The Vacuolar Transporter OsNRAMP2 Mediates Fe Remobilization During Germination and Affects Cd Distribution to Rice Grain

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Abstract

Background and aims Iron (Fe) deficiency in plants is a common problem affecting agricultural production. Cadmium (Cd) is a toxic metal that can be taken up and transported within plants by transporters for divalent metals including Fe(II). The present study aims to investigate the functions of OsNRAMP2 (Natural Resistance-Associated Macrophage Protein 2) in the remobilization and distribution of Fe and Cd in rice.

Methods The expression pattern of *OsNRAMP2* was determined by quantitative real-time PCR and *pOsNRAMP2:GUS* assay. Knockout mutants of *OsNRAMP2* were generated by using CRISPR/Cas9 gene editing. Localization of Fe in the vacuolar globoids of germinating seeds was imaged by high-resolution transmission electron microscopy coupled with energy-dispersive X-ray spectroscopy. Distributions of Fe and Cd between different plant tissues were investigated in hydroponic and soil pot experiments.

Results *OsNRAMP2* was mainly expressed in the embryo of germinating seeds, roots, leaf sheaths and leaf blades. OsNRAMP2 was localized at the tonoplast. Knockout of *OsNRAMP2* delayed seed germination and produced chlorotic seedling leaves. Remobilization of Fe stored in the protein storage vacuoles in the scutellum of germinating seeds was restricted in *osnramp2* mutants compared with wild type. Expression of genes related to Fe uptake was enhanced in the seedlings of *osnramp2* mutants. Knockout of *OsNRAMP2* significantly decreased the distribution of Cd, but not Fe, from leaves and straws to rice grains.

Conclusions OsNRAMP2 plays an important role in remobilizing vacuolar Fe during seed germination and affects translocation of Cd from vegetative tissues to rice grains.

Introduction

Iron (Fe) is an essential element for all living organisms. This transition metal plays a key role in electron transfer in both photosynthetic and respiratory reactions. Although there is abundant Fe in the Earth's crust, the bioavailability of Fe is often very limited and Fe deficiency occurs frequently both in humans and in crop plants that feed them (Takahashi et al. 2003). Iron deficiency-induced leaf chlorosis and plant growth retardation is a major agricultural problem globally (Ling et al. 1999; Gao et al. 2016).

Plants use different strategies to acquire Fe from the soil. Strategy I employed by dicotyledonous plants includes acidification of the rhizosphere, reduction of ferric iron [Fe(III)] to more soluble ferrous iron [Fe(II)] by membrane-bound Fe(III)-chelate reductases, and uptake of Fe(II) by membrane transporters such as IRT1 (Ishimaru et al. 2006; Cheng et al. 2007; Huang et al. 2020). Strategy II is employed only by graminaceous monocotyledonous species. In response to Fe deficiency, roots of strategy II plants secrete phytosiderophores, such as 2`-deoxymugineic acid (DMA), to chelate and mobilize Fe(III) in the rhizosphere, and subsequently take up Fe(III)-phytosiderophores complexes by transporters such as OsYSL15 (Yellow Stripe1-Like 15) in rice (Römheld and Marschner 1986; Inoue et al. 2009; Lee et al. 2009; Conte and Walker 2011). In Strategy II plants, nicotianamine (NA) synthase (NAS) and nicotianamine

aminotransferase (NAAT) are two key enzymes in the biosynthesis of NA and its conversion to DMA (Kobayashi et al. 2010). NA not only is the precursor of DMA, but also plays an important role in the phloem transport of Fe by complexing Fe(III) (Takahashi et al. 2003). Rice *osnaat1* mutants were not able to produce DMA and take up Fe(III) efficiently (Cheng et al. 2007; Inoue et al. 2008). Secretion of DMA is mediated by the plasma membrane transporter OsTOM1 (Transporter of Mugineic Acid 1) (Nozoye et al. 2011). Interestingly, rice appears to possess both strategies I and II (Cheng et al. 2007; Ishimaru et al. 2007). In flooded paddy soil, the major form of Fe in soil solution is Fe(II), which is present at relatively high concentrations due to reduction of Fe(III) under anoxic conditions (Wang et al. 2020). Uptake of Fe(II) may be mediated by OsIRT1 and OsIRT2 (Iron-Regulated Transporter 1 and 2) in rice roots (Bughio et al. 2002; Ishimaru et al. 2006).

Because excess Fe is highly phytotoxic, cellular Fe concentration has to be regulated strictly (Akmakjian et al. 2021). Sequestration of Fe into the vacuoles via the transporters AtVIT1, OsVIT1 and OsVIT2 (Vacuolar Iron Transporter 1 and 2) is an important mechanism of cellular Fe homeostasis in *Arabidopsis thaliana* and rice (Kim et al. 2006; Zhang et al. 2012; Che et al. 2021). In addition, OsVIT2 also regulates the distribution of Fe to the grains by sequestering Fe in the vacuoles in the mestome sheath, nodes, and aleurone layer (Che et al. 2021). Synchrotron X-ray fluorescence imaging showed that Fe is preferentially accumulated in the vacuoles of the fundamental parenchyma cells of the nodes and internodes of rice plants (Moore et al. 2014). Conversely, both AtNRAMP3 and AtNRAMP4 (Natural Resistance-Associated Macrophage Protein 3 and 4) are localized at the tonoplast and play redundant roles in the efflux of the vacuolar Fe store to support early development or under Fe-limiting conditions (Lanquar et al. 2005). OsNRAMP2 in rice is a close homologue of AtNRAMP3 and AtNRAMP4 (Supplemental Fig. S1). A recent study showed that OsNRAMP2 is a vacuolar Fe efflux transporter and plays a role in Fe translocation from the vacuole to the cytosol of rice plants (Li et al. 2021).

Cadmium (Cd) is a highly toxic element for living organisms. Because Cd has a relatively high availability in soil, it is readily taken up by food crops, posing a risk to human health (Clemens 2019; McLaughlin et al. 2021; Zhao et al. 2021). Accumulation of Cd in rice grain is of particular concern, as rice is the major dietary source of Cd for Asian populations (Meharg et al. 2013; Song et al. 2017; Wang et al. 2019). Understanding how plants take up and transport Cd is imperative for developing strategies to reduce the risk of Cd accumulation in food crops, especially rice. Owing to similarities in the physicochemical properties, Cd can hitchhike on a number of Fe and Mn transporters, e.g. AtIRT1, AtIRT2, AtNRAMP1, OsNRAMP1 and OsNRAMP5 (Thomine et al. 2000; Vert et al. 2002; Cailliatte et al. 2010; Ishikawa et al. 2012; Sasaki et al. 2012; Yang et al. 2014; Castaings et al. 2016; Chang et al. 2020a, b). Meanwhile, another key player that affects Cd translocation is OsHMA3 (Heavy Metal ATPases 3), which is a tonoplast transport responsible for the vacuolar sequestration of Cd (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016; Sui et al. 2019). Previous studies using heterologous expression in yeast also suggested that OsNRAMP2 has a transport activity for Cd (Zhao et al. 2018; Li et al. 2021). Whether OsNRAMP2 participates in the distribution of Cd in rice plants has not been elucidated.

In the present study, we performed detailed functional analysis of OsNRAMP2 with regard to its roles in Fe remobilization and Cd distribution in rice. We show that OsNRAMP2 plays a crucial role in Fe remobilization from vacuoles during seed germination and also contributes to Cd distribution to rice grains.

Materials And Methods

Plant materials and growth conditions

Rice (*Oryza sativa* L.) cultivar Nipponbare was used for gene expression experiments. Knockout lines of *osnramp2* were generated in the background of cv. Zhonghua11 (ZH11) using CRISPR/Cas9 technology as described below.

For hydroponic experiments, seeds were soaked in water for two days at 37 ^{o}C in the dark and then transferred to a black net floating on water. One week later, seedlings were transferred to 6-L plastic pots (24 plants per pot) containing ½ Kimura B solution (Chang et al. 2020a). The composition of the nutrient solution was: 0.09 mM KH_2PO_4, 0.27 mM $MgSO_4$, 0.18 mM $(NH_4)_2SO_4$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2$, 3.0 μM H_3BO_3, 0.5 μM $MnCl_2$, 1.0 μM $(NH_4)_6Mo_7O_{24}$, 0.4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, and 20 μM Fe(III)-EDTA. The pH of this solution was buffered at around 5.6 with 2 mM MES [2-(N-morpholino)-ethane sulphonic acid] buffer, and the nutrient solution was renewed every three days. Plants were grown in a growth room with a light intensity of approximately 400 μmol m^{-2} s^{-1} (sodium vapour lamps) under a photoperiod of 12/12 h at 30/25 °C.

Vector constructions and generation of transgenic plants

To generate *osnramp2* mutants, knockout vectors of *OsNRAMP2* were constructed using a CRISPR/Cas9 expression getaway system (Miao et al. 2013). The target sequences were mutated in the first exon of the coding region of *OsNRAMP2* (LOC_Os03g11010) (Supplemental Fig. S2). Specific target sequences were constructed on the sgRNA expression cassettes of OsU3 and cloned into the binary vector pOs-Cas9 by the LR Clonase (Invitrogen). To investigate tissue-specific expression pattern of *OsNRAMP2*, a 1885 bp promoter sequence upstream of the start codon of *OsNRAMP2* was amplified from the Nipponbare genomic DNA. The fragment containing *pOsNRAMP2*, the *GUS* coding sequence and *NOS* terminatorwere cloned into the pCAMBIA1301 vector at the *Kpn*I and *Nco*I sites using CloneExpress MultiS One Step Cloning Kit (Vazyme, Nanjing). All constructs were transformed into the calluses of wild-type rice (cv. ZH11) by *Agrobacterium*-mediated transformation (Hiei et al. 1997). Transgenic lines were selected by hygromycin resistance and verified by sequencing. T2 or T3 generations of homozygous transgenic lines were used in the experiments described below. Three independent lines of *osnramp2* knockout were obtained (Supplemental Fig. S1B). In some experiments, only two of the mutant lines were used due to limited availability of seeds.

Gene expression analysis

To investigate the expression pattern of *OsNRAMP2* in different tissues at different growth stages, cDNA was prepared from rice plants (cv Nipponbare) grown in a paddy field. To investigate the effects of Cd treatment or trace metal deficiency on *OsNRAMP2* expression, 21-day-old seedlings (cv. Nipponbare) were transferred to ½ Kimura B nutrient solution without Zn, Mn, Fe or Cu, or containing 0.5 μM $CdCl_2$ for 7 days. Roots and shoots were used for RNA extraction and cDNA preparation as described previously (Chang et al. 2020b).

To investigate the effect of knockout of *OsNRAMP2* on the expression of Fe transport related genes, 14-day-old *osnramp2* mutantsand wild type plants were grown in ½ Kimura B nutrient solutions containing 0, 0.2 or 20 μM EDTA-Fe(III) for 21 days. Three biological replicates were included for each treatment. At the end of experiment, roots were sampled for RNA extraction. The expression levels of *OsNRAMP2, OsIRT1, OsIRT2, OsYSL15, OsTOM1, OsNAS1* and *OsNAAT1* were quantified using an AceQTM qPCR SYBR Green Master Mix (Vazyme, Nanjing) on a BioRad CFX96. *α-tubulin* was used as the internal reference. The primer sequences used are shown in Supplemental Table S1.

Subcellular localization of OsNRAMP2

The full-length coding sequence of *OsNRAMP2* without the stop codon was amplified by PCR and inserted between the CaMV35S promoter and eGFP-NOS terminator in the pSAT6A-eGFP vector. The *OsNRAMP2-eGFP, eGFP-OsNRAMP2* and *eGFP* alone constructs were then introduced into the rice protoplasts by polyethylene-glycol method (Zhang et al. 2011). The MADS3-mCherry fusion protein was used as a nucleus marker (Gao et al. 2014). After incubation of protoplasts at 28 °C in the dark for 20 h, fluorescence signals were detected using a confocal laser scanning microscope (TCS SP8 X, Leica).

Phenotypic characterization of *osnramp2* mutants

To evaluate the effect of *OsNRAMP2* mutation on seed germination, seeds of *osnramp2* (two independent lines) and wild type were soaked in tap water for three days at 37 ^{o}C in the dark. Water was renewed every day. Germination rates were determined after 3 days.

To investigate the effect of Fe supply on growth phenotypes, 14-day-old *osnramp2* (two independent lines) and wild type plants were grown in ½ Kimura B nutrient solution containing 0, 0.2 or 20 μM EDTA-Fe(III) for 21 days. Each genotype had four biological replicates. At the end of experiment, roots were washed with tap water and deionized water for three times, and roots and shoots were separated. Plant samples were dried at 65 ^{o}C for 3 days and ground to fine powders. Samples were digested with HNO_3/$HClO_4$ (85:15, v:v) in a heating block. The concentrations of metals in the digests were determined by inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer NexIon 300x).

To investigate the effect of knockout of *OsNRAMP2* on root phenotypes, 14-day-old plants of *osnramp2* (two independent lines) and wild type were grown in ½ Kimura B nutrient solution containing 0, 0.2 or 20 μM EDTA-Fe(III) for 21 days. Each genotype and Fe treatment were replicated four times. Roots

of individual plants were imaged using a scanner (LA2400 Scanner, Canada). Total root length, total surface area, and numbers of tips were quantified using WinRHIZO software (WinRHIZO 2019a, Canada).

Electron microscopy and microanalysis

To investigate localization of Fe in the vacuolar globoids during seed germination, seeds of *osnramp2* mutants and wild type were soaked in water at 37 ^{o}Cfor 3 days. The localization of Fe in the vacuolar globoids was characterized using high-resolution transmission electron microscopy coupled with energy-dispersive X-ray spectroscopy and selected area electron diffraction (HRTEM-EDX-SAED) according to the method described previously (Lanquar et al. 2005; Jiang et al. 2021). Briefly, geminated seeds were chilled and fixed in 2.5% (v/v) triple distilled glutaraldehyde in 0.1 M phosphate buffer (PBS, pH 7.4) at 4.0°C for 24 h. The samples were fixed again with 1% (v/v) osmic acid and 0.1 M PBS (pH 7.4) at room temperature for 2 hours. Finally, they were rinsed by 0.1 M PBS (pH 7.4) three times (15 min each). The samples were embedded in resin and ultrathin sections with a thickness of 100 nm were cut using a Leica EM UC7 Ultramicrotome with a diamond knife (Diatome, Ultra 45°). The thin sections were observed under a transmission electron microscope equipped with an energy filter (Tecnai G^2 F20 system, FEI, USA) and an Energy-dispersive X-ray (EDX) spectroscopy (Aztec X-Max 150, Oxford, UK). For ESI (imaging of inelastically scattered electrons), images were captured using a slow-scan charge-coupled device (CCD) camera attached on an image filter (Gatan GIF 2000). For Fe colorization of TEM Images, the ESI images of Fe were background-subtracted, colored and superimposed on the transmission image.

Effect of knockout of *OsNRAMP2* on Cd accumulation

To test the effects of knockout of *OsNRAMP2* on Cd accumulation in rice, two independent mutation lines and wild type plants (21-day-old) were transferred to ½ Kimura B nutrient solutions containing 0.5 or 1.0 μM Cd for 10 days. Each genotype had four biological replicates. The concentrations of Cd in roots and shoots were determined by ICP-MS. To determine Cd concentration in the xylem sap, rice seedlings (28-day-old) of two *osnramp2* lines and wild type were transferred to ½ Kimura B nutrient containing 1.0 μM Cd for 12 h. Each genotype had four biological replicates. Stems were cut at 2-3 cm above the shoot-root junction, cleaned with deionized water and wrapped with an absorbent cotton ball. Xylem exudate was collected into the cotton ball for 4 h and centrifuged at 12,000 *g* for 10 min. Xylem sap was diluted with 2% HNO_3 before determination of Cd concentration by ICP-MS.

Soil pot experiment

A Cd-contaminated paddy soil was collected from a paddy field in Deyang, Sichuan Provence, China. The soil contained 3.4 mg Cd kg^{-1} and pH was 6.0. One seedling each of three *osnramp2* mutant lines and wild type were transplanted in a 15-L plastic pot filled with 8 kg soil. Four pots were prepared to represent four biological replicates. The experiment was conducted inside a net enclosure with natural sunlight and ambient temperature during the summer season of 2020. Tap water was used to flood the soil and maintain a 3 - 5 cm water level above the soil surface during the period of vegetative growth. Soil was

maintained at moist but unflooded conditions during the grain filling period. Plants were harvested at grain maturity. The concentrations of Cd and Fe in grains and straws were determined by ICP-MS.

Statistical analysis

Data were analyzed by one-way ANOVA, followed by comparisons of means using Tukey's test at $P < 0.05$. Statistical analyses were performed using OriginPro 2021.

Results

Expression pattern of *OsNRAMP2*

Quantitative reverse transcription PCR (Q-RT-PCR) was used to determine the expression levels of *OsNRAMP2* in various tissues of rice plants (cv. Nipponbare) grown in a paddy field at different growth stages. At the seedling, tillering, booting and flowering stages, *OsNRAMP2* was mainly expressed in the roots, leaf sheaths and leaf blades (Fig. 1A). The expression levels of *OsNRAMP2* in the leaf sheaths and leaf blades were higher than those in the rootsand stems at all growth stages. The expression level of *OsNRAMP2* in the leaf sheaths and leaf blades generally increased with growth stage with a peak at the flowering stage (Fig. 1A).

The responses of *OsNRAMP2* transcript level to withdrawal of micronutrients Fe, Zn and Cu or the addition of Cd were investigated in a hydroponic experiment. Withdrawal of Fe for 7 days slightly induced the expression of *OsNRAMP2* in roots, whereas other treatments had little effect (Fig. 2B). The response to Fe deficiency was further investigated in a time-course experiment. In both roots and shoots, *OsNRAMP2* expression was induced by Fe deficiency from day 2 onward, with a 1.75- and 2-fold increase, respectively, on day 5 of Fe withdrawal compared with the +Fe treatment on day 0 (Fig. 2C).

Tissue specificity and subcellular localization of OsNRAMP2

To investigate the tissue and cell specificity of *OsNRAMP2* expression, we generated transgenic rice (cv. ZH11) expressing the *GUS* reporter gene under the control of the native promoter of *OsNRAMP2*. Histochemical staining of the GUS activity in the *pOsNRAMP2::GUS* transgenic lines showed that *OsNRAMP2* was expressed mainly in the radicle and embryo of germinated seeds, as well as roots, leaf sheaths and leaf blades of seedings (Fig. 2A, B, C, H). Consistent with the Q-RT-PCR data, withdrawal of Fe from the nutrient solution for 7 days slightly increased the GUS activity in the roots (Fig. 2D, E, F, G). The GUS activity was localized in all root cells, as well as in the leaf mesophyll cells and the parenchyma cells surrounding the vascular bundles in the leaf sheaths (Fig. 2F, G, I).

To determine the subcellular localization of OsNRAMP2, *OsNRAMP2-eGFP* or *eGFP-OsNRAMP2* was transiently expressed in rice protoplasts driven by the CaMV35S promoter. MADS3-mCherry fusion protein was used as a nucleus marker (Gao et al. 2014). In the control expressing *eGFP* alone, the GFP signal was detected in the cytoplasm, whereas the GFP signal in the protoplasts expressing *OsNRAMP2-eGFP* or *eGFP-OsNRAMP2* was observed at the tonoplast with the nucleus marked by MADS3-mCherry being

outside of the GFP signal (Fig. 3). Fusion of GFP to the N- or C-terminal of OsNRAMP2 revealed the same subcellular localization at the tonoplast.

Knockout of *OsNRAMP2* inhibited germination and suppressed remobilization of vacuolar Fe during seed germination

Knockout of *OsNRAMP2* delayed seed germination (Fig. 4A, B). After 3 days of soaking at 37 ^{o}C, two *osnramp2* mutants had a germination rate of 21.2 – 27.2%, compared with 86.4% in wild type (Fig. 4C). After seedlings were grown in tap water for 8 days, shoot height of *osnramp2* mutants was 39.3% shorter than that of wild type (Fig. 4D). Moreover, *osnramp2* mutants displayed leaf chlorotic symptoms typical of Fe deficiency (Fig. 4D). The concentrations of Fe in the shoots and roots of the *osnramp2* mutants were significantly ($P < 0.05$) lower, by 41.2 - 46.9% and 41.9 - 47.8%, respectively, than wild type (Fig. 4E). The lower Fe concentrations in the mutant seedlings were not attributed to lower Fe concentrations in the seeds, because there was no significant difference in seed Fe concentration between mutants and wild type (Fig. 4F).

To investigate whether knockout of *OsNRAMP2* affects Fe remobilization during germination, we used electron microscopy coupled to energy dispersive X-ray (EDX) spectra to image the distribution of Fe in the scutellum cells of germinated seeds. In both wild type and *osnramp2*, Fe was localized to the electron dense globoids inside the protein bodies (i.e. protein storage vacuoles, Fig. 4G, H) in scutellum cells, which was confirmed by EDX analysis of individual globoids (Fig. 4L and M). This pattern of Fe localization is similar to that reported for germinated seeds of *Arabidopsis thaliana* (Lanquar et al. 2005). Compared with wild type, *osnramp2* mutant showed more Fe-containing globoids inside vacuoles (Fig. 4I and K), suggesting that remobilization of Fe from the protein storage vacuoles during germination is restricted in the mutant.

osnramp2 mutants were more sensitive to Fe deficiency at the seedling stage

Seedlings (14-day-old) of two *osnramp2* mutants and wild type were grown in nutrient solution containing 0, 0.2 or 20 μM Fe for 21 days. Under low Fe (0.2 μM) or no Fe conditions, *osnramp2* mutants grew more poorly than wild type, with significantly shorter root length and shoot height (Figs. 5A, B). Total root length, root surface area and the number of root tips (including both primary and lateral roots) of the mutants were all significantly ($P < 0.05$) smaller than those of wild type (Figs. 5B, Supplemental S3A-S3E). The youngest fully developed leaves of the mutant lines were chlorotic (Fig. 5A), and the chlorophyll content (SPAD value) was significantly lower than wild type (Fig. 5C). Meanwhile, knockout of *OsNRAMP2* significantly decreased plant total biomass (Fig. 5D). Surprisingly, *osnramp2* mutants contained significantly higher concentrations of Fe in roots and shoots than wild type under low Fe or no Fe treatments (Fig. 5E, F). Supply of 20 μM Fe alleviated the chlorotic symptoms of *osnramp2* mutants and improved their growth, although leaf chlorophyll content and plant biomass were still smaller than wild type (Fig. 5A, C, D). In addition, there was no significant difference in either shoot or root Fe concentration between mutants and wild type in the 20 μM Fe treatment (Fig. 5E, F).

Knockout of *OsNRAMP2* increased the expression of Fe transport related genes

Higher concentrations of Fe in the roots and shoots of *osnramp2* mutants could be an indirect result from altered expression of genes involved in Fe uptake and transport. To test this hypothesis, we quantified the expression of *OsIRT1, OsIRT2, OsYSL15*, *OsTOM1, OsNAS1* and *OsNAAT1* in the roots of mutants and wild type grown under different Fe (0, 0.2 and 20 μM) supply conditions for 21 days. As expected, low Fe or no Fe conditions increased the expression of *OsIRT1, OsIRT2, OsYSL15*, *OsTOM1, OsNAS1* and *OsNAAT1* markedly (Fig. 6A-F). Moreover, *osnramp2* mutants showed significantly higher expression of *OsIRT1* and *OsYSL15* than wild type under all Fe supply conditions (Fig. 6A-C). In addition, knockout of *OsNRAMP2* significantly increased the expression of *OsTOM1*, *OsNAS1*, and *OsNAAT1* in either normal or the low Fe conditions (Fig. 6D-F).

Knockout of *OsNRAMP2* decreased Cd translocation to and accumulation in rice grain

To test whether OsNRAMP2 is involved in Cd distribution, seedlings (21-day-old) of two *osnramp2* mutants and wild type were grown in nutrient solution containing 0.5 or 1.0 μM Cd for 10 days. There were no significant differences in the growth phenotypes including root length and shoot height between mutants and wild type (Supplemental Fig. 45A-C). Knockout of *OsNRAMP2* had no significant effect on the concentrations of Cd in the roots and shoots at 0.5 μM Cd (Fig. 7A, B). At 1.0 μM Cd, the concentrations of Cd in the roots and shoots of two *osnramp2* lines were significantly ($P < 0.05$) lower than those of wild type by 20.4 - 22.7% and 11.6 - 13.9%, respectively (Fig. 7A, B). After exposure to 1.0 μM Cd for 12 h, xylem sap collected from two *osnramp2* mutants also contained significantly lower concentrations of Cd than that from wild type (Fig. 7C).

To examine the effect of knockout of *OsNRAMP2* on Cd accumulation in rice grain, three *osnramp2* mutant lines and wild type were grown in a Cd-contaminated paddy soil in a pot experiment. At maturity, there was no significant difference in plant height between *osnramp2* mutants and wild type (Fig. 8A). However, knockout of *OsNRAMP2* decreased straw biomass and grain yield by 47.3 - 59.7% and 31.5 -47.0%, respectively, compared with wild type (Fig. 8B, C). Grain Cd concentration in wild type was well over the Chinese permissible limit of 0.2 mg kg^{-1}. Knockout of *OsNRAMP2* significantly decreased grain Cd concentration by 21.0 - 23.9% compared with wild type (Fig. 8D). In contrast, Cd concentrations in the flag leaves, leaf sheaths and straws of *osnramp2* mutants were significantly higher than those of wild type (Fig. 8E, F, G). In contrast, knockout of *OsNRAMP2* did not significantly affect the concentrations of Fe in the grains, flag leaves, leaf sheaths or straws, although there was a tendency for higher concentrations in the flag leaves, leaf sheaths and straws of mutants (Fig. 8H-L).

Discussion

The NRAMP family transporters have diverse subcellular localization and transport functions. For example, AtNRAMP1 is a plasma membrane transporter functioning on the uptake of Fe, Mn and Cd (Cailliatte et al. 2010; Castaings et al. 2016). OsNRAMP1 and OsNRAMP5 are plasma membrane

transporters important for the influx of Mn and Cd into root cells (Ishikawa et al. 2012; Sasaki et al. 2012; Yang et al. 2014; Chang et al. 2020a, b). AtNRAMP2 is a resident protein in the *trans*-Golgi network and functions in the remobilization of Mn in the Golgi for root growth under Mn-deficient conditions (Alejandro et al. 2017; Gao et al. 2018). AtNRAMP3 and AtNRAMP4 are two major efflux transporters localized at the tonoplast to mobilize vacuolar Fe during seed germination of *A. thaliana* (Lanquar et al. 2005). Recently, OsNRAMP2 has also been shown to be a tonoplast transporter involved in Fe efflux from vacuoles in rice (Li et al. 2021). Our study provides further evidence that OsNRAMP2 participates in Fe remobilization from vacuolar metal stores during seed germination and also affects Cd distribution to rice grain.

OsNRAMP2 is highly expressed in the radicles and embryonal of germinated seeds, as well as in leaf sheaths and leaf blades (Fig. 2), and encodes a protein localized at the tonoplast (Fig. 3). The most noticeable phenotypes of *osnramp2* mutants compared with wild type are delayed seed germination and chlorotic leaves in young seedlings grown under no Fe or low Fe conditions (Figs. 4 and 5). The chlorotic symptoms of the mutants could be alleviated by growing seedlings under a normal level of Fe supply (20 μM). Because Fe concentrations in the seeds of *osnramp2* mutants were similar, the phenotypes are likely caused by an inefficient remobilization of the Fe stored in the mutant seeds. Microanalysis of Fe using HRTEM-EDX-SAED showed that more Fe-rich globoids remained in the protein storage vacuoles in the scutellum in the germinating seeds of *osnramp2* than wild type (Fig. 4), supporting the notion that Fe remobilization from the vacuoles is impaired in the mutants. Because Fe-containing heme is a key component of cytochrome c, an electron carrier in the respiratory chain (Roschzttardtz et al. 2009; Bastow et al. 2018), inefficient Fe remobilization could lead to low energy generation and delayed germination in the mutants. Compared with wild type, roots of *osnramp2* young seedlings showed markedly enhanced expression of a number of genes involved in both the strategies I and II pathways, especially under low Fe supply (Fig. 6), indicating that the mutants experienced more acute Fe deficiency. Enhanced expression of Fe acquisition-related genes in the mutants could lead to more uptake of Fe available in the growth medium. This could partly explain higher Fe concentrations in the roots and shoots of mutant seedlings under low Fe conditions (Fig. 5). Alternatively, the higher Fe concentrations could also be attributed to smaller plant biomass of the mutant seedlings.

Despite *OsNRAMP2* being also highly expressed in the leaf sheaths and leaf blades, distribution of Fe between leaves and grains was not significantly affected (Fig. 8). These results suggest that OsNRAMP2 plays relatively little role in remobilizing Fe in the leaf vacuoles for translocation to the grains. This is not surprising, because Fe in vegetative tissues is relatively immobile, and re-translocation of Fe from one shoot tissue or plant part to another is negligible (Lieten 2001; Sawyer 2004). A possible reason is that Fe stored in the vacuoles may be precipitated with phosphate, as demonstrated by strong colocalization of Fe and phosphate in the vacuoles of fundamental parenchyma cells in the nodes and internodes of rice plants (Moore et al. 2014). In this regard, the form of Fe stored in the protein storage vacuoles in the embryo may be more mobile to allow remobilization during germination.

Because of similarities in physicochemical properties, Cd can be transported by some Fe(II) transporters, such as IRT1 (Vert et al. 2002). There is also some evidence that AtNRAMP3 and AtNRAMP4 can mediate Cd efflux out of the vacuoles in *Arabidopsis* (Thomine et al. 2000; Pottier et al. 2015). The Cd transport activity of OsNRAMP2 was demonstrated by heterologous expression assays in yeast (Zhao et al. 2018; Li et al. 2021). In the present study, knockout of *OsNRAMP2* significantly decreased grain Cd concentration but increased the concentrations of Cd in leaves and straws (Fig. 8), suggesting that OsNRAMP2 also mediates Cd efflux from the vacuoles in the vegetative tissues for translocation to grains. In contrast to Fe, Cd is much more mobile and can be readily redistributed within plants (Uraguchi and Fujiwara 2013; Zhao and Wang 2020). This difference probably explains why knockout of *OsNRAMP2* affects the distribution of Cd but not Fe. The effect of *OsNRAMP2* knockout on Cd translocation from roots to shoots was limited (Fig. 7), possibly because *OsNRAMP2* was not strongly expressed in roots. It has been well established that OsHMA3 mediates Cd influx into the vacuoles and its activity is a key determinant controlling Cd translocation from roots to shoots and grains of rice (Ueno et al. 2010; Miyadate et al. 2011; Yan et al. 2016; Sui et al. 2019). Thus, OsNRAMP2 and OsHMA3 act in the opposite directions to control Cd sequestration in the vacuoles. In a genome-wide association study, Zhao et al. (2018) identified a QTL for grain Cd with *OsNRAMP2* being within the region of this QTL. Whether *OsNRAMP2* is the causal gene for this grain Cd QTL remains to be investigated. It would also be interesting to investigate whether allelic variations in *OsNRAMP2* among rice cultivars affect the Cd transport activity and Cd translocation to rice grains.

In conclusion, the present study provides further evidence that OsNRAMP2 plays an important role in remobilizing vacuolar Fe during seed germination. Furthermore, our study reveals a new role of OsNRAMP2 in Cd distribution from vegetative tissues to rice grains.

Declarations

Acknowledgements

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Figures

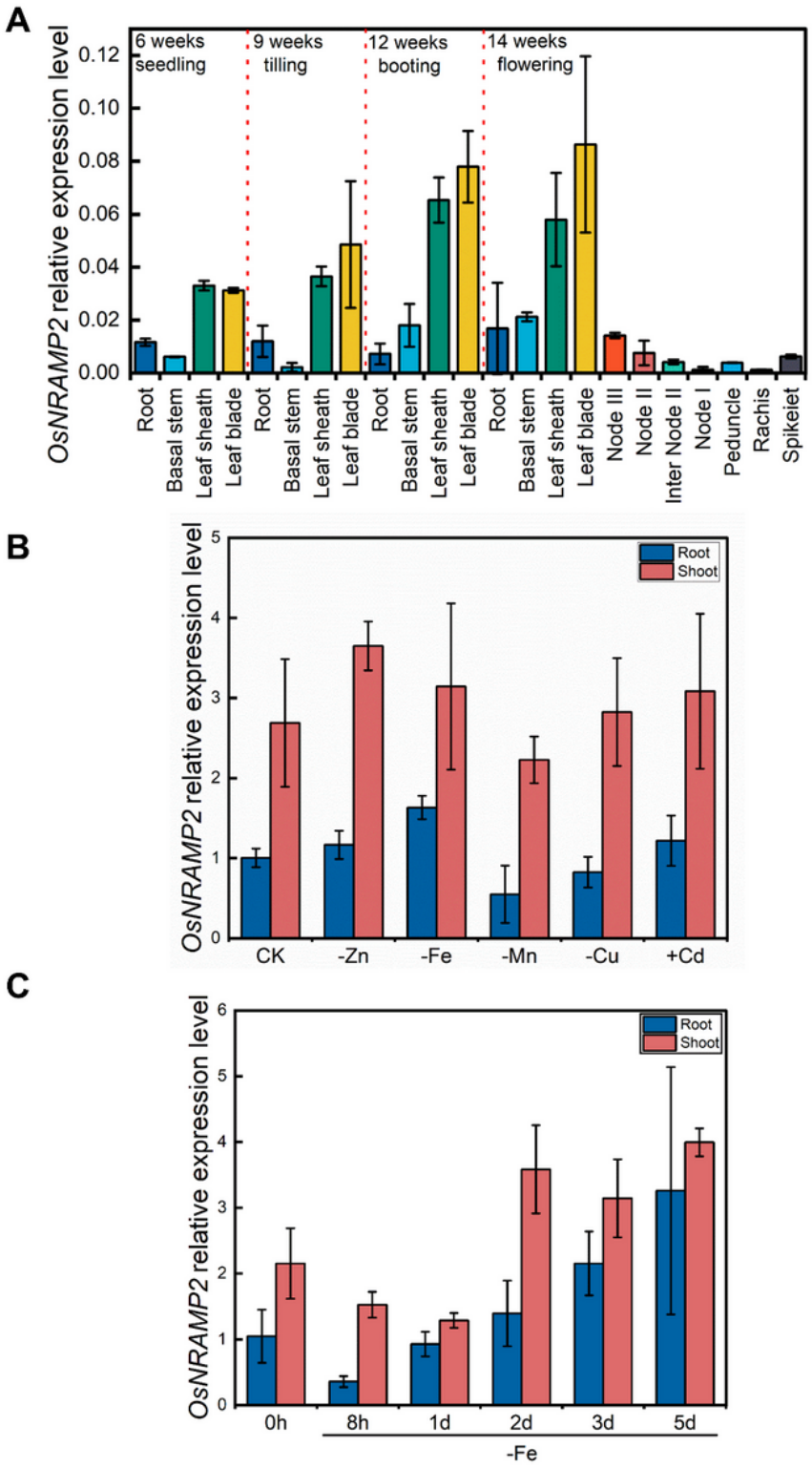

Figure 1

The expression pattern of *OsNRAMP2* in rice plants. (A) The expression levels of *OsNRAMP2* in different tissues at different growth stages of wild type rice (cv. Nipponbare) grown in a paddy soil; (B) response of *OsNRAMP2* expression in roots and shoots to withdrawal of Fe, Mn, Cu, or Zn in the nutrient solution or the addition of 0.5 μM Cd for 7 days; (C) response of *OsNRAMP2* expression in roots and shoots to

withdrawal of Fe supply in a time-course experiment. All data are means ± SD of three biological replicates.

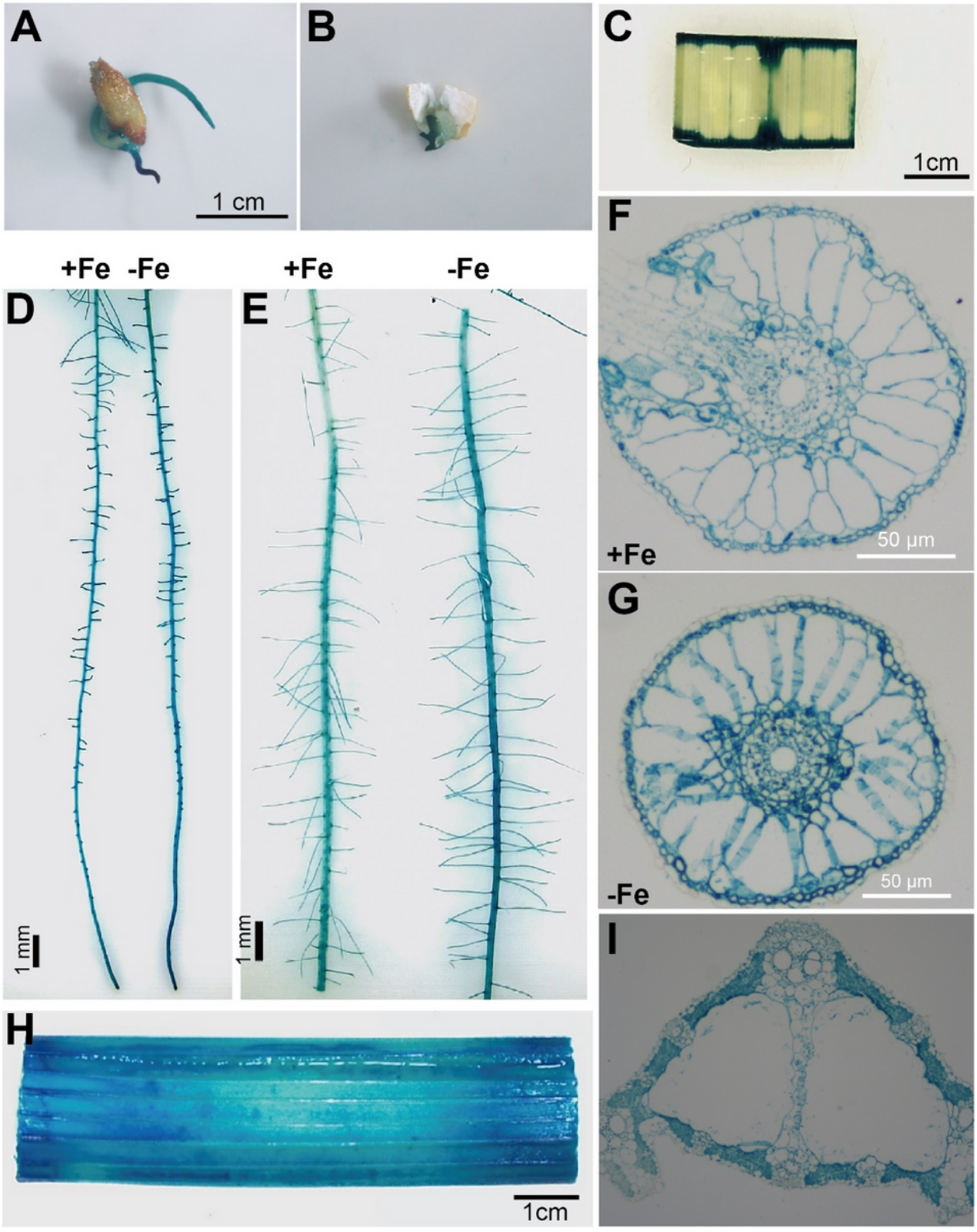

Figure 2

Tissue-specific observation of *OsNRAMP2* expression in rice. (A-B) The GUS activity in germinating seeds of 3 days after soaking in water. (C) Leaf and (H, I) leaf sheath of 28-day-old seedling were growth in

normal condition. the root (D: 0 - 75 mm from root tip; E: 75 -150 mm form root tip) of *pOsNRAMP2::GUS* transgenic plants under normal (20 μM Fe) or Fe deficiency (0 μM Fe) conditions for 7 days. (F, G, I) Cross-sections of the mature root and leaf sheath.

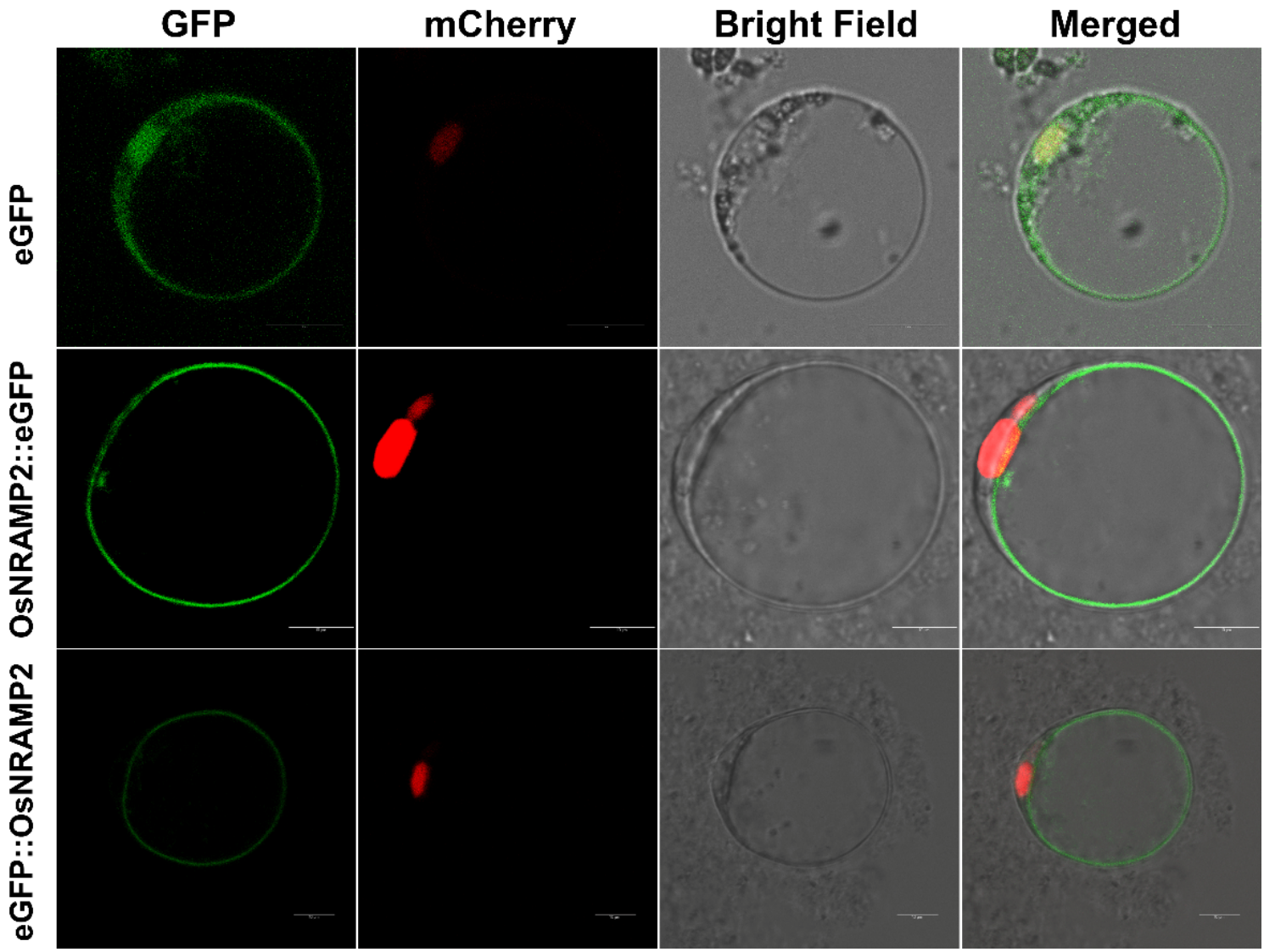

Figure 3

Subcellular localization of OsNRAMP2 in rice. Representative microscope images of rice protoplasts expressing the enhanced green fluorescence protein (eGFP, top), N-terminal or C-terminal fusion proteins of OsNRAMP2 with eGFP. Left to right: GFP fluorescence, mCherry fluorescence (nucleus marker), bright-field images and merged images. Scalar bar = 10 μm.

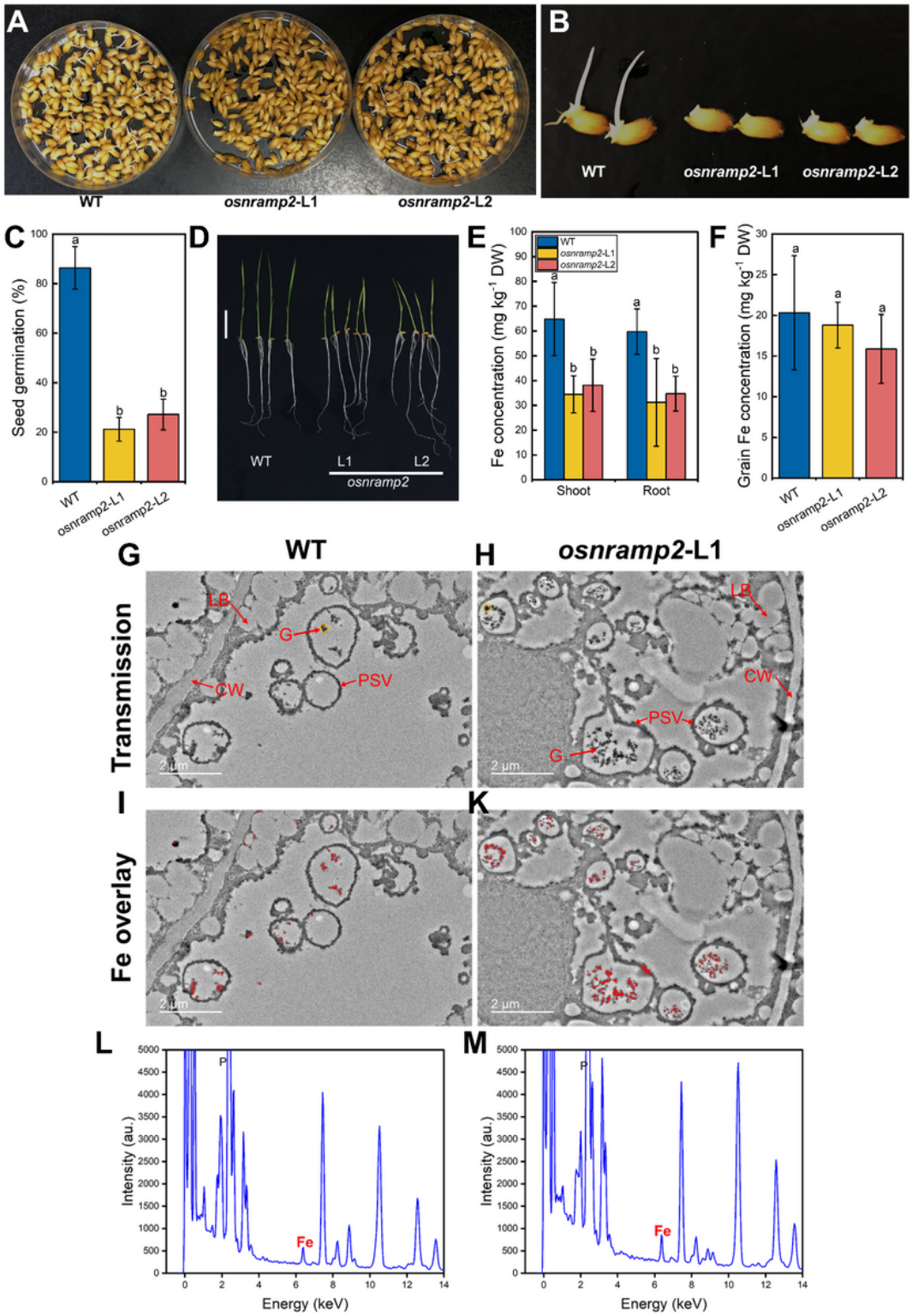

Figure 4

Knockout of *OsNRAMP2* delays seed gemination and induces Fe deficiency symptoms in seedlings. (A, B) Growth of *osnramp2* mutation lines (L1 and L2) at 3 days after sowing. (C) Germination rates of *osnramp2* mutants and wild type at 3 days after sowing. (D) Growth phenotype of *osnramp2* and wild type plants under tap water for 8 days after sowing. (E) The concentrations of Fe in roots and shoots of *osnramp2* mutants and wild type plants grown in tap water for 8 days. (F) The concentration of Fe in the

grains. Data are means ± SD of four biological replicates. Different letters indicate significant difference at $P < 0.05$. (G - K) Transmission electron microscopy images of scutellum parenchyma cells of germinating seeds of from wild type (G, I) or *osnramp2* (H, K) at 3 days after sowing. G and H panels: transmission image; I and K panels: Fe localization (red) obtained through imaging of inelastically scattered electrons (ESI) superimposed on the transmission image. Visible structures are labelled with reference to the Arabidopsis Atlas. CW: cell wall; LB: lipid body; G: globoid; PSV: protein storage vacuole. Empty globoid cavities correspond to globoid compartments from which the globoid crystal has been ejected during sectioning (Lanquar et al. 2005, EMBO J, 24:4041-4051). (L - M) Representative Energy-dispersive X-ray (EDX) spectra of globoids from wild type (L) or *osnramp2* (M) at 3 days after sowing seeds, and the collected areas are indicated by yellow circle in panel G and H.

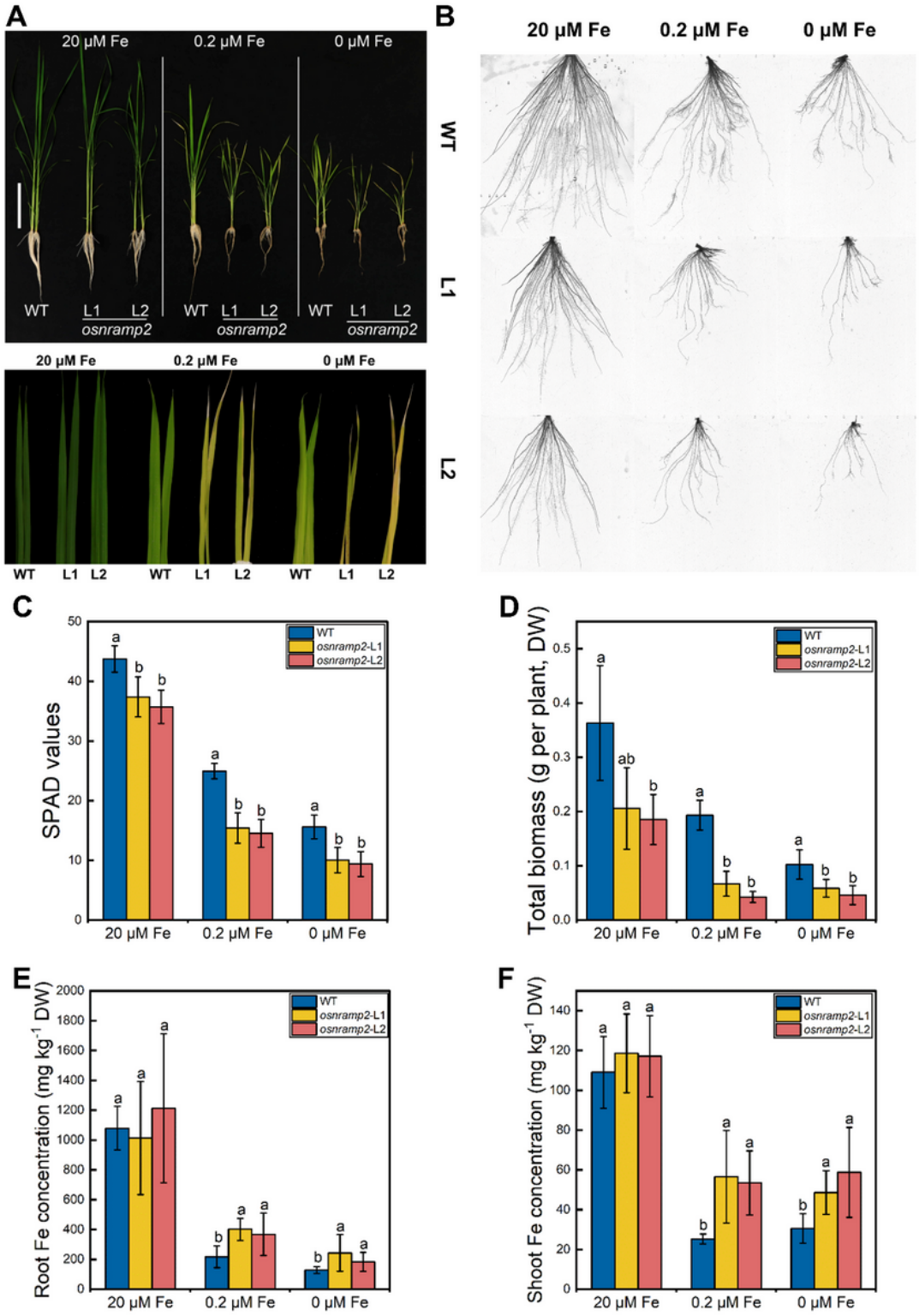

Figure 5

Phenotypic analysis of *osnramp2* mutants and wild type plants grown with different concentrations of Fe supply. (A) The growth phenotype, (B) root phenotype, (C) the SPAD values of the youngest fully developed leaves and (D) total biomass of *osnramp2* (L1, L2) and wild type. Scale bar = 15 cm. (E, F) The concentrations of Fe in roots (E) and shoots (F) of *osnramp2* mutants and wild type plants. 14-day-old plants of *osnramp2* mutants and wild type were grown in a nutrient solution containing 20, 0.2 or 0 μM

EDTA-Fe(III) for 21 days. Data are means ± SD of four biological replicates. Different letters indicate significant difference at $P < 0.05$.

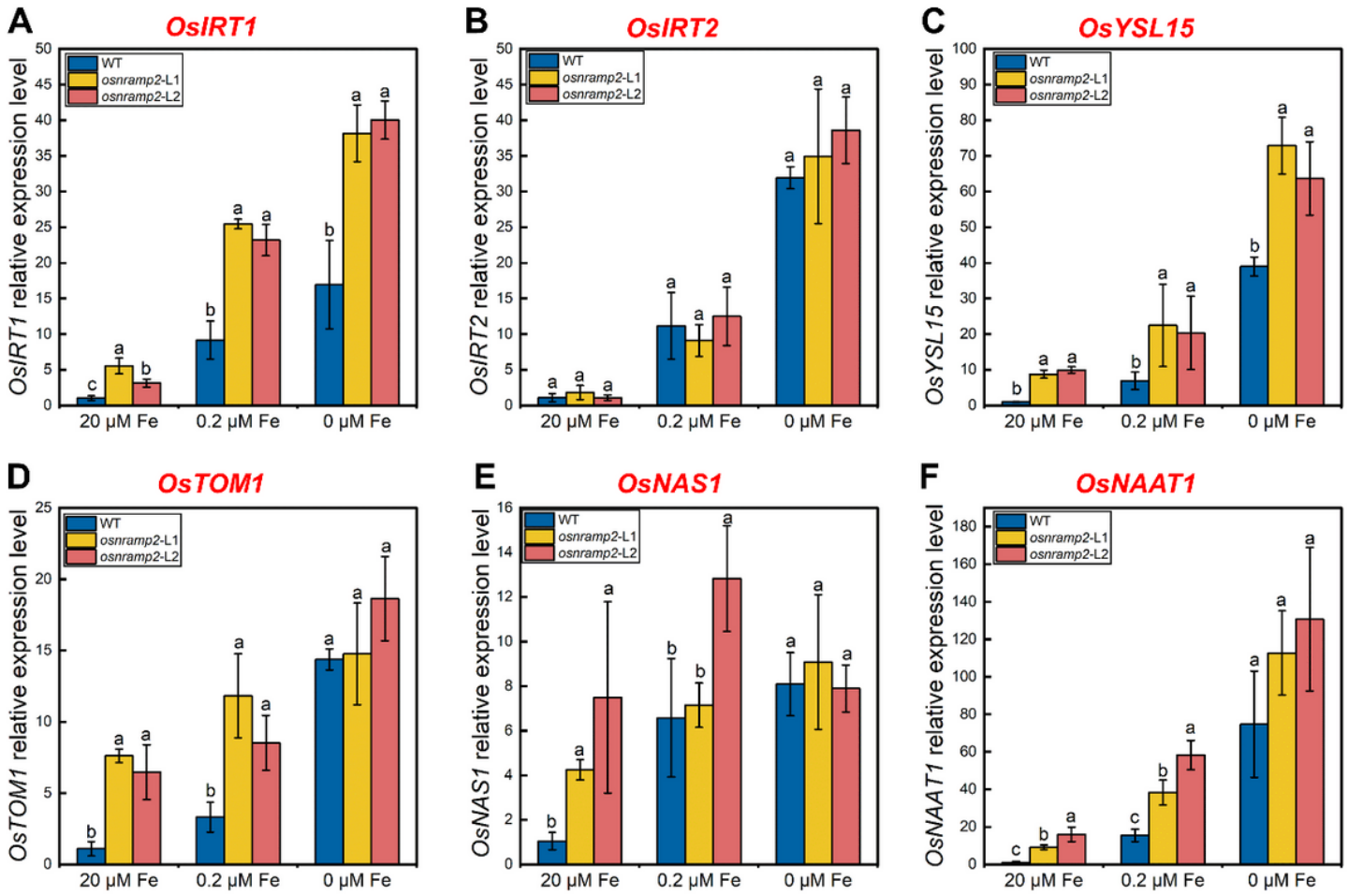

Figure 6

Responses in the expression Fe transport related genes to knockout of *OsNRAMP2* under different Fe supply conditions. The expression levels of *OsIRT1* (A), *OsIRT2* (B), *OsYSL15* (C), *OsTOM1* (D), *OsNAS1* (E), *OsNAAT1* (F) in roots of wild type and *osnramp2*. 14-day-old plants of *osnramp2* mutants (L1 and L2) and wild type grown in a nutrient solution containing 0, 0.2 or 20 μM EDTA-Fe(III) for 21 days. Data are means ± SD of three biological replicates.

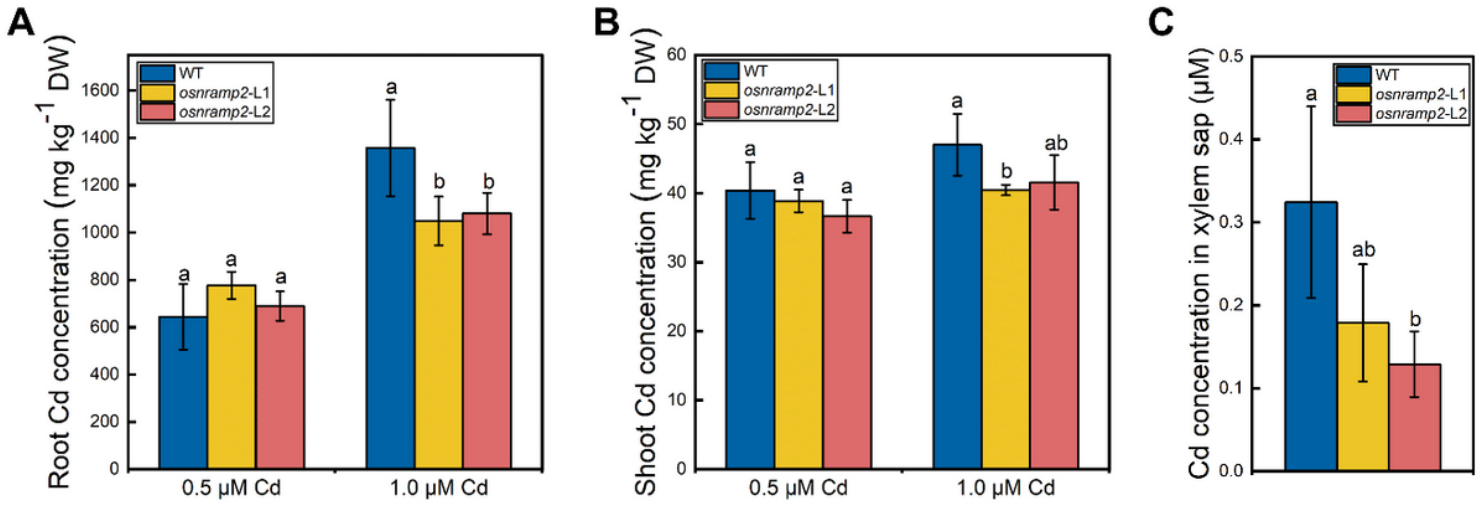

Figure 7

The concentrations of Cd in roots (A) and shoots (B) of *osnramp2* mutants and wild type grown under different Cd concentrations. 21-day-old plants of *osnramp2* mutants (L1 and L2) and wild type were grown in normal nutrient solution containing 0.5 or 1.0 μM $CdCl_2$ for 10 days. (C) Cd concentration in the xylem sap of 28-d-old plants of two independent lines of *osnramp2* mutants (L1 and L2) and wild type exposed to 1.0 μM Cd for 12 h. Data are means ± SD of four biological replicates. Different letters indicate significant difference at $P < 0.05$.

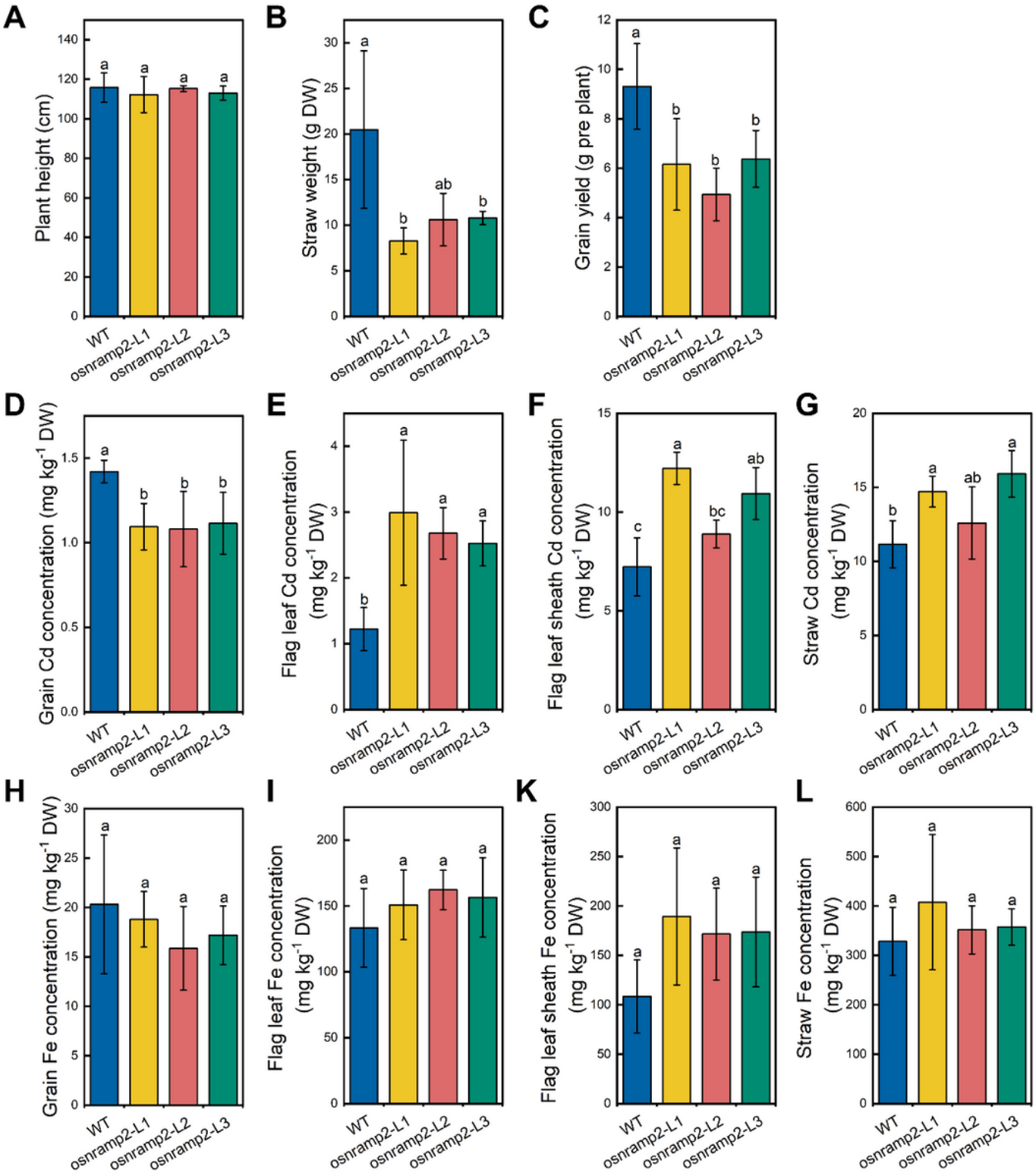

Figure 8

Growth phenotypes and the concentrations of Cd and Fe in rice grain (unpolished brown rice), flag leaves, flag leaf sheaths and straws of *osnramp2* mutants (L1 - L3) and wild type (cv. ZH11) grown in a pot experiment with a Cd-contaminated soil. (A) Plant height, (B) straw weight and (C) grain biomass of *osnramp2* mutants and wild-type. (D - G) Cd concentrations and (H - L) Fe concentrations in rice grain (D,

H), flag leaves (E, I), flag leaf sheaths (F, K) and straws (G, L). Data are means ± SD of four biological replicates. Different letters indicate significant difference at $P < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OsNRAMP2Supplementaldata.pdf