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Facilitated citrate-dependent iron translocation increases rice endosperm iron and zinc concentrations

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ABSTRACT

Iron deficiency affects one third of the world population. Most iron biofortification strategies have focused on genes involved in iron uptake and storage but facilitating internal long-distance iron translocation has been understudied for increasing grain iron concentrations. Citrate is a primary iron chelator, and the transporter FERRIC REDUCTASE DEFECTIVE 3 (FRD3) loads citrate into the xylem. We have expressed *AtFRD3* in combination with *AtNAS1* (*NICOTIANAMINE SYNTHASE 1*) and *PvFER* (*FERRITIN*) or with *PvFER* alone to facilitate long-distance iron transport together with efficient iron uptake and storage in the rice endosperm. The citrate and iron concentrations in the xylem sap of transgenic plants increased two-fold compared to control plants. Iron and zinc levels increased significantly in polished and unpolished rice grains to more than 70% of the recommended estimated average requirement (EAR) for iron and 140% of the recommended EAR for zinc in polished rice grains. Furthermore, the transformed lines showed normal phenotypic growth, were tolerant to iron deficiency and aluminum toxicity, and had grain cadmium levels similar to control plants. Together, our results demonstrate that deploying FRD for iron biofortification has no obvious anti-nutritive effects and should be considered as an effective strategy for reducing human iron deficiency anemia.

1. Introduction

Iron is an essential micronutrient for humans and iron deficiency is one of the most prevalent micronutrient deficiencies. Anemia affects around 1.6 billion people in the world, of which iron deficiency anemia is a substantial contributor [1]. Several approaches have been suggested and implemented to combat iron deficiency anemia, including food diversification, iron supplementation, food fortification and biofortification. Among these, crop biofortification is regarded as the most sustainable and cost-effective strategy. Developing high iron biofortified crops requires a thorough understanding of iron uptake, translocation and homeostasis in plants [2]. Plants need iron for several biological processes, including photosynthesis and chlorophyll biosynthesis. However, the high reactivity of iron can also generate toxic hydroxyl radicals that can damage plant cells [3,4]. Conversely, iron deficiency in plants causes chlorosis and decreased root length [5,6]. Therefore, plants have evolved complex regulatory mechanisms to maintain iron homeostasis.

Grasses use a chelation-based strategy (also known as Strategy II) for iron uptake. Mugineic acid (MA) family phytosiderophores (PS) are released into the rhizosphere by TRANSPORTER OF MUGINEIC ACID family (TOM) transporters [7]. PS chelate the ferric form of iron, forming PS-Fe^{3+} complexes that are subsequently transported into the root cells by YELLOW STRIPE (YS) and YELLOW STRIPE-LIKE (YSL) transporter families [8]. PS are synthesized from S-adenosyl-L-methionine by a conserved pathway of reactions catalyzed by NICOTIANAMINE SYNTHASE (NAS), NICOTIANAMINE AMINOTRANSFERASE (NAAT), and DEOXYMUGINEIC ACID SYNTHASE (DMAS) [9]. This leads to production of deoxymugineic acid (DMA), the precursor of all other MAs [10,11]. Rice also uses chelation based strategy for iron uptake, in addition to the direct Fe^{2+} uptake via the IRON-REGULATED TRANSPORTER 1 (OsIRT1) known as Strategy I [12].

Different translocation mechanisms distribute iron from the root to various plant organs. Citrate and nicotianamine (NA) are main iron chelators for inter- and intra-cellular translocations [13]. NA binds to both Fe^{2+} and Fe^{3+}, and YSL transporters transport metal-NA complexes in the phloem [14,15]. Among the rice YSLs, OsYSL2 transports Fe(II)-NA and is expressed in the shoot phloem companion cells, while OsYSL15, OsYSL16 and OsYSL18 transport Fe(III)-DMA [16–18]. Fe^{3+}-citrate is the main form of iron in the xylem [19–21]. Therefore, citrate has an important role in iron translocation from roots to shoots. Arabidopsis FERRIC REDUCTASE DEFFECTIVE 3 (FRD3), which belongs to the multidrug and toxin efflux (MATE) family of transporters, loads citrate into the xylem [22,23]. The *Atfrd3* mutant can translocate iron

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to the root central vascular cylinder but root to shoot transport efficiency is reduced [22]. *OsFRDL1* is the rice homolog of *AtFRD3* and also encodes a citrate efflux transporter [20,24]. *Osfrdl1* mutants show leaf chlorosis, reduced iron but increased zinc and manganese concentration in leaves, and precipitation of iron in the root stele [20]. The *Osfrdl1* mutant also has lower citrate and iron concentrations in the xylem sap. In addition to their high expression in roots, *AtFRD3* and *OsFRDL1* are also expressed in reproductive organs, indicating their importance in seed development and distribution of iron to the grains [24–26].

Iron biofortification approaches involving constitutive expression of *NAS* increased iron concentration in polished and unpolished rice grains [27–29]. Transformation of rice with *AtIRT1* also increased the endosperm iron concentration in rice [30,31]. Many of the iron biofortification approaches were combined with the endosperm specific expression of *FERRITIN* (*FER*), which encodes an iron storage protein. Combining genes facilitating iron uptake and storage in a single locus resulted in higher iron concentrations as compared to single gene strategies [32–36]. Only few studies used genes related to long distance internal iron translocation. Transgenic plants expressing *OsYSL2* under control of the *SUCROSE TRANSPORTER 1* promoter alone or combined with *HvNAS1* and *GmFER* significantly increased iron concentrations in polished grains [34,37]. Despite various studies highlighting an important role of FRD3 in internal iron translocation, engineering plants with *FRD3* to increase grain iron concentrations has not been attempted so far.

In this study, we developed transgenic plants expressing the combination of *AtFRD3*, *AtNAS1* and *PvFER*, or only *AtFRD3* and *PvFER* together. The transgenic lines have significantly increased iron and zinc concentrations in both unpolished and polished rice grains. Additionally, the transformed lines are tolerant to low iron or aluminum toxicity and did not accumulate more cadmium in polished grains than control plants.

2. Material and methods

2.1. DNA constructs, rice transformation and plant growth conditions

The full-length *AtFRD3* genome sequence fragment was amplified using a forward primer containing a *BamHI* site and a reverse primer with a *BspEI* site. The fragment was subsequently inserted into the *BamHI*- and *BspEI*-digested *OsNAS2-PbskII(−)* vector [38] to generate the *pUbi:AtFRD3:tNOS* fragment. In parallel, the *PstI* site in the *Ubi* promoter contained in the *OsNAS2-PbskII(−)* construct was changed by a point mutation in order to use *PstI* site of *pCAMBIA-1300PMI-NASFER* vector for subsequent cloning steps. The *pCAMBIA-1300PMI-NASFER* vector [39] was cut at *PstI* site and the fragment of *pUbi:AtFRD3:tNOS* was inserted to generate the NFUF cassette. For the FUF construct, *pCAMBIA-1300PMI-NASFER* was digested at *XbaI* and *PstI* sites to obtain the *pGlb:PvFERRITIN:tNOS* fragment, which was then subsequently inserted into *pCAMBIA-1300PMI* to generate the *pCAMBIA-1300PMI-FER* vector. The *pCAMBIA-1300PMI-FER* vector was cut at the *PstI* site and the *pUbi:AtFRD3:tNOS* was inserted to generate the FUF cassette. These vectors were transformed into *Oryza sativa ssp. Japonica cv.* Nipponbare using *Agrobacterium tumefaciens* strain EHA105 [40]. Transformation, selection and regeneration were conducted according to an established protocol [41]. Candidate transformants were first screened for the presence of *AtFRD3*, *AtNAS1*, and *PvFER* by PCR. Southern blot hybridization using digoxigenin (DIG) labeling was performed using *PmlI*-digested genomic DNA isolated from the transgenic lines to select the lines with single-copy cassette insertion. The PCR-amplified *PMI* DNA fragment was used as a probe to detect the transgene cassettes. The primer sequences used in cloning, sequence verification and PCR are provided in Table S2. Selected transgenic lines together with Nipponbare control plants were grown in commercial soil (Klasmann-Deilmann GmbH, Germany) under greenhouse conditions in 80% humidity at 30 °C with 12 h light and 60% humidity at 22 °C with 12 h dark. Quantification of divalent metals and transgene expression was conducted on T2 and T3 generation plants.

2.2. Iron deficiency and cadmium excess treatment in hydroponic culture

Transgenic and NB seeds were germinated *in vitro* for five days in petri dishes on a H_2O-moistened filter paper and subsequently transferred to containers (with tip holes) containing 400 ml hydroponic solutions for seven days. Solutions for hydroponic system were prepared according to established protocols [42] using 0.7 mM K_2SO_4, 0.1 mM KCl, 0.1 mM KH_2PO_4, 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 μM H_3BO_3, 0.5 μM $MnSO_4$, 0.2 μM $CuSO_4$, 0.01 μM $(NH4)_6Mo_7O_{24}$, and 0.5 μM $ZnSO_4$, with different iron concentrations added as Fe(III)-EDTA according to the treatment (normal condition: 100 μM; low iron condition: 10 μM). For the low iron condition, plants were grown in hydroponic solutions containing 10 μM Fe(III)-EDTA for 14 days. Plants grown in hydroponic solutions containing 100 μM Fe(III)-EDTA served as control. Solutions were changed every two days to avoid any precipitation and contamination. To obtain grains from cadmium-treated plants, plants were grown to maturity in a hydroponic solution containing 10 μM $CdCl_2$ in green house conditions in 80% humidity at 30 °C with 12 h light and 60% humidity at 22 °C with 12 h dark.

2.3. Metal ion measurements

Dried grains were de-husked to obtain unpolished brown grains. The de-husked grains were processed with a grain polisher (Kett grain polisher 'Pearlest', Kett Electric Laboratory, Tokyo, Japan) for one min. Shoot and root samples from plants grown in hydroponic culture were dried at 60 °C for five days. 200 mg of each ground grain sample, 50–100 mg of tissue samples and 50 μl of xylem sap samples were boiled in 15 ml of 65% v/v HNO_3 solution at 120 °C for 90 min. Three ml of 30% v/v H_2O_2 were subsequently added and continuously boiled at 120 °C for another 90 min. Metal concentrations were determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian Vista-MPX CCD Simultaneous ICP-OES). The wavelength used for iron, zinc, manganese and copper were 238.204, 213.857, 257.610, and 324.754, respectively. The National Institute of Standards and Technology (NIST) rice flour standard 1658a was treated and analyzed in the same manner and used as internal control for every measurement. Data were analyzed using Student's T-Test. The criteria of $p < 0.05$ and $p < 0.01$ was used to determine statistically significant differences among the tested lines and the control.

2.4. RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from roots and shoots of five-day-old T3 generation seedlings using Trizol® reagent (Invitrogen, USA). To obtain total endosperm and embryo RNA, endosperm and embryo were separated manually from T3 rice grains at 16 days after fertilization. Extraction buffer containing 0.15 M NaCl and 1% sarcosyl was added to the ground samples followed by purification with 8 M guanidine hydrochloride buffer. The RNA was treated with DNase I (Thermo Fisher Scientific Inc., USA) to remove genomic DNA contamination. First-strand cDNA was synthesized using the RevertAid™ cDNA synthesis kit (Thermo Fisher Scientific Inc., USA). Quantitative RT-PCR was performed as previously described [43]. In brief, qRT-PCR was performed in a 7500 Real-Time PCR System using the SYBR Green RT-PCR reagent kit following the manufacturer's protocol (Applied Biosystems, Carlsbad, CA, USA). Each reaction was performed in triplicates in a volume of 20 μl with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Data were analyzed according to the manufacturer's instructions using the 7500 System SDS Software v1.4 (Applied Biosystems). The expression level of genes of interest was normalized to the expression of rice *UBIQUITIN 5* (*OsUBQ5*).

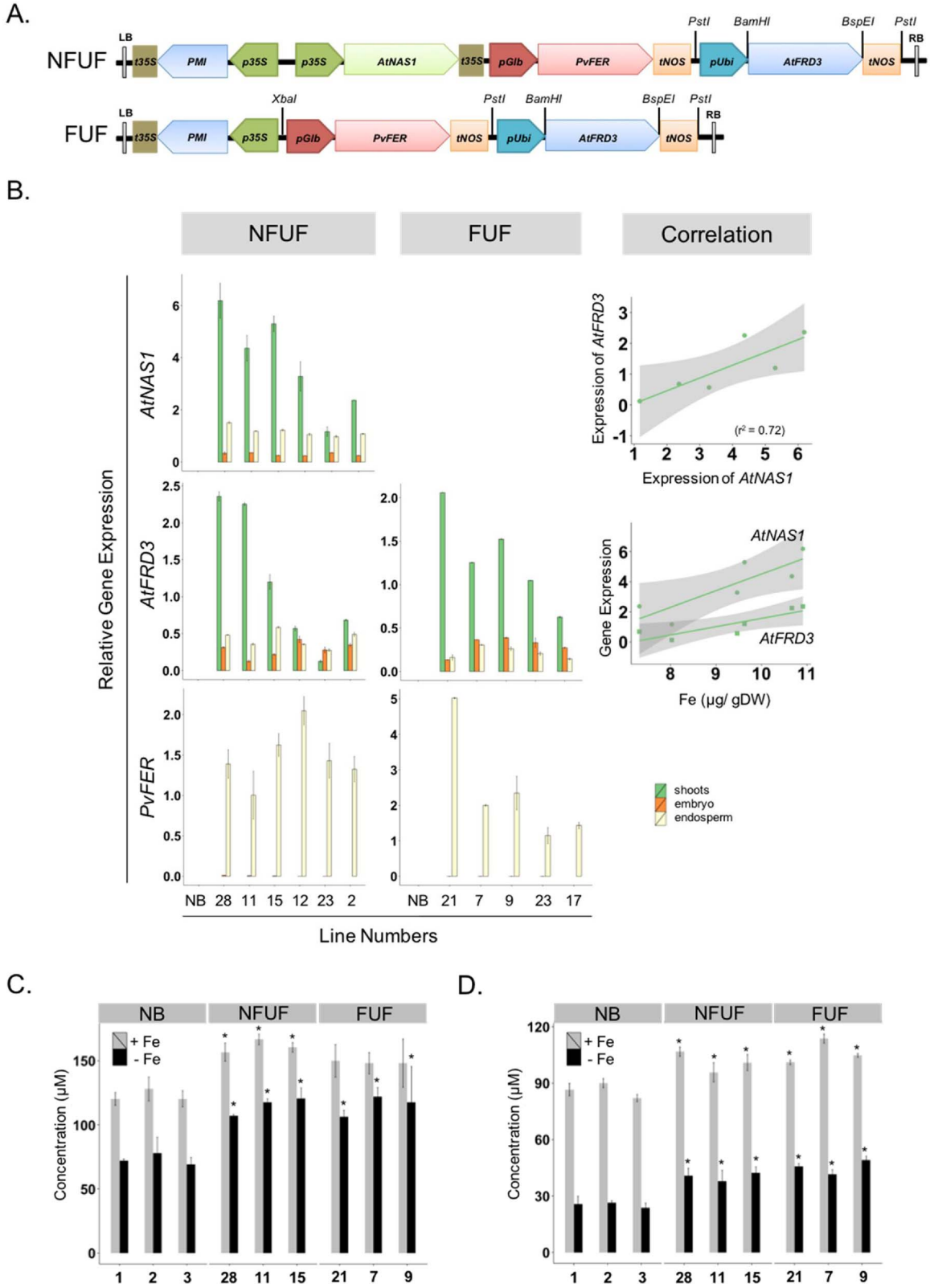

Fig. 1. Schematic representation of the transformation cassettes and the expression of transgenes in seedlings, embryo and endosperm, and citrate and iron concentration in xylem sap. (A) LB, T-DNA left border; RB, T-DNA right border; *t35S*, cauliflower mosaic virus (CaMV) 35S terminator; *PMI*, *PHOSPHOMANNOSE ISOMERASE*; *p35S*, *CaMV35S* promoter; *pUbi*, *Zea maize UBIQUITIN* promoter; *AtFRD3*, Arabidopsis *FERRIC REDUCTASE DEFECTIVE 3*; *AtNAS1*, Arabidopsis *NICOTIANAMINE SYNTHASE 1*; *tNOS*, *NOPALINE SYNTHASE* terminator; *pGlb*, rice *GLOBULIN-1* promoter; *PvFER*, *Phaseolus vulgaris FERRITIN*. BamHI, BspEI, PstI and SphI indicate restriction enzyme sites used in the construction of the transgene cassette. (B) *AtNAS1*, *PvFER* and *AtFRD3* expression level in selected transgenic lines and in Nipponbare control plants (NB). The panels on the right show the correlation between polished grain iron concentration and transgene expression in NFUF plants (lines 28, 11, 15, 12, 23, 2). Individual lines are represented by colored dots. (C) Citrate and (D) iron concentrations in the xylem sap of selected transgenic lines and NB under low iron and iron sufficient growth conditions. Black asterisks indicate statistically significant higher values calculated using Student's T-Test compared to NB (*$p < 0.05$). Material processed per sample: seedlings = 10, embryos, endosperm and xylem sap = 30. The data shown are from three replications. Error bars indicate the standard deviation (SD) of independent replicates.

Table 1
Phenotypic characterization of selected greenhouse-grown T3 transgenic lines. Performance parameters included plant height, tiller number, soil-plant analysis development (SPAD) value, days to flowering (DTF), one thousand grain weight (1000GrainWeight) and grain filling rate. Values are the average of three individual replicates. Black and red asterisks indicate statistically significantly higher and lower values compared to the NB control, respectively (*$p < 0.05$). Data is presented as mean ± SD.

Plant line	Height (cm)	Tiller number	SPAD	DTF	1000 Grain Weight (g)	Grain filling (%)
NB	63.33 (± 2.08)	7.67 (± 1.53)	36.00 (± 3.00)	81.00 (± 1.00)	24.17 (± 0.29)	82.44 (± 0.58)
NFUF						
28	55.33 (± 1.15)*	7.00 (± 1.00)	37.00 (± 1.00)	80.00 (± 1.00)	25.83 (± 0.29)	82.58 (± 1.41)
11	54.00 (± 1.00)*	9.67 (± 2.08)*	36.67 (± 0.58)	77.67 (± 1.53)	23.83 (± 0.29)	83.53 (± 1.09)
15	58.67 (± 1.53)	7.67 (± 0.58)	36.67 (± 2.08)	78.33 (± 1.53)	24.33 (± 0.58)	82.43 (± 0.29)
12	61.67 (± 0.58)	10.33 (± 1.53)*	36.33 (± 1.53)	78.00 (± 1.00)	24.33 (± 0.29)	80.03 (± 2.09)
23	57.67 (± 1.53)	7.67 (± 2.08)	36.67 (± 2.52)	78.00 (± 2.00)	24.33 (± 0.58)	83.29 (± 0.98)
2	55.00 (± 1.00)*	7.33 (± 0.58)	38.33 (± 1.53)	81.67 (± 1.53)	24.00 (± 0.50)	82.13 (± 1.11)
FUF						
21	58.33 (± 1.00)	9.67 ((± 1.00)*	37.58 (± 1.53)	81.33 (± 1.00)	25.22 (± 0.40)	82.17 (± 0.98)
9	60.00 (± 1.00)	8.00 (± 1.00)	38.67 (± 1.53)	81.00 (± 1.00)	24.83 (± 1.04)	81.07 (± 0.19)
7	59.33 (± 2.08)	5.67 (± 0.58)	39.67 (± 1.53)	80.00 (± 1.00)	24.00 (± 0.50)	80.58 (± 2.18)
23	61.00 (± 1.00)	6.67 (± 1.00)	37.33 (± 0.58)	79.67 (± 1.00)	25.04 (± 0.29)	82.19 (± 1.44)
17	58.67 (± 2.08)	7.33 (± 1.53)	39.58 (± 1.00)	80.33 (± 0.58)	24.87 (± 0.58)	81.13 (± 1.15)

2.5. Xylem sap collection

Transgenic and NB seeds were germinated *in vitro* for five days on H_2O-moistened filter paper placed in petri dishes and subsequently transferred to containers (with tip holes) containing 400 ml hydroponic solutions for seven days. The plants were then grown in hydroponic solution for two weeks with iron sufficient (100 µM Fe(III)-EDTA) or deficient (10 µM Fe(III)-EDTA) supply. The nutrient solution was renewed in the morning of the sample collection day. After six hours of solution renewal, plants were decapitated 3 cm above the roots, and xylem sap was collected using a micropipette from the cut end for all samples in about one hour time. This allowed collection of samples at the same time of the day and same number of hours into photoperiod. The first drop of xylem sap was discarded to reduce cellular contamination. The xylem sap was then stored at −80 °C for further analysis.

2.6. LC–MS analysis

Quantitative analysis of citrate and malate was performed as previously described [44]. In brief, 50 µl of xylem sap samples were extracted using chloroform-methanol. Extracted metabolites were dried in a vacuum centrifuge, dissolved in 1 ml of ddH_2O and filtered through a 0.2 µm cellulose membrane. Samples were analyzed using a UHPLC–MS/MS and the presented values were calculated based on 1 ml of total volume used for measurement.

2.7. Evaluation of aluminum tolerance and root exudate collection

Aluminium treatment was according to previously described procedure [45], with specific modifications. In brief, seeds were soaked in deionized water overnight and then germinated on filter paper moistened with 0.5 mM $CaCl_2$ (pH 4.5) solution. The solution was renewed daily for four days. After four days of growth (at 25 °C), similar sized seedlings were selected and were exposed to 0.5 mM $CaCl_2$ (pH 4.5) solution containing 0 or 50 µM $AlCl_3$ in a one-liter plastic container for 24 h. Root length was measured before and after aluminum treatment. Twenty seedlings from NFUF, FUF and NB plants were screened, and the experiment was repeated three times. To analyze citrate secreted from rice roots, root exudates from NFUF, FUF and NB plants were collected. Seeds were germinated on H_2O-moistened filter paper placed in petri dishes and five-day-old seedlings were transferred into hydroponic solutions for another five days (hydroponic solutions were prepared as explained above). After pre-culture for 10 days, roots were placed in 0.5 mM $CaCl_2$ (pH 4.5) solution overnight and then transferred to 3 ml of 0.5 mM $CaCl_2$ (pH 4.5) solution containing 50 µM $AlCl_3$. Root exudates were collected after 24 h and were stored at −80 °C for further analysis. Exudates were purified as stated in 2.6 and final sample volume of 1 ml was analysed for citrate concentration.

2.8. Accession numbers

PvFER (X58274), *AtNAS1* (At5g04950), *AtFRD3* (At3g08040), *OsIDEF1* (Os08g0101000), *OsIRO2* (Os01g0952800), *OsIRT1* (Os03g0667500), *OsIRT2* (Os03g0667300), *OsNRAMP1* (Os07g0258400), *OsFRO1* (Os04g0578600), *OsYSL2* (Os02g0649900), *OsFRDL1* (Os03g0216700), *OsNAAT1* (Os02g0306401), *OsNAS1* (Os03g0307300), *OsFER1* (Os11g0106700), *OsUBQ5* (Os01g0328400).

3. Results

3.1. Generation of rice lines expressing AtFRD3, AtNAS1 and PvFER

Rice cv. Nipponbare (NB) was transformed with two different constructs. The NFUF construct contained *pCaMV35S:AtNAS1*, *pOsGLB-1:PvFER*, and *pZmUbi:AtFRD3,* and FUF contained *pOsGLB-1:PvFER* and *pZmUbi:AtFRD3* (Fig. 1A). The ubiquitin promoter-driven expression of *AtFRD3* was combined with the constitutive *pCaMV35S:AtNAS1* expression and the endosperm-specific expression of *PvFER* under the control of *GLOBULIN-1* promoter (NFUF), or the *AtFRD3* and *PvFER* were expressed together (FUF). In total, 23 and 11 single transgene insertion lines expressing NFUF and FUF constructs, respectively, were selected and grown to the T2 generation (Fig. S1). Based on a preliminary iron concentration analysis we selected five or six lines for each of the constructs with highest endosperm iron concentrations to grow T3 generation plants (Fig. S2). The transgenic plants were phenotypically similar to NB plants, with the exception of slightly higher tiller numbers and slightly reduced plant heights found in some of the NFUF transgenic plants (Table 1). The transformed plants also did not differ significantly from the control plants in terms of quality parameters, including grain length, amylose, starch or protein content (Table S1).

Expression of *AtNAS1* and *AtFRD3* was detected in shoots, roots, embryo and endosperm of the transformed lines, whereas the expression of *PvFER* was only detected in the endosperm as expected (Fig. 1B). Furthermore, NFUF and FUF plants had up to 2-fold higher citrate concentrations in the xylem sap as compared to the NB control (Fig. 1C). These increases were paralleled by iron increases in the xylem sap in both iron deficient and sufficient conditions (Fig. 1D). The expression levels of *AtNAS1* and *AtFRD3* were positively correlated in shoots and roots and with polished grain iron concentrations in the NFUF plants. These results suggested that expression of *AtFRD3* can

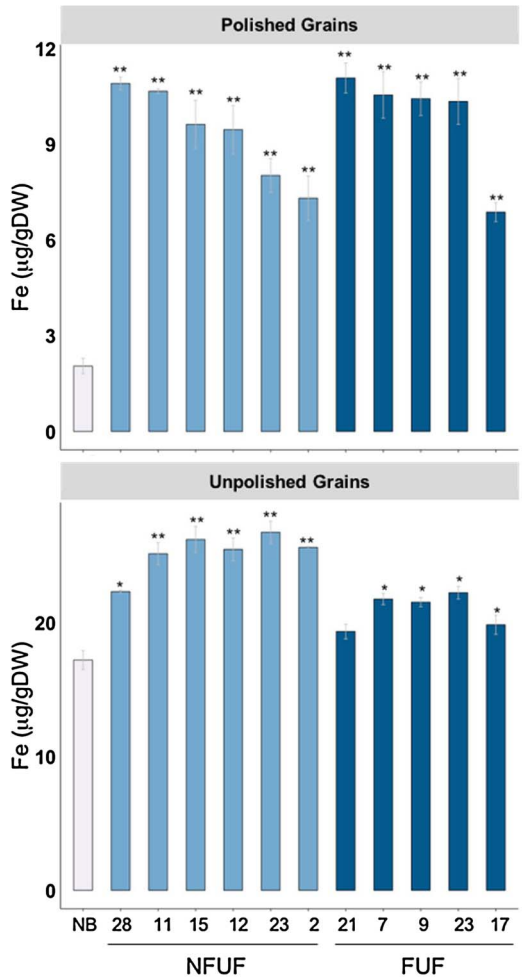

Fig. 2. Iron concentrations in polished and unpolished T3 rice grains. Values are the average of three biological replicates. For abbreviations see Fig. 1. Black asterisks indicate statistically significant higher values calculated using Student's T-Test as compared to the NB control (*p < 0.05, **p < 0.01). Error bars indicate the SD of independent replicates.

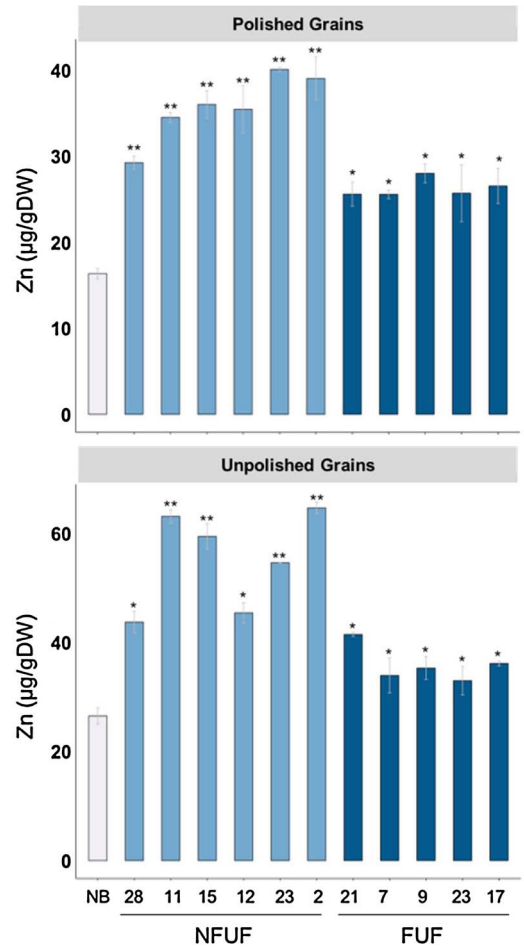

Fig. 3. Zinc concentrations in polished and unpolished T3 rice grains. Values are the average of three biological replicates. For abbreviations see Fig. 1. Black asterisks indicate statistically significant higher values calculated using Student's T-Test as compared to the NB control (*p < 0.05, **p < 0.01). Error bars indicate the SD of independent replicates.

elevate citrate levels in rice xylem sap and that *AtNAS1* and *AtFRD3* function synergistically in the transformed lines (Fig. 1B).

3.2. Concerted expression of AtFRD3, AtNAS1 and PvFER increases iron and zinc concentrations in polished rice grains

All of the NFUF and FUF lines had increased iron concentrations in polished grains, ranging from 6.88 to 11.08 µg/g DW, as compared to 2.05 µg/g DW in the NB control (Fig. 2). Iron increases were also observed in the unpolished grains of NFUF and FUF lines, although NFUF lines had comparatively higher iron concentrations ranging between 22.31–26.74 µg/g DW in comparison to 17.2 µg/g DW in the NB control (Fig. 2). Zinc concentrations in polished and unpolished grains of NFUF plants were also significantly increased, ranging from 29.27 to 40.09 µg/g DW in polished grains as compared to 16.39 µg/g DW in the NB control, and from 43.77 to 64.73 µg/g DW in unpolished grains compared to 26.54 µg/g DW in the NB control. In the FUF plants only slight increases of zinc were observed, within the range of 25.72–28.03 µg/g DW in the polished grains and of 33.05–36.19 µg/g DW in the unpolished grains (Fig. 3). Manganese and copper concentrations in grains of the transformed plants and the NB control did not vary significantly, apart from few exceptions (Fig. S3). Endosperm iron concentration and *AtFRD3* expression as well as citrate concentration was generally positively correlated in NFUF and FUF plants (Fig. 1). These results suggest that in the transgenic plants iron translocates efficiently through the xylem sap and is then subsequently transported to the rice endosperm.

3.3. NFUF and FUF plants have improved growth in iron deficient conditions

The distribution of iron and zinc to shoots and roots was determined in selected NFUF and FUF lines when grown in iron sufficient and deficient conditions. In iron sufficient conditions, iron levels were higher in shoots of all transgenic lines, with the highest 1.5-fold increase in NFUF 28. Only two of the NFUF lines had higher root iron concentrations as compared to NB control (Fig. 4A). Most of the NFUF and FUF lines also had increased iron concentrations in shoots in iron deficient conditions (Fig. 4A). Among them, NFUF 2 had the highest shoot iron concentration with 1.9-fold higher iron than the NB control. In iron deficient conditions, the root iron concentration of NFUF and FUF plants was lower compared to the NB control, with almost a 2-fold decrease in NFUF lines (Fig. 4A). Significant iron increases were also measured in the xylem sap of NFUF and FUF plants as compared to NB control in both iron deficient and sufficient conditions (Fig. 1D). In addition, root and shoot fresh weight of three selected NFUF and FUF plants was recorded. In iron sufficient conditions, shoot fresh weight was significantly higher in the NFUF than FUF and NB control plants. The root fresh weight showed no significant difference between transgenic and NB control plants in iron sufficient condition, while NFUF and FUF plants had higher shoot and root fresh weight than NB control

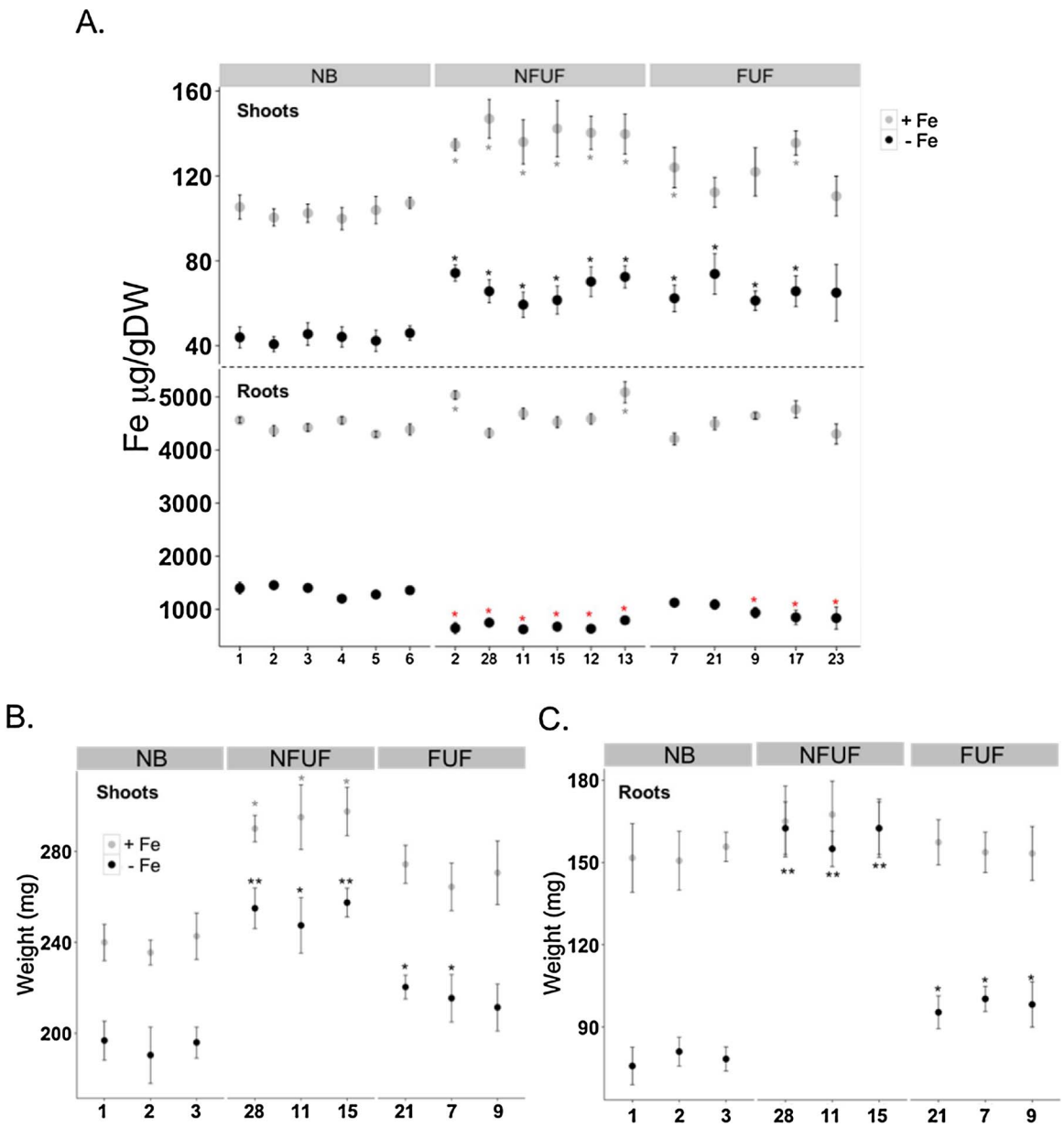

Fig. 4. Characterization of selected transgenic lines under iron sufficient and deficient growth condition. (A) Iron concentrations in shoots and roots of 28d-old seedlings in iron-sufficient and −deficient condition. (B) and (C) Fresh weight of shoots and of roots. Samples for iron and fresh weight measurements were collected from 20 individual plants of each independent line and the experiment was repeated three times. Student's T-Test was used for statistical calculation. Black and red asterisks indicate statistically significant higher and lower values as compared to the NB control, respectively (*$p < 0.05$, **$p < 0.01$). Error bars indicate the SD of independent replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plants in iron deficient conditions (Figs. 4B and C; S5). These results suggest that expression of *AtFRD3* alone and together with *AtNAS1* facilitate efficient iron translocation from roots to shoots during iron-deficient growth conditions. Most of the NFUF and FUF lines had also increased shoot and root zinc levels with up to 1.5-fold higher concentrations than NB control plants in iron-sufficient conditions. In contrast, in iron deficient conditions, the root and shoot zinc concentrations were similar to that of NB control, with the exception of NFUF 12 (Fig. S4A). The root and shoot manganese concentrations did not vary significantly between NFUF, FUF and NB plants (Fig. S4B).

3.4. Endogenous iron homeostasis-related genes are differently regulated in NFUF and FUF plants experiencing iron deficiency

We analyzed the expression of 10 endogenous genes involved in iron homeostasis in iron-deficient and −sufficient conditions (Fig. 5). The genes included those encoding enzymes for NA and DMA synthesis (*OsNAS1*, *OsNAAT1*), transcription factors (*OsIDEF1*, *OsIRO2*), iron-regulated transporters and intercellular transporters (*OsIRT1*, *OsNRAMP1*, *OsYSL2*, *OsFRO1*, *OsFRDL1*), and ferritin (*OsFER1*). *OsNAS1*, *OsNAAT1*, *OsIRT1*, *OsNRAMP1*, *OsIDEF1* and *OsIRO2* are highly induced by iron deficiency [46–48]. Most of the tested genes were also highly induced in NB shoots in iron deficiency except for *OsIDEF1* whose expression remained stable. However, induction of the genes was significantly lower in the shoots of NFUF and FUF plants in iron deficient conditions (Fig. 5A and B). The expression pattern of the genes was variable in the roots. Most had elevated expression in NFUF and FUF roots as compared to NB control in iron deficient conditions, whereas their expression did not vary significantly in iron-sufficient conditions. Overall, the transgenic plants did not vary drastically from NB control plants when the iron supply was sufficient. Together, most of the tested iron homeostasis-related genes responded to iron-deficient

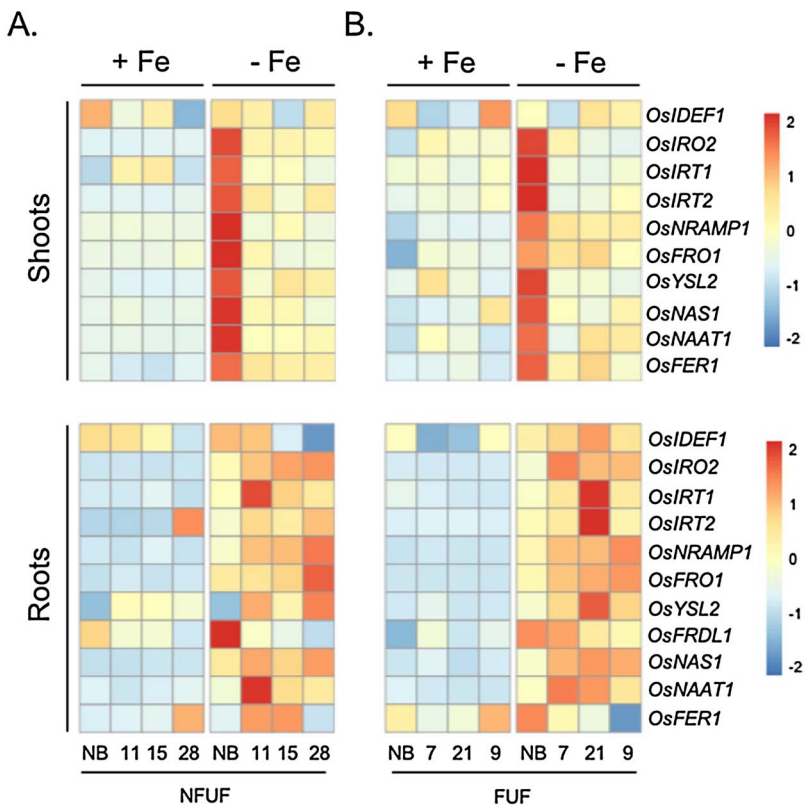

Fig. 5. Expression of selected iron homeostasis related genes in shoots and roots under iron sufficient and deficient condition. Expression of genes detected by qRT-PCR in shoots and roots of (A) NFUF plants and (B) FUF plants. For NFUF and FUF abbreviations see Fig. 1. Gene expression values were normalized to the expression of rice *UBIQUITIN 5* (*OsUBQ5*). *OsIDEF1*, rice *IDE BINDING FACTOR 1*; *OsIRO2*, rice *IRON DEFICIENCY--INDUCIBLE bHLH TRANSCRIPTION FACTOR 2*; *OsIRT1*, rice *IRON REGULATED METAL TRANSPORTER 1*; *OsIRT2*, rice *IRON REGULATED METAL TRANSPORTER 2*; *OsNRAMP1*; rice *NATURAL RESISTANCE ASSOCIATED MACROPHAGE PROTEIN 1*; *OsFRO1*, rice *FERRIC REDUCTASE OXIDASE 1*; *OsYSL2*, rice *YELLOW STRIPE-LIKE 2*; *OsFRDL1*, rice *FRD3- LIKE 1*; *OsNAAT1*, rice *NICOTIANAMINE AMINOTRANSFERASE 1*; *OsNAS1*, rice *NICOTIANAMINE SYNTHASE 1* and *OsFER1*, rice *FERRITIN 1*.

conditions in the roots but not the shoots in NFUF and FUF plants. These results suggest a concerted action between the *AtNAS1* and *AtFRD3* transgenes and different endogenous transporters to facilitate efficient iron acquisition and internal translocation during iron deficiency.

3.5. AtFRD3 expression in rice increases aluminum tolerance

Rice secretes citrate from the roots in response to aluminum (Al^{3+}) toxicity [49] and ectopic expression of *AtFRD3* conferred increased tolerance to Al^{3+} [22]. To investigate whether expressing *AtFRD3* has an effect on secretion of citrate and on growth of Al^{3+}-treated roots in rice, we compared citrate secretion and relative root elongation between selected transgenic plants and NB control. NFUF and FUF plants showed significantly elongated roots as compared to the NB control after exposure to Al^{3+} for 24 h (Fig. 6A and B). In aluminum toxicity condition, higher citrate concentrations were measured in NFUF and FUF root exudates as compared to NB root exudates (Fig. 6C and D). These results suggest that *AtFRD3* expression contributes to Al^{3+}-toxicity tolerance in the transformed rice plants, as also evidenced by a better root growth in these plants.

3.6. Transgenic plants do not accumulate more cadmium in polished grains

Cadmium (Cd^{2+}) also binds to citrate, and translocation of Cd^{2+} from roots to shoots via xylem and phloem is critical for Cd^{2+} accumulation in the rice grains [19,50]. Therefore, we determined Cd^{2+} concentrations in the grains of our transgenic plants when grown on hydroponic solution containing Cd^{2+}. The Cd^{2+} concentrations in polished NFUF and FUF grains were comparable to that of NB control grains. Cd^{2+} concentrations of up to 4.05 μg/g DW were measured in polished grains of NB control plants, while Cd^{2+} concentrations in polished grains of NFUF and FUF plants ranged between 3.78–4.4 μg/g DW (Fig. 7). This data suggested that expression of *AtFRD3* did not result in higher accumulation of Cd^{2+} in the grains of the transformed plants.

4. Discussion

More than three billion people worldwide consume rice as primary staple food every day, particularly in countries with reported medium to high prevalence of iron and zinc deficiencies. Most approaches to mitigate iron deficiency anemia, including iron supplementation, iron fortification or dietary diversification, have not always been successful to date because of economic, cultural, and/or local constraints [51]. Biofortification of staple crops is therefore considered a scientifically and economically useful strategy. Although increases of some micronutrients to date could be achieved through conventional crop breeding, improving iron content in rice grains has not been possible because of low genetic variation or negative linkages between yield and micronutrient traits [52,53]. Engineering of high-iron biofortified rice has been successful in the last few years. Several studies reported development of high-iron biofortified rice lines, with iron concentrations ranging from 6 to 19 μg/g DW in the polished grains [28,31–34]. Most of these studies focused on engineering rice with genes involved in iron uptake and storage, and only a few focusing on iron translocation within the plant. We expanded our iron biofortification strategies to iron translocation in the xylem using *AtFRD3* and combined this with efficient iron uptake (*AtNAS1*) and storage in the endosperm (*PvFER*). Overexpression of *AtNAS1* increases DMA secretion and increases NA concentrations in roots and shoots of transgenic rice plants, thus contributing to enhanced iron uptake and transport [32]. The endosperm-specific expression of *PvFER* provides a sink for iron storage in the grains. The transformed rice plants developed with the novel FRD strategy have up to 5.3-fold increased endosperm iron levels. Transgenic rice lines expressing *AtFRD3, AtNAS1* and *PvFER* have higher iron and zinc concentrations in the grains as compared to the lines expressing only the combination of *AtFRD3* and *PvFER,* particularly NFUF 28 and NFUF 23. Iron is transported to the grains from mature leaves via phloem and xylem-to-phloem transport in the nodes at early grain-

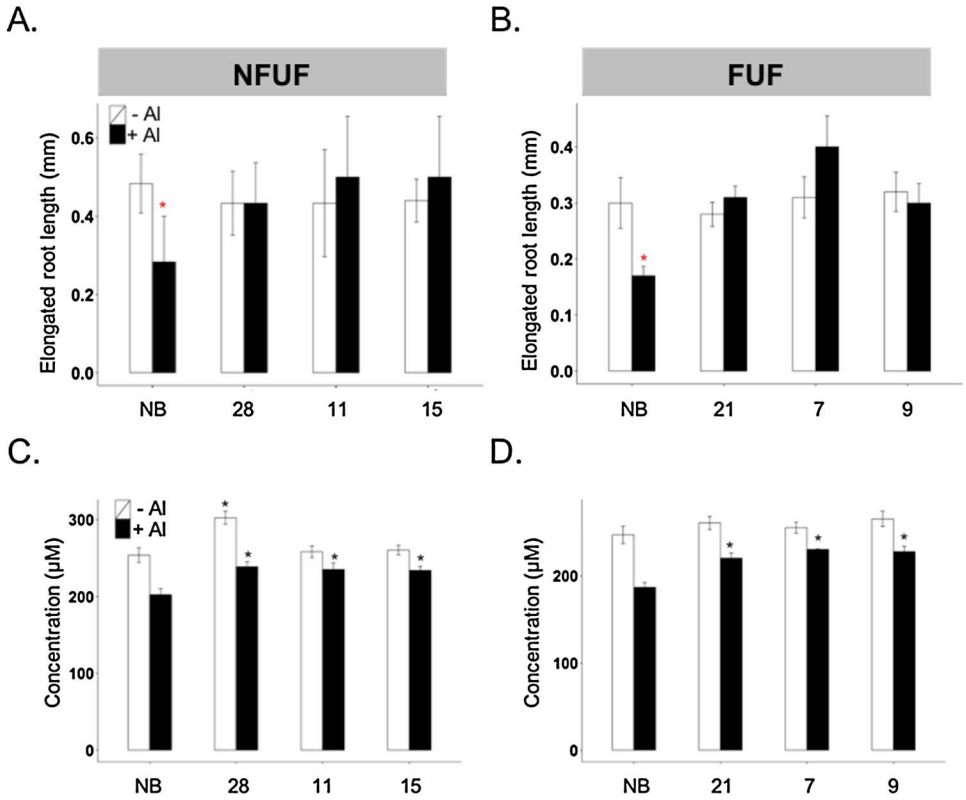

Fig. 6. Root length and citrate concentrations in root exudates of aluminum-treated plants. (A) and (B) Root lengths of 20 plants for independent NFUF and FUF lines were measured at 0 and 24 h after Al^{3+} treatment, respectively. Increase in length after the treatment is presented. For abbreviations see Fig. 1. (C) NFUF and (D) FUF root exudates were collected after a 24h-treatment of plants with Al^{3+} and citrate concentrations were analyzed by LC–MS. Black and red asterisks indicate statistically significant higher and lower values in transgenic plants compared to NB, respectively (*$p < 0.05$). Error bars indicate the SD of independent replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

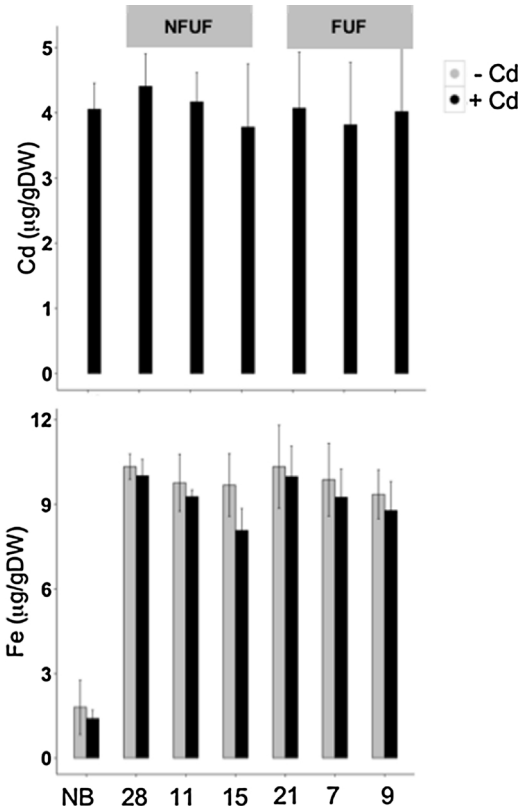

Fig. 7. Cadmium and iron concentrations in polished T4 grains when grown in a hydroponic solution containing $CdCl_2$. Cadmium and iron concentrations in polished grains of selected NFUF and FUF lines. For abbreviations see Fig. 1. Error bars indicate the SD of independent replicates.

filling stages [54–58]. In rice plants, iron in the xylem is mainly bound to citrate, while a small amount also binds to DMA [13,59]. Furthermore, in Arabidopsis *AtFRD3* is promoting iron nutrition between symplastically-disconnected tissues [26]. It is therefore likely that the increased Fe-citrate concentration we measured in the xylem sap of the transgenic rice lines expressing *AtFRD3* increases iron mobilization and transport to the grains. AtNAS1 synergistically increases Fe-NA and Fe-MA in the phloem sap. Thus, the concerted action of both these genes, together with Fe storage capacity of FERRITIN, can explain the higher endosperm iron content in the transformed rice grains.

NAS expression often leads to concurrent zinc increases in addition to the expected increase of iron in grains [30,32,60,61]. Most of our NFUF lines also had higher zinc levels in the grains. Although zinc is transported mainly as a free Zn^{2+} ion, in rice it can also chelate with NA in the phloem sap and with DMA in the xylem sap [59,62]. In addition to iron homeostasis-related genes, expression of *AtNAS1* in rice also increases the expression of endogenous genes encoding zinc transporters, including *OsZIP1*, *OsZIP3* and *OsZIP4* [46]. Together, this can explain the higher grain zinc concentrations in addition to the increases in grain iron concentrations.

NFUF and FUF lines expressing *AtFRD3* and/or *AtNAS1* have better growth during low iron availability and aluminum toxicity. In these plants transport of iron from roots to shoots is likely facilitated because they have higher shoot iron concentrations compared to NB control plants. Adjustments in the expression of iron homeostasis-related endogenous genes in the transformed plants, particularly in iron deficiency conditions, suggests that the transgenes trigger a coordinated genetic response to facilitate increased iron uptake and translocation. Similarly, tobacco plants expressing *AtNAS1* accumulated more iron in the leaves in low iron conditions and exhibited tolerance to iron deficiency [60]. The iron biofortified rice lines expressing *HvNAS1*, *HvNAAT-A* and *-B,* and *HvIDS3* from a single construct also showed low iron tolerance when grown in hydroponic culture or calcareous soil [36].

Aluminum toxicity inhibits root growth and leads to reduced uptake

of water and nutrients, which significantly limits crop production [63,64]. Secretion of citrate and malate by roots to chelate Al^{3+} is a well-known mechanism to counteract Al^{3+} toxicity [65]. Expression of *AtFRD3* in Arabidopsis and barley increased tolerance to aluminum toxicity in comparison to control plants [22,66]. The NFUF and FUF plants had longer roots when exposed to Al^{3+} and the roots released more citrate compared to NB control plants. These results demonstrate that expression of *AtFRD3* can increase tolerance to aluminum toxicity in rice by enhancing citrate efflux from the roots. Similar to Al^{3+}, Cd^{2+} could have a negative impact on plant growth and ultimately affect human health. Analysis on 69 diverse rice cultivars revealed a strong correlation between root-to-shoot Cd translocation via xylem sap and Cd accumulation in the shoots and grains [50]. Cd^{2+} concentration in the phloem sap is also correlated with grain cadmium concentrations in several rice cultivars [19]. Since Cd^{2+} can bind citrate in both xylem and phloem sap [19,50,67], it was possible that the transgenic rice lines expressing *AtFRD3* also have increased Cd^{2+} levels because of higher citrate concentrations in the xylem sap. Based on our analysis of hydroponically-grown plants treated with Cd^{2+} we confirmed, however, that NFUF and FUF lines did not have increased Cd^{2+} concentrations in the polished grains compared to NB control plants.

In summary, transgenic rice lines expressing *AtFRD3, AtNAS1* and *PvFER* have significantly increased iron and zinc but not cadmium concentrations in the grains, and these plants show increased tolerance to iron deficiency and aluminum toxicity. Importantly, the concerted expression of the three transgenes does not affect the phenotypic performance of the plants in the greenhouse. Together, the results demonstrate that enhancing citrate loading to the xylem when combined with constitutive expression of *NAS* and endosperm specific expression of *FERRITIN* genes is a novel and effective strategy for iron and zinc biofortification in rice and possibly other target crops.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

NKB conceived the study, NKB and TYW designed the experiments, TYW performed the experiments, TYW, WG and NKB interpreted the data, TYW and NKB wrote the manuscript, and WG and NKB edited the manuscript. All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.plantsci.2018.02.002.

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