

# Medicago truncatula Ferroportin2 mediates iron import into nodule symbiosomes

Viviana Escudero, Isidro Abreu, Manuel Tejada-Jiménez, Elena Rosa-Núñez, Julia Quintana, Rosa Isabel Prieto, Camille Larue, Jiangqi Wen, Julie Villanova, Kirankumar S. Mysore, et al.

# ▶ To cite this version:

Viviana Escudero, Isidro Abreu, Manuel Tejada-Jiménez, Elena Rosa-Núñez, Julia Quintana, et al.. Medicago truncatula Ferroportin2 mediates iron import into nodule symbiosomes. New Phytologist, 2020, 10.1101/630699 . hal-02997924

# HAL Id: hal-02997924 https://hal.science/hal-02997924

Submitted on 10 Nov 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

### 1 Medicago truncatula Ferroportin2 mediates iron import into nodule symbiosomes

- 2 Viviana Escudero<sup>a</sup>, Isidro Abreu<sup>a</sup>, Manuel Tejada-Jiménez<sup>a</sup>, Elena Rosa-Núñez<sup>a</sup>, Julia
- 3 Quintana<sup>b</sup>, Rosa Isabel Prieto<sup>a</sup>, Camille Larue<sup>c</sup> Jianqi Wen<sup>d</sup>, Julie Villanova<sup>e</sup>, Kirankumar
- 4 S. Mysore<sup>d</sup>, José M. Argüello<sup>b</sup>, Hiram Castillo-Michel<sup>f</sup> Juan Imperial<sup>g</sup>, Manuel González-
- 5 Guerrero<sup>a,h,1</sup>
- 6 <sup>a</sup> Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Universidad Politécnica de
- 7 Madrid, 28223 Pozuelo de Alarcón (Madrid), Spain.
- <sup>b</sup> Worcester Polytechnic Institute, Worcester MA01609, USA.
- 9 <sup>c</sup> EcoLab, Université de Toulouse, CNRS, Toulouse, France
- <sup>d</sup>Noble Research Institute, Ardmore, OK73401, USA.
- <sup>e</sup> ID16 Beamline. European Synchrotron Radiation Facility, Grenoble 38043, France.
- <sup>12</sup> <sup>f</sup>ID21 Beamline. European Synchrotron Radiation Facility, Grenoble 38043, France.
- <sup>g</sup> Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, 28006
  Madrid, Spain.
- 15 <sup>h</sup> Escuela Técnica Superior de Ingeniería Agronómica, Alimentaria y de Biosistemas,
- 16 Universidad Politécnica de Madrid, 28040 Madrid, Spain.
- 17
- 18 <sup>1</sup> Address correspondence to <u>manuel.gonzalez@upm.es</u>

19

20 Short Title: MtFPN2 delivers iron to symbiosomes

21

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instruction for Authors (www.plantcell.org) is: Manuel González-Guerrero (manuel.gonzalez@upm.es).

25

26

# 27 ABSTRACT

28 Iron is an essential cofactor for symbiotic nitrogen fixation. It is required by many of the enzymes facilitating the conversion of  $N_2$  into  $NH_4^+$  by endosymbiotic bacteria living 29 30 within root nodule cells, including signal transduction proteins, O<sub>2</sub> homeostasis systems, 31 and nitrogenase itself. Consequently, host plants have developed a transport network to 32 deliver essential iron to nitrogen-fixing nodule cells. Model legume Medicago truncatula 33 Ferroportin2 (MtFPN2) is a nodule-specific gene that encodes an iron-efflux protein. 34 MtFPN2 is located in intracellular membranes in the nodule vasculature, and in the 35 symbiosome membranes that contain the nitrogen-fixing bacteria in the differentiation and 36 early-fixation zones of the nodules. Loss-of-function of MtFPN2 leads to altered iron 37 distribution and speciation in nodules, which causes a reduction in nitrogenase activity and 38 in biomass production. Using promoters with different tissular activity to drive MtFPN2 39 expression in *MtFPN2* mutants, we determined that MtFPN2-facilitated iron delivery 40 across symbiosomes is essential for symbiotic nitrogen fixation, while its presence in the 41 vasculature does not seem to play a major role in in the conditions tested.

- 42
- 43
- 44
- 45
- 46
- 47
- 48
- 49
- 50
- -
- 51
- 52
- -
- 53

## 54 **INTRODUCTION**

55 Iron is an essential cofactor for enzymes participating in almost every plant 56 physiological process, including photosynthesis, respiration, and stress tolerance 57 (Marschner, 2011). Consequently, when lacking, a plethora of iron-dependent processes is 58 affected. Damaging as low iron levels are, so are slightly higher ones. In that situation, iron 59 becomes toxic, catalysing the production of free radicals in Fenton-style reactions and 60 competing with other essential metal nutrients for the active site of proteins (Goldstein et 61 al., 1993; Lin et al., 2011). To maintain iron homeostasis within these narrow physiological 62 levels, plants have developed a complex system to optimize iron uptake and delivery from 63 soil, while preventing toxicity. This system involves metal transporters that recover this 64 nutrient from soil and deliver it to shoots and seeds (Korshunova et al., 1999; Curie et al., 65 2001; Vert et al., 2002; Morrissey et al., 2009; Zhai et al., 2014), small soluble molecules 66 that coordinate iron and keep it soluble in plant fluids (von Wiren et al., 1999; Rogers and 67 Guerinot, 2002; Roschzttardtz et al., 2011; Flis et al., 2016), and a complex regulatory 68 network (Colangelo and Guerinot, 2004; Long et al., 2010; Sivitz et al., 2012; Kumar et al., 69 2017; Grillet et al., 2018). While in most vegetative growing plants, the main iron sinks are 70 the leaves, legumes may have additional ones in differentiated root organs known as 71 nodules (Brear et al., 2013; González-Guerrero et al., 2014). Representing just 5% of the 72 total biomass of the plant, nodules contain a large part of the plant iron content (González-73 Guerrero et al., 2014; Tejada-Jiménez et al., 2015). Therefore, it is to be expected that 74 legumes have adapted their pre-existing iron homeostasis systems to accommodate nodule 75 development and function.

76 Nodules are the result of the interaction between legumes and rhizobia, a diverse group of soil bacteria capable of converting, fixing, N<sub>2</sub> into NH<sub>4</sub><sup>+</sup> when in symbiosis 77 78 (Downie, 2014). Following a complex signal exchange between the symbionts (Vernié et 79 al., 2015), nodule primordia emerge from the roots by proliferation of pericycle and inner 80 cortex cells (Xiao et al., 2014). While the nodule develops, rhizobia in the root surface are 81 surrounded by a root hair that invaginates and directs, via an infection thread, the bacteria 82 from the root exterior to the inner nodule regions (Gage, 2002), where they are released in 83 an endocytic-like process (Limpens et al., 2009; Huisman et al., 2012). Within these 84 pseudo-organelles (symbiosomes), rhizobia differentiate into bacteroids (Sutton et al.,

85 1981; Kereszt et al., 2011), and start fixing nitrogen, transferring fixed NH<sub>3</sub> to the host 86 plant in exchange for photosynthates and mineral nutrients (Udvardi and Poole, 2013). 87 Nodules can be classified according to their development in determinate and indeterminate 88 (Sprent, 2007). The former, as illustrated by soybean nodules, has a limited growth, while 89 the later (developed by pea or *Medicago*), maintains the apical meristem and has a longer 90 growth period. Due to this indeterminate nodule growth, four nodule zones are established, 91 tracing along its length the developmental stages of nodule maturation. These zones are: the 92 meristem (zone I), the zone where the nodule cells are infected by rhizobia and they 93 differentiate into bacteroids (zone II), the  $N_2$  fixation zone (zone III), and in nodules of a 94 certain age, the senescent zone (zone IV) (Vasse et al., 1990). In addition, some authors 95 also differentiate an interzone between zones II and III in which O<sub>2</sub> levels drop and 96 nitrogenase is starting to be synthesized (Roux et al., 2014).

97 Nodule development is highly sensitive to iron levels, as suggested by its abundance 98 in these organs. Iron deficiency limits nodule number and maturation, as well as reduces 99 nitrogen fixation rates (O'Hara et al., 1988; Tang et al., 1990; Tang et al., 1992). This is the 100 consequence of many of the key enzymes involved in nodulation and nitrogen fixation 101 being ferroproteins synthesized at high levels (Brear et al., 2013; González-Guerrero et al., 102 2014). For instance, heme-containing NADPH-oxidases are important for nodule initiation 103 and development (Montiel et al., 2012; Montiel et al., 2016). Nitrogenase, the critical 104 enzyme for nitrogen fixation, is a protein complex with 34 iron atoms in the form of iron-105 sulfur (Fe-S) clusters and the unique iron-molybdenum cofactor (FeMoco) (Rubio and 106 Ludden, 2005). This enzyme is irreversibly inhibited by O<sub>2</sub>, while bacteroids must carry out 107 an aerobic metabolism (Preisig et al., 1996b). To make both processes compatible, legumes 108 express leghemoglobin (20% of the total nodule protein) which binds  $O_2$  with great affinity 109 (Appleby, 1984). This oxygen can be recovered and used by high-affinity rhizobial 110 cytochrome c oxidases (iron-copper proteins) (Preisig et al., 1996a; Preisig et al., 1996b). In 111 addition, a number of other iron-proteins are required for free radical control, cofactor 112 assembly, and general metabolic processes (Dalton et al., 1998; Rubio et al., 2004; Rubio 113 and Ludden, 2005; González-Guerrero et al., 2014).

114 To ensure the reinforced iron supply that symbiotic nitrogen fixation requires, 115 nodulation elicits the iron-deficiency response characteristic of dicots (Terry et al., 1991):

lowering the pH of the rhizosphere to increase Fe<sup>3+</sup> solubility, activating ferroreductase 116 activities to reduce  $Fe^{3+}$  to  $Fe^{2+}$ , and inducing the expression of  $Fe^{2+}$  importers of the ZIP 117 (Zrt1-, Irt1-like Proteins) and Nramp (Natural Resistance Associated Macrophage Protein) 118 119 transporter families (Andaluz et al., 2009; Kobayashi and Nishizawa, 2012). Once iron is in 120 the plant, it symplastically or apoplastically reaches the endodermis from where it will be 121 delivered by the vasculature to shoots and nodules. At the nodules, iron is released in the 122 apoplast of the infection zone of the nodule (Rodríguez-Haas et al., 2013), where it is kept 123 soluble as iron-citrate (Kryvoruchko et al., 2018), the most abundant complex in root to 124 shoot iron transport and when transferring it across symplastically disconnected tissues (Durrett et al., 2007; Roschzttardtz et al., 2011). Iron uptake into the infected cells of 125 126 Medicago truncatula is mediated primarily by MtNramp1, a transporter expressed in late 127 zone II and interzone and located in the plasma membrane (Tejada-Jiménez et al., 2015). 128 Transport into the symbiosomes is likely also facilitated by citrate, as indicated by the 129 requirement of citrate efflux protein MtMATE67 (Kryvoruchko et al., 2018). However, this 130 protein does not transport iron-citrate complexes, and therefore, another system must exist 131 for iron translocation. Different candidates have been proposed to be responsible for iron 132 delivery into the symbiosomes. The first was *Glycine max* Nramp protein GmDMT1 133 (Kaiser et al., 2003). However, although this protein was located in symbiosomes, its 134 direction of transport would be into the cytosol, as with all characterized Nramp proteins 135 (Nevo and Nelson, 2006). VIT1-CCC1-like protein SEN1 is another candidate to carry out 136 this role (Hakoyama et al., 2012), but although the direction in which iron is transported by 137 these proteins would be compatible (Li et al., 2001), there is no definitive evidence of a 138 symbiosome localization. Alternatively, a member of the ferroportin family (also known as 139 IREG proteins) could also be involved.

Ferroportins are membrane proteins that extrude iron from the cytosol, either outside the cell or into organelle (Morrissey et al., 2009; Drakesmith et al., 2015). In plants, ferroportins have been associated to iron delivery into the xylem (Morrissey et al., 2009). Interestingly, this is a process that would be coupled to MATE-mediated citrate efflux (FRD3 in *A. thaliana*) (Durrett et al., 2007), as iron is kept soluble in the xylem as an ironcitrate complex (López-Millán et al., 2000; Durrett et al., 2007). Considering the functional association of ferroportins and citrate efflux proteins in the xylem, we hypothesized that a 147 similar relationship would be established between nodule-specific citrate efflux protein 148 MtMATE67 and a ferroportin to deliver iron to nodule apoplast and to symbiosomes. As a 149 result, we have identified and characterized MtFPN2 (Medtr4g013240), one of the three 150 ferroportin-like genes encoded in the *M. truncatula* genome, and the only one with a 151 nodule-specific expression. MtFPN2 is located in the intracellular compartments in the 152 endodermis, and associated to symbiosomes in the infected cells. Mutation of MtFPN2 153 results in a severe reduction of nitrogenase activity, and in mis-localization of iron in the 154 nodules. These results support a role of MtFPN2 in iron delivery for symbiotic nitrogen fixation. 155

156

# 157 **RESULTS**

#### 158 *MtFPN2* is expressed in the nodule vasculature and interzone

159 Three ferroportin-like sequences could be found in the genome of *M. truncatula*: 160 Medtr1g084140 (MtFPN1), Medtr4g013240 (MtFPN2), and Medtr4g013245 (MtFPN3). Among them, *MtFPN2* was the only ferroportin to have a nodule-specific transcription in 161 162 the conditions tested (Figure 1A; Supplemental Figure 1). To determine the area of the 163 nodule where *MtFPN2* was expressed, the 2,144 bp DNA region immediately upstream of 164 *MtFPN2* start codon was used to drive the expression of the reporter  $\beta$ -glucuronidase (gus) 165 gene. After 28 days-post-inoculation (dpi), GUS activity was detected in a specific nodule 166 band close to the apical region, and in longitudinal bands along its major axis (Figure 1B). 167 Longitudinal sections of nodules showed that the apical GUS-activity region corresponded 168 to the late infection zone, interzone and early fixation zone (Figure 1C). Nodule cross 169 sections showed GUS activity in the nodule vasculature (Figure 1D). In these cross 170 sections, the lack of expression of MtFPN2 in the matured fixation zone was also 171 confirmed.

# 172 MtFPN2-HA is located in vascular intracellular compartments and in the 173 symbiosomes

Ferroportins have been associated to iron efflux from the cytosol (Kaplan, 2002).Their specific functional role is largely derived from the specific subcellular localization of

176 each protein. Should it be associated to the plasma membrane, its likely role would be iron 177 export for long-distance trafficking, such as FPN1 in enterocytes, or FPN1 in endodermal 178 cells (McKie et al., 2000; Morrissey et al., 2009). Alternatively, an intracellular localization 179 associated to organelle would mean a role in iron storage or metalation of specific 180 ferroproteins (Morrissey et al., 2009; Drakesmith et al., 2015). To determine the subcellular 181 distribution of MtFPN2 and gain insights into its function, three hemagglutinin (HA) 182 epitopes where fused to its C-terminus. The expression of this cassette was driven by the 183 same region previously used for the MtFPN2<sub>prom</sub>::gus fusions. Protein localization was 184 initially determined by confocal microscopy in which the epitopes were detected by an anti-185 HA monoclonal primary antibody and an Alexa594-conjugated (red) secondary antibody. 186 To facilitate visualization, the transformed plants were inoculated with a GFP-expressing 187 Sinorhizobium meliloti strain (green) and the sections stained with DAPI (blue) to visualize 188 DNA. Figure 2A shows that MtFPN2-HA localization corresponded to the expression 189 profile detected with the *MtFPN2*<sub>prom</sub>::gus fusions. A detailed view of the vascular region 190 revealed that MtFPN2-HA was located in the endodermis, pericycle and in the vascular 191 parenchyma, occupying large areas of the cytosol (Figure 2B). Visualization of infected cells in the differentiation zone also showed an intracellular localization of MtFPN2-HA, 192 193 closely related to the symbiosomes (Figure 2C). To confirm this putative symbiosome 194 localization, M. truncatula plants were transformed with a C-terminal GFP-fusion of 195 MtFPN2 driven by its own promoter and inoculated with a mCherry expressing S. meliloti. 196 Symbiosomes isolated from these nodules showed co-localization of rhizobia and MtFPN2-197 GFP (Supplemental Figure 2). To further verify the symbiosome localization of MtFPN2-198 HA, a gold-conjugated secondary antibody was used to determine the distribution of HA-199 tagged proteins using transmission electron microscopy. As indicated by the confocal 200 microscopy assays, gold particles were associated to symbiosome membranes (Figure 2D). 201 The same approach showed gold particles concentrated in large intracellular membrane 202 systems, very likely the endoplasmic reticulum in endodermal and vascular parenchyma 203 cells (Figure 2D). To confirm that none of these observations were artifactual, controls for 204 autofluorescence of the tagged proteins and specificity of the antibodies were carried out 205 (Supplemental Figure 3).

# 206 MtFPN2 is an iron efflux protein

207 To confirm that MtFPN2 was capable of transporting iron, we tested its ability to 208 restore the wild-type growth of a yeast mutant lacking the CCC1 protein. This transporter 209 introduces excess cytosolic iron into the vacuole to prevent toxicity (Li et al., 2001). Loss 210 of CCC1 function resulted in lower iron tolerance when the media was supplemented with 211 2.5 mM FeSO<sub>4</sub>. However, when this strain was transformed with a plasmid expressing the 212 coding sequence of MtFPN2, the sensitivity of the mutant strain to high iron was 213 diminished (Figure 3), suggesting that MtFPN2 was able to participate in its detoxification. 214 Addition of the HA-tag used for the immunolocalization studies did not affect MtFPN2 215 activity in yeast (Supplemental Figure 4).

# 216 MtFPN2 is required for nitrogenase activity in nodules

217 Should MtFPN2 be responsible for iron delivery to the nodules, its mutation should 218 lead to a reduction of nitrogenase activity, as a consequence of an iron-limited cofactor 219 synthesis. To explore this possibility, the *Tnt1* insertion mutant NF11374 (*fpn2-1*) was 220 obtained from the Noble Research Institute (Tadege et al., 2008). This line has an insertion 221 in the fourth exon of MtFPN2 (Figure 4A), resulting in no transcript being detected by RT-222 PCR. *MtFPN2* loss-of-function had no significant effect on plant growth or chlorophyll 223 content when the plants had not been inoculated and nitrogen was provided as ammonium 224 nitrate in the nutrient solution (Supplemental Figure 5). However, when the plants were 225 inoculated and nitrogen had to be obtained from the endosymbiotic rhizobia, removing 226 MtFPN2 function led to severe growth reduction (Figure 4B). Nodule development was 227 also altered, with a large portion (64%) of the nodules having white colour (Figure 4C, 4D), 228 indicative of non-functionality. Closer observation of these nodules using either toluidine 229 blue-stained sections or tomographic reconstructions from X-ray analyses, showed that the 230 white fpn2-1 nodules did not develop the fixation zones further than a few cell layers (Supplemental Figure 6), and that even the fewer nodules with a pink-colour had a reduced 231 232 Zone III. In addition, the symbiosomes appeared disorganized in the cytosol of *fpn2-1* 233 nodule cells, with a number of vacuoles, in contrast to the ordered, radial and peripheral 234 distribution in the wild type plants. As expected by the plant and nodule phenotypes, 235 biomass production was significantly lower in *fpn2-1* plants than in wild-type plants 236 (Figure 4E). Nitrogen fixation capabilities (nitrogenase activity) of the mutant nodules were 237 approximately 80% lower than those from wild-type plants (Figure 4F). These phenotypes

were the likely result of iron not reaching the nodules in high-enough levels, as indicated by the lower iron content of *fpn2-1* nodules (Figure 4G). Introducing into *fpn2-1* the coding sequence of *MtFPN2* regulated by the 2,144 bp region upstream its start codon restored the wild-type phenotype. However, fortifying the nutrient solution with additional iron did not have the same effect (Supplemental Figure 7).

243 Consistent with the phenotype arising from altered iron delivery to the nodules, 244 synchrotron-based micro-X-ray fluorescence studies of wild-type and *fpn2-1* nodules 245 showed altered distribution of this element (Figure 5A). MtFPN2 mutation led to less iron 246 being allocated to the early fixation zones of the nodules, in those cases where the region 247 was marginally developed (pink *fpn2-1* nodules). In the case of fully white nodules, no iron 248 distribution indicative of ordered symbiosomes (iron-rich ring-like structures) was 249 observed, consistent with no functional zone III being fully developed. This alteration in 250 iron distribution had an impact on iron speciation as determined by micro-X-ray Absorption 251 Near-Edge Spectroscopy (µXANES) on nodule sections. Different iron spectra were 252 obtained from wild-type and mutant nodules (Figure 5B). Principal component analyses of 253 the obtained spectra in the infection/differentiation zone, fixation zone and vascular region 254 showed great iron speciation variation between wild-type and white *fpn2-1* nodules (Figure 255 5B). Interestingly, iron speciation in pink fpn2-1 nodules showed an intermediate state 256 midway from wild-type and white *fpn2-1* nodules. Although the complex iron speciation in 257 nodules and the lack of a suitable number of spectra from many of these forms precludes a 258 detailed description of the precise chemical species present in these organs, it was 259 interesting to notice that the proportion of iron coordinated by sulfur (Fe-S clusters, 260 including the iron-molybdenum cofactor of nitrogenase) was different in wild type, pink 261 *fpn2-1*, and white *fpn2-1* nodules (Supplemental Table 1).

Considering the dual localization of MtFPN2 in the vasculature and in the symbiosomes, the reported phenotypes could be due to either iron not being released from the vessels and/or from it not getting across the symbiosome membrane. In an attempt to separate both causes, MtFPN2 coding sequence was cloned under the MtMOT1.3 promoter, a nodule-specific gene that we had previously located in the interzone and early fixation zone (Tejada-Jiménez et al., 2017). Mutants *fpn2-1* were transformed with this construct to test whether expressing MtFPN2 only in the nodule cortex was sufficient to restore the

269 phenotype. As shown in Figure 6,  $MOT1.3_{nrom}$  driven expression of MtFPN2 resulted in a 270 partial improvement of plant growth (Figure 6A), with the development of functional pink 271 nodules in significantly higher numbers than fpn2-1 (Figures 6B and C). Overall, these 272 plants showed improved biomass production compared to *fpn2-1* (Figure 6D), albeit not 273 enough to reach wild-type levels. The improved iron delivery to *fpn2-1* led to significantly 274 higher nitrogen fixation rates per nodule (Figure 6E). In contrast, when MtFPN2 expression 275 was driven by the promoter of vascular gene *MtNOOT1* (Magne et al., 2018), no significant 276 differences were observed in terms of biomass production, nodule development, or 277 nitrogenase activity with those that do not express it at all (Supplemental Figure 8).

278

# 279 **DISCUSSION**

280 Symbiotic nitrogen fixation carried out by the legume-rhizobia systems is one of the 281 main sources of assimilable nitrogen in natural ecosystems and in agrosystems (Vance, 282 2001; Herridge et al., 2008). A key element in crop rotation strategies to increase nitrogen 283 content in soils, symbiotic nitrogen fixation can minimize the use of polluting and 284 expensive nitrogen fertilizers. Unveiling the molecular bases of this process is critical to 285 improve nitrogen fixation rates and to introduce these capabilities to non-legume crops 286 (Oldroyd and Dixon, 2014; Mus et al., 2016). However, for symbiotic nitrogen fixation to 287 be efficiently used in sustainable agriculture or to extend it to other systems, close attention 288 has to be paid to the mechanisms of delivery of essential, limiting nutrients, such as iron.

289 Nitrogen fixation, oxygen homeostasis, reactive oxygen species production and 290 control, and several other nodule physiological processes rely on iron (Brear et al., 2013; 291 González-Guerrero et al., 2014; González-Guerrero et al., 2016), a limiting nutrient in large 292 areas of the world (Hirschi, 2009; White and Broadley, 2009). Therefore, nodules must 293 ensure a steady supply of this element. While free-living diazotrophs can obtain iron 294 directly from soil, endosymbiotic ones must obtain it from the host (Rodríguez-Haas et al., 295 2013; Udvardi and Poole, 2013). This process was likely adapted from the pre-existing 296 long-distance iron transport systems. Consequently, we might expect similar mechanisms 297 to those dedicated for iron delivery to the shoots, with the added handicap of the nodules 298 becoming a second sink of an already scarce nutrient. Further supporting this hypothesis is

the existence of a nodule-specific iron-regulated citrate efflux protein that showcases the importance of iron-citrate complexes in apoplastic iron solubility and in iron supply to symbiosomes (Kryvoruchko et al., 2018). Since ferroportins were associated to citratemediated long-distance iron supply (Morrissey et al., 2009), we speculated on a putative role for these proteins in symbiotic nitrogen fixation.

304 Model legume *M. truncatula* encodes three ferroportin-like proteins. One of them, 305 MtFPN1, closely resembles the Mar1 protein located in the chloroplasts of Arabidopsis, 306 that plays an undefined role in iron homeostasis in plants, as well as serving as entry-point 307 for antibiotics (Conte et al., 2009). Similarly to *Mar1*, *MtFPN1* was expressed in the whole 308 plant. The other two are orthologues to A. thaliana FPN1 and FPN2. While MtFPN3 was 309 expressed in most plant organs, MtFPN2 transcripts were only detected in two discrete 310 nodule regions: associated to the nodule vasculature, and from late Zone II to early Zone 311 III. This dual localization indicates a dual function related with iron efflux from the cytosol, 312 as inferred from the yeast complementation assays and from the available information on 313 the biochemistry of this family of transporters.

314 In the vasculature, MtFPN2 would introduce iron into the lumen of an 315 endomembranous compartment. Considering the relatively large amount of iron that has to 316 be transferred across the nodule vessels into the nodules (González-Guerrero et al., 2014; 317 González-Guerrero et al., 2016), it could be argued that the endomembranes would serve as 318 an iron buffer, storing it prior to being delivered to the apoplast of the infection zone or to 319 be recycled back to the host in older parts of the nodule. It could also be hypothesized that 320 the iron concentrated in intracellular compartments could be released in the apoplast by 321 exocytosis, as it has been proposed for phosphate transport mediated by the PHO1 protein 322 (Arpat et al., 2012). It is unlikely that MtFPN2 would participate in a direct translocation of 323 iron from the cytosol across the plasma membrane, since no signal was observed using 324 immunolocalization and transmission electron microscopy. In contrast, the localization of 325 MtFPN2 in the symbiosome provides a clear insight into a key function for MtFPN2: 326 mediating iron delivery to the bacteroids.

327 Several evidences in addition to the expression and localization data support this 328 role. Loss of *MtFPN2* function leads to a reduction of nitrogen fixation capabilities, with

11

329 negative effects on plant growth, biomass production, and nodule development. 330 Nitrogenase activity is highly dependent on iron delivery, due to its large requirements of 331 iron for its three cofactors (Rubio and Ludden, 2005). Altering iron uptake by rhizobia-332 infected nodule cells presented a similar phenotype, as shown when characterizing the 333 nramp1-1 M. truncatula mutant (Tejada-Jiménez et al., 2015). In addition, iron delivery to 334 fpn2-1 nodules is affected in terms of total concentration, distribution in the nodule, and 335 speciation, indicating a large disturbance in nodule iron homeostasis. Altered nodule 336 development has also been reported when legumes were grown in iron-deficient substrates 337 (O'Hara et al., 1988; Tang et al., 1990; Tang et al., 1992), or when iron delivery was altered 338 by mutating iron transporters (Hakoyama et al., 2012; Tejada-Jiménez et al., 2015), or the 339 iron-chelation system (Stephens et al., 2011; Kryvoruchko et al., 2018). The changes in iron 340 speciation in *fpn2-1* nodules would be the consequence of several factors: i) iron being 341 stored or retained by iron-detoxification systems as a consequence of not reaching the 342 proper acceptors, ii) loss of signal from ferro-proteins that have not received their cofactor, 343 iii) mismetallation of proteins due to iron being retained in different compartments. 344 Although attempts were made to fit the spectra obtained with different iron-complex 345 reference standards, the complexity and number of these species made it extremely difficult 346 to conclude anything other than a reduction of iron-sulfur species in nodules, which would 347 very likely include the iron-sulfur clusters of nitrogenase. This phenotype was stronger in 348 white non-functional nodules, but also present in those with wild type colour, a leaky 349 phenotype that was also observed with *MtNramp1* mutants (Tejada-Jiménez et al., 2015) 350 and that might indicated the existence of secondary systems able to deliver, with low 351 affinity or non-specifically, some iron for nitrogen fixation in nodules.

352 The roles of MtFPN2 in the vasculature and in the symbiosomes were not equally 353 important. Expressing *MtFPN2* only in the nodule cortical cells was enough to at least 354 partially restore the wild-type phenotype. This means that facilitating iron transport across 355 the symbiosome membrane would be the key function of MtFPN2. In contrast, when 356 MtFPN2 was only expressed in the vasculature, no significant improvements in plant 357 growth, nodule development, or nitrogenase activity were observed compared to the *fpn2-1* 358 nodules. This would also hint at the existence of mechanisms to deliver iron out of the 359 vessels that are independent of MtFPN2. In contrast, in the symbiosomes, MtFPN2 would

not be easily substituted by another iron transporter, including MtSEN1, which has also been hypothesized to be in this membrane (Hakoyama et al., 2012). This would also explain why the attempts to functionally complement *fpn2-1* by increasing iron content in the nutrient solution did not work, since it would require to: i) accumulate at high enough levels in the cell cytosol to overcome MtFPN2 absence without becoming toxic, and ii) recruit another transporter that might use iron as a substrate although at lower affinities.

366 Overall, we now have a better understanding of the mechanisms of iron delivery for 367 symbiotic nitrogen fixation. Iron would be delivered by the vessels into the apoplast of the 368 infection/differentiation zone of the nodule (Rodríguez-Haas et al., 2013). Iron release from 369 the vessels would be facilitated by MtFPN2 and some other unknown protein(s). Once in 370 the apoplast, it would form a complex with the citrate released by MtMATE67 371 (Kryvoruchko et al., 2018). Then, a yet-to-be-determined ferroreductase must work to convert Fe<sup>3+</sup> into Fe<sup>2+</sup> that is the substrate of MtNramp1, the protein responsible for iron 372 373 uptake by rhizobia-infected nodule cells (Tejada-Jiménez et al., 2015). Once in the cytosol, 374 iron will be sorted to different subcellular compartments. In the case of the symbiosome, 375 MtMATE67 would also be required to form the iron-citrate complexes that seem to be the 376 preferred iron species to be taken up by bacteroids (Moreau et al., 1995; Le Vier et al., 377 1996).

378

#### 379 **METHODS**

**Biological material and growth conditions** 

381 M. truncatula R108 and fpn2-1 seeds were scarified in concentrated sulfuric acid for 382 7.5 min (R108) or 6.5 min (fpn2-1). Excess acid was removed and washed away eight times 383 with cold water, followed by surface-sterilization in 50 % (v/v) bleach for 90 s. Sterilized 384 seeds were embedded in sterile water overnight in darkness. Seeds were stratified in water-385 agar plates for 48 h at 4 °C and germinated at 22 °C. Seedlings were planted on sterile 386 perlite pots, and inoculated with S. meliloti 2011 or the same bacterial strain transformed 387 pHC60-GFP (Cheng and Walker, 1998). Plants were grown in a greenhouse under 16 h 388 light / 8 h dark at 25 °C / 20 °C conditions. In the case of perlite pots, plants were watered 389 every two days with Jenner's solution or water alternatively (Brito et al., 1994). Nodulated

plants were harvested at 28 dpi. For hairy-root transformation experiments, *M. truncatula*seedling were transformed with *Agrobacterium rhizogenes* strain ARqua1, containing the
appropriate binary vector as described (Boisson-Dernier et al., 2001).

Yeast complementation assays were carried out using the *S. cerevisiae* strain *ccc1* (MATa *ade2-1 his3-11 leu2-3,112, trp1-1, ura3-52 can1-100, ccc1::his3*) and parental DY150 (MATa *ade2-1 his3-11 leu2-3,112, trp1-1, ura3-52 can1-100*). The strains were grown in synthetic dextrose (SD) medium, supplemented with the required auxotrophic nutrients, 2 % glucose as carbon source, and the iron concentration indicated in each particular plate.

# 399 **RNA Extraction and RT-qPCR**

400 RNA was obtained from 28 dpi plants or equivalent age non-inoculated plants using 401 Tri-Reagent (Life Technologies), treated with DNase turbo (Life Technologies), and 402 cleaned with RNeasy Mini kit (Qiagen). Samples were tested for possible DNA 403 contamination by PCR using the RNA samples as templates and primers specific for M. 404 truncatula Ubiquitin carboxyl-terminal hydrolase (Medtr4g077320.1) (Supplemental Table 405 2). RNA integrity was confirmed by electrophoresis. cDNA was synthesized from 500 ng 406 of DNA-free RNA using PrimeScript RT reagent Kit (Takara), supplemented with RNase 407 out (Life Technologies).

408 Expression studies were carried out by real-time reverse transcription polymerase 409 chain reaction (RT-qPCR) using the StepOne plus thermocycler (Applied Biosystems), 410 using the Power SyBR Green master mix (Applied Biosystems). The primers used are 411 indicated in Supplemental Table 2. RNA levels were standardized using the *M. truncatula* 412 Ubiquitin carboxyl-terminal hydrolase (Medtr4g077320.1) gene. Real time cycler 413 conditions have been previously described (González-Guerrero et al., 2010). The threshold 414 cycle (Ct) was determined in triplicate from three different assays, each with at least pooled 415 material from three different plants). The relative levels of expression were determined using the  $2^{\Delta Ct}$  method. All assays contained a non-RT control to detect possible DNA 416 417 contaminations.

# 418 **Yeast complementation assays**

Restriction sites *PstI* and *XhoI* were added to *MtFPN2* cDNA by PCR using the primers listed in Supplemental Table 2. The amplicon was inserted into the *PstI/XhoI* sites of yeast expression vector pDR196 and cloned using T4 ligation. Yeast strains were transformed with either the empty pDR196 or pDR196::*MtFPN2* using the lithium acetate method (Schiestl and Gietz, 1989) and selected by uracil autotrophy. Complementation assays were carried out in SD medium plates with the required auxotrophic nutrients. To test tolerance to iron, the plates were supplemented with 2.5 mM FeSO<sub>4</sub>.

#### 426 **GUS Staining**

427 *MtFPN2* promoter region was obtained by amplifying the 2144 pb upstream of the 428 start codon using the primers Forward MtFPN2p-GW and Reverse MtFPN2p-GW 429 (Supplemental Table 2). The amplicon was cloned in pDONR2017 (Invitrogen) by 430 Gateway Technology (Invitrogen) and transferred to destination vector pGWB3 (Nakagawa 431 et al., 2007). Hairy-root transformation of M. truncatula seedling was carried out as indicated above. After three weeks on Fahreus medium supplemented with 50 µg/ml 432 433 kanamycin, the transformed plants were transferred to sterilized perlite pots and inoculated 434 with S. meliloti 2011. GUS activity was determined in root and nodules of 28 dpi plants as 435 described (Vernoud et al., 1999).

#### 436 Immunolocalization studies

The coding sequence region of *MtFPN2* and the promoter region used in the GUS staining were fused by PCR using the primers indicated in Supplemental Table 2. This fragment was cloned in pDONR207 (Invitrogen) using Gateway Technology and transferred to pGWB13 (three HA epitopes fused in C-terminus) (Nakagawa et al., 2007). Hairy-root transformation was carried out as described previously (Boisson-Dernier et al., 2001). Transformants were inoculated with *S. meliloti* 2011 containing the pHC60 plasmid that constitutively expressed GFP. Nodules and roots were collected at 28 dpi.

Samples for immunolocalization with a confocal microscope were fixed in 4% paraformaldehyde, 2.5 % sucrose in phosphate-buffered saline (PBS) solution at 4 °C overnight (12 – 16 h). Fixative was removed and samples washed twice. Nodule and roots were included in 6 % agarose and then sectioned in a Vibratome 1000 Plus (Vibratome) in 100 - 150  $\mu$ m thick slices. Sections were dehydrated by serial incubation with methanol (30 449 %, 50 %, 70 % and 100 % in PBS) for 5 min and then rehydrated following the same 450 methanol series in reverse order. Cell wall permeabilization was carried out by incubation 451 with 2 % cellulase in PBS for 1 h and 0.1 % Tween 20 for 15 min. Sections were blocked 452 with 5 % bovine serum albumin (BSA) in PBS and then incubated with 1:50 anti-HA 453 mouse monoclonal antibody (Sigma) in PBS at room temperature for 2 h. Primary antibody 454 was washed three times with PBS for 15 min and subsequently incubated with 1:40 Alexa 455 594 - conjugated anti-mouse rabbit monoclonal antibody (Sigma) in PBS at room 456 temperature for 1 h. Secondary antibody was washed three times with PBS for 10 min, and 457 then DNA was stained using DAPI. Images were obtained with a confocal laser-scanning 458 microscope (Leica SP8) using excitation light at 488 nm and 561 nm for GFP and Alexa 459 594, respectively. DAPI-stained zones were visualized with UV light.

460 Immunolocalization in an electron microscope was carried out with 28 dpi nodules 461 from MtFPN2-HA expressing plants fixed in 1 % formaldehyde, 0.5 % glutaraldehyde, 2.5% sucrose in 50 mM potassium phosphate buffer (pH 7.4) at 4 °C for 14 – 16 h in gentle 462 463 agitation. Samples were dehydrated with ethanol series and embedded in LR-White resin 464 (London Resin Company Ltd, UK). Nodule were placed in gelatine capsules, filled with 465 LR-White resin and polymerized at 60 °C for 24 h. Serial ultrathin sections were cut using a 466 diamond knife in a Reichert Ultracut S Ultramicrotrome (Leica). Sections for 467 immunolocalization were blocked with 2 % BSA on PBS for 30 min, then incubated with 468 1:50 anti-HA rabbit monoclonal antibody (Sigma) in PBS at room temperature for 3 h. 469 Primary antibody was washed ten times with PBS for 2 min and next incubated with 1:150 470 anti-rabbit goat monoclonal antibody conjugated to 15 nm gold particles (Fisher Scientific) 471 in PBS at room temperature for 1 h. Secondary antibody was washed ten times with PBS 472 for 2 min, after that samples were washed fifteen times with water for 2 min. Sections were stained with 2 % uranyl acetate and visualized in a JEM 1400 electron microscope at 80 kV 473 474 at Centro Nacional de Microscopía Electrónica.

# 475 Acetylene reduction assays

The acetylene reduction assay was used to measure nitrogenase activity in 28 dpi plants (Hardy et al., 1968). Wild type and *fpn2-1* roots with nodules were introduced in 30 ml vials fitted with rubber stoppers. Each tube contained four or five independently transformed plants. Three milliliters of air of each bottle was replaced by the same volume of acetylene. The vials were incubated for 30 min at room temperature, when 0.5 ml gas samples were taken from each of the tube. Ethylene content in each sample was measured in a Shimadzu GC-8A gas chromatograph using a Porapak N column. The amount of the ethylene produced was determined by measuring the height of the ethylene peak relative to standards.

# 485 Metal content determination

Iron content was determined in shoots, roots, and nodules 28 dpi. Plant tissues were weighted and mineralized in 15.6 M HNO<sub>3</sub> (trace metal grade) for 1 h at 80 °C and overnight at 20 °C. Digestions were completed with 2 M  $H_2O_2$ . Samples were diluted in 300 mM HNO<sub>3</sub> prior to measurements. Element analyses were performed with Atomic Absorption Spectroscopy (AAS) in an AAnalyst 800 (Perkin Elmer), equipped with a graphite furnace. All samples were measured in duplicate.

# 492 Synchrotron-based X-ray fluorescence and XANES

493  $\mu$ XRF hyperspectral images and  $\mu$ XANES spectra were acquired on the beamline 494 ID21 of the European Synchrotron Radiation Facility (Cotte et al., 2017), at 110 K in the 495 liquid nitrogen (LN2) cooled cryostat of the Scanning X-ray Micro-spectroscopy end-496 station. A nodule from each of at least five *M. truncatula* R108 or *fpn2-1* plants (totalling 497 five R108 nodules, five pink fpn2-1 nodules, and five white fpn2-1 nodules) were 498 embedded into OCT medium and cryo-fixed by plunging in isopentane chilled with LN2. 499 25 µm-thick sections of frozen samples were obtained using a Leica LN22 cryo-microtome 500 and accommodated in a Cu sample holder cooled with LN2, sandwiched between Ultralene (SPEX SamplePrep) foils. The beam was focused to  $0.4 \times 0.9 \text{ um}^2$  with a Kirkpatrick-Baez 501 (KB) mirror system. The emitted fluorescence signal was detected with energy-dispersive, 502 503 large area (80 mm<sup>2</sup>) SDD detector equipped with Be window (XFlash SGX from RaySpec). Images were acquired at the fixed energy of 7.2 keV, by raster-scanning the sample in the 504 X-ray focal plane, with a step of  $2 \times 2 \ \mu m^2$  or  $0.5 \times 0.5 \ \mu m^2$  and 100 ms dwell time. 505 Elemental mass fractions were calculated from fundamental parameters with the PyMca 506 507 software package, applying pixel-by-pixel spectral deconvolution to hyperspectral maps 508 normalized by the incoming current (Sole et al., 2007). The incoming flux was monitored

509 using a drilled photodiode previously calibrated by varying the photon flux at 7.2 KeV 510 obtaining a response of 1927.9 charges/photon with a linear response up to 200 kcps. In 511 PyMCA the incoming flux and XRF detector parameters were set to  $2x10^9$  photons/s, 512 0.7746 cm<sup>2</sup> active area, and 4.65 cm sample to XRF detector distance. Sample matrix was 513 assumed to be amorphous ice (11% H, 89% O, density 0.92 g/cm<sup>3</sup>), the sample thickness 514 set at 25µm obtained with the use of a cryo-microtome.

515 Fe-K edge (7.050 to 7.165 keV energy range, 0.5 eV step) micro X-ray absorption 516 near edge spectroscopy (µXANES) spectra were recorded in regions of interest of the 517 fluorescence maps on ID21 beamline (ESRF, France). Individual spectra were processed 518 using Orange software with the Spectroscopy add-on (Demsar et al., 2013). The pre-519 processing step consisted of vector normalization and a second derivative Savitsky-Golay 520 filter to highlight potential differences among genotypes. Then a principal component 521 analysis combined to a linear discriminant analysis (when there were more than two 522 conditions to compare) was performed on the second derivative of the spectra to highlight 523 potential differences among genotypes. Average spectra from the regions was then fitted 524 using a linear combination routine

525

#### 526 Statistical tests

527 Data were analyzed by t-test to calculate statistical significance of observed 528 differences, between wild type plants and analyzed mutants. Test results with p-values 529 lower than 0.05 were considered as statistically significant.

530

### 531 ACKNOWLEDGEMENTS

This research was funded by a Ministerio de Economía y Competitividad grant (AGL2015-65866-P), and a European Research Council Starting Grant (ERC-2013-StG-335284), to MGG. Development of *M. truncatula Tnt1* mutant population was, in part, funded by the National Science Foundation, USA (DBI-0703285) to KSM. The X-ray experiments were performed on beamline ID21 and ID16B at the European Synchrotron Radiation Facility (ESRF), Grenoble, France. We are grateful to Juan Reyes-Herrera at the ESRF for his assistance in using beamline ID21. We would also like to acknowledge the other members

of laboratory 281 at Centro de Biotecnología y Genómica de Plantas (UPM-INIA) for their

540 support and feedback in preparing this manuscript.

541

# 542 AUTHOR CONTRIBUTION

VE carried out most of the experimental work. IA, CL, JV, and HC-M generated the synchrotron-based X-ray fluorescence, XANES, and X-ray tomographic data. JQ and JMA were responsible for metal determinations. MT-J and ER-N executed the yeast complementation assays. RP participated in *M. truncatula* transformation assays. JW and KSM performed *M. truncatula* mutant screening and isolated the *fpn2-1* allele. VE, JI, and MG-G were responsible for experimental design, data analyses, and wrote the manuscript.

549

# 550 **REFERENCES**

- Akkermans, M.D., van der Horst-Graat, J.M., Eussen, S.R.B.M., van Goudoever, J.B.,
   and Brus, F. (2016). Iron and Vitamin D deficiency in healthy young children in
   Western-Europe despite current nutritional recommendations. J. Pediatr.
   Gastroenterol. Nutr. 62, 635-642
- Andaluz, S., Rodríguez-Celma, J., Abadía, A., Abadía, J., and López-Millán, A.F.
  (2009). Time course induction of several key enzymes in *Medicago truncatula* roots
  in response to Fe deficiency. Plant Physiol. Biochem. 47, 1082-1088.
- Appleby, C.A. (1984). Leghemoglobin and *Rhizobium* respiration. Annu. Rev. Plant
  Physiol. 35, 443-478.
- Arpat, A.B., Magliano, P., Wege, S., Rouached, H., Stefanovic, A., and Poirier, Y.
  (2012). Functional expression of PHO1 to the Golgi and trans-Golgi network and its
  role in export of inorganic phosphate. Plant J. 71, 479-491.
- Boisson-Dernier, A., Chabaud, M., Garcia, F., Bécard, G., Rosenberg, C., and Barker,
  D.G. (2001). *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula*for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Mol.
  Plant Microbe Interact. 14, 695-700.
- 567 Brear, E.M., Day, D.A., and Smith, P.M.C. (2013). Iron: an essential micronutrient for
  568 the legume–rhizobium symbiosis. Front. Plant Sci. 4, 359.

- Brito, B., Palacios, J.M., Hidalgo, E., Imperial, J., and Ruíz-Argüeso, T. (1994). Nickel
  availability to pea (*Pisum sativum* L.) plants limits hydrogenase activity of *Rhizobium leguminosarum* bv. *viciae* bacteroids by affecting the processing of the
  hydrogenase structural subunits. J. Bacteriol. 176, 5297-5303.
- 573 Catalano, C.M., Czymmek, K.J., Gann, J.G., and Sherrier, D.J. (2006). *Medicago*574 *truncatula* syntaxin SYP132 defines the symbiosome membrane and infection
  575 droplet membrane in root nodules. Planta 225, 541-550.
- 576 Cheng, H.P., and Walker, G.C. (1998). Succinoglycan is required for initiation and
  577 elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J.
  578 Bacteriol. 180, 5183-5191.
- 579 Colangelo, E.P., and Guerinot, M.L. (2004). The essential basic helix-loop-helix protein
  580 FIT1 is required for the iron deficiency response. Plant Cell 16, 3400-3412.
- 581 Colangelo, E.P., and Guerinot, M.L. (2006). Put the metal to the petal: metal uptake and
  582 transport throughout plants. Curr. Opin. Plant Biol. 9, 322-330.
- 583 Conte, S., Stevenson, D., Furner, I., and Lloyd, A. (2009). Multiple antibiotic resistance
  584 in Arabidopsis is conferred by mutations in a chloroplast-localized transport protein.
  585 Plant Physiol. 151, 559.
- 586 Cotte, M. P. E., Salomé, M. Rivard, C. Nolf, W. D., Castillo-Michel, H., Fabris, T., 587 Monico, L., Janssens, K., Wang, T., Sciau, P., Verger, L., Cormier, L., 588 Dargaud, O., Brun, E., Bugnazet, D., Fayard, B., Hesse, B., del Real, A. E. P., 589 Veronesi, G., Langlois, J., Balcar, N., Vandenberghe, Y., Solé, V. A., Kieffer, J., 590 Barrett, R., Cohen, C., Cornu, C., Baker, R., Gagliardini, E., Papillon, E., 591 Susini, J. (2017). The ID21 X-ray and infrared microscopy beamline at the ESRF: 592 status and recent applications to artistic materials. J. Anal. At. Spectrom. 32, 477-593 493.
- 594 Curie, C., Panaviene, Z., Loulergue, C., Dellaporta, S.L., Briat, J.-F., and Walker,
  595 E.L. (2001). Maize yellow stripe1 encodes a membrane protein directly involved in
  596 Fe(III) uptake. Nature 409, 346-349.
- 597 Dalton, D.A., Joyner, S.L., Becana, M., Iturbe-Ormaetxe, I., and Chatfield, J.M.
  598 (1998). Antioxidant defenses in the peripheral cell layers of legume root nodules.
  599 Plant Physiol. 116, 37-43.

600	Demsar, J., Curk, T., Erjavec, A., Gorup, C., Hocevar, T., Milutinovic, M., Mozina,
601	M., Polajnar, M., Toplak, M., Staric, A., Stajdohar, M., Umek, L., Zagar, L.,
602	Zbontar, J., Zitnik, M., and Zupan, B. (2013). Orange: Data mining toolbox in
603	Python. J. Machine Learning Res. 14, 2349-2353.
604	Downie, J.A. (2014). Legume nodulation. Curr. Biol. 24, R184-R190.
605	Drakesmith, H., Nemeth, E., and Ganz, T. (2015). Ironing out Ferroportin. Cell Metab.
606	<b>22,</b> 777-787.
607	Durrett, T.P., Gassmann, W., and Rogers, E.E. (2007). The FRD3-mediated efflux of
608	citrate into the root vasculature is necessary for efficient iron translocation. Plant
609	Physiol. <b>144,</b> 197-205.
610	Flis, P., Ouerdane, L., Grillet, L., Curie, C., Mari, S., and Lobinski, R. (2016).
611	Inventory of metal complexes circulating in plant fluids: a reliable method based on
612	HPLC coupled with dual elemental and high-resolution molecular mass
613	spectrometric detection. New Phytol. 211, 1129-1141.
614	Gage, D.J. (2002). Analysis of infection thread development using Gfp- and DsRed-
615	expressing Sinorhizobium meliloti. J. Bacteriol. 184, 7042-7046.
616	Goldstein, S., Meyerstein, D., and Czapski, G. (1993). The Fenton reagents. Free Radic.
617	Biol. Med. <b>15</b> , 435-445.
618	González-Guerrero, M., Raimunda, D., Cheng, X., and Argüello, J.M. (2010). Distinct
619	functional roles of homologous Cu <sup>+</sup> efflux ATPases in Pseudomonas aeuginosa.
620	Mol. Microbiol. <b>78,</b> 1246-1258.
621	González-Guerrero, M., Matthiadis, A., Sáez, Á., and Long, T.A. (2014). Fixating on
622	metals: new insights into the role of metals in nodulation and symbiotic nitrogen
623	fixation. Front. Plant Sci. 5, 45.
624	González-Guerrero, M., V., E., Sáez, Á., and Tejada-Jiménez, M. (2016). Transition
625	metal transport in plants and associated endosymbionts. Arbuscular mycorrhizal
626	fungi and rhizobia. Front. Plant Sci. 7, 1088.
627	Grillet, L., Lan, P., Li, W., Mokkapati, G., and Schmidt, W. (2018). IRON MAN is a
628	ubiquitous family of peptides that control iron transport in plants. Nature Plants 4,
629	953-963.

630	Hakoyama, T., Niimi, K., Yamamoto, T., Isobe, S., Sato, S., Nakamura, Y., Tabata, S.,
631	Kumagai, H., Umehara, Y., Brossuleit, K., Petersen, T.R., Sandal, N.,
632	Stougaard, J., Udvardi, M.K., Tamaoki, M., Kawaguchi, M., Kouchi, H., and
633	Suganuma, N. (2012). The integral membrane protein SEN1 is required for
634	symbiotic nitrogen fixation in Lotus japonicus nodules. Plant Cell Physiol. 53, 225-
635	236.
636	Hardy, R.W., Holsten, R.D., Jackson, E.K., and Burns, R.C. (1968). The acetylene-
637	ethylene assay for n(2) fixation: laboratory and field evaluation. Plant Physiol. 43,
638	1185-1207.
639	Herridge, D.F., Peoples, M.B., and Boddey, R.M. (2008). Global inputs of biological
640	nitrogen fixation in agricultural systems. Plant Soil <b>311</b> , 1-18.
641	Hirschi, K.D. (2009). Nutrient Biofortification of Food Crops. Annu. Rev. Nutr. 29, 401-
642	421.
643	Huisman, R., Ovchinnikova, E., Bisseling, T., and Limpens, E. (2012). Endocytic
644	Accommodation of Microbes in Plants (Berlin, Heidelberg: Springer Berlin
645	Heidelberg), pp. 271-295.
646	Kaiser, B.N., Moreau, S., Castelli, J., Thomson, R., Lambert, A., Bogliolo, S., Puppo,
647	A., and Day, D.A. (2003). The soybean NRAMP homologue, GmDMT1, is a
648	symbiotic divalent metal transporter capable of ferrous iron transport. Plant J. 35,
649	295-304.
650	Kaplan, J. (2002). Mechanisms of cellular iron acquisition: Another iron in the fire. Cell
651	<b>111,</b> 603-606.
652	Kereszt, A., Mergaert, P., and Kondorosi, E. (2011). Bacteroid development in legume
653	nodules: Evolution of mutual benefit or of sacrificial victims? Mol. Plant-Microbe
654	Interact. <b>24</b> , 1300-1309.
655	Kobayashi, T., and Nishizawa, N.K. (2012). Iron uptake, translocation, and regulation in
656	higher plants. Annu. Rev. Plant Biol. 63, 131-152.
657	Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M.L., and Pakrasi, H.B. (1999).
658	The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad
659	substrate range. Plant Mol. Biol. 40, 37-44.

660	Kryvoruchko, I.S., Routray, P., Sinharoy, S., Torres-Jerez, I., Tejada-Jiménez, M.,						
661	Finney, L.A., Nakashima, J., Pislariu, C.I., Benedito, V.A., González-Guerrero,						
662	M., Roberts, D.M., and Udvardi, M.K. (2018). An iron-activated citrate						
663	transporter, MtMATE67, is required for symbiotic nitrogen fixation. Plant Physio						
664	<b>176,</b> 2315-2329.						
665	Kumar, R.K., Chu, HH., Abundis, C., Vasques, K., Rodriguez, D.C., Chia, JC.,						
666	Huang, R., Vatamaniuk, O.K., and Walker, E.L. (2017). Iron-nicotianamine						
667	transporters are required for proper long distance iron signaling. Plant Physiol. 175						
668	1254.						
669	Le Vier, K., Day D.A., and Guerinot M.L. (1996). Iron uptake by symbiosomes from						
670	soybean root nodules. Plant Physiol. 111, 893-900						
671	Li, L., Chen, O.S., Ward, D.M., and Kaplan, J. (2001). CCC1 is a transporter that						
672	mediates vacuolar iron storage in yeast. J. Biol. Chem. 276, 29515-29519.						
673	Limpens, E., Ivanov, S., van Esse, W., Voets, G., Fedorova, E., and Bisseling, T.						
674	(2009). Medicago N <sub>2</sub> -fixing symbiosomes acquire the endocytic identity marker						
675	Rab7 but delay the acquisition of vacuolar identity. Plant Cell <b>21</b> , 2811-2828.						
676	Lin, H., Li, L., Jia, X., Ward, D.M., and Kaplan, J. (2011). Genetic and biochemical						
677	analysis of high iron toxicity in yeast: Iron toxicity is due to the accumulation of						
678	cytosolic iron and occurs under both aerobic and anaerobic conditions. J. Biol.						
679	Chem. <b>286,</b> 3851-3862.						
680	Long, T.A., Tsukagoshi, H., Busch, W., Lahner, B., Salt, D.E., and Benfey, P.N.						
681	(2010). The bHLH transcription factor POPEYE regulates response to iron						
682	deficiency in Arabidopsis roots. Plant Cell 22, 2219-2236.						
683	López-Millán, AF., Morales, F.n., Abadía, A., and Abadía, J. (2000). Effects of iron						
684	deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar						
685	beet. Implications for iron and carbon transport. Plant Physiol. 124, 873-884.						
686	Magne, K., Couzigou, JM., Schiessl, K., Liu, S., George, J., Zhukov, V., Sahl, L.,						
687	Boyer, F., Iantcheva, A., Mysore, K.S., Wen, J., Citerne, S., Oldroyd, G.E.D.,						
688	and Ratet, P. (2018). MtNODULE ROOT1 and MtNODULE ROOT2 are essential						
689	for indeterminate nodule identity. Plant Physiol. 178, 295.						
690	Marschner, P. (2011). Mineral Nutrition of Higher Plants 3rd Edition. (Academic Press).						

691	McKie, A.T., Marciani, P., Rolfs, A., Brennan, K., Wehr, K., Barrow, D., Miret, S.,					
692	Bomford, A., Peters, T.J., Farzaneh, F., Hediger, M.A., Hentze, M.W., and					
693	Simpson, R.J. (2000). A novel duodenal Iron-Regulated transporter, IREG1,					
694	implicated in the basolateral transfer of iron to the circulation. Mol. Cell 5, 299-309.					
695	Montiel, J., Arthikala, MK., Cárdenas, L., and Quinto, C. (2016). Legume NADPH					
696	oxidases have crucial roles at different stages of nodulation. Int. J. Mol. Sci. 17,					
697	680.					
698	Montiel, J., Nava, N., Cárdenas, L., Sánchez-López, R., Arthikala, MK., Santana, O.,					
699	Sánchez, F., and Quinto, C. (2012). A Phaseolus vulgaris NADPH oxidase gene is					
700	required for root infection by rhizobia. Plant Cell Physiol. 53, 1751-1767.					
701	Moreau, S., Meyer J.M., and Puppo A. (1995). Uptake of iron by symbiosomes and					
702	bacteroids from soybean nodules. FEBS Lett. 361, 225-228.					
703	Morrissey, J., Baxter, I.R., Lee, J., Li, L., Lahner, B., Grotz, N., Kaplan, J., Salt, D.E.,					
704	and Guerinot, M.L. (2009). The ferroportin metal efflux proteins function in iron					
705	and cobalt homeostasis in Arabidopsis. Plant Cell 21, 3326-3338.					
706	Mus, F., Crook, M.B., Garcia, K., Garcia Costas, A., Geddes, B.A., Kouri, E.D.,					
707	Paramasivan, P., Ryu, M.H., Oldroyd, G.E., Poole, P.S., Udvardi, M.K., Voigt,					
708	C.A., Ane, J.M., and Peters, J.W. (2016). Symbiotic nitrogen fixation and					
709	challenges to extending it to non-legumes. Appl. Environ. Microbiol. 82, 3698-					
710	3710.					
711	Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka,					
712	K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007). Development of series of					
713	gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes					
714	for plant transformation. J. Biosci. Bioeng. 104, 34-41.					
715	Nevo, Y., and Nelson, N. (2006). The NRAMP family of metal-ion transporters. Biochim.					
716	Biophys. Acta <b>1763</b> , 609-620.					
717	O'Hara, G.W., Dilworth, M.J., Boonkerd, N., and Parkpian, P. (1988). Iron-deficiency					
718	specifically limits nodule development in peanut inoculated with Bradyrhizobium					
719	sp. New Phytologist <b>108,</b> 51-57.					
720	Oldroyd, G.E., and Dixon, R. (2014). Biotechnological solutions to the nitrogen problem.					
721	Curr. Opin. Biotechnol. 26, 19-24.					

722	Preisig, O., Zufferey, R., and Hennecke, H. (1996a). The Bradyrhizobium japonicum
723	fixGHIS genes are required for the formation of the high-affinity cbb(3)-type
724	cvtochrome oxidase. Arch. Microbiol. 165, 297-305.

- Preisig, O., Zufferey, R., Thony-Meyer, L., Appleby, C., and Hennecke, H. (1996b). A
   high-affinity cbb3-type cytochrome oxidase terminates the symbiosis- specific
   respiratory chain of *Bradyrhizobium japonicum*. J. Bacteriol. 178, 1532-1538.
- Rodríguez-Haas, B., Finney, L., Vogt, S., González-Melendi, P., Imperial, J., and
   González-Guerrero, M. (2013). Iron distribution through the developmental stages
   of *Medicago truncatula* nodules. Metallomics 5, 1247-1253.
- Rogers, E.E., and Guerinot, M.L. (2002). FRD3, a member of the multidrug and toxin
  efflux family, controls iron deficiency responses in Arabidopsis. Plant Cell 14,
  1787-1799.
- Roschzttardtz, H., Séguéla-Arnaud, M., Briat, J.-F., Vert, G., and Curie, C. (2011).
  The FRD3 citrate effluxer promotes iron nutrition between symplastically
  disconnected tissues throughout Arabidopsis development. Plant Cell 23, 27252737.
- Roux, B., Rodde, N., Jardinaud, M.-F., Timmers, T., Sauviac, L., Cottret, L., Carrère,
  S., Sallet, E., Courcelle, E., Moreau, S., Debellé, F., Capela, D., de CarvalhoNiebel, F., Gouzy, J., Bruand, C., and Gamas, P. (2014). An integrated analysis
  of plant and bacterial gene expression in symbiotic root nodules using laser-capture
  microdissection coupled to RNA sequencing. Plant J. 77, 817-837.
- Rubio, L.M., and Ludden, P.W. (2005). Maturation of nitrogenase: a biochemical puzzle.
  J. Bacteriol. 187, 405-414.
- Rubio, M.C., James, E.K., Clemente, M.R., Bucciarelli, B., Fedorova, M., Vance, C.P.,
  and Becana, M. (2004). Localization of superoxide dismutases and hydrogen
  peroxide in legume root nodules. Mol. Plant Microbe Int. 17, 1294-1305.
- Schiestl, R.H., and Gietz, R.D. (1989). High efficiency transformation of intact yeast cells
  using single stranded nucleic acids as carrier. Curr. Genet. 16, 339-346.
- Sivitz, A.B., Hermand, V., Curie, C., and Vert, G. (2012). Arabidopsis bHLH100 and
  bHLH101 control iron homeostasis via a FIT-independent pathway. PLoS ONE 7,
  e44843.

753	Solé, V. A.,	Papi	llon, i	E., Cotte,	М.,	Walter, P., Susini,	<b>J. A</b> . (	2007). A multi	platform
754	code	for	the	analysis	of	energy-dispersive	X-ray	fluorescence	spectra
755	Spect	rochi	mica A	Acta Part B	: Ate	omic Spectroscopy	<b>52,</b> 63-68	3.	

- 756 Sprent, J.I. (2007). Evolving ideas of legume evolution and diversity: a taxonomic
   757 perspective on the occurrence of nodulation. New Phytol. 174, 11-25.
- Stephens, B., Cook, D., and Grusak, M. (2011). Characterization of zinc transport by
   divalent metal transporters of the ZIP family from the model legume *Medicago truncatula*. Biometals 24, 51-58.
- Sutton, W.D., Pankhurst, C.E., and Craig, A.S. (1981). The Rhizobium Bacteroid State.
  In Biology of the Rhizobiaceae, K.L. Atherly and G. Gilesalan, eds (Academic
  Press CY -), pp. 149-177.
- Tadege, M., Wen, J., He, J., Tu, H., Kwak, Y., Eschstruth, A., Cayrel, A., Endre, G.,
  Zhao, P.X., Chabaud, M., Ratet, P., and Mysore, K.S. (2008). Large-scale
  insertional mutagenesis using the Tnt1 retrotransposon in the model legume *Medicago truncatula*. Plant J. 54, 335-347.
- Tang, C., Robson, A.D., and Dilworth, M.J. (1990). The role of iron in nodulation and
   nitrogen fixation in *Lupinus angustifolius* L. New Phytol. 114, 173-182.
- Tang, C.X., Robson, A.D., Dilworth, M.J., and Kuo, J. (1992). Microscopic evidence on
  how iron-deficiency limits nodule initiation in *Lupinus angustifolius* 1. New Phytol.
  121, 457-467.
- Tejada-Jiménez, M., Castro-Rodríguez, R., Kryvoruchko, I., Lucas, M.M., Udvardi,
   M., Imperial, J., and González-Guerrero, M. (2015). *Medicago truncatula* Natural Resistance-Associated Macrophage Protein1 is required for iron uptake by
   rhizobia-infected nodule cells. Plant Physiol. 168, 258-272.
- Tejada-Jiménez, M., Gil-Diez, P., Leon-Mediavilla, J., Wen, J., Mysore, K.S.,
  Imperial, J., and Gonzalez-Guerrero, M. (2017). *Medicago truncatula*molybdate transporter type 1 (MOT1.3) is a plasma membrane molybdenum
  transporter required for nitrogenase activity in root nodules under molybdenum
  deficiency. New Phytol 216, 1223-1235.

- Terry, R.E., Soerensen, K.U., Jolley, V.D., and Brown, J.C. (1991). The role of active
   *Bradyrhizobium japonicum* in iron stress response of soy-beans. Plant Soil 130,
   225-230.
- 785 Udvardi, M., and Poole, P.S. (2013). Transport and metabolism in legume-rhizobia
  786 symbioses. Annu. Rev. Plant Biol. 64, 781-805.
- 787 Vance, C.P. (2001). Symbiotic nitrogen fixation and phosphorus acquisition. Plant
  788 nutrition in a world of declining renewable resources. Plant Physiol. 127, 390-397.
- Vasse, J., de Billy, F., Camut, S., and Truchet, G. (1990). Correlation between
  ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules.
  J. Bacteriol. 172, 4295-4306.
- Vernié, T., Kim, J., Frances, L., Ding, Y., Sun, J., Guan, D., Niebel, A., Gifford, M.L.,
   de Carvalho-Niebel, F., and Oldroyd, G.E.D. (2015). The NIN transcription
   factor coordinates diverse nodulation programs in different tissues of the *Medicago truncatula* root. Plant Cell, 27, 3410-3420.
- Vernoud, V., Journet, E.P., and Barker, D.G. (1999). *MtENOD20*, a Nod factorinducible molecular marker for root cortical cell activation. Mol. Plant Microbe
  Interact. 12, 604-614.
- Vert, G., Grotz, N., Dedaldéchamp, F., Gaymard, F., Guerinot, M.L., Briat, J.F., and
  Curie, C. (2002). IRT1, an *Arabidopsis* transporter essential for iron uptake from
  the soil and the plant growth. Plant Cell 14, 1223-1233.
- von Wiren, N., Klair, S., Bansal, S., Briat, J.-F., Khodr, H., Shioiri, T., Leigh, R.A.,
  and Hider, R.C. (1999). Nicotianamine chelates both FeIII and FeII. Implications
  for metal transport in plants. Plant Physiol. 119, 1107-1114.
- White, P.J., and Broadley, M.R. (2009). Biofortification of crops with seven mineral
  elements often lacking in human diets iron, zinc, copper, calcium, magnesium,
  selenium and iodine. New Phytol. 182, 49-84.
- Xiao, T.T., Schilderink, S., Moling, S., Deinum, E.E., Kondorosi, E., Franssen, H.,
  Kulikova, O., Niebel, A., and Bisseling, T. (2014). Fate map of *Medicago truncatula* root nodules. Development 141, 3517-3528.
- Zhai, Z., Gayomba, S.R., Jung, H.-i., Vimalakumari, N.K., Piñeros, M., Craft, E.,
  Rutzke, M.A., Danku, J., Lahner, B., Punshon, T., Guerinot, M.L., Salt, D.E.,

Kochian, L.V., and Vatamaniuk, O.K. (2014). OPT3 Is a phloem-specific iron
transporter that is essential for systemic iron signaling and redistribution of iron and
cadmium in *Arabidopsis*. Plant Cell 26, 2249-2264.

- 816
- 817
- 818
- 819
- 820
- 821

### 822 FIGURE LEGENDS

823 **Figure 1.** *MtFPN2* is expressed in the vasculature and in the differentiation-fixation zones 824 of *M. truncatula* nodules. (A) *MtFPN2* expression in nodulated or nitrogen-fertilized *M*. 825 truncatula plants relative to the internal standard gene Ubiquitin carboxyl-terminal 826 *hydrolase*. Data are the mean  $\pm$  SE of two independent experiments with 4 pooled plants. 827 (B) GUS staining of 28 dpi *M. truncatula* nodules expressing the gus gene under the 828 control of *MtFPN2* promoter region. Bar = 500  $\mu$ m. (C) Longitudinal section of a GUS-829 stained 28 dpi *M. truncatula* nodule expressing the gus gene under the *MtFPN2* promoter 830 region. ZI indicates Zone I; ZII, Zone II; IZ, Interzone; and ZIII, Zone III. Bar =  $100 \mu m$ . 831 (D) Cross section of a GUS-stained 28 dpi *M. truncatula* nodule expressing the gus gene 832 under the *MtFPN2* promoter region. Bar =  $100 \,\mu\text{m}$ .

833 Figure 2. MtFPN2 is localized in intracellular membranes in the vasculature and in the 834 symbiosome membrane in S. meliloti-infected nodule cells. (A) Longitudinal section of a 835 28 dpi M. truncatula nodule expressing MtFPN2-HA under its own promoter. The three C-836 terminal HA epitopes were detected using an Alexa594-conjugated antibody (red, left 837 panel). Transformed plants were inoculated with a GFP-expressing S. meliloti strain (green, 838 middle panel). Both images were overlaid with the channel showing DAPI-stained DNA 839 (blue, right panel). Bar = 100  $\mu$ m. (B) Cross section of a vessel of 28 dpi *M. truncatula* 840 nodule expressing *MtFPN2-HA* under its own promoter. The three C-terminal HA epitopes

841 were detected using an Alexa594-conjugated antibody (red, left panel). Transillumination 842 image is shown in the centre panel, and overlaid with the Alexa594 signal (right panel). Bar 843 = 25  $\mu$ m. (C) Detail of a *S. meliloti*-colonized 28 dpi nodule cell in the differentiation zone. 844 Right panel shows the Alexa594 signal (red), middle panel the S. meliloti distribution 845 (green), and the right one overlays both images. Bar = 10  $\mu$ m. (D). Detail of symbiosomes 846 shown by transmission electron microscopy (left panel). Arrows indicate the position of the 847 colloidal gold particles conjugated to the antibody used to detect MtFPN2-HA. Middle 848 panel, *M. truncatula* nodule vessel shown with transmission electron microscopy. Asterisks 849 indicate the position of the xylem. Boxed region was amplified in the right panel to show 850 the antibody-conjugated gold particles used to position MtFPN2-HA (indicated by arrows).

Figure 3. MtFPN2 transports iron out of the cytosol. Parental yeast strain DY150 was transformed with the pDR196 empty vector, while the *ccc1* mutant was transformed with either pDR196 or pDR196 harbouring the *MtFPN2* coding sequence. Serial dilutions (10x) of the transformants were grown in YPD medium (top panel), SD medium with the required auxotrophies (centre panel), or in SD medium with the required auxotrophies and 2.5 mM FeSO<sub>4</sub> (lower panel).

857 Figure 4. MtFPN2 is required for symbiotic nitrogen fixation. (A) Tnt1 insertion in the 858 fourth exon of *MtFPN2* results in no expression being detected by RT-PCR. The *Ubiquitin* 859 carboxyl-terminal hydrolase (MtUb1) gene was used as a positive control. (B) Growth of 860 representative wild type (WT), fpn2-1, and fpn2-1 transformed with the MtFPN2 coding 861 sequence expressed under its own promoter ( $FPN2_{prom}$ :: MtFPN2). Bar = 1 cm. (C) Detail 862 of representative nodules of WT, fpn2-1, and FPN2<sub>prom</sub>::MtFPN2-transformed fpn2-1 863 plants. Bars = 500  $\mu$ m. (D) Number of pink or white nodules in 28 dpi WT, fpn2-1, and 864  $FPN2_{nrom}$ : MtFPN2-transformed fpn2-1 plants. Data are the mean  $\pm$  SE of at least 7 865 (E) Fresh weight of WT, fpn2-1, independently transformed plants. and 866  $FPN2_{prom}$ : MtFPN2-transformed fpn2-1 plants. Data are the mean  $\pm$  SE of at least 7 867 independently transformed plants. (F) Nitrogenase activity in 28 dpi nodules from WT, 868 fpn2-1, and FPN2<sub>prom</sub>::MtFPN2-transformed fpn2-1 plants. Acetylene reduction was 869 measured in duplicate from two sets of four pooled plants. Data are the mean  $\pm$  SE. (G) 870 Iron content in roots, shoot, and nodules of 28 dpi WT, fpn2-1, and FPN2<sub>prom</sub>::MtFPN2transformed *fpn2-1* plants. Data are the mean  $\pm$  SE of 3 sets of 4 pooled plants. \* indicates statistically significant differences (p < 0.05).

873 Figure 5. *MtFPN2* loss-of-function alters iron distribution and speciation in 28 dpi nodules. 874 (A). Synchrotron-based X-ray fluorescence images of wild type (left), pink fpn2-1 (middle), 875 and white *fpn2-1* (right) nodules, showing calcium (top panels) or iron (lower panels) 876 distribution. Bars = 200  $\mu$ m, intensity scale in mass fraction units (g/g). (B) XANES 877 spectra obtained from different regions of wild-type, pink *fpn2-1*, and white *fpn2-1* nodules 878 (top left panel). This signal was decomposed into its two principal components for Zone II (top right panel), Zone III (lower left panel), and the vasculature (lower right panel). Green 879 880 represents wild-type samples; red, pink *fpn2-1*; and blue, white *fpn2-1* nodules.

881 **Figure 6.** *MtFPN2* expression in cortical nodule cells partially restores the *fpn2-1* 882 phenotype. (A) Growth of representative wild type (WT), fpn2-1, and fpn2-1 transformed 883 with the MtFPN2 coding sequence expressed under the MtMOT1.3 promoter 884 ( $MOT1.3_{prom}$ :: MtFPN2). Bar = 1 cm. (B) Detail of representative nodules of WT, fpn2-1, and  $MOT1.3_{prom}$ :: MtFPN2-transformed fpn2-1 plants. Bars = 500 µm. (C) Number of pink 885 or white nodules in 28 dpi WT, fpn2-1, and MOT1.3<sub>prom</sub>::MtFPN2-transformed fpn2-1 886 887 plants. Data are the mean  $\pm$  SE of at least 11 independently transformed plants. (D) Fresh weight of WT, fpn2-1, and MOT1.3<sub>prom</sub>::MtFPN2-transformed fpn2-1 plants. Data are the 888 889 mean  $\pm$  SE of at least 11 independently transformed plants. (E) Nitrogenase activity in 28 890 dpi nodules from WT, fpn2-1, and MOT1.3<sub>prom</sub>::MtFPN2-transformed fpn2-1 plants. 891 Acetylene reduction was measured in duplicate from two sets of four pooled plants. Data 892 are the mean  $\pm$  SE. \* indicates statistically significant differences (p < 0.05).

#### **FIGURE 1**



**Figure 1.** *MtFPN2* is expressed in the vasculature and in the differentiation-fixation zones of *M. truncatula* nodules. (A) *MtFPN2* expression in nodulated or nitrogen-fertilized *M. truncatula* plants relative to the internal standard gene *Ubiquitin carboxyl-terminal hydrolase*. Data are the mean  $\pm$  SE of two independent experiments with 4 pooled plants. (B) GUS staining of 28 dpi *M. truncatula* nodules expressing the *gus* gene under the control of *MtFPN2* promoter region. Bar = 500 µm. (C) Longitudinal section of a GUS-stained 28 dpi *M. truncatula* nodule expressing the *gus* gene under the *MtFPN2* promoter region. ZI indicates Zone I; ZII, Zone II; IZ, Interzone; and ZIII, Zone III. Bar = 100 µm. (D) Cross section of a GUS-stained 28 dpi *M. truncatula* nodule expressing the *gus* gene under the MtFPN2 promoter region. Bar = 100 µm.



**Figure 2.** MtFPN2 is localized in intracellular membranes in the vasculature and in the symbiosome membrane in *S. meliloti*-infected nodule cells. (A) Longitudinal section of a 28 dpi *M. truncatula* nodule expressing *MtFPN2-HA* under its own promoter. The three C-terminal HA epitopes were detected using an Alexa594-conjugated antibody (red, left panel). Transformed plants were inoculated with a GFP-expressing *S. meliloti* strain (green, middle panel). Both images were overlaid with the channel showing DAPI-stained DNA (blue, right panel). Bar=100 µm. (B) Cross section of a vessel of 28 dpi *M. truncatula* nodule expressing *MtFPN2-HA* under its own promoter. The three C-terminal HA epitopes were detected using an Alexa594-conjugated antibody (red, left panel). Transillumination image is shown in the centre panel, and overlaid with the Alexa594 signal (right panel). Bar = 25 µm. (C) Detail of a *S. meliloti*-colonized 28 dpi nodule cell in the differentiation zone. Right panel shows the Alexa594 signal (red), middle panel the *S. meliloti* distribution (green), and the right one overlays both images. Bar = 10 µm. (D). Detail of symbiosomes shown by transmission electron microscopy (left panel). Arrows indicate the position of the colloidal gold particles conjugated to the antibody used to detect MtFPN2-HA. Middle panel, *M. truncatula* nodule vessel shown with transmission electron microscopy. Asterisks indicate the position of the xylem. Boxed region was amplified in the right panel to show the antibody-conjugated gold particles used to position MtFPN2-HA (indicated by arrows).

#### **FIGURE 3**



**Figure 3.** MtFPN2 transports iron out of the cytosol. Parental yeast strain DY150 was transformed with the pDR196 empty vector, while the *ccc1* mutant was transformed with either pDR196 or pDR196 harbouring the *MtFPN2* coding sequence. Serial dilutions (10x) of the transformants were grown in YPD medium (top panel), SD medium with the required auxotrophies (centre panel), or in SD medium with the required auxotrophies and 2.5 mM FeSO<sub>4</sub> (lower panel).

#### **FIGURE 4**



**Figure 4.** MtFPN2 is required for symbiotic nitrogen fixation. (A) *Tnt1* insertion in the fourth exon of *MtFPN2* results in no expression being detected by RT-PCR. The *Ubiquitin carboxyl-terminal hydrolase* (*MtUb1*) gene was used as a positive control. (B) Growth of representative wild type (WT), *fpn2-1*, and *fpn2-1* transformed with the *MtFPN2* coding sequence expressed under its own promoter (*FPN2<sub>prom</sub>::MtFPN2*). Bar = 1 cm. (C) Detail of representative nodules of WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed *fpn2-1* plants. Bars = 500 µm. (D) Number of pink or white nodules in 28 dpi WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed *fpn2-1* plants. Data are the mean  $\pm$  SE of at least 7 independently transformed plants. (E) Fresh weight of WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed plants. (F) Nitrogenase activity in 28 dpi nodules from WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed fpn2-1 plants. Data are the mean  $\pm$  SE. (G) Iron content in roots, shoot, and nodules of 28 dpi WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed *fpn2-1* plants. Data are the mean  $\pm$  SE. (G) Iron content in roots, shoot, and nodules of 28 dpi WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed *fpn2-1* plants. Data are the mean  $\pm$  SE. (G) Iron content in roots, shoot, and nodules of 28 dpi WT, *fpn2-1*, and *FPN2<sub>prom</sub>::MtFPN2*-transformed *fpn2-1* plants. Data are the mean  $\pm$  SE of 3 sets of 4 pooled plants. \* indicates statistically significant differences (p < 0.05).



**Figure 5.** *MtFPN2* loss-of-function alters iron distribution and speciation in 28 dpi nodules. (A). Synchrotron-based X-ray fluorescence images of wild type (left), pink *fpn2-1* (middle), and white *fpn2-1* (right) nodules, showing calcium (top panels) or iron (lower panels) distribution. Bars = 200  $\mu$ m, intensity scale in mass fraction units (g/g). (B) XANES spectra obtained from different regions of wild-type, pink *fpn2-1*, and white *fpn2-1* nodules (top left panel). This signal was decomposed into its two principal components for Zone II (top right panel), Zone III (lower left panel), and the vasculature (lower right panel). Green represents wild-type samples; red, pink *fpn2-1*; and blue, white *fpn2-1* nodules.

#### **FIGURE 6**



**Figure 6.** *MtFPN2* expression in cortical nodule cells partially restores the *fpn2-1* phenotype. (A) Growth of representative wild type (WT), *fpn2-1*, and *fpn2-1* transformed with the *MtFPN2* coding sequence expressed under the *MtMOT1.3* promoter (*MOT1.3*<sub>prom</sub>::*MtFPN2*). Bar = 1 cm. (B) Detail of representative nodules of WT, *fpn2-1*, and *MOT1.3*<sub>prom</sub>::*MtFPN2*-transformed *fpn2-1* plants. Bars = 500 µm. (C) Number of pink or white nodules in 28 dpi WT, *fpn2-1*, and *MOT1.3*<sub>prom</sub>::*MtFPN2*-transformed *fpn2-1* plants. Data are the mean ± SE of at least 11 independently transformed plants. (D) Fresh weight of WT, *fpn2-1*, and *MOT1.3*<sub>prom</sub>::*MtFPN2*-transformed plants. (E) Nitrogenase activity in 28 dpi nodules from WT, *fpn2-1*, and *MOT1.3*<sub>prom</sub>::*MtFPN2*-transformed *fpn2-1* plants. Acetylene reduction was measured in duplicate from two sets of four pooled plants. Data are the mean ± SE. \* indicates statistically significant differences (p < 0.05).