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OsNRAMP2 facilitates Cd efflux from vacuoles and contributes to the difference in grain Cd accumulation between *japonica* and *indica* rice

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ABSTRACT

Cadmium (Cd) accumulation in rice grain is of health concern. Identifying genes involved in grain Cd accumulation and performing molecular breeding may reduce it. In this study, knockout of *OsNRAMP2*, a member of the *NRAMP* family, reduced grain Cd concentrations by more than 38%, and overexpressing *OsNRAMP2* increased grain Cd concentrations by more than 50%. Physiological experiments showed that *OsNRAMP2* facilitated Cd translocation from root to shoot by positively regulating Cd efflux from the vacuoles. At filling stage, *OsNRAMP2* was highly expressed in all tissues except for husk, suggesting its role in Cd remobilization. Changes in *OsNRAMP2* expression affected the concentrations of Fe, Mn, Zn, and Cu in grain and also affected rice growth. Phylogenetic analysis showed that the distribution of *OsNRAMP2* haplotypes between *japonica* and *indica* was different. Among the four haplotypes of *OsNRAMP2*, Hap 1, with a 6-bp nucleotide insertion in exon 1, had grain Cd concentration at least 45.3% lower than any of the other three haplotypes. Almost all (99.3%) *japonica* accessions but rare *indica* accessions (4.44%) from the 3K sequenced rice genomes carry Hap 1 of *OsNRAMP2*. Our study sheds light on the molecular mechanism of grain Cd accumulation and provides a promising target for low-Cd rice breeding.

1. Introduction

Cadmium (Cd) is a heavy metal harmful to all organisms and widely released in the environment. Cd exposure has been associated with various physical problems in human including cancer, heart disease, vascular problems, osteomalacia, and bone fractures [1,2]. Rice, a staple food for more than half of the world's population, is more likely than other crops to accumulate Cd and is a major source of Cd intake [3]. Limiting grain Cd accumulation in rice would reduce human health risks.

Agronomic practices such as fertilizer, water and tillage management, microbial remediation, and bioremediation have been used to reduce Cd accumulation in rice [4–8]. However, breeding for rice with reduced Cd accumulation may be a more economical and effective way to address this problem. Rice Cd accumulation is complex, depending on absorption from soil, translocation from root to shoot, and remobilization. These processes are controlled by various genes [9]. *OsIRT1* and *OsIRT2* [10], *OsNRAMP1* [11], *OsNRAMP5* [12], *OsCd1* [13], and *OsZIP5* and *OsZIP9* [14] have been confirmed to be responsible for Cd uptake in rice. OsHMA3, a heavy metal ATPase located on the vacuolar membrane, controls root-to-shoot Cd translocation by mediating Cd compartmentation into vacuoles [15]. OsHMA2, an efflux-type metal transporter expressed in root stele and located on the plasma membrane, drives Cd loading into the xylem [16]. The defensin-like protein gene, *CAL1*, which is expressed mainly in root exodermis and xylem parenchyma cells, facilitates long-distance Cd transport via xylem vessels [17]. *OsCCX2*, expressed specifically in xylem nodes, may function in loading Cd into xylem vessels and mediate root-to-grain transport [18]. OsLCT1, a plasma membrane-localized efflux transporter, regulates phloem Cd transport in nodes and leaf blades of rice [19]. Cd in shoots distributed to grain is also regulated by OsHMA2, OsZIP7, OsPCR1, LCD, OsPCS1 and OsPCS2 [16,20–24]. Although

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progress has been made in discovery of genes and mechanisms associated with Cd accumulation in rice, the known functional genes are still limited and the mechanisms of Cd translocation, in particular to grain, remain poorly understood. Grain Cd accumulation is generally lower in *japonica* than in *indica* cultivars [25], but the genetic basis of this difference remains elusive.

In our previous study [26], a novel major QTL (*qCd3-2*) for grain Cd accumulation was identified in a genome-wide association study of a diverse rice panel. Integration of sequence comparison and yeast experiments identified *OsNRAMP2* as a candidate gene underlying *qCd3-2*. In the present study, we further confirmed the function of *OsNRAMP2* in grain Cd accumulation and investigated the gene-mediated Cd transport mechanism. We characterized allelic variations of *OsNRAMP2* in diverse rice accessions. Our results suggest that *OsNRAMP2* positively regulates grain Cd accumulation by mediating Cd efflux from the vacuoles. The large effect of *OsNRAMP2* on grain Cd accumulation may be associated with its role in Cd remobilization at the filling stage, as manifested by its high expression in all tissues but husk at this stage. Allelic variation analysis revealed that a 6-bp insertion or deletion in exon 1 of *OsNRAMP2* contributes to the difference in grain Cd accumulation between *japonica* and *indica* rice.

2. Materials and methods

2.1. Plant materials

The *japonica* rice cultivar Nipponbare was used for expression analysis, transformation, and chromogenic *in situ* hybridization (CISH) of *OsNRAMP2*. Natural variations of *OsNRAMP2* were characterized in a diverse international rice panel that was selected from the rice panel RDP2 [27].

2.2. Temporal and spatial expression patterns of OsNRAMP2

For the expression analysis of *OsNRAMP2* in different tissues throughout the growth period, Nipponbare was grown in the paddy field in Guangzhou, China. Multiple tissues, including root, basal stem, leaf blade, leaf sheath, immature panicle, lower leaf sheath, lower leaf blade, flag leaf sheath, flag leaf blade, node IV, node III, node II, node I, rachis, spikelet and husk, were sampled at several growth stages. All samples were frozen in liquid nitrogen. Total RNA was extracted with Trizol reagent (Takara, Dalian, Liaoning, China). First-strand cDNA was synthesized from 0.5 μg total RNA using the PrimeScriptTM RT reagent kit (Takara). Gene expression was evaluated by qRT-PCR (Biorad CFX96, Pleasanton, CA, USA). The primer sequences for qRT-PCR of *OsNRAMP2* were 5′-CATCACACTTGTCTCAAAGGAGCA-3′ and 5′-GGACTTCAGTGGCA TAGAAGGACA-3′.

2.3. Tissue localization of OsNRAMP2

For tissue-specific expression analysis of *OsNRAMP2*, roots of one-week-old seedlings grown in sterile medium, and the leaf blade, node II, and internode II of rice plants at the filling stage, grown in soil were sampled and stored immediately in 50% formaldehyde-acetic acid–ethanol fixative. These samples were used for paraffin section. *In situ* hybridization of *OsNRAMP2* was performed with a CISH kit (BersinBio, Guangzhou, Guangdong, China). The samples from paraffin section were immersed in C_8H_{10} and dewaxed for 3 times, 6 min each time. Then rehydration was performed with 100%, 90%, 80% and 70% ethanol for 5 min at each concentration. Endogenous enzymes were inactivated by immersion in H_2O_2 solution (30% H_2O_2: water, 1:9) at room temperature for 10 min. Digestion was completed by addition of pepsin in a 37 °C water bath for 10 min. The probe and the hybridization solution (in the ratio of 1:40) were dropped onto the sections. After co-denaturation at 75 °C for 5–10 min, the samples were quickly transferred to 42 °C for hybridization overnight, followed by washing with 2 × Saline Sodium Citrate buffer (SSC), 2 × SSC (containing 0.1% NP-40 lysis buffer), 0.5 × SSC, 0.2 × SSC solution for 5 min each. The samples were then treated one by one with sealing fluid, biotinylated digoxin, streptAvidin Biotin Complex, and peroxidase, and washed with phosphate buffered saline for 4 times, 5 min each time. Color development was initiated by addition of 10–20 μL 3-amino-9-ethylcarbazole solution and stopped by immersion in water. The samples were dyed with hematoxylin solution for 10 min and then washed with running water. A high-resolution slide-scanning system (Pannoramic MIDI, 3DHISTECH Ltd., Hungary) was used to scan the samples and obtain images. The probe sequence of *OsNRAMP2* was

ttcagaagttccaagtgtcaacagcaattctgcagatcatcatcatccaaaaaatgagaa aggtaacaaagttatctactgctgtttataacctcaaacacatgaatgccactgtggtttatccc aaccctaagtggagaagcccaaccaattcgctaatgcacttatacaacagcagaggaatata cagtgtagttcatcggtaggc.

2.4. Development of OsNRAMP2 knockout (Cr) and overexpression (OE) lines

OsNRAMP2 knockout lines were created using CRISPR/Cas9 editing. The sequences of two small guide RNA (sgRNA) (*OsNRAMP2*-PS1 and *OsNRAMP2*-PS2) were respectively 5′-GCCGAGAGCCTCCTCCCCGTGGG-3′ and 5′-CGCGTACGACTCCG ACGACAAGG-3′, with underlining showing the trinucleotide NGG. The amplification primers used to identify positive knockout lines were 5′-GAGAGCACAACTTCCCATCC-3′ and 5′-GAGAGACCAACAG GATCGAC-3′.

The coding sequence of *OsNRAMP2* was amplified from Nipponbare and subcloned into the pOx overexpression vector under control of the ubiquitin promoter. The amplification primers were 5′-gtgttacttctgcagggtaccATGGCGTCGCGCGACCTCGC-3′ and 5′- taagcttggcgcgccggtaccTCATGTGCTCTTTGTCATTG-3′. After sequencing, the plasmids were transferred into Nipponbare by *Agrobacterium*-mediated genetic transformation. The amplification primers used for mutation site identification were 5′-GAGAGCACAACTTCCCATCC-3′ and 5′-GAGAGACCAACAGGATCGAC-3′.

2.5. Hydroponic culture and treatment

Rice seeds of Nipponbare, Cr, and OE lines were sown in sterile medium. After germination, the seeds were transferred to Kimura B solution containing KNO_3, $(NH_4)_2SO_4$, $Ca(NO_3)_2{\cdot}4H_2O$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, Fe-EDTA and micronutrients $MnCl_2{\cdot}4H_2O$, H_3BO_3, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, pH 5.6. After growth in this solution for one week, treatments with 5 μmol L^{-1} $CdCl_2$ and 100 nmol L^{-1} $CdCl_2$ were applied for another week. The seedlings were cultivated in a 28 °C incubator and the nutrient solution was changed every two days. Roots and shoots were collected separately. All samples were dried at 80 °C for 6 h and their Cd concentrations were determined as described below. All assays were repeated at least twice. The root-to-shoot Cd translocation ratio was defined as (shoot Cd concentration × shoot weight) × 100%/(shoot Cd concentration × shoot weight + root Cd concentration × root weight).

2.6. Pot experiments

Pot experiments were performed in the greenhouse of Rice Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, Guangdong, China. Three-week-old rice seedlings of

Nipponbare (wild type), and Cr and OE lines pre-cultured in pots were transplanted to pots with irrigation water containing 2 mg L^{-1} $CdCl_2$ [28] and grown until grain ripening. For environmental uniformity, transgenic and wild-type plants were planted in the same pot. The seeds and straw of each line were collected separately in 4–6 replications for determination of metal concentration. The Cd translocation ratio (in aboveground parts) was defined as (seed Cd concentration × seed weight) × 100%/(seed Cd concentration × seed weight + straw Cd concentration × straw weight).

2.7. Determination of metal concentration

The plant samples were dried in an oven at 80 °C for 6 h. The dried samples from hydroponic experiments and leaves at the seedling stage were directly digested with an acid mixture (HNO_3:$HClO_4$, 5:1). In the pot experiments, the grain was dehusked manually and the straw was ground in a mill, followed by digestion with HNO_3-$HClO_4$. The concentrations of Cd, Fe, Mn, Zn, Cu, K, Mg, Ca and P were determined by inductively coupled plasma mass spectrometry (ICP-MS) (Agilent Technologies 7700 Series, Santa Clara, CA, USA).

2.8. Alleles and phylogenetic analysis of OsNRAMP2

Sequences spanning the 2-kb promoter and coding regions of *OsNRAMP2* were extracted from the resequencing data (unpublished data) of 141 *japonica* and 209 *indica* accessions selected from the diverse rice panel RDP2 [27]. All accessions were sequenced to 50× coverage on the Illumina NovaSeq6000 platform (BerryGenomics, Beijing, China). Genomic variations were called with the GATK Best Practices pipeline [29], and all variations in *OsNRAMP2* were extracted and used for phylogenic analysis with MEGAX [30]. According to the biological significance of these variations, three variations resulting in amino acid changes and one variation resulting in a 5′ UTR sequence change were selected for haplotype analysis. The 6-bp variation information of *OsNRAMP2* in the 3K sequenced rice genomes was retrieved from the RFGB database (https://www.rmbreeding.cn/Index/).

3. Results

3.1. Functional confirmation of OsNRAMP2 in grain Cd accumulation

To confirm the function of *OsNRAMP2* in grain Cd accumulation, gene knockout mutants were generated using CRISPR/Cas9 gene editing. Two sequence-specific sgRNA target sites (*OsNRAMP2*-PS1 and *OsNRAMP2*-PS2) located at base positions 18 and 98, respectively, from ATG of exon 1 (Fig. 1A), and transformed into Nipponbare. Three Cas9-positive lines (Cr-1, Cr-2, and Cr-3), which carried either a deletion or an insertion of one or few bases in the target sequences resulting in frameshift mutations in *OsNRAMP2*, were selected for further study (Fig. 1A). Gene overexpression lines were developed in which the coding sequence of *OsNRAMP2* was driven by the ubiquitin promoter. Three *OsNRAMP2* overexpression lines (OE-1, OE-2, and OE-3) that showed higher expression than wild-type plants were used for subsequent analysis (Fig. 1B).

The brown rice Cd concentrations and the Cd translocation ratios (aboveground part) of Cr lines were lower than those of wild-type plants, while the brown rice Cd concentrations and the Cd translocation ratios (in aboveground parts) of OE lines were higher than those of wild-type plants (Fig. 1C, D).

3.2. Effects of knockout and overexpression of OsNRAMP2 on Cd uptake and root-to-shoot translocation

To investigate the mechanism of *OsNRAMP2*-mediated Cd transport in rice, we grew transgenic lines in hydroponic solution and investigated the roles of *OsNRAMP2* in Cd uptake and translocation from root to shoot. Shoot and root lengths did not differ among Cr, OE and wild type plants (Fig. S1). After treatment with 5 μmol L^{-1} $CdCl_2$ for one week, the shoot lengths, root lengths, Cd concentrations of shoots and roots, and root-to-shoot Cd translocation ratios of the Cr lines did not differ significantly from those of wild-type plants (Figs. 2A–C, and S1). In contrast, the shoot Cd concentrations and the root-to-shoot Cd translocation ratios of the OE lines were higher than those of wild-type plants, whereas the root Cd concentrations and the shoot lengths of OE lines were lower than those of wild-type plants (Figs. 2A–C, and S1). The root lengths of OE lines and wild-type plants did not differ (Fig. S1).

To avoid possible damage caused by 5 μmol L^{-1} $CdCl_2$, we further investigated Cd uptake and transportation in the transgenic lines and wild-type plants under 100 nmol L^{-1} $CdCl_2$, a nontoxic Cd concentration according to the previous study. As with the treatments with 5 μmol L^{-1} $CdCl_2$, OE lines displayed higher Cd concentrations in shoots and root-to-shoot Cd translocation ratios than wild-type plants (Fig. 2D, F). Cr lines showed lower Cd concentrations in shoot and lower root-to-shoot Cd translocation ratios than wild-type plants (Fig. 2D, F). The root Cd concentrations of Cr, OE and wild type plants did not differ significantly (Fig. 2E). The whole-plant Cd contents of Cr, OE, and wild-type plants did not differ significantly (Fig. S2).

3.3. Temporal and spatial expression patterns of OsNRAMP2 in rice

To further understand the mechanisms of *OsNRAMP2*-mediated Cd translocation from tissues to grains, we investigated the expression patterns of *OsNRAMP2* by qRT-PCR in several tissues throughout the growth period of rice plants grown in paddy field. The expression of *OsNRAMP2* varied with tissue and growth stage (Fig. 3A). *OsNRAMP2* showed higher expression in leaf blades, but lower expression in basal stems and leaf sheaths from vegetative to booting stages. At the flowering stage, *OsNRAMP2* displayed lower expression in all tissues tested. However, high expression of *OsNRAMP2* was observed in all tissues tested except for husk at the filling stage.

To further investigate the expression of *OsNRAMP2* in cells and tissues, CISH was performed in roots of one-week-old seedlings, and in node II, internode II and leaf blade at the filling stage. The positive expression of *OsNRAMP2* was detected in exodermis, sclerenchyma, cortex, endodermis, and stele cells in root (Fig. 3B). The cross section of a leaf blade showed that *OsNRAMP2* was expressed mainly in the epidermis, mesophyll cells and xylem, but less in phloem (Fig. 3C). At node, *OsNRAMP2* was expressed mainly in epidermal tissue, parenchymal cell, xylem and bundle sheath cell, and less in phloem (Fig. 3D). In stem, *OsNRAMP2* was expressed mainly in epidermal tissue, xylem and bundle parenchyma cells, and less in phloem (Fig. 3E).

3.4. Effects of knockout and overexpression of OsNRAMP2 on rice growth and interactions between divalent cations and OsNRAMP2

Cd is not an essential element for plant growth and it is generally believed [31] that Cd is co-transported with divalent cations. To confirm that *OsNRAMP2* regulates rice growth and the accumulation of other required divalent cations in grain, *OsNRAMP2* transgenic lines and wild type plants were grown in soil until grain ripening and their yield traits were investigated. The growth, seed

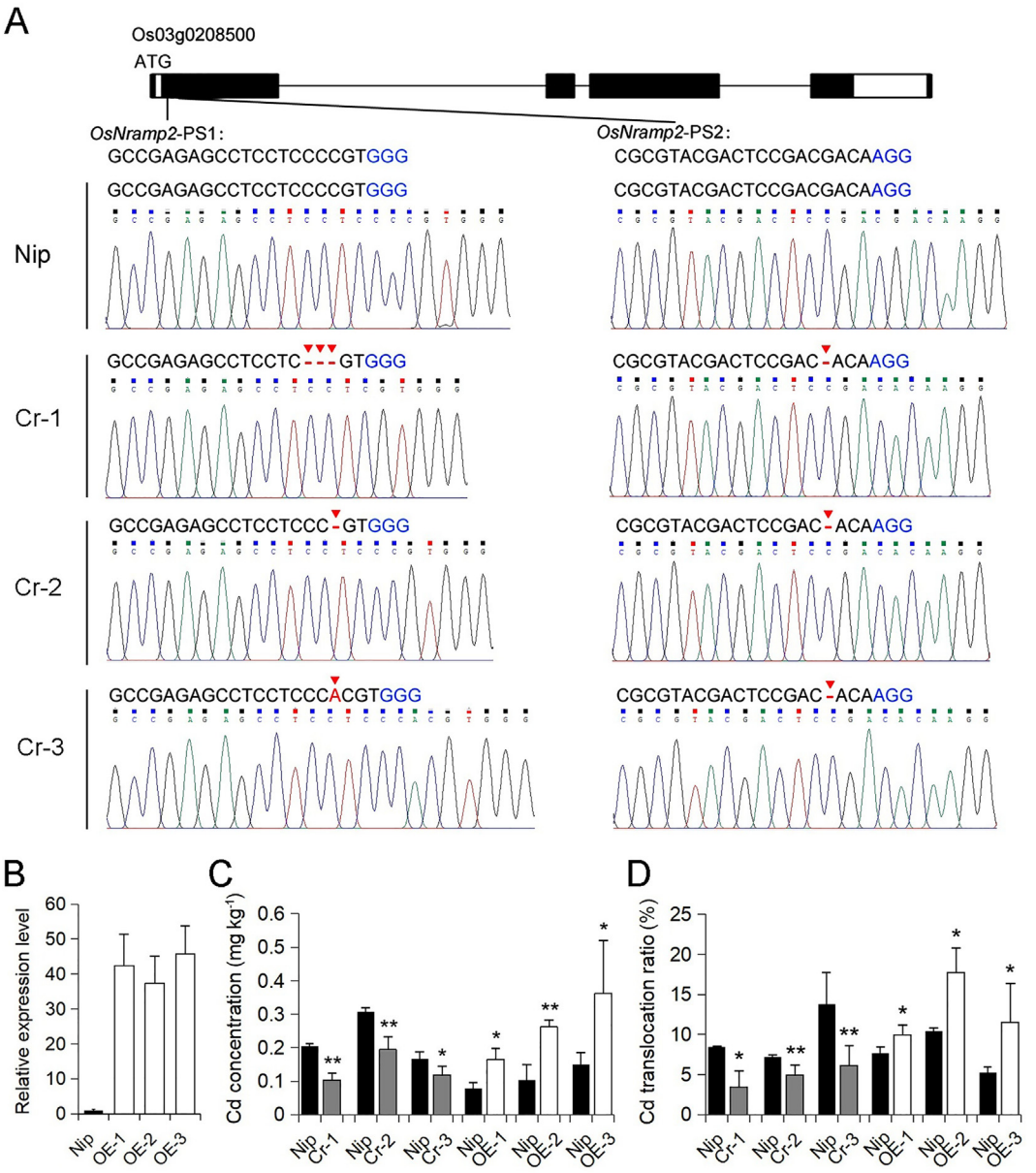

Fig. 1. *OsNRAMP2* contributes to grain Cd accumulation in rice. (A) Sequences of CRISPR-*OsNRAMP2* alleles in Cr lines. sgRNA target sequences (*OsNRAMP2*-PS1 and *OsNRAMP2*-PS2) are showed, with mutation positions indicated by red arrows. (B) Expression of *OsNRAMP2* in OE lines. The transcript level of *OsNRAMP2* in wild type was used as control and set to a value of 1. (C) Brown rice Cd concentrations of the transgenic lines and wild-type plants in pot experiments. (D) Cd translocation ratios (in aboveground parts) of transgenic lines and wild-type plants. Plants were grown in pot soil containing 2 mg L^{-1} $CdCl_2$ until grain ripening. Brown rice and straw were harvested and used separately for determination of Cd concentration.

weight, plant height and seed setting rate of Cr and OE lines were decreased in comparison with wild-type plants (Fig. 4A–C, F). There was no significant difference in grain number per plant or 1000-grain weight between transgenic and wild-type plants (Fig. 4D, E).

Fe, Mn, and Zn concentrations of Cr lines were lower than those of wild-type plants, but no significant difference in Cu concentration was observed (Fig. 4G–J). In contrast, Fe, Mn, Zn, and Cu concentrations of OE lines were higher than those of wild-type plants (Fig. 4G–J).

Fe, Mn, Zn, and Cu are required minerals for all known living organisms and function as cofactors for a wide variety of enzymes with various functions [32,33]. Disruption of *OsNRAMP2* is likely to influence the rice growth owing to reduced Fe, Mn, and Zn concentrations, ultimately leading to reduced yield and plant height (Fig. 4). But although the OE lines showed higher enrichment of Fe, Mn, Zn, and Cu, they showed poorer growth than wild-type plants (Fig. 4). To investigate why overexpression of *OsNRAMP2* impaired rice growth, we monitored the growth of transgenic lines throughout the growth period. We found severe discoloration in old leaves of one-month-old OE seedlings but none in Cr lines and wild-type plants (Fig. S3). Zn concentrations of OE lines were higher than those of wild-type plants, but no significant and consistent difference in the other element concentrations in transgenic lines and wild-type plants was observed (Fig. S3). Based on the symptoms of Zn^{2+} toxicity and its role in plant growth [34], we speculate that the toxic effects of excessive Zn in leaves accounted for the poorer growth of OE lines.

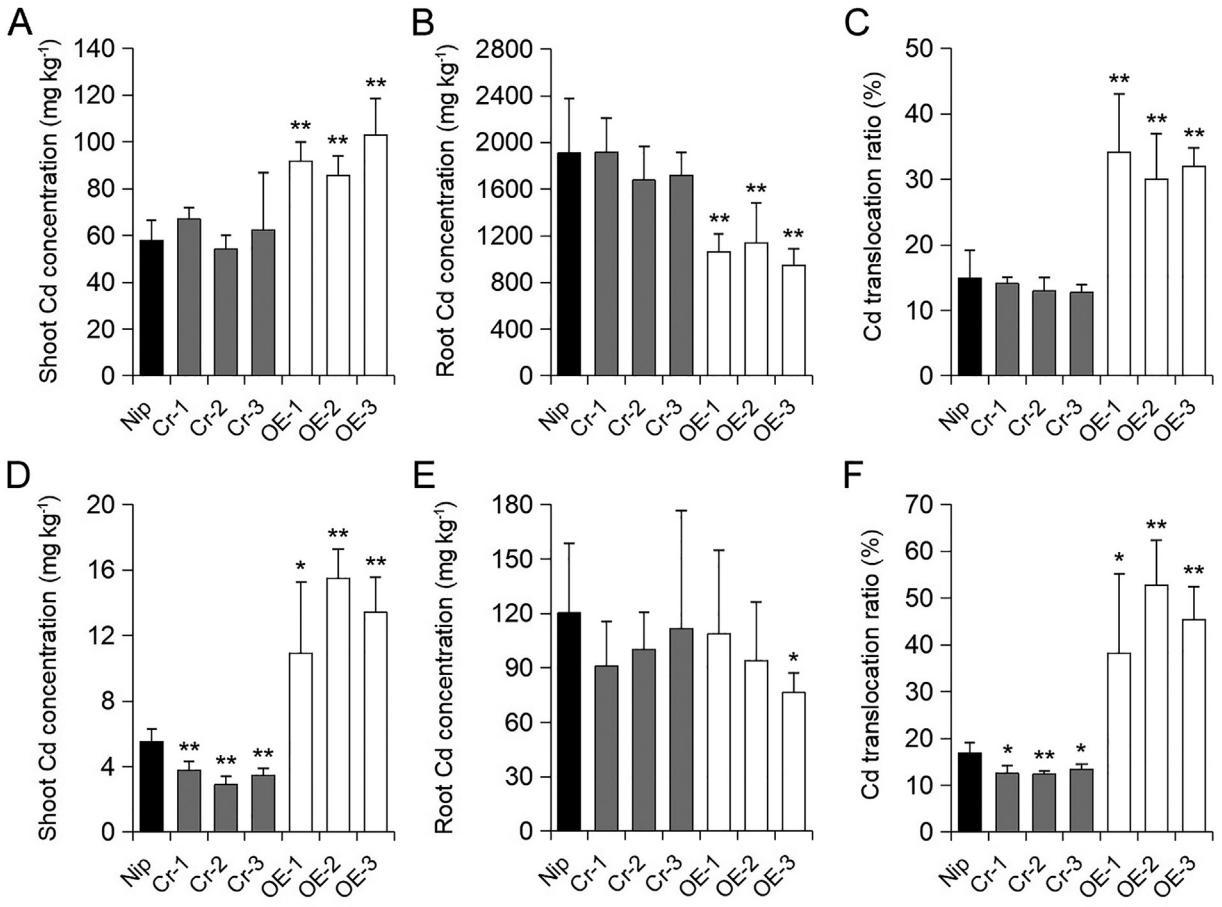

Fig. 2. OsNRAMP2 contributes to root-to-shoot Cd translocation in rice. (A–C) Cd concentrations in the shoot and root, and root-to-shoot Cd translocation ratios after treated with 5 μmol L^{-1} $CdCl_2$ for one week. (D–F) Cd concentrations in the shoot and root, and root-to-shoot Cd translocation ratios after treatment with 100 nmol L^{-1} $CdCl_2$ for one week. Three biological replicates were measured in these tests. In every biological replicate, 4–6 replications of each line were used to measure Cd concentration. Nip, Nipponbare (wild type); Cr, CRISPR-osnramp2 lines; OE, OsNRAMP2 overexpression lines. Error bars indicate standard deviation. Means comparisons employed one-tailed *t*-tests (*, $P < 0.05$; **, $P < 0.01$).

3.5. Phylogenetic analysis and allelic variations of OsNRAMP2 in rice germplasm

To investigate whether *OsNRAMP2* contributes to the difference in Cd accumulation between *japonica* and *indica*, we resequenced 350 diverse international rice accessions consisting of 141 *japonica* accessions and 209 *indica* accessions (unpublished data) and compared the sequence differences of *OsNRAMP2* among these rice accessions. In the phylogenetic tree based on the single nucleotide polymorphisms (SNPs) of *OsNRAMP2*, the 350 rice accessions were clearly separated into two clades, with *japonica* accessions in one clade and *indica* accessions in another clade (Fig. 5A). In comparison with the genome of Nipponbare, four major sequence variations in *OsNRAMP2* among 350 rice accessions were observed, including two deletions and two SNPs with obvious biological significance in the 5′ UTR and the coding region. Four haplotypes were identified (Hap 1 to 4) (Fig. 5B). We further associated the four haplotypes with grain Cd concentrations of 235 accessions reported in our previous study [26]. Almost all *japonica* accessions (98.73%) but few *indica* accessions (1.28%) harbored Hap 1. The average grain Cd concentrations of Hap 1, Hap 2, Hap 3 and Hap 4 were respectively 0.29, 0.53, 0.53 and 0.59 mg kg^{-1}. The average grain Cd concentration of the rice accessions with Hap 1 was lower than those of the rice accessions with any of the other three haplotypes, and no significant difference in grain Cd concentration among Hap 2, Hap 3, and Hap 4 was detected (Fig. 5C). Integrated analysis of haplotypes and Cd concentrations showed that a 6-bp nucleotides variations in exon 1 resulting in a deletion or insertion of two amino acids (threonine and alanine) accounted for the difference in grain Cd concentration between Hap 1 and the other three haplotypes (Fig. 5B, C). We further analyzed the distribution of the 6-bp variation in the 3 K rice genomes. As with the 235 rice accessions, 820 of the 826 *japonica* accessions (99.27%) and 76 of 1710 *indica* accessions (4.44%) carried Hap 1 (Fig. 5D).

4. Discussion

4.1. OsNRAMP2 positively regulates grain Cd accumulation in rice

Reducing grain Cd accumulation in rice is an urgent task. Identifying genetic components controlling Cd accumulation and elucidating their regulatory mechanisms are essential for development of effective strategies to reduce grain Cd accumulation in rice. Although several genes responsible for Cd uptake, compartmentation and remobilization in rice have been identified, known functional genes are still limited and the mechanisms of Cd transport to grain are not well understood [9]. To identify the genes responsible for grain Cd accumulation in rice, a genome-wide association study was conducted in a diverse rice panel in our previous study [26] and a major QTL (*qCd3-2*) for grain Cd accumulation was identified. By integration of sequence comparison, subcellular localiza-

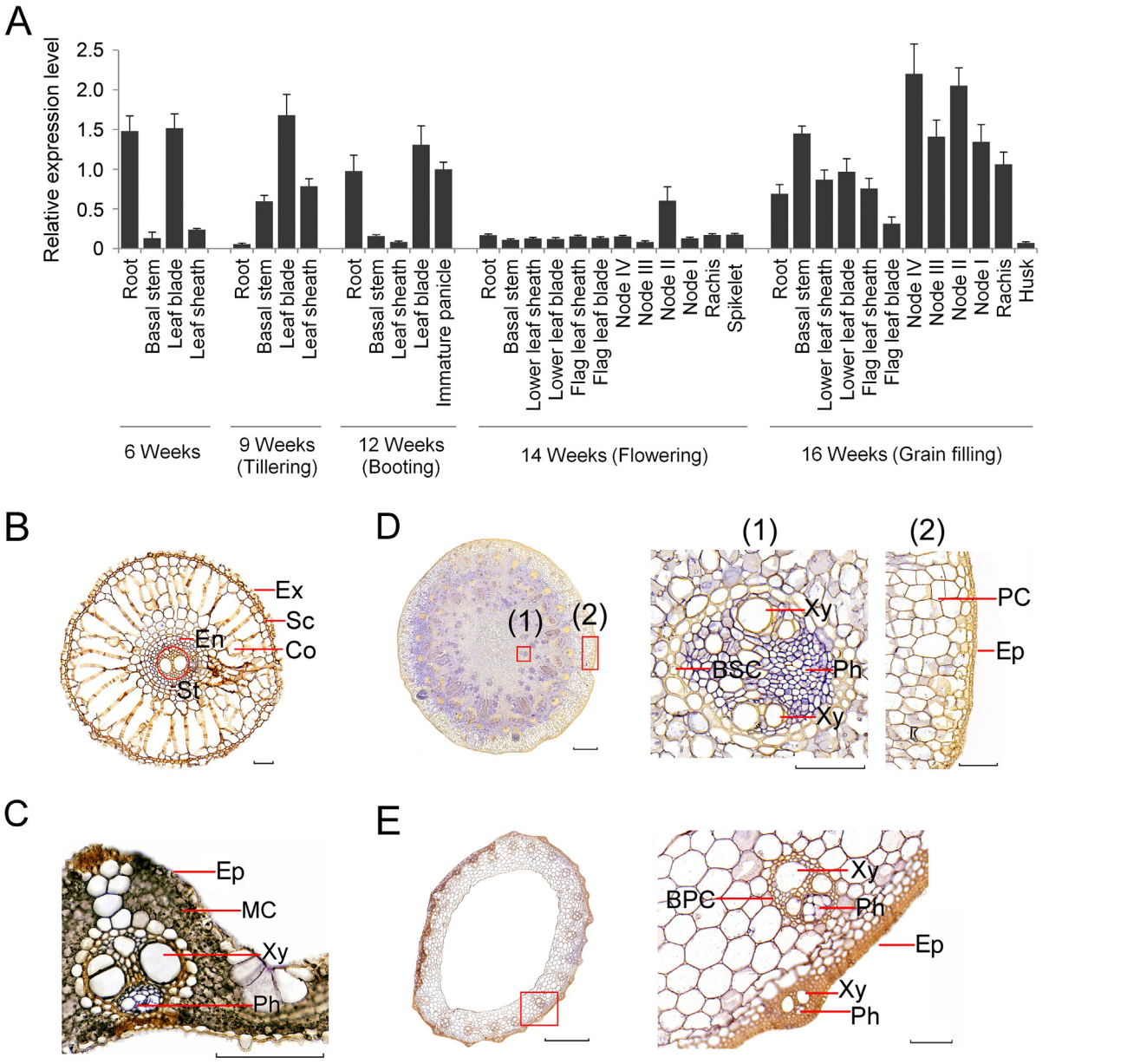

Fig. 3. Temporal and spatial expression patterns of OsNRAMP2 in rice. (A) Relative expression in various tissues at several growth stages. Rice (Nipponbare) was grown in a paddy field until ripening and various tissues were sampled for RNA analysis. The relative expression of OsNRAMP2 was measured by qRT-PCR using the reference gene EF1α as control. (B–E) *In situ* hybridization of OsNRAMP2: Cross section of root (B), leaf blade (C), node II (D) and internode II (E) by CISH. The positive expression of OsNRAMP2 is shown in yellow-brown. Ex, exodermis; Sc, sclerenchyma; Co, cortex; En, endodermis; St, stele; Xy, xylem; Ph, phloem; MC, mesophyll cell; BSC, bundle sheath cell; PC, parenchymal cell; Ep, epidermis; BPC, bundle parenchyma cells. Scale bars, 50 μm in (B), 50 μm in (C), 500, 100 and 100 μm in (D), 500 and 50 μm in (E).

tion, and yeast experiments, *OsNRAMP2* was identified as a candidate gene underlying *qCd3*. *OsNRAMP2* belongs to the *NRAMP* gene family. Among the seven *NRAMP* genes in rice, *OsNRAMP1* and *OsNRAMP5* are expressed mainly in roots and act as Cd uptake transporters [11,12]. In the present study, we further confirmed the function of *OsNRAMP2* in regulating grain Cd accumulation in rice by transgenic experiments. Brown rice Cd concentrations of the *OsNRAMP2* knockout mutants were lower than those of wild-type plants, whereas brown rice Cd concentrations of *OsNRAMP2*-overexpressing transgenic plants were higher than those of wild-type plants (Fig. 1C). A recent study also confirmed that *OsNRAMP2* affected Cd distribution to rice grain [35]. Thus, both our results and the results from the earlier study suggest that *OsNRAMP2* positively regulates grain Cd accumulation in rice.

4.2. OsNRAMP2 regulates Cd translocation from root to grain via different routes at various stages

OsNRAMP1 and OsNRAMP5 are plasma membrane transporters mediating Cd uptake in root cells [11,12]. Our previous study localized OsNRAMP2 to the tonoplast [26] and this finding was confirmed by two recent studies [35,36]. The differing sub-cellular localizations of OsNRAMP1 and OsNRAMP5 suggest that OsNRAMP2 might use a different mechanism in regulating Cd translocation in rice. In the present study, the root-to-shoot Cd translocation ratios were higher in OE lines than in wild-type plants, whereas the root-to-shoot Cd translocation ratios were lower in Cr lines than in wild-type plants (Fig. 2). In a recent study [36], OsNRAMP2 was localized to the vacuolar membrane and

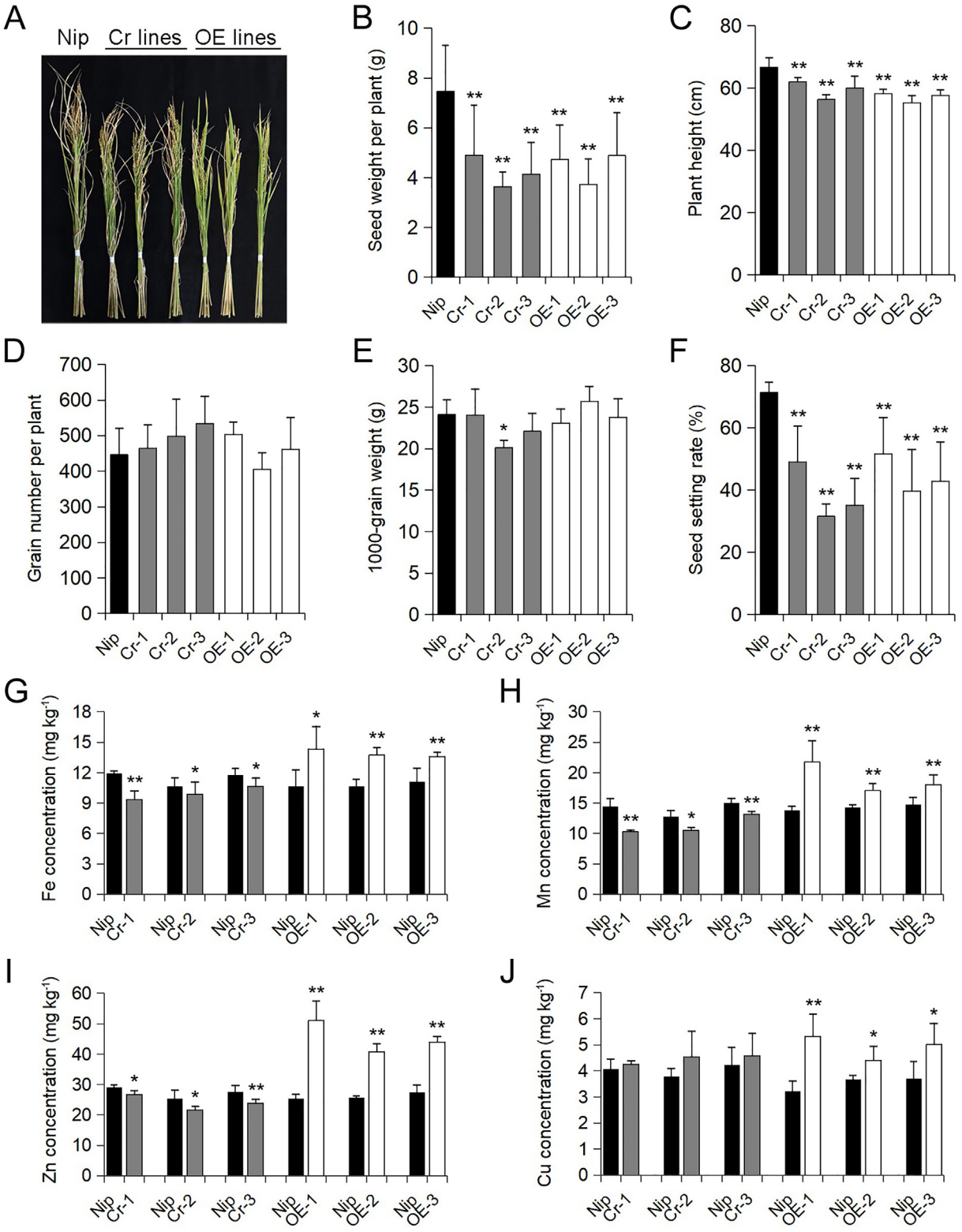

Fig. 4. OsNRAMP2 regulates rice growth and metal concentration. (A) Growth phenotypes. (B) Seed weight per plant. (C) Plant height. (D) Grain number per plant. (E) 1000-grain weight. (F) Seed setting rate. (G) Fe, (H) Mn, (I) Zn and (J) Cu concentrations of wild-type plants and OsNRAMP2 transgenic lines grown in potting soil. Transgenic lines and wild type plants were grown in potting soil until grain ripening. Poor growth of transgenic lines was observed with irrigation water containing 2 mg L^{-1} $CdCl_2$ or without $CdCl_2$. More than 10 plants of each line were used to measure agronomic traits. Nip, Nipponbare (wild type); Cr, CRISPR-osnramp2 lines; OE, OsNRAMP2 overexpression lines. Error bars indicate standard deviation. Statistical comparisons employed one-tailed *t*-tests (*, $P < 0.05$; **, $P < 0.01$).

could transport Fe from vacuole to cytosol. Cd is generally co-transported by divalent cations [31] and the present study showed that *OsNRAMP2* could also regulate grain Fe accumulation (Fig. 4G). These results together suggest that OsNRAMP2 mediates Cd efflux from vacuoles. OsHMA3 is also localized in the vacuolar membrane, but mediates Cd influx into vacuoles and controls Cd

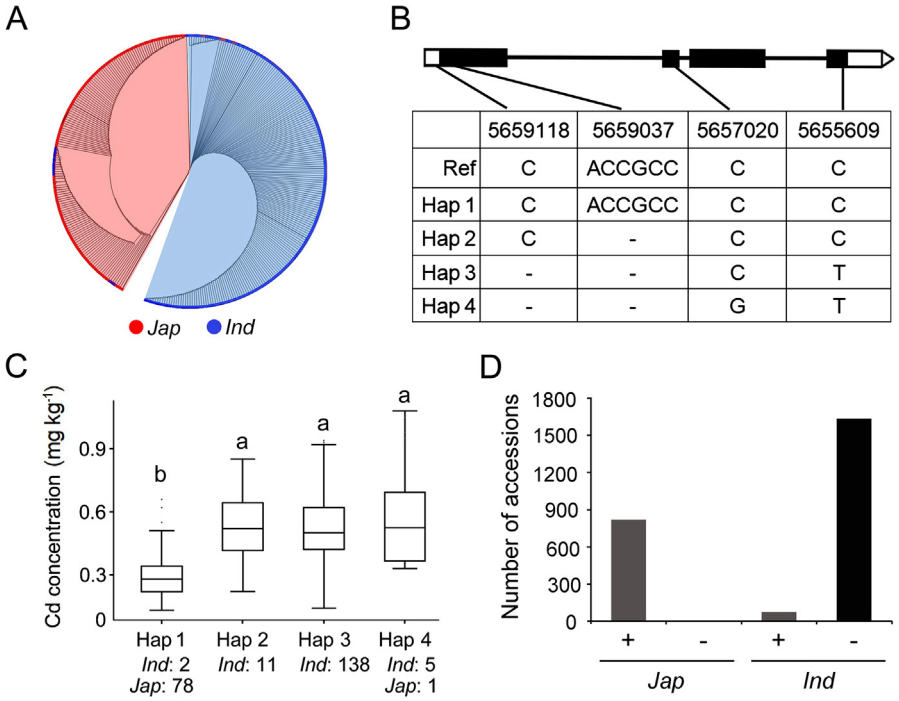

Fig. 5. Alleles and phylogenetic analysis of OsNRAMP2 in rice germplasm. (A) A phylogenetic tree of the 350 rice accessions was constructed based on their SNPs in OsNRAMP2. (B) Haplotypes of OsNRAMP2 among rice natural variations. Ref, Reference genome of Nipponbare. 5,659,118, 5,659,037, 5,657,020 and 5,655,609 refer to the locations of sequence variations in the OsNRAMP2 region on chromosome 3. (C) Comparison of grain Cd concentration among accessions harboring various haplotypes of OsNRAMP2. "Ind: 2" means that the number of *indica* accession in this test is 2 (the same below). (D) The distribution of 6-bp variation in the 3K rice genomes. "+" indicates carrying the 6-bp insertion; "-" indicates lacking the 6-bp deletion. Different letters indicate significant difference at $P < 0.01$.

translocation from roots to shoots and grain of rice [15]. Cd vacuolar sequestration operates in Cd detoxification in plants [9]. Thus, OsNRAMP2 and OsHMA3 act in opposite directions to control Cd sequestration in vacuoles and function in Cd accumulation in rice.

Grain Cd accumulation depends on Cd absorption in roots from soil, translocation from roots to shoots, and remobilization. These processes are controlled by different genes [9]. *OsIRT1* [10], *OsNRAMP1* [11], *OsNRAMP5* [12], *OsCd1* [13], and *OsZIP9* [14] are responsible for Cd uptake. *OsHMA3* [15] and *CAL1* [17] are reported to mediate Cd root-to-shoot transport. *OsHMA2* [16], *LCD* [20], *OsPCR1* [21] and *OsZIP7* [24] function in regulating distribution of Cd in shoots to grain. In the present study, the shoot Cd concentrations and root-to-shoot Cd translocation ratios in the *OsNRAMP2* overexpression lines were higher than those in wild-type plants, whereas the shoot Cd concentrations and root-to-shoot Cd translocation ratios in the *OsNRAMP2* knockout lines were lower than those in wild-type plants (Fig. 2D, F). Cr, OE and wild-type plants did not differ in whole-plant Cd content (Fig. S2). Because root Cd uptake determines total Cd in rice plants [37] and similar whole-plant Cd contents among the Cr, OE and wild type plants were detected, we infer that *OsNRAMP2* does not function in regulating Cd uptake in roots. These results suggest that OsNRAMP2 functions mainly in mediating Cd translocation from root to shoot rather than in root Cd uptake.

The temporal and spatial expression analysis of *OsNRAMP2* revealed that the expression levels of *OsNRAMP2* varied with developmental growth stage and tissue (Fig. 3A). At the flowering stage, *OsNRAMP2* displayed very low expression in tested tissues. At the filling stage, *OsNRAMP2* appeared to be activated and showed high expression in all tissues tested except for the husk. Grain filling is the key growth stage for grain Cd accumulation in rice [38]. We speculate that activation of *OsNRAMP2* at the filling stage strongly promotes Cd remobilization in various tissues and that *OsNRAMP2*-mediated Cd remobilization at the filling stage functions in grain Cd accumulation in rice.

OsNRAMP2 was expressed in multiple cells including xylem but less expressed in phloem in leaf blade, node and stem at the filling stage (Fig. 3C–E), suggesting that OsNRAMP2-mediated xylem Cd transport might function in Cd distribution from tissues to grain at the filling stage. *OsCCX2* was highly expressed in xylem at nodes [18], and may participate in Cd loading into xylem vessels and regulate direct root-derived Cd transport to grain. We speculate that OsNRAMP2 mediates Cd translocation to grains in rice via two pathways. OsNRAMP2 functions in root-to-shoot Cd translocation by regulating Cd efflux from root vacuoles, and then mediates Cd remobilization from tissues to grain at filling stage. The second pathway could be OsNRAMP2-mediated Cd direct translocation from roots to grains via the xylem. Based on the present study and other studies [9,39–41], a model for Cd translocation from root to grain in rice is illustrated in Fig. 6.

4.3. OsNRAMP2 has potential value in low-Cd rice breeding

Breeding for rice cultivars with low Cd accumulation has been proposed [9] as one of the most economical and effective strategies to reduce Cd accumulation in rice. The effectiveness of this strategy depends on the genes used. *qCd3-2* is a major QTL for grain Cd accumulation identified in a diverse international rice panel in our previous study [26]. In the present study, we confirmed that *OsNRAMP2* is the functional gene underlying *qCd3-2*. Disrupting *OsNRAMP2* led to more than 38% reduction in grain Cd concentration (Fig. 1C). The low Cd-accumulating haplotype (Hap 1) of *OsNRAMP2* showed at least 45.3% lower grain Cd concentration than any of the other three haplotypes (Fig. 5C). The distribution of Hap 1 in the sequenced 3 K rice genomes is consistent with the difference in grain Cd accumulation between *japonica* and *indica*

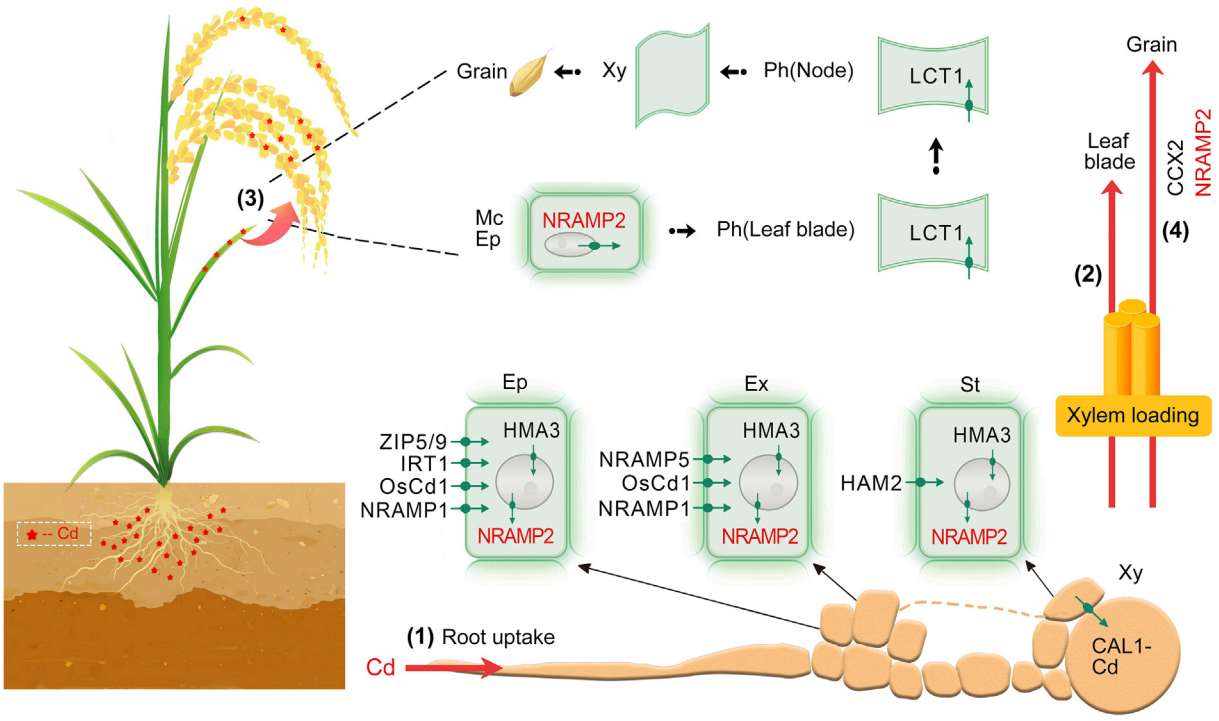

Fig. 6. A molecular model of Cd transport processes from soil to grain in rice. Transport of Cd from soil to grain can be divided into four steps: (1) uptake by roots; (2) xylem loading and root-to-shoot translocation; (3) Cd remobilization from leaves to grain via phloem; (4) newly absorbed Cd transport to grain via xylem. The OsNRAMP2 identified in this study is highlighted in red. Cd transporters that have been reported are shown. Details of each transporter can be found in the main text. Xy, xylem; Ph, phloem; St, stele; Ep, epidermis; Ex, exodermis; MC, mesophyll cell.

(Fig. 5D), suggesting that *OsNRAMP2* determines the difference in grain Cd accumulation between *japonica* and *indica*. Given that *OsNRAMP2* has a large phenotypic effect, it has high potential value for low-Cd rice breeding.

Although disrupting *OsNRAMP2* effectively reduced grain Cd accumulation in rice (Fig. 1), it led to poor growth (Fig. 4). This effect may be due partly to reduced accumulation of Fe, Mn, and Zn (Fig. 4), which are required for plant growth and development [32,33]. Similar effects have been observed in previous studies of *OsNRAMP5* and *OsCd1* [12,13]. Thus, simply disrupting *OsNRAMP2* is not an optimal approach to developing low-Cd rice. Using the natural variation of *OsNRAMP2* for low-Cd rice breeding could be a good strategy. Based on our results, Hap 1 of *OsNRAMP2* showed the lowest grain Cd accumulation among the four haplotypes and a 6-bp insertion or deletion in exon 1 accounted for the difference in grain Cd accumulation in rice. The 6-bp variations resulted in a deletion or insertion of two amino acids (threonine and alanine), of which threonine is a polar amino acid (Fig. 5). During the transport reaction cycle, some amino acids, especially charged or polar amino acids at N and C terminals, form hydrogen bonds with substrates and transport the substrates via the "rocker-switch" mechanism [42]. The two amino acid variations are near the N terminal of the OsNRAMP2 protein. The presence or absence of the two amino acids may be associated with Cd transport ability. The 6-bp variation in exon 1 could be used for development of functional markers and marker-assisted selection could be used for low-Cd rice breeding.

CRediT authorship contribution statement

Wu Yang: Funding acquisition, Project administration, Writing - original draft. **Luo Chen:** Investigation, Validation. **Yamei Ma:** Investigation, Validation. **Rui Hu:** Data curation, Visualization. **Jian Wang:** Data curation, Visualization. **Wenhui Li:** Data curation, Visualization. **Jingfang Dong:** Data curation, Visualization. **Tifeng Yang:** Methodology. **Lian Zhou:** Project administration. **Jiansong Chen:** Project administration. **Dilin Liu:** Project administration. **Ning Yu:** Project administration. **Zhixia Liu:** Project administration. **Lingyan Zhou:** Project administration. **Shaohong Zhang:** Software, Supervision. **Junliang Zhao:** Software, Funding acquisition, Writing - review & editing. **Bin Liu:** Funding acquisition, Conceptualization, Writing - review & editing.

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.

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Appendix A. Supplementary data

Supplementary data for this article can be found online at https://doi.org/10.1016/j.cj.2022.09.013.

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