A receptor-like protein RMC is involved in regulation of iron acquisition in rice

An Yang¹, Yansu Li², Yunyun Xu³ and Wen-Hao Zhang¹,4,*

¹ State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, PR China
² The Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, PR China
³ Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, PR China
⁴ Research Network of Global Change Biology, Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing, PR China

* To whom correspondence should be addressed. E-mail: whzhang@ibcas.ac.cn

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Abstract

Iron (Fe) is one of the essential mineral elements for plant growth and development. Acquisition of Fe by plants is mediated by a complex network involving Fe mobilization, uptake by root cells, and transport within plants. Here, we evaluated the role of a previously clarified gene encoding a receptor-like protein from rice, OsRMC, in the regulation of Fe acquisition by comparing Fe concentration, biomass, and expression patterns of genes associated with Fe mobilization and transport in wild-type (WT) rice with those in OsRMC overexpression and RNA interference (RNAi) knockdown transgenic rice plants. Expression of OsRMC was upregulated in both shoots and roots upon exposure of WT to Fe-deficient medium. Expression levels of OsRMC were positively correlated with Fe concentration in rice plants under both Fe-sufficient and Fe-deficient conditions such that overexpression and RNAi lines had higher and lower Fe concentration in both roots and shoots than WT plants, respectively. Moreover, overexpression of OsRMC conferred greater accumulation of Fe in mature seeds under Fe-sufficient conditions. OsRMC may also play a role in regulation of Fe deficiency-induced changes in root growth, as evidenced by greater and smaller root systems of OsRMC overexpression lines and RNAi lines than WT under Fe-deficient conditions, respectively. Several Fe deficiency-responsive genes including OsDMAS1, OsNAS1, OsNAS2, OsNAAT1, OsIRT1, OsYSL15, and OsIRO2 were up- and downregulated in OsRMC-overexpressing and RNAi plants compared with WT rice plants. These novel findings highlight an important role of OsRMC played in mediation of Fe acquisition and root growth in rice, particularly under Fe-deficient conditions.

Key words: Fe concentration, iron deficiency, OsRMC, rice (Oryza sativa), root system, seed Fe concentration.

Introduction

Mineral deficiency is the most widespread dietary problem, affecting more than half the world’s population, particularly in developing countries (Lee et al., 2009). Rice is a major crop and primary food source in many countries, and thus increasing its mineral content is an effective way to improve the nutritional quality of plants (Lee and An, 2009). Among the mineral elements, iron (Fe) is one of the essential nutrients for plant growth and human health (Curie and Briat, 2003; Lee et al., 2009). Fe deficiency results in chlorosis and reduced crop yield and quality. Deficiency in bioavailable Fe in food affects a large proportion of the world population. For instance, anaemia due to deficiency in Fe nutrition is one of the most serious problems in humans (Hell and Stephan, 2003; Lee et al., 2009).

Abbreviations: CHL, chlorophyll; JA, jasmonic acid; MA, mugineic acid; RNAi, RNA interference; WT, wild type.

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Although Fe is the second most abundant metal element in the earth’s crust, its bioavailability to plants is often limited, especially in neutral-to-alkaline soils where Fe occurs largely in the form of insoluble hydroxides and oxides (Guerinot and Yi, 1994; Romera and Alcántara, 2004). To cope with Fe deficiency in these soils, plants have evolved two distinct strategies to mobilize and acquire Fe, classified as strategy I in non-graminaceous monocots and dicots, and strategy II in graminaceous monocots (Marschner et al., 1986; Mori, 1999). In strategy I plants, Fe$^{3+}$ is reduced to Fe$^{2+}$ and then transported into root cells by an Fe$^{2+}$ transporter, IRON-REGULATED TRANSPORTER 1 (IRT1) (Eide et al., 1996; Robinson et al., 1999; Vert et al., 2002). In contrast, in strategy II graminaceous plants, acquisition of Fe is involved in exudation of phytosiderophores, Fe$^{3+}$ chelators of the mungoenic acid (MA) family (Takagi et al., 1984; Inoue et al., 2009; Lee et al., 2012). In addition to uptake of Fe$^{3+}$, rice plants can also uptake Fe$^{2+}$ via IRT transporters in the waterlogged paddy field conditions where Fe$^{2+}$ is abundant because of the low redox potential (Ishimaru et al., 2006).

Exudation of MAs is an important step for acquisition of Fe by strategy II plants. MAs are synthesized from S-adenosyl-1-methionine catalysed by three sequential enzymes: nicotianamine (NA) synthase (NAS), nicotianamine aminotransferase (NAAT), and deoxymugineic acid synthase (DMAS) (Inoue et al., 2003; Bashir et al., 2006; Lee et al., 2012). In rice, genes encoding these enzymes have been isolated (Inoue et al., 2003, 2008; Bashir et al., 2006). Among them, expression of OsNAS1, OsNAS2, and OsNAS3 that encode rice NAS is differentially regulated by Fe deficiency (Inoue et al., 2003). Bashir et al. (2006) reported that expression of OsDMAS1 is upregulated under Fe-deficient conditions in shoots and roots. OsYSL15 is responsible for the acquisition of Fe$^{3+}$-MA from the rhizosphere and strongly upregulated by Fe deficiency (Inoue et al., 2009; Lee et al., 2009). OsIR1, an Fe$^{2+}$ transporter, is expressed mainly in roots and induced under Fe-limited conditions (Ishimaru et al., 2006). In addition, several transcription factors have been identified to play a role in regulation of Fe homeostasis. These include OsIDEF1 (Kobayashi et al., 2007, 2009, 2012), OsIDEF2 (Ogo et al., 2008), OsIRO2 (Ogo et al., 2007), OsIRO3 (Zheng et al., 2010), and OsbHLH133 (Wang et al., 2013). However, the overall signalling networks associated with Fe-deficiency responses in plants in general and in rice in particular remain largely to be dissected.

The receptor-like kinases are characterized by an N-terminal hydrophobic signal peptide, and an internal hydrophobic sequence followed by a basic ‘stop-transfer’ sequence (Hardie, 1999). These receptors, which are localized at the cell surface, are often involved in sensing signals associated with developmental, environmental, and hormonal cues, and with transducing the signals to trigger adaptive responses (Torii, 2000; Morris and Walker, 2003; Afzal et al., 2008). OsRMC is a member of the domain unknown function 26 (DUF26) (cysteine-rich repeat) subfamily (Jiang et al., 2007). Knockdown of the OsRMC gene by RNA interference (RNAi) led to altered root development by targeting jasmonic acid (JA) signalling (Jiang et al., 2007). Zhang et al. (2009) reported that RNAi knockdown of OsRMC in transgenic rice makes the transgenic rice plants more tolerant to salt stress, implying that OsRMC plays a role in regulation of the plant response to salt stress. Moreover, a recent study reported that the expression of a putative OsRMC is downregulated by phosphorus deficiency using a comparative proteome approach (Torabi et al., 2009). However, there have been no detailed physiological studies to evaluate the role of OsRMC in regulation of mineral nutrients in plants so far. In the present study, we demonstrated that OsRMC positively regulated Fe acquisition in rice plants, such that overexpression and knockdown of OsRMC led to an enhanced and suppressedFe accumulation in roots, shoots, and seeds, respectively. We further explored the physiological mechanisms by which the transgenic rice plants with altered expression levels of OsRMC differed from wild-type (WT) plants in terms of their responses to Fe deficiency.

Materials and methods

Plant materials and growth conditions

Rice plants (Oryza sativa L. ssp. japonica, cv. Zhonghua 10) were used as WT controls relative to the transgenic plants (overexpression and knockdown lines of OsRMC) by RNAi, OER3, R11, and R41, respectively) in physiological experiments. The transgenic seeds used in the present study were kindly provided by Professor Kang Chong (Jiang et al., 2007). Seeds of WT and transgenic lines were germinated in dark at 28 °C for 3 d. Thereafter, the rice seedlings were pre-cultured hydroponically in one-half strength Kimura B solution containing 0.37 mM (NH$_4$)$_2$SO$_4$, 0.18 mM KH$_2$PO$_4$, 0.18 mM KNO$_3$, 0.55 mM MgSO$_4$, 0.09 mM K$_2$SO$_4$, 0.37 mM Ca(NO$_3$)$_2$, 46.2 × 10$^{-3}$ mM HBO$_3$, 3.2 × 10$^{-4}$ mM CuSO$_4$, 7.7 × 10$^{-3}$ mM ZnSO$_4$, 9.1 × 10$^{-3}$ mM MnCl$_2$, 4H$_2$O, 3.6 × 10$^{-3}$ mM H$_2$MoO$_4$, 0.70 mM Na$_2$SiO$_3$·9H$_2$O, and 10.0 × 10$^{-3}$ mM EDTA-Fe in a growth room with a 16 h light (30 °C)/8 h dark (22 °C) photoperiod, and the relative humidity was controlled at ~70%. The solution was refreshed every 3 d.

For analysis of OsRMC expression in transgenic plants, 2-week-old rice seedlings grown hydroponically in normal conditions were harvested. To determine the time course of Fe deficiency-induced changes in gene expression, 2-week-old rice seedlings grown in the control solution with a sufficient supply of Fe (100 μM EDTA-Fe) were transferred to Fe-deficient solution by omitting EDTA-Fe. To minimize the supply of Fe from seed endosperm, the seed endosperm was removed prior to transferring of rice seedlings to Fe-deficient medium. Shoots and roots were harvested for RNA extraction after exposure of rice seedlings to Fe-deficient medium for varying periods (0, 6, 12, 24, 72, and 168 h). To determine the effect of deprivation of other mineral nutrients, including nitrogen (N), phosphate (P), potassium (K), and sulfur (S) on OsRMC expression, 2-week-old rice seedlings grown in the control solution with a sufficient supply of nutrients were transferred to solutions containing no nitrogen (−N), phosphate (−P), potassium (−K), and sulfur (−S), respectively. Plants were harvested for RNA extraction after the treatments for 5 d. To investigate the phenotypes of the transgenic rice plants with overexpression and knockdown of OsRMC and WT rice plants, 1-week-old seedlings were germinated and grown in both Fe-sufficient (100 μM EDTA-Fe) and Fe-deficient (0 μM EDTA-Fe) medium. After 15 d of treatment, height, shoot biomass, and root biomass were measured. Shoots and roots were dried for 3 d at 80 °C before being weighed.

Measurement of metal content

To determine Fe content in the transgenic and WT rice plants, elemental analysis was conducted on seedlings grown under both
Fe-sufficient and Fe-deficient conditions. Shoot and root samples were harvested, ground to a fine powder, and digested in 6 ml of concentrated nitric acid and 2 ml of hydrogen peroxide with a CEM MARS system. Total metal contents were determined by inductively coupled plasma mass spectrometry. To determine the concentrations of metals in seeds of WT and transgenic plants, seeds that were harvested over the previous 3 years were used to measure the content of Fe, Zn, Mn, and Cu. WT and transgenic plants were grown in a paddy field located at the Institute of Botany, Chinese Academy of Sciences.

**Measurement of chlorophyll (CHL) content**

To determine CHL content in both WT and transgenic rice plants, leaves were harvested, weighed, and extracted with aqueous ethanol (95% v/v) overnight. Newly formed leaves were weighed and then ground with aqueous acetone (80% v/v) and centrifuged at 10,000g for 5 min. Absorbance (A) readings of the supernatant were recorded at wavelengths of 646 and 665 nm. Total CHL content was calculated as 7.18 A_{665} + 17.32 A_{646}, and was expressed as μg CHL mg⁻¹ of fresh weight.

**RNA isolation and real-time RT-PCR**

Total RNA was isolated from leaves and roots using RNAsiso reagent (Takara) and was reverse-transcribed into first-strand cDNA with a PrimeScript® RT Reagent kit (Takara) and was reverse-transcribed into first-strand cDNA (Takara) and was reverse-transcribed into first-strand cDNA with a PrimeScript® RT Reagent kit (Takara). Real-time PCR was performed in an optical 96-well plate with an Applied Biosystems StepOne™ Real-Time PCR system. Each reaction contained 7.5 μl of 2× SYBR Green Master mix, 0.5 μl of cDNA samples, and 0.6 μl of 10 μM gene-specific primers in a final volume of 15 μl. The thermal cycle used was as follows: 95 °C for 10 min, and 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The following primers were used: OsRMC, 5′-TCGGAGGTGTACCCGTTCTACA-3′ and 5′-ACTCTTTAATT TGTGCCATTTTTAATCGCT-3′ (Zhang et al., 2009); OsNAS1, 5′-GTCTAACAGCAGCGGACAGCAGAAAG-3′ and 5′-TTCCTC ACTGCTCATAACAGAGATGCC-3′; OsNAS2, 5′-TGAGTGCGTG CAGATGATCGGC-3′; 5′-CAGACGTTGACCAAACACCT TCTTCG-3′ (Inoue et al., 2003); OsIRT1, 5′-CGGTCTTGTCTTC TCCACACGAC-3′ and 5′-GAGCTGTAGCTGAGTCTGAGT C-3′; OsDM81, 5′-TCTCTTCGACACCACTCCTCC-3′ and 5′-CC TTGCCTCAGACCCACCTCCT-3′; OsNAA1, 5′-AGACCAGG CTACCCAAACTTG-3′ and 5′-CAGCTTGGATAGCCGTC ATCC-3′; OsYSL1, 5′-ACTCCTTCTCTGACTCAGAACAAT-3′ and 5′-GC AATGTGCTAAGCAAGAAG-3′; OsIBR02, 5′-CTTCCATCGT TCGGCTACCT-3′ and 5′-GCTGGGCACCTCCGTTGTGAC-3′; and actin, 5′-ACCACTATGTTATCTGTTGACTC-3′ and 5′-AG ACATATCCTTCTATAGATGGG-3′. Relative quantification (ΔΔCt method) was used to evaluate quantitative variation between the replicates. Amplification of actin (GenBank accession no. AB047313) was used as an internal control to normalize data. The expression level of genes in the WT under Fe-sufficient conditions was defined as 1.

**Results**

**Expression profiles of OsRMC under conditions of different nutrient deficiencies**

A gene, OsRMC, which encodes a receptor-like protein, was isolated in rice by Jiang et al. (2007). OsRMC has been shown to be involved in the regulation of JA-dependent root development and tolerance of rice plants to salt stress (Jiang et al., 2007; Zhang et al., 2009). To evaluate the role of OsRMC in Fe acquisition in rice, the responsiveness of OsRMC to Fe deficiency was studied. Upregulation of OsRMC expression in both shoots and roots was observed upon exposure of WT rice seedlings to Fe-deficient medium (Fig. 1). For instance, an
increase in OsRMC transcripts in the shoot was detected after 6h of exposure to Fe-deficient medium, and the increase was more evident with prolonged exposure to Fe-deficient medium (Fig. 1A). Expression of OsRMC peaked by 24h of Fe-deficient treatment, and declined thereafter (Fig. 1A). A similar Fe deficiency-induced expression pattern of OsRMC in roots was observed (Fig. 1B). In addition, the expression of OsRMC was also upregulated by deprivation of P, K, and S, but not by N starvation (Supplementary Fig. S1 at JXB online).

Expression levels of OsRMC in transgenic lines

The expression levels of OsRMC-overexpressing and RNAi knockdown lines were examined by real-time RT-PCR. Expression of OsRMC was enhanced in the overexpression lines (OE3 and OE6) and was suppressed significantly in the knockdown lines (Ri1, Ri4, and Ri5) (Fig. 2). One overexpression line (OE3) and two knockdown lines (Ri1 and Ri4) were chosen based on their expression levels of OsRMC and their seed availability to study the physiological function of OsRMC using T5 generations of these transgenic plants as well as WT rice plants.

Transgenic rice plants exhibit a different response to Fe deficiency from WT plants

The observation that expression of OsRMC was induced by Fe deficiency prompted us to examine whether the transgenic rice plants with altered expression levels of OsRMC differed from their WT counterpart in response to Fe deficiency. As shown in Fig. 3A, no evident differences in phenotypes between WT and the OsRMC-overexpressing line OE3 were observed when grown in Fe-sufficient medium. However, the overexpressing line OE3 exhibited greater growth than WT plants when grown in Fe-deficient medium (Fig. 3B, C). Accordingly, the overexpressing line and WT plants had comparable shoot and root biomass when grown in Fe-sufficient medium (Table 1). By contrast, the shoot and root biomass of the two RNAi lines was significantly lower than that of WT plants under Fe-sufficient conditions (Table 1). In addition to biomass, overexpression of OsRMC led to a higher height of transgenic plants than WT plants under Fe-deficient conditions, while WT and OE3 plants did not differ in their height under Fe-sufficient conditions (Table 1). In contrast, the plant height of the two RNAi lines was significantly shorter than that of WT and the overexpression line OE3 under both Fe-sufficient and Fe-deficient conditions (Table 1).

No differences in CHL content between WT and the overexpressing line OE3 were observed under Fe-sufficient conditions, while the RNAi lines had lower CHL contents (Table 1). A marked reduction in CHL content in both WT and the transgenic rice plants was observed when grown in Fe-deficient medium (Table 1). However, the Fe deficiency-induced reduction in CHL content was less and greater in overexpressing and RNAi lines, respectively, than in WT plants, leading to higher and lower CHL content in the overexpression and RNAi lines, respectively, than in WT plants under Fe-deficient conditions.

Overexpression and suppression of OsRMC makes transgenic rice plants more and less efficient, respectively, at acquiring Fe than WT plants

The observations that transgenic rice plants overexpressing OsRMC exhibited greater biomass, a greater height, and less...
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reduced CHL contents than WT plants under Fe-deficient conditions suggested that the expression level of OsRMC might be positively correlated with tolerance of rice plants to Fe deficiency. To test this hypothesis, we measured Fe concentrations in both shoots and roots of rice plants at the seedling stage. Under Fe-sufficient conditions, Fe concentrations in the OsRMC-overexpressing plants were marginally, but statistically insignificantly, higher than those in WT. However, OsRMC-overexpressing plants accumulated a greater amount of Fe than WT plants under Fe-deficient conditions (Fig. 4). In contrast to the overexpression line, Fe concentrations in RNAi lines were much less than WT plants grown under the same conditions (Fig. 4). In addition to Fe, we also compared other divalent metal concentrations in the transgenic plants with those in WT plants under both Fe-sufficient and Fe-deficient conditions. Among the metals examined, Cu concentrations in shoots of the two RNAi lines were significantly lower than in WT plants under both Fe-sufficient and Fe-deficient conditions, while the OE3 line had a higher Cu concentration in shoots than in WT plants under Fe-deficient conditions (Fig. S2 at JXB online). In contrast, no significant differences in concentrations of Mn and Zn in both shoots and roots of the transgenic plants and WT under both Fe-sufficient and Fe-deficient conditions were observed (Fig. S2).

Transgenic rice plants with altered expression of OsRMC and WT plants exhibit different root phenotypes

Root phenotype is an important trait for plants to efficiently utilize Fe under Fe-deficient conditions. Under Fe-deficient conditions, the total root length and root surface area of the OsRMC-overexpressing rice seedlings were significantly higher than those of WT seedlings (Fig. 5). In contrast, knockdown of OsRMC by RNAi in the transgenic rice plants reduced root growth compared with WT plants grown in Fe-sufficient and Fe-deficient medium, as evidenced by the significantly reduced total length and surface areas of RNAi plants compared with WT plants (Fig. 5). In addition, the primary root length and total length of the three longest adventitious roots as well as number of adventitious roots in WT, OE, and RNAi plants were measured. Under Fe-sufficient conditions, no significant differences were observed in primary root length and total length of the three longest adventitious roots between WT and the OsRMC-overexpressing rice seedlings (Fig. 6). However, the number

Table 1. Plant height, dry shoot biomass, dry root biomass, and CHL content of WT and transgenic plants

One-week-old seedlings were grown hydroponically for 15 d in Fe-sufficient (100 μM EDTA-Fe) or Fe-deficient (0 μM EDTA-Fe) medium were sampled for measurements. Data are means ±SD of three independent experiments. Asterisks (*) indicate significant differences at P<0.05 with regard to WT by Student’s t-test. DW, dry weight; FW, fresh weight.

| Genotype | Shoot biomass (mg DW per plant) | Root biomass (mg DW per plant) | Plant height (cm) | CHL content (μg mg⁻¹ FW) |
|----------|----------------------------------|---------------------------------|-------------------|------------------------|
|          | Fe-sufficient medium              |                                 |                   |                        |
| WT       | 47.42 ± 3.45                     | 7.45 ± 1.23                     | 25.78 ± 2.23      | 3.34 ± 0.14            |
| OE3      | 45.58 ± 4.73                     | 7.68 ± 1.33                     | 24.56 ± 2.18      | 3.36 ± 0.22            |
| Ri1      | 30.79 ± 3.13*                    | 5.47 ± 1.17*                    | 20.24 ± 1.01*     | 2.79 ± 0.43*           |
| Ri4      | 29.57 ± 2.46*                    | 5.28 ± 1.37*                    | 20.04 ± 1.17*     | 2.98 ± 0.16*           |
|          | Fe-deficient medium               |                                 |                   |                        |
| WT       | 30.13 ± 1.41                     | 5.34 ± 1.23                     | 18.13 ± 0.89      | 0.80 ± 0.13            |
| OE3      | 34.61 ± 1.37*                    | 6.10 ± 0.98                     | 20.68 ± 0.32*     | 1.47 ± 0.21*           |
| Ri1      | 16.34 ± 1.78*                    | 3.98 ± 1.44*                    | 12.13 ± 1.65*     | 0.34 ± 0.22*           |
| Ri4      | 15.78 ± 1.59*                    | 3.76 ± 0.61*                    | 11.43 ± 0.78*     | 0.31 ± 0.12*           |

Fig. 4. Quantification of metal content in shoots, roots, and seeds of WT and transgenic plants. (A) Shoot tissue. (B) Root tissue. One-week-old seedlings were grown hydroponically for 15 d in Fe-sufficient or Fe-deficient medium and the plants were sampled for measurements. Significant differences from WT were determined by Student’s t-test (*P<0.05).
of adventitious roots in OE and RNAi plants was greater and less than in WT plants, respectively, under Fe-sufficient conditions (Fig. 6). Knockdown of OsRMC by RNAi led to the shorter primary root length, total root length of the three longest adventitious roots and less adventitious root number compared with WT plants under both Fe-sufficient and Fe-deficient conditions (Fig. 6). These results indicated that disruption of OsRMC expression may alter root development under both Fe-sufficient and Fe-deficient conditions. The large root systems can facilitate Fe acquisition by increasing root surface area for Fe uptake and exploring more soil space.

Overexpression of OsRMC results in greater accumulation of Fe in rice seeds

In addition to in shoots and roots, the content of Fe and other mineral nutrients in mature seeds of WT and overexpressing and RNAi lines was also determined. Seeds of the OE3 line contained a greater amount of Fe than those of WT seeds (Fig. 7A). In contrast, Fe contents in the RNAi seeds were significantly less than in WT seeds (Fig. 7A). For instance, the Fe content in seeds of the overexpressing line OE3 was 8.9% higher than that in WT seeds, while the Fe content in seeds of the RNAi line R1 was 17.1% lower than in WT seeds. In addition to Fe content, the Zn content in the OE3 seeds was also significantly higher than that in WT seeds (Fig. 7B). In contrast, the Zn content in seeds of the two RNAi lines was reduced compared with those in WT seeds (Fig. 7B). Compared with WT and RNAi grains, the contents of Mn and Cu were higher in overexpressing plants than in WT plants (Fig. 7C, D).

Expression of Fe deficiency-responsive genes is altered in transgenic rice plants

Given the important role of MA in Fe acquisition in strategy II rice plants (Takagi, 1976; Takagi et al., 1984), we monitored the changes in transcript levels for the genes involved in MA biosynthesis in WT and transgenic rice plants grown in Fe-sufficient and Fe-deficient medium. These include genes encoding nicotianamine synthase (OsNAS1, OsNAS2), nicotianamine aminotransferase (OsNAAT1) and deoxymugineic acid synthase (OsDMASI). In addition to biosynthesis of MAs, the effect of OsRMC on expression patterns of OsIRT1 and OsYSL15 encoding transporters of Fe\(^{2+}\) ions (Ishimaru et al., 2006) and Fe\(^{3+}\)-MA (Inoue et al., 2009) in rice, respectively, was evaluated. We also examined the role of OsIRO2, which is an Fe-responsive transcription factor involved in regulation of Fe homeostasis (Ogo et al., 2007), in OsRMC-dependent Fe acquisition by comparing OsIRO2 expression in WT and the transgenic rice plants under Fe-sufficient and Fe-deficient conditions. Overexpression of OsRMC and knockdown of OsRMC by RNAi led to enhanced and suppressed expression of OsNAS1 in rice plants grown in Fe-sufficient medium, respectively (Fig. 8A). Fe deficiency induced upregulation of OsNAS1 and OsNAS2 in WT plants, and the Fe deficiency-induced upregulation of OsNAS1 and OsNAS2 was markedly potentiated and inhibited in the overexpression and RNAi lines, respectively. The expression level of OsDMASI in the OE3 line was marginally higher than in WT plants under Fe-sufficient conditions, while OsDMASI transcripts in the two RNAi lines (Rii and Rii4) were substantially lower than in WT plants under Fe-sufficient conditions (Fig. 8C). Exposure of WT plants to Fe-deficient medium led to upregulated expression of OsDMASI, and the upregulation was enhanced and suppressed by overexpression and RNAi of OsRMC, respectively (Fig. 8C). As a consequence, transcript levels of OsDMASI in the overexpression and RNAi lines were higher and lower than in WT under Fe-deficient conditions. A similar expression pattern of OsNAAT1, OsIRT1, OsYSL15, and OsIRO2 was found in overexpression and RNAi lines such that overexpression and knockdown of OsRMC led to greater and less expression of OsIRT1, OsYSL15, and OsIRO2, respectively, and the effect was more pronounced under Fe-deficient conditions than under Fe-sufficient conditions (Fig. 8D–G).

Discussion

Fe deficiency is one of the limiting factors affecting production and nutritional quality of crops due to low solubility of Fe(III)-oxides that often occur in soils, especially in
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In addition to Fe deficiency in plants, more than 2 billion people have been reported to be at risk of Fe deficiency-induced anaemia worldwide (WHO, 2007). Given that plants are a primary source of Fe nutrition for humans, understanding the mechanisms by which plants efficiently acquire Fe from soil, particularly under Fe-deficient conditions, could help to alleviate Fe malnutrition of humans. Rice is a major crop and primary source of food in the majority of Asian regions (Khush, 2005). Therefore, identification of key regulators that contribute to greater accumulation of Fe in rice would be of significance for improvement of Fe nutrition in food by breeding rice cultivars with a high Fe nutritional quality.

There are reports demonstrating that Fe-fortified grains can be obtained by molecular manipulation of genes associated with Fe mobilization and transport in plants (Kobayashi and Nishizawa, 2012). For example, an elevated Fe level in rice has been achieved by increasing the amount of metal chelators (Takahashi et al., 2001; Lee et al., 2009). Overexpression of specific transporters involved in absorption and translocation of Fe has also been used to enhance Fe content in plants (Lee and An, 2009; Lee et al., 2009; Ishimaru et al., 2010). In addition, manipulation of central transcription factors involved in regulation of the Fe-deficiency response can also improve rice growth under Fe-deficient conditions (Kobayashi et al., 2007; Ogo et al., 2007; Ogo et al., 2008). However, the signalling networks responsible for Fe homeostasis remain largely to be dissected. In the present study, we demonstrated that a gene encoding a receptor-like protein, OsRMC, plays an important role in the regulation of Fe acquisition in rice. More specifically, we found that overexpression of OsRMC made rice seedlings more tolerant to Fe deficiency than WT rice plants, while RNAi knockdown of OsRMC rendered the seedling growth more sensitive to Fe deficiency. Most importantly, our results revealed that, in addition to improving the growth of overexpressing rice plants under Fe-deficient conditions, overexpression of OsRMC also led to enhanced accumulation of Fe and Zn in mature seeds under Fe-sufficient conditions. These findings highlight a novel function of OsRMC in the regulation of mineral nutrients in both plants and seeds. To the best of our knowledge, this is the first report demonstrating the involvement of receptor-like proteins in the control of mineral nutrient acquisition in higher plants.

Fig. 6. (A, B) Root phenotypes of 1-week-old WT and transgenic rice plants grown in Fe-sufficient (A) and Fe-deficient medium (B) for 8 d. (C–E) Primary root length (C), total length of three longest adventitious roots (D), and number of adventitious roots (E) for WT and transgenic rice plants grown in Fe-sufficient and Fe-deficient medium were measured after exposure to Fe-sufficient and Fe-deficient medium for 8 d. Significant differences from WT were determined by Student’s t-test (*P<0.05). Bars, 2 cm.
To elucidate the mechanism underlying the efficient acquisition of Fe in transgenic rice plants overexpressing OsRMC under Fe-deficient conditions, physiological processes associated with MA synthesis and IRT-mediated Fe\(^{2+}\) transport were studied at the transcriptional level. Our results revealed that expression levels of OsRMC were positively correlated with expression of genes (OsNAS1, OsNAS2, OsNAAT1, OsDMAS1, and OsYSL15) involved in MA synthesis and uptake of MA-Fe\(^{3+}\), particularly under Fe-deficient conditions (Fig. 8). The upregulation of these genes would allow the transgenic plants overexpressing OsRMC to have more effective exudation of MAs from rice roots, which in turn act as phytosiderophores to chelate Fe\(^{3+}\) in the rhizosphere, thus facilitating Fe mobilization. OsIRO2 is an Fe deficiency-inducible basic helix–loop–helix (bHLH) transcription factor and is responsible for regulating the genes involved in Fe homeostasis in rice, including OsNAS1, OsNAS2, OsNAAT1, OsDMAS1, and OsYSL15. Here, we showed that the transcriptional level of OsIRO2 was also upregulated by overexpression of OsRMC, indicating that OsRMC is a potential upstream target of OsIRO2. In addition to enhanced biosynthesis of MAs, our results also showed that overexpression of OsRMC upregulated expression of the Fe\(^{2+}\) transporter OsIRT1, particularly under Fe-deficient conditions. As OsIRT1-mediated Fe\(^{2+}\) transport is involved in Fe\(^{2+}\) uptake in rice (Ishimaru et al., 2006), enhanced expression of OsIRT1 under Fe-deficient conditions would make the rice plants more efficient at taking up Fe\(^{2+}\) in the Fe-deficient medium, thus contributing to the observed high Fe concentrations in both shoots and roots.

Previous studies have shown that RNAi knockdown of OsRMC alters root development (Jiang et al., 2007) and confers enhanced tolerance to salt stress (Zhang et al., 2009). The two RNAi lines (Ri1 and Ri4) used in the present study displayed shorter primary roots and fewer adventitious roots than WT plants (Jiang et al., 2007). Our results showing that total root length of the two RNAi lines was less than that of WT plants grown in Fe-sufficient conditions (Fig. 5A) are consistent with the results of Jiang et al. (2007). Torabi et al. (2009) showed that expression of OsRMC is suppressed in response to P deficiency. In contrast, we detected a higher expression level of OsRMC in response to P deficiency (Fig. S1). We do not have explanation for the difference between low P-induced expression of OsRMC in our studies and those reported by Torabi et al. (2009). We speculate that the differences may be due to the rice genotypes and the experimental protocols used in the two studies. In previous studies, OsRMC was found to be responsive to JA treatment, salt stress, and P\(_7\)-starvation (Jiang et al., 2007; Torabi et al., 2009; Zhang et al., 2009). In the present study, we demonstrated that deprivation of Fe, K, and S from the growth medium altered the expression patterns of OsRMC, suggesting that OsRMC has diverse roles in the control of rice growth and development, particularly by regulating physiological processes associated with nutrient acquisition. As a secreted protein, OsRMC can function as a ligand for receptor-like kinases (Chen 2001;
Fig. 8. Expression patterns of some Fe deficiency-responsive genes in the roots of transgenic and WT plants. The following genes were analysed: OsNAS1 (A), OsNAS2 (B), OsDMAS1 (C), OsNAAT1 (D), OsIRT1 (E), OsYSL1 (F), and (G) OsIRO2. Total RNA was extracted from 2-week-old rice seedlings grown under control and Fe-limited stress conditions for 24 h. Transcript levels were measured by real-time PCR. Actin was used as an internal control. Error bars are calculated based on three biological replicates.
Therefore, we speculate that OsRMC may regulate the Fe-deficiency response and root development by interacting with some receptor-like kinases.

Another important finding in the present study was that overexpression and suppression of OsRMC significantly enhanced and reduced Fe and Zn content, respectively, in mature seeds under normal growth conditions. It has been reported that overexpression of OsIRT1 in rice plants leads to elevated Fe and Zn level in the mature seeds (Lee and An, 2009). Constitutive overexpression of OsNAS genes also results in increases in the content of Fe and Zn in rice grains (Lee et al., 2009; Masuda et al., 2009; Johnson et al., 2011). In addition to increased Fe content in shoots, overexpression of OsIRO2 conferred greater accumulation of Fe in seeds than WT rice plants when grown on calcareous soil (Ogo et al., 2011). Therefore, it is conceivable that the increased concentrations of Fe and Zn in OsRMC-overexpressing grains of the OE3 line may result from upregulation of the OsIRT1, OsNAS1, OsNAS2, and OsIRO2 genes.

In addition to changes in physiological processes, plants can also modify their root morphological traits to maximize their Fe acquisition by increasing the root surface area (Guerinot and Yi, 1994; López-Bucio et al., 2003). Examples include increased formation and branching of root hairs, root-tip swelling, and enhanced lateral root formation (Schmidt, 2002; Müller and Schmidt, 2004). In the present study, we found that transgenic rice plants with overexpression of OsRMC exhibited greater root systems than WT rice plants under Fe-deficient conditions, such that the total root length and root surface area in the OE3 line were greater than those in WT rice plants (Figs 5 and 6). In contrast, the total root length and surface area in the transgenic rice plants with underexpression of OsRMC by RNAi were smaller than those in WT rice plants in Fe-deficient medium. The increased root systems in the OsRMC-overexpressing line would facilitate exudation of Fe phytosiderophores and uptake of Fe due to a greater root surface area, thus making the overexpressing line more efficient at mobilization and uptake of Fe by the roots in Fe-deficient medium.

JA is an oxylipin-based plant hormone originating from polyunsaturated fatty acids that acts in response to both developmental and environmental stimuli. A recent study reported that jasmonate may act as an inhibitor to fine-tune Fe-deficiency responses, but that it is not involved in the systemic downregulation of Fe-deficiency responses in the roots of Arabidopsis (Maurer et al., 2011). RNAi knockdown of OsRMC led to altered root development and coiling, which are mediated by JA signalling in rice (Jiang et al., 2007). In the present study, we found that OsRMC played a role in adaptation of rice plants to Fe deficiency. Therefore, future work to investigate the role of JA in OsRMC-mediated Fe-deficiency responses is warranted.

In summary, our results demonstrated that OsRMC is likely to play a significant role in the regulation of Fe acquisition by upregulating biosynthesis of MAs, Fe-MA transport, IRT1-dependent Fe transport, and root development in rice under Fe-deficient conditions. In addition, we found that overexpression of OsRMC in rice plants resulted in greater accumulation of Fe in mature rice seeds. This observation has important implications in molecular manipulation of Fe contents in seeds. The enhanced mobilization and transport of Fe and increased root surface area due to greater root growth and development in transgenic rice plants overexpressing OsRMC may underpin the observed greater Fe use efficiency under Fe-deficient conditions. These novel findings will be valuable for dissecting the signalling networks associated with the response of plants to Fe deficiency in general and rice plants in particular.

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