

Functional Analysis of Transgenic Rice (*Oryza sativa* L.) Transformed with an *Arabidopsis thaliana* Ferric Reductase (*AtFRO2*)

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Iron deficient soils limit crop production on 25–30% of the world's arable land. Both grasses (Strategy II) and dicotyledonous crops (Strategy I) are susceptible to iron deficiency, but each respond to iron stress by different mechanisms. In order to acquire iron from the soil, Strategy I plants utilize an iron reduction and Fe²⁺ transporter system at the root level, whereas Strategy II plants use a phytosiderophore-based system. Unfortunately, in some grasses such as rice, the production of phytosiderophores is low, and thus their ability to survive in iron-deficient conditions is limited. To determine whether a Strategy I root reductase can function in a Strategy II plant, and enhance its iron acquisition, we inserted the *FRO2* gene from *Arabidopsis thaliana* (*AtFRO2*) into rice (*Oryza sativa*). Root reductase activity was determined and was found to be low in both transgenic and control plants grown at different iron concentrations. The low activity levels were attributed to the release of soluble reductants in the assay and not to membrane-localized root reductase activity. RT-PCR analysis of rice roots and shoots of plants grown hydroponically at different iron concentrations revealed no expression of the transgene. In this paper, we discuss the lack of functionality of the *AtFRO2* gene in rice, and we perform a comparative study of the 0.6 kb promoter region by PlantCARE and PLACE analysis.

Key Words: *AtFRO2*, iron, promoter, reductase, rice.

Rice is one of the major food crops worldwide, and for many it is the main source of caloric intake. Rice is a very diverse species, and there is still no agreement if it was a dry-land crop that adapted to wet conditions or vice versa (Bandyopadhyay and Roy 1992). Because it is such a diverse crop, rice can be grown in many types of ecosystems such as irrigated, rain-fed lowland, upland, and flood-prone soils. Although upland rice constitutes a relatively small proportion of the total rice area worldwide (around 13%), it is the predominant method of rice culture in Latin America and West Africa. For this culture method, there are sometimes problems of nutrient deficiencies, in particular of iron deficiency. In Africa, Bangladesh and India, rice soils are often calcareous and have severe problems of iron deficiency (Vose 1982). Rice, amongst the grass species, is one of the crops most susceptible to iron deficiency, especially during the early stages of plant development, requiring considerable amounts of available iron during the nursery period, with a second peak during panicle emergence (Mori et al. 1991).

In order to overcome this deficiency problem, plants

have developed different response mechanisms to increase the solubility of iron and its uptake from the rhizosphere. Two different strategies evolved, that separated the graminaceous monocots from the dicots and non-graminaceous monocots. The first group of plants relies on the secretion of low molecular weight compounds called phytosiderophores, or mugineic acids (MAs), and there seems to be a strong positive correlation between the amount of MAs released and the tolerance of the plants to iron limiting conditions (Römheld and Marschner 1990). It is for this reason that rice, amongst the graminaceous species, seems to be particularly prone to iron deficiency symptoms. In rice, the release of MAs is not very robust, especially during early developmental stages (Mori et al. 1991; Hansen and Jolley 1995; Yin et al. 2000).

In the second group of plants, low iron availability induces an upregulation of iron reduction, ferrous iron transport, and ATPase-dependent release of protons. This combination of responses is referred to as Strategy I. Several genes related to iron metabolism have been isolated from plants of both Strategy I and II (Okumura

et al. 1991, 1994; Eide et al. 1996; Higuchi et al. 1999; Takahashi et al. 1999; Curie et al. 2001; Yun et al. 2001; Waters et al. 2002). In particular, one of the best-described genes is the *FRO2* gene from *Arabidopsis thaliana* (*AtFRO2*) (Robinson et al. 1999; Connolly et al. 2003). This gene expresses a root ferric chelate reductase that is induced in iron deficient conditions, and its regulation has been well characterized. It has been suggested that the reduction of ferric iron to ferrous iron is the rate-limiting step in iron acquisition, at least in Strategy I plants like *Pisum sativum* L. (Grusak et al. 1990) and *Arabidopsis thaliana* (Connolly et al. 2003).

In rice, a Strategy I root iron reductase is not believed to function in roots, although there is evidence for Fe^{2+} uptake (Bugchio et al. 2002). Additionally, recent genomic sequencing of rice suggests that iron reductase genes may be present in the genome (Gross et al. 2003), although no functional evidence for the proteins are yet available. Nonetheless, the presence of these features raises the possibility that rice could benefit from an increased activity of the ferric chelate reductase to generate more available iron when the plants are grown in upland conditions, where iron is often less available and insufficient to sustain proper development of the plant.

In an attempt to try to enhance the rice plant's ability to cope with iron deficient conditions, and to assess the function of a Strategy I gene in a Strategy II model plant, we transformed the rice variety IR68144 with the *AtFRO2* gene from *Arabidopsis thaliana*. We used a genomic-derived construct that included a 0.6 kb upstream region believed to correspond to the *Arabidopsis* promoter region that confers iron-dependent root expression. We grew plants in hydroponics at different iron concentrations and analyzed ferric chelate reductase activity and localization. Finally, we characterized the *Arabidopsis* 0.6 kb upstream region and its functionality in rice.

MATERIALS AND METHODS

Plant material and transformation. Homozygous seeds of the indica rice variety IR68144 and control seeds were used in the hydroponics study. The plasmid *pGL2* containing the *AtFRO2* genomic fragment with a 0.6 kb upstream promoter region and 2.4 kb downstream terminator region, and with *hpt* as selectable marker, was used for biolistic-mediated transformation of immature embryos (Fig. 1). Transformation was conducted as previously described (Vasconcelos et al. 2003).

Plant growth conditions. For the hydroponics study, seeds of control plants and three homozygous

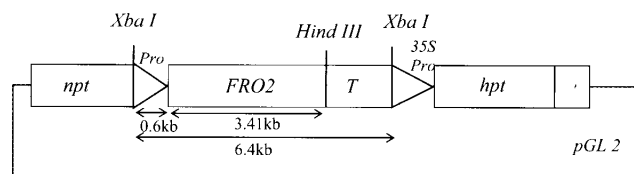


Fig. 1. Plasmid *pGL2* carrying the 3.41 kb fragment corresponding to the *AtFRO2* gene, with 0.6 kb upstream region corresponding to the *Arabidopsis* root-specific promoter (Robinson et al. 1999). *hpt*, hygromycin phosphotransferase; *npt*, neomycin phosphotransferase; *Pro*, promoter; *T*, terminator; *Xba I* and *Hind III*, restriction enzyme cleavage sites.

independent transgenic lines of variety IR68144 in T_3 generation (lines 18.7, 19.7, and 19.11) were germinated in Petri dishes with filter paper for 8 d before being transferred to hydroponic solution with different iron treatments. The filter paper was wet daily with de-mineralized water in order to avoid drying of the seeds. Plants were grown in a controlled environment chamber with 16-h, 20°C-day and 8-h, 15°C-night. Relative humidity was maintained at 50% and photon flux density during the day was $350 \mu\text{mol m}^{-2} \text{s}^{-1}$, supplied by a mixture of incandescent bulbs and fluorescent lamps. The standard solution for hydroponically grown plants contained: 1 mM $\text{Ca}(\text{NO}_3)_2$, 3 mM KNO_3 , 0.5 mM MgSO_4 , 0.75 mM K_2SO_4 , 0.5 mM KH_2PO_4 , 25 μM CaCl_2 , 25 μM MnSO_4 , 0.5 μM ZnSO_4 , 0.5 μM CuSO_4 , 0.5 μM H_2MoO_4 , 0.1 μM NiSO_4 , and 0.1 mM K_2SiO_3 . Plants were grown at 0, 4, 16, and 64 μM $\text{Fe}(\text{III})\text{-HEDTA}$ (*N*-hydroxyethylethylenediaminetriacetic acid). All nutrients were buffered with 2 mM MES (2,4-morpholino-ethane sulfonic acid), pH 5.5 and growth solutions were changed weekly. Three control plants and three plants of each transgenic line were grown for 14 d at each of the above-described concentrations, and the experiments were repeated twice. Plants were grown six per pot in 4.5 L of solution.

Molecular analysis for transgene detection.

PCR and Southern analysis were conducted to identify the presence of the transgene. Specific primers for *AtFRO2* were used (*AtFRO2F*: 5'-CAGTGTTGGGGATGCTCTGAA-3', *AtFRO2R*: 5'-GGTCATTGTTGCGATCTGCCA-3') in order to avoid amplifying the endogenous *FRO2* gene from rice. For Southern analysis, total genomic DNA was isolated by the method of Dellaporta et al. (1983), and *XbaI*-cleaved DNA of the transformed lines was hybridized with the *AtFRO2* probe, giving a 6.4 kb band corresponding to the 3.41 kb genomic fragment plus the 0.6 kb upstream promoter region and 2.3 kb downstream terminator region.

RNA isolation and cDNA synthesis. Total RNA was isolated from individual shoots and roots using the RNA-easy kit from Qiagen (Qiagen Inc., Valencia, California, USA) according to the manufactur-

er's instructions. Two μg total RNA were subjected to reverse transcription (RT) with an anchored oligo (dT) primer and 200 units Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) in a volume of 20 μL according to manufacturer's instructions. The experiments and the PCRs were repeated twice. PCR reactions were carried out with 1.5 μL of the RT reaction solutions using *AtFRO2* specific primers (RTatFRO2F: 5'-GCCACATCTGCGTATCAAGTT-3', RTatFRO2R: 5'-TCCCAAACAAGCTACGACCA-3'). Primers were designed to amplify a specific 100 bp fragment that spanned an intron region of genomic *AtFRO2*, and this way avoiding false positives potentially due to genomic contamination.

Root iron reductase assays. Iron reduction was measured in intact roots via the spectrophotometric measurement of Fe(II)-chelated to BPDS. The assay solution contained: 1.5 mM KNO_3 , 1 mM $\text{Ca}(\text{NO}_3)_2$, 3.75 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 0.25 mM MgSO_4 , 25 μM CaCl_2 , 25 μM H_3BO_3 , 2 μM MnSO_4 , 2 μM ZnSO_4 , 0.5 μM CuSO_4 , 0.5 μM H_2MoO_4 , 0.1 μM NiSO_4 , 100 μM Fe(III)-EDTA (ethylenediaminetetraacetic acid), and 100 μM BPDS (bathophenanthrolinedisulfonic acid). All nutrients were buffered with 1 mM MES, pH 5.5. The assay was conducted under low light conditions at 20 to 22°C and was terminated after 50 min by removal of the roots. Root fresh weight was measured before the material was frozen in liquid nitrogen for subsequent RNA extraction. Absorbance values for aliquots of the assay were obtained spectrophotometrically at 535 nm and an aliquot of the solution that had no roots during the assay was used as blank. Rates of iron reduction were determined using a molar extinction coefficient of 22.14 $\text{mM}^{-1} \text{cm}^{-1}$.

To assess the possible contribution of soluble reductants released from roots to overall root iron reduction, additional assays were conducted with plants grown at 64 μM Fe(III)-HEDTA. Roots were placed for 30 min in buffered nutrient solution with no iron source or BPDS, and aliquots of the solutions were collected prior to transferring the roots to regular assay solution for 30 min supplemented with 100 μM Fe(III)-EDTA and 100 μM BPDS. An aliquot of the solution from each root system collected prior to iron addition was added to a solution containing 100 μM Fe(III)-EDTA and 100 μM BPDS and left for 30 min; absorbance was then read at 535 nm as described above.

Reductase activity localization. For spatial visualization of iron reduction, intact roots of plants grown at 0, 4, 16, and 64 μM Fe(III)-HEDTA were placed in a gel composed of: 1.5 mM KNO_3 , 1 mM $\text{Ca}(\text{NO}_3)_2$, 3.75 mM $\text{NH}_4\text{H}_2\text{PO}_4$, 0.25 mM MgSO_4 , 100 μM Fe(III)-EDTA, 100 μM BPDS, and 0.75% (w/v) agarose (Sea-Plaque, FMC Corporation, Rockland, ME, USA). All nutrients were buffered with 5 mM MES, pH 5.5. Roots

were left in the dark for 45 min before visualization of Fe(II)-BPDS₃ formation (red coloration) along the roots.

Promoter sequence analysis. In order to study the nucleotide sequence motifs found in the *cis*-acting regulatory DNA elements in the 0.6 kb upstream genomic region of *AtFRO2*, PlantCARE (Rombauts et al. 1999) and PLACE databases (Higo et al. 1998) were used. Both databases provide indirect estimation of the mode of the regulation for the target gene, the regions involved in such regulation, and the plant species in which these elements have been found.

RESULTS

Transgene expression analysis

Southern blot analysis of putative transgenic plants was conducted and three independent lines carrying the *AtFRO2* gene were identified (Fig. 2). For RT-PCR analysis the roots and shoots of transgenic and control plants that were grown at different iron concentrations for 14 d were collected immediately after the reductase assay, and mRNA was extracted from these tissues. The roots and shoots of 2 month-old plants that were hydroponically grown in iron sufficient conditions and iron starved

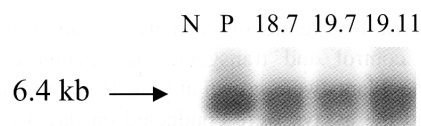


Fig. 2. Southern blot analysis of 6.4 kb fragment corresponding to the *AtFRO2* genomic sequence with the 0.6 kb promoter region and 2.4 kb terminator downstream region in transgenic T₃ plants 18.7, 19.7, and 19.11. N, negative control; P, positive control.

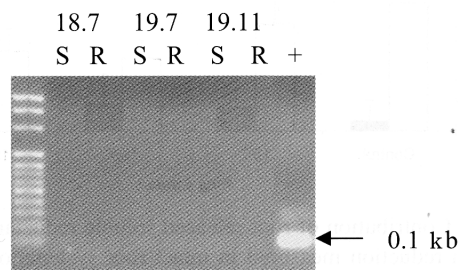


Fig. 3. RT-PCR analysis of *AtFRO2* in transgenic rice shoots (S) and roots (R) of plants grown for 14 d at 64 μM Fe(III)-HEDTA. The same pattern was observed in plants grown at 0, 4.0, 16.0 μM Fe(III)-HEDTA and also in plants grown hydroponically in greenhouse conditions for 1 month in iron starvation (data not shown). +, positive control.

for 1 month were also harvested to check for transgene expression. The *AtFRO2* mRNA expression pattern was investigated and no expression of the transgene was found (Fig. 3).

Root reductase activity and localization in transgenic and control plants

Iron reductase activity measurements in intact root systems of transgenic and control plants grown for 2 weeks at different iron concentrations showed a consistent low reduction capacity in both control and transgenic plants, regardless of iron concentration (Fig. 4). Spatial localization of iron reduction was determined by

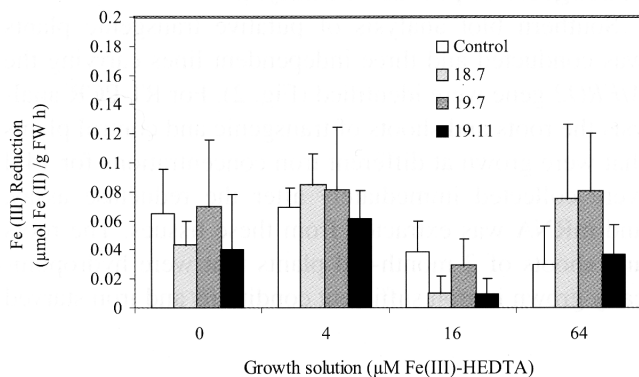


Fig. 4. Ferric iron reduction capacity measured in intact root systems of control and transgenic rice plants carrying the *AtFRO2* gene. Plants were grown at 0, 4, 16, and 64 μM Fe(III)-HEDTA, and the assays were conducted on day 14. Error bars represent standard error of the mean for three replicates.

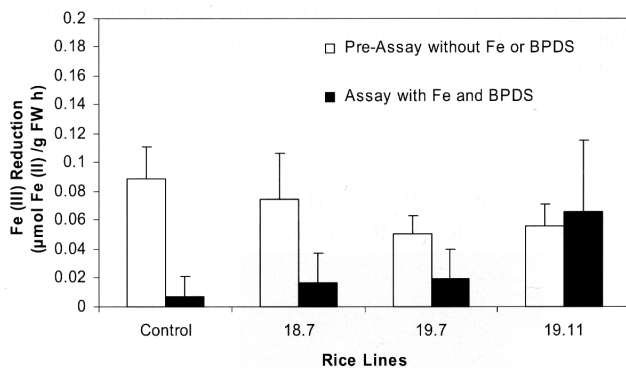


Fig. 5. Contribution of root-released soluble reductants to the ferric iron reduction measured in intact root systems of control and transgenic plants (lines 18.7, 19.7, and 19.11). Plants were grown at 64 μM Fe(III)-HEDTA and assays were conducted on day 14. Roots were first placed in a pre-assay solution for 30 min without Fe or BPDS; roots were removed and solution was combined with an Fe plus BPDS solution. Then, roots were assayed with Fe and BPDS. Error bars represent standard error of the mean for three replicates.

placing intact roots in soft agarose containing Fe(III)-EDTA plus Na₂-BPDS. The intensity and the extent of coloration, which is indicative of iron reduction, was found to be very low for all transgenic lines and control plants, regardless of the iron concentration at which the plants were grown (data not shown). Furthermore, the minimal staining that did occur was seen throughout most of the root system in all cases. Images are not presented due to the very weak staining, and the poor ability to show this either in a color or black and white image.

Iron reduction due to soluble reductants

The contribution of soluble reductants to the overall assay reduction values was tested in plants grown for 2 weeks at 64 μM Fe(III)-HEDTA (Fig. 5). Soluble reductant activity was similar to or higher than the membrane-associated root reductase activity, measured sequentially in the same root systems.

AtFRO2 promoter region analysis

PlantCARE analysis of the 0.6 kb upstream region of the *AtFRO2* gene revealed 40 types of *cis*-acting regulatory elements. Of these, some were related to light response (16), hormone response (2), heat stress response (1), methyl jasmonate response (2), and wound response (1).

Additionally, three other types of regulatory elements were found: the CAAT-box, TATA-box, and the *cis*-acting element *as-1*. Some elements with no attributed function were also identified (11). PLACE database search revealed 46 *cis*-acting regulatory DNA elements, of which five also belonged to CAAT-box-type elements, and three to TATA-box domains.

DISCUSSION

Rice, amongst the monocot species, has a low ability to secrete phytosiderophores, and for this reason, it suffers from severe problems of iron deficiency when it is grown in upland, alkaline soil conditions. This is especially true during the first weeks of the rice plant's developmental stages (Mori et al. 1991). The recent advances in molecular biology have allowed us to devise potentially useful biotechnological strategies in order to improve iron nutrition in plants. One strategy is to introduce foreign genes responsible for the iron reduction, absorption, uptake, transport, or accumulation of iron. Another strategy is to over-express endogenous genes and hope that by enhancing one of the steps in iron absorption, the others will follow. It has been described before that the rate-limiting physiological step in iron acquisition for dicots is the reduction of iron (Grusak et

al. 1990). Therefore, if this step can be enhanced, the likelihood is high that the plant will absorb more iron. We hypothesized that by introducing the *AtFRO2* gene into rice, which is responsible for the iron reduction step in *Arabidopsis thaliana*, we could produce rice plants that when challenged with iron deficient conditions would produce more available iron for absorption.

Ferric chelate reductase measurements in intact roots of *AtFRO2* rice grown hydroponically in iron limiting conditions revealed low rates of reductase activity, and these rates were similar to non-transformed plants (Fig. 4). Furthermore, the low values obtained in all plants were due to the release of soluble reductants in the assay solution and not to membrane-localized root-reductase activity (Fig. 5). Reductase localization along the roots by the BPDS-agarose system revealed very faint coloring, indicating low reduction activity in all plants, that was apparently also due to the excretion of soluble reductants.

Moreover, RT-PCR in the roots of transgenic rice showed no expression of the *AtFRO2* transgene (Fig. 3), even though the plants were successfully transformed (Fig. 2). This can be explained either by the fact that the genomic construct containing *AtFRO2* does not include a complete promoter sequence, or, if the promoter is present, it may not be functional in rice. Alternatively, the mRNA may have been rapidly degraded.

If the *Arabidopsis* promoter is not functional in rice, then perhaps there are differences between *Arabidopsis* and rice in the endogenous *cis*-acting elements that confer root specific expression of iron-related genes induced by iron deficiency. However, *cis*-acting elements of other iron-related genes have been shown to express transgenes in different plant species. Transgenic rice showed expression of several genes when transformed with barley genomic clones for *IDS3*, *HvNAS1*, *HvNAAT-A*, and *HvNAAT-B* with no additional promoters (Higuchi et al. 2001a; Kobayashi et al. 2001; Takahashi et al. 2001). This showed that rice can be transformed with genomic clones isolated from other monocot species that also utilize the Strategy II type mechanism for iron absorption. Moreover, tobacco transformed with *HvNAS1*, or *GUS* driven by the *IDS2*, *IDE1*, and *IDE2* barley promoters showed expression of the transgenes (Higuchi et al. 2001b; Kobayashi et al. 2003; Yoshihara et al. 2003), suggesting that it is possible also to transform a Strategy II plant with genomic clones and *cis*-acting elements of Strategy I plants and have expression of the transgene without using any additional promoters. However, we did not get expression of the *Arabidopsis thaliana FRO2* gene in rice when using the endogenous 0.6 kb promoter region. It could be argued that this region is not long enough to induce expression of the transgene. However, in other

root-specific genes, such as the *TobRB7* gene from tobacco, the 0.6 kb upstream region was enough to induce its expression (Yamamoto et al. 1991). In fact, a longer sequence inhibited transgene expression, due to the presence of a negative regulatory element between 813 bp and the 636 bp 5' of the transcription start site. Moreover, it has been shown before that by complementing the *frd1-1 Arabidopsis* mutant with the *AtFRO2* genomic sequence and the 0.6 kb sequence in the 5' upstream region of the *AtFRO2* ATG start codon, this was enough to functionally complement the plant mutation (Robinson et al. 1999). These are indications that the promoter region is complete and enough for expression of the transgene and restoration of ferric reductase activity in *Arabidopsis*.

PlantCARE and PLACE analysis of the promoter region showed that there were over 40 types of *cis*-acting elements in the region, and that at least one, the *cis*-acting element *as-1*, has been shown to target genes that are preferentially expressed in root tip meristems of tobacco (Klinedinst et al. 2000). The *as-1*-type *cis* elements were first identified in the 35S promoter of the cauliflower mosaic virus (Lam et al. 1989; Benfey and Chua 1990). Abiotic and biotic stress differentially stimulates *as-1* element activity in *Arabidopsis* (Redman et al. 2002). These elements may be involved in light-dependent regulation (Terzaghi and Cashmore 1995). However, there is no reference yet of *as-1* having a role in rice. Two other important elements strongly associated with promoter and enhancer regions were also identified: the CAAT-box element, found in different plant species such as *Arabidopsis thaliana*, *Nicotiana tabacum*, *Brassica rapa*, *Zea mays*, *Pisum sativum*, and *Glycine max*, and the TATA-box element, a core promoter element around -30 bp of transcription start, also described in different plant species, including rice (Rombauts et al. 1999). Most of the remaining types of *cis*-acting elements were related to light regulation and hormone responsive regulation.

Despite the fact that this region contains several *cis*-acting elements to confer promoter activity, we found no expression of the transgene in the *AtFRO2* rice. Perhaps the transcription factors that regulate the promoter region and the *cis*-acting elements of this gene are not present in rice, in this way preventing the initiation of transcription by the RNA polymerase. On the other hand, if rice has such transcription factors, perhaps they are only available under specific conditions. It is also possible that during transgene integration, the promoter region might have been integrated far from additional distal or proximal enhancer regions that are needed to stimulate transgene transcription.

A future approach to confirm if this promoter is in fact not functional in rice could be to transform rice

with a GUS fusion construct carrying the 0.6 kb promoter region, and to check for transient gene expression. Additionally, further efforts involving a 35S-promoter strategy could be taken to assess the viability of using a *FRO2* gene in rice, and to determine if its protein product is functional in a Strategy II system.

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