 -16505. https://doi.org/10.1073/pnas.1005396107.

Wairich, A., Vitali, A., Adamski, J., Lopes, K., Duarte, G., Ponte, L., et al., 2023. Enhanced expression of OsNAC5 leads to up-regulation of OsNAC6 and changes rice (Oryza sativa L.) ionome. Genet mol biol 46, e20220190-e20220190. https://doi.org/10.1590/1678-4685-GMB-2022 -0190.

Xie, K., Yang, Y., 2013. RNA-Guided Genome Editing in Plants Using a CRISPR–Cas System. Mol Plant 6, 1975-1983. https://doi.org/10.1093/mp/sst119.

Xie, K., Zhang, J., Yang, Y., 2014. Genome-Wide Prediction of Highly Specific Guide RNA Spacers for CRISPR–Cas9-Mediated Genome Editing in Model Plants and Major Crops. Mol Plant 7, 923-926. https://doi.org/10.1093/mp/ssu009.

Yamaji, N., Xia, J., Ueno, N., Yokosho, K., Ma, J., 2013. Preferential Delivery of Zinc to Developing Tissues in Rice Is Mediated by P-Type Heavy Metal ATPase OsHMA2. Plant Physiol 162, 927-939. https://doi.org/10.1104/pp.113.216564.

Yan, J., Wang, P., Wang, P., Yang, M., Lian, X., Tang, Z., et al., 2016. A loss□of□function allele of OsHMA3 associated with high cadmium accumulation in shoots and grain of Japonica rice cultivars. Plant Cell Environ 39, 1941-1954. https://doi.org/10.1111/ pce.12747.

Yang, C., Zhang, Y., Huang, C., 2019. Reduction in cadmium accumulation in japonica rice grains by CRISPR/Cas9-mediated editing of OsNRAMP5. J Integr Agr 18**,** 688-697. https://doi.org/10.1016/S2095-3119(18)61904-5.

Yang, M., Zhang, Y., Zhang, L., Hu, J., Zhang, X., Lu, K., et al., 2014. OsNRAMP5 contributes to manganese translocation and distribution in rice shoots. J Exp Bot 65, 4849-4861. https://doi.org/10.1093/jxb/eru259.

Zhang, H., Liu, Y., Wen, F., Yao, D., Wang, L., Guo, J., et al., 2014. A novel rice C2H2-type zinc finger protein, ZFP36, is a key player involved in abscisic acid-induced antioxidant defence and oxidative stress tolerance in rice. J Exp Bot 65, 5795-809. https://doi.org/ 10.1093/jxb/eru313.

Zhang, P., Wang, R., Ju, Q., Li, W., Tran, L., Xu, J., 2019. The R2R3-MYB Transcription Factor MYB49 Regulates Cadmium Accumulation. Plant Physiol 180, 529-542. https://doi.org/ 10.1104/pp.18.01380.

Zhang, Q., Cai, W., Ji, T., Ye, L., Lu, Y., Yuan, T., 2020. WRKY13 Enhances Cadmium Tolerance by Promoting D-CYSTEINE DESULFHYDRASE and Hydrogen Sulfide Production1. Plant physiol

183, 345-357. https://doi.org/10.1104/pp.19.01504.

Zhao, F., Ma, Y., Zhu, Y., Tang, Z., McGrath, S., 2015. Soil Contamination in China: Current Status and Mitigation Strategies. Environ Sci Technol 49, 750-759. https://doi.org/ 10.1021/es5047099.

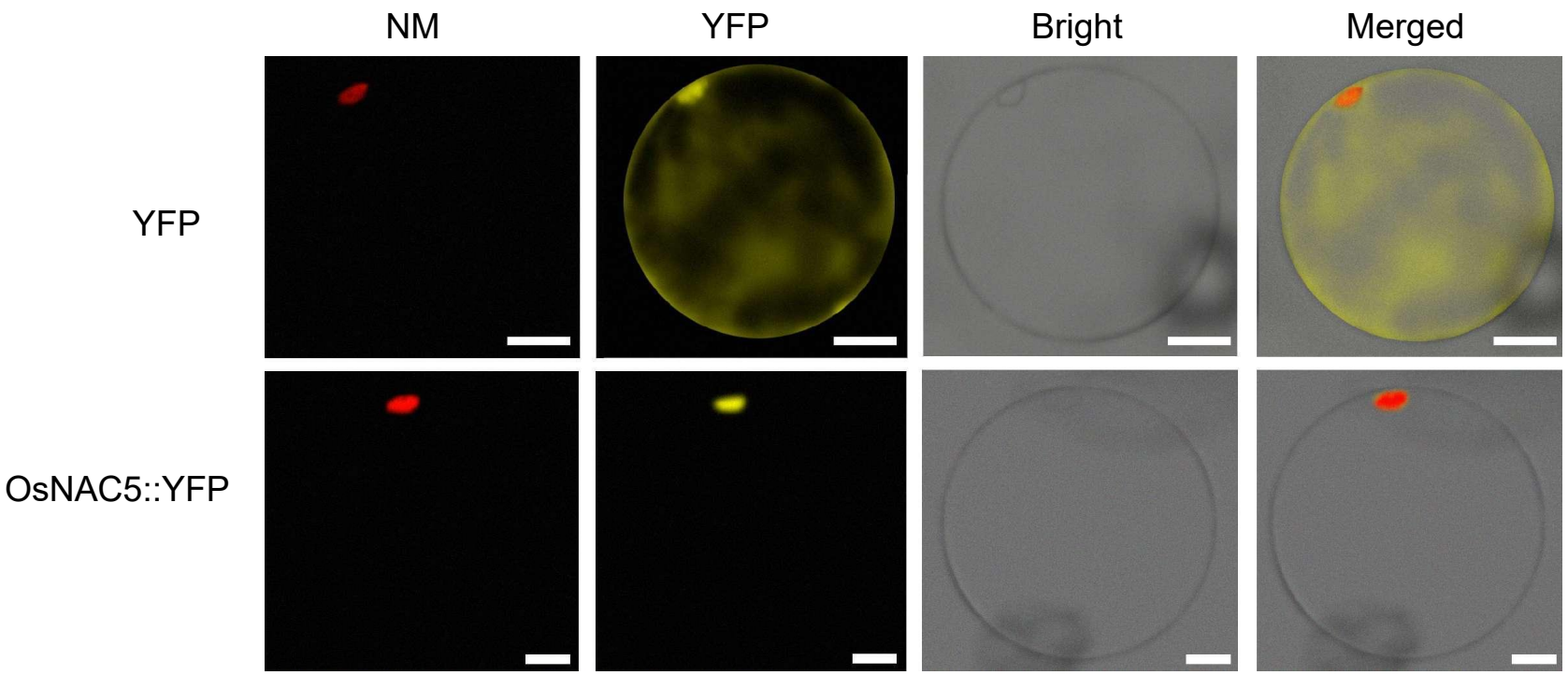

Fig. 1. Subcellular localization of OsNAC5. Confocal images of rice protoplast expressiong yellow fluorescent protein gene (*YFP*) or *OsNAC5-YFP*. NM (nuclear marker) is a nuclear localization tag, red fluorescent protein (RFP) linked to Histone 2A (RFP-H2A). Scale bars = 20 μM.

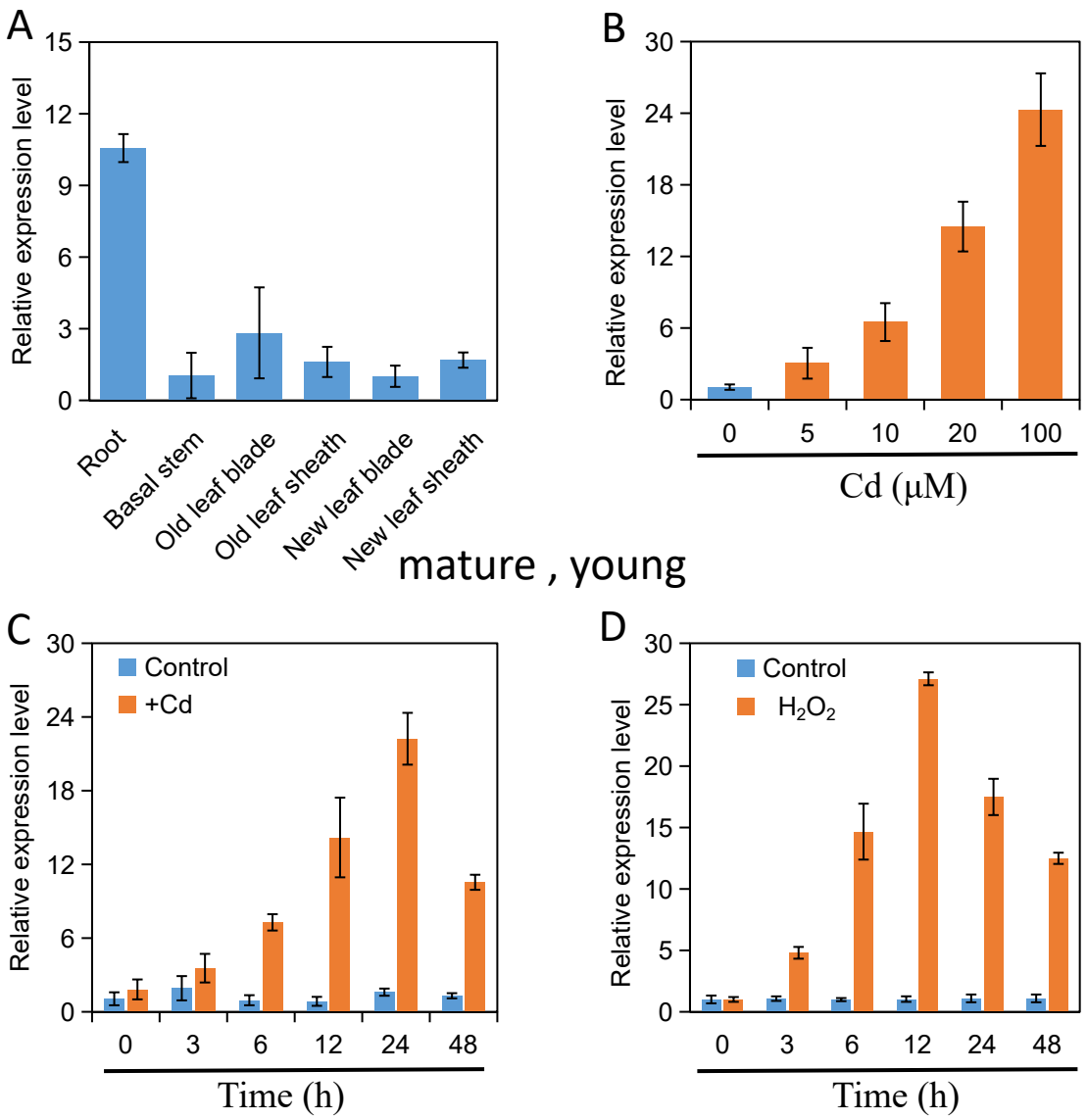

Fig. 2. Gene expression pattern of *OsNAC5*. (A) Relative expression of the *OsNAC5 gene* in various tissues of the 2-week-old wild-type rice seedlings. (B) Dose-dependent expression of the *OsNAC5* gene in the roots of wild-type rice in response to Cd stress. Rice seedlings were exposed to a $Cd(NO_3)_2$ solution with varying concentrations (0, 5, 10, 20, 100 μM) of for 24 hours. (C, D) Time-dependent expression of the *OsNAC5* gene in the roots of wild-type rice in response to Cd stress or H_2O_2. Rice seedlings were exposed to a solution containing 100 μM $d(NO_3)_2$ 10 mM H_2O_2 (to induce stress) for different durations. The expression of the *OsNAC5* gene was determined using RT-qPCR. *Actin* was used as an internal standard. The expression levels were normalized to seedlings without the addition of Cd or H_2O_2. Data are represent the means ±standard deviation (SD) of four biological replicates.

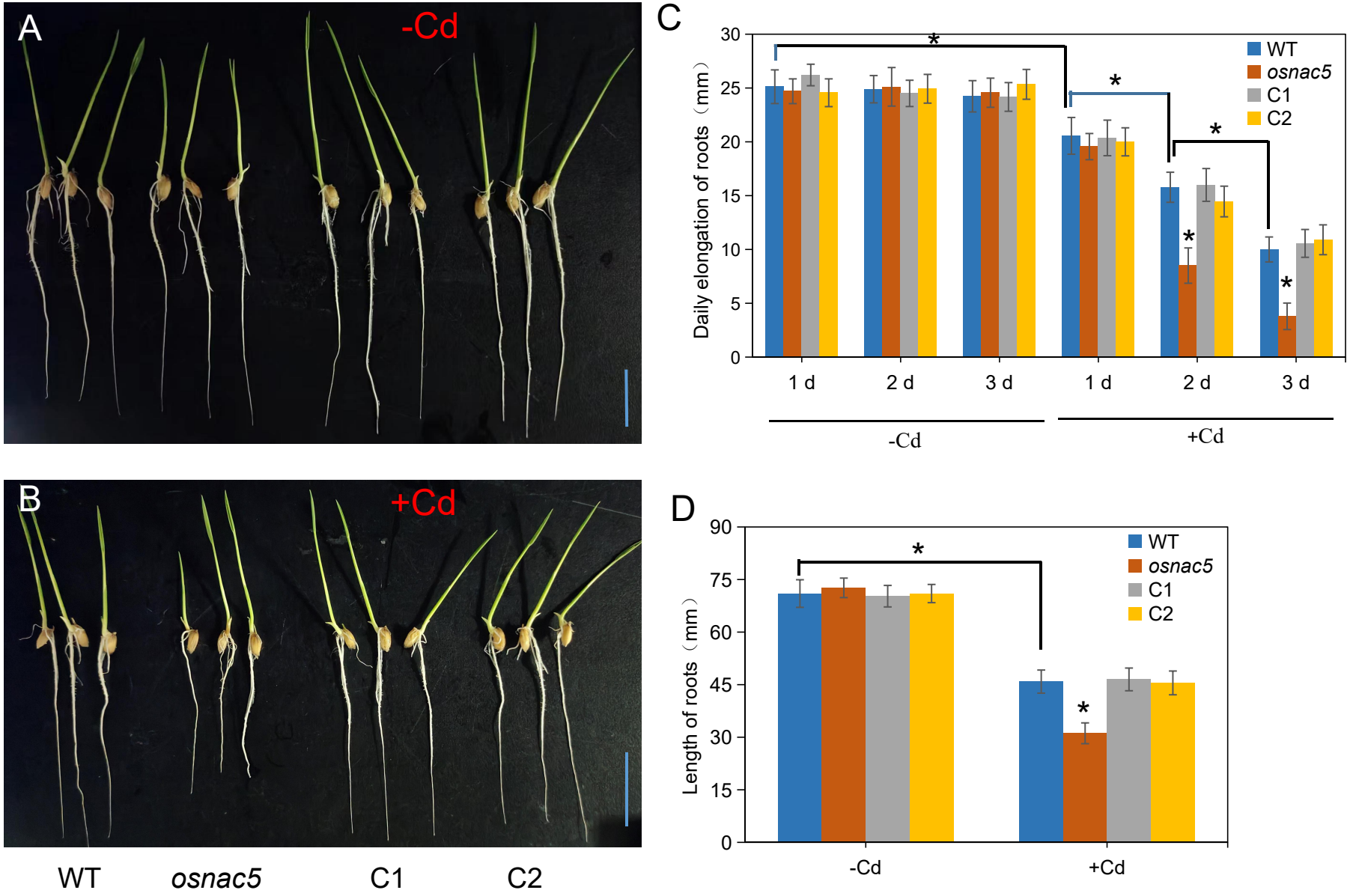

Fig. 3. The root elongation of *osnac5* mutant is hypersensitive to Cd stress. (A, B) The growth phenotypes of rice seedling roots treated for 3 days in solutions containing 0.5 mM $CaCl_2$ (-Cd) or 0.5 mM $CaCl_2$+5 μM $Cd(NO_3)_2$ (+Cd), respectively. (C) Daily elongation rate of the roots of rice seedlings under -Cd and +Cd conditions. (D) Length of rice seedling roots after 3 days under -Cd and +Cd conditions. WT refers to wild-type rice, *osnac5* denotes rice with the *OsNAC5* gene knockout line, and C1 and C2 represent complemented lines of *osnac5* mutant line. Mean values ± SD are shown. The values of the indicated genotypes were compared to that of the wild-type (WT) plants (Student's *t* test, * $P < 0.05$, n = 8). Bar=20 mm.

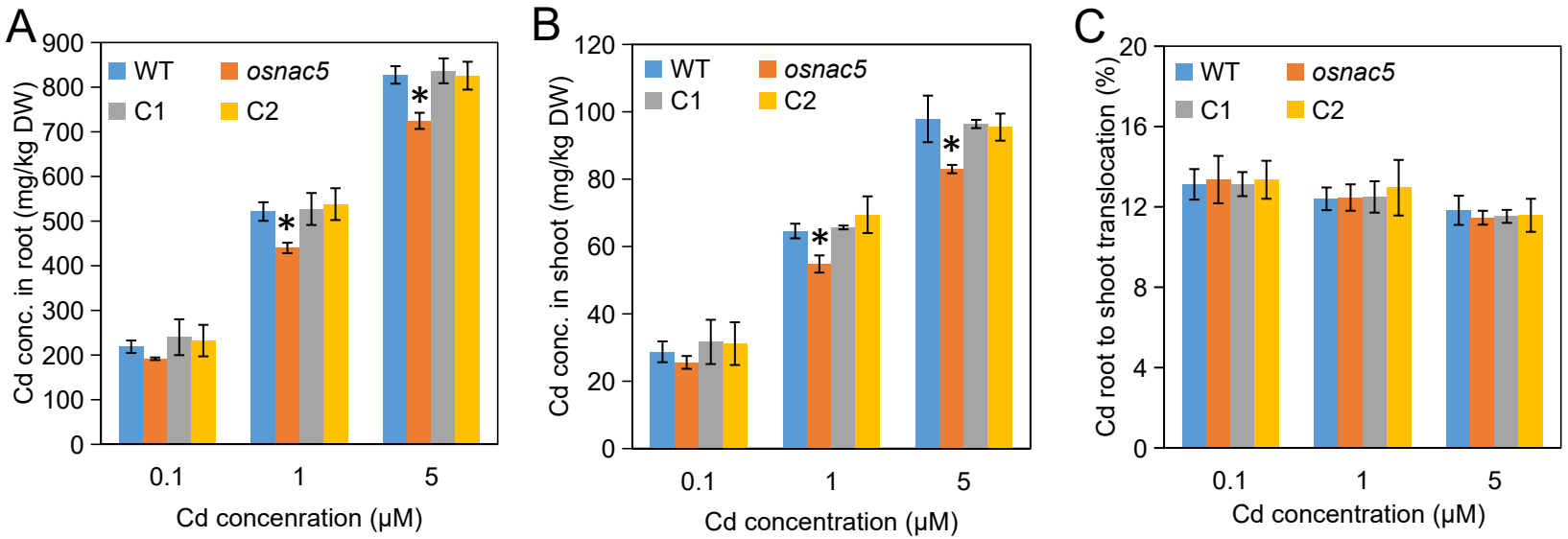

Fig. 4. Effect of *OsNAC5* knockout on rice Cd accumulation. The *OsNAC5* knockout line (*osnac5*), two complemental lines (C1 and C2) and wild type (WT, cv. ZH11) were exposed to 0.1, 1 or 5 μM $Cd(NO_3)_2$ for 7days. (A,B) Concentration of Cd in the roots (A) and the shoots (B). (C) The percentage of Cd distributed to the shoot. Roots and shoots were harvested separately and subjected to determination of Cd by ICP-MS. All data were based on comparisons with WT plants. DW: dry weight. Values represent means ± SD of biological replicates (n = 4). Statistical comparison was performed by Tukey's test (*P<0.05).

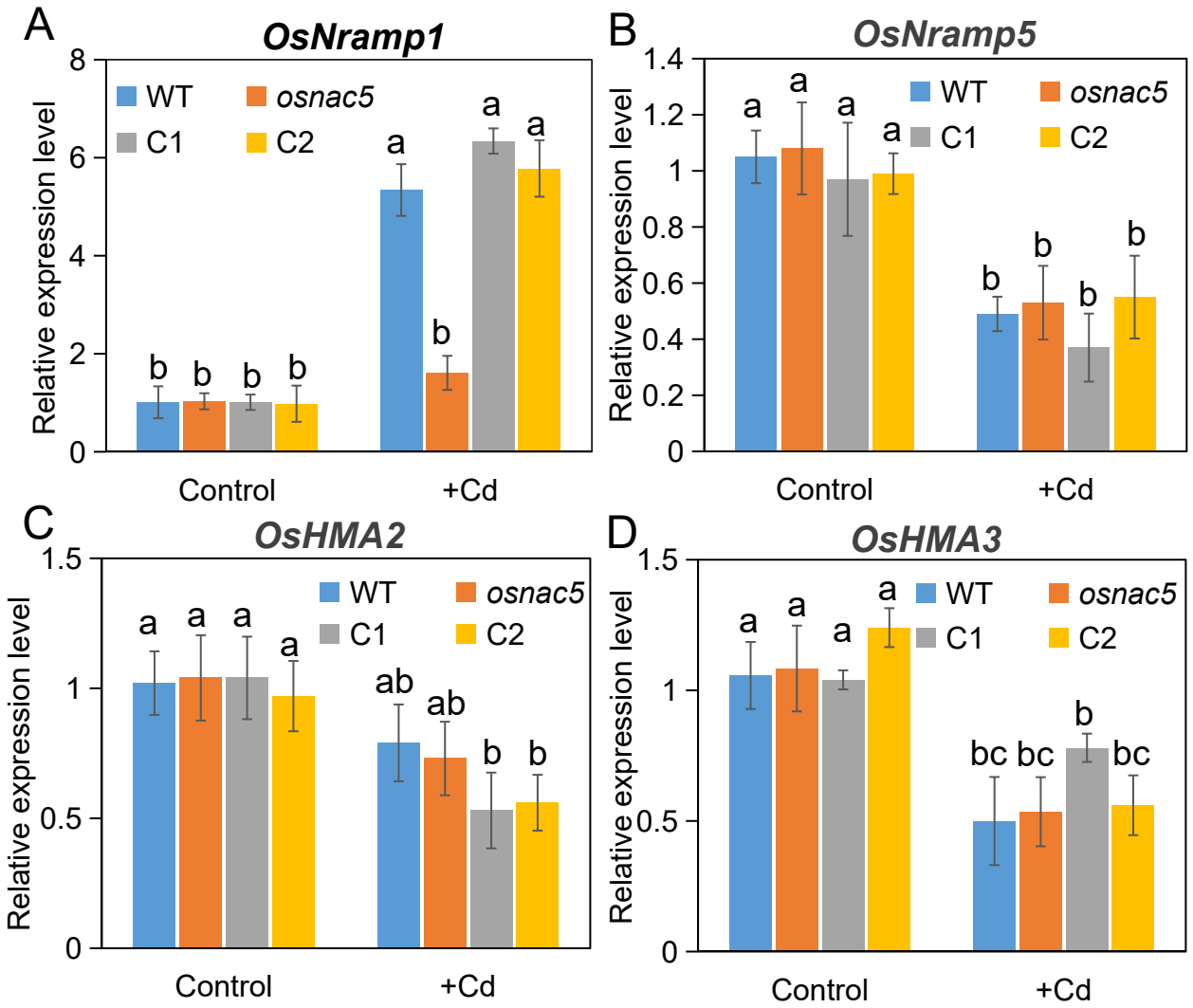

Fig. 5. Transcript levels of Cd transporter genes *OsNramp1* (A), *OsNramp5* (B), *OsHMA2* (C) and *OsHMA3* (D) in roots of Cd-treated rice plants. Two-week-old wild-type rice seedlings were exposed to 0.1 μM Cd(Control) or 10 μM Cd(+Cd) for 12 h. Real-time qPCR analysis was used to determine the relative transcript levels shown. *Actin* was used as internal standards. Values are mean ± SD (n = 4). Different letters above bars indicate significant differences among rice plants lines under control or +Cd treatment ($P<0.05$ by Tukey's test).

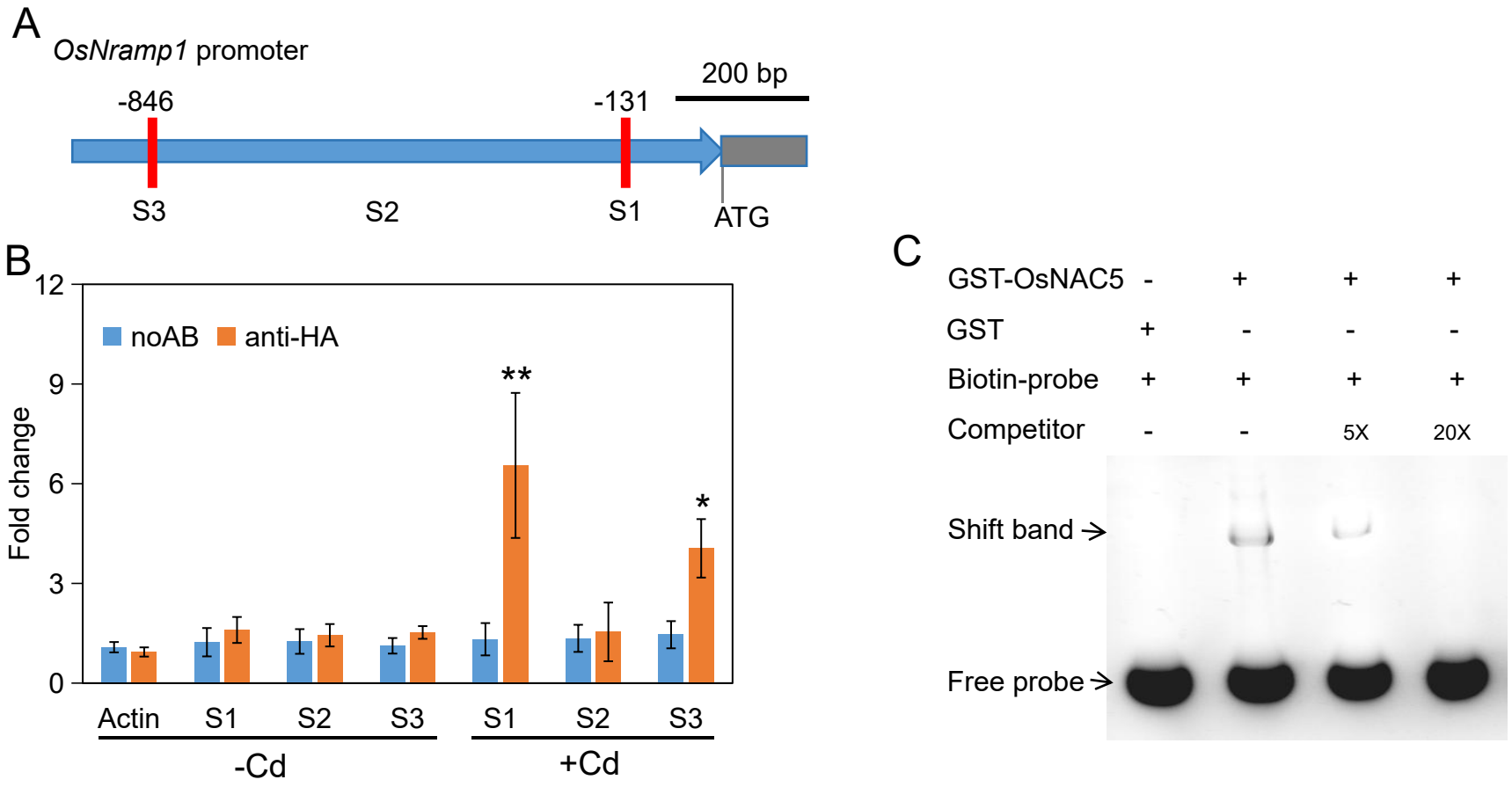

Fig. 6. OsNAC5 binds directly to the promoter of *OsNramp1*. (A) Diagram of *OsNramp1* promoter with two "CATGTG" motifs. Red vertical bars represents "CATGTG" motif. Site 1 and site2 indicates genomic DNA fragments around *OsNramp1* promoter for ChIP-qPCR. (B) ChIP-qPCR assay. Binding of OsNAC5 protein to specific regions of the *OsNramp1* promoter was examined with 2-week-old *OsNAC5Pro::NAC5-HA* transgenic seedlings grown to 1/2 Kimura (-Cd) and 1/2 Kimura 100 μM Cd (+Cd) for 6 h. The noAB (no antibody) was used as the control and *Actin* in 'noAB' samples was used as the internal standard. Data are means ± SD ($n = 4$). Statistical comparison between noAB and anti-HA was performed by Student's *t* test. Asterisks indicates significant differences from the control (*$p \leq 0.05$, **$p \leq 0.01$). ChIP, chromatin immunoprecipitation; HA, the human influenza hemagglutinin tag protein; GST, gulathione S-transferase. (C) Electrophoretic mobility shift assay. Biotin-labeled probe contains the "CATGTG" motif in the *OsNramp1* promoter. Competition is unlabeled probe. Black arrows indicate the shifted bands and free probes.

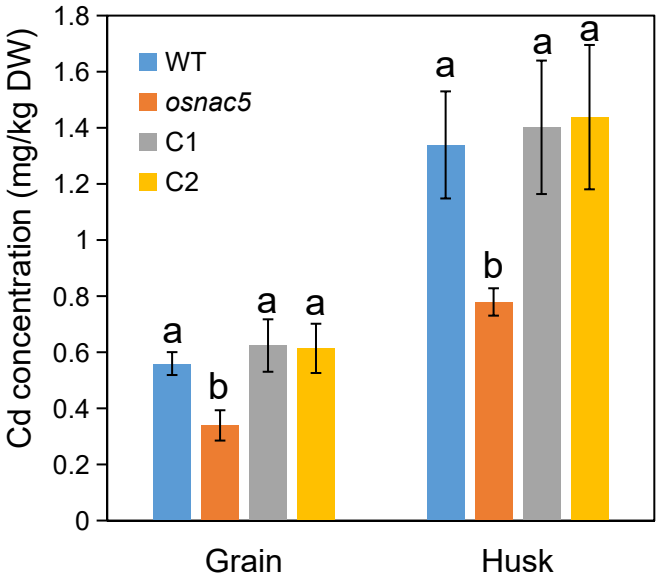

Fig. 7. The concentrations of Cd in rice grain (unpolished brown rice) and husk. Wild type (cv. ZH11) , *osnac5* mutant and two complementation lines(C1 and C2) grown in a Cd-contaminated paddy soil. Values are mean ± SD (n = 4). Different letters above bars indicate significant differences among rice plants lines (Student's *t* test, $P < 0.05$).

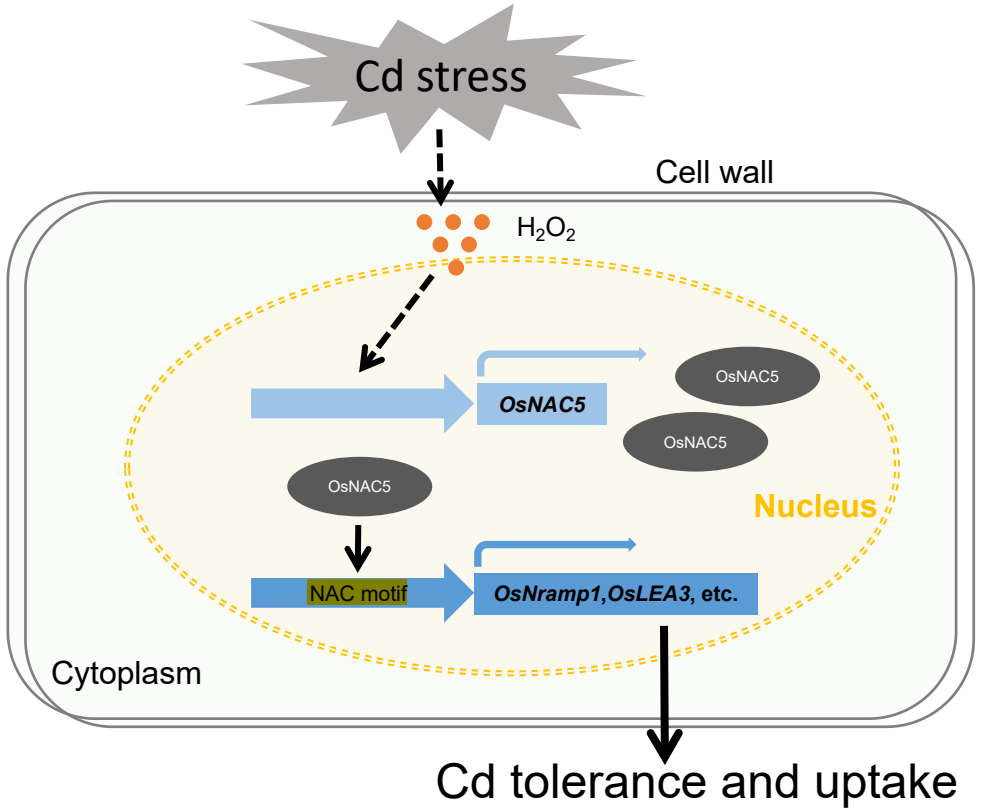

Fig. 8. Proposed working model for OsNAC5 regulating of Cd stress response. Cd stress induces an increase in OsNAC5 expression levels, which is dependent on hydrogen peroxide (H_2O_2) production triggered by Cd exposure. Subsequently, OsNAC5 directly upregulates the transcription of *OsNramp5* and *OsLEA3*, key genes involved in the cellular response to Cd stress.