MtNramp1 mediates iron import in rhizobia-infected *Medicago truncatula* cells.

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ABSTRACT
Symbiotic nitrogen fixation is a process that requires relatively high quantities of iron provided by the host legume. Using synchrotron-based X-ray fluorescence, we have determined that this iron is released from the vasculature into the apoplast of zone II of *M. truncatula* nodules. This overlaps with the distribution of MtNramp1, a plasma membrane iron importer. The importance of MtNramp1 in iron transport for nitrogen fixation is indicated by the 60% reduction of nitrogenase activity observed in knock-down lines, most likely due to deficient incorporation of this essential metal cofactor at the necessary levels.

INTRODUCTION
Iron is an essential growth-limiting micronutrient for plants (Grotz and Guerinot, 2002). Most of the proteins (nitrogenase, leghemoglobin, etc…) involved in symbiotic nitrogen fixation require iron cofactors (Udvardi and Day, 1997). In spite of this very little is known about how iron reaches the nodule and how it is incorporated by rhizobia-containing cells. Here we show that iron is released in the infection/maturation areas of indeterminate nodules, and that a Nramp transporter introduces it into the infected cells.

MATERIALS AND METHODS
Synchrotron-based X-ray fluorescence
X-ray fluorescence assays were performed as described by Rodríguez-Haas *et al* (2013).
Plant transformation.
M. truncatula plants were transformed with *Agrobacterium rhizogenes* as indicated (Boisson –Dernier *et al.* 2001).
MtNramp1 localization.
Promoter:GUS and confocal immunofluorescence studies were performed as described (Limpens *et al.* 2009).
Generation of knock-down plants
The first 450 bp of *MtNramp1* cDNA sequence were cloned in pENTR1A and transferred using Gateway Technology to pFRN.
Nitrogenase activity measurements.
Nitrogenase activity was measured as described (Ruíz-Argüeso *et al.* 1979).

RESULTS AND DISCUSSION
There are three possible mechanisms for iron transfer to the nodule: i) increased uptake from the epidermis, ii) use of accumulated stores from the nodule primordia, and iii) long-distance transport from the vasculature. These possibilities would present different iron distribution patterns, and these could be determined by means of synchrotron-based

X-ray fluorescence. The data showed that iron is transported by the vasculature and accumulates in the apoplast of the zone II of the nodule (Fig. 1A). This also indicates that plasma membrane iron importers must be present in these cells to introduce apoplastic iron into the cytosol.

In a previous microarray analysis we identified *MtNramp1* as a metal transporter gene induced more than 7-fold by nodulation. This result was verified by qPCR. MtNramp1 was able to complement the phenotype of the *fet3/fet4* yeast mutant that has a reduced capability of incorporating iron from the medium. Promoter:GUS studies of MtNramp indicated that it is expressed in the apical region of the nodule, coincidental with zone II, where iron is being released into the apoplast. This was verified with immunolocalization of HA-tagged MtNramp1 using confocal microscopy (Fig. 1B). It appeared that MtNramp1 is localized in the plasma membrane of cells of nodule zone II. All these results suggest that MtNramp1 could be the transporter that introduces apoplastic iron into the infected cells that would be used for nitrogen fixation. This was tested by obtaining knock-down lines and determining the nitrogenase activity of their nodules, since nitrogenase is one of the most abundant iron proteins in the nodule. On average, a 90 % reduction of expression levels was achieved that caused a 60 % reduction of nitrogenase activity (Fig. 1C), supporting a role of MtNramp1 in symbiotic iron transport.

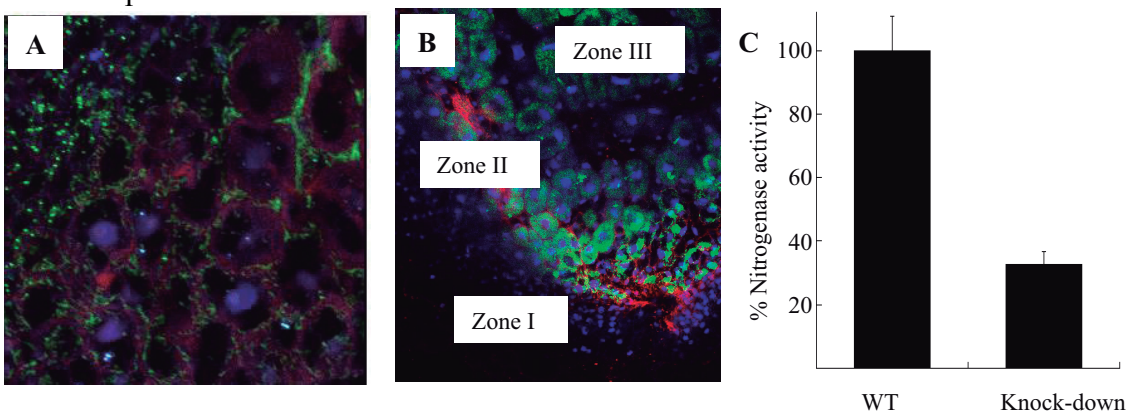

Figure 1. Iron transport in *M. truncatula nodules*. (A) Elemental distribution in Zone II. Iron is in green, zinc in blue and calcium in red. (B) Immunolocalization of MtNramp1. Blue is DAPI stained DNA, green indicates GFP expressing rhizobia and red is MtNramp1-HA labelled with anti-HA mouse antibody conjugated to Alexa594. (C) Nitrogenase activity in wild type and *MtNramp1* knock-down nodules.

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