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Research article

Expression of peanut Iron Regulated Transporter 1 in tobacco and rice plants confers improved iron nutrition

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ABSTRACT

Iron (Fe) limitation is a widespread agricultural problem in calcareous soils and severely limits crop production. Iron Regulated Transporter 1 (IRT1) is a key component for Fe uptake from the soil in dicot plants. In this study, the peanut (*Arachis hypogaea* L.) *AhIRT1* was introduced into tobacco and rice plants using an Fe-deficiency-inducible artificial promoter. Induced expression of *AhIRT1* in tobacco plants resulted in accumulation of Fe in young leaves under Fe deficient conditions. Even under Fe-excess conditions, the Fe concentration was also markedly enhanced, suggesting that the Fe status did not affect the uptake and translocation of Fe by *AhIRT1* in the transgenic plants. Most importantly, the transgenic tobacco plants showed improved tolerance to Fe limitation in culture in two types of calcareous soils. Additionally, the induced expression of *AhIRT1* in rice plants also resulted in high tolerance to low Fe availability in calcareous soils.

1. Introduction

Iron (Fe) plays essential roles in plant growth and development in a series of biochemical processes, including respiration, photosynthesis and DNA synthesis. Thirty percent of the global cultivated soils are calcareous with low Fe availability because Fe is present in insoluble oxidized forms (Guerinot and Yi, 1994; Mori, 1999). Fe limitation in calcareous soils is a worldwide agricultural problem. Therefore, production of plants that can acquire Fe from alkaline soils is of great importance.

Dicots and non-grass monocots have developed the Strategy I response to Fe deficiency stress (Römheld and Marschner, 1986; Marschner and Römheld, 1994). This response includes acidification of the rhizosphere by releasing protons, subsequent induction of Fe(III)-chelate reductase activity that reduces Fe(III) to Fe(II) and acquisition of Fe(II) across the plasma membrane of root epidermal cells (Römheld, 1987). Arabidopsis Ferric Reductase Oxidase 2 (FRO2) encodes ferric chelate reductase (Robinson et al., 1999), and the Iron Regulated Transporter 1 (IRT1) is a high-affinity Fe(II) transporter for Fe uptake from the soil (Eide et al., 1996; Connolly et al., 2002; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). IRT1, the first identified member of the ZIP family (Guerinot, 2000), also transports other divalent metals (Korshunova et al., 1999; Rogers et al., 2000). Arabidopsis *IRT1* mRNA was induced within 24 h after exposure to Fe deficiency and was undetectable after plants were returned to Fe-sufficient conditions for 12 h. Analysis of *AtIRT1* over-expressing plants with the 35S promoter indicated that the IRT1 protein was present in the roots only when Fe was limited, and the Fe levels were unchanged in transgenic plants compared with the wild type (Connolly et al., 2002). A more recent study demonstrated that overexpression of *AtIRT1* led to constitutive IRT1 protein accumulation and metal overload (Barberon et al., 2011). *AtIRT1* was also regulated by monoubiquitination-dependent endocytosis for proper Fe uptake (Barberon et al., 2011).

Graminaceous plants use the Strategy II mechanism, which involves synthesis and secretion of mugineic acid family

Abbreviations: Cd, Cadmium; Cu, Copper; Fe, iron; FRO, Ferric Reductase Oxidase; IDE1/2, iron-deficiency-responsive elements 1/2; IDS2, Fe-deficiency-specific clone no. 2; IRT1, Iron Regulated Transporter 1; MAs, mugineic acid family phytosiderophores; Mn, Manganese; MS, Murashige and Skoog; Ni, Nickel; NRAMP1, the Natural Resistance-Associated Macrophage Protein; NT, Non-transgenic; SPAD, Soil and Plant Analyzer Development; TOM1, Transporter of Mugineic acid 1; YS1, Yellow Stripe 1; YSL, Yellow Stripe 1-Like; ZIP, ZRT1/IRT-like protein; Zn, Zinc.

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phytosiderophores (MAs) to dissolve insoluble Fe(III) in the rhizospheres (Römheld and Marschner, 1986). The synthesized MAs are secreted by Transporter of Mugineic acid 1 (TOM1) (Nozoye et al., 2011) and the resulting Fe(III)-MAs complex is taken up by the YS1 (YSL) transporter (Curie et al., 2001). The genes encoding the enzymes of the MAs biosynthetic pathway have been identified. The Fe-deficiency-inducible *IDS2* gene, isolated from barley, encodes a putative dioxygenase that hydroxylates the C-3 position of MAs (Okumura et al., 1994; Nakanishi et al., 2000). By analysis of the *IDS2* promoter of barley, two cis-acting elements, iron-deficiency-responsive elements 1 (IDE1) and IDE2, which drive strong Fe-deficiency expression in transgenic tobacco roots, were identified, the cis-acting elements were found to be conserved among various genes and species (Kobayashi et al., 2003, 2005).

Peanut (*Arachis hypogaea* L.) is an important food legume and also the major oilseed crop in China, accounting for 30% of the total production. However, Fe deficiency frequently limits both crop yields and the quality of peanut grown in the calcareous soils of northern China (Zuo et al., 2000). By suppression subtractive hybridization between intercropped and monocropped peanuts, the Fe acquisition transporter, Natural Resistance-Associated Macrophage Protein 1 (NRAMP1) gene, was isolated from peanuts. Induced expression of *AhNRAMP1* by a Fe deficiency-inducible artificial promoter in tobacco plants resulted in enhanced tolerance to Fe deprivation (Xiong et al., 2012). In addition, the key Fe uptake transporter AhIRT1 gene was cloned, and this gene was found to be induced by Fe deficiency in both roots and shoots. Yeast complementation assay indicated that AhIRT1 was a functional Fe transporter (Ding et al., 2010). However, the biological function of *AhIRT1* in plant is not yet identified.

With the aim to produce plants tolerant to Fe-limited calcareous soils, the *AhIRT1* gene was highly expressed in transgenic tobacco and rice plants using an Fe deficiency-inducible artificial promoter. We found that transgenic plants showed high tolerance to low Fe availability in calcareous soils.

2. Materials and methods

2.1. Plant materials and growth conditions

For germination, transgenic plants were grown on Murashige and Skoog (MS) medium containing hygromycin B (50 mg L^{-1}), or non-transgenic (NT) seeds on MS without hygromycin B under 16 h light/8 h dark conditions at 28 °C for 2–3 weeks. Following an acclimation period of 3 days, the plants were transferred to hydroponics or calcareous soils in a greenhouse under natural light conditions for analysis.

2.2. Generation of the transgenic construct

A root sample from peanuts after 11 days of Fe deprivation in hydroponics was used for total RNA extraction with TRIzol reagent (Invitrogen, USA), and the first-strand cDNA was synthesized by ReverTraAce reverse transcriptase (Toyobo, Tokyo, Japan). The open reading frame (ORF) of the *AhIRT1* gene was amplified with the following primers: 5′- TCTAGAATGGGTACTAATTCAGAAGTAAAAC -3′ and 5′- GAGCTCTTAATTCCATTTTGCCATGA -3′. The E-90Ω plasmid, which contains the backbone of the pIG121Hm binary vector (Hiei et al., 1994), and an Fe-deficiency-inducible artificial promoter with a high basal expression level (Kobayashi et al., 2004) were used for generation of the construct. The *GUS* gene of the E-90Ω plasmid was replaced by the *AhIRT1* ORF using *Xba*I and *Sac*I restriction sites. The resultant construct drives *AhIRT1* under the control of the −272/−131 region of the barley *IDS2* gene containing the Fe-deficiency-responsive elements 1 (IDE1) and 2 (IDE2) fused to the −90/+8 region of the cauliflower mosaic virus 35S promoter and tobacco mosaic virus 5′ leader (Ω) sequence.

2.3. Plant transformation

The above construct was introduced into *Agrobacterium tumefaciens* strain (C58) by electroporation. The tobacco (*Nicotiana tabacum* L.) cultivar Petit-Havana SR1 was used for tobacco transformation by the standard leaf-disk method (Helmer et al., 1984). Rice transformation was conducted via *Agrobacterium*-mediated transformation according to Hiei et al. (1994) and Kobayashi et al. (2001) by using the rice cultivar Tsukinohikari.

2.4. Analysis of transgenic plants

For Fe-deficiency treatment, transgenic lines and NT plants with at least three replicates were grown in 20 L boxes containing the nutrient solution (0.70 mM K_2SO_4, 0.10 mM KCl, 0.10 mM KH_2PO_4, 2.0 mM $Ca(NO_3)_2$, 0.50 mM $MgSO_4$, 10 μM H_3BO_3, 0.50 μM $MnSO_4$, 0.50 μM $ZnSO_4$, 0.20 μM $CuSO_4$ and 0.01 μM $(NH_4)_6Mo_7O_{24}$) as described previously (Xiong et al., 2012) for about 1 week and then treated with Fe deprivation for 9 days. The tobacco plants were grown in a greenhouse under a 14 h light (25 °C, 300 μmol m^{-2} s^{-1}) and 10 h dark (20 °C) regimen. Roots or young leaves (non-expanded and the first expanded leaves) were sampled for RNA extraction and metal content measurement, respectively. For Fe excess treatment, the tobacco plants were treated with 500 μM Fe(III)-EDTA for 4 days. Roots or leaves were harvested for metal content measurement. In addition, a small portion of roots or young leaves were sampled for RNA extraction. For tobacco plant growth in calcareous soils, two calcareous soils from different locations were used: one was obtained from Takaoka City (Toyama, Japan) (Takahashi et al., 2001; Ishimaru et al., 2007) and the other from Shahe (Beijing, China). The former (0.5 kg, pH 8.5, $CaCO_3$ 35.48%) was used for each pot and a 1/10 nutrient solution without Fe was used for daily watering. The chlorophyll contents were recorded in tobacco plants at 34, 42, 49 and 56 days. Young leaves and other leaves were harvested for metal content measurement after 56 days. In the case of the other soil sample (pH 8.1, $CaCO_3$ 8.67%), 1 kg was used for each pot and amended with basal fertilizers [composition (mg kg^{-1} soil): N 100 ($Ca(NO_3)_2 \cdot 4H_2O$), P 150 (KH_2PO_4), K 100(KCl), Mg 50($MgSO_4 \cdot 7H_2O$), Cu 5($CuSO_4 \cdot 5H_2O$), and Zn 5($ZnSO_4 \cdot 7H_2O$)]. Rice plants were also grown in this calcareous soil containing basal fertilizers.

2.5. Measurement of chlorophyll content and metal concentration

The SPAD meter index of the youngest leaf was measured using a SPAD-502 chlorophyll meter (Konica-Minolta, http://www.konicaminolta.com). The harvested plant samples were dried for 2–3 days at 80 °C. Portions of ~100 mg were digested with 4 ml of 4.4 M HNO_3 and 6.5 M H_2O_2 for 30 min at 220 °C using a MarsX-press oven (CEM, http://www.cem.com/). Metal concentrations were measured by inductively coupled plasma optical emission spectrometry (SPS3000; Seiko, Tokyo, Japan).

2.6. Quantitative real-time PCR

Total RNA was extracted by the SDS-phenol method and then treated with RNase-free DNase I (Takara, Tokyo, Japan) to remove genomic DNA contamination. First-strand cDNA was synthesized by ReverTraAce reverse transcriptase (Toyobo, Tokyo, Japan). Quantitative real time PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the SYBR Premix Ex Taq (Perfect Real Time) reagent (Takara,