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RESEARCH PAPER

AhNRAMP1 iron transporter is involved in iron acquisition in peanut

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Abstract

Peanut/maize intercropping is a sustainable and effective agroecosystem to alleviate iron-deficiency chlorosis. Using suppression subtractive hybridization from the roots of intercropped and monocropped peanut which show different iron nutrition levels, a peanut gene, *AhNRAMP1*, which belongs to divalent metal transporters of the natural resistance-associated macrophage protein (NRAMP) gene family was isolated. Yeast complementation assays suggested that *AhNRAMP1* encodes a functional iron transporter. Moreover, the mRNA level of *AhNRAMP1* was obviously induced by iron deficiency in both roots and leaves. Transient expression, laser microdissection, and *in situ* hybridization analyses revealed that *AhNRAMP1* was mainly localized on the plasma membrane of the epidermis of peanut roots. Induced expression of *AhNRAMP1* in tobacco conferred enhanced tolerance to iron deprivation. These results suggest that the *AhNRAMP1* is possibly involved in iron acquisition in peanut plants.

Key words: *AhNRAMP1*, intercropping, iron acquisition, peanut, tobacco, transporter.

Introduction

Iron (Fe) is an essential nutrient for plant growth and development, participating in a series of biochemical processes such as DNA biosynthesis, respiration, and photosynthesis. Although Fe is abundant in soil, the availability of Fe is often very limited because of the insoluble oxidized form (Guerinot and Yi, 1994). Higher plants have developed two unique mechanisms to acquire Fe in response to Fe deprivation (Römheld and Marschner, 1986). Graminaceous species secrete phytosiderophores to mobilize Fe in the rhizosphere (Takagi, 1976). Subsequently, the Fe(III)–phytosiderophore complexes are absorbed by specific membrane transporters yellow stripe1/yellow stripe like (YS1/YSL) (Curie *et al.*, 2001; Murata *et al.*, 2006; Inoue *et al.*, 2009; Lee *et al.*, 2009). Dicots and non-graminaceous monocots reduce Fe(III) to the more soluble Fe(II) by a membrane-bound ferric chelate reductase (Robinson *et al.*, 1999) and the Fe(II) is then taken up via a high affinity Fe(II) uptake transporter, iron-regulated transporter 1 (IRT1) (Eide *et al.*, 1996; Connolly *et al.*, 2002; Vert *et al.*, 2002; Varotto *et al.*, 2002). IRT1 transports other divalent metals as well (Korshunova *et al.*, 1999; Rogers *et al.*, 2000). Disruption of *AtIRT1* leads to a severe growth defect, which is rescued by application of Fe, indicating that IRT1 is the major transporter for Fe uptake from the soil (Vert *et al.*, 2002). Interestingly, graminaceous plants such as rice can take up Fe^{2+} by OsIRT1 and OsIRT2 even without inducible Fe^{3+} chelate reductase activity, in addition to the Fe(III)–phytosiderophore system (Ishimaru *et al.*, 2006).

In addition to *IRT1*, the *NRAMP* (natural resistance-associated macrophage protein) gene family encodes integral membrane proteins that transport a broad range of metal ions including Fe. The evolutionarily conserved *NRAMP* genes have been identified in various species from bacteria to human (Williams *et al.*, 2000; Nevo and Nelson, 2006). In mammals, DCT1 (divalent cation transporter)/NRAMP2 is essential for intestinal Fe absorption and endosomal recycling of Fe (Fleming *et al.*, 1997, 1998; Gunshin *et al.*, 1997). *SMF* and *MntH*, *NRAMP* homologues in yeast and bacteria, respectively, are involved in manganese (Mn) accumulation (Makui *et al.*, 2000; Portnoy *et al.*, 2000). In plants, the NRAMP family transporters have been identified in various species and show diverse functions (Belouchi *et al.*, 1997; Curie *et al.*, 2000; Thomine *et al.*, 2000; Bereczky *et al.*, 2003; Kaiser *et al.*, 2003; Mizuno *et al.*, 2005; Xiao *et al.*, 2008; Oomen *et al.*, 2009; Wei *et al.*, 2009; Xia *et al.*, 2010; Takahashi *et al.*, 2011; Ishimaru *et al.*, 2012). In *Arabidopsis*, heterologous expression of AtNRAMP1, AtNRAMP3, and AtNRAMP4 in yeast mutants indicated that these proteins could transport Fe, Mn, and cadmium (Cd) (Curie *et al.*, 2000; Thomine *et al.*, 2000). AtNRAMP3 and AtNRAMP4 are responsible for mobilization of vacuolar Fe stores (Thomine *et al.*, 2003; Lanquar *et al.*, 2005). Meanwhile, both genes also function in export of vacuolar Mn into photosynthetic tissues of adult plants (Lanquar *et al.*, 2010). AtNRAMP6 contributes to Cd toxicity (Cailliatte *et al.*, 2009). Recently, it has been proposed that AtNRAMP1 is a high-affinity Mn transporter and essential for uptake of Mn from the soil in low Mn conditions (Cailliatte *et al.*, 2010). The NRAMP1 transporters have also been identified in other plant species. In tomato, LeNRAMP1 localizes in the vascular parenchyma of the root hair zone as well as in the root epidermis and the cortex behind the root tip, and is thought to play a role in distribution of Fe in the vascular parenchyma upon Fe deficiency (Bereczky *et al.*, 2003). The identification of MbNRAMP1 in a fruit tree (*Malus baccata*) suggested that MbNRAMP1 was involved in Fe, Mn, and Cd trafficking (Xiao *et al.*, 2008). Rice OsNRAMP1 rescues the growth of an Fe-defective yeast mutant and is related to Cd accumulation in rice (Takahashi *et al.*, 2011). Recent characterization of rice OsNRAMP5 revealed its involvement in Mn, Fe, and Cd uptake and transport (Ishimaru *et al.*, 2012). Therefore, the biological functions of *NRAMP* are diverse in different plant species and need to be further clarified.

It has been reported that peanut/maize intercropping alleviates Fe-deficiency chlorosis of peanut compared with monocropped peanut (Zuo *et al.*, 2000; Inal *et al.*, 2007; Zuo and Zhang, 2008, 2009). In the present report, a peanut *NRAMP* gene, designated as *AhNRAMP1*, was isolated by suppression subtractive hybridization (SSH) from the roots of intercropped and monocropped peanut, which represented different Fe nutrition levels. Further functional identification of *AhNRAMP1* showed that AhNRAMP1 is a functional Fe transporter, and might be responsible for Fe acquisition and distribution in peanut plants.

Materials and methods

Plant materials and growth conditions

The seedlings of peanut (*Arachis hypogaea* L.cv. Luhua 14) were grown in 5 litre boxes containing continuously aerated nutrient solution. The composition of the nutrient solution was as follows: 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 $CuSO_4$, 0.01 μM $(NH_4)_6Mo_7O_{24}$, and 100 μM Fe(III)-EDTA. The plants were grown in a greenhouse with 30 °C light/25 °C dark cycles under natural light conditions.

Cloning of the AhNRAMP1 *gene*

Peanut monocropping and intercropping with maize in a pot experiment were performed as previously described (Zuo and Zhang, 2008). The roots of intercropped and monocropped peanut were harvested after intercropping with maize for ~1 month. Total RNA was extracted with Trizol reagent (Invitrogen, USA) and then mRNA was isolated using an Oligotex mRNA Purification Kit (Qiagen, http://www.qiagen.com). SSH between intercropped and monocropped peanut roots was performed using a Clontech PCR-Select™ cDNA Subtraction Kit (Clontech, http://www.clontech.com) following the manufacturer's instructions. The clones were sequenced and one clone containing the fragment of the *NRAMP* gene was found by BLAST alignment (http://www.ncbi.nlm.nih.gov/). Based on the sequence of the cloned fragment, the full-length cDNA, which was designated as *AhNRAMP1*, was generated by the rapid amplification of cDNA ends (RACE) technique (SMART™ RACE cDNA amplification kit; Clontech) using the following primers: 5′-GCCAATCCACGAAGGCAGTGATGAGG-3′ (5′ RACE) and 5′-AACACAGCAATGCAAACCCATGTGGA-3′ (3′ RACE). The open reading frame (ORF) of *AhNRAMP1* cDNA was amplified by PCR using primers AhNRAMP1-F, 5′-GACTCAT-CACTTGGATTGACTGT-3′ and AhNRAMP1-R, 5′-CTCATA-CATACATAGCTCAAGTCACT-3′. After cloning, the plasmid content of *AhNRAMP1* was confirmed by sequencing. The phylogenic tree was constructed after multiple alignment using BioEdit v7.0.5 (Hall, 1999). The accession number of *AhNRAMP1* in GenBank is JQ581595.

Quantitative real-time PCR

Total RNA was extracted from the roots and leaves of Fe-deficient or Fe-sufficient peanut in hydroponics 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 ReverTra Ace reverse transcriptase (Toyobo, Tokyo, Japan) by priming with the $d(T)_{17}$-adaptor primer. Quantitative real-time PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq (Perfect Real Time) reagent (Takara) using gene-specific primers QAhNRAMP1-F, 5′-CCTCATCACTGCCTTCGT-3′ and QAhNRAMP1-R, 5′-ATTGCTGTGTTATCCTTGGTC-3′. The PCR products were confirmed by DNA sequencing (3130 Genetic Analyzer, Applied Biosystems, Tokyo, Japan). The transcript abundance was normalized against peanut *Ubiquitin* (Luo *et al.*, 2005) transcript levels.

Yeast functional complementation

The full-length coding sequence of *AhNRAMP1* with restriction sites was amplified by PCR using primers *Xba*I-NRAMP-F, 5′-TCTAGAATGGCAAGCGTTCTTAGACA-3′ and *Sac*I-NRAMP-R, 5′-GAGCTCTTATTCCGGTAGTGGGATAT-3′. The PCR product of *AhNRAMP1* cDNA was then subcloned into pCR®-Blunt II-TOPO® vector (Invitrogen) and the sequence was confirmed. After digestion, the *AhNRAMP1* cDNA was introduced into yeast expression vector pDR195 (Schaaf *et al.*, 2005;

kindly provided by Dr Nicolaus von Wirén, University of Hohenheim, Germany). *Saccharomyces cerevisiae* strain DEY1453 (*fet3fet4* mutant, MAT_/MAT_ ade2/_ can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::His3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2) (Dix *et al.*, 1994; kindly provided by Dr David Eide, University of Minnesota School of Medicine, USA and Dr Toshihiro Yoshihara, CRIEPI, Japan) was transformed with pDR195-AhNRAMP1, empty vector pDR195, and pDR195-OsNRAMP1 (Takahashi *et al.*, 2011) as a positive control, using the LiAc/SS-DNA/PEG method (Gietz and Schiestl, 1995). The yeast cells were selected on solid synthetic defined (SD) media plates without uracil. The transformed cells were diluted to OD 1 to 0.001 at 600 nm and spotted onto the plate containing SD medium at pH 5, 6, and 7. The spotted yeast cells were then incubated at 30 °C for 2–3 d.

Subcellular localization of AhNRAMP1

The *AhNRMAP1* ORF without a stop codon and containing *Xho*I and *Bgl*II restriction sites was amplified by PCR using primers 5′-CTCGAGATGGCAAGCGTTCTTAGACA-3′ and 5′-AGATCTGTTCCGGTAGTGGGATATCAG-3′. The PCR product was then subcloned into the *Cauliflower mosaic virus* (CaMV) 35S-sGFP(S65T)-NOS3′ vector (kindly provided by Dr Yasuo Niwa, University of Shizuoka, Japan). The AhNRAMP1–sGFP (synthetic green fluorescent protein) fusion construct or the CaMV35S–sGFP construct was transiently expressed in onion epidermal cells transformed by DNA particle bombardment as described by Mizuno *et al.* (2003). After 4–6 h, FM4-64 (Invitrogen, Molecular Probes, www.invitrogen.com) was added to the onion epidermal cells at a final concentration of 2 μM. The fluorescent cells were imaged by confocal microscopy (LSM5Pascal; Carl Zeiss, Göttingen, Germany).

Laser microdissection (LMD) and expression analysis

LMD was performed as previously described (Takahashi *et al.*, 2010). Briefly, peanut roots from Fe sufficiency and deficiency conditions were dissected into 5 mm strips in the fixative solution (ethanol:acetic acid 3:1) on ice. The fixative was infiltrated into the tissues under vacuum three times for 5 min on ice and then overnight at 4 °C. The samples in fixative solution were further fixed by microwave at 37 °C for 15 min, and this was repeated three times with fresh and pre-chilled fixative solution. The samples were then dehydrated by 70, 80, 90, and 100% ethanol at 58 °C (1.5 min each time) in the microwave. The paraffin-embedded blocks were prepared by gradually exchanging butanol with melted paraffin wax at 58 °C. The sections were laser-microdissected using the Veritas Laser Microdissection System LCC1704 (Molecular Devices) and the dissected content was confirmed. Total RNA was extracted from the laser-microdissected samples using a Pico-Pure™ RNA isolation kit (Molecular Devices) and quantified by a Quant-iT™ RiboGreen RNA reagent and kit (Invitrogen). About 10 ng of RNA was used for quantitative real-time PCR.

In situ hybridization

A gene-specific fragment of *AhNRAMP1* at the 3′-untranslated region was amplified by primers IAhNRAMP-F, 5′-ACCACTTCACACTGCTTTTTAGG-3′ and IAhNRAMP-R, 5′-GATCTTCAAAGGAGAAATTGTCAC-3′. The PCR product was then subcloned into pCR®-Blunt II-TOPO® vector (Invitrogen) and the content was confirmed by sequencing. The correct plasmids with two directions of insert fragments for sense or antisense probes were individually linearized with *Bam*HI. The sense or antisense probe was generated by T7 RNA polymerase and labelled with digoxigenin-11-UTP (Roche, Mannheim, Germany). Peanut plants were treated without Fe for 1 week. The roots were fixed in FAA (50% ethanol:acetic acid:37% formaldehyde solution 18:1:1) and embedded in paraffin after dehydration with an ethanol and *t*-butanol series. The root tissues were sectioned to 10 μm and mounted on slides. *In situ* hybridization was performed according to Ishimaru *et al.* (2005) with some modifications. In brief, after pre-treatment of sections with proteinase K and acetylation, the slides were hybridized for 16 h with sense or antisense probes at 50 °C and washed. The tissues were then incubated with anti-digoxigenin–alkaline phosphatase conjugate (Roche) and stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

Generation of transgenic plants

The coding region of the *AhNRAMP1* gene was amplified with the following primers: 5′-TCTAGAATGGCAAGCGTTCTTAGACAG-3′ and 5′-GAGCTCTTATTCCGGTAGTGGGATATC-3′. *Xba*I and *Sac*I restriction sites were used to replace *AhNRAMP1* cDNA with the *GUS* (β-glucuronidase) gene of E-90Ω plasmid (Kobayashi *et al.*, 2004). The resultant plasmid has the backbone of the pIG121Hm binary vector (Hiei *et al.*, 1994) and drives *AhNRAMP1* cDNA under the control of the –272/–131 region of the barley *IDS2* gene containing Fe-deficiency-responsive element 1 (IDE1) and IDE2, flanked by the –90/+8 region of the CaMV35S promoter and the *Tobacco mosaic virus* 5′ leader (Ω) sequence. The constructed plasmid was introduced into *Agrobacterium tumefaciens* strain C58 by electroporation and then transformed to tobacco (*Nicotiana tabacum* L. cv. Petit-Havana SR1) according to the method of Helmer *et al.* (1984).

Analysis of transgenic tobacco plants

Nine T_2 transgenic tobacco lines were generated, and germinated on Murashige and Skoog (MS) medium containing hygromycin B (50 mg l^{-1}). Non-transgenic seeds were germinated on MS medium lacking hygromycin B. After 1 week the seedlings were transferred to MS medium without Fe for 6 d. The expression level of the *AhNRAMP1* gene in all lines treated with Fe deficiency was analysed by quantitative real-time PCR. The transcript abundance was normalized against tobacco *Actin* (Yoshihara *et al.*, 2006) transcript levels. Two lines of transgenic tobacco plants with a high expression level of *AhNRAMP1* were selected for further analysis. For hydroponics, after 2–3 weeks of growth in MS medium followed by an acclimation period of 3 d, the plantlets were transferred to nutrient solution (the same composition as described above for peanut) in a greenhouse under natural light conditions at 25 °C. The transgenic lines and wild-type plants with at least three replicates were grown in hydroponics for ~1 week and then subjected to Fe deficiency for 9 d. Roots and young leaves were harvested for metal content measurement. Samples were dried for 2–3 d at 80 °C, and 100–200 mg portions were then wet-ashed with 4 ml of 4.4 M HNO_3 and 6.5 M H_2O_2 for 30 min at 220 °C using a MarsXpress oven (CEM, http://www.cem.com/). Metal concentrations were measured by using inductively coupled plasma optical emission spectrometry SPS3000 (Seiko, Tokyo, Japan).

Results

Identification of the NRAMP1 *gene from peanut*

Using SSH between intercropped (with maize) and monocropped peanut, a partial sequence of a 490 bp cDNA fragment showing 73% identity with *LeNRAMP1* was obtained. By additional 5' and 3' RACE analysis of cDNA from Fe-deficient peanut roots, a 2033 bp cDNA sequence containing the full-length ORF was identified, and designated as *AhNRAMP1*. The *AhNRAMP1* gene was predicted

to encode 545 amino acids, consisting of 12 putative transmembrane domains (TMs) (Supplementary Fig. S1 available at *JXB* online). Phylogenetic tree analysis showed that among several *NRAMP* genes from *Arabidopsis*, rice, and tomato, AhNRAMP1 was most similar to LeNRAMP1, with 78% identity (Fig. 1).

AhNRAMP1 *is induced by Fe deficiency in both roots and leaves*

The transcriptional abundance of *AhNRAMP1* was assessed in peanut supplemented or not with Fe. The expression of *AhNRAMP1* was higher in roots than in leaves, and markedly induced by Fe deficiency in both roots and leaves (Fig. 2). The transcript level of *AhNRAMP1* increased at least 5-fold after treatment without Fe for 4, 7, and 11 d in roots (Fig. 2A). In comparison with roots, the induction time of expression was delayed in young and old leaves. In leaves, only at 11 d did *AhNRAMP1* show remarkably higher expression under Fe limitation (Fig. 2B, C). Interestingly, after transferring the Fe-starved plants to a solution containing an excess amount of Fe (500 μM) for 1 d, the expression of the *AhNRAMP1* gene was not down-regulated in roots (Fig. 2A), but was significantly suppressed in young and old leaves (Fig. 2B, C).

AhNRAMP1 *functionally complements the growth defect of Fe-deficient yeast mutant*

In order to investigate whether AhNRAMP1 transports Fe, a yeast functional complementation assay was performed by using the *fet3fet4* yeast double mutant (strain DEY1453; Dix *et al.*, 1994), which is defective in high- and low-affinity Fe-uptake systems. The full-length cDNA of the *AhNRAMP1* gene was subcloned into the yeast expression vector pDR195. Meanwhile, the rice *OsNRAMP1* gene (Curie *et al.*, 2000) was used as a positive control. Expression of *AhNRAMP1*, as well as *OsNRAMP1*, significantly improved yeast growth compared with the control strain transformed with empty vector under various pH values tested (pH 5, 6, and 7) (Fig. 3), suggesting that the peanut *AhNRAMP1* transports Fe.

AhNRAMP1 *is a plasma membrane protein*

The subcellular localization of *AhNRAMP1* was investigated by expressing an AhNRAMP1–GFP fusion protein in onion epidermal cells. The dye FM4-64 was used as a plasma membrane marker (Uraguchi *et al.*, 2011). AhNRAMP1–GFP fluorescence was observed in the plasma membrane, overlapping the FM4-64 fluorescence immediately after staining (Fig. 4A–C). The green fluorescence of

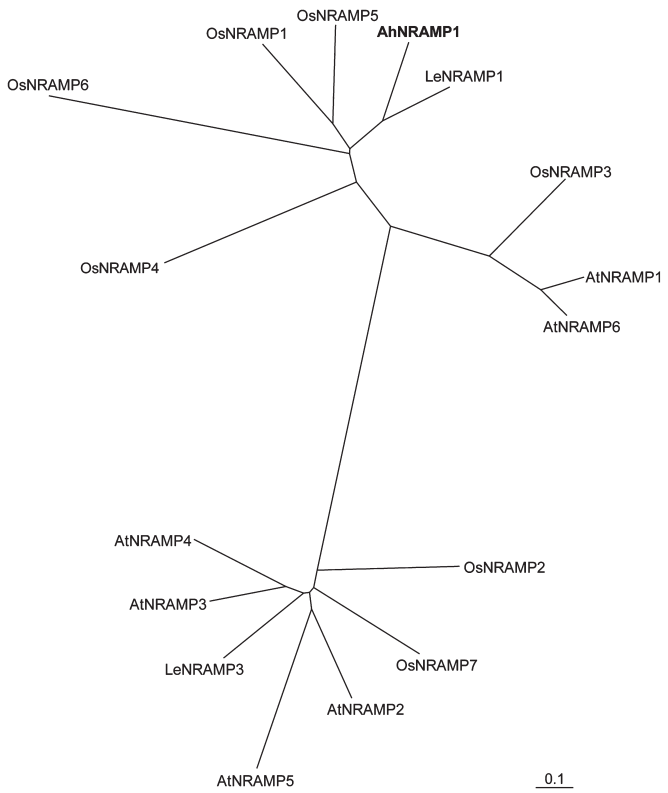

Fig. 1. Phylogenetic tree of the NRAMP proteins from *Arabidopsis*, rice, tomato, and peanut. Accession numbers are as follows: AhNRAMP1, JQ581595; AtNRAMP1, AEE36455.1; AtNRAMP2, AEE32142.1; AtNRAMP3, AAF13278.1; AtNRAMP4, AAF13279.1; AtNRAMP5, NP_193614.1; AtNRAMP6, AEE29390.1; OsNRAMP1, AAB36424.1; OsNRAMP2, Q10Q65; OsNRAMP3, Q653V6; OsNRAMP4, Os02g0131800; OsNRAMP5, Os07g0257200; OsNRAMP6, Os01g0503400; OsNRAMP7, Os12g0581600; LeNRAMP1, NP_001234318; LeNRAMP3, NP_001233770.

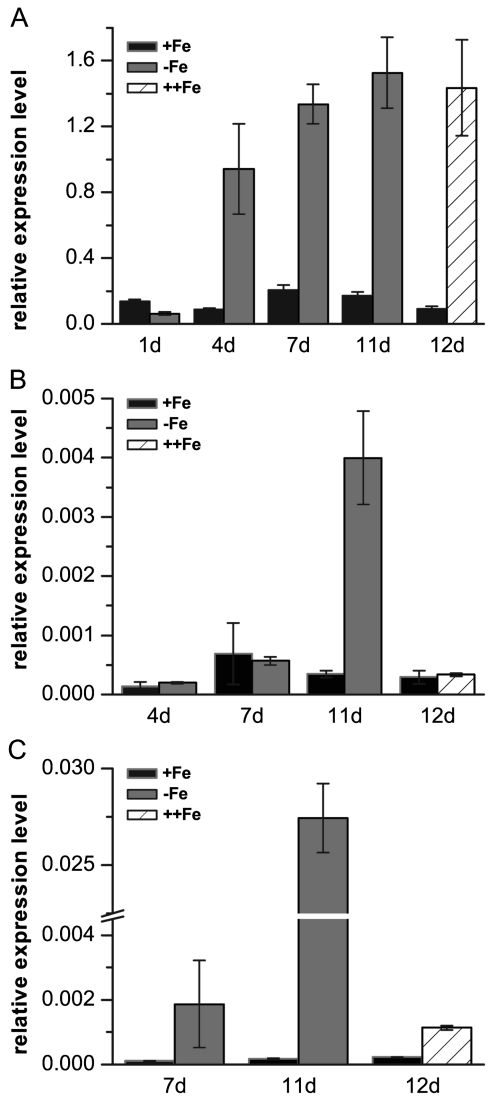

Fig. 2. Expression pattern of *AhNRAMP1* in response to Fe-deficient and Fe-sufficient conditions. Peanut plants were treated without Fe or with normal amounts of Fe (100 μM) for 1, 4, 7, and 11 d. 1d, 4d, 7d, and 11d represent the day of treatments. On day 12 (12d), the Fe-deficient peanut plants were transferred to 500 μM Fe for the treatment with excess Fe (++Fe) for 1 d. The samples were harvested from roots (A), young leaves (B), and old leaves (C) of the treated peanut plants. The vertical bars indicate the expression level of the genes relative to that of the control *AhUbiquitin* gene. Values are the means of three replications. Error bars indicate the SD.

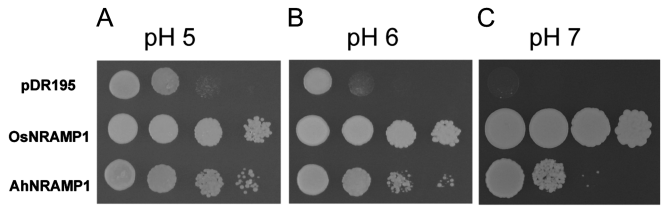

Fig. 3. Functional complementation of the *fet3fet4* yeast mutant with *AhNRAMP1*. Empty pDR195 vector was used as a negative control and *OsNRAMP1* cDNA as a positive control. Serial dilutions of the transformed yeast cells with $OD_{600\ nm}$ 1 to 0.001 were plated onto SD medium at pH 5 (A), pH 6 (B), and pH 7 (C).

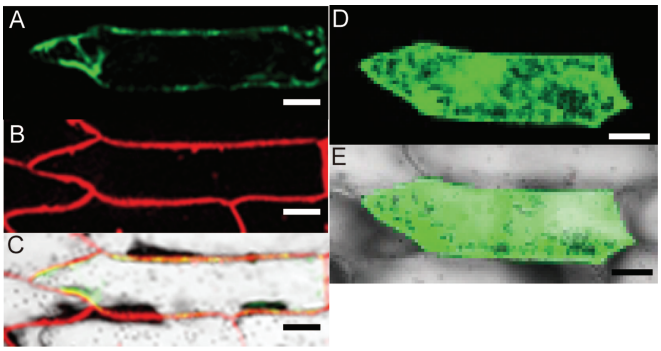

Fig. 4. Subcellular localization of *AhNRAMP1* in onion epidermal cells. (A) *AhNRAMP1* fused to GFP was transiently expressed in onion epidermal cells. (B) The red fluorescence detected from the FM4-64 dye (2 μM), as a plasma membrane marker. (C) Confocal microscopy images illustrating AhNRAMP1–GFP co-localization with the FM4-64 dye at the plasma membrane. (D) GFP alone was transiently expressed in onion epidermal cells. (E) Overlay with the transmission image shown in (D). Scale bars represent 50 μm.

GFP alone, as control, was observed mainly in the cytosol and nucleus (Fig. 4D, E).

AhNRAMP1 *is mainly localized in the epidermis of peanut roots*

The peanut root tissues from Fe-deficient and Fe-sufficient conditions were separated into epidermis/exodermis, cortex, and stele by LMD. Quantitative real-time PCR was performed to identify the expression abundance of the *AhNRAMP1* gene in the different parts of root tissues. The expression abundance of *AhNRAMP1* was the highest in the epidermis/exodermis, and the lowest in the stele, irrespective of the Fe status (Fig. 5A). Consistent with the expression pattern in whole roots (Fig. 2A), the expression of *AhNRAMP1* was strongly induced by Fe deprivation in all parts of root tissues (Fig. 5A).

Further, *in situ* hybridization was employed to confirm the tissue localization of the *AhNRAMP1* gene in peanut root using a specific probe. Young roots of peanut under Fe deficiency were analysed. In good agreement with the expression in LMD sections, the staining of *AhNRAMP1* antisense probe was more visible on the cells of the root epidermis in both transverse (Fig. 5B) and longitudinal (Fig. 5D) sections compared with that of the sense probe as a negative control (Fig. 5C, E).

Induced expression of AhNRAMP1 *in tobacco results in tolerance to Fe deprivation*

To evaluate further the function of *AhNRAMP1 in planta*, this gene was introduced into tobacco, a model dicot plant. Since constitutive overexpression of *NRAMP* genes can cause a growth defect (Takahashi *et al.*, 2011; Ishimaru *et al.*, 2012), an Fe deficiency-inducible artificial promoter

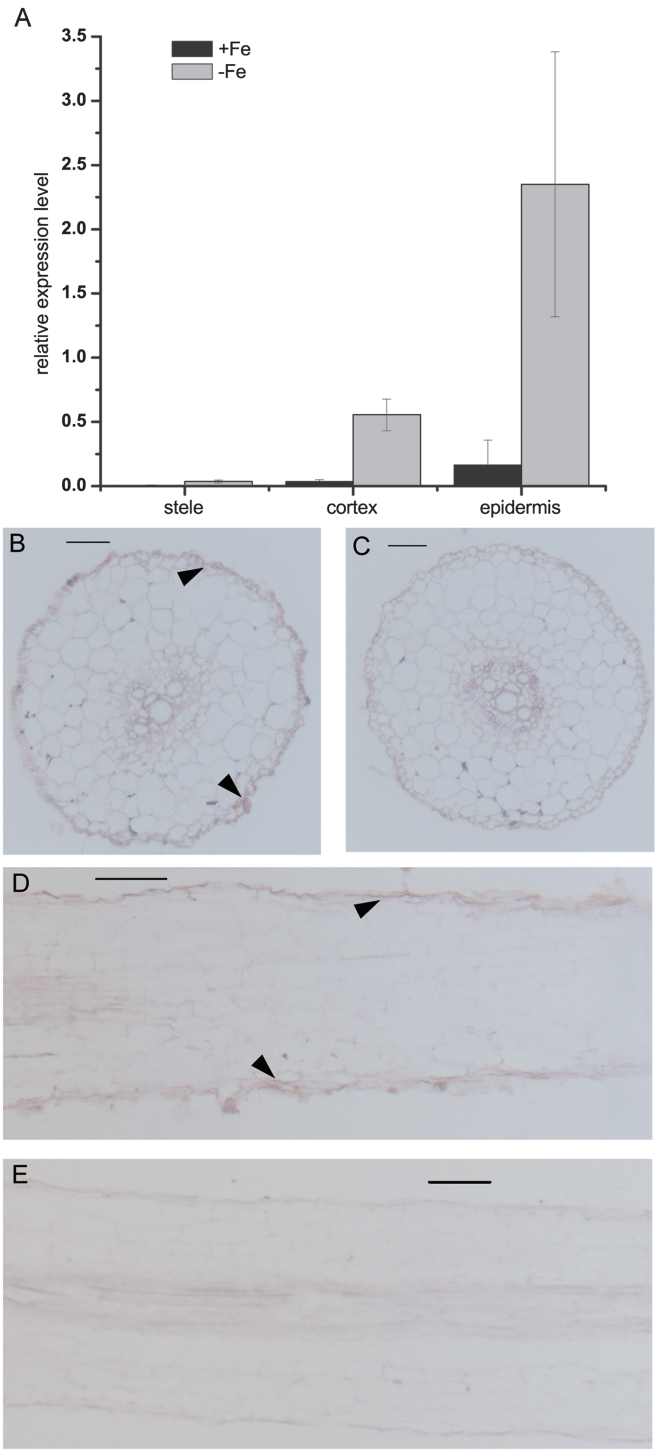

Fig. 5. Tissue localization of *AhNRAMP1* in peanut roots by LMD analysis and *in situ* hybridization. (A) LMD and expression analysis of *AhNRAMP1*. Peanut plants were cultured with or without Fe for 7 d. Stele, cortex, and epidermis/endodermis of peanut roots were separated by LMD. The vertical bars indicate the relative expression level of *AhNRAMP1* as compared with the control *AhUbiquitin* gene. Three biological replications were performed for each treatment. Error bars represent the SD. (B–E) *In situ* hybridization analysis of *AhNRAMP1* in the roots of Fe-deficient peanut. *AhNRAMP1* antisense probes were hybridized in a cross-section (B) or longitudinal section (D) of peanut root. Sense probes of *AhNRAMP1* were used as a control (C, E). Bars = 100 μm. Arrowheads indicate representative hybridization signals.

(Kobayashi *et al*., 2004) was utilized to drive high expression of *AhNRAMP1* under Fe deficiency in tobacco. When grown in Fe-deprivation conditions on both MS medium and hydroponics, non-transgenic (NT) plants exhibited more chlorosis than *AhNRAMP1*-induced transformants (Fig. 6A, B). The Fe concentration in young leaves of *AhNRAMP1*-induced plants was higher than in NT plants (Fig. 6C). The Mn and Zn levels of young leaves were similar between the induced lines and NT plants (Fig. 6D, Supplementary Fig. S3 at *JXB* online). In the roots, no significant changes in the level of any metals were observed (Supplementary Fig. S4 at *JXB* online). Taken together, these results indicate that the induced expression of *AhNRAMP1* in tobacco facilitates the availability and accumulation of Fe in young leaves and thus leads to tolerance under Fe deficiency.

Discussion

SSH is a powerful approach for isolation of differentially expressed genes between two samples (Diatchenko *et al*., 1996). In the present study the *AhNRAMP1* cDNA fragment was obtained by SSH between intercropped and monocropped peanut roots in calcareous soils. It has been clearly elucidated that peanut intercropped with maize enhances the Fe nutrition of peanut in calcareous soils (Zuo *et al*., 2000; Zuo and Zhang, 2008, 2009). The phytosiderophores released by maize may play a role in improving the Fe nutrition of peanut in the intercropping system (Zuo and Zhang, 2009), and the molecular mechanisms involved in this phytosiderophore-based Fe improvement are being clarified (H. Xiong *et al*., unpublished results). Since the monocropped peanut suffers from less Fe availability, peanut under monocropping changes the expression of Fe-responsive genes, such as *AhIRT1*, encoding an Fe-uptake transporter (Ding *et al*., 2010), and *AhFRO1*, encoding ferric-chelate reductase (Ding *et al*., 2009). Therefore, the distinctly different expression levels of *AhNRAMP1* between intercropped and monocropped peanut roots lead us to hypothesize that *AhNRAMP1* plays a role in Fe nutrition. Moreover, the transcript level of *AhNRAMP1* was specifically higher in the roots and increased at least 5-fold after treatment without Fe for 4, 7, and 11 d in the roots (Fig. 2A). In tomato, *Arabidopsis*, *M. baccata*, and rice, *LeNRAMP1*, *AtNRAMP1*, *MbNRAMP1*, and *OsNRAMP1* genes also show higher expression in the roots under Fe deficiency compared with the Fe-sufficient condition (Curie *et al*., 2000; Bereczky *et al*., 2003; Xiao *et al*., 2008; Takahashi *et al*., 2011). Therefore, these *NRAMP* genes are thought to belong to an Fe deficiency-induced subclass, suggesting a possible conserved function in Fe homeostasis.

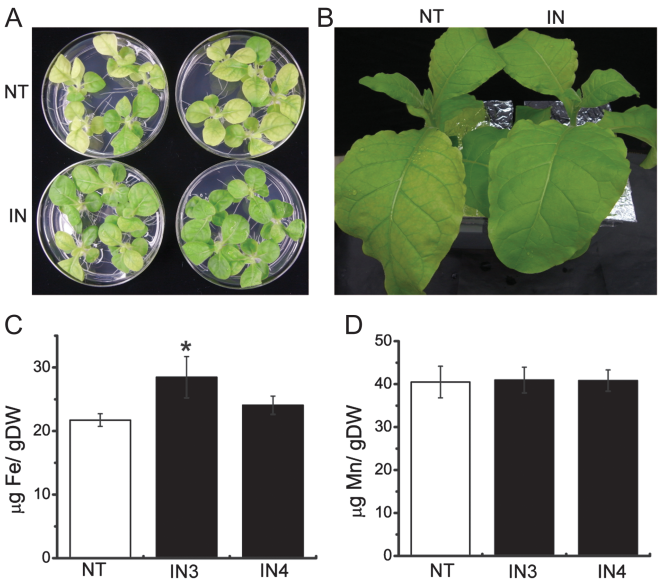

Fig. 6. The response to Fe deficiency in *AhNRAMP1*-induced tobacco lines. The phenotype of *AhNRAMP1*-induced lines grown on MS medium (A) or hydroponics (B) without Fe. NT represents non-transformed tobacco. IN is *AhNRAMP1*-induced tobacco. (C) Fe and (D) Mn concentration in the new leaves of NT and IN tobacco in hydroponics treated under Fe deficiency for 9 d. The results are presented as the means ±SD of triplicate samples. Asterisks indicate a significant difference at $P < 0.05$ by *t*-test.

By clustal analysis, AhNRAMP1 was closer to LeNRAMP1, with 78% identity (Fig. 1). Similar to *LeNRAMP1*, expression of *AhNRAMP1* restored growth of the high- and low- affinity Fe uptake-defective yeast mutant (Fig. 3), suggesting that AhNRAMP1 is also a functional Fe transporter. *AhNRAMP1* expression was strongly induced under Fe deficiency, and rapidly decreased to nearly basal levels after 1 d of Fe resupply only in leaves but not in roots (Fig. 2). It has been reported that the expression level of *AhIRT1* increased ~70-fold after exposure to Fe deficiency for 6 d in the roots and was suppressed rapidly by transfer to Fe-sufficient conditions (Ding *et al.*, 2010). Comparing the expression pattern of *AhNRAMP1* with that of *AhIRT1*, *AhIRT1* is thought to be more sensitive in response to Fe deficiency and resupply. AhNRAMP1 is a plasma membrane protein (Fig. 4) and is mainly localized in the epidermis of peanut roots, as indicated by LMD and *in situ* hybridization assay (Fig. 5). *LeNRAMP1*, on the other hand, is expressed in the vascular parenchyma of the root hair zone and root epidermis and the cortex behind the root tip, and is thought to distribute Fe in the vascular parenchyma upon Fe deficiency (Bereczky *et al.*, 2003). Therefore, in contrast to *LeNRAMP1*, the localization of *AhNRAMP1* in the epidermis suggested that the Fe-regulated transporter AhNRAMP1 might function in Fe acquisition from the soil, in addition to the major Fe uptake system AhIRT1. This speculation is supported by a previous study showing that *Arabidopsis AtNRAMP1* partially rescued the growth and Fe content of the *irt1* mutant, which is defective in essential Fe uptake (Cailliatte *et al.*, 2010). *AhNRAMP1* expression was also induced by Fe starvation in the leaves (Fig. 2B, C), suggesting that *AhNRAMP1* might also be involved in Fe distribution in the leaves.

To clarify its physiological function *in planta*, *AhNRAMP1* was introduced into tobacco plants by using an artificial promoter containing IDE1 and IDE2, which are *cis*-acting elements conferring Fe-deficiency-specific expression in tobacco roots (Kobayashi *et al.*, 2003), fused to the –90/+8 region of the CaMV35S promoter and the 5′ leader (Ω) sequence of *Tobacco mosaic virus* to enhance the expression level (Kobayashi *et al.*, 2004). The *AhNRAMP1*-induced tobacco accumulated a higher concentration of Fe in young leaves and was more tolerant to Fe deficiency compared with the NT plants (Fig. 6). It has been shown that the IDE1 and IDE2 elements fused to the –90/+8 region of the 35S promoter drive expression in the whole root tissues under Fe deficiency in tobacco, including the epidermis (Kobayashi *et al.*, 2003). Therefore, it is reasonable to consider that the high expression of *AhNRAMP1* in the whole roots of tobacco results in the acquisition of more Fe and thus these plants are resistant to Fe starvation.

Recent studies showed that AtNRAMP1 and OsNRAMP5 transporters play important roles in Mn uptake from the soil (Cailliatte *et al.*, 2010; Ishimaru *et al.*, 2012). In rice, OsNRAMP1 and OsNRAMP5 transporters are also involved in Cd accumulation (Takahashi *et al.*, 2011; Ishimaru *et al.*, 2012). It has been well established that the *NRAMP* gene family transports a broad range of metal ions. Yeast complementation tests suggested that AtNRAMP1, AtNRAMP3, and AtNRAMP4 transport Fe, Mn, and Cd (Curie *et al.*, 2000; Thomine *et al.*, 2000). *AtNRAMP3* and *AtNRAMP4* function in mobilization of vacuolar Fe pools and export of vacuolar Mn into photosynthetic tissues of adult plants (Thomine *et al.*, 2003; Lanquar *et al.*, 2005). Hence, it is possible that AhNRAMP1 also transports other metals, such as Mn and Cd.

In conclusion, in the present study novel functions for *AhNRAMP1* in peanut are proposed. Several lines of evidence indicate a putative function for *AhNRAMP1* in acquisition of Fe, namely: (i) AhNRAMP1 is an Fe transporter and is strongly induced by Fe deficiency in the roots; (ii) AhNRAMP1 is a plasma membrane protein and is mainly localized in the epidermis of peanut roots; and (iii) induced expression of *AhNRAMP1* in tobacco results in Fe accumulation and tolerance of Fe deprivation.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Multiple alignments of the amino acid sequences of AhNRAMP1, LeNRAMP1, AtNRAMP1, and OsNRAMP1.

Figure S2. The expression level of *AhNRAMP1* in non-transformant (NT) and the *AhNRAMP1*-induced (IN) tobacco lines treated under Fe-deficient MS medium for 6 d.

Figure S3. Zn concentration in new leaves of NT and *AhNRAMP1*-induced (IN) tobacco lines treated under Fe deficiency for 9 d in hydroponics.

Figure S4. Fe (A), Mn (B), Zn (C), and Cu (D) concentration in roots of NT and *AhNRAMP1*-induced (IN) tobacco lines treated under Fe deficiency for 9 d in hydroponics.

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