Cloning an iron-regulated metal transporter from rice

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Abstract

Rice cDNA and genomic libraries were screened in order to clone an Fe(II) transporter gene. A cDNA clone highly homologous to the Arabidopsis Fe(II) transporter gene IRT1 was isolated from Fe-deficient rice roots. The cDNA clone was named OsIRT1. A genomic clone corresponding to the cDNA was also obtained, sequenced and analysed. When expressed in yeast cells, OsIRT1 cDNA reversed the growth defects of the yeast iron-uptake mutant. Northern blot analysis revealed that OsIRT1 mRNA was predominantly expressed in roots and was induced by Fe- and Cu-deficiency. This suggests that OsIRT1 is a functional metal transporter for iron, and is actively engaged in Fe uptake from soils, especially under limiting conditions.

Key words: Copper, Fe(II) transporter, iron, IRT1, rice.

Introduction

Decades of research on plants have established that there are two distinct iron-uptake systems based on the response of plants to Fe-deficiency. These are known as Strategy I and Strategy II (Roèmheld, 1987; Roèmheld and Marschner, 1986). Strategy I plants include all dicots and non-graminaceous monocots. These plants respond to Fe-deficiency by decreasing rhizosphere pH and reducing sparingly soluble ferric iron. These mechanisms employ releasing protons and reductants, and morphological changes in the roots. The most important step of the mechanism is the reduction of ferric iron at the root surface by membrane-resident ferric chelate reductase (Chaney et al., 1972). In Arabidopsis, this reductase is encoded by FRO2 (Robinson et al., 1999). Reduced ferrous iron is absorbed into root cells by the Fe(II) transporter. IRT1 in Arabidopsis, a member of the ZIP metal transporter family, was the first metal transporter involved in Fe(II) transport to be identified in higher plants (Eide et al., 1996). IRT1 was also the first member of the ZRT, IRT-like protein (ZIP) family to be described (Guerinot, 2000). To date, IRT1-like Fe(II) transporters have been isolated from several dicotyledonous species (Eckhardt et al., 2001; Vert et al., 2001).

Strategy II iron-uptake systems are limited to graminaceous monocots. These plants release mugineic acid-family phytosiderophores to the rhizosphere, where they solubilize sparingly soluble iron by chelation. The chelated complex is then absorbed into the roots. Genes for the synthesis of phytosiderophores have been isolated from barley (Higuchi et al., 1999; Kobayashi et al., 2001; Okumura et al., 1994; Takahashi et al., 1999). The first step of phytosiderophore synthesis is also crucial in Strategy I plants (Ling et al., 1999), which is conversion of three molecules of S-adenosyl methionine to nicotianamine. Recently, a strong candidate for the transporter of the iron–phytosiderophore complex was cloned from maize (Curie et al., 2001).

It is generally believed that plants depend on a single system (either Strategy I or II) for iron acquisition. In graminaceous plants, such as barley and maize, the inducible Fe-deficiency Fe(II) transport system is either absent or is expressed only at very low levels (Zaharieva...
and Römheld, 2001). It is possible that some plants have both uptake systems. Rice is a strategy II plant and releases phytosiderophores under Fe-deficiency. In fact, rice is the first plant in which the root-secreted iron-solubilizing substance, the phytosiderophore, was observed (Takagi, 1976). Under submerged conditions, such as in a paddy field where rice is normally grown, however, ferrous iron is more abundant than ferric iron. Up until now, quite limited information has been available as to the existence of an Fe(II) transport system for iron uptake in rice (Eide et al., 1996).

This paper reports the isolation of cDNA and a genomic clone for a functional Fe(II) transporter from rice. This is the first report of the isolation of a functional Fe(II) transporter from a Strategy II plant. It is proposed that the Fe(II) transport system participates in iron uptake in rice in the reductive environment in which rice is normally grown.

Materials and methods

Plant material

Two varieties of rice plants, Oryza sativa cv. Notohikari or Nihonbare, were used for cDNA library construction or Northern analysis, respectively. Seeds were germinated and seedlings were transferred to nutrient solution without Cu, Fe, Mn, or Zn, and grown for three more weeks.

cDNA cloning

The OsIRT1 cDNA clone, identical to a rice expression sequence tag (EST) (accession no. D49213) homologous to IRT1, was isolated as follows. RNA was extracted from the roots of rice plants grown under Fe-deficient conditions for 2 weeks. A cDNA library was constructed according to the SUPER SCRIPT Plasmid System for Plasmid purification, subcloning, electrophoresis, blotting, and screening allowed an EST clone for a functional Fe(II) transporter from rice. This is the first report of the isolation of a functional Fe(II) transporter from a Strategy II plant. It is proposed that the Fe(II) transport system participates in iron uptake in rice in the reductive environment in which rice is normally grown.

Materials and methods

Plant material

Two varieties of rice plants, Oryza sativa cv. Notohikari or Nihonbare, were used for cDNA library construction or Northern analysis, respectively. Seeds were germinated and seedlings were hydroponically grown as previously described (Mori and Nishizawa, 1987). For micronutrient deficiency treatment, 3-week-old plants were transferred to nutrient solution without Cu, Fe, Mn, or Zn, and grown for three more weeks.

cDNA cloning

The OsIRT1 cDNA clone, identical to a rice expression sequence tag (EST) (accession no. D49213) homologous to IRT1, was isolated as follows. RNA was extracted from the roots of rice plants grown under Fe-deficient conditions for 2 weeks. A cDNA library was constructed according to the SUPER SCRIPT Plasmid System for cDNA Synthesis and Plasmid Cloning Instruction manual (Gibco-BRL, USA). The OsIRT1 cDNA clone was isolated using a PCR-based strategy. Approximately 10\(^5\) independent colonies of Escherichia coli from the library were divided into 96 sublibraries. Ninety-six pools of plasmid were prepared, and each sublibrary was screened for the EST clone by PCR using specific primers (5’-CGAAGCTGAGGATCCGAGGCTCGAGATCG and 5’-GGGGACGCCCGTCTGAAC). Any sub-library in which a fragment was amplified was further analysed. Repeated subdivision and screening allowed an OsIRT1 cDNA clone to be isolated.

Cloning a genomic sequence

A commercial genomic library of rice Oryza sativa L. cv. IR36 was used for screening (CLONTECH Inc., USA) by plaque hybridization. A DNA fragment used as a probe was amplified using the above primer set from a cDNA pool prepared from RNA isolated from 3-month-old rice leaves.

DNA and RNA procedures

Plasmid purification, subcloning, electrophoresis, blotting, and hybridization were carried out according to standard protocols (Sambrook et al., 1989). For Northern analysis, total RNA was extracted from plant materials by the SDS-phenol method (Naito et al., 1988). Total RNA (20 µg) was electrophoresed and blotted onto nylon membranes. The membranes were hybridized with 32P-labelled probes specific for the cDNA fragments for OsIRT1.

Yeast strains and growth media

The following strains of the yeast, Saccharomyces cerevisiae, were used in this study: CM3260 (parent strain) MAT&alpha trp1-63 leu2-3,112 cnr1-411 his3-165 ura3-52, YH001 MAT&alpha trp1-63 leu2-3,112 cnr1-411 his3-609 ura3-52 Δ&alpha1::URA3, YH003 MAT&alpha trp1-63 leu2-3,112 cnr1-411 his3-609 ura3-52 Δ&alpha1::URA3 Δ&alpha1::HIS3 Δ&alpha4::TRP1, M3 MAT&alpha trp1-63 leu2-3,112 cnr1-411 his3-609 ura3-52 FRE1::HIS3::URA3 ctn1-3, FTPRUNB1 MAT&alpha trp1-63 leu2-3,112 cnr1-411 his3-609 ura3-52 Δ&alpha1::URA3. Yeast disruption mutant strains, YH001 and YH003, were made by homologous recombination.

Yeast cells were grown in 1% yeast extract, 2% peptone, and 2% glucose (YPD), 1% yeast extract, 2% peptone, and 2% glycerol (YPG), and synthetic defined medium (SD) supplied with appropriate amino acids. Two per cent agar was added for solid plate media (Sherman, 1991). For iron- or copper-depleted media, bathophenanthroline disulphonic acid disodium salt (BPDS) or bathocuproine disulphonic acid disodium salt (BPCS) (Wako Pure Chemical Industries, Ltd., Japan) was added, respectively.

Functional expression in yeast

A yeast expression vector (pYH23) was constructed as follows. The plasmid YEPlac181 (Gietz and Sugino, 1988) was cleaved with XhoI and EcoRI, blunt ended with T4 DNA polymerase, and re-ligated in order to remove most of the multiple cloning sites. The HindIII site was similarly deleted. The plasmid was digested with Sphi and the 728 bp fragment from pV100U (Vernet et al., 1987) containing the ADH1 expression cassette was inserted. To insert a NotI site in the multicloning site of the cassette, the plasmid was cleaved with BamHI and blunt ended with T4 DNA polymerase and the phosphorylated linker AGCGGCCGCT (TaKaRa Shuzo, Japan) was inserted. The new plasmid pYH23 has HindIII, PvuII, PstI, XhoI, SrlI, XbaI, and NotI sites in the ADH1 expression cassette. For constitutive expression of OsIRT1 in yeast cells, OsIRT1 cDNA was inserted into the XhoI–NotI site in pYH23 to form pYH23-OsIRT1. Yeast transformation was carried out using the Li-acetate transformation method (Gietz and Schiestl, 1995).

Results

Cloning the cDNA and genomic sequences of OsIRT1

The database contains expression sequence tag (EST) from rice (accession no. D49213) that is highly homologous to the metal transporter IRT1 from Arabidopsis (Eide et al., 1996). Specific PCR primers were designed based on the EST sequence in order to isolate a corresponding cDNA clone. These primers were used to amplify a fragment (280 bp) clone from cDNA prepared from leaves of rice with 91% identity to the EST. The PCR-based strategy successfully allowed the isolation of a cDNA clone identical to the amplified fragment from a cDNA library made from Fe-deficient rice roots. This cDNA clone had high sequence similarity to IRT1. The cDNA was designated as OsIRT1. A rice genomic library was also screened by hybridization using the 280 bp amplified fragment as a probe. After several rounds of screening, a clone containing the genomic sequence of OsIRT1 cDNA was isolated.
The first (699 bp) and second (679 bp) exons of OsIRT1 cDNA are separated by an intron (4.3 kb). Sequence of OsIRT1 cDNA and the deduced amino acid sequence of OsIRT1 protein

The OsIRT1 cDNA is 1395 bp long and contains an open reading frame of 374 amino acids (accession no. AB070226). OsIRT1 protein has a strong similarity to the metal transport protein IRT1 from Arabidopsis (53% amino acid identity, accession no. U27590). IRT1 and related metal transporters comprise the ZIP metal transporter family (Guerinot, 2000). Within the ZIP family, Rit1, an iron transport protein from pea, has the highest amino acid identity to OsIRT1 (55%, AF065444). LeIRT1 and LeIRT2 from tomato have a strong similarity as well (55% and 55%, AF26266 and AF2/6266, respectively) (Eckhardt et al., 2001). The amino acid alignment of these proteins is shown in Fig. 1. The hydrophobicity profile reveals that OsIRT1 has eight transmembrane domains with a ‘variable region’ between domains 3 and 4. These are typical features found in the ZIP metal transporter family (Guerinot, 2000). The variable region was less similar among these proteins. The region of OsIRT1 contains a potential metal-binding domain rich in histidine residues, which was also found in IRT1. OsIRT1 has only one intron, while IRT1 has two. However, the position of the first intron of IRT1 is same as OsIRT1 (Fig. 1).

OsIRT1 reversed growth defect of ferrous uptake mutants of yeast

The yeast strain YH003 (Δftr1 Δfet4 Δfre1) has disrupted null mutations in the genes for high- and low-affinity iron transporters and ferric reductase (Dancis et al., 1992; Dix et al., 1994; Stearman et al., 1996). YH003 could not be grown on SD media containing 50 μM of BPDS, a strong chelator of the ferrous iron. Heterologous expression of OsIRT1, however, reversed the growth defect of the mutant on iron-depleted media (Fig. 2). The effect of OsIRT1 expression in copper uptake mutant ctr1 was also checked. Two strains, M3 (ctr1-3) and FTRUNB1 (Δctr1) (Dancis et al., 1994) were tested. However, no significant effect on the growth defect of these strains could be found on media containing non-fermentable carbon source (YPG) or SD media containing 50 μM or 100 μM BPCS, a strong chelator of the copper ion (data not shown).

Expression of OsIRT1 under trace metal nutrient deficiency

Induction of OsIRT1 expression in rice roots and leaves under trace metal-limiting conditions was examined by Northern hybridization (Fig. 3). Transcripts of OsIRT1 from plants grown under nutrient-sufficient conditions were very faint in roots, and not visible in shoots. In roots, Fe-deficiency significantly increased the level of OsIRT1 transcripts. Cu-deficiency also increased the transcript level. Under Mn- and Zn-deficiency, induction was not observed. In shoots, however, no increase in transcript level was found under any metal deficiency conditions.
the growth defects of et al. experiments, OsIRT1 may participate in copper uptake (1994), while OsIRT1, an IRT1-like metal transport protein from rice is reported. OsIRT1 has features typical of the ZIP metal transporter family, such as eight transmembrane domains and a variable region with a histidine-rich metal binding domain (Guerinot, 2000). So far, several members of this family have been isolated from dicotyledonous species (Eckhardt et al., 2001; Eide et al., 1996; Vert et al., 2001). OsIRT1 is the first member of the ZIP metal transporter family to be isolated from a graminaceous plant.

It was also shown that OsIRT1 cDNA reversed the growth defect of the yeast iron-uptake mutant frl1 fet4 fre1 on iron-depleted media when expressed in the yeast cells. This suggests that OsIRT1 encodes a functional iron transporter. OsIRT1 transcripts were predominantly expressed in the roots, and were induced by Fe-deficiency. The fact that expression of OsIRT1 is limited to the roots and is induced by Fe-deficiency indicates the direct involvement of OsIRT1 in uptake of iron from soil.

It has been reported that IRT1 is a metal transporter with a broad substrate specificity (Eide et al., 1996; Korshunova et al., 1999; Rogers et al., 2000). In addition to ferrous iron, IRT1 can transport manganese, zinc, and probably cadmium and cobalt, when expressed in yeast cells. LeIRT1 and LeIRT2 from tomato can also complement yeast mutants defective in the uptake of iron, zinc, manganese, and copper (Eckhardt et al., 2001). Although IRT1 has a broad substrate specificity, the transcript level was not increased by any of these metal deficiency conditions other than iron (Eide et al., 1996; Korshunova et al., 1999). Therefore, it is interesting that expression of OsIRT1 was also induced by Cu-deficiency in addition to Fe-deficiency. It was reported that LeIRT1 and LeIRT2 from tomato could reverse the growth defects of the yeast high-affinity copper uptake mutant, ctrl (Dancis et al., 1994), while IRT1 from Arabidopsis could not (Eckhardt et al., 2001). Although no effects of OsIRT1 expression on the growth defects of ctrl could be found in these experiments, OsIRT1 may participate in copper uptake when it is Cu-limited. The effects of OsIRT1 expression on manganese or zinc uptake yeast mutants were not tested. Although OsIRT1 was not induced by these metal deficiency conditions, it may have a broad substrate specificity for these metals similar to IRT1, LeIRT1, and LeIRT2.

To date, it has been generally accepted that graminaceous plants can utilize only ferric iron in the rhizosphere in a phytosiderophore-dependent manner (Römheld, 1987; Römheld and Marschner, 1986). Compared with dicotyledonous plants, Fe-deficiency inducible Fe(II) transport systems are absent or expressed at lower levels in the roots of barley and maize (Zaharieva and Römheld, 2001). This report on the isolation of a root-specific IRT1-like Fe(II) transporter clearly demonstrates that rice possesses an iron uptake system for Fe(II), in addition to a phytosiderophore-mediated Fe(III) transport system. This is a new mechanism of iron uptake that combines both Strategy I and Strategy II. Rice is grown under submerged conditions in which the dominant form of soil iron is ferrous iron (Yoshida, 1981). Therefore, it seems reasonable that rice may have an iron uptake system for Fe(II) as well as Fe(III) in its roots. Rice also transports oxygen from the shoots to the roots, and secretes oxygen from roots to oxidize the rhizosphere. Therefore, rice plants may induce one or the other of these iron transport systems by sensing the concentration of ferrous iron or the redox potential around their rhizoplane. Perhaps rice is unique among graminaceous plants in possessing a transport system for Fe(II).

There is another class of metal transporters, the Nramp family, involved in Fe(II) transport in higher plants. Nramp genes are widely distributed in mammals, yeasts, and higher plants, and are known to be involved in a variety of processes, including metal transport and resistance to microbial infection (Cellier et al., 1996; Cohen et al., 2000; Gunshin et al., 1997). AtNramp1 and OsNramp1 can complement the fet3 fet4 yeast mutant defective both in low- and high-affinity iron transporters. The expression of AtNramp1, however, did not enhance iron uptake in yeast mutant cells. These proteins were speculated to be localized to intracellular compartments, such as chloroplasts, and to facilitate intracellular iron transport (Curie et al., 2000). In another paper, it was reported that AtNramp3 and AtNramp4 could complement the yeast metal uptake-deficient strain snf1, in addition to the fet3 fet4 mutant. The authors suggested the involvement of Arabidopsis Nramp proteins in the transport of iron, manganese, and cadmium, including Fe-deficiency inducible iron transport, especially AtNramp3 (Thomine et al., 2000). There are at least three isologues of Nramp proteins in rice (Belouchi et al., 1997). However, the function of these proteins remains unclear. Recently, yellow stripe 1 (YS1), a strong candidate for the gene for the transport...
protein for the ferric phytosiderophore complex, was isolated from maize (Curie et al., 2001). Several other rice YS1 homologues were found in the database. Further study might elucidate the function of each of these three classes of metal transport proteins (IRT, Nramp, and YS1), and how the expression of these proteins is regulated developmentally and spatially. Analysis of these genes will allow an understanding of iron nutrition from the viewpoint not only of iron uptake from soils, but iron transport \textit{in planta}, as well as organellar iron transport.

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