

Iron-based nanomaterials reduce cadmium toxicity in rice (*Oryza sativa* L.) by modulating phytohormones, phytochelatin, cadmium transport genes and iron plaque formation

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1 **Iron-based nanomaterials reduce cadmium toxicity in**
2 **rice (*Oryza sativa* L.) by modulating phytohormones,**
3 **phytochelatin, cadmium transport genes and iron**
4 **plaque formation**

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22 **Abstract**

23 Rice is known to accumulate cadmium (Cd) in its grains, causing a severe threat to billions of
24 people worldwide. The possible phytotoxicity and mechanism of 50-200 mg/L hydroxyapatite NPs
25 (nHA), iron oxide NPs (nFe₂O₃) or nano zero valent iron (nZVI) co-exposed with Cd (100 μM) in
26 rice seedlings were investigated. Three types of nanoparticles significantly reduced the
27 bioaccumulation of Cd in rice shoots by 16-63%, with nZVI showing the greatest effect, followed by
28 nHA and nFe₂O₃. A decrease in Cd content in the roots was observed only in the nZVI treatment,
29 with values ranging from 8-19%. Correspondingly, nZVI showed the best results in promoting plant
30 growth, increasing rice plant height, shoot and root biomass by 13%, 29% and 42%. *In vitro* studies
31 showed that nZVI reduced the content of Cd in the solution by 20-52% through adsorption, which
32 might have contributed to the immobilization of Cd in root. Importantly, the nZVI treatment resulted
33 in 267% more iron plaques on the root surface, which acted as a barrier to hinder the entry of Cd.
34 Moreover, all three nanoparticles significantly reduced the oxidative stress induced by Cd by
35 regulating phytohormones, phytochelatin, inorganic homeostasis and the expression of genes
36 associated with Cd uptake and transport. Overall, this study elucidates for the first time the multiple
37 complementing mechanisms for some nanoparticles to reduce Cd uptake and transport in rice and
38 provides theoretical basis for applying nanoparticles for reducing Cd accumulation in edible plants.

39 **Keywords:** Nanoparticles; Cadmium; Phytohormones; Antioxidant system; Iron plaque

Abbreviations

Abbreviation	Full name
NPs	Nanoparticles
TEM	Transmission Electron Microscope
CAS	Chinese Academy of Sciences
DI	Deionized water
ELISA	Enzyme linked immunosorbent assay
ZR	Zeatin riboside
DH-ZR	Dihydrozeatin riboside
IPA	Isopentenyl adenosine
BR	Brassinolide
IAA	Indole-3-acetic acid
GA3	Gibberellic acid 3
GA4	Gibberellic acid 4
JA-ME	Methyl jasmonate
ABA	Abscisic acid
SOD	Superoxide dismutase
POD	Peroxide dismutase
MDA	Malondialdehyde
CAT	Catalase
GSH	Glutathione
GSH-PX	Glutathione peroxidase
PCs	Phytochelatin
NPTH	Non-protein thiols

41 **1 Introduction**

42 Human health risks caused by heavy metals have attracted attention worldwide (Lu et al., 2015).
43 Cadmium (Cd) is a toxic heavy metal commonly found in agricultural soils (Rehman et al., 2018).
44 The World Health Organization (WHO) designated Cd and Cd-containing compounds as group I
45 carcinogens due to their high toxicity and bioaccumulation potential (International Agency for
46 Research on Cancer, 2012). Cd pollution in some countries such as China is particularly severe. The
47 risk threshold of Cd in China is 0.3 mg/ kg, while the over standard rate of Cd pollution in
48 agricultural soils is 7.0%, which is the highest inorganic pollutant (The Ministry Of Environmental
49 Protection and The Ministry Of Land Resources, 2014; Zhao et al., 2015). Cd in soil displays a long
50 decomposition cycle, high mobility and toxicity, resulting in reduced seed germination, plant growth
51 inhibition and yield reduction (Kaya et al., 2019; Kaya et al., 2020; Rossi et al., 2018). Moreover,
52 phytoaccumulation of Cd in the food chain may endanger human health (Zhou et al., 2022).
53 Therefore, there is an urgent need of innovative and sustainable strategies to control the absorption of
54 cadmium in edible crops.

55 Nanotechnology is the convergence of various sciences to provide disruptive technologies for
56 manipulating nano at the atomic scale, creating new structures and applying nanomaterials (Poole Jr
57 and Owens, 2003). Among them, nanoparticles (NPs) have been widely used in recent decades due to
58 their unique chemical and physical properties (Ali et al., 2021; Mansoor et al., 2022; Zhao et al.,
59 2022). Compared with traditional materials, NPs exhibit greater adsorption capacity, and catalytic
60 and magnetic properties (Adeel et al., 2021; Shakoor et al., 2022). A recent report has revealed that
61 the interaction between Cd and NPs and may alter the absorption, transfer and accumulation of Cd in
62 rice (Zhou et al., 2021). Ji et al., (2017) reported that 100 mg/L of titanium dioxide alleviated the
63 phytotoxicity of Cd, mainly because Cd was adsorbed by TiO₂. Similarly, (Wang et al., 2019)
64 demonstrated that CeO₂ NPs alleviated the Cd stress and mitigated DNA damage in rice.

65 Cd is non-essential and no specific transporter proteins were identified for Cd uptake by rice
66 (Tang et al., 2017). Instead, Cd uptake and transfer is mainly through the iron transporter, which
67 involves *NRAMP* and *IRT* family transporters (Yang et al., 2014). Therefore, the competition
68 between Cd and Fe absorb may cause an imbalance of Fe homeostasis in rice, leading to chlorosis of
69 leaves (Zhang et al., 2020). Improving Fe homeostasis in plants using iron-based NPs thus
70 potentially can help to relieve Cd phytotoxicity (Rizwan et al., 2016). In addition, the strong

71 adsorption of NPs for Cd may effectively reduce the bioavailability of Cd in the environment
72 (Anjum et al., 2019). Some NPs made of nutrient element (e.g., Fe in Fe-based NPs and Ca, P in
73 hydroxyapatite) may dissolve and release these nutrients thereby enhancing the tolerance of plants to
74 the Cd induced toxicity. Fe-based NPs could also reduce Cd uptake by regulating Fe uptake
75 transport-related proteins due to their shared transporters. Moreover, Fe^{2+} produced by the
76 dissolution of iron-based NPs can contribute to the formation of iron plaques in rice roots, which can
77 effectively prevent the uptake of Cd by plant (Sebastian and Prasad, 2016).

78 Three types of NPs including two Fe-based NPs (nano zero-valent iron (nZVI) and ferric oxide
79 (nFe_2O_3)) and one natural inorganic mineral NPs (hydroxyapatite, nHA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) were
80 chosen to evaluate their impact on Cd accumulation in rice tissues due to their known beneficial
81 effects on plants. For example, a whole life cycle culture study showed that Fe_2O_3 NPs reduced the
82 harm of Cd to wheat growth and yield (Adrees et al., 2020a). Due to its high elemental P, nHA
83 resulted in a 16.5 and 8-fold increase in aboveground and root fresh biomass of sunflower grown in
84 phosphorus-deficient soils (Xiong et al., 2018). In addition, soil application of nHA promoted potato
85 growth and reduced the Cd content in tubers by 17% compared to Cd treatment alone (Liu et al.,
86 2018). All three NPs exhibited excellent biocompatibility at environment-relevant concentrations and
87 were inexpensive, making them promising tools for Cd-contaminated soil. However, comprehensive
88 studies concerning the physiological and biochemical effects of NPs and Cd on rice are still lacking.

89 In this study, we hypothesized that the addition of NPs could alter the uptake, translocation, and
90 subsequent effects of Cd on rice, the most important food crop in many Asian countries (Zhang et al.,
91 2021). Specifically, we investigated the effects of different NPs on rice growth under Cd stress by
92 measuring a range of phenotypic and physiological parameters, including the biomass, root
93 morphology, photosynthetic system, and inorganic nutrients. The stress status and plant tolerance to
94 stress were also assessed by measuring the response of antioxidant systems and phytohormones in
95 plants. In addition, the adsorption capacity of different NPs for Cd, the blockage of Cd by iron
96 plaques and the expression of Cd transport genes were determined and their correlations with the
97 observed biological effects were investigated.

98 **2. Materials and methods**

99 *2.1 Characterization of NPs*

100 Fe₂O₃ NPs, nZVI and nHA were all obtained at Pantian Co., Shanghai. The
101 morphologies and primary sizes of all NPs were characterized using TEM (Tecnai
102 F20, FEI, USA). The zeta potential and hydrodynamic sizes of NPs in Kimura B
103 nutrient solution were analyzed with a Zetasizer Nano (ZS90, Malvern, UK). Detailed
104 characterization of the NPs is shown in **Figures S1 and S2**.

105

106 *2.2 Sorption of cadmium by NPs*

107 Briefly, NPs powder were added to a glass vial containing Cd solution and
108 subjected to ultrasonic treatment to disperse the NPs. After 14 days of incubation in
109 the same environment as the plant culture, 10 ml of suspension was taken from the
110 vial for the Cd determination. The detailed experiments are shown in Section 1, S1.

111

112 *2.3 Plant culture and NPs treatment*

113 Seeds (Y Liangyou 900) of rice (*Oryza sativa* L.) were acquired from the CAS.
114 Rice seeds were disinfected with 10% H₂O₂ for 20 min and flushed completely using
115 DI water. The seeds were placed evenly on a plastic tray containing a layer of gauze,
116 then covered with a sheet of filter paper and immersed in 15 ml of DI water. The tray
117 was then sealed with a film tray to avoid water loss. And germinate in a climatic
118 incubator under darkness for 7 days (25°C). Similarly sized seedlings were anchored
119 in sponge and planted into a plastic bottle with 250 ml ¼ strength Kimura B.
120 Seedlings were cultivated in a climatic incubator (25/22 °C and a 16 h photoperiod).
121 After 7 days of acclimation, rice seedlings were individually exposed to 50 and 200
122 mg/L nHA, nFe₂O₃ or nZVI for 14 days or 100 µM Cd + NPs. Seedlings treated with
123 100 µM Cd without addition of NPs were used as a control. Seedlings without any
124 treatment were used as blank control. The Kimura B was added regularly to an initial
125 volume of 250 ml every other day, followed by the determination of Cd levels in the
126 hydroponic system. At day 14, all seedlings were harvested and rinsed thoroughly
127 with 10% of nitric acid and DI water to remove the NPs and Cd attached on root

128 surface. After length and biomass measurements, fresh roots and leaves were stored at
129 -80°C for subsequent analysis.

130

131 *2.4 Root morphology and iron plaque extraction*

132 An automated scanner (Epson expression 1680, Japan) was used to scan the rice
133 roots, followed by analysis of root parameters using RHIZO software (Pro 2007d,
134 Quebec, Canada). The extraction of iron plaque on the root surface was performed by
135 the citric acid bicarbonate dithionate (DCB) method as described by Liu et al., (Liu et
136 al., 2006). Briefly, fresh roots were soaked in 20 ml of 125 mM NaHCO₃ and 30 mM
137 Na₃C₆H₅O₇ mixture, then added 0.4 g Na₂S₂O₄ powder. The mixture containing roots is
138 stirred and placed at 25 °C for 1 hour. The roots were rinsed with DI water, and all
139 eluents were collected and diluted to 50 ml with DI water, followed by Fe and Cd
140 measurements. The digestion and determination methods of elements (Cd and other
141 mineral elements) are shown in **Section 1, S2**.

142

143 *2.5 Determination of antioxidative system, metal chelators, chlorophyll content and* 144 *phytohormones*

145 The phytochelatin (PCs), glutathione (GSH), non-protein thiol (NPTH) that can
146 bind Cd²⁺ were measured by testing kit. MDA content and the activities of CAT, SOD,
147 POD and GSH-PX were determined using testing kit. Spectrophotometric methods
148 were used to determine chlorophyll in leaves. ELISA was used to quantify the level of
149 nine phytohormones in the roots, according to the previously described methods (Ma
150 et al., 2020). Detailed information is shown in **Section 1, S3, S4 and S5**.

151

152 *2.6 Extraction of subcellular Cd fractions*

153 Cells were isolated in three fractions three fractions using centrifugation
154 according to the method of Wan et al., (Wan et al., 2019). Briefly, the tissue samples
155 were homogenized in buffer and centrifuged at different speeds to collect three cell
156 fractions. These three parts were digested with nitric acid and determined by ICP-MS.
157 Details are presented in **Section1, S6**.

158

159 *2.7 RT-qPCR Analysis of Cd Transporters*

160 Total RNA extraction, complementary DNA synthesis and RT-qPCR were
161 conducted according to the method of Ma et al., (Ma et al., 2021). The relative
162 expression of four genes related to Cd absorption and transportation, including *IRT1*,
163 *Nramp5*, *HMA3* and *LCT1*, was computed by a $2^{-\Delta\Delta C_t}$ method. Details are presented
164 in **Section 1, S8**.

165

166 *2.8 Data analysis*

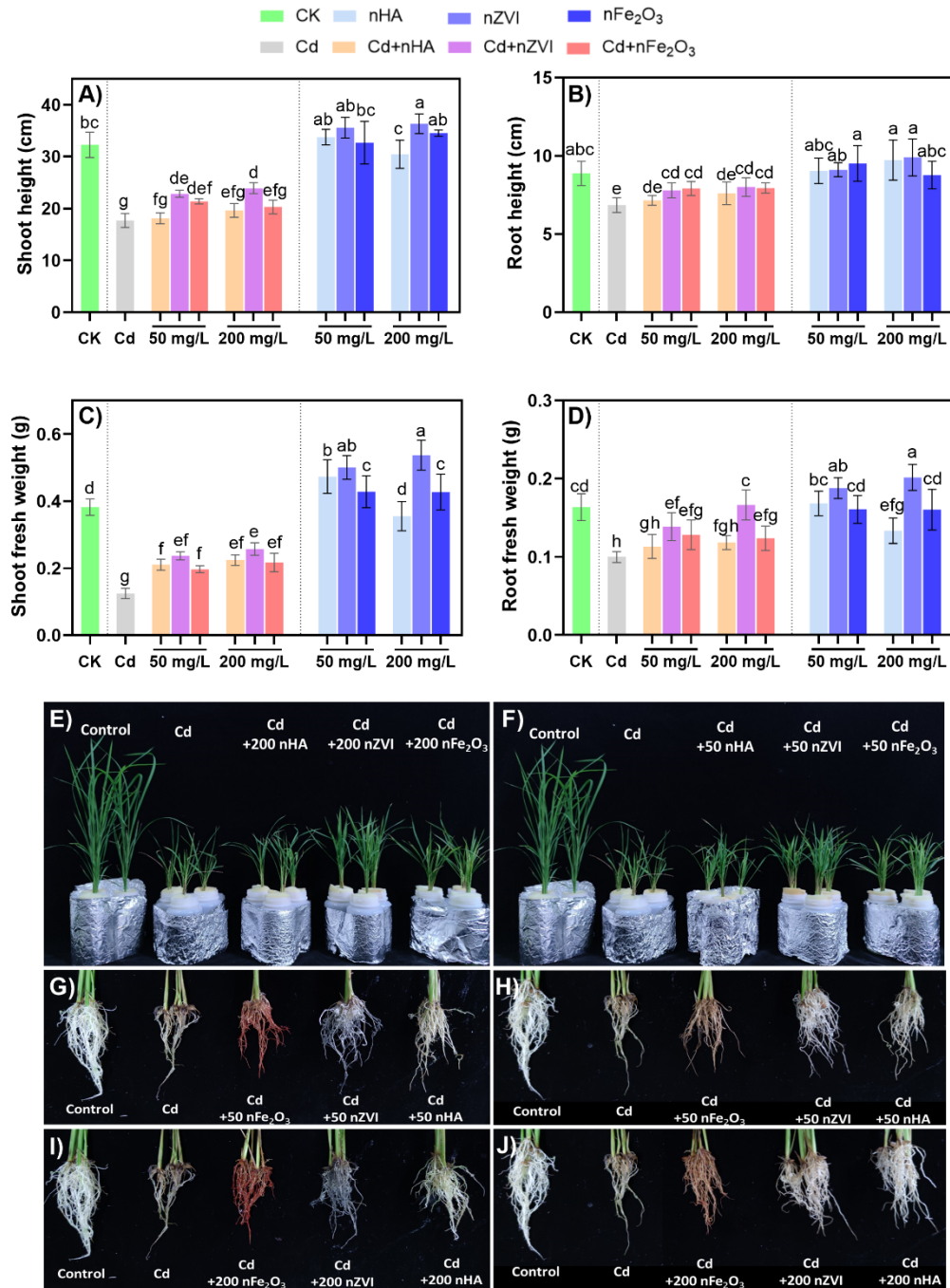
167 SPSS 20.0 was used to conduct the data analysis. Statistical significance was
168 executed through one-way ANOVA. The average values of each treatment were
169 compared using the Tukey's HSD at $p < 0.05$ confidence level.

170 **3 Results**

171 *3.1 Different impacts of nanoparticles on rice phenotypes under cadmium exposure*

172 All rice seedlings survived after 14 days of exposure (**Figure 1**). Inhibitive effect
173 was observed only for 200 mg/L of nHA treatment which slightly reduced the root
174 fresh weight by 7% (**Figure 1D**). nZVI showed stronger effects than other NPs both at
175 low and high concentrations, and 200 mg/L of nZVI increased the plant height, shoot
176 weight and root weight by 13%, 29% and 42%, respectively. Cd (100 μ M)
177 significantly inhibited the rice seedling growth. Cd treatment reduced the shoot and
178 root lengths, shoot and root biomass by 45%, 23%, 67% and 30%, respectively
179 (**Figure 1**). The presence of NPs mitigated the phytotoxicity of Cd and improved rice
180 growth parameters (e.g., biomass and root morphology) in a dose-dependent manner
181 (**Figure 1E, F and S3**). Specifically, all three NPs increased the fresh shoot and root
182 biomass by 58.27%-106.51% and 13.32%-66.52%, respectively, compared to the Cd
183 treatment (**Figure 1C, D**). Again, nZVI at 200 mg/L showed the strongest positive
184 effects on alleviating the Cd toxicity, resulting in 35%, 17%, 107% and 67% increase
185 in plant height, root length, shoot biomass and root biomass, correspondingly.
186 Furthermore, nHA and nZVI at 200 mg/L also improved all the root parameters

187 (Figure S4). In particular, nZVI at 200mg/L enhanced the total root length, average
 188 root diameter, total surface area and total root volume by 135, 82, 127 and 163%,
 189 respectively.

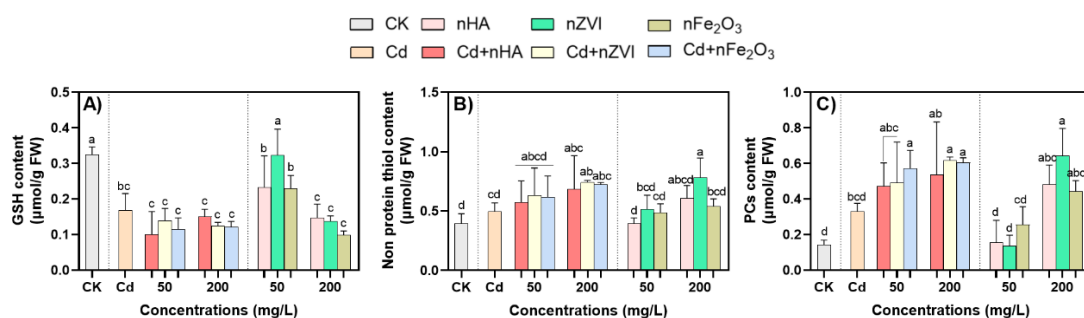


190
 191 **Figure 1.** Length (A and B) and fresh biomass (C and D) of rice seedlings cultivated
 192 to Cd, NPs or Cd + NPs for 2 weeks. Values indicate the mean \pm SD of 6 replicates.
 193 Significant differences at $p < 0.05$ are indicated by different lowercase letters.
 194 Representative physiological pictures of rice exposed to Cd and NPs. (E and F)

195 represent rice images as affected by 50 and 200 mg/L NPs for 14 days, respectively.
196 Root images of rice treated with Cd and NPs. (**G** and **I**) taken before cleaning NPs on
197 the root surface. (**H** and **J**) taken after cleaning NPs on the root surface.

198 ***3.2 Nanoparticles regulate photosynthesis, antioxidative system and metal chelators***

199 Compared with Cd treatment, the addition of NPs enhanced the leaf moisture
200 (77%-127%), Cha (52%-131%), Chb (109%-295%) and SPAD values (39%-82%).
201 High concentration of nZVI (200mg/L) showed the strongest positive effects,
202 resulting in an increase of 127%, 131%, 295% and 82% in leaf moisture, Cha, Chb
203 and SPAD values, respectively (**Figure S5**). MDA contents in shoots with Cd
204 exposure alone was significantly increased 48% relative to the control, which means
205 Cd exposure caused lipid peroxidation (**Figure S6A**). Cd stress activated the plant
206 antioxidant system, elevating POD, SOD and GSH-PX activities by 99%, 108% and
207 25%, respectively. Notably, the addition of NPs further elevated POD and SOD
208 activities by 169%-308% and 91%-209%, and reduced the MDA contents (65%-76%).
209 Additional discussions about photosynthesis and enzymatic antioxidant system are
210 provided in the **Section1, S9 and S10**. These results suggest that NPs treatments
211 enhanced the enzymatic antioxidant systems. Under Cd stress, the non-enzymatic
212 antioxidant system such as GSH and non-protein thiols (NPTH) were not significantly
213 altered by NPs supplement (**Figure 2A and 2B**), except for ZVI alone at 200 mg/L
214 enhanced NPTH level (98%). Previous evidence has suggested that phytochelatins
215 (PCs) play important roles in the detoxification of Cd in plants (Guha et al., 2020).
216 Interestingly, PCs in rice increased by 208-346% as compared with the control after
217 exposure to high concentrations of NPs alone (**Figure 2C**). The same trend in PCs
218 was also observed for Cd + NPs co-exposure, with nZVI showing the strongest effects.
219 Specifically, nZVI at 200 mg/L elevated the content of NPTH and PCs by 88% and 49%
220 compared to the Cd treatment (**Figure 2B and 2C**). nFe₂O₃ also significantly elevated
221 PC content (73%-84%), but it did not significantly elevate NPTH (24%-46%). The
222 above results indicate that the addition of NPs can modulates the antioxidant system
223 and PCs levels in plants, which protects plant from Cd stress.



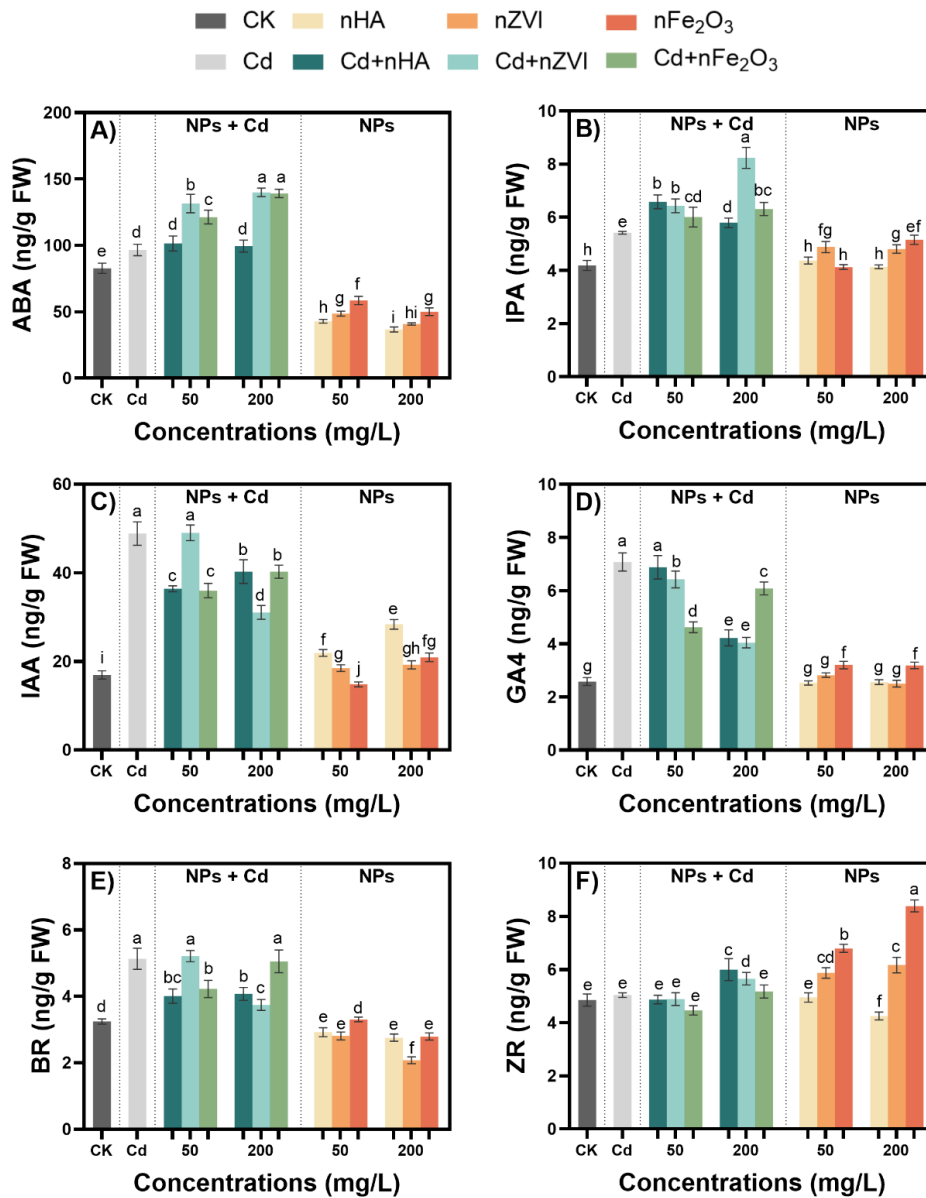
224

225 **Figure 2.** The content of GSH (A), Non protein thiol (B) and phytochelatin (C) in rice
 226 shoots exposed to Cd, NPs or Cd + NPs for 2 weeks. Values indicate the mean \pm SD
 227 of 4 replicates. Significant differences at $p < 0.05$ are indicated by different lowercase
 228 letters.

229 3.3 Regulation of phytohormones by nanoparticles

230 Under Cd stress, all phytohormones except for ZR were significantly up-
 231 regulated compared to the control (**Figure 3**). The phytohormones displayed similar
 232 trends for NPs alone and Cd+NPs except for ABA and ZR. The ABA content
 233 significantly decreased by 29-55% when exposed to NPs alone, indicating that the
 234 exposure to NPs alone did not cause a significant stress response. A previous report
 235 demonstrated that excess ABA was associated with plant growth inhibition
 236 (Brookbank et al., 2021). However, iron-based NPs resulted in a significant 20-72%
 237 increase of ZR, which is considered an antagonist of ABA, and our results are
 238 consistent with this trend (Zhang et al., 2021). Overall, a more significant up-
 239 regulation of ABA and IPA occurred in the case of Cd + NPs. The addition of nZVI
 240 and nFe₂O₃ significantly up-regulated ABA by 36%-45% and 25%-44% compared to
 241 the Cd control (**Figure 3A**). Similarly, nZVI and nFe₂O₃ at 200 mg/L resulted in a
 242 significant increase in IPA by 52% and 16% (**Figure 3B**), consistent with the
 243 physiological results. In contrast, a decreasing trend was observed for IAA, GA4 and
 244 BR after NPs exposure (**Figure 3C-3E**). Once again, nZVI at 200 mg/L showed the
 245 strongest effect, indicating its greatest effectiveness in mitigating Cd stress among the
 246 three NPs investigated in this study. For IAA, the addition of 200 mg/L nHA, nZVI
 247 and nFe₂O₃ decreased IAA by 17%, 36% and 17%, respectively (**Figure 3C**). Overall,
 248 our results suggest that some NPs can mitigate Cd toxicity, at least in part, by

249 regulating defense-related phytohormone levels.



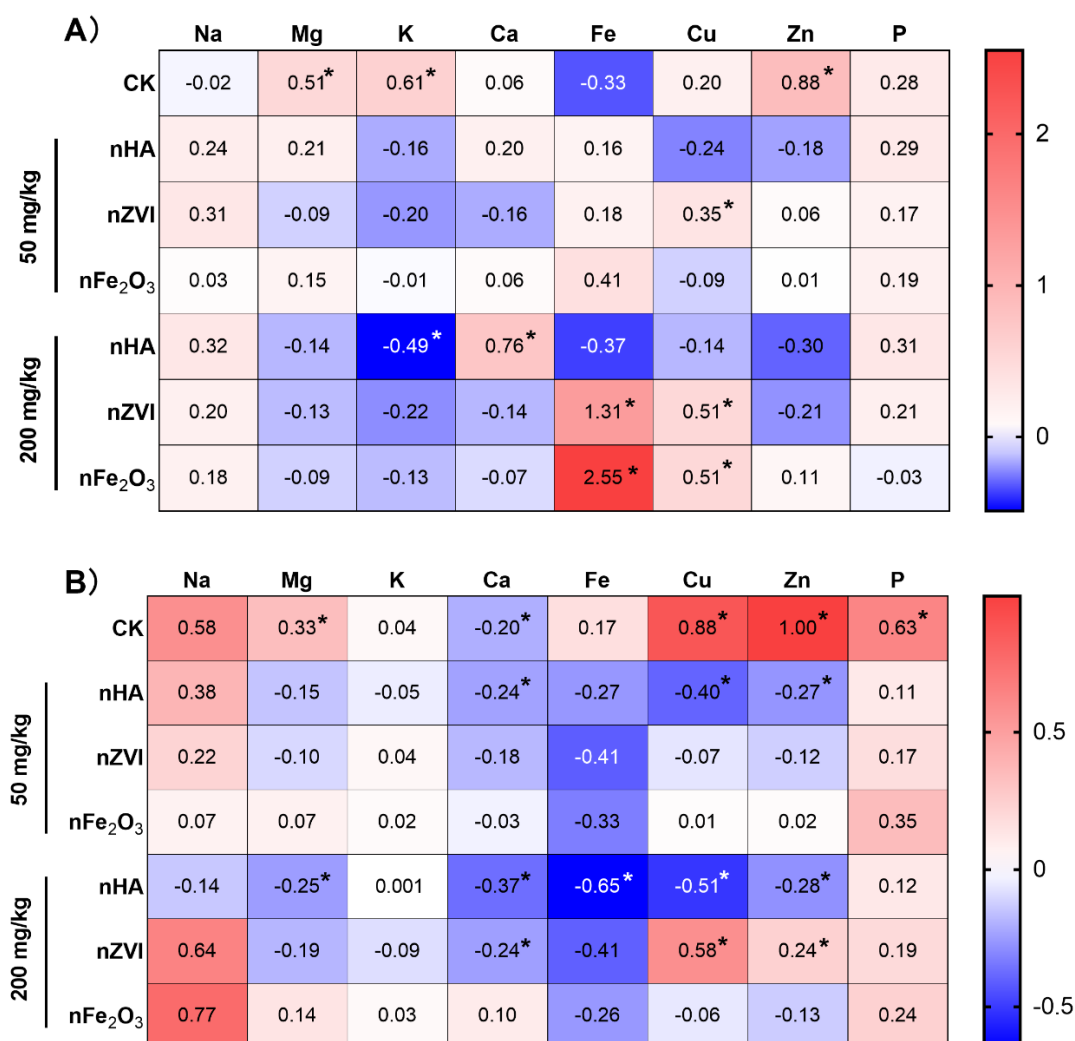
250

251 **Figure 3.** Phytohormone contents including ABA, IPA, IAA, GA4, BR and ZR in rice
 252 roots exposed to Cd, NPs or Cd + NPs for 2 weeks. Values indicate the mean \pm SD of
 253 4 replicates. Significant differences at $p < 0.05$ are indicated by different lowercase
 254 letters.

255 **3.4 Homeostasis of inorganic mineral nutrients**

256 The inorganic homeostasis was severely imbalanced in rice under Cd stress, resulting
 257 in significant alterations in three elements (Mg, K and Zn) in roots and five elements
 258 (Mg, Ca, Cu, Zn and P) in shoots (**Figure 4**). High concentrations of nZVI and

259 nFe₂O₃ resulted in 131% and 255% increases of Fe in roots (**Figure 4A**). Similarly,
 260 200 mg/L of nHA resulted in 76% and 31% increase in Ca and P in the root. However,
 261 the increase of P was not significant (29%-31%), probably due to the high content of
 262 P in the nutrient solution. Notably, both nZVI and Fe₂O₃ NPs at 200 mg/L resulted in
 263 a 51% increase in Cu content in the roots, with no effect on other inorganic elements
 264 except for Fe. Inorganic nutrient homeostasis in shoots was more sensitive to NPs
 265 (**Figure 4B**) than roots. In the treatment of Fe-based NPs, 200 mg/L nZVI resulted in
 266 58% and 24% increases in Cu and Zn elements. At 200 mg/L, nHA reduced the
 267 content of all five elements in the leaves, i.e., Mg, Ca, Fe, Cu, and Zn by 25%, 37%,
 268 65%, 61%, and 28%, respectively. The results suggested that the addition of nHA
 269 might have blocked the *in-planta* transport of inorganic nutrients from underground to
 270 aboveground.

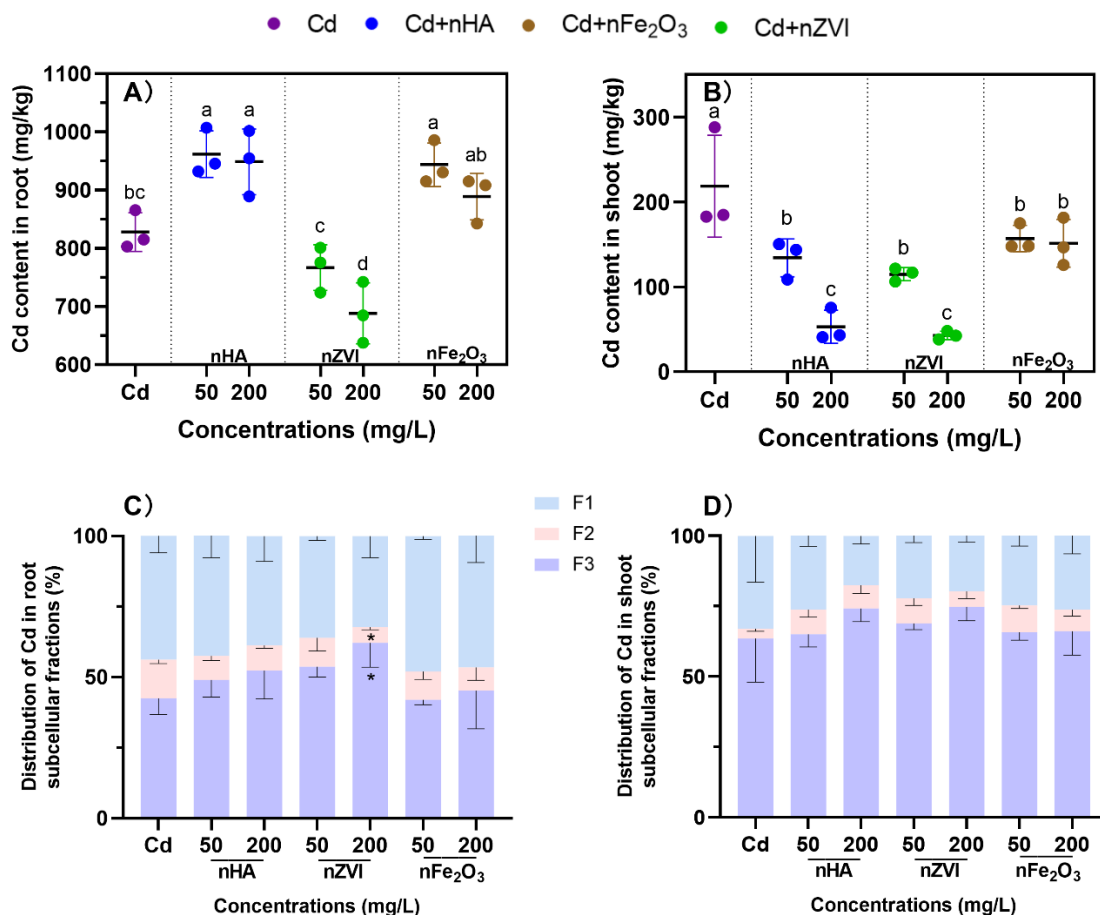


271

272 **Figure 4.** Thermogram illustrating the homeostasis of inorganic nutrients in rice root
273 (A) and shoot (B) modulated by Cd and Cd + NPs after two weeks. Numbers represent
274 the multiplicity of change in content of elements compared to the Cd alone treatment.
275 Negative values denote a decrease in content, while positive values are the opposite.
276 Values indicate the mean \pm SD of 6 replicates. Significant differences compared with
277 the Cd alone control at $p < 0.05$ (n=4) are indicated by *.

278 **3.5 Total cadmium and subcellular distribution in rice tissues**

279 The absorb and subcellular distribution of Cd in plant were further investigated
280 to explore the link between Cd accumulation and plant physiological regulators. Cd
281 accumulation in the roots of plants treated with Cd+NPs was not significantly reduced
282 except for the 200 mg/L nZVI treatment (**Figure 5A**). However, Cd accumulation in
283 the shoots was more significantly reduced after NPs exposure (**Figure 5B**).
284 Specifically, rice exposed to 200 mg/L nHA and nZVI resulted in a 75% and 80%
285 reduction in Cd in shoots. The effects of different NPs on Cd accumulation of shoots
286 upon exposure to Cd are consistent with the results of growth differences in **Figure 1**.
287 Further investigation of the mechanisms related to the effect of NPs on the absorb and
288 translocation of Cd was conducted by measuring the Cd subcellular distribution. As
289 shown in **Figure 5C-D**, the majority of Cd was in the soluble fraction (F3, 42–74%),
290 followed by the cell wall fraction (F1, 17–48%) and organelle fraction (F2, 4–13%),
291 both for shoots and roots. The soluble fraction consists mainly of vesicles, which are
292 the main location to preferentially bind Cd to minimize its damage to the cell wall or
293 entry into the organelle (Wang et al., 2009). Compared with Cd alone treatment, nZVI
294 at 200 mg/L significantly enhanced the percentage of Cd in soluble fractions of roots
295 by 20% and decreased the cell wall and organelles fractions by 8% and 12%,
296 respectively (**Figure 5C**). A similar phenomenon was observed in shoots (**Figure 5D**),
297 although the difference was not significant.



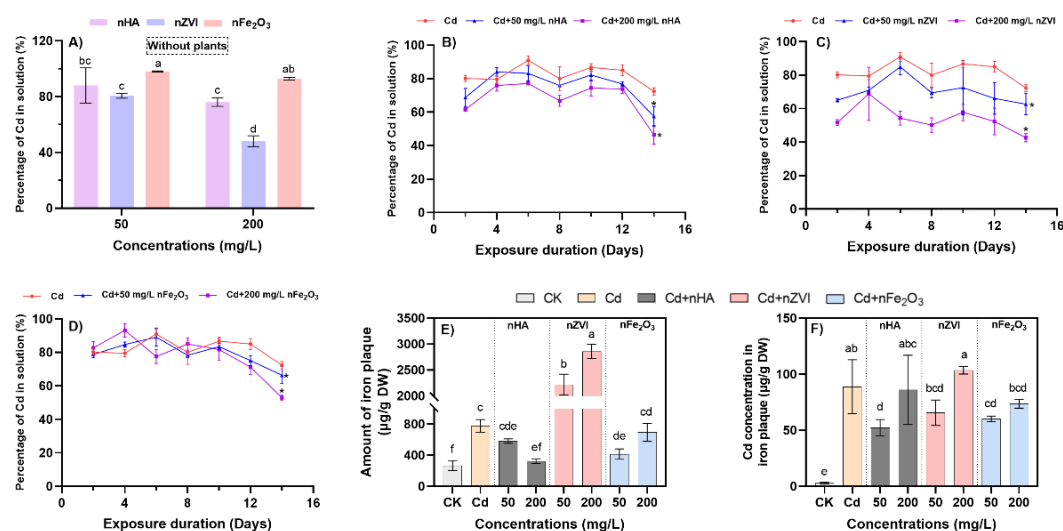
298

299 **Figure 5.** Total Cd content in rice seedlings (A-B). Cd in root (A), and shoot (B) after
 300 14 days of exposure to Cd + NPs. Cd distribution of subcellular components in root
 301 (C) and shoot (D). F1, F2 and F3 refer to the cell wall, organelle and soluble fractions.
 302 Values indicate the mean \pm SD of 3 replicates. Significant differences at $p < 0.05$ are
 303 indicated by * or different lowercase letters.

304 **3.6 Cadmium sorption in solution and cadmium content in iron plaque**

305 Dynamic chemical changes between Cd and NPs in the nutrient solution may be
 306 another important factor affecting Cd uptake by plants. The findings indicated that the
 307 sorption capacity of nFe₂O₃ for Cd was much less than that of nHA and nZVI, and
 308 only 3-8% of the initial Cd content in the nutrient solution was adsorbed on nFe₂O₃
 309 (**Figure 6A**). However, nHA and nZVI significantly reduced the Cd content in the
 310 nutrient solution by 12%-24% and 20%-52%, respectively. The zeta potential of NPs
 311 in nutrient solution is critical to the adsorption of NPs. Our data showed that the zero
 312 charge points (nHA, nZVI and nFe₂O₃) of the three NPs were 4.4, 4.9 and 7.3,

313 respectively (**Figure S2**). Therefore, at the beginning of Cd exposure, Cd²⁺ will
 314 strongly adsorb to the negatively charged nHA and nZVI through electrostatic
 315 interactions. However, nFe₂O₃ has a zeta potential of about +14.5 mV at pH 5.5, and
 316 the electrostatic repulsion prevents the approach of Cd and further interactions.
 317 Therefore, the adsorption capacity of nFe₂O₃ for Cd is much lower than the other two
 318 NPs. In addition to Cd adsorption via NPs, Cd uptake by plants is a primary process
 319 leading to the reduction of Cd in nutrient solutions (**Figure 6B-D**). The uptake of Cd
 320 by plants was around 28% of its initial concentration in the Kimura B containing only
 321 Cd. With the addition of 200 mg/L nZVI, the plant uptake of Cd only accounted for 6%
 322 of its initial content. The remaining 46% and 48% of Cd were either adsorbed by
 323 nZVI or retained in the nutrient solution, respectively, emphasizing the essential role
 324 of nZVI in reducing the Cd bioavailability (**Figure 6C**).



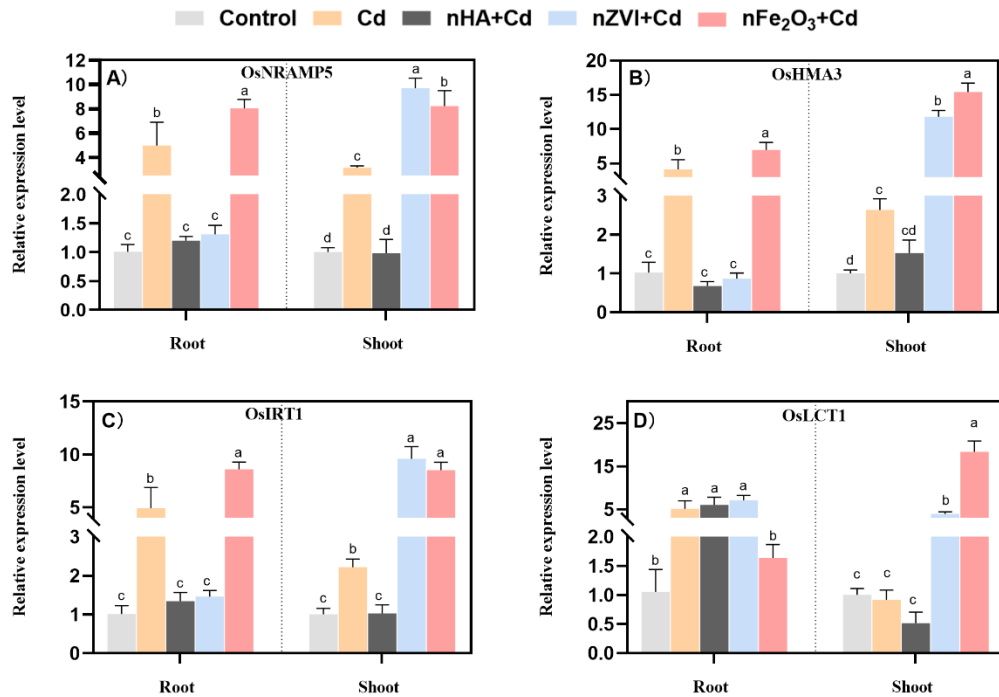
325
 326 **Figure 6.** The residual percentage of cadmium in nutrient solution after 14 days of
 327 exposure without plants (A). Curves of Cd concentration in the nutrient solution
 328 during the exposure with plants (B-D). (B) nHA; (C) nZVI; (D) nFe₂O₃. The amount
 329 of iron plaque (E) on the root surfaces and cadmium content in iron plaque (F) after
 330 14 days exposure. Values indicate the mean ± SD of 4 replicates. Significant
 331 differences at *p* < 0.05 are indicated by * or different lowercase letters.

332 In order to accommodate the flooded conditions, rice can deliver O₂ from the
 333 shoot to the inter-root environment, causing the oxidation of Fe²⁺ and Mn²⁺ to form a

334 reddish-brown iron oxide film (Iron plaque) (Amaral et al., 2017). The content of iron
335 plaque and the Cd in the plaques were determined to explore the effect of NPs on iron
336 plaque formation and how iron plaques influence the absorption of Cd by roots. The
337 total iron plaque amount was significantly increased by 190% in the Cd exposure
338 alone compared to the blank control. At 200 mg/L nHA, iron plaques were markedly
339 decreased by 58% compared to the Cd control (**Figure 6E**). Further analysis indicated
340 that the total iron plaque produced on the root surface was positive correlated with the
341 Cd content in the nutrient solution in the Kimura B for the non-Fe-based NPs added
342 system ($R^2 = 0.786$, **Figure S9**). For the system with Fe-based NPs addition, the total
343 amount of iron plaques was increased with increasing dose of Fe-based NPs. In
344 particular, nZVI significantly increased the mass of iron plaques by 185% and 267%
345 relative to the Cd control, which was in agreement to the results for the Cd content in
346 roots. In the presence of 50 mg/L nHA and nFe₂O₃, Cd levels in iron plaques were
347 significantly reduced by 32-41% relative to the Cd-exposed system alone; the
348 remaining treatments had no significant effect on Cd levels in iron plaques (**Figure**
349 **6F**). In addition, the Cd content in iron plaques was positively correlated with NPs
350 concentration. These discoveries indicate that the rise in iron plaques might be a
351 fundamental cause of the decline in Cd content in rice.

352 **3.7 Relative expression of Cd transporters**

353 The relative expression of Cd uptake and translocation-related proteins
354 (*OsNRAMP5*, *OsHMA3*, *OsIRT1*, and *OsLCT1*) in rice shoots and roots were
355 measured to explore the molecular mechanisms for altered Cd accumulation by NPs
356 (**Figure 7**). *NRAMP5*, a resistance- related macrophage protein, is known to be the
357 main transporter protein for Cd²⁺ and Fe²⁺ uptake in rice roots (Yang et al., 2014).
358 Similarly, Cd could be taken up through the Fe (II) transporter, the Fe -regulated
359 transporter 1 (*IRT1*) in rice roots (Lee and An, 2009). *OsHMA3* localized on the
360 tonoplast is a member of the heavy metal ATPase (*HMA*) family, which could
361 sequester Cd into vacuoles to reduce the transport of Cd to shoots (Lu et al., 2019).
362 Interestingly, we found that the expression of *OsNRAMP5*, *OsHMA3* and *OsIRT1*
363 genes showed a similar trend both in the roots and shoots. Expression of these three
364 genes in the roots of Cd treated rice was upregulated about 500%, whereas co-
365 exposure to nHA and nZVI with Cd restored their expression back to control levels.
366 Surprisingly, co-exposure to nFe₂O₃ with Cd caused an approximately 800% increase
367 in *OsNRAMP5*, *OsHMA3* and *OsIRT1* expression in rice roots (**Figure 7A, B, C**). To
368 better understand the potential effects of NPs on Cd accumulation in the aboveground
369 tissues, the expression of low-affinity cationic transport protein (*LCT1*) was
370 determined. *LCT1* regulates the transport of Cd through the roots to the aboveground
371 tissues (Uraguchi et al., 2011). An approximately 500% increase in expression was
372 observed in roots in all treatments except for the Cd+nFe₂O₃ exposure (**Figure 7D**).
373 However, nZVI and nFe₂O₃ up-regulated *LCT1* expression in shoots by approximately
374 500% and 1800%, respectively.



375

376 **Figure 7.** Relative expressions of genes involved in Cd assimilation and transport in
 377 rice root and shoot under Cd + 200mg/L NPs application. *NRAMP5* (A), *HMA3* (B),
 378 *IRT1*(C), *LCT1* (D). Values indicate the mean \pm SD of 4 replicates. Significant
 379 differences at $p < 0.05$ are indicated by different lowercase letters.

380 4 Discussion

381 4.1 Regulation of plant physiological and biochemical systems by NPs

382 The NPs treatment alone either did not affect or increased the plant height and
 383 biomass except for nHA, whereas Cd treatments significantly inhibited rice growth.
 384 All different types of NPs reduced the phytotoxicity of Cd and improved the growth
 385 parameters of rice in a dose-dependent manner. Both at low or high concentrations of
 386 treatment, nZVI showed the best results for growth promotion. Similar results were
 387 also observed in one of our previous Fe-based NPs studies, where the “fertilizer effect”
 388 and “nano stimulus effect” of nZVI treatment resulted in a rise in chlorophyll,
 389 hormone regulation, and enhanced expression of iron transporter protein in rice (Li et
 390 al., 2021). To investigate the potential mechanisms of NPs to alleviate Cd stress, we
 391 conducted studies on both the physiological and biochemical systems of plants, the

392 formation of iron plaque and the adsorption behavior of NPs.

393 Cd is known to cause membrane lipid peroxidation and inhibit photosynthesis
394 (Ma et al., 2020; Rossi et al., 2017). Our results suggest that the enhanced biomass
395 and seedling elongation may be partly attributed to the enhanced photosynthesis by
396 NPs treatments. Compared with Cd treatment, the addition of NPs enhanced the leaf
397 moisture, chlorophyll content and antioxidative system. The positive effects of NPs
398 might be related to the release of mineral elements (Fe, P, Ca) which can support
399 photosynthesis and plant growth. Compared to conventional ion treatment, the slow-
400 release effect of nanoparticles can effectively avoid plant toxicity caused by a large
401 supply of ions in a short period of time (Cao et al., 2022). Iron is the central metal for
402 several vital enzymes (e.g., cytochromes) involved in electron transfer during
403 photosynthesis (Tagawa et al., 1963). Similar results were reported previously that
404 foliar application of 20 mg/L nFe₂O₃ increased chlorophyll a, b and carotenoid
405 concentrations by 70%, 139% and 119%, respectively (Hussain et al., 2019). Similarly,
406 P is a component of chloroplast bilayer membrane, grana and adenosine triphosphate
407 (ATP), which plays an essential role in photosynthesis energy conversion (Veneklaas
408 et al., 2012). The increase in photosynthetic pigments caused by the supply of mineral
409 elements indirectly led to an increase in photosynthetic rate, thus enabling rice to
410 accumulate more organic matter (Kaya et al., 2020).

411 Under the Cd stress, plants produce excessive harmful reactive oxygen species
412 (ROS), leading to an increase in MDA content, an important biomarker of lipid
413 peroxidation (Zhou et al., 2022). We found that the MDA content was relatively high
414 in rice shoots not treated with NPs due to heavy metal toxicity. The addition of all
415 types of NPs significantly increased the POD and SOD activity, followed by the
416 reduction of MDA contents, indicating that the addition of NPs could further trigger
417 the defense mechanism of antioxidant enzymes. In agreement with our study, nZVI
418 has been shown to increase SOD and POD in sunflower leaves, followed by the
419 decrease of MDA (Micháľková et al., 2017). Adress et al., (2020b) also demonstrated
420 that the soil application of iron-based NPs promoted wheat growth and the SOD and

421 POD activities under Cd stress. Recent studies reported the specificity of NPs, not
422 only as key substances for regulating ROS homeostasis, but also for directly
423 scavenging excess ROS to enhance crop stress resistance (Altaf et al., 2022; Zhao et
424 al., 2022). We also explored non-enzymatic antioxidant systems, but the differences
425 after NPs treatment were not significant (NPTH), suggesting that enzymatic
426 antioxidant systems are the main mechanism of action. GSH further decreased after
427 treatment with NPs, demonstrating the involvement of GSH in the elimination of ROS
428 and alleviation of oxidative stress (Kaya et al., 2021). In addition, the important role
429 of PC in the detoxification of plants against Cd was clearly indicated in several
430 reports (Guha et al., 2020; Kaya et al., 2022). PC could form complexes with toxic
431 metal ions in cytoplasmic solution and subsequently translocate them to the vesicles
432 (Yadav, 2010). A significant rise in PC was observed in the case of Cd + NPs co-
433 exposure, with nZVI showing the strongest effect. Notably, the PC-induced rise in Cd
434 content in the vesicles was consistent with that observed in the subcellular fractions,
435 which provides strong evidence that NPs induced PC production is one of the
436 important mechanisms for the mitigation of Cd stress. Overall, our results suggest that
437 regulation of the enzymatic antioxidant system and PC production are important
438 mechanisms for NPs-induced relief of Cd stress.

439 Phytohormones are crucial in plant growth and their regulation is an important
440 mechanism for abiotic stress alleviation (Wani et al., 2016). The present study
441 identified the important role of ABA and IPA in the mitigation of Cd stress. ABA is a
442 'stress phytohormone', which is typically increased when plants tried to cope with
443 stresses (Vishwakarma et al., 2017). The supplementation of NPs under Cd stress led
444 to a further increase of ABA indicating that NPs could enhance the Cd stress tolerance
445 of rice. IPA is a plant growth regulator with similar biological activity as IAA but is
446 defined as a cytokinin (Mano and Nemoto, 2012). Interestingly, the adding of NPs
447 under Cd stress seemed to promote the synthesis of IPA. This might be attributed to
448 the NPs promoted production of new rice roots which contain large number of
449 cytokinin. It is important to point out that the contents of phytohormones in plants are

450 often interrelated. For instance, GA4 is usually positively correlated with the content
451 of IAA. The IAA content is also correlated with BR because IAA is regulated by BR
452 through the inhibition of the auxin oxidase activity. These connections among the
453 three phytohormones explained the similar trend observed in this study (Kandhol et
454 al., 2022; Tripathi et al., 2022; Wang et al., 2022). In addition to those reported in the
455 present study, some previous reports have also demonstrated that NPs can modulate
456 plant phytohormones to alleviate environmental stress, such as TiO₂ NPs alleviated
457 Cd stress (Ji et al., 2017) and CeO₂ NPs alleviated N overload or deficiency (Wang et
458 al., 2020). Undoubtedly, further studies are required to gain more insight into Cd and
459 NPs induced phytohormonal responses to optimize this potentially important abiotic
460 stress management strategy.

461 Cd stress leads to a serious imbalance in plant mineral element homeostasis,
462 which may be one of the important causes of stunting in rice. We found that the Cd
463 content in rice decreased after the addition of NPs, and correspondingly the mineral
464 homeostasis was altered. This may be partly due to the uptake of ions by plants in the
465 form of chemical equivalents, and the competing uptake between ions (Cui et al.,
466 2008; Qin et al., 2020). In addition, previous reports also showed that the small size of
467 NPs may affect ion transporters, thus changing the mineral homeostasis (Zhou et al.,
468 2021). Thus, the reduction of Cd induced by nZVI (**Figure 5B**) may have elevated the
469 concentrations of Cu and Zn in shoots. Interestingly, nHA displayed different effects
470 on nutrient homeostasis in plant tissues from Fe-based NPs. We found a further
471 reduction of the five mineral elements in the stems resulting from the addition of high
472 concentrations of nHA compared to the Cd treatment alone. The greater interference
473 of ion transport by nHA than nZVI might be an important reason why nHA was less
474 effective than nZVI in alleviating Cd stress. The growth and development of rice are
475 dependent on a variety of inorganic nutrients. Deficiency or excess of these elements
476 can even lead to poor development (Zhang et al., 2021). The results indicate that the
477 regulation of inorganic nutrient homeostasis may be another meaningful way for NPs
478 to alleviate Cd stress. High concentrations of nZVI significantly elevated the Fe and

479 Cu contents in the roots and Cu and Zn elements in the shoots to a comparable level
480 in the control plants. Despite the rise of P after nHA addition, nHA might result in a
481 further imbalance in the homeostasis of the remaining inorganic nutrients. Fe is an
482 essential component of Fe oxygen reduction protein in plants, which is involved in
483 plant photosynthesis, nitrate reduction, biological nitrogen fixation and other electron
484 transfer (Hussain et al., 2019). Similarly, Cu plays a crucial role in electron transfer
485 during photosynthesis, and it is as well an essential part of SOD (Ma et al., 2020).
486 Therefore, the high Fe and Cu content induced by nZVI treatment may effectively
487 promote photosynthesis, improve nutrient transport (Pishkar et al., 2022) and promote
488 growth of rice under Cd stress.

489 ***4.2 Regulation of iron plaques and influence on Cd uptake and distribution by NPs***

490 The results of Cd uptake and distribution showed that co-exposure of nZVI with
491 Cd induced significantly lower Cd accumulation in the rice compared to the other two
492 NPs. It should also be noted that all NPs except nZVI resulted in elevated Cd content
493 in the roots, which may be due to the entrance of Cd adsorbed on NPs (This fraction
494 of cadmium or other elements can be desorbed again in the plant). However, this
495 fraction of Cd will be mostly retained in the roots because of the low upward transport
496 of NPs, resulting in a relatively high Cd content in the root compared to the shoot.
497 Gong et al. (2017) also demonstrated that nZVI at 1 µg/L promoted Cd accumulation
498 by 50% in roots of ramie. The significant reduction of Cd in shoots when Cd and NPs
499 were co-exposed suggests that the effect of NPs in mitigating phytotoxicity is a
500 function of reduced Cd absorb and translocation. The Cd content in the root system
501 was significantly reduced by nZVI and most of the residual Cd was translocated to the
502 vesicular fraction. In addition, the high concentration of nZVI significantly enhanced
503 the length and weight of the root system, indicating that it effectively relieved the Cd
504 stress in rice roots. Interestingly, more severe Cd toxicity may significantly lower Cd
505 in the soluble fraction in plant roots, resulting in more Cd accumulation in the cell
506 walls than shoot (Lu et al., 2017). The constituents of plant cell walls could provide
507 numerous potential ligands with different functional group, like hydroxyl, carboxyl,

508 aldehyde and amino groups (Wang et al., 2009), that can provide adsorption sites or
509 form complexes with Cd. Compared with Cd alone treatment, the relatively higher
510 integrity of the cell wall treated with nZVI reduced the number of Cd ions entering
511 the organelles, thus reducing the damage of Cd to the root cells. It should be noted
512 that the destructive method (Differential centrifugation method, detailed in
513 Supplementary materials S6) that used in this study may cause redistribution of Cd in
514 vacuole to cell walls during the sample preparation. However, this potential drawback
515 may not significantly change the overall observations in this study even though it may
516 underestimate the actual soluble fraction of Cd in the vacuoles.

517 The adsorption results further demonstrated that the final Cd concentration in the
518 Kimura B after 14 days of nZVI exposure was much lower than that of nHA and
519 nFe₂O₃, which can be attributed to the strong adsorption capacity of nZVI for Cd. The
520 surface charge of NPs and the solution pH are important factors affecting the uptake
521 of NPs. The adsorption capacity of nFe₂O₃ for Cd is much lower than that of the other
522 two NPs due to electrostatic repulsion (nFe₂O₃ has a zeta potential of about +14.5 mV
523 at pH 5.5) (Gao et al., 2004). Plants may release protons or alkaline secretion to
524 balance Cd uptake as a response to environmental stress, which would also alter
525 nutrient solution pH, Cd morphology, and NPs adsorption effects (Ryan et al., 2001).
526 In addition, the nanomaterials themselves also have the potential to alter the
527 environmental pH (Adeel et al., 2021; Shakoor et al., 2023). pH is a key factor
528 affecting the bioavailability of Cd, which usually shows a tendency to enhance
529 bioavailability with decreasing pH (Van Gestel and Koolhaas, 2004). High
530 concentration of nZVI induced a slight increase in the pH of rice solution compared to
531 the initial pH, while the opposite was observed for nFe₂O₃ and nHA treatments,
532 suggesting that nZVI can alleviate Cd stress by changing the pH. For a detailed
533 discussion of the dynamics of pH in solution see Section 1, S11.

534 Iron plaque function as a barrier to prevent the entry of phytotoxic compounds
535 such as Cd into plant roots (Yu et al., 2020), and reduce their uptake by roots (Bao et
536 al., 2019). The major constituents of iron plaques are reported to be fibrous iron ore

537 (γ -FeOOH) and needle iron ore (α -FeOOH), which easily incorporate Cd via hydroxyl
538 groups (Chen et al., 2017). Significant increase in iron plaques content when exposed
539 to Cd alone. The reason may be that the plants were stimulated to promote iron plaque
540 production under a high concentration of Cd stress, thereby alleviating the oxidative
541 stress effect. Similarly, (Wu et al., 2021) demonstrated a positive correlation between
542 the As content and the iron plaques. For the system with Fe-based NPs addition, the
543 total amount of iron plaques was further increased with increasing dose of Fe-based
544 NPs, indicating that the rise in iron plaques might be a fundamental cause of the
545 decline in Cd content in rice. It is noteworthy that the effect of reddish-brown iron
546 plaques caused by 200 mg/L nFe₂O₃ was seen to be more pronounced in the pictures
547 (**Figure 1G**), yet the highest iron concentration in the DCB extracts was in the nZVI
548 group. Some of the NPs are difficult to be washed by running water or have entered
549 the epidermal plant cells. Therefore, we speculate that the DCB may extracted this
550 part of iron-based NPs, leading to the high iron plaques concentration. In conclusion,
551 our study suggests that iron plaques are a natural and effective barrier to Cd
552 accumulation in the rice root system and nZVI can further strengthen this barrier to
553 lower Cd accumulation in rice shoots.

554 ***4.3 Regulation of iron transport protein expression by NPs***

555 The mechanism was further determined by measuring the genes for Cd uptake and
556 translocation. We found that the expression of three genes, OsNRAMP5, OsHMA3,
557 and OsIRT1, returned to control levels after nHA and nZVI treatments, indicating that
558 Cd uptake was effectively inhibited. However, these three genes were further elevated
559 after exposure to nFe₂O₃, which is consistent with the results of Cd accumulation in
560 rice. The release of Fe ions from nFe₂O₃ is likely a reason for the increase of the
561 expressions of these proteins (**Figure S8A**). Importantly, the protein expression data
562 align with the low Cd accumulation in rice shoots exposed to Cd+NPs, as compared
563 to Cd treatment. The expression of LCT1 in shoots showed a striking increase under
564 nFe₂O₃ induction, emphasizing the overload of Fe transporter proteins (LCT1). It is
565 speculated that the release of Fe ions from Fe-based NPs may have caused the up-

566 regulation of *LCT* in shoots due to the positive correlation of *LCTI* expression and
567 iron ions(Huang et al., 2022). These results demonstrated that nZVI and nHA NPs
568 suppressed Cd-induced expression of Cd uptake and transport genes in rice roots
569 under Cd stress. Therefore, 200 mg/L nZVI could be an efficient agent to lower Cd
570 accumulation in rice seedlings.

571 ***5 Conclusions***

572 Exposure to the three NPs alone did not have any significant adverse effects on
573 rice development, except for high concentrations of nHA. Under Cd stress, the
574 amendment of all three NPs significantly reduced the phytotoxicity of Cd in rice. The
575 NPs reduced the bioavailability of Cd and mitigated its phytotoxicity a range of
576 mechanisms including adsorption, promotion of iron plaque formation, activating
577 plant antioxidant defense systems, regulation of PC production, hormone homeostasis,
578 mineral homeostasis, and Cd transporter protein expression. In this study, nZVI at 200
579 mg/L was the strongest in decreasing phytotoxicity and Cd accumulation in rice,
580 mainly by enhancing Cd chelation in vesicles and reducing Cd transport capacity..
581 Thus, nZVI serves as a sustainable and safe nano-strategy to reduce the accumulation
582 of Cd in food crops. Since Cd accumulation in seeds is a significant human health
583 concern, the findings in this study offer important implications for the influence of
584 nanotechnology on food safety. The present study elucidated the mechanisms of
585 altered Cd uptake, translocation and accumulation by three different NPs and
586 highlighted their potential roles in lowering Cd risks from food consumption. More
587 work focusing on the interaction of NPs and heavy metals in complex environmental
588 media over long periods will further illustrate the potential of this novel sustainable
589 agricultural strategy.

590

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596

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603

604 ***Conflict of interest***

605 The authors declare no conflict of interest.

606

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