A weak allele of OsNRAMP5 for safer rice

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The dilemma between Mn and Cd

NRAMPs (natural resistance-associated macrophage proteins) are a family of membrane transporters for divalent transition metals. NRAMP transporters are present in all organisms, including bacteria, fungi, plants, and animals (Bozzi and Gaudet, 2021). The first NRAMP gene (NRAMP1) was cloned from mice varying in natural resistance to infection with intracellular pathogens (Vidal et al., 1993). NRAMP1 encodes a di-valent metal efflux pump at the phagosomal membrane of macrophages that functions to extract essential metals such as Mn$^{2+}$ and Fe$^{2+}$ from phagosomes to help kill engulfed pathogens (Forbes and Gros, 2001; Bozzi and Gaudet, 2021). NRAMP2 (also called DCT1 or DMT1) was cloned from rat and encodes a transporter for the uptake of Fe$^{2+}$ and other divalent transition metals in the proximal duodenum of the small intestines (Gunshin et al., 1997). The genome of rice contains seven NRAMP genes, some of which have been characterized functionally (Table 1). Most mammalian and prokaryotic NRAMP transporters are promiscuous regarding their transport substrates, often including Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$ (Gunshin et al., 1997; Bozzi and Gaudet, 2021). Mn$^{2+}$, Fe$^{2+}$, and Cd$^{2+}$ are also the common substrates for plant NRAMPs (Table 1), except OsNRAMP4 (OsNRAT1) which unusually transports Al$^{3+}$ (Xia et al., 2010). While Mn, Fe, Zn, Co, and Ni are essential for life, Cd is highly toxic and a carcinogen to humans. The reason why evolution has not resulted in Cd-discriminating NRAMPs may be because Cd usually is not present at levels in the environment high enough to exert a strong and persistent selective pressure on organisms to evolve transporter proteins that can discriminate it from other essential trace metals (Bozzi and Gaudet, 2021; Zhao et al., 2022).

Ten years ago, OsNRAMP5 was identified as the major transporter for the uptake of Mn and Cd in rice (Ishikawa et al., 2012; Sasaki et al., 2012). OsNRAMP5 is polarly localized to the distal side of the plasma membranes of the exodermal and endodermal cells of rice roots responsible for transporting Mn$^{2+}$ and Cd$^{2+}$ into the root cells (Sasaki et al., 2012). Knockout of OsNRAMP5 resulted in dramatic decreases, often >90%, in the plant uptake of Mn and Cd and their accumulation in the grains (Ishikawa et al., 2012; Sasaki et al., 2012; Yang et al., 2014). Because Mn is an essential micronutrient functioning, among others, in PSI, knockout mutants of OsNRAMP5 grew poorly under conditions of low Mn supply and exhibited typical Mn deficiency symptoms (Sasaki et al., 2012; Yang et al., 2014; Chang et al., 2020). The growth defect can be rescued by supplying a relatively high concentration of Mn in the nutrient solution (8 μM) (Yang et al., 2014). When paddy soils are flooded for growing rice, the
Table 1. Functions of NRAMPs in rice (Oryza sativa).

| NRAMP family | Location | Transport substrates | Functions | References |
|--------------|----------|----------------------|-----------|------------|
| OsNRAMP1     | Plasma membrane | Mn, Cd | Uptake of Mn and Cd | Takahashi et al. (2011); Chang et al. (2020) |
| OsNRAMP2     | Tono plast | Fe, Cd | Exporting Fe and Cd from the vacuole to the cytosol | Li et al. (2021); Chang et al. (2022) |
| OsNRAMP3     | Plasma membrane | Mn | Distribution of Mn in the nodes | Yamaji et al. (2013) |
| OsNRAMP4(OsNRAT1) | Plasma membrane | Al | Uptake of Al | Xia et al. (2010) |
| OsNRAMP5     | Plasma membrane | Mn, Cd | Uptake of Mn and Cd | Ishikawa et al. (2012); Sasaki et al. (2012) |
| OsNRAMP6     | Plasma membrane | Fe, Mn | Negatively regulates resistance to the rice blast fungus in rice plants | Peris-Peris et al. (2017) |

A weak transporter offers a compromise solution

To circumvent the above problems, Kuramata et al. (2022) looked for mutant alleles of OsNRAMP5 that may lead to lower Cd uptake but without causing severe Mn deficiency. They identified several mutant alleles from a mutagenized pool of rice. Among these alleles, one with a substitution of glutamine at the 337th position by lysine (Q337K) in the protein sequence showed a Cd and Mn accumulation phenotype intermediate between that of the wild type and the knockout lines. Heterologous expression in yeast and physiological studies of the Q337K mutant confirmed that this mutation significantly weakened, but did not abolish, the transport activities of OsNRAMP5 for both Mn$^{2+}$ and Cd$^{2+}$. The OsNRAMP5-Q337K mutant was less susceptible to Mn deficiency than the knockout lines and could tolerate an Mn concentration in the nutrient solution as low as 0.1 μM without showing Mn deficiency symptoms in a hydroponic experiment. When grown in a paddy field with a Cd-contaminated soil, the OsNRAMP5-Q337K mutant produced 50% and 30% lower grain concentrations of Cd and Mn, respectively, than the wild type, whereas grain yields were comparable. Thus, the OsNRAMP5-Q337K mutant is a compromise between acquiring enough Mn and not accumulating too much Cd.

How to increase substrate selectivity?

An ideal solution to solve the problem of Cd contamination in rice would be to enhance the selectivity of OsNRAMP5 towards Mn$^{2+}$. While the OsNRAMP5-Q337K allele decreases the uptake of both Mn$^{2+}$ and Cd$^{2+}$, it does not affect the transporter’s selectivity towards these two competing substrates (Kuramata et al., 2022). In fact, substitutions of Q337 with other amino acids affect the transport activities of Mn$^{2+}$ and Cd$^{2+}$ similarly, suggesting that the 337th residue affects the rate of transport but not the substrate selectivity (Kuramata et al., 2022). Q337 is located in transmembrane helix 8 (TM8), which is not directly involved in metal binding (Bozzi and Gaudet, 2021; Fig. 1). Based on homology modelling of the protein structure, Kuramata et al. (2022) suggest that amino acid substitutions of Q337 affect the conformational dynamics of the protein by changing the structural flexibility of TM8 and the stability of the loop structure between TM7 and TM8.

Studies on the structures of mammalian and prokaryotic NRAMPs have yielded a wealth of information regarding the transport mechanisms of these proteins (Ehrnstorfer et al., 2022). In an attempt to increase the selectivity of OsNRAMP5 towards Mn$^{2+}$, the consensus for the Q337K mutation was changed to Q337E, which is predicted to stabilize the protein structure and the loop TM7-TM8. The Q337E mutant was found to have a higher Mn$^{2+}$ uptake than the Q337K mutant, suggesting that it may be a more effective transporter for Mn$^{2+}$.

In conclusion, the OsNRAMP5-Q337K mutant represents a promising strategy for combating the Cd contamination problem in rice. However, further studies are needed to understand the molecular mechanisms behind the increased Mn$^{2+}$ selectivity of this mutant, and to explore other approaches for enhancing substrate selectivity in OsNRAMP5 and other members of the NRAMP family.
The highly conserved metal-binding site is located within TMs 1 and 6. Based on homology modelling, five amino acid residues in OsNRAMP5, namely A57, D60, and N63 in TM1, and A232 and M235 in TM6, provide coordination to the metal ion. Interestingly, the methionine residue in TM6 plays a crucial role in metal transport and substrate selectivity. In the NRAMP of the bacterium Deinococcus radiodurans, the presence of methionine (M230, corresponding to M235 in OsNRAMP5) makes Cd$^{2+}$ the preferred substrate. When M230 was mutated to alanine (A), Cd$^{2+}$ uptake decreased greatly but uptake of Mn$^{2+}$ and Fe$^{2+}$ was still robust, although this mutation also allowed Ca$^{2+}$ and Mg$^{2+}$ to be transported (Bozzi et al., 2016). The importance of methionine for Cd$^{2+}$ transport is consistent with the ‘soft’ metal Cd preferring S-containing ligands. M235 in OsNRAMP5 may be a target to manipulate the selectivity between Mn$^{2+}$ and Cd$^{2+}$. A potential pitfall is that Ca$^{2+}$ and Mg$^{2+}$, which are usually abundant in soils, may then compete with Mn$^{2+}$ uptake (Bozzi et al., 2016).

Apart from the metal-binding site, mutations in other amino acid residues may also affect substrate selectivity. An elegant study was presented by Pottier et al. (2015), who randomly mutated cDNA of the Arabidopsis AtNRAMP4, which encodes a tonoplast efflux transporter with transport activities for Fe$^{2+}$, Mn$^{2+}$, Zn$^{2+}$, and Cd$^{2+}$. They then screened the mutated AtNRAMP4 in the yeast mutant fet3fet4 defective in Fe uptake for mutant alleles that could rescue the growth of the mutant (i.e. restoring Fe$^{2+}$ uptake) but with a suppressed sensitivity to Cd (i.e. decreased Cd$^{2+}$ uptake). They found that the mutations L67I, L67V, E401K, and F413I restored Fe$^{2+}$ uptake but with suppressed Cd sensitivity compared with the wild-type AtNRAMP4. Further experiments showed that L67V and L67I mutations specifically impaired Cd$^{2+}$ transport by AtNRAMP4. These results provide a proof of concept that NRAMPs could be manipulated to discriminate Cd$^{2+}$ while maintaining the transport activities for essential trace metals. It would be interesting to evaluate whether mutations in the corresponding positions in OsNRAMP5 produce the same effects.

While the study of Kuramata et al. (2022) represents a step forward in the direction towards low Cd rice, a more perfect solution by altering metal substrate preference awaits further investigations.

Conflict of interest
The authors declare they have no conflicts of interest.

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