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REVIEW

Generation and Field Trials of Transgenic Rice Tolerant to Iron Deficiency

Takanori Kobayashi · Hiromi Nakanishi · Michiko Takahashi · Satoshi Mori · Naoko K. Nishizawa

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Abstract Iron deficiency is a major cause of reduced crop yields worldwide, particularly in calcareous soils. Unlike barley, rice is highly susceptible to iron deficiency because of a low capacity to secrete mugineic acid family phytosiderophores (MAs), which are iron chelators secreted by graminaceous plants. We present an approach toward the generation along with field trials of transgenic rice lines exhibiting increased tolerance to iron deficiency. Cloning barley genes that encode biosynthetic enzymes for MAs enabled us to produce transgenic rice plants by introducing barley MAs biosynthesis-related genes. We tested three transgenic lines possessing barley genomic fragments responsible for MAs biosynthesis in a paddy field experiment on calcareous soil, which revealed tolerance of these lines to low iron availability. We also applied new approaches to generate iron-deficiency-tolerant rice lines, including the introduction of an engineered ferric-chelate reductase gene and manipulation of transcription factor genes regulating the iron deficiency response.

Keywords Iron deficiency · Field trial · Mugineic acid family phytosiderophores · Transgenic rice plants

T. Kobayashi · H. Nakanishi · M. Takahashi · S. Mori · N. K. Nishizawa (✉)
Graduate School of Agricultural and Life Sciences,
The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku,
Tokyo 113-8657, Japan
e-mail: annaoko@mail.ecc.u-tokyo.ac.jp

Introduction

Iron (Fe) is essential for most living organisms including plants. Although abundant in mineral soils, Fe is sparingly soluble under aerobic conditions at high soil pH, especially in calcareous soils, which account for about 30% of the world's cultivated soils. Fe deficiency is a widespread agricultural problem that reduces plant growth and crop yields [33, 36]. To take up and utilize Fe from the rhizosphere, higher plants have evolved two major strategies [34]: reduction (strategy I) and chelation (strategy II). The strategy I mechanism, utilized by nongraminaceous plants, includes induction of ferric-chelate reductase to reduce Fe at the root surface to the more soluble ferrous form and transport the ferrous ions generated across the root plasma membrane. In contrast, the strategy II mechanism, which is specific to graminaceous plants, is mediated by natural Fe chelators, the mugineic acid family phytosiderophores (MAs). Graminaceous plants synthesize and secrete MAs from their roots to solubilize Fe(III) in the rhizosphere [59], and the resulting Fe(III)–MAs complexes are taken up by roots through a specific transporter in the plasma membrane [5, 59].

In calcareous soils, the strategy II mechanism is more efficient than that of strategy I [33]. Tolerance to Fe deficiency is divergent among graminaceous plants and is thought to be dependent on the amount and kinds of MAs that they secrete. Rice, sorghum, and maize secrete only small amounts of 2'-deoxymugineic acid (DMA) among the MAs and thus are susceptible to low Fe availability. In contrast, barley secretes large amounts of MAs, including mugineic acid (MA), 3-epihydroxy-2'-deoxymugineic acid (epiHDMA), and 3-epihydroxymugineic acid (epiHMA), in addition to DMA, under Fe deficiency; therefore, it is more

tolerant to Fe deficiency than other graminaceous plants [32, 37, 52, 59].

We therefore hypothesized that introducing barley genes responsible for MAs biosynthesis into rice would lead to enhanced MAs production and tolerance in calcareous soils. We successfully produced various transgenic rice lines showing enhanced tolerance to low Fe availability by introducing barley MAs biosynthesis genes. Using selected lines, we carried out field trials on calcareous soil that revealed tolerance of these lines to low Fe availability. We also produced Fe-deficiency-tolerant rice lines using two other strategies: introduction of a reconstructed ferric-chelate reductase gene and manipulation of transcription factor genes controlling the expression of Fe-deficiency-induced genes. Future perspectives on generating further favorable and commonly acceptable transformants are described.

Generation of Fe-deficiency-tolerant transgenic rice by introducing barley MAs biosynthesis genes

Identification of genes responsible for MAs biosynthesis

The biosynthetic pathway of MAs (Fig. 1) has been identified through extensive biochemical and physiological studies [21, 30, 32, 37, 54]. Methionine is the precursor of MAs [37] and is adenosylated by *S*-adenosylmethionine (SAM) synthetase. Nicotianamine synthase (NAS) catalyzes the trimerization of SAM to nicotianamine (NA) [11]. All higher plants, including nongraminaceous plants, have the biosynthetic pathway to synthesize NA [29, 42], which serves as a common metal chelator involved in the internal transport of various micronutrients including Fe and zinc (Zn) [9, 62]. NA aminotransferase (NAAT) catalyzes the first step specific to graminaceous plants: transamination of NA to produce the 3''-oxo intermediate [20, 54]. DMA synthase (DMAS) subsequently reduces the 3''-oxo form to DMA [2]. All MAs share their biosynthetic pathway from methionine to DMA, which is then hydroxylated to form other MAs in some species, including barley.

Attempts to isolate the genes responsible for MAs biosynthesis have included "direct" approaches via enzyme purification and "indirect" approaches through screening the genes and proteins specifically induced in Fe-deficient roots. The former approach was applied to NAS and NAAT. *NAS* genes were first isolated from barley (*HvNAS1–7*) through the establishment of a NAS activity assay [11] and enzyme purification from Fe-deficient barley roots [13]. Two barley *NAAT* genes, *HvNAAT-A* and *HvNAAT-B*, were also cloned through the establishment of an enzyme activity assay [46] and enzyme purification [20, 60]. Expression of *HvNAS1*, *HvNAAT-A*, and *HvNAAT-B* is strongly induced by Fe deficiency and occurs almost exclusively in the roots [13, 60], suggesting direct involvement in MAs biosynthesis for the acquisition of Fe from the rhizosphere. Detection of NAS and NAAT enzyme activities in Fe-deficient roots of various graminaceous species revealed that NAS and NAAT activities are positively correlated with both the amounts of MAs secreted and Fe-deficiency tolerance [12, 19].

In the latter "indirect" approach, the differential hybridization method was applied with mRNA from Fe-deficient and Fe-sufficient barley roots. We cloned iron-deficiency-specific (*IDS*) genes specifically expressed in Fe-deficient barley roots [39, 49, 50]. Among these, *IDS2* and *IDS3* are

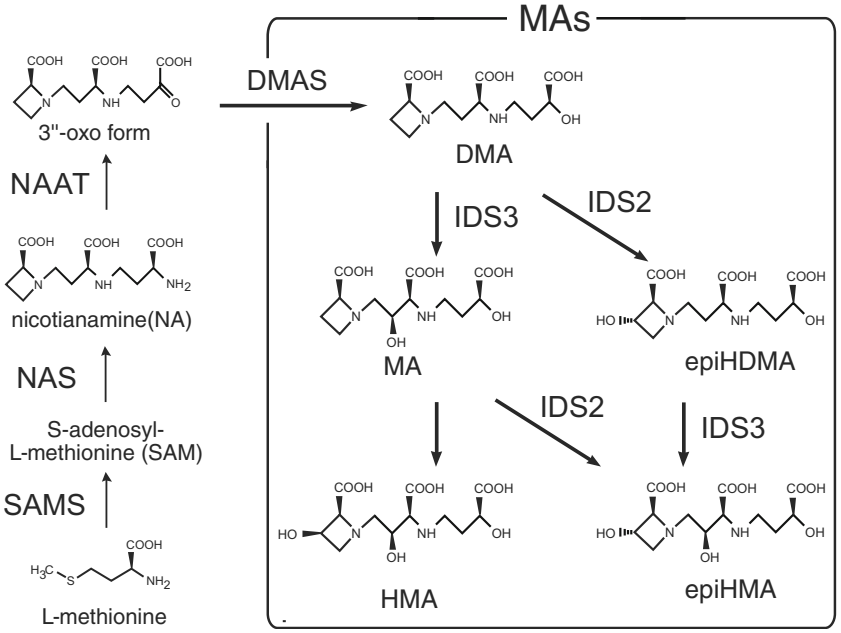

Fig. 1 Biosynthesis pathway of mugineic acid family phytosiderophores (*MAs*). *SAMS*, *S*-adenosylmethionine synthetase; *NAS*, nicotianamine synthase; *NAAT*, nicotianamine aminotransferase; *DMAS*, deoxymugineic acid synthase; *IDS2*, iron-deficiency-specific clone no. 2; *IDS3*, iron-deficiency-specific clone no. 3; *DMA*, 2'-deoxymugineic acid; *MA*, mugineic acid; *HMA*, 3-hydroxymugineic acid; *epiHDMA*, 3-epihydroxy-2'-deoxymugineic acid; *epiHMA*, 3-epihydroxymugineic acid.

homologous to 2-oxoglutarate-dependent dioxygenases, suggesting their possible involvement in the hydroxylation of MAs. By interspecies correlation between the expression of *IDS2–IDS3* and the capacity to secrete hydroxylated MAs, we deduced that IDS3 is the enzyme that hydroxylates the C-2' positions of DMA and epiHDMA, while IDS2 hydroxylates the C-3 positions of DMA and MA (Fig. 1; [40]). IDS3 was further confirmed to be the "MA synthase" by introducing the barley *IDS3* gene into rice [23]: transgenic rice plants secreted MAs in addition to DMA, while nontransformants secreted only DMA.

We also compared proteins of Fe-sufficient and Fe-deficient barley roots using two-dimensional polyacrylamide gel electrophoresis. Peptide sequencing of the induced proteins revealed that formate dehydrogenase (FDH) and adenine phosphoribosyltransferase (APRT), as well as the IDS3 protein, were induced in Fe-deficient roots [55]. The corresponding genes, *HvFDH* and *HvAPT*, were subsequently cloned [18, 55]. Both FDH and APRT are thought to function in scavenging the by-products (formate and adenine) that are released during the methionine cycle [36], thus supporting the production of MAs. Indeed, the methionine cycle works vigorously in roots to meet the increased demand for methionine in the synthesis of MAs [31]. We also applied a revised differential hybridization screening, identifying iron-deficiency-induced (*IDI*) genes from barley roots [70–72]. *IDI1* and *IDI2* putatively encode enzymes catalyzing steps in the methionine cycle [26, 56].

Recent application of microarray techniques reconfirmed the induction of the abovementioned genes involved in MAs biosynthesis in Fe-deficient barley roots [41, 56]. The microarray approach also resulted in cloning of *DMAS* genes from rice (*OsDMAS1*), barley (*HvDMAS1*), wheat (*TaDMAS1*), and maize (*ZmDMAS1*). All of the corresponding encoding proteins were confirmed to possess the reductase activity to produce DMA [2].

Introduction of barley genes responsible for MAs biosynthesis into rice

To produce transgenic rice plants with enhanced tolerance to Fe deficiency by increasing MAs production capacity, we introduced barley *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, and/or *IDS3* genes using either their genomic fragments or the *IDS3* gene promoter to confer inducibility to Fe deficiency. To introduce barley genomic fragments, we utilized the pBIGRZ1 vector [1], which was developed as a modified binary vector capable of transferring large-size DNA fragments into the rice genome. Rice cultivar Tsukinohikari was subjected to *Agrobacterium*-mediated transformation [10]. The transformants included those introduced with (a) a 13.5-kb genome fragment of the *HvNAS1* gene [14], designated "gNAS1"; (b) an 11-kb genome fragment of *HvNAAT* in which the *HvNAAT-A* and *HvNAAT-B* genes are tandemly located [61], designated "gNAAT"; (c) a 20-kb genome fragment of the *IDS3* gene [23], designated "gIDS3"; (d) a 7.6-kb genome fragment of *HvNAS1* plus an 11-kb genome fragment containing the *HvNAAT-A* and *HvNAAT-B* genes, designated "gNAS1-gNAAT"; (e) an *HvAPT* cDNA fragment fused downstream of the 2.2-kb *IDS3* promoter; (f) *HvNAS1*, *HvNAAT-A*, and *HvAPT* cDNA fragments each fused downstream of the 2.2-kb *IDS3* promoter; and (g) an *IDS3* cDNA fragment fused downstream of the cauliflower mosaic virus *35S* promoter [23]. Expression analysis revealed that the Fe-deficiency-induced expression was strongly conferred by genome fragments of the *HvNAS1*, *HvNAAT-A*, *HvNAAT-B*, or *IDS3* genes [14, 23, 61], confirming the potency of the barley promoter elements included in the genome fragments to drive Fe-deficiency-induced expression in rice plants. Although the barley promoters exhibit induction almost exclusively in Fe-deficient roots in native barley, they induced moderate expression in Fe-deficient leaves and prominent expression in Fe-deficient roots when introduced into rice.

To examine whether the transformants have enhanced tolerance to low Fe availability, the plants were cultured in pots filled with calcareous soils (pH 8.5–9.0; [61]) under controlled conditions in a greenhouse. Of the 36 gNAAT lines evaluated, ten showed remarkable tolerance to calcareous soils [61]. Nontransformants exhibited reduced growth and severe leaf chlorosis caused by Fe deficiency, whereas the gNAAT lines had greener and larger shoots. At harvest, the gNAAT lines possessed 4.2 and 4.1 times higher shoot dry weight and grain yield per pot than the nontransformants [61]. We also examined tolerance in calcareous soils for the other transformants. We found rice lines showing some tolerance to calcareous soils from all transgenes (a)–(g). In these lines, increased amounts or kinds of MAs secreted were thought to have contributed to enhanced Fe availability under Fe-limiting conditions.

Field trials of Fe-deficiency-tolerant rice lines

Approval for using transgenics in field trials

For the field experiment, we first selected one line from each of (a) to (f) described above. Prior to culture in a quarantine field in Japan, we needed approval for Type 1 Use Regulations for living modified organisms from the Ministry of Agriculture, Forestry, and Fisheries in Japan. For that purpose, we performed evaluation tests on their priority in competition, possible production of harmful substances, and influence on biological diversity in relation to interspecific crossing. We performed the following tests on six lines of transgenic rice and the nontransformant: growth comparison in Andosol,

stability of the transgene and expression beyond the first generation, determination of the interspecific crossing rate from transformants to nontransformants, evaluation of soil microorganism populations, evaluation of the residual effect of harmful compounds in postharvest soil, plowing-under effect of the dead transformants, carryover of *Agrobacterium*, tests on pollen shape, fertility, and dispersal distance, and tests on the germination rates of the seeds and tolerance to low temperature during early growth.

We confirmed stable inheritance of every transgene over at least three generations and found no harmful impacts on the environment in any of the abovementioned tests. Moreover, we detected no interspecific crossing from transformants to nontransformants. Based on these results, the transformants were approved for the quarantine field trials following the Type 1 Use Regulations.

Field trials of the selected lines

A calcareous subsoil from Toyama Prefecture containing fossil shells (pH ~9.2; [38]) was used to establish a paddy field in the quarantine area of the Field Science Center of Tohoku University (Osaki, Miyagi, Japan; 38° 44' N; 140° 45' E). The paddy field in the first-year experiment was 7 m long, 14 m wide, and 0.5 m deep, with the external ridges completely covered with a vinyl sheet to avoid contamination from the surrounding Andosol at the site. The first-year experiment was conducted from April to October 2005, using the six transformant lines (a)–(f) and nontransformants (cv. Tsukinohikari). The following year, from April to October 2006, the second-year experiment was performed using the three most promising lines: gNAS1 (a), gIDS3 (c), and gNAS1–gNAAT (d). Experimental procedures of the second-year experiment were described by Suzuki et al. [57]. The paddy field in the second-year experiment was 6 m long and 4 m wide, and the experimental plots were arranged in a completely randomized design (Fig. 2b) including the three transgenic rice lines (gNAS1, gIDS3, and gNAS1–gNAAT) and nontransformants (NTs). Germinated seeds were grown for 45 days in a greenhouse, and seedlings were then transplanted (three per hill) into the calcareous paddy field.

Sixteen days after transplanting (DAT), chlorosis and growth retardation began to appear. By 42 DAT, the three transgenic rice lines were clearly superior to the NTs (Fig. 2a) both in leaf color and growth, although differences in performance were observed in individual plots. Using gIDS3 as an example, we saw no evident difference from NT on 16 DAT (Fig. 3a); chlorotic symptoms appeared in NTs but not in gIDS3 at 30 DAT (Fig. 3b). The clearest difference between gIDS3 and NTs was evident at 42 DAT (Fig. 3c). One week later (50 DAT), leaf chlorosis began to disappear, especially in NT plants close to the plot boundary adjacent to the transgenic gIDS3 rice plants (Fig. 3d), which suggests that NTs utilized MAs secreted by the transgenic rice.

From 16 to 42 DAT, plant height and the SPAD value (leaf color) of the three transformant lines were higher than those of NTs. In addition, the number of tillers per plant was higher in gIDS3 than in the other lines. By 42 DAT, however, all lines had about 15 tillers per plant. After 42 DAT, when soil Eh fell below 0 mV, all plant lines recovered their leaf color, and, consequently, the SPAD value of NT plants rose up to levels similar to that of transformants. The decrease in soil redox potential with time is thought to have resulted in the absorption of generated ferrous ion via the ferrous transporter OsIRT1 ("Introducing an engineered ferric-chelate reductase gene"; [16]).

At the time of grain harvest, the number of grains, 1,000-grain weights, and the grain yield of gNAS1 were higher than those of the NT and other lines. Plant height and the proportion of fully matured grains showed no significant difference among the lines. Timing of the decrease in soil redox potential might account for the relatively small differences in grain parameters between transformants and NTs, despite the clearly inferior performance of NTs during early growth. Indeed, in a prior experiment, NT seedlings grown in the same calcareous paddy field showed severe chlorosis, and many seedlings died in the early stages before the Eh fell below 0 mV [38]. Therefore, it is crucial for rice in calcareous paddy fields to survive the early stages of growth, when enhanced MAs production greatly supports Fe acquisition.

Interestingly, the concentrations of Fe and Zn in the rice grains of gIDS3 were significantly higher than those of NTs and the other lines, suggesting that MA synthesized by IDS3 contributed not only to improved Fe uptake from the soil but also to increased translocation to the grain. MAs have been suggested to be involved in long-distance transport of Fe and Zn inside rice plants [15, 58]. Since more hydroxylated MAs exhibit higher stability under mildly acidic conditions [67], MA synthesized by IDS3 would have been favorable for internal translocation of Fe and Zn.

In conclusion, our field trial of the transformants demonstrated that a transgenic approach to increase the tolerance of rice to low Fe availability is practical for improving agricultural productivity in calcareous paddy soils.

Production of other transgenic rice plants tolerant to Fe deficiency

Introducing an engineered ferric-chelate reductase gene

In strategy I plants, Fe uptake from the rhizosphere is mediated by ferrous ion transporters. Eide et al. [7] isolated

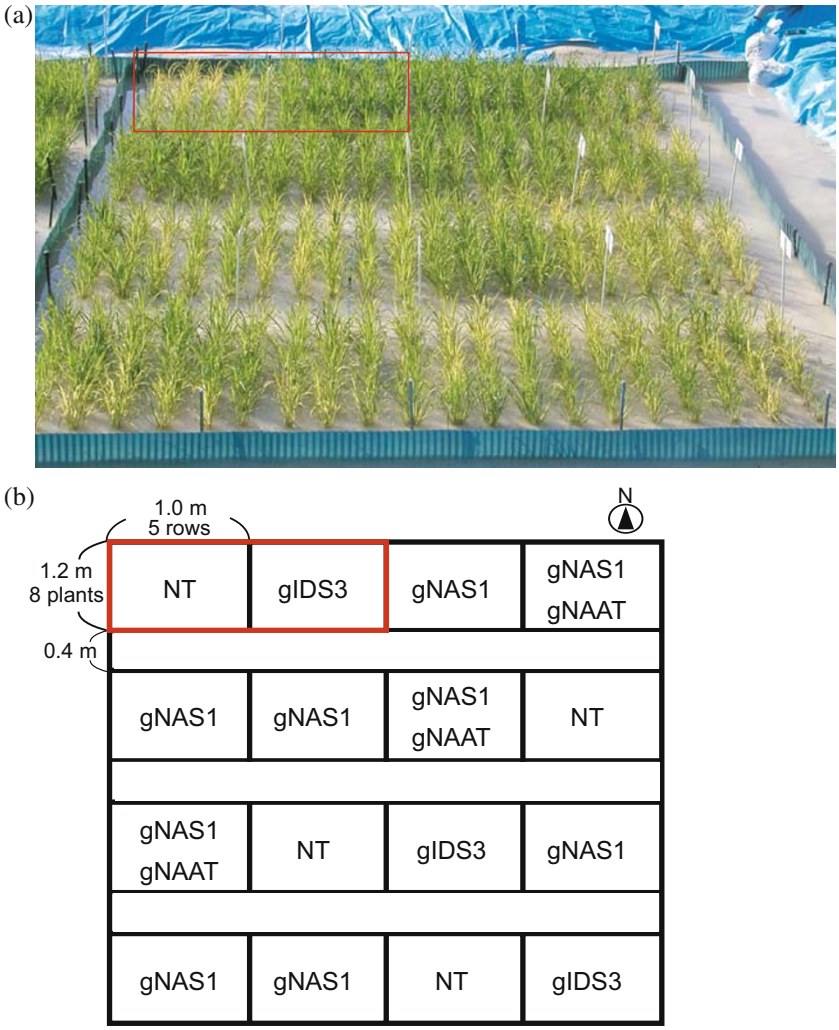

Fig. 2 **a** Photograph (42 DAT) of the rice lines tested in a paddy field in the quarantine area of the Field Science Center of Tohoku University (Osaki, Miyagi, Japan) and **b** field layout. Each population contained five 1.2-m-long rows of rice with 20 cm between rows and 15 cm between hills. The *box* in the *upper left* indicates the two plots photographed on several occasions (Fig. 3). *NT*, non-transformant. Original figure: Suzuki et al. [57].

the *Arabidopsis IRT1* gene, which is the dominant ferrous transporter in the Fe-uptake process [65]. Rice, in spite of being a strategy II plant, possesses homologs of the *Arabidopsis IRT1* gene, *OsIRT1* and *OsIRT2*, the ferrous transport capacity of which was demonstrated by functional complementation in yeast [3, 16]. *OsIRT1* expression is strongly induced in Fe-deficient roots, and *OsIRT2* is expressed similarly but at lower levels. Promoter β-glucuronidase (*GUS*) analysis indicated that *OsIRT1* is mainly expressed in the epidermis, exodermis, and inner layer of the cortex in Fe-deficient roots, as well as in companion cells of shoots. Moreover, an analysis using a positron-emitting tracer imaging system (PETIS) revealed that rice is able to take up both Fe(III)–DMA and Fe^{2+}. Thus, rice plants possess a system other than the MAs-based strategy II for Fe uptake [16]. In contrast to their ferrous-transporting ability, Fe-deficient rice roots do not induce ferric-chelate reductase activity [16], which is a hallmark of the strategy I response.

To take up ferrous ion directly using OsIRT1, without reducing ferric chelates, seems to be a consequence of adaptation of rice to waterlogged soils, in which the concentration of soluble ferrous iron increases with the decrease in soil redox potential [16, 57]. Because of the presence of OsIRT1, severe Fe deficiency is relatively rare in irrigated rice systems. Nevertheless, rice plants grown in calcareous soils exhibit Fe deficiency symptoms even under waterlogged conditions as noted previously because of their inability to induce ferric-chelate reductase and their low capacity to synthesize MAs. Therefore, we hypothesized that introducing ferric-chelate reductase into rice would enhance Fe deficiency tolerance, creating a complete strategy I system in addition to the rice endogenous strategy II.

For functional expression in plants, we modified and completely reconstructed the yeast ferric reductase gene, *FRE1*, to produce *refre1* (reconstructed *FRE1*; [47]). Since ferric-chelate reductase activity is inhibited by high pH, we then screened reductases with improved enzymatic activity at

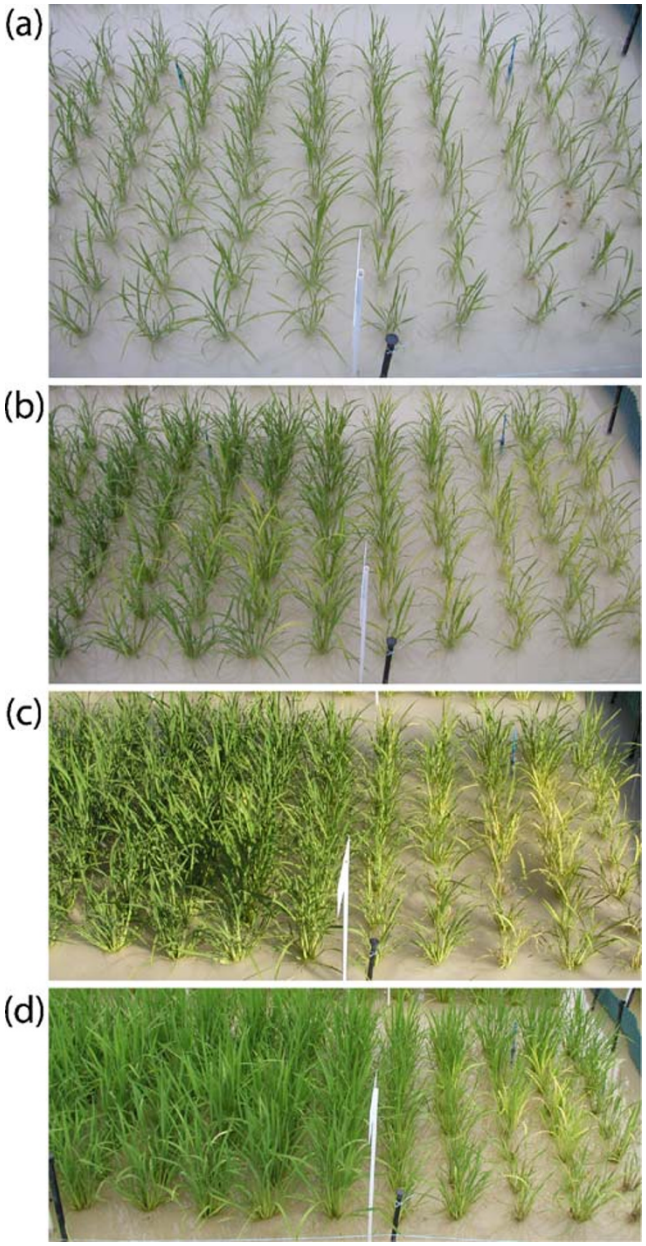

Fig. 3 Visual comparison between gIDS3 (*left*) and NT (*right*) from 16 to 50 DAT, as illustrated in Fig. 2 but photographed from the opposite direction. **a** 16 DAT, **b** 30 DAT, **c** 42 DAT, and **d** 50 DAT. Original figure: Suzuki et al. [57].

high pH [48]. Through screening of randomly mutagenized *refre1* derivatives, we obtained a variant designated *refre1/372*, whose encoding protein maintained strong reductase activity at pH 8–9. Transgenic tobacco plants with the introduced *refre1/372* under control of the *35S* promoter exhibited enhanced ferric-chelate reductase activity in roots and better growth when grown in calcareous soils [48].

Another concern in relation to the introduction of exogenous reductase genes into rice was the choice of an appropriate promoter. Vasconcelos et al. [64] introduced the *Arabidopsis* ferric-chelate reductase gene *FRO2* with its own promoter but observed no transgene mRNA expression. In *Arabidopsis*, expression of ferric-chelate reductase FRO2 and ferrous transporter IRT1 is similarly and coordinately regulated at transcriptional and posttranscriptional levels [4, 66]. Therefore, we chose the promoter of the rice ferrous transporter gene *OsIRT1* to drive the exogenous ferric-chelate reductase gene *refre1/372* [17].

Transgenic rice plants with the introduced *OsIRT1* promoter connected to *refre1/372*, successfully induced ferric-chelate reductase expression and activity in Fe-deficient roots, leading to higher Fe uptake than by vector controls, as revealed by a PETIS analysis. The transformants exhibited enhanced tolerance to low Fe availability in both hydroponic culture and calcareous soil (Fig. 4a). When grown in calcareous soil until harvest, the transformants had a 7.9 times higher grain yield than vector controls (Fig. 4b,c; [17]), demonstrating that creating a complete strategy I system in rice by enhancing ferric-chelate reductase activity is extremely effective in improving Fe deficiency tolerance.

Manipulating transcription factors regulating the Fe deficiency response

The above studies have shown that introduction of only a single or a few genes is effective in conferring Fe deficiency tolerance if appropriate promoter(s) and gene(s) are utilized. However, further enhancement of Fe availability might be achieved by engineering multiple genes in a coordinated manner. The genetic enhancement of a wide range of related genes requires manipulation of basal regulatory systems, including transcription factors. Therefore, we also aimed to clarify the regulation mechanism controlling the Fe deficiency response in graminaceous plants.

Under low Fe availability, graminaceous plants induce various genes, many of which are involved in Fe acquisition and utilization [2, 26, 28, 36, 41]. Despite the number of Fe-deficiency-inducible genes isolated, little is known about the regulation of gene expression in response to Fe deficiency. Therefore, we applied a stepwise strategy to identify the molecular components regulating the expression of Fe-deficiency-responsive genes: establishment of a promoter assay system, identification of *cis*-acting elements, and identification of *trans*-acting factors that interact with the elements.

We introduced the promoter region of the barley *IDS2* gene connected to the *GUS* gene as a reporter into tobacco plants [73]. Transgenic tobacco plants induced GUS expression in Fe-deficient roots, basically reflecting the regulation pattern in native barley. Precise deletion and mutation analyses using numerous lines of transgenic tobacco identified the novel Fe-deficiency-responsive *cis*-acting elements, iron-deficiency-responsive element 1 and 2 (IDE1 and IDE2; [24]); these are the first identified elements related to micronutrient deficien-

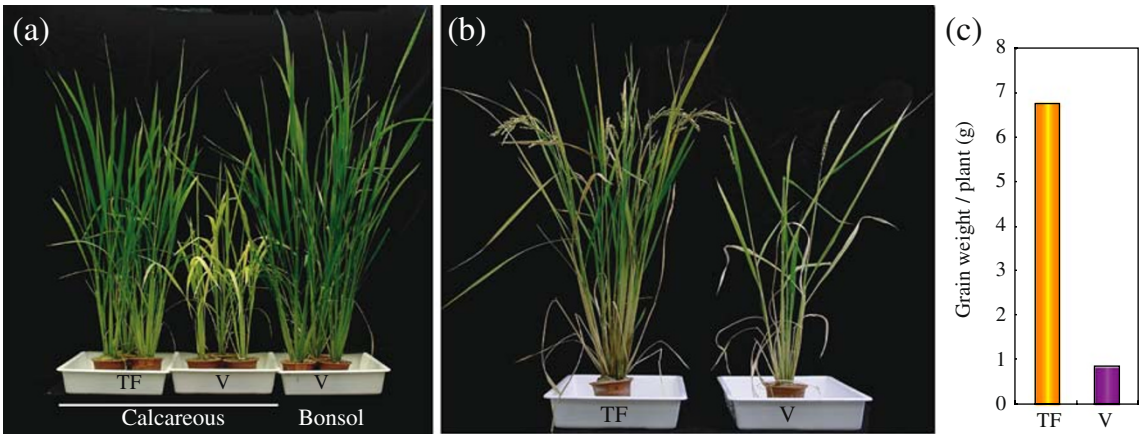

Fig. 4 Tolerance to Fe deficiency in transformants with the introduced *OsIRT1* promoter *refre1/372* grown in calcareous soil. **a** Transformants (*TF*, *left*) and vector controls (*V*, *center*) after 4 weeks of growth in a calcareous soil; vector controls (*V*, *right*) in bonsol (normal cultivated soil). **b** Transformant (*TF*, *left*) and vector control (*V*, *right*) after 17 weeks of growth in calcareous soil. **c** Grain yield after cultivation for 17 weeks in calcareous soil. Original figure: Ishimaru et al. [17].

cies in plants. IDE1 and IDE2 synergistically induce Fe-deficiency-responsive expression in tobacco roots. When introduced into rice, the pair IDE1 and IDE2 is able to induce Fe-deficiency-responsive expression both in roots and leaves [25]. Sequences similar to IDE1 or IDE2 were found in various Fe-deficiency-inducible promoters of barley, rice, tobacco, and *Arabidopsis* [6, 24, 26]. This suggests that gene regulation mechanisms involving IDEs are not only conserved among graminaceous (strategy II) plants but are also functional in nongraminaceous (strategy I) plant species.

Next, we searched for transcription factors that interact with IDEs. Very recently, we successfully identified two rice transcription factors, IDE-binding factor 1 (IDEF1) and IDEF2, which specifically bind to IDE1 and IDE2, respectively [27, 45]. IDEF1 and IDEF2 belong to uncharacterized branches of plant-specific transcription factor families ABI3/VP1 and NAC, respectively, and exhibit novel properties of sequence recognition. IDEF1 recognizes the CATGC sequence within IDE1, whereas IDEF2 predominantly recognizes CA[A/C]G[T/C][T/C/A][T/C/A] within IDE2 as the core binding site. Both *IDEF1* and *IDEF2* transcripts are constitutively expressed in rice roots and leaves.

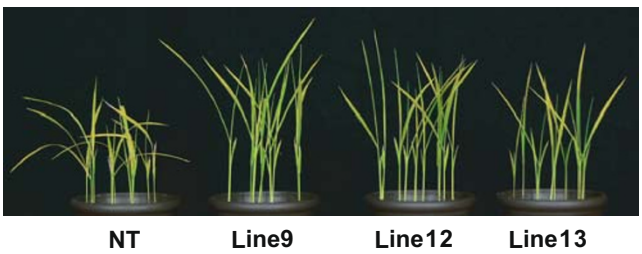

Fig. 5 Tolerance to Fe deficiency in seedlings with the introduced *IDS2* promoter *IDEF1* germinated in a calcareous soil (*lines 9*, *12*, and *13*) compared to nontransformants (*NT*) 17 days after sowing. Original figure: Kobayashi et al. [27].

In an attempt to improve Fe deficiency tolerance by modulating IDEF1 expression, we introduced *IDEF1* cDNA fused to either the constitutive *35S* promoter or the Fe-deficiency-inducible *IDS2* promoter. Transgenic rice

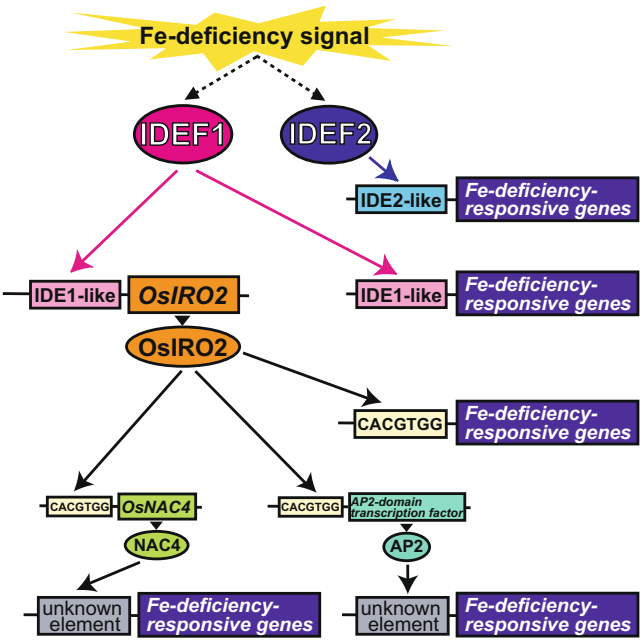

Fig. 6 Proposed regulatory network for the induction of Fe-deficiency-responsive genes via IDEF1, IDEF2, and OsIRO2. Under Fe-deficient conditions, IDEF1 and IDEF2 transactivate the expression of Fe-deficiency-responsive genes by binding to the IDE1-like and IDE2-like elements, respectively [27, 45]. OsIRO2, which is induced by Fe deficiency and is positively regulated by IDEF1, binds to the CACGTGG element to activate another subset of Fe-deficiency-responsive genes, including two transcription factor genes: *OsNAC4* and the AP2 domain-containing gene. These transcription factors may then regulate Fe-deficiency-responsive genes lacking IDEs and CACGTGG in their promoter regions [44]. The induced expression of *IDEF1* in transgenic rice plants would effectively strengthen the overall regulatory pathway to confer tolerance to Fe deficiency.

seedlings with the introduced *35S* promoter *IDEF1* showed severe growth retardation during early growth, while those carrying the *IDS2* promoter *IDEF1* showed healthy growth. Notably, the *IDS2* promoter *IDEF1* transformants exhibited slower progression of leaf chlorosis in Fe-free hydroponic culture and also showed better growth when germinated on calcareous soil (Fig. 5; [27]).

To clarify the molecular mechanisms that regulate Fe acquisition, we also characterized Fe-deficiency-induced transcription factors. Microarray analyses revealed the upregulation of several transcription factor genes in barley and rice [41, 43], among which a bHLH transcription factor gene, *IRO2*, is of particular interest because of its pronounced transcriptional upregulation by Fe deficiency in shoots and roots of barley and rice [43]. The core sequence for OsIRO2 binding was determined to be CACGTGG [43].

We produced transgenic rice plants with enhanced or repressed OsIRO2 expression by introducing the *35S-OsIRO2* cassette or using the RNA interference technique [44]. In Fe-deficient hydroponic culture, OsIRO2-overexpressing lines showed enhanced MAs secretion and slightly better growth compared to nontransformants, whereas OsIRO2-repressed lines resulted in lower MAs secretion and hypersensitivity to Fe deficiency. Microarray and Northern blot analyses revealed that the expression level of OsIRO2 is positively related to various Fe-deficiency-induced genes in roots, including those responsible for MAs biosynthesis (*OsNAS1*, *OsNAS2*, *OsNAAT1*, *OsDMAS1*, and various genes involved in the methionine cycle) and Fe(III)–MAs uptake (*OsYSL15*). OsIRO2 also affects the expression of some Fe-deficiency-inducible transcription factor genes that possess OsIRO2-binding core sequences in their promoter regions [44]. Importantly, *OsIRO2* itself possesses multiple IDEF1-binding core sequences in its promoter region and is positively regulated by IDEF1 [27]. Based on these results, a sequential link in the Fe deficiency response involving IDEF1, IDEF2, OsIRO2, and its downstream Fe-deficiency-inducible transcription factors is proposed (Fig. 6; [27, 44, 45]).

In contrast to growth retardation observed in the *35S* promoter *IDEF1* transformants, the *35S* promoter *OsIRO2* transformants were healthy, not exhibiting any obvious defects. These differences in phenotypes of the transformants are thought to be related to the distinct nature of the two transcription factors.

Future perspectives

We produced various lines of transgenic rice plants with enhanced tolerance to low Fe availability. Among these, tolerance of three selected lines (gNAS1, gIDS3, and gNAS1–gNAAT) in calcareous soil was demonstrated in field trials (Figs. 2, 3). Availability of Fe in rice fields is severely affected by soil type and redox potential, as well as numerous other environmental factors. An elaborate combination of previously adopted or new strategies will be needed to produce rice lines with even more tolerance to low Fe availability in problematic soils without loss of favorable agricultural traits. Manipulation of *DMAS* genes, which were recently cloned and thus have not been genetically modified, in the steps of MAs biosynthesis [2] would be of special interest. In addition, further clarification of the underlying mechanisms involved in Fe homeostasis is extremely important, including expressional regulation, secretion of MAs, and metal translocation inside the plants.

Understanding metal homeostasis also paves the way to fortifying rice grains with Fe and Zn. Previous efforts to enhance Fe in grains were performed by overexpressing ferritin, a common Fe storage protein in rice grain [8, 51, 63]. Our field trials revealed that the gIDS3 line is capable of accumulating more Fe in grains in both calcareous and Andosol paddy fields [35, 57]. Production and characterization of transgenic rice lines with introduced biosynthetic genes for MAs and ferritin genes in combination to enhance both Fe uptake and storage is in progress (Masuda et al. unpublished). Other advanced applications of our knowledge on Fe nutrition include the production of novel antihypertensive substrates. NA, the precursor of MAs, inhibits angiotensin-I-converting enzyme in humans and consequently reduces high blood pressure [22, 53]. We produced a yeast strain that highly accumulates NA by introducing the *Arabidopsis NAS* gene, *AtNAS2* [69]. Production and selection of rice lines with elevated levels of NA in grain by introducing the *HvNAS1* gene under the control of a seed-specific promoter of the rice glutelin gene is now under way [68].

Public acceptance of genetically modified organisms is still low. As a technical way to improve public acceptance, we modified the "marker-free vector" of the Cre/*loxP* DNA excision system [74] to construct a high-capacity binary vector for the transformation of rice, from which the sequence sandwiched between two *loxP* sites (including the selectable marker) can be removed by 17β-estradiol administration [68]. Many other approaches may aid public acceptance of transgenic plants, which have such high potential to increase food production, preserve the environment, and improve human health.

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