Title

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

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

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

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

[c]College of Life Sciences, Nanjing Agricultural University, Nanjing, 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

[f] College of Computer and Information, Anqing Normal University, Anqing 246003, China

Corresponding author: Ganlin Wu and Hannie Zha

E-mail address: wugl@sina.cn and zhahn@aqnu.edu.cn

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

Abstract: Cadmium (Cd) is a toxic heavy metal that endangers human health through contaminated rice consumption. Reducing Cd uptake in rice is essential for minimizing grain Cd accumulation. However, the transcriptional regulation of Cd uptake in rice 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 upregulated by Cd-induced H_2O_2. Knocking out *OsNAC5* reduces Cd levels in both shoots and roots and increases Cd sensitivity. *OsNRAMP1* expression, enhanced by Cd stress, depends on OsNAC5, which binds to "CATGTG" motifs in the *OsNRAMP1* promoter 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.

However, the transcriptional regulatory mechanisms controlling Cd accumulation in crops remain poorly understood due to a lack of research in this area. Overexpression of *OsNAC5* in transgenic plants has been shown to provide increased tolerance to high salinity (Takasaki et al., 2010). As a

104 transcriptional activator, OsNAC5 enhances stress tolerance by increasing the expression of
105 stress-responsive genes in rice, such as late embryogenesis abundant 3 (*OsLEA3*) (Takasaki et al.,
106 2010). Song et al. (2011) reported that OsNAC5 plays a significant role in enhancing tolerance to
107 abiotic stress in rice by modulating downstream targets associated with the accumulation of
108 compatible solutes, sodium ions (Na^+), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA).
109 Jeong et al. (2013) showed that root-specific overexpression of *OsNAC5* leads to substantial root
110 enlargement, which in turn improves drought tolerance and increases grain yield under field
111 conditions. However, the roles of OsNAC5 in Cd tolerance and accumulation have yet to be fully
112 explored.

113 In this study, we aimed to investigate the role of OsNAC5 in the regulation of Cd tolerance and
114 uptake in rice. Through analysis of its expression pattern, subcellular localization, phenotypic response,
115 and Cd concentration, we provide compelling evidence that OsNAC5 significantly influences Cd
116 tolerance and accumulation in rice. Our findings shed light on the importance of OsNAC5 in the
117 response of rice to Cd stress, highlighting its potential as a target for improving Cd tolerance in rice
118 crops. Moreover, we demonstrate that OsNAC5 directly interacts with "CATGTG" motifs in the
119 promoter of OsNRAMP1, promoting its expression.

120

121 **2. Materials and Methods**

122 *2.1. Generation of rice genetic materials and plant growth conditions*

123 Generation of The *osnac5* Knockout Line: The *osnac5* knockout line was generated using the
124 CRISPR/Cas9 genome editing system in the 'ZH11' rice cultivar background. A specific guide RNA
125 (gRNA) sequence (297–319 bp) was used for this purpose (Supplementary Table S1). Agrobacterium
126 tumefaciens-mediated transformation was performed on actively dividing calli derived from mature
127 rice embryos (Nishimura et al., 2006). Homozygous mutant lines were confirmed by sequencing
128 PCR-amplified genomic fragments from putative mutants.

129 Construction of Genetic Complementation Lines: For the genetic complementation of the *osnac5*
130 mutant, a genomic fragment encompassing the *OsNAC5* promoter (1011 bp upstream of the start codon)
131 and codon sequence (990 bp starting from 'ATG') was PCR-amplified from rice root DNA. This
132 fragment was subsequently cloned into the binary vector pCAMBIA1300-HA.The primers used for

133 amplification are listed in Supplementary Table S1. The resulting construct,
134 ProOsNAC5::OsNAC5-HA, was introduced into the *osnac5* mutant plants (free of selection marker)
135 via Agrobacterium-mediated transformation. Two independent complementation lines
136 (ProOsNAC5::OsNAC5-HA, T3 generation) were selected based on immunoblotting analysis using
137 anti-HA antibody. These lines are used for further phenotypic characterization and chromatin
138 immunoprecipitation (ChIP) assays.

139 Growth Conditions and Nutrient Solution: Seeds of both wild-type 'ZH11' and *osnac5* mutant rice
140 were initially soaked in water for 48 hours, followed by germination in a 0.5 mM $CaCl_2$ solution. After
141 five days, seedlings were transferred to a half-strength Kimura B solution (pH 5.5) and grown in a
142 greenhouse with controlled conditions at 25—30 ℃. The nutrient solution was composed of 0.18 mM
143 $(NH_4)_2SO_4$, 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,
144 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
145 $CuSO_4 \cdot 5H_2O$, and 100 μM Fe(III)-EDTA. This solution was refreshed every two days to ensure
146 optimal nutrient availability.

147 2.2. Subcellular localization

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

159 *2.3. Gene expression analysis*

160 To elucidate the tissue-specific expression pattern of *OsNAC5* in wild-type (WT) rice, samples

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

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

176 *2.4. Phenotypic analysis*

177 To evaluate the impact of Cd stress on plant phenotype, seeds from wild-type (WT) rice, the
178 *osnac5* mutant, and two independent *osnac5* complementation lines were germinated. Post-germination,
179 seedlings were placed on nets floating in 4-liter containers of solution. Two-week-old seedlings were
180 then exposed to 0, 10, and 100 μM $Cd(NO_3)_2$ solutions for 5 days. Plant phenotype was photographed
181 and recorded, and measurements of plant height, root length, and biomass were taken. To further assess
182 Cd stress on root elongation, 3-day-old seedlings were exposed to a control solution (0.5 mM $CaCl_2$)
183 and a treatment solution (0.5 mM $CaCl_2$ with 5 μM $Cd(NO_3)_2$) at 30 °C for three days. Root lengths
184 were measured at 0, 24, 48, and 72 hours post-exposure. Each rice line and Cd treatment condition had
185 eight biological replicates.

186 *2.5. Measurement of Malondialdehyde (MDA)*

187 Lipid peroxidation was assessed by measuring MDA levels with thiobarbituric acid (TBA) assay.
188 Fresh leaf samples (0.6 g) were homogenized in 6 mL of 0.1% (w/v) trichloroacetic acid (TCA). The

homogenate was centrifuged at 4500×g for 8 minutes. A 3 mL portion of the supernatant was mixed with 3 mL of 0.6% TBA and incubated in boiling water for 10 minutes. After rapid cooling on ice, the mixture was centrifuged again. The absorbance of the supernatant was measured at 450 nm, 532 nm, and 600 nm, and MDA concentration was calculated according to the TBA reaction as described by Hu et al., 2017.

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(NO3)2 for a duration of 7 days. Post-exposure, the roots were meticulously washed twice with 5 mM CaCl2 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 °C 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, consisting of a minimum of 0.1 g of dry tissue, was digested in a heating block with a mixture of perchloric acid (HClO4) and nitric acid (HNO3) in a 13:87 (v/v) ratio. The digestion

217 process involved a temperature gradient: the samples were heated at 110 ℃ for 2 hours, followed by
218 140 ℃ for 1.5 hours, then 160 ℃ for 0.5 hours, and finally maintained at 180 ℃ until complete
219 dryness was achieved.After digestion, the residue was dissolved in 2.5% HNO3 for analysis. The
220 concentration of metal element in the digested samples was quantified using inductively coupled
221 plasma mass spectrometry (ICP-MS, Perkin-Elmer NexION 300x, Waltham, MA, USA).

222 *2.7. Chromatin Immunoprecipitation Quantitative PCR assay (ChIP-qPCR)*

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

229 A total of 100 μL of the sonicated chromatin solution was then immunoprecipitated with 5 μL of
230 anti‐HA antibody (Santa Cruz Biotechnology) under gentle agitation at 50 rpm and 4 ℃ overnight.
231 The immunocomplexes were processed to release and purify the DNA fragments associated with the
232 OsNAC5 protein, which were then utilized as templates for qPCR analysis.Genomic fragments from
233 regions not expected to bind OsNAC5 served as negative controls. Quantification of the relative
234 abundance of target DNA sequences was performed using the △△Ct (cycle threshold) method.Actin
235 was used as the internal reference gene, and its expression in the 'no antibody' (noAB) samples was
236 used as the baseline for normalization.

237 *2.8. Electrophoretic mobility shift assay (EMSA)*

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

243 For EMSA, a DNA fragment encompassing the “CATGTG” motif within the OsNramp1
244 promoter was synthesized and labeled at 5’ end with biotin. Additionally, an unlabeled version of the

245 same DNA fragment was synthesized to function as a competitor in the binding assay (Supplementary
246 Table S1).

247 2.8. Transient Luciferase (LUC) Reporter Assay

248 The 1214 bp promoter region of *OsNramp1* was amplified using specific primers (Supplemental
249 Table S1). This fragment was cloned into the pCam1381-LUC binary vector to create
250 proNramp1::LUC via a single BamH I restriction site. The construct, along with pCam1300-OsNAC5,
251 was then introduced into A. tumefaciens (strain EHA105). Transient luciferase reporter assays were
252 performed as described by Hu et al., 2021.

253 *2.12 Statistical analysis*

254 To analyze the data, we used two-way ANOVA with Tukey's HSD post hoc tests and Student's *t*
255 tests when applicable. These analyses were conducted using IBM SPSS Statistics version 25.0. Results
256 are shown as means ± standard deviation, with different letters indicating significant differences ($p \leq$
257 0.05). Significance levels are marked as $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

258 *2.9. Gene numbers*

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

262 **3. Results**

263 *3.1. Sequence and subcellular localization of Analysis of OsNAC5*

264 The full-length open reading frame (ORF) of *OsNAC5* was cloned from rice root cDNA,
265 consistent with the sequence in the Rice Annotation Project Database. Structural analysis identified
266 three exons and two introns in *OsNAC5* gene, which encodes a protein of 329 amino acids with a
267 molecular weight of approximately 35.53 kDa. Phylogenetic analysis placed OsNAC5 in the rice NAC
268 transcription factor family, noted for a conserved NAM domain involved in DNA binding
269 (Supplementary Fig. S1A). OsNAC6, the closest homolog, shares 65.23% sequence identity with
270 OsNAC5 (Supplementary Fig. S1B). Previous studies have shown that OsNAC6 responds to stress
271 factors like drought, abscisic acid (ABA), and jasmonic acid (JA), and its overexpression enhances

272 drought and high salinity tolerance in rice (Nakashima et al., 2007; Lee et al., 2017; Rachmat et al.,
273 2014).

274 Bioinformatic analysis using ProtComp 9.0 program (http://www.softberry.com/) predicted that
275 OsNAC5 is a nuclear protein with a nuclear localization signal (NLS) peptide. This was experimentally
276 confirmed through a transient expression assay in rice protoplasts using an OsNAC5-YFP (yellow
277 fluorescent protein) fusion construct. Confocal laser scanning microscopy showed that the YFP-tagged
278 OsNAC5 colocalized with the red fluorescent protein-tagged Histone 2A (RFP-H2A) nuclear marker
279 (Hu et al., 2017). In contrast, YFP alone was distributed throughout the cell (Fig. 1). These findings
280 align with previous research showing nuclear localization of OsNAC5-GFP in rice roots (Takasaki et
281 al., 2010). Collectively, these results confirm that OsNAC5 is localized in the nucleus.

282 *3.2. Expression pattern of OsNAC5*

283 The expression pattern of *OsNAC5* was examined by RT-qPCR in various tissues of
284 hydroponically grown rice plants (cv. ZH11). The analysis showed that *OsNAC5* was mainly expressed
285 in the roots (Fig. 2A). This observation contrasts with Song et al. (2011), who reported that *OsNAC5*
286 was primarily expressed in the leaves of cv. Zhonghua10, indicating that the expression of *OsNAC5*
287 varies among cultivars.

288 To explore the response of *OsNAC5* to Cd or hydrogen peroxide (H_2O_2) stress, hydroponic
289 experiments were conducted. Cd exposure led to a dose-dependent increase in *OsNAC5* expression in
290 rice roots (Fig. 2B). A time-course analysis of Cd-treated roots revealed a rapid rise in *OsNAC5*
291 transcript levels, peaking at 24 hours post-treatment (Fig. 2C). Previous research has shown that
292 *OsNAC5* is inducible by various abiotic stresses such as drought, high salinity, and cold (Takasaki et al.,
293 2010; Jeong et al., 2013; Song et al., 2011). Considering the role of H_2O_2 as a crucial signaling
294 molecule (Mittler et al., 2011; Ni et al., 2019), we also assessed its effect on *OsNAC5* expression. The
295 results demonstrated a rapid up-regulation of *OsNAC5* following H_2O_2 treatment, with peak expression
296 levels at 12 hours (Fig. 2D), suggesting a quicker response to H_2O_2 than to Cd stress.

297 To explore the potential role of H_2O_2 in the Cd-induced *OsNAC5* expression pathway, rice plants
298 were pre-treated with two reactive oxygen species (ROS) modulators, dimethylthiourea (DMTU), an
299 H_2O_2 scavenger, and diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, before Cd exposure

300 (Zhang et al., 2014). These pre-treatments significantly reduced the Cd-induced increase in *OsNAC5*
301 expression (Supplementary Fig. S2). Indicating that intracellular H_2O_2 is a crucial mediator for the
302 Cd-induced up-regulation of *OsNAC5* transcript levels.

303 *3.3. Knockout of OsNAC5 results in a Cd-sensitive phenotype in rice*

304 To clarify the physiological role of *OsNAC5* in rice's response and tolerance to Cd stress, we
305 generated an *osnac5* mutant in the ZH11 background using the CRISPR/Cas9 system. Sanger
306 sequencing confirmed a homozygous *osnac5* mutant line with a 1 bp thymine insertion causing a
307 frameshift mutation at the target gRNA site (Supplementary Fig. S3). To verify the function of
308 *OsNAC5*, we conducted a genetic complementation assay by introducing *OsNAC5* genomic sequence
309 (ProNAC5::NAC5-HA) into the *osnac5* mutant. This resulted in the generation of two
310 complementation lines (C1 and C2), which were confirmed through immunoblotting analysis with
311 anti-HA antibody. These lines were then used for subsequent phenotypic analysis and chromatin
312 immunoprecipitation (ChIP) assay (Supplementary Fig. S4).

313 Under control conditions, no noticeable differences were observed among WT, the *osnac5*
314 mutant, complementation lines C1 and C2 at two-week-old plants (Figure 3A). However, when
315 exposed to 10 μM Cd（+Cd）or 100 μM Cd（++Cd）for 5 days, the *osnac5* mutant plants showed more
316 severe growth inhibition and leaf necrosis, unlike WT and complementation lines (Figure 3B,C). We
317 measured shoot height, root length, biomass, and MDA concentration under these conditions. Under
318 normal conditions, there were no visible differences in these parameters among the WT, the *osnac5*
319 mutant, and complementation lines (Figure 3D-I). However, under +Cd or ++Cd conditions, the shoot
320 height, root length, and biomass of the *osnac5* mutant were lower than those of the WT and
321 complementation lines. Additionally, the MDA concentration in the shoots and roots of the *osnac5*
322 mutant was higher (Figure 3D-I).

323 Further investigation showed that under normal conditions, the root length of the *osnac5* mutant
324 seedlings was similar to WT (Supplementary Fig. S5A). Under 5 μM Cd for 3 days, the root length of
325 mutants was significantly reduced compared to WT, but complementation lines maintained root length
326 similar to WT under both conditions (Supplementary Fig. S5B). Daily root elongation was about 25
327 mm for all lines under normal conditions, but Cd stress inhibited elongation on the first day across all

328 lines. On the second and third days, *osnac5* mutants showed more pronounced inhibition compared to
329 WT and complementation lines (Supplementary Fig. S5C). After 3 days of Cd treatment, WT rice had a
330 36.5% inhibition rate of root elongation compared to control, while the *osnac5* mutants showed a lower
331 rate of 57.1% (Supplementary Fig. S5D). These findings strongly indicate that *OsNAC5* plays a crucial
332 role in the response of rice roots to Cd stress, as its disruption leads to increased Cd sensitivity.

333 *3.4 Knockout of OsNAC5 decreased Cd uptake in rice*

334 To explore the role of *OsNAC5* in mediating Cd accumulation in rice, two-week-old plants of WT,
335 the *osnac5* mutant, two complementation lines C1 and C2 were subjected to 0.1, 1 or 5 μM $Cd(NO_3)_2$
336 for 7 days. At Cd concentration of 0.1 μM, the *osnac5* mutant plants showed a marginal decrease in Cd
337 accumulation in both the roots and shoots compared to the WT and complementation lines, however,
338 this reduction was not statistically significant (P>0.05; Fig. 4A，B). In contrast, at 1 μM Cd exposure,
339 the *osnac5* mutant plants exhibited a significant reduction in Cd concentration by 15.6% in roots and
340 15.2% in shoots relative to the WT and two complementation lines (both P < 0.05; Fig. 4A, B). This
341 trend persisted at the higher concentration of 5 μM Cd, with the *osnac5* mutants displaying a
342 significant decrease of 15.2% in root Cd concentration and 15.1% in shoot Cd concentration compared
343 to the WT and complementation lines (both P < 0.05; Fig. 4A, B).

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

350 *3.5 OsNRAMP1 is induced by Cd stress dependent on OsNAC5.*

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

356 complementation lines C1 and C2, however, showed a strong induction of *OsNRAMP1* expression,
357 mirroring the response seen in the WT rice (Fig. 5A).

358 Under the same Cd exposure, the expression levels of *OsNRAMP5* and *OsHMA3* in WT rice roots
359 were significantly downregulated to about 0.5-fold of the control, whereas *OsHMA2* expression
360 remained unchanged (Fig. 5B–D). Notably, there were no significant differences in the expression
361 levels of *OsNRAMP5*, *OsHMA2*, and *OsHMA3* between the WT, the *osnac5* mutant, and
362 complementation lines (Fig. 5B–D). Additionally, under control conditions, the transcript levels of
363 these four Cd transport-related genes were similar across the roots of WT, the *osnac5* mutant, and
364 complementation lines (Fig. 5A–D). These observations suggest that of the four Cd transporters
365 analyzed, only *OsNRAMP1* expression is induced by Cd stress in a manner dependent on the functional
366 presence of *OsNAC5*.

367 We also assessed whether the *OsNAC5* knockout would alter the gene's own expression response
368 to Cd stress. Real-time qPCR analysis indicated that under Cd stress, the expression level of *OsNAC5*
369 in the *osnac5* mutant was similar to that in the WT rice (Supplementary Fig. S6A). Given that the
370 reduced Cd accumulation in the *osnac5* mutant does not account for its increased sensitivity to Cd
371 stress, it is postulated that *OsNAC5* may regulate the expression of other stress-related genes. The "late
372 embryogenesis abundant" gene *OsLEA3*, known to be directly upregulated by OsNAC5 and involved in
373 various stress responses (Takasaki et al., 2010), was found to be strongly induced by Cd stress in an
374 OsNAC5-dependent manner (Supplementary Fig. S6B). This may partly explain the *osnac5* mutant's
375 heightened sensitivity to Cd stress. Furthermore, Jeong et al. (2013) showed that overexpression of
376 *OsNAC5* enhances the expression of genes involved in the homeostasis of divalent metals, such as
377 *OsNAS3* and *OsYSL2*. In our study, we found that *OsNAS3* expression was suppressed by Cd stress, but
378 it was unaffected by the *OsNAC5* knockout (Supplementary Fig. S6C). On the other hand, *OsYSL2*
379 expression was not influenced by either Cd stress or OsNAC5 status (Supplementary Fig. S6C). These
380 findings suggest that OsNAC5's biological function is multifaceted, encompassing the regulation of Cd
381 transport as well as broader stress response mechanisms.

382 *3.6 OsNAC5 binds directly to the promoter of OsNramp1*

383 The binding of transcription factors (TFs) to cis-regulatory elements in gene promoters is crucial
384 for gene expression regulation. OsNAC300 has a strong affinity for the NAC transcription factor
385 binding element motif (CATGTG) (Hu et al., 2021).In the ~1.0 kb *OsNRAMP1* promoter region, two
386 "CATGTG" motifs were identified at -846 to -841 bp and -131 to -126 bp upstream of the transcription
387 start site (Fig. 6A). To examine if OsNAC5 interacts with these motifs in vivo, ChIP-qPCR assays were
388 performed on ProOsNAC5::OsNAC5-HA transgenic rice using an anti-HA monoclonal
389 antibody.Specific promoter region amplicons (S1, S2, and S3) were enriched. Fragments S1 and S3
390 containing "CATGTG" motifs showed significant enrichment compared to controls, with Cd exposure
391 resulting in higher enrichment than in the absence of it (Fig. 6B). To verify OsNAC5's binding affinity
392 to the "CATGTG" motif in vitro, EMSA was conducted using recombinant GST-OsNAC5 fusion
393 protein and synthetic oligonucleotides from *OsNRAMP1* promoter. The EMSA results demonstrated a
394 strong DNA binding complex with the "CATGTG" motif, confirming OsNAC5's specific interaction
395 with the motif in vitro (Fig. 6C). These findings indicate that OsNAC5 directly binds to "CATGTG"
396 motifs in *OsNRAMP1* promoter, regulating gene expression in response to Cd stress.

397 To determine if OsNAC5 activates *OsNramp1* promoter, a luciferase (LUC) assay was conducted
398 in tobacco leaves using the firefly LUC gene as a reporter. *OsNAC5* was placed under the control of the
399 CaMV 35S promoter. Coexpression of 35S::OsNAC5 with proNramp1::LUC increased the fluorescent
400 signal (Fig. 6D) and showed higher LUC relative expression levels than the control (Fig. 6E).This
401 demonstrates that OsNAC5 directly enhances the transcriptional activity of the *OsNramp1* promoter.

402 *3.7 Knockout of OsNAC5 reduced Cd accumulation in rice grain*

403 To assess the impact of *OsNAC5* knockout on Cd accumulation in rice grains (unpolished
404 brown rice), a pot experiment was conducted with the WT, the *osnac5* mutant, and complementation
405 lines (C1 and C2) grown in Cd-contaminated paddy soil (total Cd concentration of 1.13 mg/kg, pH
406 5.77). Upon reaching maturity, no significant differences were observed among the four lines in terms
407 of plant height, effective panicle number per plant, seed-setting rate, or grain yield per plant
408 (Supplementary Table S2). However, the *osnac5* mutant displayed a marked reduction in Cd
409 concentrations, showing a 39.4% decrease in grains and a 41.8% decrease in husks compared to the

410 WT (Fig. 7). In contrast, the Cd concentrations in the grains and husks of the two complementation
411 lines were similar to those observed in the WT (Fig. 7).

412 Given that OsNRAMP1 is also known to transport Mn (Chang et al., 2020), we measured Mn
413 concentrations in rice grains and detected no significant differences among the four rice lines
414 (Supplementary Fig. S7A). Recent studies have indicated that overexpression of the *OsNAC5* gene
415 enhances the translocation of Fe and Zn from vegetative tissues to seeds (Wairich et al., 2023).
416 Consequently, we measured the concentrations of Fe and Zn in rice grains and found no significant
417 differences among the four lines (Supplementary Fig. S7B, C). These results are consistent with the
418 notion that Fe, Zn, and Mn are essential micronutrients, and plants have developed intricate regulatory
419 mechanisms to maintain their homeostasis, avoiding both deficiency and toxicity.

420 **4. Discussion：**

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

430 Previous work has shown that overexpression of *OsNAC5* under the maize ubiquitin promoter
431 significantly enhances salt tolerance in rice (Takasaki et al., 2010). Conversely, rice lines with reduced
432 *OsNAC5* expression through RNA interference (RNAi) display decreased tolerance to cold, drought,
433 and high salinity, while overexpression of *OsNAC5* in rice or *Arabidopsis* confers increased resistance
434 to these stressors (Song et al., 2010). Additionally, driving *OsNAC5* expression in rice roots with the
435 root-specific promoter RCc3 leads to increased root diameter, improved drought tolerance, and higher
436 yield (Jeong et al., 2013). In our study, we utilized CRISPR-Cas9 technology to generate an *osnac5*
437 mutant and found that this mutant exhibited increased sensitivity to Cd stress compared to WT plants

438 (Fig. 3B, D). However, no phenotypic differences were detected between the *osnac5* mutant and WT
439 plants under normal growth conditions (Fig. 3A, D).

440 Both *OsNAC5* and *OsNRAMP1* are induced by Cd stress (Fig. 2B, 5A). Notably, in the *osnac5*
441 mutant, *OsNRAMP1* does not respond to Cd stress induction (Fig. 5A). However, in the
442 complementation lines, *OsNRAMP1* expression is restored to WT levels (Fig. 5A). Interestingly, the
443 loss of *OsNAC5* function does not influence the extent of Cd stress-induced suppression of *OsNRAMP5*
444 expression (Fig. 5B). Considering the established transcriptional activation function of the OsNAC5
445 protein (Takasaki et al., 2010), our findings indicate that OsNAC5 exerts a distinct positive
446 transcriptional regulatory influence on *OsNRAMP1* in response to Cd stress, while it does not appear to
447 regulate *OsNRAMP5* in the same manner. This specificity underscores the nuanced role of OsNAC5 in
448 the plant's adaptive response to heavy metal stress, selectively modulating the expression of key
449 transporters involved in Cd uptake.

450 Moreover, our observations revealed that under non-stress conditions (0.1 μM Cd, serving as the
451 control), the loss of OsNAC5 function does not affect the Cd concentration in the roots and shoots of
452 rice plants (Fig. 4A, B). Correspondingly, the expression level of *OsNRAMP1* is not impacted by the
453 loss of *OsNAC5* function under these conditions (Fig. 5A). These findings provide additional support
454 for the hypothesis that OsNAC5 specifically increases *OsNRAMP1* expression under Cd stress
455 conditions. Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) experiments
456 confirmed that OsNAC5 interacts more strongly with the NAC binding elements in the *OsNRAMP1*
457 promoter in the presence of Cd stress in vivo (Fig. 6B). Consequently, we propose that OsNAC5
458 directly associates with *OsNRAMP1* promoter to positively influence its expression during Cd stress,
459 highlighting a targeted regulatory mechanism activated by heavy metal exposure (Fig. 8).

460 OsHMA2, a plasma membrane transporter for Cd, is localized in the pericycle of roots and is
461 constitutively expressed during the vegetative stage (Yamaji et al., 2013). Loss-of-function mutations
462 in OsHMA2 lead to diminished translocation of Cd from roots to shoots, as previously reported
463 (Satoh-Nagasawa et al., 2012; Takahashi et al., 2012; Yamaji et al., 2013). Our study indicates that the
464 expression of *OsHMA2* is minimally influenced by Cd stress and does not appear to be associated with
465 the function of *OsNAC5* (Fig. 4C). OsHMA3, located in the tonoplast of all root cells, has been shown

466 to have its expression modestly reduced by Cd in a cultivar-dependent manner (Ueno et al., 2010; Yan
467 et al., 2016). In line with these findings, we observed that Cd stress suppresses the expression of
468 *OsHMA3*, but this suppression is independent of *OsNAC5* function (Fig. 4D).

469 Recent molecular biology strategies have aimed to mitigate Cd accumulation in rice grains.
470 CRISPR/Cas9-mediated knockout of *OsNRAMP5* in both japonica and indica rice varieties has been
471 demonstrated to significantly lower Cd accumulation in seeds (Tang et al., 2017; Yang et al., 2019).
472 Overexpression of *OsNRAMP5* has been shown to disrupt radial Cd transport into the stele, thereby
473 reducing Cd accumulation in rice grains (Chang et al., 2020). Similarly, CRISPR/Cas9-mediated
474 knockout of *OsNRAMP1* in japonica rice (ZH11) has been effective in significantly reducing Cd
475 content in both grains and husks (Chang et al., 2020). Alterations in *OsHMA2* expression have also
476 been reported to significantly decrease Cd levels in rice seeds (Takahashi et al., 2012). Moreover,
477 overexpression of *OsHMA3a* from japonica rice can reduce grain Cd content by over 90% without
478 compromising essential nutrient levels such as Zn and Fe (Ueno et al., 2010). In our research, we found
479 that knockout of the transcription factor *OsNAC5* similarly results in reduced Cd content in japonica
480 rice grains (ZH11) (Fig. 7). Unlike the transporters previously mentioned, transcription factors such as
481 OsNAC5 do not directly mediate Cd uptake or transport. This discovery provides an alternative
482 molecular biology strategy to regulate Cd content in rice grains, expanding the toolkit for food safety.

483 **4. Conclusion**

484 In addition to its established roles in mediating responses to drought, cold, high salinity, and ABA,
485 our research has unveiled a novel function of the rice transcription factor OsNAC5 in responding to Cd
486 stress. We have demonstrated that OsNAC5 is pivotal in modulating rice's response to Cd stress and its
487 subsequent accumulation in plant tissues. The expression of the *OsNAC5* gene is upregulated in the
488 presence of Cd stress, a response that is contingent upon the production of H_2O_2. Notably, OsNAC5
489 specifically interacts with the "CATGTG" motifs within the promoter region of *OsNRAMP1*, thereby
490 orchestrating its expression in response to Cd exposure (Fig. 8). Crucially, the knockout of *OsNAC5*
491 leads to a reduction in Cd accumulation in rice grains, underscoring its potential utility in mitigating
492 heavy metal content in crops. These insights not only enhance our comprehension of the molecular
493 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 Anhui Provincial University Innovation Team Project, Digital Agriculture Innovation Team (2023AH010039); the National Natural Science Foundation of China (41907145); key project support from the Anhui Provincial Department of Education (2022AH051048, 2023AH050484); a research project of Anqing Normal University (100001195); and the Open Project Program of the 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 were conducted to compare 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 identified through immunoblotting analysis using an anti-HA antibody.

Supplementary Fig. S5. The root elongation of the *osnac5* mutant is hypersensitive to Cd stress.

Supplementary Fig. S6. Transcript levels of Cd transporter genes *OsNAC5* (A), *OsLEA3* (B), *OsNAS3* (C), and *OsYSL2* (D) were analyzed in the roots of Cd-treated rice plants.

Supplementary Fig. S7. The concentrations of Mn (A), Zn (B), and Fe (C) in unpolished brown rice grains of wild type (cv. ZH11), the *osnac5* mutant, and two complementation lines (C1 and C2) were measured.

Supplementary Table S1.Primers sequence used in this study.

Supplementary Table S2. The analysis of agronomic traits in WT plants (ZH11), the *osnac5* mutant, 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.

566 Han, Y., Fan, T., Zhu, X., Wu, X., Ouyang, J., Jiang, L., et al., 2019. WRKY12 represses GSH1
567 expression to negatively regulate cadmium tolerance in Arabidopsis. Plant Mol Biol 99, 149-159.
568 https://doi.org/10.1007/s11103-018-0809-7.
569 Hu, S., Du B, Mu, G., Jiang, Z., Li, H., Song, Y., et al., 2024. The transcription factor OsbZIP48
570 governs rice responses to zinc deficiency. Plant Cell Environ https://doi.org/10.1111/pce.14825.
571 Hu, S., Shinwari, K., Song, Y., Xia, J., Xu, H., Du, B., et al., 2021. OsNAC300 Positively Regulates
572 Cadmium Stress Responses and Tolerance in Rice Roots. Agronomy 11, 95.
573 https://doi.org/10.3390/agronomy11010095.
574 Hu, S., Yu, Y., Chen, Q., Mu, G., Shen, Z., Zheng, L., 2017. OsMYB45 plays an important role in rice
575 resistance to cadmium stress. Plant Sci 264, 1-8. https://doi.org/10.1016/j.plantsci.2017.08.002.
576 Ishikawa, S., Ishimaru, Y., Igura, M., Kuramata, M., Abe, T., Senoura, T., et al., 2012. Ion-beam
577 irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium
578 rice. Proc Natl Acad Sci 109, 19166-19171. https://doi.org/10.1073/pnas.1211132109.
579 Ishimaru, Y., Takahashi, R., Bashir, K., Shimo, H., Senoura, T., Sugimoto, K., et al., 2012.
580 Characterizing the role of rice NRAMP5 in Manganese, Iron and Cadmium Transport. Sci Rep
581 2:286. https://doi.org/ 10.1038/srep00286.
582 Jeong, J., Kim, Y., Redillas, M., Jang, G., Jung, H., Bang, S., et al., 2013. OsNAC5 overexpression
583 enlarges root diameter in rice plants leading to enhanced drought tolerance and increased grain
584 yield in the field. Plant Biotechnol J 11, 101-114. https://doi.org/10.1111/pbi.12011.
585 Lee, D., Chung, P., Jeong, J., Jang, G., Bang, S., Jung, H., et al., 2017. The rice OsNAC6 transcription
586 factor orchestrates multiple molecular mechanisms involving root structural adaptions and
587 nicotianamine biosynthesis for drought tolerance. Plant Biotechnol J 15, 754-764.
588 https://doi.org/10.1111/pbi.12673.
589 Li, F., Deng, Y., Liu, Y., Mai, C., Xu, Y., Wu, J., et al., 2023. Arabidopsis transcription factor WRKY45
590 confers cadmium tolerance via activating PCS1 and PCS2 expression. J Hazard Mater 460,
591 132496.
592 Li, R., Song, Y., Wang, X., Zheng, C., Liu, B., Zhang, H., et al., 2023. OsNAC5 orchestrates OsABI5
593 to fine-tune cold tolerance in rice. J Integr Plant Biol. https://doi.org/10.1111/jipb.13585.
594 Lin, T., Yang, W., Lu, W., Wang, Y., Qi, X., 2017. Transcription Factors PvERF15 and PvMTF-1 Form
595 a Cadmium Stress Transcriptional Pathway. Plant Physiol 173, 1565-1573. https://doi.org/
596 10.1104/pp.16.01729.
597 Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V., Vandepoele, K., et al., 2011. ROS
598 signaling: the new wave? Trends Plant Sci 16, 300-309.
599 https://doi.org/10.1016/j.tplants.2011.03.007.
600 Nakashima, K., Tran, L., Van Nguyen, D., Fujita, M., Maruyama, K., Todaka, D., et al., 2007.
601 Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic
602 stress-responsive gene expression in rice. Plant J 51, 617-630.
603 https://doi.org/10.1111/j.1365-313X.2007.03168.x.
604 Ni, L., Fu, X., Zhang, H., Li, X., Cai, X., Zhang, P., et al., 2019. Abscisic Acid Inhibits Rice Protein
605 Phosphatase PP45 via H_2O_2 and Relieves Repression of the Ca^{2+}/CaM-Dependent Protein Kinase
606 DMI3. Plant Cell 31, 128-152. https://doi.org/10.1105/tpc.18.00506.
607 Nishimura, A., Aichi, I., Matsuoka, M., 2006. A protocol for Agrobacterium-mediated transformation in
608 rice. Nat Protoc 1, 2796-2802. https://doi.org/10.1038/nprot.2006.469.
609 Nordberg, G., 2009. Historical perspectives on cadmium toxicology. Toxicol Appl Pharm 238, 192-200.

610 https://doi.org/10.1016/j.taap.2009.03.015.
611 Rachmat, A., Nugroho, S., Sukma, D., Aswidinnoor, H., Sudarsono, 2014. Overexpression of OsNAC6
612 transcription factor from Indonesia rice cultivar enhances drought and salt tolerance. Emir J Food
613 Agr 26, 519. https://doi.org/10.9755/ejfa.v26i6.17672.
614 Sasaki, A., Yamaji, N., Yokosho, K., Ma, J., 2012. Nramp5 Is a Major Transporter Responsible for
615 Manganese and Cadmium Uptake in Rice. Plant Cell 24, 2155-2167. https://doi.org/10.1105/
616 tpc.112.096925.
617 Sheng, Y., Yan, X., Huang, Y., Han, Y., Zhang, C., Ren, Y., et al., 2019. The WRKY transcription factor,
618 WRKY13, activates PDR8 expression to positively regulate cadmium tolerance in Arabidopsis.
619 Plant Cell Environ 42, 891-903. https://doi.org/10.1111/ pce.13457.
620 Shi, B., Ni, L., Liu, Y., Zhang, A., Tan, M., Jiang, M., 2014. OsDMI3-mediated activation of OsMPK1
621 regulates the activities of antioxidant enzymes in abscisic acid signalling in rice. Plant Cell
622 Environ 37, 341-352. https://doi.org/10.1111/pce.12154.
623 Shim, D., Hwang, J., Lee, J., Lee, S., Choi, Y., An, G., et al., 2009. Orthologs of the Class A4 Heat
624 Shock Transcription Factor HsfA4a Confer Cadmium Tolerance in Wheat and Rice. Plant Cell 21,
625 4031-4043. https://doi.org/10.1105/tpc.109.066902.
626 Song, S., Chen, Y., Chen, J., Dai, X., Zhang, W., 2011. Physiological mechanisms underlying
627 OsNAC5-dependent tolerance of rice plants to abiotic stress. Planta 234, 331-345. https://doi.org/
628 10.1007/s00425-011-1403-2.
629 Sui, F., Chang, J., Tang, Z., Liu, W., Huang, X., Zhao, F., 2018. Nramp5 expression and functionality
630 likely explain higher cadmium uptake in rice than in wheat and maize. Plant Soil 433, 377-389.
631 https://doi.org/10.1007/s11104-018-3849-5.
632 Takahashi, R., Ishimaru, Y., Senoura, T., Shimo, H., Ishikawa, S., Arao, T., et al., 2011. The
633 OsNRAMP1 iron transporter is involved in Cd accumulation in rice. J Exp Bot 62, 4843-4850.
634 https://doi.org/10.1093/jxb/err136.
635 Tang, L., Mao, B., Li, Y., Lv, Q., Zhang, L., Chen, C., et al., 2017. Knockout of OsNramp5 using the
636 CRISPR/Cas9 system produces low Cd-accumulating indica rice without compromising yield. Sci
637 Rep 7:14438. https://doi.org/10.1038/s41598-017-14832-9.
638 Ueno, D., Yamaji, N., Kono, I., Huang, C., Ando, T., Yano, M., et al., 2010. Gene limiting cadmium
639 accumulation in rice. Proc Natl Acad Sci 107, 16500 -16505.
640 https://doi.org/10.1073/pnas.1005396107.
641 Wairich, A., Vitali, A., Adamski, J., Lopes, K., Duarte, G., Ponte, L., et al., 2023. Enhanced expression
642 of OsNAC5 leads to up-regulation of OsNAC6 and changes rice (Oryza sativa L.) ionome. Genet
643 mol biol 46, e20220190-e20220190. https://doi.org/10.1590/1678-4685-GMB-2022 -0190.
644 Xie, K., Yang, Y., 2013. RNA-Guided Genome Editing in Plants Using a CRISPR–Cas System. Mol
645 Plant 6, 1975-1983. https://doi.org/10.1093/mp/sst119.
646 Xie, K., Zhang, J., Yang, Y., 2014. Genome-Wide Prediction of Highly Specific Guide RNA Spacers
647 for CRISPR–Cas9-Mediated Genome Editing in Model Plants and Major Crops. Mol Plant 7,
648 923-926. https://doi.org/10.1093/mp/ssu009.
649 Yamaji, N., Xia, J., Ueno, N., Yokosho, K., Ma, J., 2013. Preferential Delivery of Zinc to Developing
650 Tissues in Rice Is Mediated by P-Type Heavy Metal ATPase OsHMA2. Plant Physiol 162,
651 927-939. https://doi.org/10.1104/pp.113.216564.
652 Yan, J., Wang, P., Wang, P., Yang, M., Lian, X., Tang, Z., et al., 2016. A loss-of-function allele of
653 OsHMA3 associated with high cadmium accumulation in shoots and grain of Japonica rice

654 cultivars. Plant Cell Environ 39, 1941-1954. https://doi.org/10.1111/ pce.12747.
655 Yang, C., Zhang, Y., Huang, C., 2019. Reduction in cadmium accumulation in japonica rice grains by
656 CRISPR/Cas9-mediated editing of OsNRAMP5. J Integr Agr 18, 688-697.
657 https://doi.org/10.1016/S2095-3119(18)61904-5.
658 Yang, M., Zhang, Y., Zhang, L., Hu, J., Zhang, X., Lu, K., et al., 2014. OsNRAMP5 contributes to
659 manganese translocation and distribution in rice shoots. J Exp Bot 65, 4849-4861.
660 https://doi.org/10.1093/jxb/eru259.
661 Zhang, H., Liu, Y., Wen, F., Yao, D., Wang, L., Guo, J., et al., 2014. A novel rice C2H2-type zinc finger
662 protein, ZFP36, is a key player involved in abscisic acid-induced antioxidant defence and
663 oxidative stress tolerance in rice. J Exp Bot 65, 5795-809. https://doi.org/ 10.1093/jxb/eru313.
664 Zhang, P., Wang, R., Ju, Q., Li, W., Tran, L., Xu, J., 2019. The R2R3-MYB Transcription Factor
665 MYB49 Regulates Cadmium Accumulation. Plant Physiol 180, 529-542. https://doi.org/
666 10.1104/pp.18.01380.
667 Zhang, Q., Cai, W., Ji, T., Ye, L., Lu, Y., Yuan, T., 2020. WRKY13 Enhances Cadmium Tolerance by
668 Promoting D-CYSTEINE DESULFHYDRASE and Hydrogen Sulfide Production1. Plant physiol
669 183, 345-357. https://doi.org/10.1104/pp.19.01504.
670 Zhao, F., Ma, Y., Zhu, Y., Tang, Z., McGrath, S., 2015. Soil Contamination in China: Current Status and
671 Mitigation Strategies. Environ Sci Technol 49, 750-759. https://doi.org/ 10.1021/es5047099.
672

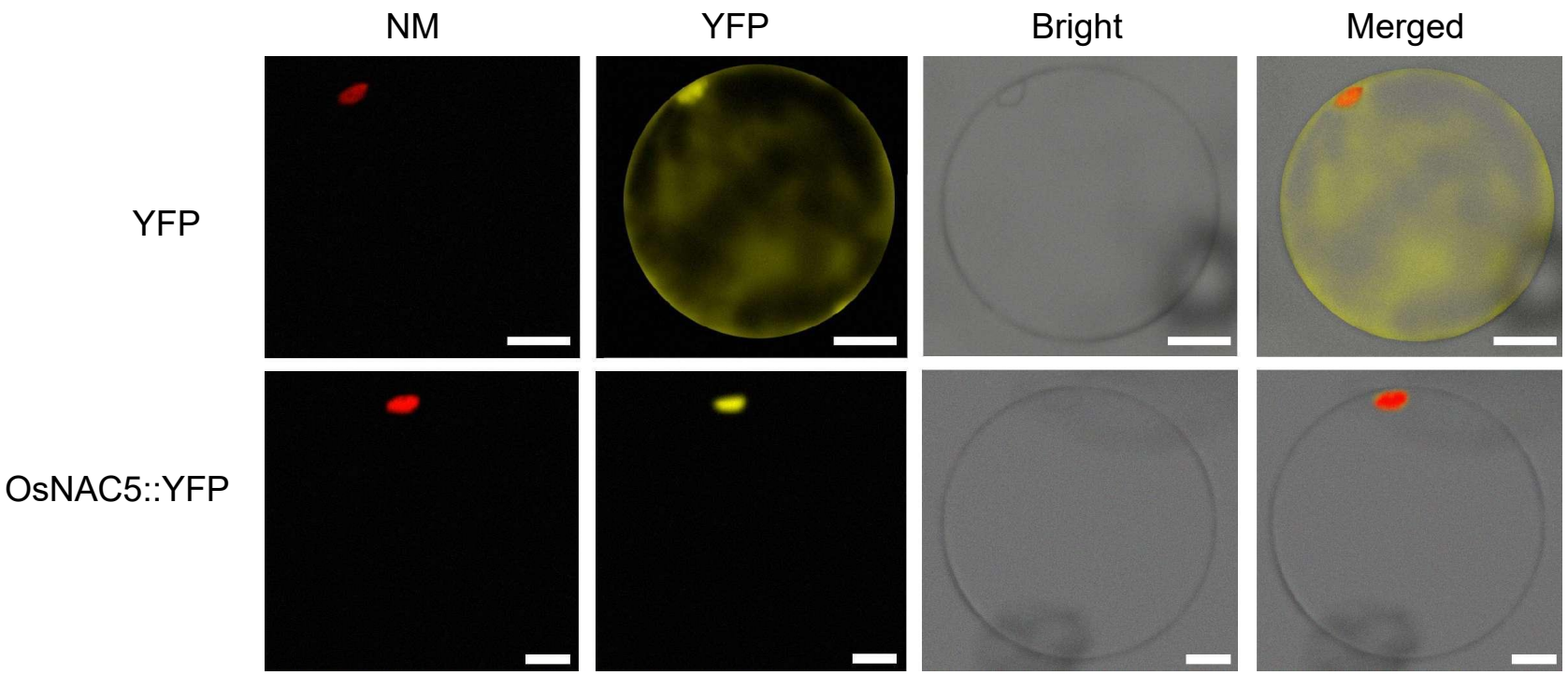

Fig. 1. Subcellular localization of OsNAC5. Confocal images of rice protoplast expressiong yellow fluorescent protein gene (*YFP*) or *OsNAC5-YFP*. NM (nuclear marker) is a nuclear localization tag, red fluorescent protein (RFP) linked to Histone 2A (RFP-H2A). Scale bars = 20 μM.

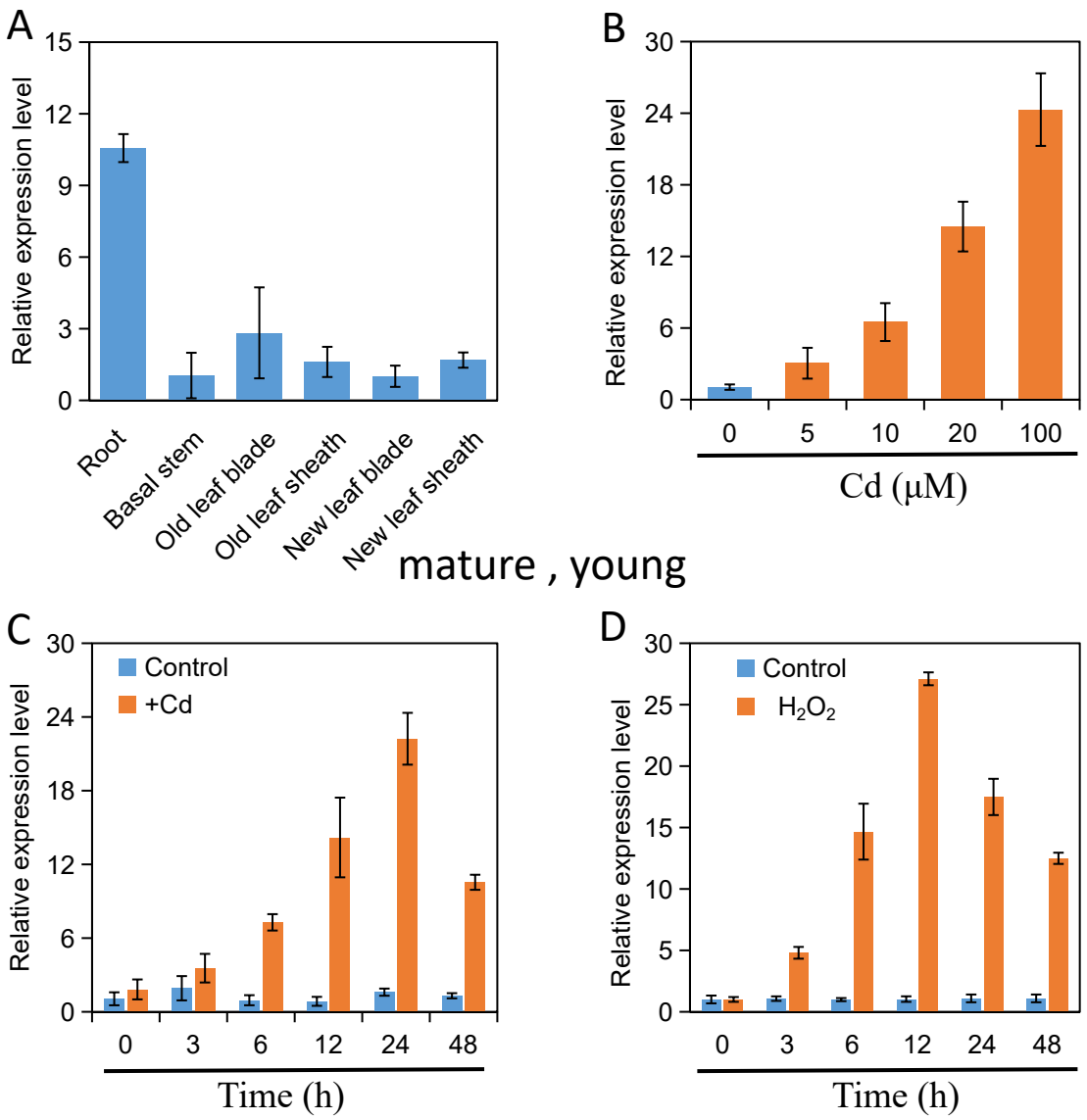

Fig. 2. Gene expression pattern of *OsNAC5*. (A) Relative expression of the *OsNAC5 gene* in various tissues of the 2-week-old wild-type rice seedlings. (B) Dose-dependent expression of the *OsNAC5* gene in the roots of wild-type rice in response to Cd stress. Rice seedlings were exposed to a $Cd(NO_3)_2$ 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.

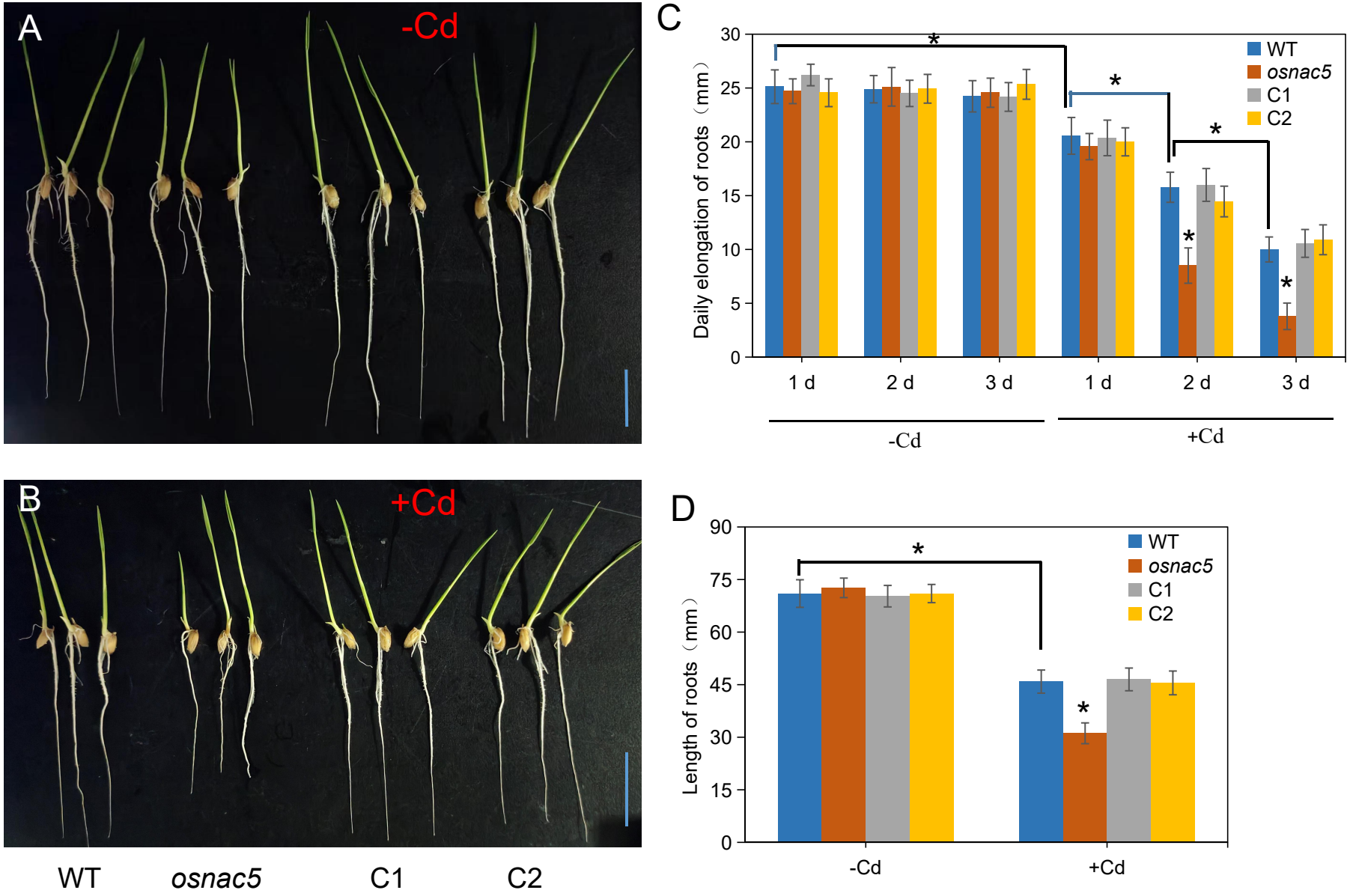

Fig. 3. The root elongation of *osnac5* mutant is hypersensitive to Cd stress. (A, B) The growth phenotypes of rice seedling roots treated for 3 days in solutions containing 0.5 mM $CaCl_2$ (-Cd) or 0.5 mM $CaCl_2$+5 μM $Cd(NO_3)_2$ (+Cd), respectively. (C) Daily elongation rate of the roots of rice 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.

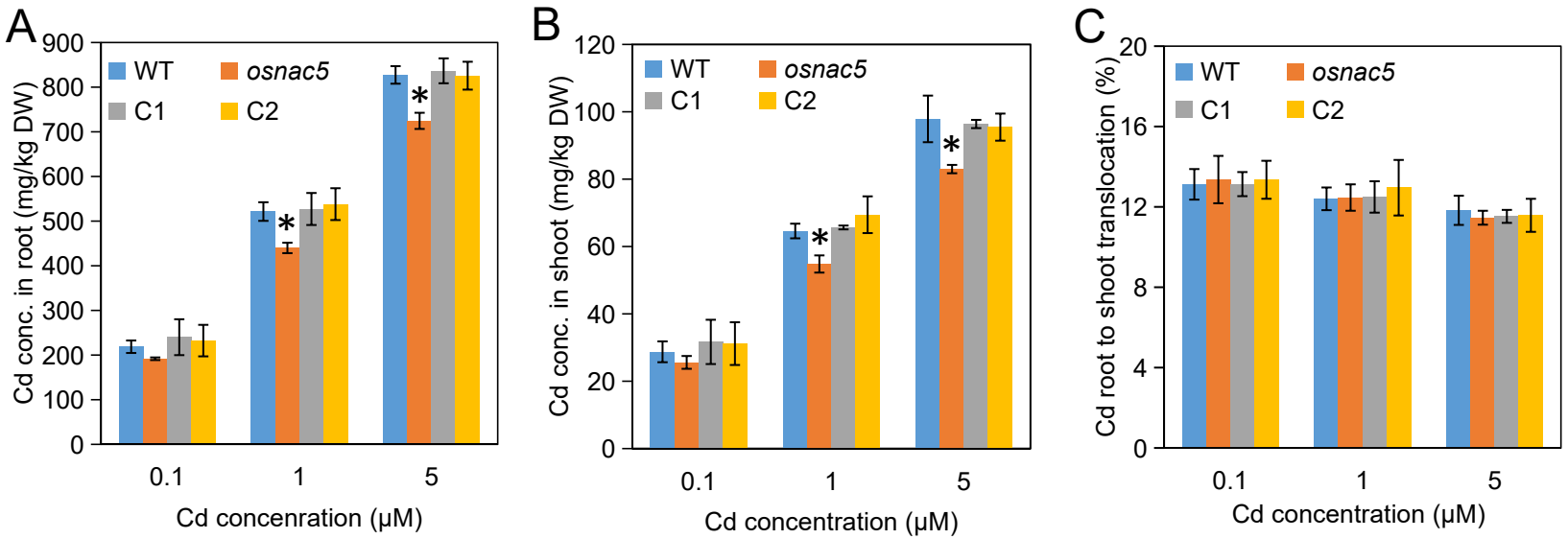

Fig. 4. Effect of *OsNAC5* knockout on rice Cd accumulation. The *OsNAC5* knockout line (*osnac5*), two complemental lines (C1 and C2) and wild type (WT, cv. ZH11) were exposed to 0.1, 1 or 5 μM $Cd(NO_3)_2$ for 7days. (A,B) Concentration of Cd in the roots (A) and the shoots (B). (C) The percentage 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).

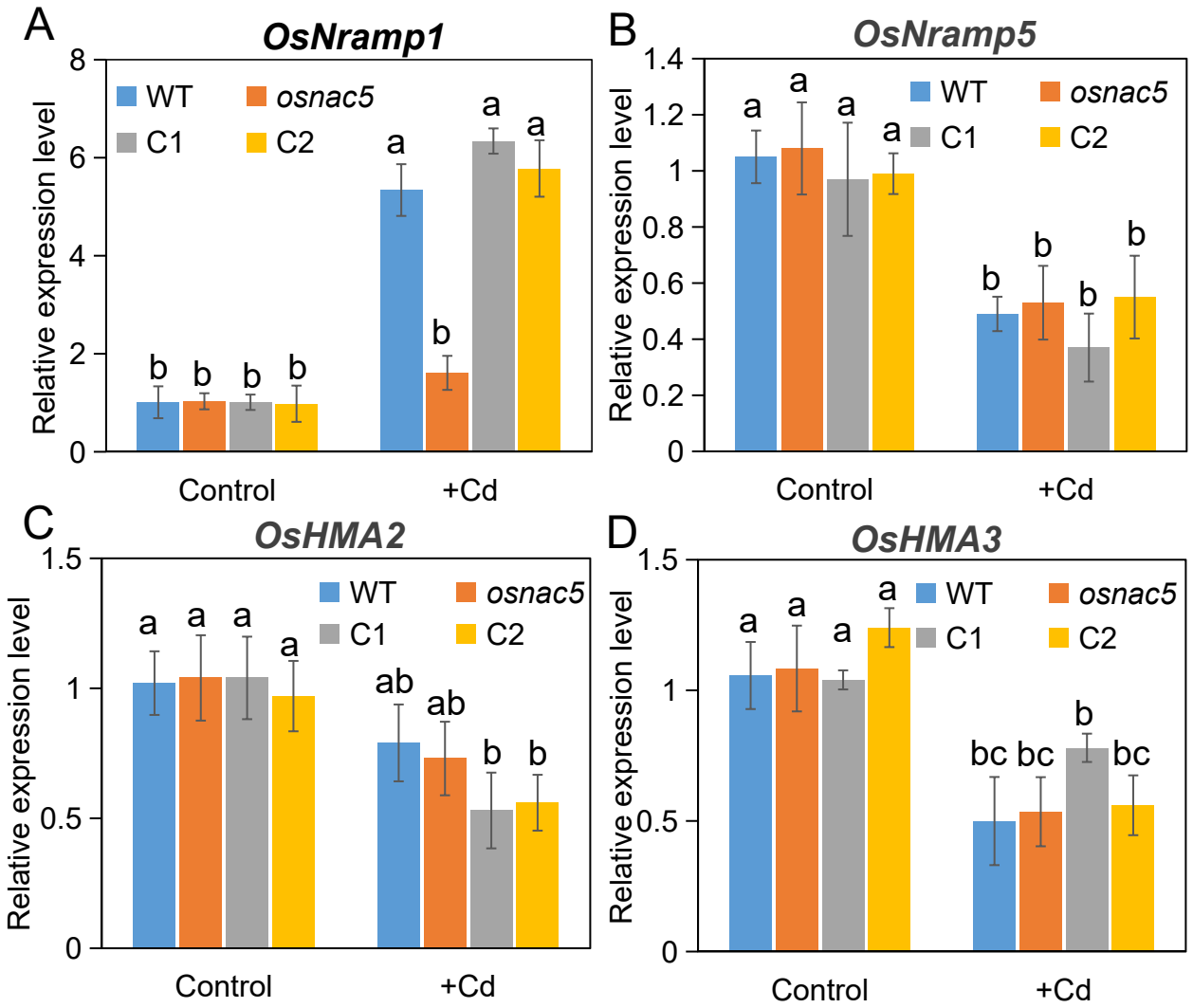

Fig. 5. Transcript levels of Cd transporter genes *OsNramp1* (A), *OsNramp5* (B), *OsHMA2* (C) and *OsHMA3* (D) in roots of Cd-treated rice plants. Two-week-old wild-type rice seedlings were exposed to 0.1 μM Cd(Control) or 10 μM Cd(+Cd) for 12 h. Real-time qPCR analysis was used to determine the relative 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).

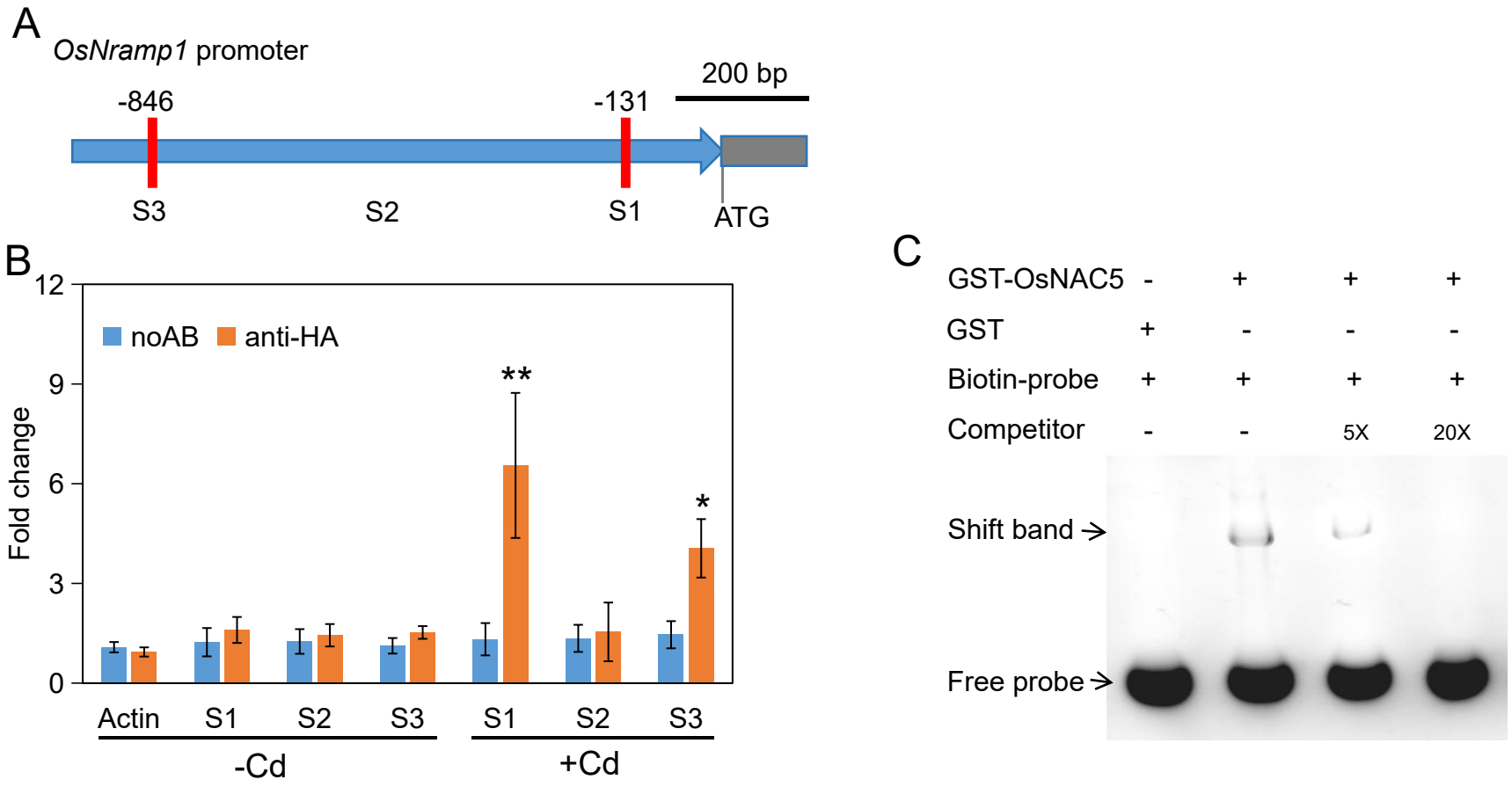

Fig. 6. OsNAC5 binds directly to the promoter of *OsNramp1*. (A) Diagram of *OsNramp1* promoter with two "CATGTG" motifs. Red vertical bars represents "CATGTG" motif. Site 1 and site2 indicates genomic DNA fragments around *OsNramp1* promoter for ChIP-qPCR. (B) ChIP-qPCR assay. Binding of OsNAC5 protein 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.

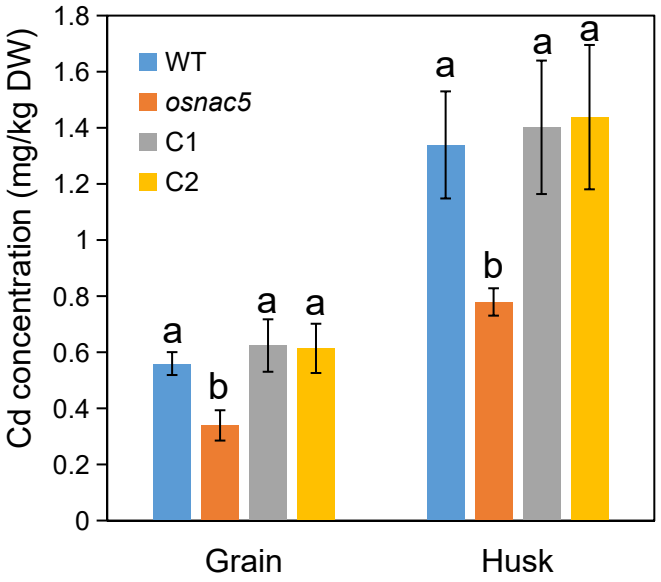

Fig. 7. The concentrations of Cd in rice grain (unpolished brown rice) and husk. Wild type (cv. ZH11) , *osnac5* mutant and two complementation lines(C1 and C2) grown in a Cd-contaminated paddy soil. Values are mean ± SD (n = 4). Different letters above bars indicate significant differences among rice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

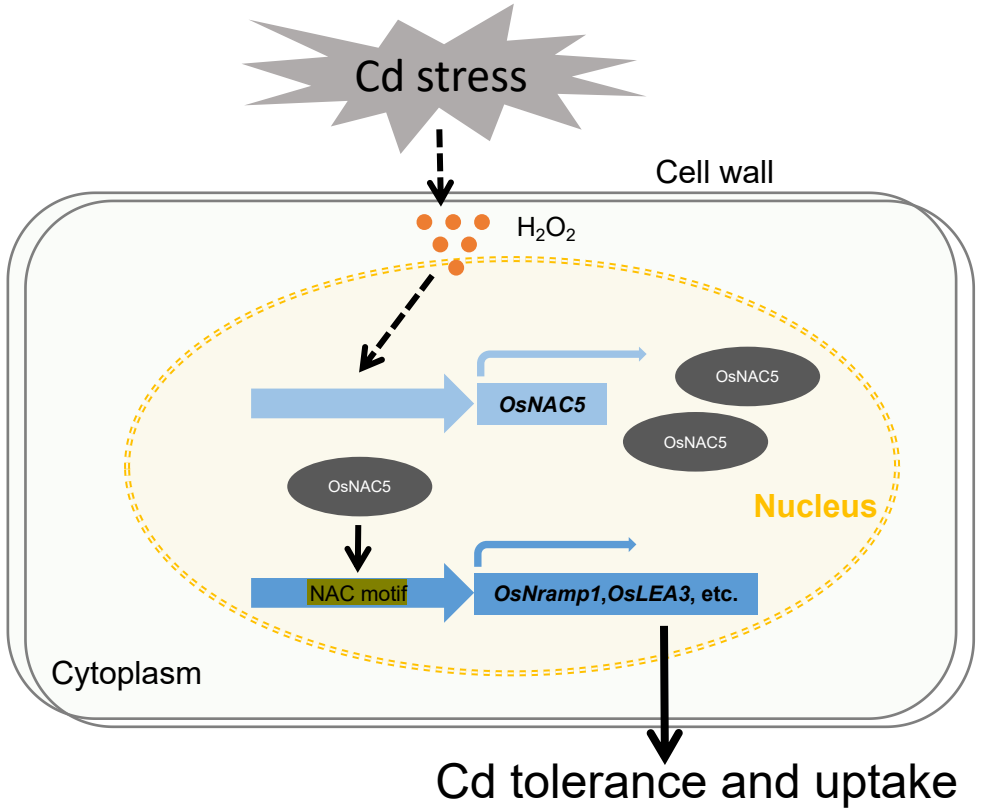

Fig. 8. Proposed working model for OsNAC5 regulating of Cd stress response. Cd stress induces an increase in OsNAC5 expression levels, which is dependent on hydrogen peroxide (H_2O_2) production triggered by Cd exposure. Subsequently, OsNAC5 directly upregulates the transcription of *OsNramp5* and *OsLEA3*,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