Characterizing the Crucial Components of Iron Homeostasis in the Maize Mutants \textit{ys1} and \textit{ys3}

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Abstract

To acquire iron (Fe), graminaceous plants secrete mugineic acid family phytosiderophores through the phytosiderophore efflux transporter TOM1 and take up Fe in the form of Fe(III)–phytosiderophore complexes. Yellow Stripe 1 (\textit{ys1}) and \textit{ys3} are recessive mutants of maize (\textit{Zea mays} L.) that show typical symptoms of Fe deficiency, i.e., interveinal chlorosis of the leaves. The \textit{ys1} mutant is defective in the Fe(III)–phytosiderophore transporter YS1 and is therefore unable to take up Fe(III)–phytosiderophore complexes. While the \textit{ys3} mutant has been shown to be defective in phytosiderophores release, the causative gene has not been identified. The present study was performed to characterize the expression profiles of the genes in \textit{ys1} and \textit{ys3} mutants to extend our understanding of Fe homeostasis in maize. Using quantitative real-time polymerase chain reaction, we assessed changes in the levels of gene expression in response to Fe deficiency of genes involved in Fe homeostasis, such as those related to phytosiderophores biosynthesis and Fe transport. As with other crops, these Fe deficiency-inducible genes were also upregulated in maize. In addition, these Fe deficiency-inducible genes were upregulated in both the \textit{ys1} and \textit{ys3} mutants, even under Fe-sufficient conditions. Indeed, the Fe concentrations in the roots of \textit{ys1} and \textit{ys3} plants were lower than that of wild-type controls. These results suggest that \textit{ys1} and \textit{ys3} are Fe-deficient during growth in the presence of Fe. In agreement with previous reports, the level of \textit{YS1} expression decreased in the \textit{ys1} mutant. Moreover, the expression level of a homolog of \textit{TOM1} in maize decreased significantly in the \textit{ys3} mutant. Unspliced introns of \textit{ZmTOM1} were detected only in \textit{ys3}, and not in \textit{YS1YS3} or \textit{ys1}, suggesting that \textit{ZmTOM1} may be involved in the \textit{ys3} phenotype.

Introduction

Iron (Fe) is an essential nutrient for virtually all living organisms. Fe plays a key role in electron transfer in both photosynthetic and respiratory reactions in chloroplasts and mitochondria. Although abundant in mineral soils, Fe is sparingly soluble under aerobic conditions at high soil pH and exists mainly as oxidized insoluble Fe(III) compounds. Consequently, plants grown on calcareous soils often exhibit severe chlorosis due to Fe deficiency, which results in reduced crop yields [1].

Higher plants have two strategies for the uptake of Fe(III) from the rhizosphere [2]. All higher plants, with the exception of graminaceous plants, take up Fe using ferric-chelate reductases (FROs) to reduce Fe(III) to Fe(II), which is subsequently taken up by ferrous Fe transporters (IRTs; Strategy I; [3–5]). Alternatively, graminaceous plants secrete Fe chelators called mugineic acid family phytosiderophores (MAs) from their roots via Transporter Of MAs (TOM1) to solubilize Fe in the rhizosphere (Strategy II; [6,7]). Graminaceous plants then take up Fe as Fe(III)–MAs complexes from the rhizosphere through the action of Yellow Stripe 1 (YS1) transporters at the plasma membrane [8,9].

The biosynthetic pathway for MAs in graminaceous plants has been elucidated [7,10–13]. Methionine, which is a precursor of MAs [10], is converted to 2'-deoxymugineic acid (DMA) via a series of reactions. While \textit{maize} (\textit{Zea mays} L.) and rice (\textit{Oryza sativa} L.) secrete DMA, other species, including barley (\textit{Hordeum vulgare} L.) and rye (\textit{Secale cereale} L.), further hydroxylate DMA to other MAs. The genes that encode the biosynthetic enzymes responsible for converting S-adenosyl methionine to MAs have been identified in barley (\textit{HeNASI-7}, \textit{NASHORI} and 2, \textit{HeNAAT-A} and \textit{B}, \textit{HeDMASI}; [14–18], maize (\textit{ZnNASI-3}, \textit{ZnNAAT1}, \textit{ZnDMASI} [14,19–20]), and rice (\textit{OsNASI-3}, \textit{OsNAAT1}, \textit{OsDMASI} [14,20–22]). Expression of these genes is strongly induced by Fe deficiency. The non-proteinogenic amino acid nicotianamine (NA), which serves as an intermediate in the MAs biosynthetic pathway, also functions as a transition metal chelator in plants [23–25].

\textit{TOM1}, which is a major facilitator superfamily (MFS) antiporter, was identified as an efflux transporter of DMA in rice [26]. \textit{TOM1} expression is strongly induced in Fe-deficient roots. DMA secretion from rice roots is increased by the overexpression of \textit{TOM1} and decreased by its repression, indicating that \textit{TOM1} encodes the efflux transporter of DMA in plants [26].
Maize belongs to the C₄ grasses; it has high photosynthetic efficiency and is important not only as a staple crop but also for adaptation to the environment. Under Fe-deficient conditions, as in calcareous soils, maize is highly susceptible to Fe deficiency and its growth decreases dramatically. However, as little information is available regarding genes in maize compared to other crops, such as rice, the molecular components involved in Fe homeostasis are not well understood. Two maize mutants, yellow stripe-1 (ys1) and yellow stripe-3 (ys3), are considered to be defective in Fe uptake mainly because they exhibit typical Fe-deficiency chlorosis (yellowing between the veins), which can be rescued by the administration of ferric chelates to the leaves [27–29]. The ys1 mutant has been shown to be defective in the uptake of Fe(III)–DMA complexes [30]. The causative gene of the ys1 mutant is YSI, which encodes the specific transporter responsible for the uptake of Fe-chelated DMA complexes from the rhizosphere into root cells [50]. While YSI expression increases in both roots and shoots under conditions of Fe deficiency, it is not regulated by zinc (Zn) or copper (Cu) deficiency [9,31]. YS1 functions as a proton-coupled symporter for various DMA-bound metals, including Fe(III), Zn(II), Cu(II), and nickel (Ni)(II) [32]. YS1 also transports NA-chelated Ni(II), Fe(II), and Fe(III) complexes. Eighteen YSL-like (YSL) genes have been identified in rice [33]. Among these, OsYSL15 encodes an Fe(III)–DMA transporter that appears to be an ortholog of YSI [34,35]. OsYSL18 and OsYSL16 also transport Fe(III)–DMA [36,37]. OsYSL2 transports Fe(II)–NA and manganese (Mn)(II)–NA, but not Fe(III)–DMA, and has been suggested to be responsible for phloem transport of Fe and Mn [33,38]. Non-graminaceous plants also possess YSL genes, which encode transporters that are considered to play important roles in internal metal homeostasis by transporting metal-NA complexes, as non-graminaceous plants synthesize NA but not MAs [39–43]. In contrast, the ys3 mutant has been reported to show impaired DMA secretion [44], although the causative gene has not yet been identified.

In the present study, quantitative real-time polymerase chain reaction (PCR) was performed to determine the regulation of genes involved in Fe homeostasis in maize. First, we confirmed that the expression of genes homologous to those involved in Fe homeostasis in rice were induced in maize under Fe-deficient conditions. The expression levels of the genes putatively involved in Fe homeostasis, such as the MAs biosynthetic pathway, as well as those for transcription factors and transporters, were induced in maize under Fe-deficient conditions. Second, the gene expression profiles of the ys1 and ys3 mutants were analyzed. The expression levels of the Fe-inducible genes were higher in both ys1 and ys3 compared to YSI, YS3 [wild type (WT), i.e., (YS3WT), which is the same cultivar and has the same genetic background as the ys3 mutant] even under Fe-sufficient conditions. These results suggest that ys1 and ys3 experience Fe deficiency even under Fe-sufficient conditions. In agreement with previous reports, the expression level of YSI in ys1 decreased in comparison to the WT. Moreover, the expression level of the TOM1 homolog (ZmTOM1) in ys3 decreased compared to the WT or ys1. Unspliced TOM1 mRNAs were detected only in ys3. ZmTOM1 is located in the quantitative trait locus (QTL) of the ys3 phenotype, and our results suggested that ZmTOM1 may be involved in the ys3 phenotype.

**Results**

**Phenotyping of ys1 and ys3**

Maize plants grown hydroponically under Fe-sufficient or Fe-deficient conditions for 5 days (Figure 1) were harvested at the same time. WT plants and the ys1 and ys3 mutants were grown together in a 20-L plastic container. The cultivar backgrounds used for the WT and the ys1 and ys3 mutants differed from each other. Thus, while comparing the mutants, note that the differences in phenotype are the cumulative effects of the differences in root Fe concentrations when plants were grown under control conditions (Figure 2A). No significant differences were observed in root Fe concentrations when plants were grown under Fe-deficient conditions (Figure 2A). In the shoot tissues, the Fe concentration was higher in the ys1 plants than in the WT and ys3 plants when grown under Fe-deficient conditions, while no significant differences in shoot Fe concentration were noted under Fe-sufficient conditions (Figure 2B). In addition to Fe, significant differences in the concentrations of other metals were also detected. In the roots, the concentration of Zn was significantly lower in the ys1 and ys3 mutants than in the WT plants under Fe-deficient conditions (Figure 2C). In the shoot tissues, the ys1 and ys3 plants showed higher Zn concentrations under Fe-sufficient conditions, while the Zn concentration was significantly lower in the ys1 mutant than in the WT and ys3 plants under Fe-deficient conditions (Figure 2D). In the roots, the ys1 and ys3 mutants showed significantly lower Cu concentrations under both Fe-sufficient and Fe-deficient conditions (Figure 2E). In shoot tissues, no significant differences were observed in the Cu concentrations when the plants were grown under Fe-sufficient conditions, while the ys1 and ys3 shoots showed higher Cu concentrations under Fe-deficient conditions (Figure 2F). In the roots, the concentration of Mn in the ys1 mutants was higher than in the WT under both Fe-sufficient and Fe-deficient conditions (Figure 2G). When the plants were grown under Fe-sufficient conditions, the Mn concentration in the ys3 mutants was slightly higher than those in WT and ys1 plants, while the Mn concentrations in the ys1 and ys3 mutants were higher in the shoots under Fe-deficient conditions (Figure 2H). These results clearly indicate that metal homeostasis is significantly impaired in ys1 and ys3 mutants.

**Changes in the Expression of Fe Homeostasis-related Genes**

In Fe-deficient rice, the genes that encode the enzymes involved in the MAs biosynthetic pathway, including the methionine cycle, as well as those for transcription factors and transporters involved in Fe homeostasis, are induced, and their functions have been characterized [14,24,26,34,45–49]. Therefore, the differences in expression levels of the maize homologs of these genes between Fe-sufficient and Fe-deficient conditions were examined by quantitative real-time PCR. The expression levels of maize homologs that participate in the methionine cycle (ZmMTN, GRMZM2G171111; ZmAPT, GRMZM2G093347; ZmMTK, GRMZM2G464137; ZmIDI2, GRMZM2G139533; ZmFDH, GRMZM2G094901; ZmMHT, GRMZM2G067265; ZmRPI, GRMZM2G035599) and MAs biosynthesis (ZmNAS1, GRMZM2G049811; ZmDMAS1, GRMZM2G034956; ZmDMAS3, GRMZM2G060952) were markedly elevated in roots under Fe-deficient conditions (Figure 3). The expression level of ZmNAS3 was higher in the shoots than the roots, and its expression decreased under Fe-deficient conditions (Figure 3; ZmNAS3, GRMZM2G4748568). The expression levels of the homologs of the OsIRO2 and OsIRO3 genes
which are Fe deficiency-induced transcription factors that regulate the Fe-deficiency response [45–49], were markedly elevated in both roots and shoots under Fe-deficient conditions (Figure 3). YS1 was also induced in both roots and shoots under Fe-deficient conditions (Figure 3; YS1, GRMZM2G156599). Transporter genes with similarities to TOM1 and TOM3 were induced in the roots under Fe-deficient conditions (Figure 3; ZmTOM1, GRMZM2G063306; ZmTOM3, GRMZM2G141081). In contrast, the TOM2 homolog was induced only in the shoots, and not in the roots, under Fe-deficient conditions (Figure 3; ZmTOM2, GRMZM5G877788). The expression levels of the OsIRT1 homolog, which is involved in the transport of ferrous Fe [50,51], were not induced in either the roots or shoots by Fe deficiency (Figure 3; ZmIRT1, GRMZM2G118821). Among the NRAMP family members, which transport various divalent metals, including Fe, Mn, and cadmium [52–54], the expression levels of OsNRAMP2 homologs increased in both roots and shoots under Fe-deficient conditions (Figure 3; ZmNRAMP1, GRMZM2G178190). The expression levels of the phenolics efflux zero PEZ [55–57] homologs increased strongly in both roots and shoots under Fe-deficient conditions (Figure 3; ZmMATE2/ ZmPEZ1, GRMZM2G170128).

**Gene Expression Profiles of ys1 and ys3 Mutants**

The ys1 and ys3 mutants exhibit the Fe-deficient phenotype. The ys1 mutant was reported to have a defect in the YS1 gene and is unable to take up Fe–DMA from the soil [9,30], while the ys3 mutant was reported to have a defect in the secretion of DMA from the roots, although the causative gene has not been identified [43]. Changes in the expression levels of genes involved in biosynthesis and transport of MAs were examined in ys1 and ys3 (Figures 4, 5). As the background used in the present study differed for the WT and ys1 and ys3, the sizes and sequences of the cDNA products for each gene were checked and confirmed to be the same. In addition, many of the genes linked to the methionine cycle were upregulated in ys1 and ys3, especially under Fe-sufficient conditions (Figure 4; ZmMTN, ZmAP, ZmMTK, ZmI2, ZmFDH, ZmID4, ZmRPI, ZmPRP). The expression levels of genes encoding NA synthase (ZmNAS1) and DMA synthase (ZmDMAS1) were also higher in the Fe-sufficient roots of ys1 and ys3 than in the WT (Figures 4, 5). In contrast, the expression level of ZmNAS3 decreased in the shoots of the ys1 mutant under both Fe-sufficient and Fe-deficient conditions (Figure 4), while it increased in ys3 Fe-sufficient shoots. The expression levels of the ZmIRO3 were also higher in the Fe-sufficient roots of ys1 and ys3 than in the WT (Figures 4, 5). As reported previously, the expression level of YS1 markedly decreased in ys1, although it was not different in ys3 compared to the WT (Figure 5). The expression levels of ZmIRO2 in Fe-sufficient and Fe-deficient roots were higher in ys1 and ys3 than in the WT (Figure 5). The expression levels of ZmI1 in Fe-sufficient and Fe-deficient roots were higher in ys1 and ys3 than in the WT (Figure 5). The expression level of ZmMATE2 (homolog of PEZ1, ZmPEZ1) was lower in ys1 compared to the WT and ys3.

As noted in the previous section, ys3 was reported to have a defect in DMA secretion. In rice roots, the efficiency of DMA secretion is lower than in maize roots. In contrast, the roots of maize plants used in the analysis.

**Figure 1. Maize plants used in the analysis.** Maize seeds were germinated for 4 days on filter paper and then grown hydroponically for 17 days. (A) Fe-sufficient conditions. Twelve days after germination, the plants were transferred to Fe-free culture medium for 5 days. (B) Fe-deficient conditions. doi:10.1371/journal.pone.0062567.g001
secretion was diminished by the repression of \textit{TOM1}. The \textit{ys3} mutants and \textit{TOM1} repression rice both showed similar DMA secretion profiles, and the level secretion was reduced relative to the WT. Therefore, we analyzed the expression of homologs of \textit{tom1} mutants and \textit{TOM1} expression level was lower in both \textit{ys1} and \textit{ys3} compared to the WT. In contrast, the expression level of \textit{ZmTOM2} was higher in Fe-deficient shoots of \textit{ys1}, \textit{ys3}, and the WT, and no significant differences were observed among them. According to the Maize Genetics and Genomics Database (MaizeGDB), the \textit{ys3} mutation is located within the interval of 85,750,522–114,783,939 on chromosome 3 (http://maizegdb.org/cgi-bin/locus_lookup_gensrev2.cgi?locus=ys3&lid=IBM2); \textit{ZmTOM1} (GRMZM2G063306) is also found within this interval (chromosome 3: 112,042,104–112,047,482). Therefore, we also confirmed the differences in size and sequence of the \textit{ZmTOM1} cDNA between the WT and \textit{ys3} by semiquantitative reverse transcription (RT)-PCR analysis (Figure 6). Consistent with quantitative real-time PCR analysis, the expression of \textit{ZmTOM1} was lower in \textit{ys3} than in the WT and \textit{ys1} in Fe-deficient roots (Figure 6A). In \textit{ys3}, three additional bands were detected in addition to the band that was of the same size as the \textit{ZmTOM1} cDNA. The cultivars used for the WT and the \textit{ys1} and \textit{ys3} mutants differed from each other in the above experiment, and the difference in genetic background between the WT and the \textit{ys1} and \textit{ys3} mutants may have affected \textit{ZmTOM1} expression. Therefore, we examined \textit{ZmTOM1} expression in \textit{YS3WT}, which is the same cultivar and has the same genetic background as the \textit{ys3} mutant. The expression of \textit{ZmTOM1} was also induced in \textit{YS3WT} under Fe-deficient conditions (Figure S3 in File S1). In Fe-deficient roots, the expression of \textit{ZmTOM1} was higher in \textit{YS3WT} than in \textit{ys3}. The expression levels of other genes, such as \textit{ZmTOM2} and \textit{ZmTOM3}, were not different in \textit{ys3} compared to \textit{YS3WT}. The larger bands observed in \textit{ys3} were not detected in \textit{YS3WT} (Figure 6A). In addition, we cultivated an additional three \textit{ys3} mutant lines (304A, 311F, and 311G) provided by the Maize Stock Center (http://www.maizegdb.org/cgi-bin/displayvarrecord.cgi?id=15373). As the seeds were all from test crosses, they should show 1:1 segregation. We extracted mRNA from the Fe-deficient roots of five plants of each line, and \textit{ZmTOM1} expression was analyzed by quantitative real-time PCR (Figure 6B). In all three lines, the progeny was segregated into plants in which the expression level of \textit{ZmTOM1} was comparable to the WT or significantly lower than the WT. Furthermore, we sequenced the PCR products of \textit{ZmTOM1} (GRMAM2G0633306_T02) in the WT and the \textit{ys1} and \textit{ys3} mutants. The larger bands observed only in \textit{ys3} contained unspliced introns of \textit{ZmTOM1}. Three patterns of intron insertions were noted (Figures S4, S5, S6 in File S1). These results suggest that \textit{ZmTOM1} is involved in the phenotype of \textit{ys3}.

**Discussion**

Homologs of Genes known to be Involved in Fe Homeostasis in Rice are also Induced by Fe Deficiency in Maize

Under Fe-deficient conditions, plants experience Fe-deficiency signals that trigger cellular activities to acquire and transport Fe into the plant body. In agreement with the observations in rice, the expression levels of genes homologous to those that participate in the methionine cycle (\textit{ZmMTN}, GRMZM2G171111; \textit{ZmAPT}, GRMZM2G093347; \textit{ZmMTK}, GRMZM2G464137; \textit{ZmIDF2}, GRMZM2G139533; \textit{ZmFDH}, GRMZM2G049811; \textit{ZmIDF4}, GRMZM2G067265; \textit{ZmRPI}, GRMZM2G035599), Ma biosyn-
Figure 3. Expression change of maize homologs of genes involved in Fe homeostasis in rice. Quantitative real-time PCR of homologs of genes involved in the methionine cycle (ZmMTN, GRMZM2G171111; ZmAPT, GRMZM2G093347; ZmMTK, GRMZM2G464137; ZmID12, GRMZM2G139533; ZmFDH, GRMZM2G049811; ZmDI4, GRMZM2G067265; ZmRPI, GRMZM2G035599; ZmPRPPs, GRMZM2G065030), transcription (ZmIRO2, GRMZM2G057413; ZmIRO3, GRMZM2G350312), MAs biosynthesis (ZmNAS1, GRMZM2G060952), and transport (ZmYS1, GRMZM2G156599; ZmTOM1, GRMZM2G063306; ZmTOM2, GRMZM2G065878; ZmTOM3, GRMZM2G141081; ZmIRT1, GRMZM2G118821; ZmNRAMP1, GRMZM2G170128; ZmMATE2/ZmPEZ1, GRMZM2G170128) was performed with appropriate primers (Table S1 in File S1). These data are shown as ratios relative to the expression in Fe-sufficient shoots. The ubiquitin gene (UBQ) was used to normalize data. S.D. was calculated from experimental replicates (n = 3). Column bars followed by different letters are significantly different from each other according to the Tukey-Kramer HSD test (n = 3, P < 0.05). Biological replicates were confirmed by repeating the experiment (Figure S1 in File S1). +Fe, Fe-sufficient conditions; –Fe, Fe-deficient conditions.

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thesis (ZmNAS1, GRMZM2G034956; ZmDMAS1, GRMZM2G060952), transcription (ZmIRO2, GRMZM2G057413; ZmIRO3, GRMZM2G350312), and Fe transport (YS1, GRMZM2G156599; ZmTOM1, GRMAM2G063306; ZmTOM2, GRMZM5G877788; ZmTOM3, GRMZM2G141081; ZmIRT1, GRMZM2G118821; ZmNRAMP1, GRMZM2G178190; ZmMATE2/ZmPEZ1, GRMZM2G170128) was performed with appropriate primers (Table S1 in File S1). The data are shown as ratios relative to the expression in Fe-sufficient WT shoots. The ubiquitin gene (UBQ) was used to normalize data. S.D. was calculated from experimental replicates (n = 3). Biological replicates were confirmed by repeating the experiment (Figure S3 in File S1).

Figure 5. Differences in expression levels of Fe deficiency-inducible genes among WT, ys1, and ys3. The differences in gene expression between the nonmutant [wild-type (WT) cv. Alice] and ys1 or ys3 mutants were analyzed by quantitative real-time PCR. Quantitative real-time PCR of the genes involved in MAs biosynthesis (ZmDMAS1, GRMZM2G060952) and transport (YS1, GRMZM2G156599; ZmTOM1, GRMAM2G063306; ZmTOM2, GRMZM5G877788; ZmTOM3, GRMZM2G141081; ZmIRT1, GRMZM2G118821; ZmNRAMP1, GRMZM2G178190; ZmMATE2/ZmPEZ1, GRMZM2G170128) was performed with appropriate primers (Table S1 in File S1). The data are shown as ratios relative to the expression in Fe-sufficient WT shoots. The ubiquitin gene (UBQ) was used to normalize data. S.D. was calculated from experimental replicates (n = 3). Biological replicates were confirmed by repeating the experiment (Figure S3 in File S1).

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transcription (RT)-PCR analysis of expression profiles of expression of the (Figure 3). These results support the suggestion that Fe deficiency in maize were increased by Fe deficiency. 

Figure 6. TOM1 family in maize. (A) Semiquantitative reverse transcription (RT)-PCR analysis of ZmTOM1 (GRMZM2G063306_T02) in Fe-deficient roots of ys1, ys3, the WT, and YS3WT. Arrowheads represent the three bands only detected in ys3. (B) Quantitative real-time PCR of ZmTOM1 in Fe-deficient roots of three ys mutant lines (304A, 311F, and 311G). The ubiquitin gene (UBQ) was used to normalize data. These data are shown as ratios relative to the expression in 304A line #1. Five plants in each line were analyzed. The seeds were all from test crosses and should show 1:1 segregation. (C) Phylogenetic tree of the TOM family. doi:10.1371/journal.pone.0062567.g006

GRMZM2G170128) in maize were increased by Fe deficiency (Figure 3). These results support the suggestion that Fe deficiency triggers similar responses in rice and maize. Moreover, the expression of the OsIRT1 homolog was not strongly induced in maize by Fe deficiency. In rice, ferrous Fe was reported to be acquired by the OsIRT1 transporter [50,51], and the expression of OsIRT1 is strongly induced in Fe-deficient roots. Maize is an upland plant, while rice grows under submerged conditions, where ferrous Fe is abundant. This difference suggests that maize acquires Fe mainly by chelation, while rice absorbs ferrous Fe via OsIRT1 in addition to chelation under Fe-deficient conditions.

Expression Profiles of ys1 and ys3

The expression levels of genes involved in the methionine cycle [ZmMTN, GRMZM2G171111; ZmAPT, GRMZM2G099347; ZmMTK, GRMZM2G064137; ZmID1, GRMZM2G139533; ZmFDH, GRMZM2G049811; ZmIDH, GRMZM2G067265; ZmRPI, GRMZM2G053599; ZmPRPP, GRMZM2G065090] and MAbs biosynthesis [ZmNAS1, GRMZM2G034956; ZmDMAS1, GRMZM2G060952] were higher in the Fe-sufficient roots of ys1 and ys3 plants than in those of the WT (Figures 4, 5). These results suggest that ys1 and ys3 are unable to acquire sufficient Fe in the roots, which triggers the Fe deficiency response, even under Fe-sufficient conditions. The expression levels of ZmMTN, ZmAPT, ZmMTK, ZmID1, ZmFDH, ZmIDH, ZmRPI, ZmPRPP, ZmNAS1, ZmDMAS1, ZmRPI3, and TOM1 were higher in ys1 than in ys3 and the WT, suggesting that ys1 senses Fe deficiency more strongly than ys3. This difference in gene expression may be linked to the observation that the shoots of ys1 were much smaller than those of the WT or ys3 under Fe-sufficient conditions (Figure 1). Fe concentration and shoot biomass would suggest that ys1 mutants are far less capable of Fe uptake than either ys3 mutants or WT plants. The ys1 mutant has been shown to have a mutation in YS1 [9]. As described previously, the expression level of YS1 had decreased in ys1. These observations confirm that YS1 is defective in the ys1 mutant and that this defect is responsible for the ys1 phenotype. The YSL family has been suggested to be important not only for the acquisition of Fe(III)-DMA from the soil but also for the translocation of Fe from the root to shoot and seeds in rice [24,33,34,36–38]. In addition, YS family genes were reported to be involved in the translocation of other metals, including Zn, Cu, and Mn [34,35,38,41,58]. In this study, ys1 was smaller than the WT and ys3 under both Fe-sufficient and Fe-deficient conditions (Figure 1). Moreover, in addition to Fe, the concentrations of other metals are altered in ys1 mutants. These results suggest that ys1 mutants suffer from Fe deficiency even under Fe-sufficient conditions and induce the expression of Fe deficiency-responsive genes.

In contrast to ys1, ys3 showed no Fe-related visible phenotype under Fe-sufficient conditions. Instead, ys3 showed slightly better growth than the WT. The Fe deficiency-inducible genes were slightly induced in ys3 compared to the WT, but less than in ys1 (Figures 4, 5). These results suggest that ys3 suffers from Fe deficiency under Fe-sufficient conditions but not as strongly as ys1. In this experiment, ys3 plants were grown beside the WT and ys1 plants. ys3 plants may have absorbed DMA secreted from the roots of WT and ys1 plants, and grew better than ys1. In rice, OsNRAMP1 and OsNRAMP5 have been reported to play important roles in the absorption and translocation of ferrous Fe in rice [52–54]. In the present study, the maize plants were grown hydroponically where ferrous Fe was comparatively abundant. The expression level of the OsNRAMP2 homolog (OsNRAMP1; GRMZM2G178190) was upregulated in ys3 as compared to the WT. The ys3 mutant has been speculated to experience Fe deficiency in the roots as it cannot secrete DMA [44]. However, ys3 could acquire ferrous Fe through NRAMP family transporters. As the level of DMA in the roots of ys3 may have been high, Fe may have been efficiently transported from the roots to the shoots via YSL family members, perhaps because ys3 has larger shoots than the WT.

The Expression Level of DMA Efflux Transporter Decreased in ys3

The ys3 mutant was reported to be defective in the secretion of DMA, although the causative gene has not been identified [44]. Recently, TOM1 was identified as a DMA efflux transporter in rice that mainly secretes DMA from the roots into the soil [26]. TOM1 and ENA1 belong to the major facilitator superfamily, and maize has many homologous genes (Figure S7 in File S1). In the present study, the expression level of the TOM1 homolog (ZmTOM1; GRMZM2G063306_T02) decreased in ys3 but not in ys1 as compared to the WT and YS3WT. Similar to TOM1, ZmTOM1 was mainly expressed in the roots and strongly induced by Fe deficiency. ZmTOM1 is localized on chromosome 3; 112,042,104–112,047,482. In MaizeGDB, the ys3 QTL is located
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on chromosome 3; 83,750,522–114,783,939 (http://maizegdb.org/cgi-bin/locus_lookup_refGenev2.cgi?locus = ys3&kid = IBM2). Semiquantitative RT-PCR showed larger bands of this gene in ys3, but not in ysl or the WT (Figure 6). These larger bands were not observed in YS3WT, suggesting that the unspliced cDNA in ys3 was not due to its genetic background. These observations suggest that ZmTOM1 is responsible for the ys3 phenotype. The larger bands corresponded to the unspliced mRNA of TOM1 (Figures S4, S5, S6 in File S1). These results suggested that some mutation or insertion affect the splicing of ZmTOM1 in the ys3 mutant. The GRAMENE database shows that the genome has not been completely sequenced and part of the ZmTOM1 genome information is missing (Figure S5 in File S1; http://www.gramene.org/Zeama_zs/Genes/Sequence?g = GRMZM2G063306;r = 3:112042104-112047482). Further analysis is needed to determine why several patterns exist for the splicing of ZmTOM1.

In conclusion, using transcriptomic analyses, we identified the maize genes involved in the response to Fe deficiency. Furthermore, transcriptomic analyses revealed candidate genes for the ys3 mutant. Further analysis may provide additional data to conclude that a defect in ZmTOM1 is involved in the phenotype of the ys3 mutant.

Materials and Methods

Plant Materials

The ysl and ys3 mutant plants were grown from homozygous seeds. A WT cultivar (Alice) was used as a control, as even though it has a different genetic background from ysl and ys3, this line was previously used in a study of the ysl mutant [30]. To confirm the expression of ZmTOM1, another WT line (YS3WT) with the same genetic background as ys3 was used [44]. Three additional lines of ys3 mutants (304A, 311F, and 311G) were also analyzed.

Hydroponic Culture

Seeds were germinated for 4 days in the dark at 25°C on filter paper soaked with distilled water. Seedlings were then grown hydroponically in a nutrient solution containing 0.7 mM K$_2$SO$_4$, 0.1 mM KCl, 0.1 mM KH$_2$PO$_4$, 2.0 mM Ca(NO$_3$)$_2$, 0.5 mM MgSO$_4$, 10 μM H$_2$BO$_3$, 0.5 μM MnSO$_4$, 0.2 μM CuSO$_4$, 0.5 μM ZnSO$_4$, 0.05 μM Na$_2$MoO$_4$, and 0.1 mM Fe(III)–EDTA. The pH of the nutrient solution was adjusted daily to 5.5 with 1 M HCl. Plants were grown in a 5-L plastic container for 4 days and then transferred to a 20-L plastic container with air-bubbling. Fe deficiency was initiated 8 days after germination by transfer of the plants to Fe(III)–EDTA-free culture medium. Maize plants grown hydroponically under Fe-sufficient or Fe-deficient conditions for 5 plants to Fe(III)–EDTA-free culture medium. Maize plants grown hydroponically under Fe-sufficient or Fe-deficient conditions for 5 days were harvested at the same time.

Metal Determination

The roots or shoots of dried maize plants grown hydroponically were ground, and samples of 50 μg were used for metal determination. The samples were digested in 2 ml of 13 M HNO$_3$ (Wako Pure Chemical, Osaka, Japan) at 210°C for 20 min with MARS Xpress (CEM Corp., Matthews, NC). After digestion, the samples were diluted to a volume of 5 ml and analyzed by inductively coupled plasma atomic emission spectrometry (SPS1200VR; Seiko, Tokyo, Japan). All experiments were performed in triplicate.

RNA Extraction

The maize plants grown hydroponically were immediately frozen in liquid nitrogen. Total RNA was extracted from the shoots and roots of three plants per treatment using an RNase-free Plant Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. The yield and purity of the RNA were determined spectrophotometrically. To confirm the biological replicates, RNA was separately extracted from the shoots and roots of three to five plants per treatment.

Quantitative Real-time PCR and Semiquantitative RT-PCR

Total RNA (3 μg) was treated with RNase-free DNase I (Takara, Kyoto, Japan) to remove contaminating genomic DNA. First-strand cDNA was synthesized using ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan) by priming with oligo-d(T)$_{30}$. For quantitative RT-PCR, a fragment was amplified by PCR in a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA) with SYBR Green I and ExTag™ Real-Time PCR Version (Takara) according to the manufacturer’s instructions. The template concentration was adjusted to 30 ng per reaction. The primers used for real-time PCR are described in Table S1 in File S1. The primers used as the internal control (ZmUbiquitin, GRMZM2G118637) in RT-PCR were as follows: ZmUbiquitin forward, 5′-GGTGAAGCTGCTGCTG-3′ and ZmUbiquitin reverse, 5′-GGCGGTGCGACGATAGTTTG-3′. Normalization of quantitative real-time PCR was performed by the comparative Ct method calculation according to the manufacturer’s instructions (Applied Biosystems StepOnePlus™ Real-Time PCR system). The data show the relative increase or decrease of the gene expression level in each sample compared to the gene expression levels in Fe-sufficient shoots of the non-transformant (NT) in three experimental replicates and three to five biological replicates. The standard deviation of the nonmutant segregant plants (YS3WT) was also calculated from three biological replicates. The sizes and sequences of the amplified fragments were confirmed by agarose gel electrophoresis and with an automated sequencer (3130 Genetic Analyzer; Applied Biosystems), respectively. Analysis of variance with the Tukey-Kramer HSD test was used to compare data. The statistical software package JMP9 (SAS Institute, Cary, NC) was used in all analyses. All methods and data were confirmed to follow the MIQE guidelines [59]. For semiquantitative RT-PCR, 30 ng of cDNA was used for each reaction. PCR was performed using the GeneAmp PCR system 9700 (Applied Biosystems).

Supporting Information

The raw data for quantitative real-time PCR in this study have been deposited in GEO (Accession No. GSE44557; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE44557). The nucleotide sequence data reported in this paper have been deposited in the GRAMENE database under the accession numbers ZmTOM1, GRAMZM2G063306; ZmTOM2, GRAMZM2G877788; ZmTOM3, GRAMZM2G141081; ZmMTX, GRAMZM2G171111; ZmAPT, GRAMZM2G093347; ZmMTK, GRAMZM2G464137; ZmMDF2, GRAMZM2G393533; ZmFDH, GRAMZM2G049811; ZmMD4, GRAMZM2G067265; ZmRPI, GRAMZM2G035599; ZmMPRPP, GRAMZM2G063030; ZmIRO2, GRAMZM2G057413; ZmRO3, GRAMZM2G530312; ZmXAS1, GRAMZM2G034956; ZmXAS3, GRAMZM2G478568; ZmDMAS1, GRAMZM2G660952; ZmS1, GRAMZM2G135699; ZmRHT1, GRAMZM2G118821; ZmNRAMP1, GRAMZM2G178190; ZmMATE2/ZmPEZ1, GRAMZM2G170128.
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Supporting Information

File S1 Supporting Information. Figure S1. Biological replicates for quantitative real-time PCR for the expression changes in maize of genes homologous to those involved in Fe homeostasis in rice. The expression changes in maize [wild type (YS3WT), which was the same cultivar and had the same genetic background as the ys3 mutant], of genes homologous to those involved in Fe homeostasis in rice. Quantitative real-time PCR of the genes homologous to those involved in the methionine cycle (ZmNAS1, GRMZM2G171111; ZmAPT, GRMZM2G093347; ZmMTK, GRMZM2G64137; ZmID12, GRMZM2G139533; ZmFDH, GRMZM2G049811; ZmID14, GRMZM2G067265; ZmRPI, GRMZM2G035599; ZmPRPPs, GRMZM2G065030), transcription (ZmIR2O2, GRMZM2G057413; ZmIR3, GRMZM2G350312), MAs biosynthesis (ZmNAS3, GRMZM2G034956; ZmNAS3, GRMZM2G174858; ZmDMS1, GRMZM2G060959), and transport (ZmYS1, GRMZM2G156599; ZmTOM1, GRMAM2G663096; ZmTOM2, GRMZM5G877788; ZmTOM5, GRMZM2G141081; ZmRT1, GRMZM2G118821; ZmNRAMP1, GRMZM2G178190; ZmMATE2/ZmPEZ1, GRMZM2G170128) was performed with appropriate primers (Table S1 in File S1). The data are shown as ratios relative to the expression in Fe-sufficient shoots. The ubiquitin gene (UBQ) was used to normalize data. S.D. was calculated from the biological replicates (n = 3–6). Column bars followed by different letters are significantly different from each other according to the Tukey-Kramer HSD test (n = 3–6, P < 0.05).

+Fe, Fe-sufficient conditions; –Fe, Fe-deficient conditions. Figure S4. Unspliced introns of ZmTOM1 in ys3. GRMZM2G663306_T02orf, ZmTOM1 cDNA sequence predicted by GRAMENE; ZmTOM1pcr, partial sequence of ZmTOM1 used for quantitative real-time PCR; ZmTOM1orf, sequenced sequence of ZmTOM1 from the WT; Pattern_1, intron insertion version of ZmTOM1 cDNA in ys3; Pattern_2, intron insertion version of ZmTOM1 cDNA in y3; Pattern_3, intron insertion version of ZmTOM1 cDNA in y3. Figure S5. Genomic sequence of GRMZM2G0663306. Colored and bold font indicates the assembled plant exon in this region. Pink font shows the region where sequencing was not perfect. Figure S6. Full-length cDNA of ZmTOM1. GRMZM2G663306_T02orf, ZmTOM1 cDNA sequence predicted by GRAMENE; ZmTOM1pcr, partial sequence of ZmTOM1 used for quantitative real-time PCR; ZmTOM1orf, sequenced sequence of ZmTOM1 from the WT. Figure S7. Phylogenetic tree of TOM1 and ENAI homologs in maize. GRAMENE was searched for homologous genes of ENAI and TOM1. Scale bar, 0.3 substitutions/site. Table S1. (PDF)

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Author Contributions

Conceived and designed the experiments: TN HN NKN. Performed the experiments: TN. Analyzed the data: TN. Wrote the paper: TN.

References


