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The rice mitochondrial iron transporter is essential for plant growth

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In plants, iron (Fe) is essential for mitochondrial electron transport, heme, and Fe-Sulphur (Fe-S) cluster synthesis; however, plant mitochondrial Fe transporters have not been identified. Here we show, identify and characterize the rice mitochondrial Fe transporter (MIT). Based on a transfer DNA library screen, we identified a rice line showing symptoms of Fe deficiency while accumulating high shoot levels of Fe. Homozygous knockout of *MIT* in this line resulted in a lethal phenotype. MIT localized to the mitochondria and complemented the growth of Δ*mrs3*Δ*mrs4* yeast defective in mitochondrial Fe transport. The growth of *MIT*-knockdown (*mit-2*) plants was also significantly impaired despite abundant Fe accumulation. Further, the decrease in the activity of the mitochondrial and cytosolic Fe-S enzyme, aconitase, indicated that Fe-S cluster synthesis is affected in *mit-2* plants. These results indicate that MIT is a mitochondrial Fe transporter essential for rice growth and development.

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Like other organisms, plants require iron (Fe) to complete their life cycle. Fe is essential for several cellular processes, such as respiration, chlorophyll biosynthesis and photosynthetic electron transport[1]. Fe exists in multiple redox states, readily accepting and donating electrons, permitting it to serve as a cofactor for several proteins such as components of the electron transport chain in mitochondria and chloroplast. In mitochondria, Fe is essential for the synthesis of heme by ferrochelatase[2], and for the synthesis of Fe–sulphur cluster-containing proteins (Fe-S proteins) of both the matrix (for example, aconitase and homoaconitate hydratase) and the inner membrane[3,4]. Thus, limiting the supply of Fe to mitochondria impairs the metabolic and respiratory activities of this organelle and also affects Fe-S proteins in the cytoplasm[5]. On the other hand, excess Fe is toxic through the generation of reactive oxygen species. Free Fe ions might be particularly harmful to mitochondria, where free reactive oxygen species are generated as a side reaction of electron transport. In yeast, the alteration of mitochondrial activity by the inhibition of mitochondrial Fe transport leads to Fe accumulation in vacuoles and Fe deficiency in the cytoplasm. Mrs3 and Mrs4 are yeast (*Saccharomyces cerevisiae*) mitochondrial solute carrier family proteins transporting Fe into mitochondria under conditions of low-Fe availability[6,7]. *MRS3-MRS4* knockout yeast (Δ*mrs3*Δ*mrs4*) accumulates more Fe compared with wild-type (WT) strain and unable to grow well in Fe-limiting medium. The disturbance in mitochondrial Fe transport may result in high Fe accumulation in cytoplasm. To avoid this, the activity of yeast vacuolar Fe transporter Ccc1 increases resulting in increased Fe accumulation in vacuole and rendering the cytoplasm Fe deficient[8]. As a result, the Fe uptake system is triggered ultimately accumulating more Fe[7].

Although Fe is abundant in soils, it is not easily available, as it is mainly present as oxidized compounds which are poorly soluble in neutral to alkaline soils. Thus the uptake, translocation and storage/recycling of Fe is extremely important for normal growth of plants. Plants have developed sophisticated mechanisms to acquire Fe from soil[9]. Graminaceous plants use a chelation strategy to acquire Fe from soil and secrete mugineic acid family phytosiderophores. Furthermore, rice is capable of absorbing Fe as Fe(II)[10] as well as Fe(III)-deoxymugineic acid (DMA). The biosynthetic pathway of mugineic acid (MA) has been studied in detail and all the genes involved in the biosynthesis of DMA have been cloned[11]. In addition, transporters involved in the absorption and translocation of Fe in rice have been characterized. OsYSL15 (ref. 12) and OsIRT (ref. 10) were reported to be Fe(III)-DMA and Fe(II) transporters, respectively, which take up Fe from the rhizosphere. OsYSL2 is an Fe(II)-nicotianamine and manganese (Mn) (II)-nicotianamine transporter responsible for the phloem transport of Fe and Mn[13]. Arabidopsis acquires Fe from rhizosphere through IRT[14]. Vacuolar Fe transporter (AtVIT1 (ref. 15)) and PIC1, the chloroplast Fe transporter, have also been characterized[16]. Additionally, several proteins involved in Fe homeostasis in plants have been characterized[17,18]. Despite the rapid progress in understanding Fe uptake and homeostasis, the protein transporting Fe into the mitochondria has not been identified in plants.

Here we report the cloning and characterization of a mitochondrial iron-transporter (*MIT*) gene in rice. Our results suggest that *MIT* is essential for plant growth and development. The cloning of *MIT* is an important advancement in understanding plant-cellular-Fe transport and for developing strategies to mitigate wide spread Fe deficiency problem.

Results

Screening T-DNA library and identification of MIT. We screened a rice transfer DNA (T-DNA) library consisting of 3,993 independent lines for symptoms of Fe deficiency (that is, chlorotic new leaves and green old leaves) to identify genes with a significant role in Fe transport. This criterion was set to avoid the identification of plants defective in chlorophyll biosynthesis and/or related defects.

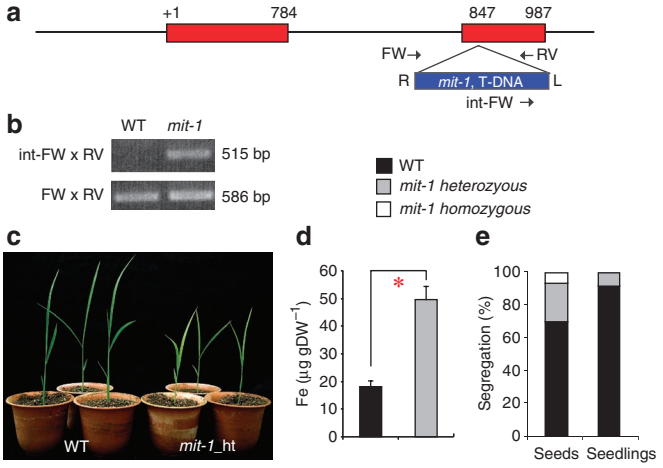

Figure 1 | Screening of a T-DNA library and identification of *mit-1* plants. (**a**) Genomic organization of *MIT* and T-DNA integration in *mit-1* plants. Red boxes represent exons. (**b**) Confirmation of heterozygous status of *mit-1* plants. The position of primers is shown in (**a**), FW, forward; RV, reverse are located on genomic DNA; int-FW, internal forward for T-DNA. (**c**) Phenotype of *mit-1* heterozygous plants. (**d**) Fe concentration of *mit-1* heterozygous plants; μg per gDW^{-1}: μg per gram dry weight. (**e**) Segregation analysis of *mit-1* heterozygous plants using *mit-1* seeds ($n = 27$) and seedlings ($n = 32$). The graph shows mean ± s.d.; *$P < 0.05$, ANOVA followed by a Student-Newman-Keuls test, $n = 3$.

We identified a line harbouring a T-DNA insertion in the second exon of *Os03g0296800* (later named mitochondrial Fe transporter, *MIT*; Fig. 1a,b). The respective T-DNA insertion line (*mit-1*) was analysed; however, we failed to find any plants homozygous for this knockout. Growth of the *mit-1* heterozygous plants was also significantly affected (Fig. 1c), although they accumulated 2.5 times more shoot Fe than WT plants, when grown in soil (Fig. 1d). We analysed individual seeds collected from *mit-1* heterozygous plants to examine the segregation of homozygous and heterozygous plants in the progeny. Surprisingly, only 7 and 23% of the seeds were homozygous and heterozygous for T-DNA integration, compared with the expected segregation ratio of 25 and 50%, respectively (Fig. 1e). After germination, no homozygous seedling was identified, and the ratio of heterozygous seedlings also dropped (Fig. 1e).

MIT localizes to mitochondria and complements the growth of Δ*mrs3*Δ*mrs4*. A homology search (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed the presence of the conserved regions of mitochondrial solute carrier family (MSC) proteins in MIT (amino acids from 134–166 and 171–223). MIT-green fluorescent protein (GFP) localized to mitochondria, when expressed in tobacco BY-2 cells whereas GFP alone, used as control, localized to the cytoplasm (Fig. 2a–f).

MIT expressed in Δ*mrs3*Δ*mrs4* yeast mutants complemented the growth defect of the mutant yeast (Fig. 2g). In Δ*mrs3*Δ*mrs4* yeast, the expression of Fe uptake genes is upregulated[7]. The upregulation of FIT2 and FET3 is reverted to wild-type levels upon expression of the rice gene (Fig. 2h,i). Moreover, it also reversed the changes in accumulation of Fe and Cu (Fig. 2j–m). These results suggested a clear role for MIT in Fe homeostasis.

Expression pattern of *MIT* in rice. Quantitative RT–PCR revealed that the transcripts of *MIT* were three times lower in the roots and shoots of plants exposed to Fe-limited conditions compared with plants grown hydroponically in the presence of 100 μM Fe. *MIT* transcripts were increased in the roots and shoots when the plants were exposed to excess Fe (500 μM Fe; Fig. 3a,b). To further understand its role during germination and seed development, the *MIT*

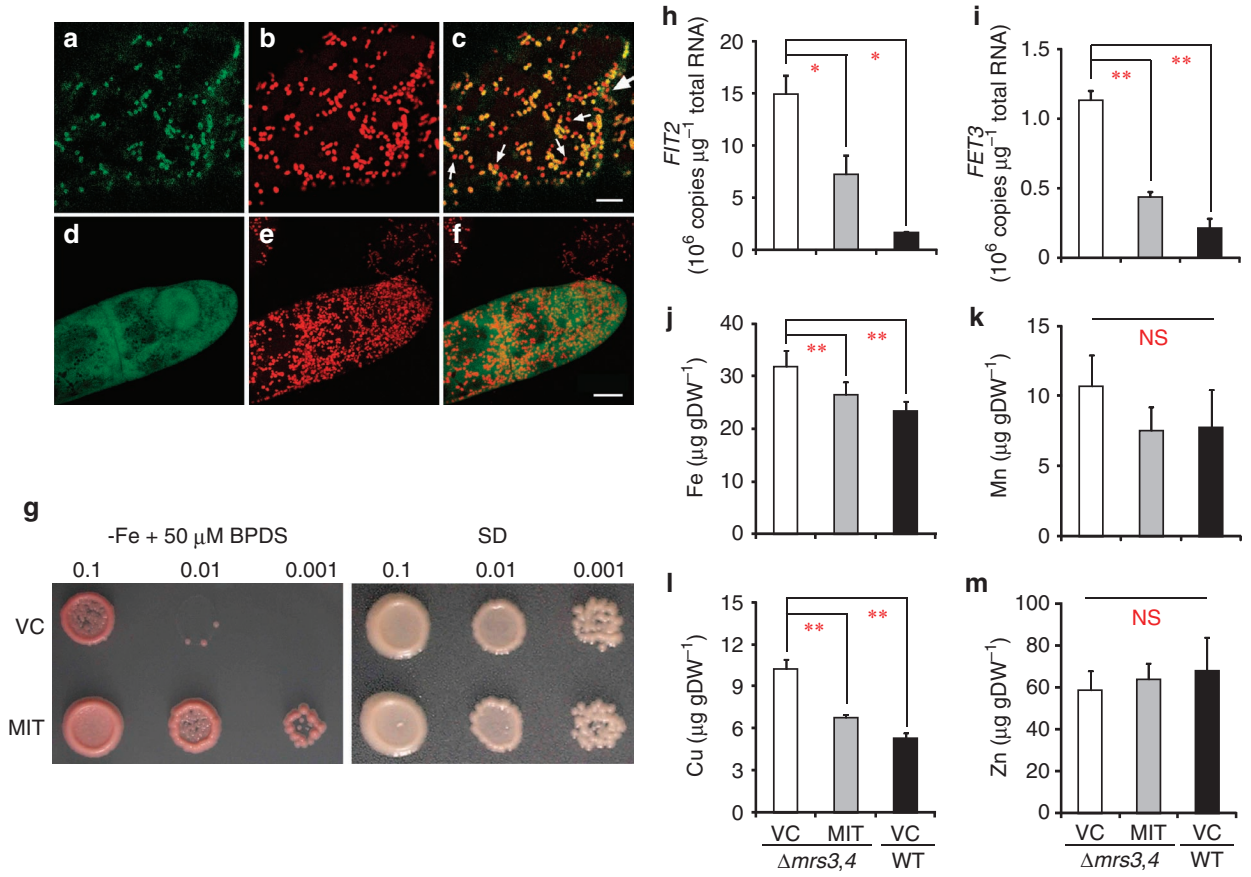

Figure 2 | Subcellular localization and yeast complementation assay of MIT. (**a**–**f**). MIT-GFP expression in suspension cultured tobacco BY2 cells. (**a**, **d**): GFP fluorescence; (**b**, **e**): MitoTracker red fluorescence; (**c**, **f**): merged image. (**a**–**c**): MIT : : sGFP; (**d**–**f**): sGFP. Scale, **a**–**c**, 5 μM, **d**–**f**, 10 μM. (**g**) Representative of serial dilutions of Δ*mrs3*Δ*mrs4* cells transformed with MIT or empty vector placed onto control SD medium or SD medium with 50 μM BPDS ($n = 6$). (**j**–**m**): Metal concentration of Δ*mrs3*Δ*mrs4* and WT yeast transformed with MIT or VC; μg g DW^{-1}: μg per gram dry weight. The graph shows mean ± s.d.; $^{*}P < 0.05$, $^{**}P < 0.001$; NS, not significant; ANOVA followed by a Student-Newman-Keuls test, $n = 3$. BPDS, batho-phenanthroline disulfonic acid; SD, synthetic defined medium; VC, vector control/empty vector.

promoter was used to drive the expression of β-*glucuronidase* in rice. *MIT* expression was observed during germination and at all stages of seed development. During germination, *MIT* expression was specific to the embryo (Fig. 3c–f). Expression was observed in the leaf primordia and coleorhizae 1 day after germination (Fig. 3d). It increased subsequently and, 3 days after germination, was observed in whole embryo (Fig. 3f). Expression was also observed from anthesis through seed development (Fig. 3g, left to right), supporting the hypothesis that MIT has a crucial role in these growth stages. Further the steady state transcripts of *MIT* were observed during all growth stages of rice plant including root, leaves, stem, anther, pistil, lemma, palea, ovary, embryo and endosperm as revealed by microarray analysis (Supplementary Fig. S1).

Characterizing the *MIT* knock down mutant. As the homozygous knockout of MIT in *mit-1* plants proved lethal, we characterized *MIT*-knockdown (*mit-2*) plants (Fig. 4). Hydroponically grown *mit-2* plants were smaller than similarly grown WT plants (Fig. 4a; Supplementary Fig. S2). PCR analysis confirmed the integration of the T-DNA 604 bp upstream of the start codon and the homozygous status of *mit-2* plants (Supplementary Fig. S2a,b). Moreover, quantitative RT-PCR analysis confirmed that the expression of *MIT* in the *mit-2* plants was reduced compared with WT plants (Fig. 4b). There was a significant reduction in root and shoot dry weight as well as in the root and shoot length, leaf width and chlorophyll content (Supplementary Fig. S2c–h). Hydroponically grown *mit-2* plants accumulated 51% more Fe than WT plants in the shoots (Fig. 4c). The accumulation of Mn was also changed (Fig. 4d) whereas no change in the accumulation of Cu and Zinc (Zn) was observed (Supplementary Fig. S2i,j).

Mitochondria isolated from shoot tissue of *mit-2* plants accumulated less Fe compared with WT mitochondria. Mitochondrial Fe concentration of *mit-2* plants was 49% less, compared with mitochondria isolated from WT plants (Fig. 4f). Changes in Mn and Cu accumulation were also observed (Fig. 4g; Supplementary Fig. S2k). The expression of aconitase genes was not changed in *mit-2* plants (Table 1), whereas the total and mitochondrial aconitase activity decreased in the *mit-2* plants compared with WT plants. In shoot tissue, the total aconitase activity of *mit-2* was 39% less compared with WT plants, whereas a reduction of 42% was observed for the *mit-2* aconitase activity from isolated mitochondria (Fig. 4e,h).

The growth of the *mit-2* plants in soil was also significantly impaired compared withWT plants. The average number of tillers in the *mit-2* plants was 8 compared with 20 in the WT plants; in addition, the mutants were compromised in terms of plant height (Supplementary Fig. S2m,n). Flowering was delayed in the *mit-2* plants, and fertility was also significantly lower, reducing the yield by 59% compared with that in WT plants (Fig. 4i–k). Although the growth of soil-grown *mit-2* heterozygous plants was impaired compared with that of WT plants, it was superior to that of *mit-2* homozygous plants for all these characteristics (Fig. 4i–k; Supplementary Fig. S2m,n), confirming that the phenotype was specific to the *mit-2* plants.

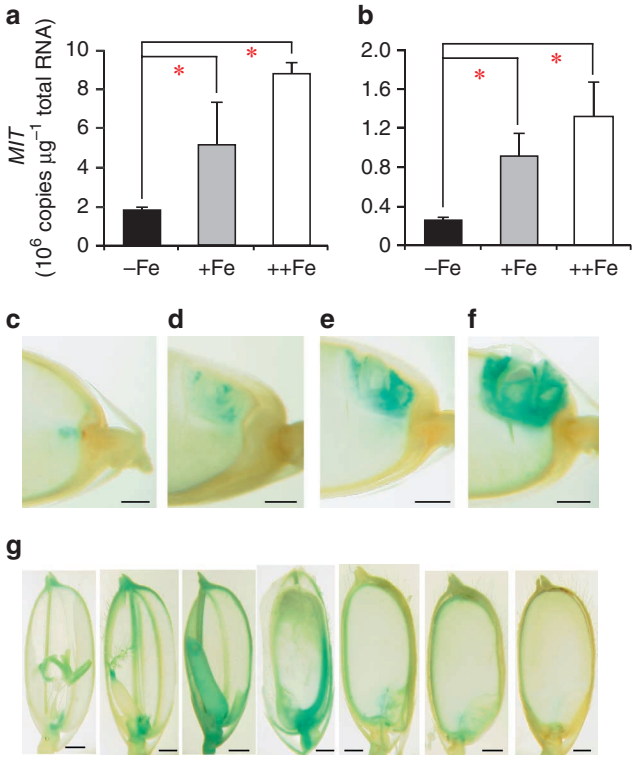

Figure 3 | Expression analysis of *MIT*. (**a**, **b**) The changes in expression of *MIT* in response to Fe availability −Fe: 0 µM Fe; +Fe: 100 µM Fe; ++Fe: 500 µM Fe. (**a**) Root. (**b**) Shoot. (**c**–**g**) *MIT* promoter driven β-*glucuronidase* expression during germination (**c**–**f**) and seed development (**g**); (**c**) 0; (**d**) 1; (**e**) 2; (**f**) 3 days after germination. (**g**) From left to right, before anthesis 1, 4, 12, 16, 20 and 25 days after anthesis. Scale, 500 µm. The graph shows mean ± s.d.; *P < 0.05, ANOVA, n = 3.

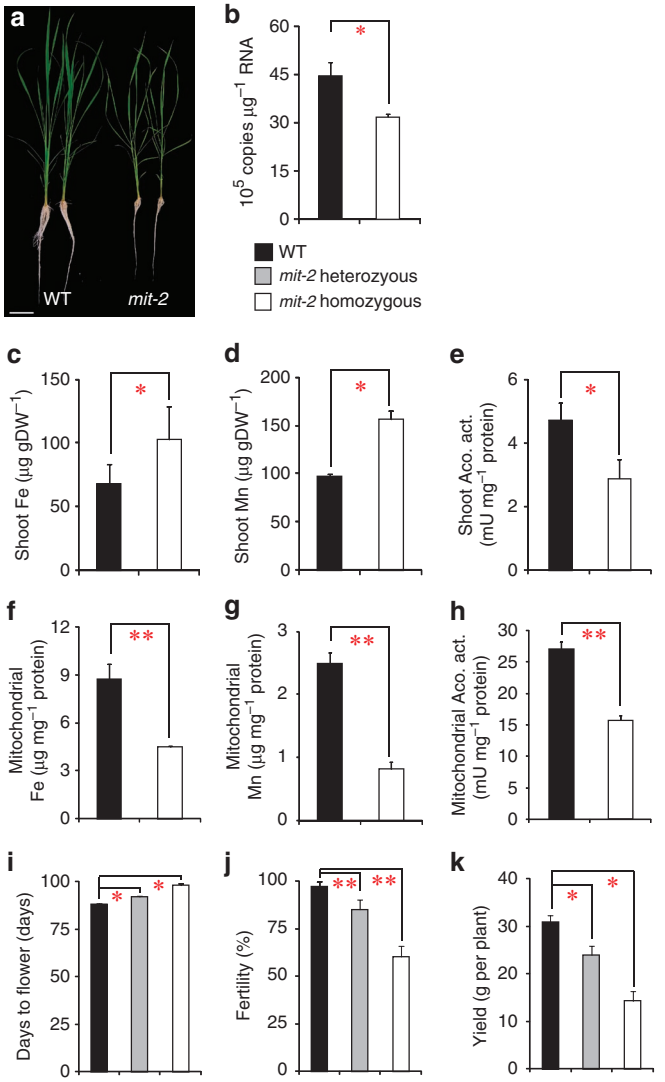

Figure 4 | Characterization of *mit-2* plants. (**a**). Phenotype of hydroponically grown WT and *mit-2* plants. Scale, 10 cm. (**b**) *MIT* expression in *mit-2* shoots. (**c**, **d**). Fe and Mn contents of WT and *mit-2* shoots; µg g DW^{-1}: µg per gram dry weight. (**e**). Total aconitase activity of WT and *mit-2* shoots; 1 mU: n mol min^{-1} per mg protein). (**f**, **g**). Fe and Mn contents of WT and *mit-2* isolated mitochondria. (**h**). Mitochondrial aconitase activity. (**i**–**k**) Morphological characteristics of soil grown WT and *mit-2* plants. The graph shows mean ± s.d.; *P < 0.05, **P < 0.001; ANOVA followed by a student-Newman-Keuls test, n = 3.

As mitochondrial activity is important for cell division, we generated calli from WT and *mit-2* plants; the size and weight of the calli was significantly reduced in the *mit-2* calli compared with the WT (Supplementary Fig. S2o).

Complementing *mit-2* with *MITp-MIT*. To determine whether the *mit-2* phenotype is specifically caused by the reduced expression of *MIT*, *mit-2* plants were transformed with *MIT* open reading frame (ORF) governed by the *MIT* promoter. As the *mit-2* plants were previously transformed with T-DNA and were selected on hygromycin, second transformation was done using rice ALS gene[19] as selection marker. The expression of *MIT* in *mit-2* plants significantly reversed the growth defect in terms of plant height, chlorophyll content as well as Fe and Mn accumulation (Fig. 5).

Microarray analysis. Microarray analysis of *mit-2* plants was performed to determine whether the change in *MIT* expression affected other Fe homeostasis-related genes in rice plants. Our results indicate that *MIT* expression was significantly reduced in the *mit-2* plants (Table 1). Moreover, the expression of a homologue of the *Arabidopsis* vacuolar Fe and Mn transporter[15] (*OsVIT1*), homologue of *Arabidopsis* Fe efflux transporter[20] (*OsFerroportin*) was significantly upregulated in the *mit-2* plants, and the expression of ferritin, an Fe storage protein[21], was also slightly upregulated. On the other hand, expression of the genes upregulated by Fe deficiency was downregulated; for example, expression of the Fe(II) transporters *OsIRT1*, *OsIRT2* (ref. 6) and Fe(III)-DMA transporter *OsYSL15* (ref. 12) was slightly downregulated in the roots. Similarly, the expression of rice ferric chelate reductase *OsFRO2* (ref. 10) was decreased in the shoots, whereas expression of the Fe transporter *OsNramp1* (ref. 22), and Fe(II)-nicotianamine transporter *OsYSL2* (ref. 13) was downregulated in the roots.

Discussion

Like other eukaryotes, plant mitochondria depend much on Fe for normal function; however, the proteins transporting cytoplasmic Fe across the mitochondrial membrane have not been characterized. We screened a rice T-DNA mutant library and identified a plant showing Fe-deficiency symptoms when grown under Fe-sufficient conditions, accumulating higher Fe compared with WT plants, and resulting in a lethal phenotype. Further studies revealed that T-DNA is integrated in to a gene encoding MIT. *MIT* knock out mutation caused a growth defect during seed development as revealed by the analysis of *mit-1* heterozygous plants examined for the segregation of homozygous and heterozygous plants in the

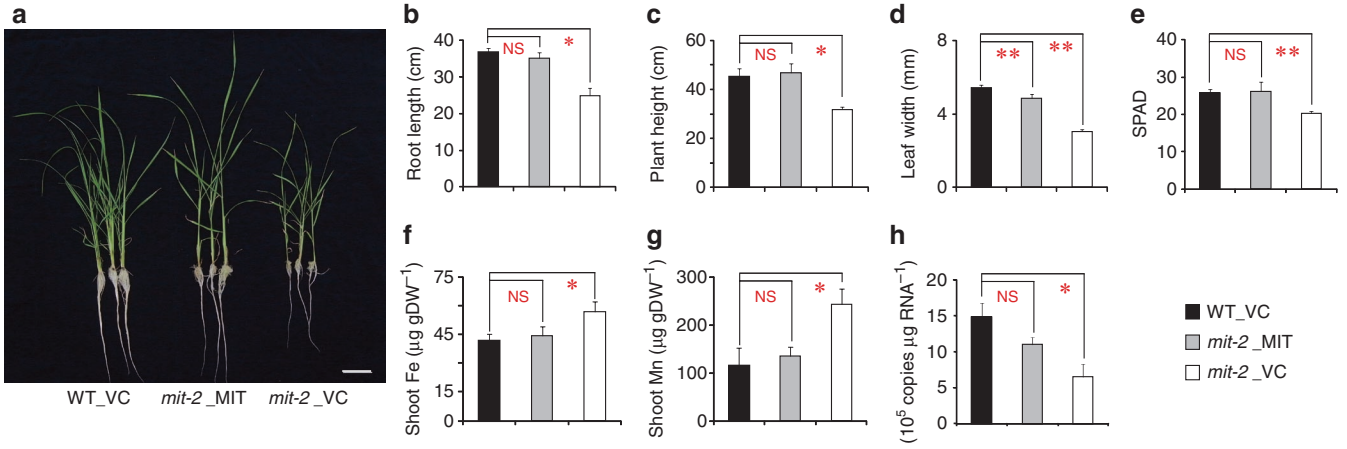

Figure 5 | Characterization of *mit-2* plants complemented with MITp-MIT. (**a**–**d**) Morphological characteristics of hydroponically grown WT plants transformed with empty vector (WT_VC) and *mit-2* plants transformed with MIT ORF (*mit-2*_MIT) or empty vector (*mit-2*_VC). (**a**) Phenotype of hydroponically grown plants. Scale, 10 cm. (**b**) Root length. (**c**) Shoot length. (**d**) Leaf width. (**e**–**g**) Fe and Mn content of hydroponically grown plants. (**h**) Expression of MIT in hydroponically grown plants. The graph shows mean ± s.d.; $*P<0.05$, $**P<0.001$; NS, not significant; ANOVA followed by a student-Newman-Keuls test, WT and *mit-2* plants; $n=3$, *mit-2-MIT* plants; $n=5$.

Table 1 | Summary of microarray analysis of MIT knockdown plants (*mit-2*).

Gene name	Accession no.	mit-2/WT (+Fe)		WT (−Fe/+Fe)	
		Shoot	Root	Shoot	Root
MIT	AK058471	0.4	0.4	0.5	1.1
OsVIT1	AK071589	3.3	4.0	0.0	0.1
OsFerroportin	AK101863	1.1	4.4	1.1	1.7
OsFerritin	AK102242	1.5	1.1	0.2	0.1
OsIRT1	AK107681	0.9	0.7	2.8	2.3
OsIRT2	CI162465	1.5	0.1	22.4	7.0
OsYSL2	CI446246	0.7	0.3	82.2	12.7
OsYSL15	AK063464	1.0	0.8	8.8	197
OsNRAMP1	AK103557	0.7	0.3	245	6.9
OsFRO2	AK068159	0.2	0.8	132	1.1
Aconitase	AK067183	0.9	1.1	0.8	0.9
Aconitase	AK061677	0.9	1.1	0.6	0.8

progeny. These analyses further revealed that, although to a lesser extent, MIT also has a role in germination.

Besides producing a lethal phenotype for knockout mutant, the knockdown mutation also resulted in a severe phenotype and the growth of *mit-2* was also significantly impaired when grown hydroponically, or in soil. Although growth was also impaired in *mit-2* heterozygous plants compared with WT plants, soil grown *mit-2* heterozygous plants were superior to *mit-2* homozygous plants for agronomical characteristics such as average number of tillers, fertility and yield. The heterozygous plants were also superior to *mit-2* homozygous plants in terms of plant height and days to flowering confirming that the *mit-2* phenotype is specific to *MIT* knockdown plants. Both *mit-1* heterozygous and *mit-2* homozygous plants accumulated higher Fe in shoot tissue compared with WT plants. The impaired growth, despite the higher accumulation of Fe, indicated that plants may not be able to utilize the accumulated Fe.

Metal profiling of mitochondria isolated from shoot tissue of *mit-2* plants was significantly different from WT for Fe accumulation, whereas changes in Mn and Cu accumulation were also observed. Thus, in *mit-2* plants, reduced *MIT* expression leads to low Fe transport to mitochondria, leaving the cytoplasm with slightly elevated Fe levels. Changes in mitochondrial Fe accumulation could affect the Fe-S cluster synthesis in mitochondria. In line with this, the total and mitochondrial aconitase activity decreased in the *mit-2* plants compared with WT plants, showing that Fe-S cluster synthesis was significantly affected in *mit-2* plants. The reduction in the chlorophyll soil-plant analysis development (SPAD) value may be due to problems in mitochondrial Fe-S cluster synthesis that indirectly affect the chloroplasts. It has already been shown that knockout plants for the mitochondrial-synthesized Fe-S cluster exporter exhibit chlorosis[23].

The lethal phenotype of *mit-1* plants and the significantly impaired growth of the *mit-2* plants, complementation of *mit-2* plants with MIT and phenotype of *mit-2* heterozygotes highlight the importance of MIT in plant growth and development. On the other hand, the Fe-related phenotype and reduced aconitase activity in *mit-2* plants indicate that MIT is essential for Fe homeostasis in rice. A complete loss of MIT may be lethal, whereas a reduction in *MIT* expression (as in *mit-1* heterozygous and *mit-2* plants) significantly affects mitochondrial activity. This reduced activity may affect several cellular functions, which force the plant to compromise on important agronomic parameters.

MIT belongs to the MSC family of proteins. The members of this family localize to the inner mitochondrial membrane, have conserved regions and transport a wide range of substrates, including Fe[24,25]. The MSC family members transporting Fe into the mitochondria have been characterized in several organisms including yeast (Mrs3-Mrs4 (ref. 7)), mouse[26], *Drosophila*[27] and zebrafish[28] (mitoferrin). The mitoferrin has been characterized in detail and the interactions of ferrochelatase, mitoferrin-1 and Abcb10 has also been revealed[29]. MIT has 39, 41 and 42% homology to Mrs3, Mrs4 and mitoferrin, respectively; whereas phylogenetic analysis revealed the absence of any close homologue in rice (Supplementary Fig. S3). The yeast Δ*mrs3*Δ*mrs4* phenotype is only significant under low-Fe conditions (in the presence of Fe a substantial amount of Fe is imported into mitochondria), indicating that it is not the only MIT, although it is the only high-affinity MIT in yeast[30]. Besides Fe, Mrs3–Mrs4 also transport copper (Cu)[31]. In the yeast Δ*mrs3*Δ*mrs4* double mutant, activity of the yeast vacuolar Fe and Mn transporter Ccc1 increases to avoid Fe toxicity in the cytoplasm, resulting in increased Fe and Mn accumulation in the vacuole and Fe deficiency in the cytoplasm[7,8]. As a result, the Fe-uptake system is triggered leading to a greater accumulation of these metals in the cells compared with the WT strain.

MIT localized to mitochondria and complemented the growth defect of Δ*mrs3*Δ*mrs4* yeast mutants confirming that MIT has the same function as Mrs3p/Mrs4p. The expression of MIT in

yeast reversed the upregulation of FIT2 and FET3 found in the Δ*mrs3*Δ*mrs4* mutant and also reversed the changes in accumulation of Fe, and Cu. These results also suggested a clear role of MIT in cellular Fe homeostasis. The mutation in rice MIT is lethal, as Fe participates in vital processes in the mitochondria, including the electron transport chain and the synthesis of haem and Fe-S proteins[2–4]. Zebrafish expressing a mutant version of mitoferrin had severely hypochromic erythrocytes[28], whereas *Arabidopsis* defective in frataxin which is involved in Fe-S cluster assembly, had a lethal phenotype[32]. Mutation in rice specific mitochondrial iron-regulated gene also resulted in severe phenotype[17].

The transcripts of *MIT* increased in the roots and shoots when the plants were exposed to excess Fe (500 μM Fe), and decreased under Fe-limiting conditions, indicating that the steady state MIT transcript levels are regulated by iron. Further expression of *MIT* was observed during germination, through vegetative stage in roots and leaves, and during seed development to support the hypothesis that MIT has a crucial role in these growth stages.

Microarray analysis of *mit-2* plants revealed significant changes in the expression of genes regulated by Fe. The *mit-2* plants accumulated more Fe than the WT plants, thus, it is not surprising that genes related to Fe sequestration were upregulated and expression of genes involved in Fe uptake decreased. In yeast Δ*mrs3*Δ*mrs4*, the expression of genes regulated by Fe deficiency as well as Ccc1 is upregulated. It appears that rice uses a different strategy compared with yeast to tackle the reduced expression of *MIT* (Supplementary Fig. S4). This difference may be explained by the fact that higher plants need more complex control of Fe homeostasis compared with yeast, a unicellular eukaryote.

In brief, through library screening, we cloned a major MIT essential for plant growth and development. Reduced *MIT* expression affected mitochondrial activity, including Fe-S cluster synthesis, and impaired plant growth and development, especially at the reproductive stage. As rice is a staple food for a large portion of the human population, understanding Fe homeostasis and improving the Fe content of rice are major objectives for rice researchers[33] to mitigate widespread Fe deficiency in humans. The characterization of MIT is a significant step in advancing these efforts.

Methods

Identification and characterization of the mutant lines. Approximately 30,000 seeds, from 3,993 independent lines of a rice T-DNA library of japonica cultivar Dongjing transformed with p2715 (ref. 34) were grown hydroponically and screened for the presence of mutants showing symptoms of Fe deficiency. Three plants were selected, and iPCR was performed, as described in ref. 35 to identify the point of T-DNA integration. Finally, one plant was identified that harboured the T-DNA in the second exon of *Os03g0296800*. Further, the respective T-DNA line (*mit-1*) was analysed for integration of the T-DNA by PCR using a T-DNA-specific primer (5′-AATATCTGCATCGGCGAACTGATCG-3′), and an *mit-1*-specific FW primer (5′-GCTGTCATTGAGTTGTGCAC-3′) and *mit-1*-specific RV primer (5′-TGAATGTTCAGATTATTTCC-3′). This line was further analysed for the presence of homozygous *mit-1* plants, and seed DNA extracted from 27 independent seeds were analysed with the following primers: T-DNA internal forward (5′-CTCGTATGTTGTGTGGAATTGTGA-3′) and T-DNA internal reverse (5′-GTAGAACGGTTTGAGGTTAATCAGGAAC-3′), and with primers specific for the borders of the T-DNA integration site (forward [5′-GCTGTCATTGAGTTGTGCAC-3′] and reverse [5′-GAACAGCATTCTTGGCTTCC-3′]). T-DNA integration in the *mit-2* plants was confirmed using the T-DNA-specific primer 5′-AATATCTGCATCGGCGAACTGATCG-3′ and *mit2*-specific RV primer 5′-GTAGGGTTAGGGTTTAGACGC-3′. To confirm the homozygous status of the *mit-2*, primers located near the T-DNA integration site, *mit-2*-specific FW (5′-CCAACTTGGTCACTAGATCTGG-3′ and *mit-2*-specific RV were used as forward and reverse primers, respectively. Similarly, the seeds were germinated on tissue paper and analysed 1 day after germination for segregation.

Subcellular localization of MIT. The full-length ORF of *MIT* was amplified with forward and reverse primers (5′-caccATGGCCGCCGACTACCGCACACC-3′ and 5′-TTATTTCCTTCTTTTCTCGTTGA-3′, respectively), subcloned into pENTR/D-TOPO (Invitrogen), and sequenced. The *MIT* ORF was then subcloned into pH7WGF2 (ref. 36). Tobacco BY-2 cells were transformed, and the expression of GFP was observed as described previously[37].

Complementation of Δ*mrs3*Δ*mrs4* mutant yeast. Δ*mrs3*Δ*mrs4* mutant yeast and the respective WT strain (BY4741)[7] were transformed with empty vector (pHY23) or vector containing the *MIT* ORF. The yeast strains were grown on synthetic defined (SD) medium as described[7]. For complementation assays, agarose was used instead of agar to avoid Fe contamination. Yeast strains were grown in liquid SD medium, and serial dilutions were prepared (OD_{600}) as 0.1, 0.01 and 0.001 and plated onto SD plates. The plates were incubated at 30 °C for 6 days. RNA was extracted using Qiagen RNeasy total RNA extraction kit (QIAGEN). For quantitative RT-PCR, following primers were used: FIT2 forward, 5′-GACACCGCTGACCCTATCAT-3′, and reverse as 5′-AGGCAGAAGAGGAGGAGGAG-3′, FET3 forward 5′-ACGGTGTGAATTACGCCTTC-3′, and reverse as 5′-TGGAAAGCGTGACCATGTAA-3′. For normalizing data, the following UBC6 specific primers were used: forward 5′-GATACTTGGAATCCTGGCTGGTCTGTCTC-3′, and reverse as 5′-AAAGGGTCTTCTGTTTCATCACCTGTATTTGC-3′.

Rice transformation and growth conditions. The 1.7-kb 5′-flanking region of *MIT* was amplified by PCR using genomic DNA as template with the forward primer 5′-CACCCTATCTTTCAAGCTTGGAGCTTGCCTTTTCG-3′ and reverse primer 5′-CGGCGGCCATTCTAGAGGAGGGCGGCTGCGGTAGG-3′, which contain a *Hin*dIII restriction site. The amplified fragment was fused into the pBluescript II SK + vector, and its sequence confirmed. The *MIT* promoter was digested with *Xho*I and *Bgl*II, and the digested 1.7-kb fragment was subcloned upstream of the *uid*A ORF, which encodes β-glucuronidase, in the pIG121Hm vector[38]. *Oryza sativa* L. cv. Tsukinohikari was transformed by using the agrobacterium-mediated transformation method. The plants were grown in a greenhouse; the histochemical localization of *MIT* was observed in three independent T2 plants as described[10].

Characterization of the mutants. *mit-1* and *mit-2* plants were either grown hydroponically or in soil. The morphological characters like plant height, average number of tillers, days to flower and fertility percentage were recorded as described[39]. An aconitase assay was performed using an aconitase assay kit (Biovision) as suggested by the manufacturer. For measuring metal concentration in WT and mutants, Leaf or root samples were digested with 3 ml of 13 M HNO_3 at 220 °C for 20 min using a MARS XPRESS microwave reaction (CEM) in triplicate. After digestion, samples were collected and diluted to 5 ml and elemental analysis of the WT, *mit-1* and *mit-2* plants were performed using inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko) as described[40]. Mitochondria were isolated from WT and *mit-2* plants grown hydroponically for three weeks as described[41]. Briefly, shoot tissue from 120 WT and *mit-2* plants, grown hydroponically for 3 weeks, were grinded in the presence of buffer (450 mM Sucrose, 1.5 mM EGTA, 15 mM MOPS, pH 7.4, 0.2% BSA, 0.6% PVP, 10 mM DTT and 0.2 mM PMSF), purified through three layers of mira cloth, centrifuged at 3,500 *g* twice (first time 10 min and second time 5 min); the supernatant was centrifuged at 6,000 *g* for 5 min and mitochondria were pelleted by centrifugation at 17,000 *g* for 10 min. The pellet was dissolved in buffer containing 300 mM sucrose, 1 mM EGTA, 10 mM MOPS, pH 7.2 and loaded on to a Percoll gradient and centrifuged for 45 min at 70,000 *g* and mitochondria were recovered between 29% and 45% percoll. All the steps were performed at 4 °C.

For microarray analysis, seeds of the *mit-2* plants were germinated on Murashige and Skoog (MS) medium and transferred to hydroponic solution 10 days after germination and grown for three weeks in the presence of 100 μM Fe. Roots and shoots were collected, frozen in liquid nitrogen, and stored at −80 °C until use. RNA was extracted from the roots and shoots of three plants, and total RNA (200 ng) from the *mit-2*, and WT plants were labelled with Cy3 or Cy5 using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies), and microarray analysis was performed in duplicate, according to the manufacturer's instructions, using a rice 44 K oligo-DNA microarray (Agilent). The data has been deposited in GEO under the accession number GSE28428. Points with a *P*-value <0.001, and a ratio >2 in both the Cy3 and Cy5 channels, were considered to be significantly upregulated. The data regarding expression of MIT was generated through Rice global gene expression profile data sets[42] (http://www.ricexpro.dna.affrc.go.jp/index.html).

Quantitative RT-PCR for *MIT* was performed with the forward and reverse primers 5′-CAAGACTCAGCTGCAGTGTC-3′ and 5′-GAACAGCATTCTTGGCTTCCA-3′, respectively, as described[43]. The primers used for internal control in RT-PCR were α-*tubulin* forward, (5′-TCTTCCACCCTGAGCAGCTC-3′) and α-*tubulin* reverse (5′-AACCTTGGAGACCAGTGCAG-3′).

Complementation of *mit-2* with MIT. For complementation analysis, *MIT* ORF was subcloned into pR-5 (Kumiai Chemicals), and the 1.7 kb promoter region was subcloned with *Xba*1 and *Hin*dIII restriction sites. As the *mit-2* plants contain T-DNA and were selected on hygromycin, for complementation analysis, plants were transformed using *Oryza sativa* W548L/S627I ALS gene[19] and sodium 2,6-bis(4,6-dimethoxypyrimidin-2-yloxy) benzoate as selection marker. WT plants transformed with empty vector and *mit-2* plants with either empty vector, or pR5 containing *MITp-MIT* were grown hydroponically for two weeks and metal profiling and morphological analysis were performed.

Statistical analysis. To determine whether the observed differences between the WT and *mit* plants were statistically significant, one-way ANOVA was performed

using Costat 6.400 (Cohort, USA) with a completely randomized design followed by a Student-Newman-Keuls test ($P < 0.05$).

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Author contributions

K.B., Y.I., H.N. and N.N. designed research, discussed the data and wrote the paper. T-DNA library was developed by G.A. T-DNA screening was performed by Y.I. Yeast complementation assay was performed by K.B., Y.I. GFP analysis was performed by K.B., Y.I., M.F. and N.T. Mitochondria isolation was performed by K.B., H.T. and N.T. Aconitase activities were analysed by K.B. Rice transformations were performed by K.B., Y.I. and H.S. Metal concentration analysis was performed by K.B., Y.I. and S.N. Microarray and expression analysis were performed by K.B. and Y.I. Statistical analysis was performed by K.B.

Additional information

Accession codes: The microarray data have been deposited in Gene Expression Omnibus database under the accession codes GSE28428.

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