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**Title**

Overexpression of *OsIRO2* improves both iron uptake and translocation to seeds in rice

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**Authors**

Ogo, Yuko  
Itai, Reiko Nakanishi  
Kobayashi, Takanori  
et al.

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## INTRODUCTION

Plants often exhibit iron (Fe)-deficiency symptoms, as manifested by chlorosis (yellowing caused by chlorophyll deficiency) in calcareous soils, which constitute about 30% of the world's cultivated soil. For humans, Fe deficiency is also a serious nutritional problem. Fe-deficiency anemia afflicts an estimated 30% of the world's population, especially where vegetable-based diets predominate. To solve these problems, the mechanisms of Fe uptake and translocation in plants have been investigated. Graminaceous plants absorb Fe using natural Fe chelators, the mugineic acid family phytosiderophores (MAs). MAs and their precursor nicotianamine (NA) are also thought to be involved in Fe homeostasis in plants, e.g., in the long-distance transport of Fe. The genes that encode the key enzymes for MAs biosynthesis and transporter genes are coordinately upregulated in response to Fe deficiency. We previously reported that the Fe deficiency-inducible bHLH transcription factor, *OsIRO2*, is responsible for regulating key genes involved in MAs biosynthesis and Fe transport, e.g., *OsYSL15* (Ogo et al., 2007). Transgenic rice in which *OsIRO2* is repressed by RNA interference (RNAi) secretes less deoxymugineic acid (DMA) than non-transgenic (NT) rice and is hypersensitive to Fe deficiency. In contrast, *OsIRO2*-overexpressing (OX) rice induces the genes involved in Fe uptake more strongly and secretes more DMA than NT rice (Ogo et al., 2007). In the present report, we demonstrate that *OsIRO2* overexpression enhances Fe-deficiency tolerance of rice and improves translocation of Fe to seeds when grown on calcareous soil by activating the expression of several genes involved in Fe uptake.

## MATERIALS AND METHODS

Generation and line selection of the OX plants were described previously (Ogo et al., 2007). The T<sub>2</sub> seeds of OX and NT plants were grown in calcareous soil (pH 8.5) without waterlogging. The Fe concentrations in the shoot and seed were measured as previously described (Ishimaru et al., 2006). For histochemical analysis, 2.0 kb of the 5' upstream region from the transcriptional initiation site was used as the promoter sequence of *OsIRO2*. Rice (cultivar Tsukinohikari) was transformed using the *OsIRO2* promoter-*GUS* with the *Agrobacterium*-mediated method. T<sub>1</sub> seeds were germinated and cultured in Fe-sufficient soil with fertilizer, and developing seeds were progressively sampled for *GUS* expression. *GUS* staining was performed as previously described (Inoue et al., 2003).

## RESULTS AND DISCUSSION

*The OX plant is tolerant to Fe deficiency in calcareous soil*—The OX plants were generated utilizing the constitutive cauliflower mosaic virus 35S promoter from which two lines with strong and constitutive expression of *OsIRO2* were selected (OX1, OX2) (Ogo et al., 2007). The two OX

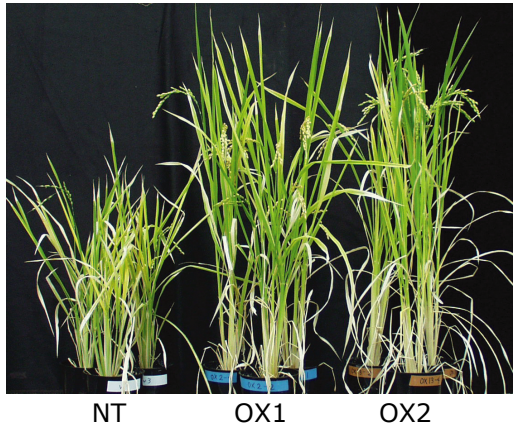


Figure 1. The OX and NT plants after 90 days of growth in calcareous soil.

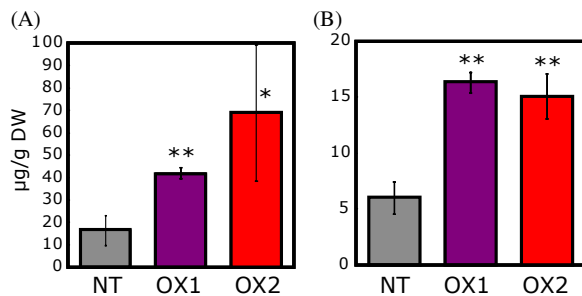


Figure 2. Fe concentration in the shoot (A) and seeds (B) of plants grown in calcareous soil. The values with asterisks indicate significant differences compared to NT plants according to t-test (\*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ;  $n = 3$ ). Error bars represent the SD.

considerable Fe-deficiency tolerance to rice. Furthermore, in *OsIRO2* OX plants, the higher expression of *OsYSL15* and many other genes regulated by *OsIRO2* may assist Fe uptake of plants, in addition to the increase in DMA secretion. The Fe concentrations in shoots of OX plants were two to four times higher than those of NT plants (Fig. 2A). Notably, the Fe concentrations in seeds (brown rice) of OX plants were more than twice those in NT plants (Fig. 2B).

*OsIRO2* is expressed during reproductive development in rice—We investigated the localization of expression of *OsIRO2* in flowers and developing seeds through histochemical localization in the *OsIRO2* promoter–*GUS* transformants. The transformants were cultured in Fe-sufficient soil and developing seeds were progressively sampled for *GUS* expression. The *OsIRO2* promoter was active in the vascular bundles of spikelets before anthesis (Fig. 3A), in anthers (Fig. 3A, J), and in pollen grains (Fig. 3K). As the ovary grew, the *GUS* staining intensified, especially in the outer layer of the endosperm, where storage proteins and minerals accumulate (Fig. 3B–G). After 30 days, the strong staining remained in dorsal vascular bundles (Fig. 3H) and in the radicle of the embryo (Fig. 3I). Fe is thought to be transported to seeds as Fe–DMA and

lines and NT plants were cultured in calcareous soil (pH 8.5) to examine tolerance to low Fe availability. The two OX lines showed remarkable tolerance to low Fe availability and grew better than NT plants (Fig. 1). In the OX plants, the genes involved in MAs biosynthesis, the Fe(III)–DMA transporter gene *OsYSL15* and many other genes whose function have not been established, were expressed more strongly than in the NT plants under Fe-deficient conditions (Ogo et al., 2007). The OX plants secreted 1.7 times more DMA than NT rice under Fe-deficient conditions (Ogo et al., 2007). In growth evaluation on calcareous soil, OX plants were extremely tolerant to Fe deficiency in spite of only a slight increase in DMA secretion. Takahashi et al. (2001) reported that rice with the introduced barley NA aminotransferase genes secreted 1.8 times more DMA than NT rice and showed extensive tolerance to Fe deficiency on calcareous soil. These results indicate that only a small increase in DMA secretion confers

Fe–NA complexes. Thus, enzymes involved in DMA synthesis, as well as transporters of Fe–DMA and Fe–NA complexes, are likely to play essential roles in the import of Fe to seeds during maturation in rice. In the present report, we demonstrated that *OsIRO2* is expressed in developing seeds (Fig. 3). In particular, *OsIRO2* is strongly expressed in the outer layer of the endosperm (Fig. 3E–G). This part probably includes the aleurone layer, which plays an important role in the import of storage products, including minerals, into seeds. *OsIRO2* is known to regulate the expression of genes involved in MAs biosynthesis and *OsYSL15* during the vegetative stage (Ogo et al., 2007), and is also considered to regulate these genes during seed development. The Fe concentrations in seeds of OX plants were significantly higher than those of NT rice (Fig. 2A). Increased expression of the enzymes involved in MAs biosynthesis and Fe transport resulting from overexpression of *OsIRO2*, especially in developing seeds, may improve Fe transport into seeds.

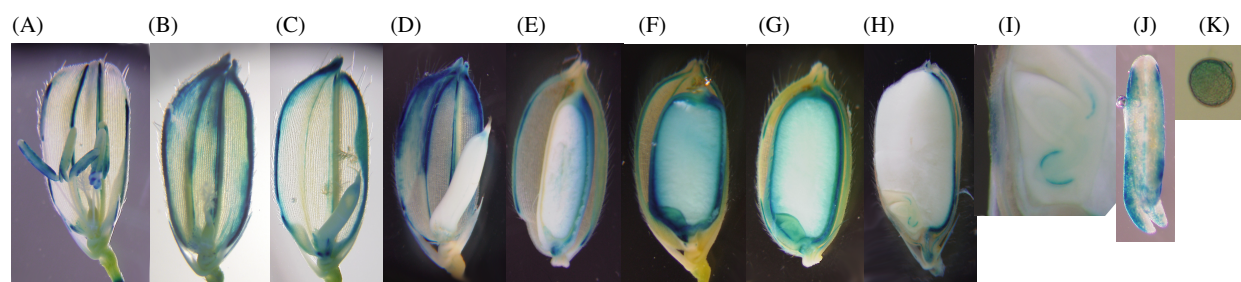


Figure 3. Histochemical localization of the *OsIRO2*-promoter GUS activity in developing seeds. Before anthesis (A, J) and after fertilization (B), 2–4 (C), 5–7 (D), 12–16 (E), 18–20 (F), 21–25 (G), and 30–35 (H, I) days after fertilization. Embryo (I), anther (J), pollen (K).

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