**LjMATE1: A Citrate Transporter Responsible for Iron Supply to the Nodule Infection Zone of *Lotus japonicus***

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Symbiotic nitrogen fixation by intracellular rhizobia within legume root nodules requires the exchange of nutrients between host plant cells and their resident bacteria. While exchanged molecules imply nitrogen compounds, carbohydrates and also various minerals, knowledge of the molecular basis of plant transporters that mediate these metabolic exchanges is still limited. In this study, we have shown that a multidrug and toxic compound extrusion (MATE) protein, LjMATE1, is specifically induced during nodule formation, which nearly paralleled nodule maturation, in a model legume *Lotus japonicus*. Reporter gene experiments indicated that the expression of LjMATE1 was restricted to the infection zone of nodules. To characterize the transport function of LjMATE1, we conducted a biochemical analysis using a heterologous expression system, *Xenopus* oocytes, and found that LjMATE1 is a specific transporter for citrate. The physiological role of LjMATE1 was analyzed after generation of *L. japonicus* RNA interference (RNAi) lines. One RNAi knock-down line revealed limited growth under nitrogen-deficient conditions with inoculation of rhizobia compared with the controls (the wild type and an RNAi line in which LjMATE1 was not suppressed). It was noteworthy that Fe localization was clearly altered in nodule tissues of the knock-down line. These results strongly suggest that LjMATE1 is a nodule-specific transporter that assists the translocation of Fe from the root to nodules by providing citrate.

**Keywords:** Citrate • Fe translocation • *Lotus japonicus* • MATE • Nodulation.

**Abbreviations:** ARA, acetylene reduction activity; DAB, diaminobenzidine; dpi, days post-inoculation; GUS, β-glucuronidase; ICP-MS, inductively coupled plasma mass spectrometry; MATE, multidrug and toxic compound extrusion; MBS, modified Barth’s saline; NAS, nicotianamine synthase; *pLjMATE1, LjMATE1* promoter region; RNAi, RNA interference; SNF, symbiotic nitrogen fixation.

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**Introduction**

Symbiotic nitrogen fixation (SNF) between legume plants and soil bacteria called rhizobia is one of the most important symbioses on earth. SNF provides not only a major source of fixed nitrogen in natural ecosystems, but also the single largest natural source of nitrogen for agriculture (Smil 1999). SNF in legumes takes place in specialized organs called nodules that develop from dedifferentiated root cells after infection by rhizobia in the soil in a species-specific manner. Via infection threads rhizobia colonize in developing nodule tissue (Brewin 1991) and ultimately enter cortical cells by endocytosis (Verma and Hong 1996). The nodule development is accompanied by coordinated differentiation of both plant and bacterial cells, which results in the formation of a mosaic of both infected and uninfected plant cells. During nodulation, global changes in gene expression were shown to occur in both partners (Colebatch et al. 2004, Kouchi et al. 2004, Uchiumi et al. 2004). Many of these changes strongly affect both plant and bacterial metabolism and transport processes, which become specialized for supporting the exchange of reduced carbon and other nutrients from the plant to bacteroids, and fixed nitrogen from bacteroids to the plant (White et al. 2007). It is postulated that many transporters are involved in the exchange of these nutrients, and thus far several transporters have been identified, e.g. SST1, a symbiotic sulfate transporter in *Lotus japonicus* (Krusell et al. 2005), and an allantoin transporter, PvUPS1, in French bean (*Phaseolus vulgaris*) (Pelissier et al. 2004), but the overall understanding of membrane transport systems in plant nodules is still very limited.

Multidrug and toxic compound extrusion (MATE) proteins occur widely in bacteria, fungi, mammals and also in plants (Omote et al. 2006). Apparently plants have a higher diversity
of MATE-type transporters than bacteria and animals, e.g., there are 56 MATE orthologs in Arabidopsis thaliana, while only two members are found in the human genome. AtDTX1, the first identified plant MATE, was isolated from an Arabidopsis cDNA library using a bacterial mutant defective in multidrug resistance (Li et al. 2002). AtDTX1 showed efflux activity for a variety of xenobiotics. Further studies on other plant MATEs revealed that many of them showed rather restricted substrate specificities for their own metabolites such as flavonoids, alkaloids, and citrate. In particular, MATE-type transporters that transport citrate are reportedly involved in iron (Fe) translocation or aluminum (Al) detoxification in plants. For instance, FRD3, a citrate transporter from Arabidopsis, is localized in the pericycle and vascular cylinder in roots, and is required for Fe translocation from the roots to the shoots (Green and Rogers 2004, Durrett et al. 2007). Defects in this transporter resulted in the precipitation of Fe in the root vasculature (Green and Rogers 2004, Durrett et al. 2007). Also in monocots, a rice MATE transporter, OsFRDL1, transports citrate in the pericycle cells of roots, and was suggested to be involved in Fe translocation in the xylem (Yokoshio et al. 2009). On the other hand, two studies in 2007 showed that Al-induced secretion of citrate in barley (Hordeum vulgare) and sorghum (Sorghum bicolor) is mediated through MATE transporters (Furukawa et al. 2007, Magalhaes et al. 2007). Secretion of citrate from the roots is a mechanism of Al resistance in many plant species (Ma et al. 2001, Kochian et al. 2005, Delhaize et al. 2007). In addition, more recent studies have shown that two soybean MATE-type transporters, GmFRD3a and GmFRD3b, as well as ScFrDL1 from Secale cereale, mediated Fe translocation (Rogers et al. 2009, Yokoshio et al. 2010).

In order to comprehend the membrane transporters involved in SNF, we have performed transcriptome analysis following laser microdissection in a model legume L. japonicus. As a result, a MATE-type transporter (LjMATE1) was identified as a nodule-specific gene in L. japonicus (Takanashi et al. 2012b). In this study, we demonstrate that LjMATE1 can transport citrate and assist the translocation of Fe to the nodule infection zone, which is essential to support nodule function.

Results

Expression analysis of LjMATE1 during nodulation

Based on our microarray analysis in nodules of L. japonicus, a MATE-type transporter gene (LjMATE1) is suggested to be involved in nitrogen fixation in this plant due to the high level of expression in the infection zone. To confirm the in silico data experimentally, expression analysis was carried out. Using gene-specific primers, we found that the expression of LjMATE1 was specifically observed in nodules of L. japonicus (Fig. 1A). Time-course experiments at five different nodulation stages revealed that LjMATE1 gene expression was dramatically increased at 7 days post-inoculation (dpi) with Mesorhizobium loti and remained high during SNF (Fig. 1B).

To analyze the tissue-specific expression of LjMATE1 in nodules in more detail, we isolated a 1.7 kb fragment upstream of the translational initiation codon from L. japonicus genomic DNA, which contains the putative LjMATE1 promoter region (pLjMATE1), and fused it to the β-glucuronidase (GUS) reporter gene. The binary vector containing the pLjMATE1::GUS reporter system was then introduced into L. japonicus using the hairy root transformation method. GUS expression analysis clearly showed that LjMATE1 was expressed only in nodules, and no expression was seen in root tissues throughout nodulation (Fig. 1C–H). To identify the cell type expressing LjMATE1, nodule sections were prepared after GUS staining. Microscopic observation revealed that GUS staining was present mainly in infected cells of nodules at 7 and 21 dpi (Fig. 1D, F), while GUS staining decreased in the central infection zone at 35 dpi (Fig. 1H).

LjMATE1 has transport activity for citrate

Plant MATE proteins can be divided into two distinct groups based on their amino acid sequences, which seems to reflect their transport substrate, i.e. citrate or other metabolites. LjMATE1 belongs to a clade in which all reported citrate-transporting MATE proteins are clustered, having high amino acid sequence similarity to GmFRD3a (72%) and GmFRD3b (71%), which are involved in Fe translocation from the root to aerial parts (Rogers et al. 2009) (Fig. 2). To evaluate whether LjMATE1 can transport citrate as a substrate, we expressed LjMATE1 in Xenopus oocytes. Fig. 3 shows the transport assay using radioisotope-labeled citrate. The data indicated that the efflux activity for citrate was significantly higher in oocytes pre-injected with LjMATE1 cRNA than in oocytes injected with water as a negative control. This efflux activity of LjMATE1 was almost the same with HvAACT1, a previously reported citrate-transporting MATE (Furukawa et al. 2007), as shown in Supplementary Fig. S1. We also examined the transport activity for malate, which is used as an energy source in bacteroids (Price et al. 1987); however, oocytes pre-injected with LjMATE1 cRNA did not show specific transport activity for malate compared with control (Fig. 3).

Growth phenotype of an LjMATE1 knock-down line

To determine the physiological role of LjMATE1, we suppressed its expression in transgenic plants by RNA interference (RNAi). Among 20 stable transformants, appreciable suppression of LjMATE1 expression was found in an RNAi line (ljmate1) at the T2 generation (Fig. 4A). As controls, we used an RNAi line (ljmate1) in which LjMATE1 gene expression was not suppressed, in addition to wild-type plants. The T2 RNAi line grew normally with nitrogen supplementation, and no apparent phenotypic difference was observed compared with the wild type. In contrast, when plants were inoculated with M. loti under nitrogen-deficient conditions, the RNAi plant showed a clear growth defect phenotype (Fig. 4B, C), suggesting that the ability for SNF was reduced in the knock-down line. Then we observed the nodule phenotype of the ljmate1 line.
Instead of pink nodules that were normally seen in control plants, about one-third of nodules in *ljmate1* revealed a greenish appearance (Fig. 5A–D). The nodule size of *ljmate1* was significantly smaller than that of control plants (Fig. 5E), and the acetylene reduction activity (ARA) of nitrogenase as well as the expression level of *leghemoglobin* was also significantly reduced in *ljmate1* (Fig. 5F, G).

**Fe accumulation in *LjMATE1* knock-down nodules**

MATE-type transporters that have transport activity for citrate are known to be involved in either Fe translocation or Al resistance. To investigate whether or not *LjMATE1* mediates Fe translocation, Fe localization in nodules of RNAi plants was examined with Perls staining (Green and Rogers 2004). While no visible Fe precipitation was detected inside wild-type nodules (Fig. 6A, B), strong Perls staining was observed at the root–nodule junction area and the nodule vascular bundle of RNAi nodules (Fig. 6C, D), indicating altered Fe localization in *ljmate1* nodules. This staining pattern was not observed in *LjMATE1* control nodules (Fig. 6E, F). To observe Fe localization in more detail with nodule sections, the iron staining was enhanced by secondary reaction with diaminobenzidine (DAB) and hydrogen peroxide (Roschttardtz et al. 2009). Fig. 7A and B clearly shows that Fe was accumulated mainly in the infection zone of wild-type nodules, especially in infected cells, where a large amount of *leghemoglobin* is accumulated. It is to be noted that almost no Fe accumulation was observed in the root vascular bundle of wild-type plants (Fig. 7C). In contrast, Fe localization in nodules of *ljmate1* was seen in infected cells, but the level was much lower, in particular in the central zone (Fig. 7D, E). Another clear difference was observed in the root vascular bundle of *ljmate1*, where high Fe deposition was detected (Fig. 7F). The Fe accumulation pattern in nodules of *LjMATE1* control plants (Fig. 7G–I) was almost the same as seen in wild-type nodules. Similar phenotypes were also observed in the hairy root transformants (Supplementary Fig S2).

For quantitative comparison, we determined the Fe content in nodules using inductively coupled plasma mass spectrometry (ICP-MS). The Fe content in the nodules of stable *ljmate1* was significantly lower compared with that in wild-type plants (Fig. 8), whereas the content of other metals examined was not significantly different between wild-type and *ljmate1* plants (Fig. 8). These results strongly suggested that *LjMATE1* is involved in Fe translocation in nodules by releasing citrate, which is essential to supporting SNF.

**Discussion**

Fe storage is of a special importance in nodules, especially in infected cells, because of a high level of accumulation of...
leghemoglobin and nitrogenase, both of which require Fe for their activity. Several studies demonstrated partial mechanisms of Fe accumulation in nodules. For instance, Fe (II) transport activity across the peribacteroid membrane was measured in soybean nodules (Moreau et al. 1998), and later a transporter that mediates Fe movement at the peribacteroid membrane was identified as GmDMT1 (Kaiser et al. 2003). Fe (III) is also provided across the peribacteroid membrane when chelated with several organic acids such as citrate, and accumulates in the peribacteroid space, where Fe (III) reductase activity is present (LeVier et al. 1996). In contrast to Fe transport across the peribacteroid membrane, the mechanisms of Fe translocation from root tissues to nodules, especially to infected cells, have been unknown. In this study, we characterized LjMATE1 and found that this MATE-type protein mediates Fe translocation into infected cells by releasing citrate into the apoplast in the infection zone.

Expression analysis by quantitative PCR showed that LjMATE1 expression was induced at 7 dpi and reached a maximum at 12 dpi (Fig. 1B). This induction pattern is similar to the level of leghemoglobin and nitrogenase activity. Moreover, promoter analysis revealed that expression of LjMATE1 was restricted to the infection zone of nodules, suggesting that LjMATE1 is directly involved in SNF.

The physiological role of LjMATE1 was demonstrated by generating transgenic plants in which the expression of LjMATE1 was suppressed by RNAi. Previous reports on Fe-deficient mutants in Arabidopsis frd3 and rice osfrd1 showed chlorosis in cotyledons or leaves, especially under low Fe conditions (Durrett et al. 2007, Yokosho et al. 2009). However, the knock-down line ljmate1 generated in this study did not show such a phenotype in nitrogen-sufficient conditions, as LjMATE1 functions exclusively in nodules as indicated in the expression analysis (Fig. 1A). When ljmate1 plants were inoculated with rhizobia under nitrogen-deficient conditions, growth was apparently suppressed compared with the wild type, while growth was rescued by supplementation with nitrogen (Fig. 4C). In addition, ljmate1 showed many greenish nodules (Fig. 5B, D), which are known to be inactive in SNF. Indeed, ARA of ljmate1 nodules was significantly reduced (Fig. 5F), indicating that LjMATE1 is required for SNF, and suppression of LjMATE1 caused fix⁻ nodules.

DAB-enhanced Perls staining was first applied in plant tissue to detect Fe accumulation in Arabidopsis embryos, in which single Perls staining alone was too weak (Roschtztardt et al. 2009). In this study, we have employed DAB enhancement to monitor the Fe accumulation in nodule sections. In wild-type nodules, Fe accumulation was detected mainly in the infection zone.
zone, which is consistent with the physiological functions of Fe in nodules (Fig. 7A, B). In contrast, in ljmate1 nodules, Fe staining was apparently fainter in the infection zone of nodules, particularly in the central region (Fig. 7D, E). We then determined Fe contents in nodules using ICP-MS, and found a reduced Fe concentration in ljmate1 nodules (Fig. 8). These results strongly suggest the involvement of LjMATE1 in Fe accumulation in nodules by increasing Fe mobility. Moreover, unusual Fe deposition was seen in root vascular bundles in ljmate1 plants (Figs. 6D, 7F), which may be a secondary phenomenon of stagnant Fe delivery into nodules, as the expression of LjMATE1 is restricted to the infection zone of nodules (Fig. 1C–H). A spatial difference between the relevant gene expression and Fe accumulation in tissues was also observed in rice (Kobayashi et al. 2010), i.e. rice OsYSL2 functioning as a transporter of the Fe–nicotianamine complex is expressed mainly in the phloem cells of leaves and leaf sheaths, whereas the RNAi lines of OsYSL2 showed increased Fe accumulation in roots (Koike et al. 2004, Ishimaru et al. 2010).

Plants utilize highly sophisticated mechanisms for Fe uptake and translocation among organs. As naturally abundant Fe (III) is hardly water soluble, plants secrete various molecules to increase Fe solubility, for instance nicotianamine and citrate to form Fe–chelator complexes (Conte and Walker 2011). The accumulation of nicotianamine in nodules has not been reported; however, Hakoyama and co-workers showed that a nicotianamine synthase (NAS), which catalyzes the conversion of S-adenosylmethionine to nicotianamine, was expressed in a late stage of nodulation in L. japonicus, and they postulated that LjNAS2 is involved in Fe reuse from inactive nodules to other organs (Hakoyama et al. 2009). It is reported that citrate, which also serves as a chelator of Fe, accumulates in nodules, while the citrate content decreases to approximately 60% when the plant is inoculated by ineffective Rhizobium (Rosendahl et al. 1990). In this study, we could not demonstrate the transport direction of citrate by LjMATE1, but we presume that LjMATE1 effluxes citrate at the plasma membrane into the apoplast for the following reasons. (i) Fe accumulation was decreased in the infected cells when LjMATE1 expression was suppressed (Fig. 7, 8); and (ii) Fe movement in the peribacteroid space is possible without citrate, where Fe (III)–chelator complex can be reduced to Fe (II) and/or bound with siderophores released by the bacteroid (LeVier et al. 1996, Wittenberg et al. 1996). For the uptake of the Fe–citrate complex into infected cells, other metal transporters may be involved. In fact, metal transporter genes, such as Zrt/Irt-like protein (ZIP) and natural resistance-associated macrophage protein (Nramp), were up-regulated during nodulation in our transcriptomic analysis, in addition to LjMATE1 (Takanashi et al. 2012b). Detailed characterization of these transporters will enable us to decipher the overall scheme of Fe exchange between bacteroids and plant cells.
Materials and Methods

Plant materials and growth conditions

Lotus japonicus MG-20 Miyakojima was used in this work. Seeds were surface-sterilized with a 1% sodium hypochloride solution for 10 min, rinsed five times with sterile distilled water, then germinated on water agar plates (0.8%). Five-day-old seedlings were transferred on sterile vermiculite with liquid 1/2 B&D medium (Broughton and Dilworth 1971) in a plant box and grown in a cultivation chamber under a 16 h day/8 h night cycle at 23°C/14°C.

Expression analysis with rhizobia

To obtain RNA samples, 9-day-old seedlings were inoculated with *M. loti* MAFF303099, which was cultured overnight in TY medium at 28°C. For organ-specific expression analysis, plants at 19 dpi were sampled. Time-course analysis was conducted by collecting aerial parts and underground parts, respectively, at 0, 2, 4, 7, 12 and 19 dpi. Reverse transcription was carried out using SuperscriptIII Reverse Transcriptase (Invitrogen), followed by semi-quantitative PCR (95°C for 1 min, 32 cycles at 95°C for 20 s, 55°C for 30 s and 72°C for 20 s) using Go Taq DNA polymerase (Promega) with a set of primers specific to LjMATE1 (forward primer, 5’-GCTACACAAACCCATCAAT CG-3’ and reverse primer, 5’-TGCAATGAGACCACATCAC CA-3’). Ubiquitin (forward primer, 5’- ATGCAGATCTTCGAAGACCTTGAC-3’ and reverse primer, 5’- ACCTCCCCTCAGACGAAGGA-3’) was used as an internal control. For quantitative PCR (95°C for 15 min, 40 cycles at 94°C for 10 s, 56°C for 20 s and 72°C for 25 s), a DyeNAMO HS SYBR Green qPCR Kit (Finnzymes) was used. Real-time detection of PCR products was performed using Roter-Gene 3000A (Corbett Research).

Histochemical analysis of the promoter–GUS transformant of *L. japonicus*

A fragment of 1.7 kb upstream of the start codon of LjMATE1 was amplified from genomic DNA of MG-20 using Phusion High-Fidelity DNA Polymerase (Finnzymes) with a set of primers specific to LjMATE1 (forward primer, 5’-GCTACACAAACCCATCAAT CG-3’ and reverse primer, 5’-TGCAATGAGACCACATCAC CA-3’). Ubiquitin (forward primer, 5’- ATGCAGATCTTCGAAGACCTTGAC-3’ and reverse primer, 5’- ACCTCCCCTCAGACGAAGGA-3’) was used as an internal control. For quantitative PCR (95°C for 15 min, 40 cycles at 94°C for 10 s, 56°C for 20 s and 72°C for 25 s), a DyeNAMO HS SYBR Green qPCR Kit (Finnzymes) was used. Real-time detection of PCR products was performed using Roter-Gene 3000A (Corbett Research).
specific primers: forward primer, 5′-ACAAGTTTGTACAAAAAAGCAGGCTCTTGGAAGGGGCTGTCTTTT-3′; reverse primer, 5′-ACCACTTTGTACAAGAAAGCTGGGTGATATCCTAAATCTTATGTAA-3′ (the underlined positions are non-native sequences of the attB recombination sites). The PCR product was subcloned into pDONR/Zeo (Invitrogen), and then transferred into a Gateway-compatible binary vector, pGWB3 (Nakagawa et al. 2007). The construct was then introduced into *L. japonicus* using hairy root transformation mediated by *Agrobacterium rhizogenes* LBA1334, as previously reported (Kumagai and Kouchi 2003, LBA1334 was imported on license from Ministry of Agriculture, Forestry and Fisheries of Japan).

The transformed plants were inoculated with *M. loti* and grown in a cultivation chamber under the conditions mentioned above. Nodules were sampled at 7, 21 and 35 dpi, respectively, and GUS staining was performed as described previously (Takanashi et al. 2011).

**Transport assay in Xenopus oocytes**

The full-length cDNA of *LjMATE1* was cloned into the oocyte vector pXbG-evil. The plasmid was linearized with *SacII*, and cRNA was transcribed in vitro with T3 RNA polymerase (mMESSAGE mMACHINE kit; Ambion). Oocytes were isolated from adult female *Xenopus laevis* as described before (Ma et al.)
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vector: forward primer, 5'-ACAAGTTTGTACAAAAAAGCAGGC-TGTACAAGAAAGCTGGGT-3'. Reverse primer, 5'-ACCACTTGTTAGTGCAACCAATTGTTGT-3'. The PCR product was subcloned into pDONR/Zeo and then transferred into a Gateway-compatible binary vector, pUB-GWS-Hyg (Maekawa et al. 2008). The construct was used for plant transformation as described previously (Thykjaer et al. 1997). For phenotypic analysis, the wild type and T2 generation were grown either with supplementation of nitrogen without rhizobia inoculation, or under nitrogen-deficient conditions with rhizobia inoculation.

**LjMATE1 RNAi transformants of L. japonicus**

For LjMATE1 suppression by RNAi, a 245 bp fragment (position 226–471) was amplified from the LjMATE1 cDNA using a set of primers containing attB recombination sites for the Gateway vector: forward primer, 5'-TGTAGACCTGCTTGGGAGTAGCT-3' and reverse primer, 5'-AGGGCATGCAAAAACCAGAAAAC-3'. The quantitative PCR conditions were as described above. For hairy root transformation, the RNAi fragment in pDONR/Zeo was transferred into a binary vector, pUB-GWS-GFP (Maekawa et al. 2008). Transformed hairy roots, selected with green fluorescent protein (GFP) signal, were inoculated with M. loti and sampled at 28 dpi.

**Perls staining**

For Perls staining, pink nodules were vacuum-infiltrated with equal volumes of 4% (v/v) HCl and 4% (w/v) potassium ferrocyanide for 15 min, and incubated for 30 min at room temperature (Roschttardtz et al. 2009). For signal intensification with DAB and H2O2, Perls-stained nodules were fixed and embedded in Technovit 7100 as previously reported (Takanashi et al. 2012a). Cut sections (15 µm) were placed on glass slides, and then the intensification procedure was performed as described by Meguro et al. (2007).

**Measurement of the Fe content in nodules**

Seeds were germinated on a water agar plate. Five-day-old seedlings were transferred to sterile vermiculite with liquid 1/2 B&D medium in a plant box. After 2 d, plants were inoculated with bacteria. At 28 dpi, pink nodules were collected and dried in an oven at 70°C for 2 d. Samples were then subjected to digestion.

Fig. 8 The content of Fe and other metals in L. japonicus nodules determined by ICP-MS. Nodules of wild-type and ljmate1 plants were sampled at 28 dpi. The Fe concentration of ljmate1 was reduced to 56.8% of that of the wild type. Values represent the mean ± SD (n = 3). *P < 0.05 compared with the wild type by Student’s t-test.
with 0.5 ml of 61% HNO3 in a 2 ml plastic tube. The samples were heated to 125°C for 5 h and then diluted with 5% HNO3. The metal concentration was determined by ICP-MS (7700X; Agilent Technologies).

**Supplementary data**

**Supplementary data** are available at PCP online.

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