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Author(s):
Wu, Ting-Ying; Gruissem, Wilhelm; Bhullar, Navreet K.

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

Ting-Ying Wu, Wilhelm Gruissem, Navreet K. Bhullar

Department of Biology, ETH Zurich, Universitaetsstrasse 2, 8092 Zurich, Switzerland

A R T I C L E   I N F O

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- Rice
- Iron
- Zinc
- Biofortification
- AtFRD3

A B S T R A C T

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 PsFER 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 NICOTIAMINE SYNTHASE (NAS), NICOTIAMINE 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 nicotiamine (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...
to the root central vascular cylinder but root to shoot transport efficiency is reduced [22]. OsFRD3 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 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::NOS fragment. In parallel, the PsI site in the Ubi promoter contained in the OsNAS2-PbskII (−) construct was changed by a point mutation in order to use PsI site of pCAMBIA-1300PMI-NASER vector for subsequent cloning steps. The pCAMBIA-1300PMI-NASER vector [39] was cut at PsI site and the fragment of pUbI:AtFRD3::NOS was inserted to generate the NFUF cassette. For the FUF construct, pCAMBIA-1300PMI-NASER was digested at XbaI and PsI sites to obtain the pGUb:PsFERRITIN::NOS 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 PsI site and the pUbI:AtFRD3::NOS was inserted to generate the FUF cassette. These vectors were transformed into Oryza sativa ss. 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 pml-digested genomic DNA isolated from the transgenic lines to select the lines with single-copy cassette insertion. The plant 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 H2O-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 K2SO4, 0.1 mM KCl, 0.1 mM KH2PO4, 2 mM Ca(NO3)2, 0.5 mM MgSO4, 10 μM H3BO3, 0.5 μM MnSO4, 0.2 μM CuSO4, 0.01 μM (NH4)2MoO4, and 0.5 μM ZnSO4, 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 CdCl2 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 ‘Pearles’, 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 HNO3 solution at 120°C for 90 min. Three ml of 30% v/v H2O2 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 ReverTraAce® 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 UBQUITIN 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; \textit{t35S}, cauliflower mosaic virus (CaMV) 35S terminator; \textit{PMI}, PHOSPHOMANNOSE ISOMERASE; \textit{p35S}, CaMV35S promoter; \textit{pUbi}, Zea mays UBIQUITIN promoter; \textit{AtFRD3}, Arabidopsis FERRIC REDUCTASE DEFECTIVE 3; \textit{AtNAS1}, Arabidopsis NICOTIANAMINE SYNTHASE 1; \textit{tNOS}, NOPALINE SYNTHASE terminator; \textit{pGb}, rice GLOBULIN-1 promoter; \textit{PvFER}, Phaseolus vulgaris FERRITIN. BamHI, BspEI, PstI and SphI indicate restriction enzyme sites used in the construction of the transgene cassette. (B) \textit{AtNAS1}, \textit{PvFER} and \textit{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.
Phenotypic characterization of selected greenhouse-grown T3 transgenic lines. Performance parameters included plant height, tiller number, soil-plant analysis development (SPAD) single and multi-compartmental model (SPAD), and one thousand grain weight (1000 Grain Weight) 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.

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<th>DTF</th>
<th>1000 Grain Weight (g)</th>
<th>Grain filling (%)</th>
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<td>7.67 (± 1.53)</td>
<td>36.00 (± 3.00)</td>
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<td>24.17 (± 0.29)</td>
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<td>7.00 (± 1.00)</td>
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<td>80.00 (± 1.00)</td>
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<td>9.67 (± 2.08)*</td>
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<td>39.58 (± 1.00)</td>
<td>80.33 (± 0.58)</td>
<td>24.87 (± 0.58)</td>
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2.8. Accession numbers

- **pVz** (X58274), AtNAS1 (At5g04950), AtFRD3 (At3g08040), OsIDEF1 (Os08g0101000), OsIRI2 (Os01g0952800), OsIRT1 (Os03g0667500), OsIRT2 (Os03g0667300), OsNRAMP1 (Os07g0258400), OsFRD1 (Os04g0578600), OsYSL2 (Os02g0649900), OsFRD1 (Os03g0216700), OsNAT1 (Os02g0306400), OsNAS1 (Os03g0307300), OsFRR1 (Os11g0106700), OsUBQ5 (Os1g0328400).

3. Results

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

Rice cv. Nipponbare (NB) was transformed with two different constructs. The NFUS construct contained pCaMV35S:AtNAS1, pOsGLB-1-pVzFER, and pZmUb1:AtFRD3, and FUF construct contained pOsGLB-1-pVzFER and pZmUb1:AtFRD3 (Fig. 1A). The ubiquitin promoter-driven expression of AtFRD3 was combined with the constitutive pCaMV3SS:AtNAS1 expression and the endosperm-specific expression of pVzFER under the control of GLOBULIN-1 promoter (NFUS), or the AtFRD3 and pVzFER were expressed together (FUF). In total, 23 and 11 single transgene insertion lines expressing NFUS 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 lines were phenotypically similar to NB plants, with the exception of slightly higher tiller numbers and slightly reduced plant heights found in some of the NFUS 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, embryonic and endosperm of the transformed lines, whereas the expression of pVzFER was only detected in the endosperm as expected (Fig. 1B). Furthermore, NFUS 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 NFUS plants. These results suggested that expression of AtFRD3 can
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.
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, FUF and NB plants (Fig. S4B).
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\(^{3+}\)-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-
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 Zn²⁺ 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\(^{3+}\) and 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 \(AtFRD3\), \(AnNAS1\) and \(PpFER\) 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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