Research Note

Comparison of Cd concentration in rice grain between *OsNramp5* knock-out mutants and *OsHAM3*-overexpressing line

Yuejing Gui[1], Joanne Teo[1], Dongsheng Tian[1], Raji Mohan[1] and Zhongchao Yin[1,2,*]

[1]Temasek Life Sciences Laboratory, 1 Research Link, National University of Singapore, Singapore 117604, Republic of Singapore.

[2]Department of Biological Sciences, 14 Science Drive, National University of Singapore, Singapore 117543, Republic of Singapore.

[*]Author for correspondence: Zhongchao Yin (Email: yinzc@tll.org.sg)

ABSTRACT

Cadmium (Cd) is a highly toxic element that causes cancer and serious diseases in human beings. Rice is prone to accumulate Cd in its grain, which causes health concerns for rice consumers. OsNramp5 is a major Cd and Mn transporter in rice, whereas OsHMA3 is a tonoplast-localized transporter that is involved in Cd sequestration in vacuoles. We generated an *OsNramp5* knock-out mutant in T5105 genetic background by gene editing approach. Cd and Mn concentrations in the grain of *OsNramp5* knock-out mutants were measured and compared with those in the grain of an *OsHMA3*-overexpressing line developed in a previous study. We found that the *OsHMA3* overexpression approach worked more efficiently than the *OsNramp5* knock-out method in generating low-Cd rice grain without affecting Mn uptake and transport in rice.

KEY WORDS: Rice, cadmium, OsNramp5, OsHMA3, low-Cd rice, transgenic rice, gene editing

Cadmium (Cd) is a highly toxic metal for most living organisms, including humans. Exposure to Cd may cause cancer and severe disease in the human body systems. Rice growing in paddy fields is prone to accumulate Cd in its grain, which causes health concerns to rice consumers. Several genes on Cd uptake, transport, and detoxification in rice have been isolated and characterized in recent years (Ueno et al., 2010; Miyadate et al., 2011; Ishikawa et al., 2012; Ishimaru et al., 2012; Sasaki et al., 2012). Among these, OsNramp5 is a major Cd and manganese (Mn) transporter in rice (Ishikawa et al., 2012; Ishimaru et al., 2012; Sasaki et al., 2012). *OsNramp5* knock-out mutant significantly reduces Cd concentration in rice grains (Ishikawa et al., 2012). The mutant alleles of *OsNramp5* derived from gene editing were used to generate low-Cd rice (Tang et al., 2017; Hu et al., 2024). However, the *OsNramp5* knock-out mutation also decreased Mn uptake, which might result in impaired growth and yield, especially when grown at low Mn concentrations (Sasaki et al., 2012). OsHMA3 is a tonoplast-localized transporter that is involved in Cd detoxification by sequestering it into the vacuoles of root cells, which

effectively inhibits Cd loading into the xylem for long-distance transport to the shoot and the grain (Ueno et al., 2010; Miyadate et al., 2011). Overexpression of OsHMA3 efficiently reduced Cd accumulation in the grain and enhanced Cd tolerance in rice (Ueno et al., 2010; Sasaki et al., 2014; Shao et al., 2018). Following a similar approach, we recently generated low-Cd rice in T5105 genetic background, an improved rice line of Thai fragrant rice cultivar KDML 105, by overexpressing *OsHMA3* under the control rice *OsActin1* promoter (Gui et al., 2024). To compare the efficiency of *OsHAM3* overexpression and *OsNramp5* knock-out mutation in grain Cd reduction, we generated three independent knock-out mutants of *OsNramp5* by using CRISPR-Cas9 technology (Fig. 1A). Compared to the wild-type T5105, the Cd concentration in the grain of *OsNramp5* mutants was reduced by 54.2% to 56.6% when they were grown in Cd-contaminated soil (Fig. 1B). The Cd reduction in the grain of the *OsNramp5* knock-out mutants was significantly lower than that of the *OsHMA3*-overexpressing lines reported in our recent study (Gui et al., 2024). It was also much lower than that of the *OsNramp5* mutants obtained through mutagenesis or gene editing in the previous reports (Ishikawa et al., 2012; Tang et al., 2017). As reported in the previous study (Sasaki et al., 2012), the Mn concentration in the grain of the *OsNramp5* knock-out mutants was significantly decreased when they were grown in either control or Cd-contaminated soils (Fig. 1C). However, no significant changes in growth and development, or seed setting rate were observed in the *OsNramp5* knock-out mutants. Nramp5-L45, a representative *OsNramp5* mutant in this study, was selected for further comparison with HMA3-L3, a representative *OsHMA3*-overexpressing line in the previous study (Gui et al., 2024). When grown in Cd-contaminated soil, the Cd concentration in the grain of HMA3-L3 was decreased by 98.4% over that of T5105, whereas that in the grain of Nramp5-L45, it was decreased by only 39.7% (Fig. 1D). 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 by NH, did not result in any further significant Cd reduction in grain when compared to HMA3-L3 (Fig. 1D). The grain Cd concentrations of HMA3-L3 and NH were 0.027±0.005 mg/kg and 0.020±0.003 mg/kg, respectively (Fig. 1D). Both grain Cd concentrations were significantly lower than the maximum level (ML) of Cd in rice at 0.4 mg/kg set by the Codex Alimentarius Commission (Codex) (Commission, 2006). The gain Cd concentration of Nramp5-L45 was 1.038±0.079 mg/kg, which was lower than that of T5105 at 1.720 ±0.137 mg/kg but much higher than the set ML of Cd in rice by Codex (Fig. 1D). There was no significant difference in grain Mn concentration between HMA3-L3 and T5105, indicating that the *OsHMA3* overexpression did not significantly affect Mn uptake and transport in rice (Fig. 1E). However, both Nramp5-L45 and NH had lower grain Mn concentration than T5105, suggesting that the *OsNramp5* knock-out mutation impaired Mn uptake and transport (Fig. 1E). The partial impairment of Cd and Mn accumulation in the grain of *OsNramp5* knock-out mutants also suggests that, in addition to OsNramp5, rice should have other transporter(s) or redundant pathway(s) for Cd and Mn uptake. Indeed, transporter OsNramp1 was found to contribute to Cd and Mn uptake in rice (Chang et al., 2020). The promoters of *OsNramp5*

genes in different rice varieties could be classified into three haplotypes (Haplotype I, Haplotype II and Haplotype III) based on single nucleotide polymorphisms (SNPs) in the promoter regions (Liu et al., 2017). *OsNramp5* with Haplotype I promoter showed higher expression than *OsNramp5* with Haplotype II or Haplotype III promoter (Liu et al., 2017). Rice varieties carrying *OsNramp5* with Haplotype I promoter also accumulated more Mn in brown rice (Liu et al., 2017). T5105 carries an *OsNramp5* with Haplotype II promoter in its genome (Fig. 2A). qRT-PCR analysis revealed that the expression of *OsNramp5* in the root of T5105 was significantly lower than that in the root of Nipponbare, a cultivar carrying an *OsNramp5* with Haplotype I promoter (Fig. 2B) (Liu et al., 2017). Both the gene redundancy between *OsNramp1* and *OsNramp5* and the low transcription activity of *OsNramp5* in T5105 may affect the contribution of *OsNramp5* to Cd and Mn accumulation in grain. In this scenario, it is clear that the *OsHMA3* overexpression approach worked more efficiently than the *OsNramp5* knock-out method in generating low-Cd rice grain without affecting Mn uptake and transport in rice.

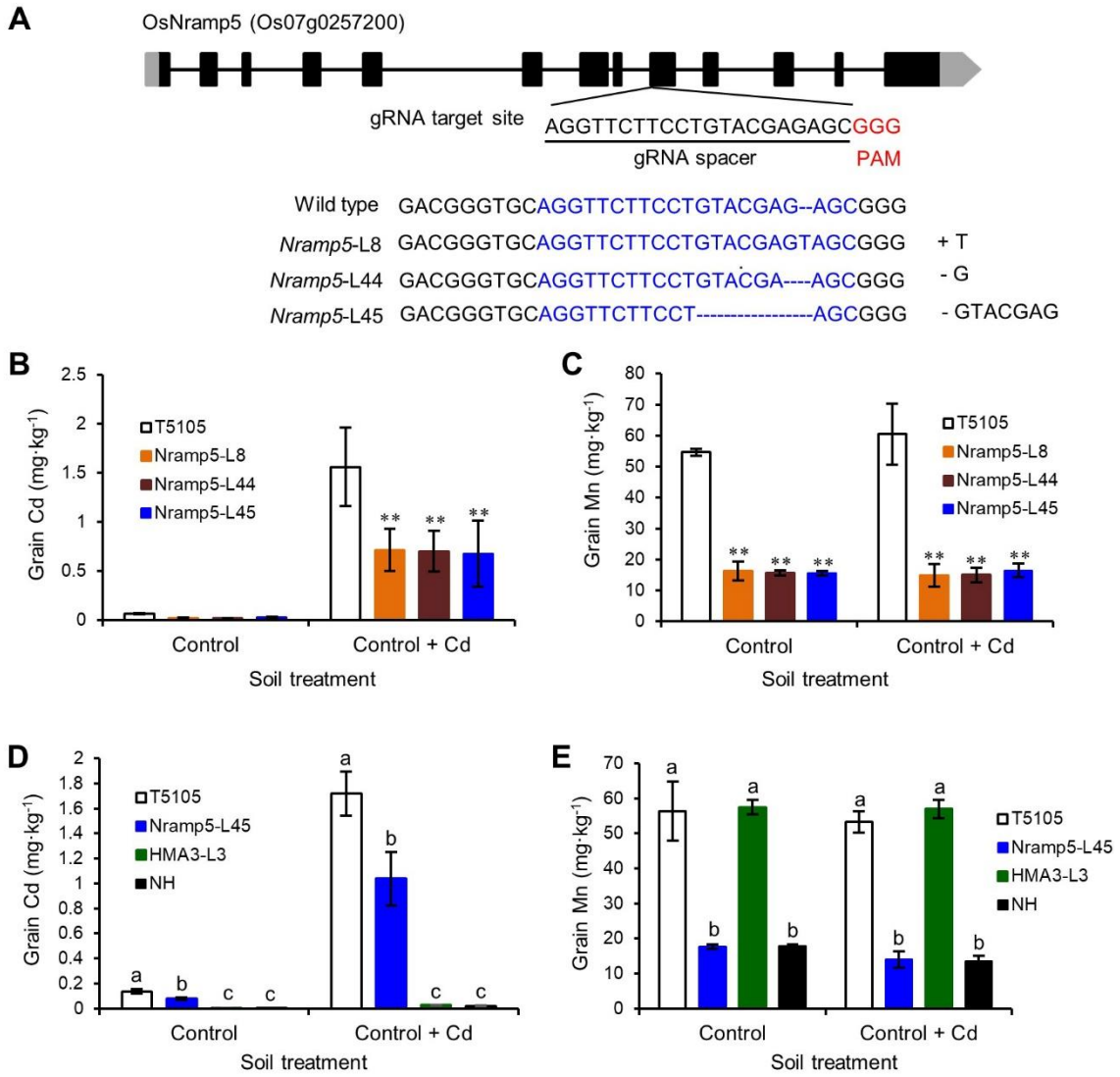

Fig. 1. Characterization of *OsNramp5* knock-out mutants and *OsHAM3*-overexpressing line

(**A**) *OsNramp5* knockout mutants obtained through CRISPR-Cas9-mediated gene editing. (**B**) Grain Cd concentration of T5105 and *OsNramp5* mutants. (**C**) Grain Mn concentration of T5105 and *OsNramp5* mutants. (**D**) Grain Cd concentration of T5105, Nramp5-L45, HMA3-L3 and NH. (**D**) Grain Mn concentration of T5105, Nramp5-L45, HMA3-L3 and NH. For soil treatment in (**B**) to (**E**), the soil was supplied with $CdSO_4$ to the concentration of 3 mg/kg Cd. Asterisks in (**B**) and (**C**) indicate a significant difference between T5105 and *OsNramp5* mutants at ** $P < 0.01$ by Student's *t*-test. Different letters in (**D**) and (**E**) indicate statistically different at $P < 0.05$ according to LSD's test. All data are means ± SD of at least three biological replicates. T5105, non-transgenic control; Nramp5-L8, Nramp5-L44 and Nramp5-L45, three independent Nramp5 knock-out mutants; HMA-L3, a representative *OsHMA3*-overexpressing line derived from the previous study (Gui et al., 2024); NH, a double homozygous line harbouring the *OsNramp5* mutant allele from Nramp5-L45 and the P_{Actin1}:*cHMA3*:T_{Nos} gene from HMA3-L3. Gene editing on *OsNramp5* in T5105 was done according to the method described previously (Tang et al., 2017). Cd and Mn concentration in grains were measured by ICP-MS as described in the previous report (Gui et al., 2024).

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	-

B

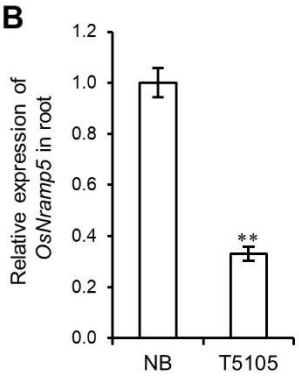

Fig. 2. SNPs in *OsNramp5* promoters and the expression of *OsNramp5* in rice root

(**A**) SNPs in different haplotypes of *OsNramp5* promoter. The SNPs in Haplotype I to Haplotype III, published by Liu et al., (2017), were shown in the top three rows, whereas the SNPs in *OsNramp5* promoters of Nipponbare (NB) and T5105 are listed below. (**B**) Relative expression of *OsNramp5* in the root of Nipponbare and T5105 detected by qRT-PCR. qRT-PCR analysis was conducted according to the method described in the previous report (Gui et al., 2024). 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.

ACKNOWLEDGEMENT

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).

CONFLICT OF INTEREST

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*-overexpressing line HMA3-L3 used in this study was disclosed in the patent.

REFERENCES

Chang JD, Huang S, Yamaji N, Zhang W, Ma JF, Zhao FJ (2020) OsNRAMP1 transporter contributes to cadmium and manganese uptake in rice. Plant Cell Environ **43:** 2476-2491

Commission CA (2006) Report of the 29th session of the Codex Alimentarius Commission. FAO/WHO. Geneve. Switzerland

Gui Y, Teo J, Tian D, Yin Z (2024) Genetic engineering low-arsenic and low-cadmium rice grain. J Exp Bot **75:** 2143-2155

Hu S, Zhou L, Wang J, Mawia AM, Hui S, Xu B, Jiao G, Sheng Z, Shao G, Wei X (2024) 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

Ishikawa S, Ishimaru Y, Igura M, Kuramata M, Abe T, Senoura T, Hase Y, Arao T, Nishizawa NK, Nakanishi H (2012) Ion-beam irradiation, gene identification, and marker-assisted breeding in the development of low-cadmium rice. Proc Natl Acad Sci USA **109:** 19166-19171

Ishimaru Y, Takahashi R, Bashir K, Shimo H, Senoura T, Sugimoto K, Ono K, Yano M, Ishikawa S, Arao T (2012) Characterizing the role of rice NRAMP5 in manganese, iron and cadmium transport. Sci Rep **2:** 286

Liu C, Chen G, Li Y, Peng Y, Zhang A, Hong K, Jiang H, Ruan B, Zhang B, Yang S, Gao Z, Qian Q (2017) Characterization of a major QTL for manganese accumulation in rice grain. Sci Rep **7:** 17704

Miyadate H, Adachi S, Hiraizumi A, Tezuka K, Nakazawa N, Kawamoto T, Katou K, Kodama I, Sakurai K, Takahashi H, Satoh-Nagasawa N, Watanabe A, Fujimura T, Akagi H (2011) OsHMA3, a P1B-type of ATPase affects root-to-shoot cadmium translocation in rice by mediating efflux into vacuoles. New Phytol **189:** 190-199

Sasaki A, Yamaji N, Ma JF (2014) Overexpression of OsHMA3 enhances Cd tolerance and expression of Zn transporter genes in rice. J Exp Bot **65:** 6013-6021

Sasaki A, Yamaji N, Yokosho K, Ma JF (2012) Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. Plant Cell **24:** 2155-2167

Shao JF, Xia J, Yamaji N, Shen RF, Ma JF (2018) Effective reduction of cadmium accumulation in rice grain by expressing OsHMA3 under the control of the OsHMA2 promoter. J Exp Bot **69:** 2743-2752

Tang L, Mao B, Li Y, Lv Q, Zhang L, Chen C, He H, Wang W, Zeng X, Shao Y, Pan Y, Hu Y, Peng Y, Fu X, Li H, Xia S, Zhao B (2017) Knockout of OsNramp5 using the CRISPR/Cas9 system produces low Cd-accumulating indica rice without compromising yield. Sci Rep **7:** 14438

Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF (2010) Gene limiting cadmium accumulation in rice. Proc Natl Acad Sci USA **107:** 16500-16505