BMC Research Notes

RESEARCH NOTE Open Access

OsHMA3 overexpression works more efficiently in generating low-Cd rice grain than *OsNramp5* knockout mutation

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Abstract

Objective Cadmium (Cd) is a highly toxic metal element and a carcinogen to humans. Rice is prone to taking up Cd from paddy fields and accumulating it in grain, which raises health concerns for rice consumers. OsNramp5 is a major transporter for Cd and manganese (Mn) uptake in rice, whereas OsHMA3 is a tonoplast-localized transporter involved in Cd detoxification in vacuoles. In this study, we compared the efficiency of *OsNramp5* knockout mutation and *OsHMA3* overexpression in reducing Cd content in rice grain.

Results The grain Cd content of the *OsNramp5* knockout mutants was significantly lower than that of the wild-type rice T5105. However, these mutants still had much higher grain Cd content than the previously reported *OsNramp5* mutants or the *OsHMA3* overexpression lines developed in our previous study. Pyramiding the *OsNramp5* mutant allele and the *OsHMA3* transgene in a single line did not result in an additional reduction in grain Cd content. The *OsNramp5* gene in T5105 has a haplotype II promoter, and its knockout mutation also partially reduced Mn content in rice grain. Our results demonstrate that *OsHMA3* overexpression works more efficiently in generating low-Cd rice grain than *OsNramp5* knockout mutation without affecting Mn uptake in rice.

Keywords Rice, Cadmium, Cd transporter, *OsNramp5*, *OsHMA3*, low-Cd rice

Introduction

Cadmium (Cd) and its compounds are highly toxic for most living organisms. Long-term exposure of the human body to Cd may lead to cancer and toxicity in organ systems. Rice is a staple cereal crop for over 40% of the world's population. Compared to other crops, rice is prone to taking up Cd from paddy fields and accumulating it in grain, which makes rice the major source of dietary Cd exposure. Several genes on Cd uptake, transport, and detoxification in rice have been isolated and characterized in recent years [1–5]. Among these, OsNramp5 is a major transporter for Cd uptake in rice [1–3]. The *OsNramp5* knockout mutant derived from physical mutagenesis significantly reduces Cd content in the rice grain [1]. The *OsNramp5* knockout mutants derived from gene editing were used to generate low-Cd rice [6, 7]. Since OsNramp5 is also a major transporter for manganese (Mn) uptake in rice and Mn is essential for rice growth and development, the *OsNramp5* knockout mutation might result in impaired growth, development, reproduction and stress response, especially when grown at low Mn concentrations [3, 8].

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OsHMA3 is a tonoplast-localized transporter belonging to the P1B-type Heavy Metal ATPase family [4, 5]. OsHMA3 transports Cd from the cytosol into vacuoles in root cells for Cd detoxification, which effectively restricts Cd loading into the xylem for long-distance transport to shoot and grain. Overexpression of the *OsHMA3* gene in rice selectively and significantly reduced Cd accumulation in grain and enhanced Cd tolerance [4, 9, 10]. We recently developed *OsHMA3* overexpression lines in the genetic background of rice variety T5105 [11]. The Cd concentration in the grain of the *OsHMA3* overexpression lines was significantly reduced, while the concentrations of other nutrient metal elements in the transgenic rice were not affected [11]. In this study, we generated *OsNramp5* knockout mutants using CRISPR/Cas9 technology and compared them with the *OsHMA3* overexpression lines on their efficiency in reducing Cd content in rice grain. The results would provide helpful information and guidelines on generating low-Cd rice through genetic engineering.

Main text

Materials and methods

Plant materials, growth conditions, and cd treatment

T5105 is an improved aromatic rice line in the KDML105 genetic background [12]. Nipponbare is a japonica cultivar. HMA3-L3 is an *OsNramp5* overexpression line carrying the P_{Actin1}:*cHMA3*:T_{Nos} gene in T5105 genetic background [11]. Rice plants were grown in pot soil in a greenhouse at 24–33 °C under natural light with a day length of 12 h. The soil used for rice plantation was a 1:1 mixture (by weight) of garden soil and BVB Peatmoss (Kekkilä-BVB), containing background levels of Cd at 0.436 mg/kg and Mn at 734.774 mg/kg, respectively. For the Cd treatment, the control mixed soil was supplemented with 3 mg/kg of Cd supplied as $CdSO_4$ [11].

Constructs and rice transformation

Gene editing on *OsNramp5* was carried out according to the method described previously [6]. A specific DNA target sequence and its adjacent protospacer adjacent motif (PAM) (AGGTTCTTCCTGTACGAGAGCGG G) were designed for CRISPR/Cas9-mediated gene editing of exon 9 in *OsNramp5*. The expression cassette, containing the target sequence and the gRNA under the rice snRNA U3 promoter in vector pYLsgRNA-OsU3, was amplified and cloned into the vector pYLCRISPR/Cas9P_{Ubi}-H to make binary construct pYLCas9-Nramp5. pYLCas9-Nramp5 was introduced into the *Agrobacterium tumefaciens* strain AGL1 by electroporation. The *Agrobacterium*-mediated transformation of T5105 was performed according to the procedures described previously [11].

ICP-MS

The contents of Cd and Mn in the de-husked but unpolished brown rice were determined by ICP-MS (7700 S, Agilent Technologies, USA) as described in the previous study [11].

qRT-PCR

Total RNA extraction from rice root tissues, the first-strand cDNA synthesis and qRT-PCR analysis were carried out according to the methods described in our previous report [11]. The qRT-PCR results were normalized against the rice elongation factor gene *OsEF-1α* (Os03g0178000). The relative expression levels of *OsNramp5* were determined using the $2^{-\Delta\Delta Ct}$ relative quantification method with the expression level in Nipponbare arbitrarily set to 1. The oligo DNA primer pairs are 5'GTCGCCGTCGTCTACCT3'/5'GTACGGCAAGGGC TCGT3' for the *OsNramp5* gene.

and 5'GCACGCTCTTCTTGCTTTC3'/5'AGGGAAT CTTGTCAGGGTTG3' for the *OsEF-1α* gene.

Results and discussion

We recently developed low-Cd rice lines in the genetic background of rice variety T5105 by overexpressing *OsHMA3* under the control of rice *OsActin1* promoter [11]. To suppress Cd uptake, we generated three independent knockout mutants of *OsNramp5* by using CRISPR-Cas9 technology (Fig. 1A and B). The Cd contents in the grain of these knockout mutants (0.678±0.336 mg/kg to 0.715±0.217 mg/kg) were significantly lower than that of T5105 (1.561±0.398 mg/kg) when grown in Cd-contaminated soil (Fig. 1C). However, the Cd content in the grain of the *OsNramp5* knockout mutants was still significantly higher than that in the grain of the *OsNramp5* knockout mutants obtained through mutagenesis or gene editing in the previous reports [1, 6]. As for the efficiency in grain Cd reduction, the Cd content in the grain of the *OsNramp5* knockout mutants was partially reduced by 54.2–56.6% over that of T5105, which was also significantly lower than that in the previous reports [1, 6]. Similar to the *OsNramp5* mutants reported in the previous studies [3], the knockout mutation in this study partially affected Mn uptake and resulted in lower grain Mn content than the wild-type rice when they were grown in either control or Cd-contaminated soil (Fig. 1D). However, no significant change in the phenotype including seed setting rate was observed in the *OsNramp5* knockout mutants. Thus, the partial reduction in Mn uptake did not affect plant growth and development, possibly due to the high background level of Mn concentration (734.774 mg/kg) in the soil [3].

The representative *OsNramp5* mutant line *nramp5-L45* was then selected for comparative analysis with HMA3-L3, a representative *OsHMA3* overexpression

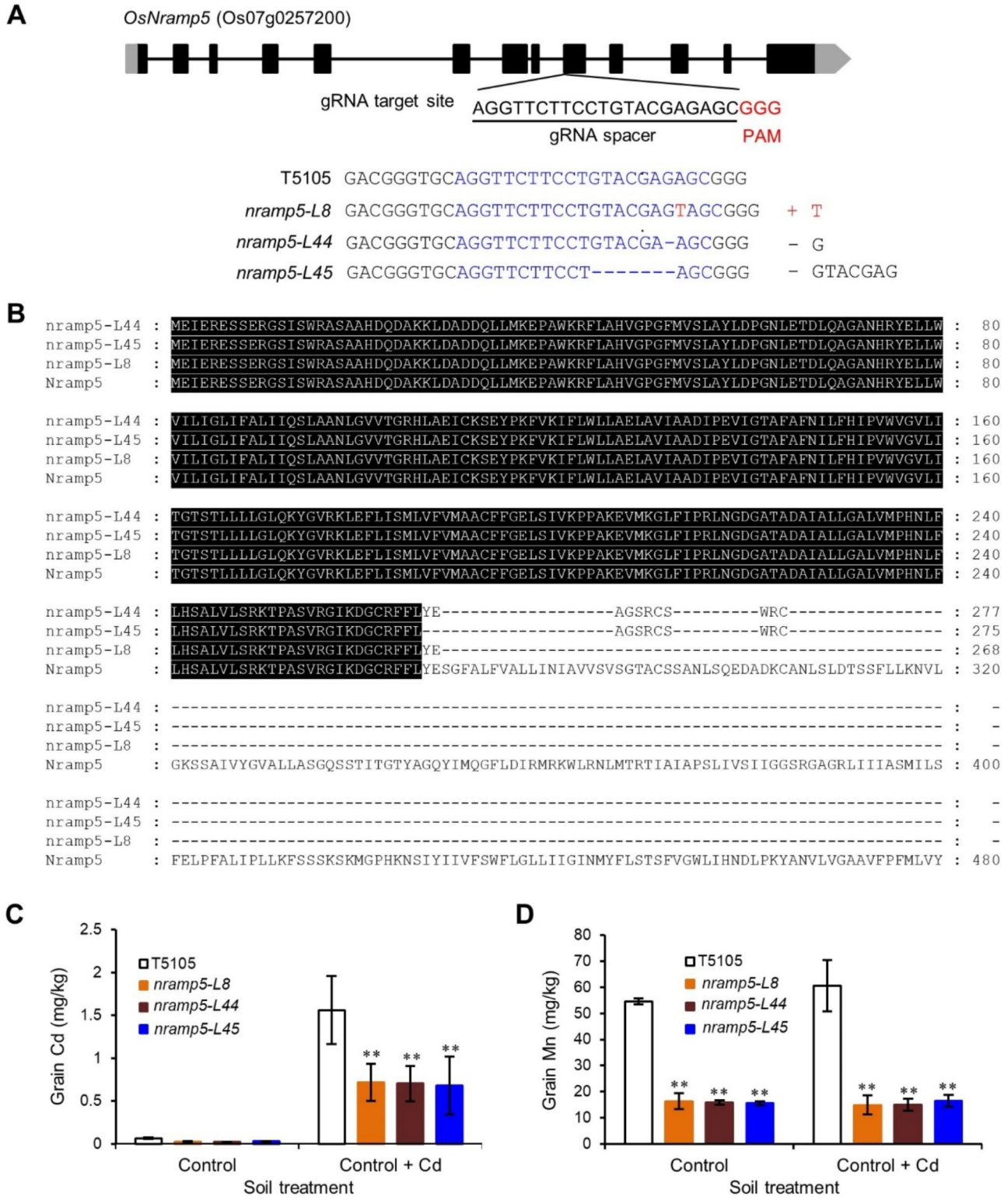

Fig. 1 Generation of *OsNramp5* knockout mutants. (**A**) Schematic diagram of *OsNramp5* gene structure. UTRs, exons, and introns are indicated by grey blocks, black blocks, and lines, respectively. The nucleotide sequences of the CRISPR/Cas9 target together with PAM (GGG) and the gene edited *OsNramp5* mutants are shown at the bottom of the gene structure. Deletions and insertions are indicated by dashes and red letters, respectively. (**B**) Alignment of amino acid sequences of OsNramp5 and its variants. The amino acid sequences were aligned by Clustral W method under the software MegAlign and viewed by software GeneDoc. The identical residues among all aligned proteins were highlighted in black. (**C**) Grain Cd content of T5105 and *OsNramp5* knockout mutants. (**D**) Grain Mn content of T5105 and *OsNramp5* knockout mutants. Asterisks in (**C**) and (**D**) indicate a significant difference between T5105 and *OsNramp5* mutants at ** $P < 0.01$ by Student's *t*-test. All data are means ± SD of at least three biological replicates

line developed in the previous study [11]. The Cd content in the grain of *nramp5-L45* was 1.038±0.079 mg/kg when it was grown in Cd-contaminated soil, which was lower than that of T5105 at 1.720±0.137 mg/kg, but still much higher than the maximum level (ML) of Cd in rice at 0.4 mg/kg set by the Codex Alimentarius Commission (Codex) (Fig. 2A) [13]. Pyramiding the *OsNramp5* mutant allele from *nramp5-L45* and the P_{Actin1}:*cHMA3*:T_{Nos} gene from HMA3-L3 in a double homozygous line, designated as NH, did not result in any significant Cd reduction in the grain of NH when compared to that of HMA3-L3 (Fig. 2A). The grain Cd contents of HMA3-L3 and NH were 0.027±0.005 mg/kg and 0.020±0.003 mg/kg, respectively (Fig. 2A). Both were significantly lower than the set ML of Cd in rice by Codex. Both *nramp5-L45* and NH had lower grain Mn contents than T5105 (Fig. 2B). However, there was no significant difference in Mn content in the grain between HMA3-L3 and T5105, indicating that the *OsHMA3* overexpression did not affect Mn uptake in rice (Fig. 2B) [11]. The partial impairment of Cd and Mn accumulation in the grain of the *OsNramp5* knockout mutants suggests the existence of other transporters for Cd and Mn uptake. One example could be the iron transporter OsNramp1, a plasma membrane-localized transporter for Mn and Cd [14, 15]. OsNramp1 was found to play a complementary, but not redundant, function to OsNramp5 in the uptake of Mn and Cd based on their differences in gene expression patterns to Cd exposure, subcellular localization of proteins in root cells and relative distribution of Mn and Cd between root and shoot in knockout mutants [14].

Based on single nucleotide polymorphisms (SNPs) in the promoters, the *OsNramp5* genes in different rice varieties could be classified into three haplotypes: Haplotype I, Haplotype II and Haplotype III [16]. *OsNramp5* carrying a Haplotype I promoter has a higher expression level than those carrying a Haplotype II or Haplotype III promoter [16]. Rice varieties carrying *OsNramp5* with a Haplotype I promoter also accumulate more Mn in brown rice [16]. Genome sequencing revealed that the *OsNramp5* gene in T5105 carries a Haplotype II promoter (Fig. 3A) [11]. Further qRT-PCR analysis confirmed that the *OsNramp5* gene in T5105 had a lower expression level in root than the *OsNramp5* gene in Nipponbare, a cultivar harbouring an *OsNramp5* gene with a Haplotype I promoter (Fig. 3B) [16]. Since OsNramp1 plays a similar and complementary function to OsNramp5 in the uptake of Mn and Cd [14], the relatively low expression level of the *OsNramp5* gene in T5105 might weaken its contribution to Cd and Mn uptake. Therefore, the single knockout mutation of *OsNramp5* may only partially impair Cd and Mn uptake in rice (Figs. 1C and D and 2A and B). Since Mn is essential for rice, *OsNramp5* knockout mutants may affect rice growth, development, yield and tolerance to high temperatures under insufficient Mn supply from soil [3, 8]. Nevertheless, the *OsNramp5* knockout mutants and the *OsHMA3* overexpression lines developed in the present and previous studies enable farmers to produce low-Cd rice in Cd-contaminated paddy fields, where the soil Cd contents might be around or above 3 mg/kg [1, 6, 11, 17]. Compared to the *OsNramp5* knockout mutants, the *OsHMA3* overexpression lines performed more efficiently in reducing Cd content in rice grain without affecting Mn uptake in rice.

Limitations

The contribution of *OsNramp5* to Cd and Mn uptake and transport may work in a haplotype- or variety-dependent manner. The conclusion in this report was based on results obtained from the *OsNramp5* knockout mutants and the *OsHMA3* overexpression lines in T5105 genetic background. It remains to be further investigated whether this also applies to other rice varieties or cultivars.

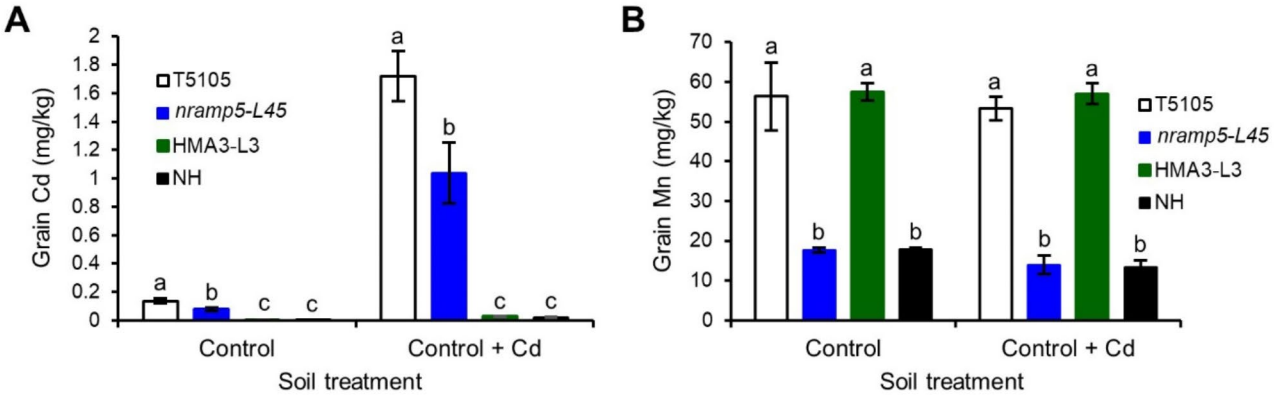

Fig. 2 Cd and Mn contents in the grain of rice lines. (**A**) Cd content in the grain of T5105, *nramp5-L45*, HMA3-L3 and NH. (**B**) Mn content in the grain of T5105, *nramp5-L45*, HMA3-L3 and NH. Different letters in (**A**) and (**B**) indicate statistically different at $P<0.05$ according to LSD's test. All data are means ± SD of at least three biological replicates

A

Haplotype	Position												
	-1950	-1866	-1800	-1725	-1692	-1586	-1550	-1385	-1365	-1214	-930	-832	-50
I	T	G	T	A	C	T	G	C	A	C	G	T	C
II	C	A	A	C	T	C	G	T	G	T	T	C	-
III	C	T	A	C	T	C	T	T	G	T	T	C	-
NB	T	G	T	A	C	T	G	C	A	C	G	T	C
T5105	C	A	A	C	T	C	G	T	G	T	T	C	-

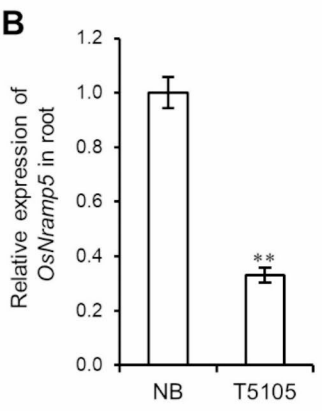

Fig. 3 Haplotypes of *OsNramp5* promoters in rice varieties and their relative expression levels. (**A**) Haplotypes of *OsNramp5* promoters and SNPs. The SNPs in Haplotype I to Haplotype III promoters of *OsNramp5* genes published by Liu et al., (2017) were listed as the references above the SNPs in the promoters of the *OsNramp5* genes in Nipponbare (NB) and T5105. (**B**) Relative expression of the *OsNramp5* genes in the root of Nipponbare and T5105 detected by qRT-PCR. The expression level of *OsNramp5* in the root of Nipponbare was set as 1. Asterisks in (**B**) indicate a significant difference between Nipponbare and T5105 at ** $P < 0.01$ by Student's *t*-test

Acknowledgements
The authors thank Prof. Yao-Guang Liu for providing gene editing constructs pYLsgRNA-OsU3 and pYL-CRISPR/Cas9PUbi-H.

Author contributions
YZ conceived the research. GY, TJ and TD conducted the experiments. YZ, GY, TD and RM wrote the article. All authors reviewed and approved the manuscript.

Funding
This work was supported by intramural research funds for Temasek Life Sciences Laboratory from Temasek Trust and a fund from the National Research Foundation (NRF), Prime Minister's Office, Singapore, on the Disruptive & Sustainable Technology for Agricultural Precision (DiSTAP).

Data availability
The OsNramp5 knockout mutants developed in this study are available from the corresponding author for non-commercial research purposes upon signing a Material Transfer Agreement defined by the Intellectual Property Office of Temasek Life Sciences Laboratory Ptd, Singapore. The data that support the findings of this study are presented in the article. There is no additional digital data.

Declarations

Ethics approval and consent to participate
Not appliable.

Consent for publication
Not applicable.

Competing interests
Temasek Life Sciences Laboratory Ltd has filed a patent on the method of generating low-As and low-Cd rice grains with ZY and YG as the inventors (PCT APPLICATION NO. PCT/SG2023/050847). The OsHMA3 overexpression line HMA3-L3 used in this study was included in the patent.

Received: 29 July 2024 / Accepted: 20 January 2025
Published online: 05 February 2025

References

1. Ishikawa S, Ishimaru Y, Igura M, Kuramata M, Abe T, Senoura T, Hase Y, Arao T, Nishizawa NK, Nakanishi H. Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. Pro Natl Acad Sci USA. 2012;109(47):19166–71.
2. Ishimaru Y, Takahashi R, Bashir K, Shimo H, Senoura T, Sugimoto K, Ono K, Yano M, Ishikawa S, Arao T. Characterizing the role of rice NRAMP5 in manganese, iron and cadmium transport. Sci Rep. 2012;2(1):286.
3. Sasaki A, Yamaji N, Yokosho K, Ma JF. Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. Plant Cell. 2012;24(5):2155–67.
4. Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF. Gene limiting cadmium accumulation in rice. Pro Natl Acad Sci USA. 2010;107(38):16500–5.
5. Miyadate H, Adachi S, Hiraizumi A, Tezuka K, Nakazawa N, Kawamoto T, Katou K, Kodama I, Sakurai K, Takahashi H, et al. OsHMA3, a P1B-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. New Phytol. 2011;189(1):190–9.
6. Tang L, Mao B, Li Y, Lv Q, Zhang L, Chen C, He H, Wang W, Zeng X, Shao Y, et al. Knockout of OsNramp5 using the CRISPR/Cas9 system produces low Cd-accumulating indica rice without compromising yield. Sci Rep. 2017;7(1):14438.
7. Hu S, Zhou L, Wang J, Mawia AM, Hui S, Xu B, Jiao G, Sheng Z, Shao G, Wei X. Production of grains with ultra-low heavy metal accumulation by pyramiding novel alleles of OsNramp5 and OsLsi2 in two-line hybrid rice. Plant Biotechnol J. 2024;22:2921–31
8. Dong J, Wu T, Sun Y, He H, Li Y, Peng Y, Ji Z, Meng Q, Zhao B, Tang L. Effects of OsNRAMP5 mutation on heat tolerance and main economic traits of rice under the conditions of different manganese concentration. Hybrid Rice. 2021;36(2):79–88.
9. Sasaki A, Yamaji N, Ma JF. Overexpression of OsHMA3 enhances cd tolerance and expression of zn transporter genes in rice. J Exp Bot. 2014;65(20):6013–21.
10. Shao JF, Xia J, Yamaji N, Shen RF, Ma JF. Effective reduction of cadmium accumulation in rice grain by expressing OsHMA3 under the control of the OsHMA2 promoter. J Exp Bot. 2018;69(10):2743–52.
11. Gui Y, Teo J, Tian D, Yin Z. Genetic engineering low-arsenic and low-cadmium rice grain. J Exp Bot. 2024;75(7):2143–55.
12. Luo Y, Yin Z. Marker-assisted breeding of Thai fragrance rice for semi-dwarf phenotype, submergence tolerance and disease resistance to rice blast and bacterial blight. Mol Breed. 2013;32(3):709–21.
13. Commission CA. Report of the 29th session of the Codex Alimentarius Commission. FAO/WHO Geneve Switzerland; 2006.
14. Chang JD, Huang S, Yamaji N, Zhang W, Ma JF, Zhao FJ. OsNRAMP1 transporter contributes to cadmium and manganese uptake in rice. Plant Cell Environ. 2020;43(10):2476–91.
15. Takahashi R, Ishimaru Y, Senoura T, Shimo H, Ishikawa S, Arao T, Nakanishi H, Nishizawa NK. The OsNRAMP1 iron transporter is involved in cd accumulation in rice. J Exp Bot. 2011;62(14):4843–50.

16. Liu C, Chen G, Li Y, Peng Y, Zhang A, Hong K, Jiang H, Ruan B, Zhang B, Yang S, et al. Characterization of a major QTL for manganese accumulation in rice grain. Sci Rep. 2017;7(1):17704.
17. Kubier A, Wilkin RT, Pichler T. Cadmium in soils and groundwater: a review. Appl Geochem. 2019;108:104388.

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