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1 **Short title: LjNPF8.6 controls symbiotic nodule functioning**

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35 **The nitrate transporter family protein NPF8.6 controls the N-fixing nodule**
36 **activity**

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62 **One sentence summary:**

63 Nitrate Transporter 1/Peptide Transporter Family protein NPF8.6 plays a key role in
64 nodulation in *Lotus japonicus*.

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73

74 **Abstract**

75 **N-fixing nodules are new organs formed on legume roots as result of the beneficial interaction**
76 **with soil bacteria, rhizobia. The nodule functioning is still a poorly characterized step of the**
77 **symbiotic interaction as only few of the genes induced in N-fixing nodules have been**
78 **functionally characterized. We present here the characterization of the member of the *Lotus***
79 ***japonicus* nitrate transporter 1/peptide transporter family *LjNPF8.6*. The phenotypic**
80 **characterization carried out in independent *L. japonicus* LORE1 insertion lines indicates a**
81 **positive role of *LjNPF8.6* on nodule functioning as knock out mutants display N-fixation**
82 **deficiency (25%) and increased nodular superoxide content. The partially compromised**
83 **nodule functioning induces two striking phenotypes: anthocyanin accumulation already**
84 **displayed four weeks after inoculation and shoot biomass deficiency, which is detected by**
85 **long-term phenotyping. *LjNPF8.6* achieves nitrate uptake in *Xenopus laevis* oocytes both at**
86 **0.5 mM and 30 mM external concentrations and a possible role as nitrate transporter on the**
87 **control of N-fixing nodule activity is discussed**

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105 **Introduction**

106 Nitrate (NO_3^-) represents together with ammonium (NH_4^+) the main forms of inorganic nitrogen
107 source for plant growth and metabolism with NO_3^- being the largely dominant supply form in
108 temperate climates (Miller & Cramer, 2005). Higher plants possess two NO_3^- transport systems to
109 cope with a wide range of external concentrations, the Low Affinity Transport System (LATS; >
110 0.5 mM) and the High Affinity Transport System (HATS; < 0.5 mM), both including constitutive
111 and inducible types of transport (Tsay *et al.*, 2007). In higher plants LATS proteins are mainly
112 represented by the Nitrate Transporter 1/Peptide Transporter Family (NPF), which include a large
113 number of genes (53 members in *Arabidopsis*, 80 in rice), divided in eight subfamilies and able to
114 transport different substrates (Léran *et al.*, 2014). To date, nitrate transport activity has been
115 reported for 17 out of 53 NPF proteins in *Arabidopsis thaliana* (Corratgé-Faillie & Lacombe,
116 2017), with AtNPF6.3 being the only exception as it displays dual affinity for nitrate in the high and
117 low concentration ranges (Liu *et al.* 1999). Dual affinity for NO_3^- uptake in *Xenopus* oocytes has
118 also been reported for the *Medicago truncatula* MtNPF6.8 (Morère-Le Paven *et al.*, 2011) and
119 MtNPF1.7 (previously named Numerous Infection and Polyphenolics/Lateral root-organ Defective;
120 NIP-LATD) proteins (Bagchi *et al.* 2012). Moreover, *Mtnpf1.7* KO mutant plants display a more
121 defective lateral root responses *in planta* at low KNO_3 concentrations than at higher concentrations
122 indicating a high affinity transport physiological function (Bagchi *et al.* 2012). The specificity of
123 spatio-temporal patterns of *AtNPFs* expression and of their regulatory profiles ensure nitrate uptake
124 from soil, long-distance transport within the plant body and distribution from source to sink tissues
125 (Krapp *et al.*, 2014; Noguero & Lacombe, 2016).

126 It is well known that nitrate plays also a role as signalling molecule involved in the control of
127 many physiological processes including gene regulation (Wang *et al.*, 2004) and root development
128 (Walch-Liu *et al.*, 2006). A crucial role in the nitrate signalling pathway governing root system
129 architecture and modulation of expression of many genes has been demonstrated for *AtNPF6.3* that
130 functions as a nitrate transceptor (Ho *et al.*, 2009; Krouk *et al.*, 2010). In particular, the control
131 exerted by AtNPF6.3 on lateral root development, in response to different external nitrate
132 concentrations, is mediated by its action as auxin transport facilitator (Krouk *et al.*, 2010; Bouguyon
133 *et al.*, 2015). Plant NPFs members encompass proteins capable of transporting different substrates
134 other than nitrate such as: di/tri-peptides, amino acids, glucosinolates, malate, auxin, abscisic acid
135 (ABA), gibberellic acid and jasmonic acid (Frommer *et al.* 1994; Jeong *et al.* 2004; Waterworth &
136 Bray, 2006; Krouk *et al.* 2010; Kanno *et al.* 2012; Nour-Eldin *et al.* 2012; Saito *et al.*, 2015; Tal *et*

137 *al.*, 2016). In particular, the multitransport feature recently reported for some NPF proteins
138 displaying phytohormones transport capacities, may suggest additional roles played by these
139 proteins on regulatory cross-talks linking different physiological signals (Krouk *et al.* 2010; Kanno
140 *et al.* 2012; Saito *et al.*, 2015; Chiba *et al.*, 2015; Tal *et al.*, 2016). However, the different transport
141 capacities are distributed among the eight NPF subclades identified in plants (Leràn *et al.*, 2014) as
142 sequence homologies do not correlate with substrate specificity and the determination of the
143 transported substrate cannot be determined from the sequences data alone.

144 Symbiotic Nitrogen Fixation (SNF) is part of a a multi-step mutualistic relationship, mainly
145 restricted to legumes, in which plants provide a niche (represented by root nodule organs) and fixed
146 carbon to the microorganism partner (*Rhizobium*), in exchange of fixed nitrogen. The establishment
147 and functioning of an effective SNF consists of: reciprocal recognition of symbiotic partners,
148 penetration, stimulation of cortical cell division (nodule primordium), invasion of divided cells,
149 differentiation of the endosymbiont, N-fixation, nodule senescence. As for root system, nitrate both
150 as nutrient and signal, plays a regulative role on the nodulation program and high external
151 concentrations inhibite different steps of SNF, although the mechanisms involved are still
152 controversial (Carroll & Gresshoff, 1983; Carroll & Mathews, 1990; Fujikake *et al.* 2003;
153 Barbulova *et al.* 2007; Omrane & Chiurazzi, 2009; Jeudy *et al.* 2010). Addition of 5 mM nitrate
154 quickly stops nodule growth and this effect seems to be linked to a decrease in photoassimilate
155 supply to growing nodules (Fujikake *et al.*, 2003). Furthermore, N-fixation activity is almost
156 completely lost after a short exposure to high nitrate concentrations (Schuller *et al.*, 1988; Vessey &
157 Waterer, 1992) and several hypotheses have been done to explain such a strong impact of nitrate on
158 nodule activity (Vessey & Waterer, 1992; Minchin, 1997; Naudin *et al.* 2011; Cabeza *et al.*, 2014).

159 In N-fixing nodules bacteria entered into root nodule cells are surrounded by a plant-derived
160 membrane, the peri-bacteroidal membrane (PBM), which encloses the intracellular bacteria in a
161 symbiosome (SM). Inside the SM bacteria differentiate into bacteroids with the ability to fix
162 atmospheric N₂ via nitrogenase activity. A primary nutrient exchange across the PBM is the
163 transport of a carbon energy as products of plant photosynthates to bacteroid in exchange for fixed-
164 nitrogen. N-fixation is an energy intensive process that requires also O₂ for respiration to generate
165 ATP and reducing equivalents for reduction of N₂ to NH₃. At the same time, as bacteroid
166 nitrogenase is inactivated by O₂, a micro-aerophilic condition must be maintained in rhizobia-
167 containing nodule cells. This is achieved by limiting the rate of O₂ influx through the outer
168 uninfected cells layers of the nodule (Witty & Minchin, 1998) and by maintaining high rates of
169 respiration in mitochondria and bacteroids of invaded cells (Bergensen 1996). High respiration rates
170 are mainly ensured by the presence at millimolar concentrations of the high affinity O₂⁻ binding

171 protein leghemoglobin (Lb), which delivers O₂ efficiently to mitochondria and bacteroids for
172 respiration while buffering free O₂ at the required level (Appleby 1984). This extremely high rate of
173 respiration in the invaded nodule cells is the main reason of Reactive Oxygen Species (ROS)
174 generation, whose steady-state concentrations must be strictly controlled as these represent not only
175 toxic by-products of aerobic metabolism but also key signals for nodulation. Nodule specific
176 metabolic pathways are completed by redox reactions involved in the control of concentrations of
177 ROS generated in N₂-fixing nodules (Evans *et al.*, 1999; Hernandez-Jimenez *et al.*, 2002; Puppo *et*
178 *al.*, 2005; Becana *et al.*, 2010; Matamoros *et al.*, 2013). The distinct metabolic pathways of N₂-
179 fixing nodules reflect changes in gene expression for related metabolic enzymes. Genome-wide
180 transcriptomic analyses have allowed the classification of genes induced in nitrogen-fixing nodules
181 and among these, a significant percentage of genes encoding for transporter proteins have been
182 found (Colebatch *et al.*, 2004; Hogslund *et al.*, 2009; Takanashi *et al.*, 2012). NPF proteins are
183 largely represented in this category of transporters and at least eight members have been recently
184 sub-classified as nodule induced (NI) genes in *L. japonicus* (Valkov & Chiurazzi, 2014).

185 Here we report the functional characterization of *LjNPF8.6*, a NI gene that plays a specific
186 positive role on nodule functioning controlling nitrogenase activity and nodular ROS content.

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188

189 **Results**

190

191 **LjNPF8.6 expression is progressively induced in N-fixing nodules**

192

193 We have previously reported the identification of a large *L. japonicus* NPF family consisting of
194 more than 70 members, 39 of which have a complete sequence that can be retrieved from the *L.*
195 *japonicus* whole-genome sequence resource (Sato *et al.* 2008; Valkov and Chiurazzi 2014;
196 <http://www.kazusa.or.jp/lotus/>). Transcriptomic data provided through gene specific and GeneChip
197 approaches (Hogslund *et al.* 2009; Criscuolo *et al.* 2012; Takanashi *et al.* 2012) allowed the
198 identification of a subclass of eight NPF genes with a clear-cut nodule induced (NI) level of
199 expression (Valkov & Chiurazzi, 2014). Among these, the gene Lj3g3v2681670.1 (genomic
200 assembly build 3.0, classified as chr2LjT15I01.230.r2.d in the build 2.5) has been sub-classified in
201 the clade 8 and provisionally named *LjNPF8.6* (Valkov & Chiurazzi, 2014), which encodes for a
202 561 amino acid protein with a molecular mass of 62.4 kDa.

203 In order to further characterize the profile of expression of *LjNPF8.6*, we have first analyzed the
204 distribution of the *LjNPF8.6* transcript in different organs of *L. japonicus* by qRT-PCR. Seedlings
205 have been germinated on Gamborg-B5 medium without N sources, inoculated with *M. loti* and
206 RNA extracted from different organs after four weeks. The *LjNPF8.6* gene is strongly expressed in
207 mature nodules, with an amount of transcript about 10-fold higher than in roots, whereas it is barely
208 detectable in stems, leaves and flowers (Fig. 1A). *LjNPF8.6* expression has been also tested through
209 a time course experiment in roots of *L. japonicus* inoculated with *M. loti* at one week after sowing.
210 *LjNPF8.6* expression pattern is not induced in roots at early times after *M. loti* inoculation, when
211 compared to the well-known early symbiotic marker *NODULE INCEPTION* (*LjNIN*) gene
212 (Schauser *et al.* 1999). Moreover, a progressive increase of the amount of *LjNPF8.6* transcript is
213 detected in nodule tissue at 10 and 28 days after inoculation (Fig. 1B). This profile of induction
214 starting after the onset of N-fixation and progressively induced during nodule maturation resembles
215 the one of the late nodulin genes, suggesting an involvement in nodule functioning rather than
216 development.

217

218 **Isolation of LORE1-insertion null mutants and phenotypic characterization**

219

220 To determine the *in vivo* function of *LjNPF8.6*, three independent LORE1 insertion mutants
221 have been isolated from the LORE1 lines collection (Fukai *et al.* 2012; Urbanski *et al.* 2012;
222 Malolepszy *et al.* 2016). Lines 53155, 49638 and 19899, bearing retrotransposon insertions in the

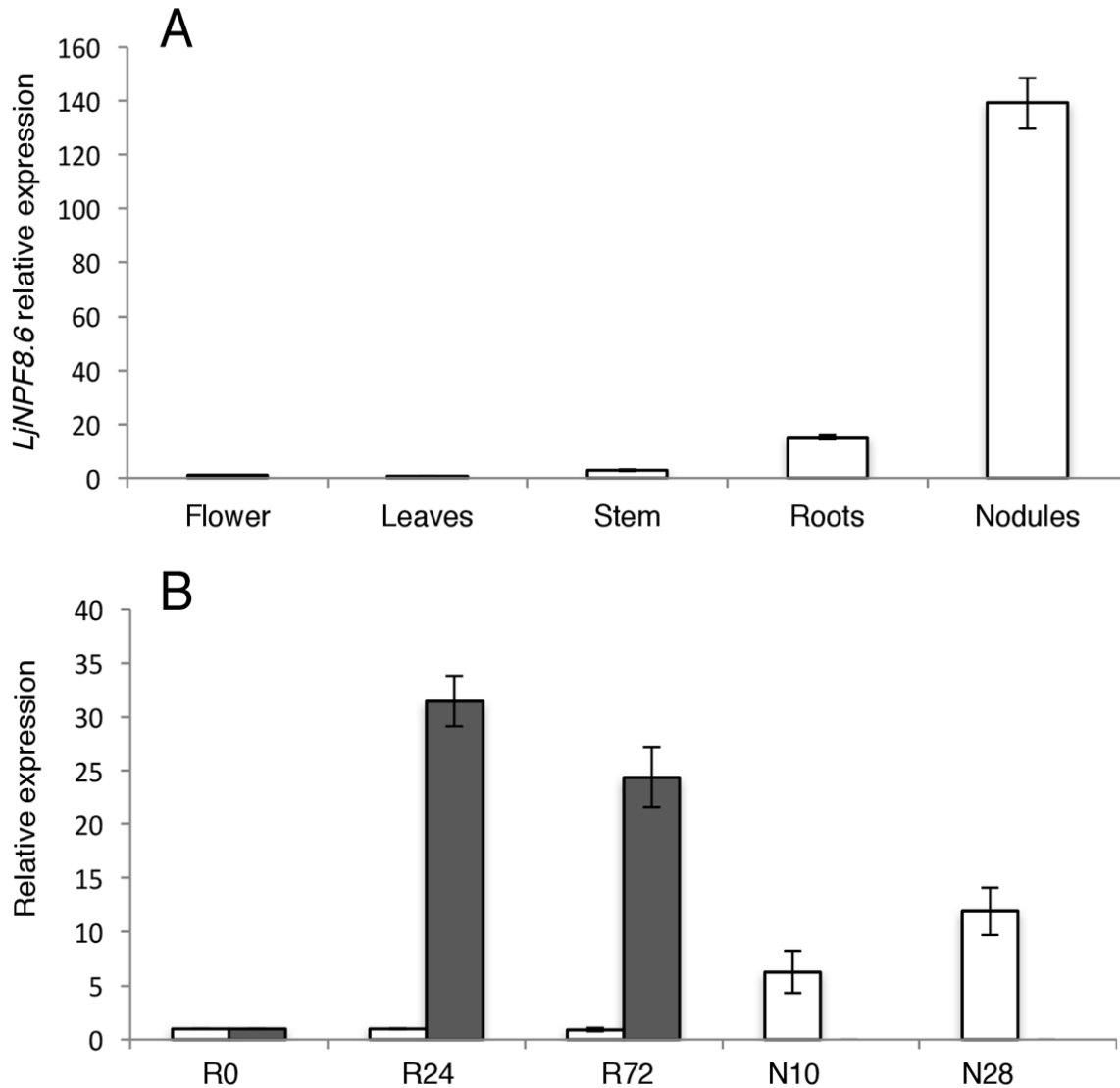


Figure 1. *LjNPF8.6* transcriptional regulation. A, Expression in different organs. RNAs have been extracted by wild type plants grown on Gamborg-B5 derivative medium without N source at four weeks after inoculation. Mature flowers have been obtained from Lotus plants propagated in the growth chamber. B, Time course of expression in root and nodule tissues after *M. loti* inoculation. RNAs have been extracted from roots of wild type seedlings grown in N starvation conditions at different times after inoculation (R0, 24 hrs, 72 hrs) and from young (10 dpi) and mature nodules (28 dpi). Expression levels are normalized with respect to the internal control ubiquitin (*UBI*) gene and plotted as relative to the expression of flowers (A) and T0 (B). White bars, *LjNPF8.6*; grey bars, *LjNIN*. Data bars represent the mean and standard deviations of data obtained with RNA extracted from three different sets of plants and 3 real-time PCR experiments.

223 second and third exon (Fig. 2), have been genotyped by PCR and plants homozygous for the
 224 insertion event into the *LjNPF8.6* gene, selected and transferred to the plant chamber for seeds

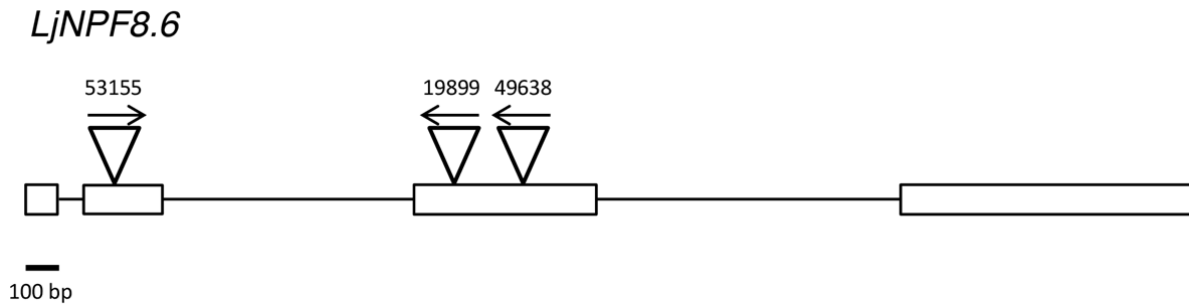


Figure 2. Exon/intron organization of the *LjNPF8.6* gene. Insertion sites and relative orientations of the LORE1 retrotransposon element in the 53155, 19899 and 49638 lines are indicated.

225 production. Endpoint RT-PCRs have revealed no detectable *LjNPF8.6* mRNA in mature nodules of
 226 homozygous plants screened from lines 53155, 49638 and 19899 and hence, these can be
 227 considered null mutants (Supplemental Fig. S1). Initially, two individual homozygous mutant plants
 228 from each line have been selected for analyses and because their growth phenotypes did not
 229 significantly differ, the data obtained with the selected individual mutants have been pooled in this
 230 study. The initial phenotyping of the three *LORE1* mutant lines included measurements of shoot
 231 lengths and fresh weights of 4-week-old plants with/out inoculation with *M. loti*. As shown in Fig. 3
 232 in the absence of N sources (no N) or in the presence of KNO_3 concentrations (100 μM ; 1 mM)
 233 compatible with full nodulation capacity, the three lines did not present significant differences when
 234 compared to wild type plants, in terms of shoot biomass and nodules number (Fig. 3A-C). In order
 235 to test whether *LjNPF8.6*, which is also expressed at a significant level in the root tissue, could be
 236 involved in the nitrate dependent inhibitory pathways controlling the nodule formation process,
 237 nodulation capacity has been also tested in the presence of high external concentrations of KNO_3
 238 (10 mM). As expected the number of nodules is strongly reduced in *L. japonicus* wild type plants
 239 (85%; Barbulova et al., 2007) and an identical inhibitory response is observed in the *Ljnpf8.6*
 240 mutants (Fig. 3C).

241 However, a careful analysis of phenotypes of the inoculated plants has allowed to detect visible
 242 accumulation of anthocyanin, conferring deep purple colour, in stems of mutant plants in symbiotic
 243 conditions when compared to wild type plants (Fig. 4A, B). The anthocyanin accumulation starts to
 244 be easily detectable in inoculated *Ljnpf8.6* plants at 17-20 days after inoculation and the spreading
 245 of pigments increases progressively up to the third internode (40-45 % of the stem length) at 4 wpi,
 246 whereas in wild type plants traces of pigmentation are observed only at the base of stem structure
 247 (Fig. 4A, B). A quantitative analysis performed through anthocyanin extraction from stem tissues at
 248 4 wpi has revealed a content 210-250% higher in nodulated KO than wild type plants grown either
 249 under no N as well as 1 mM KNO_3 conditions (Fig. 4C). A significant systemic increase of

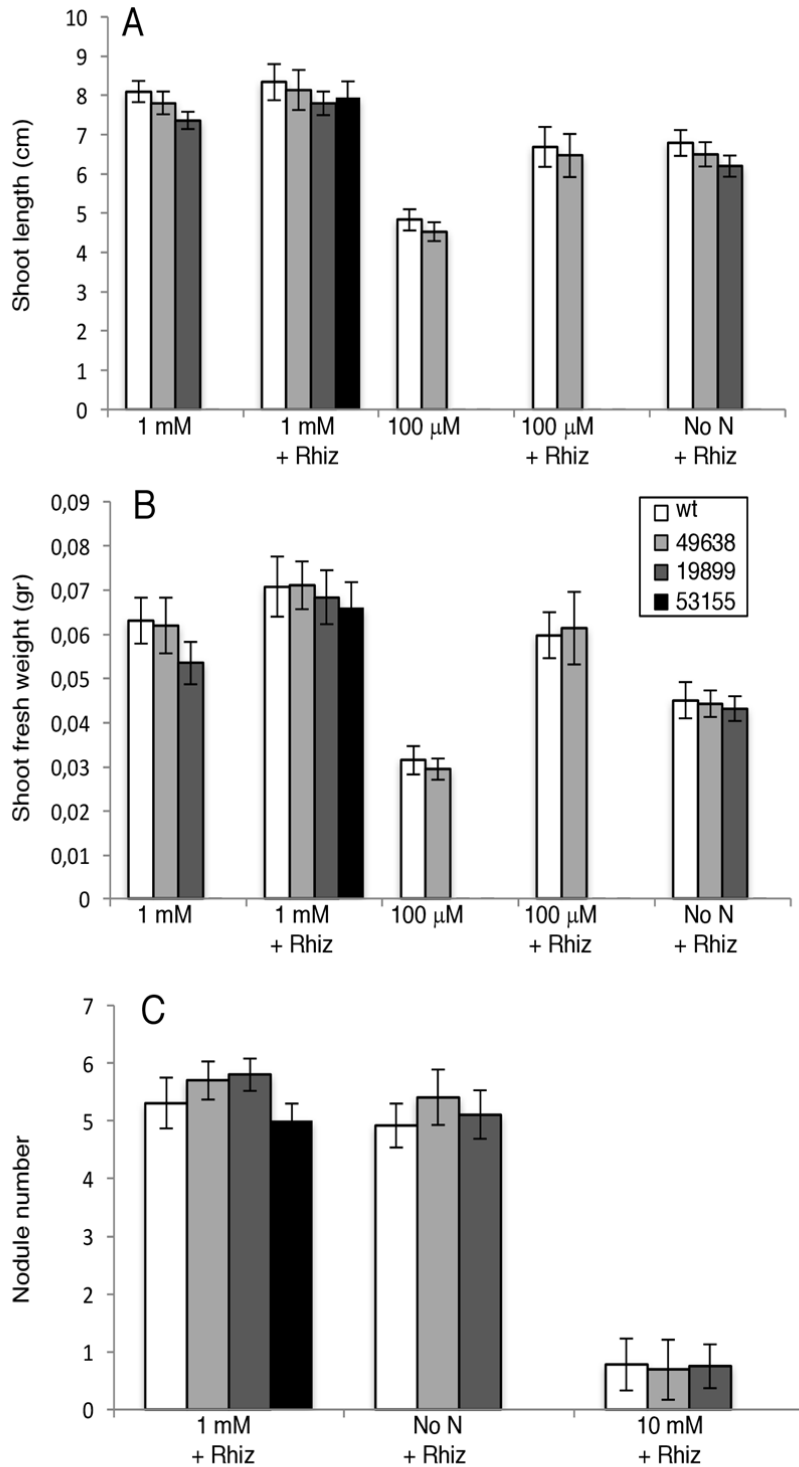


Figure 3. Quantitative analysis of shoot biomass and nodulation capacity of *L. japonicus* wild type and *LjNPF8.6* null mutants, grown in the presence of different KNO_3 concentrations, in symbiotic and non symbiotic conditions. A, Shoot length per plant. B, Fresh shoot weight per plant. C, Nodule numbers per plant. The different KNO_3 concentrations and when performed, *M. loti* inoculations are indicated. Bars corresponding to wild type and different LORE1 plants are indicated. Data bars represent means and SE of measures from three experiments (12 plants per experiment per condition). Data in A and B have been scored 25 days after sowing (21 days after transferring the plants from H_2O agar). Data in (c) have been scored 28 days after inoculation.

250 anthocyanin content is also revealed in roots of plant mutants inoculated with *M. loti* (Supplemental

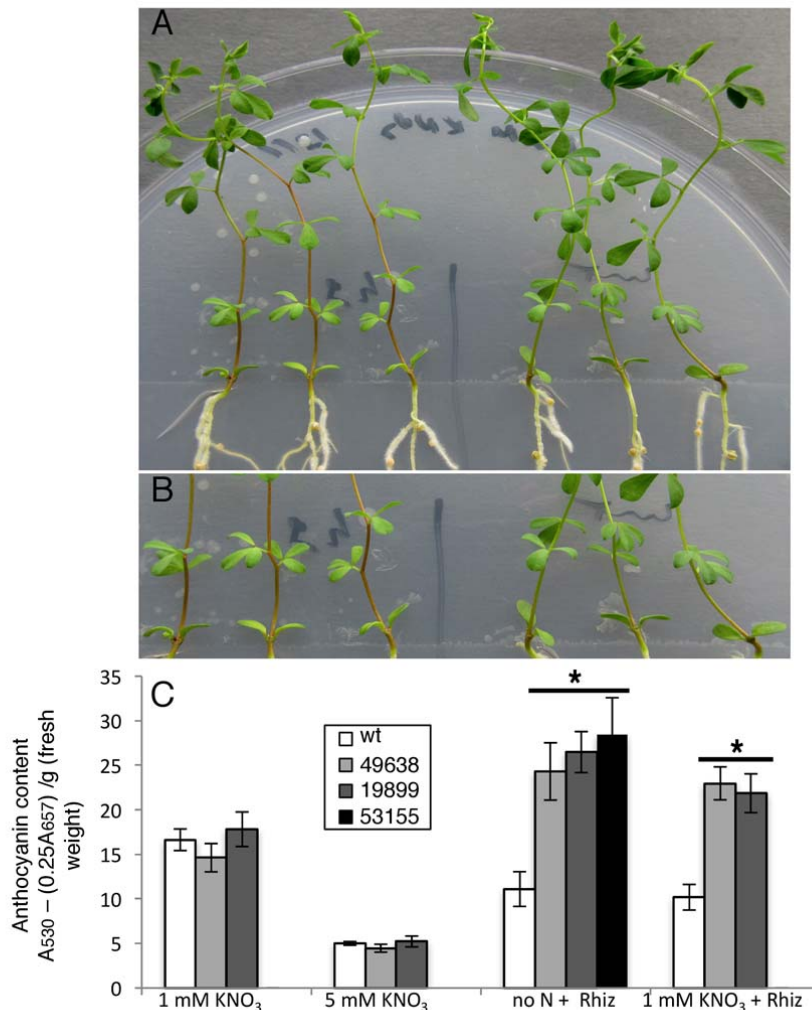


Figure 4. Analysis of anthocyanin content. A, Three representative wild type and 49638 plants are shown on the right and left of the Petri dish, respectively. B, Higher magnification showing intense purple colours in the stems of mutant plants (on the left). C, Anthocyanin content in stems of wild type and LOR1 lines. The different KNO₃ concentrations and when performed, *M. loti* inoculations are indicated. Bars corresponding to wild type and different LOR1 plants are indicated. Data bars represent means and SE of measures from three experiments (12 plants per experiment per condition). Data in C have been scored 28 days after inoculation. Asterisks indicate significant differences ($p < 0.001$) with wild type levels.

251 Fig. 2A). Conversely, un-inoculated plant mutants did not display anthocyanin accumulation in
 252 stems and roots as no quantitative differences have been detected in wild type and mutants plants
 253 grown in the presence of 1 mM or 5 mM KNO₃ (Fig. 4C; Supplemental Fig. S2A). Line 53155 has
 254 been analyzed only for plants inoculated on 1 mM KNO₃ conditions and not further utilized for
 255 phenotypic characterization because of the segregation of the *nod*⁻ phenotype due to the additional
 256 LOR1 insertion in the *CERBERUS* gene (Yano *et al.* 2009). However, the identical phenotype
 257 displayed by the 53155, 49638 and 19899 lines confirms that the LOR1 insertion in the *LjNPF8.6*
 258 gene is the causal mutation of the increased anthocyanin content observed exclusively in symbiotic
 259 conditions. In addition, heterozygous plants for the LOR1 insertion in the *LjNPF8.6* gene, isolated
 260 in the three lines, did not display high level of anthocyanin in the stem (data not shown).
 261

262 ***Ljnpf8.6* nodule mutants display nitrogenase activity deficiency under permissive low nitrate**
 263 **conditions, associated to a long-term shoot biomass reduction phenotype**

264

265 Accumulation of anthocyanin is a clear marker of plant response to different stress conditions
 266 such as low N availability (Diaz *et al.* 2006). In order to investigate whether the anthocyanin
 267 accumulation detected in the *Ljnpf8.6* mutant in symbiotic conditions is correlated to a reduced
 268 nodule functionality, we have compared N-fixation activity in nodules of wild type and mutant
 269 plants at four weeks after inoculation. A significant 25% decrease of acetylene reduction activity
 270 (ARA) is detected in nodules of *Ljnpf8.6* mutants grown either on absence of N or in the presence
 271 of 1 mM KNO₃ (Fig. 5A). However, the reduction of N-fixation capacity detected in the *Ljnpf8.6*
 272 KO genetic background is not correlated to any evident shoot phenotype other than antocyanin
 273 accumulation during *in vitro* growth, which must be limited to a short period of analysis (4 weeks
 274 post inoculation; Fig. 3A, B). In order to check whether a more severe shoot biomass phenotype
 275 could be displayed by the *Ljnpf8.6* mutants, 4 weeks old nodulated plants have been transferred on
 276 growth conditions compatible with long term phenotypic analyses. We have first tried to transfer
 277 the 4-weeks old nodulated plants to pots filled with inert material but the phenotyping analyses have
 278 been biased by a random, genotype-independent stress response due to bad adaptation to the new
 279 conditions of growth. Conversely, the transfer of nodulated plants to hydroponic conditions
 280 minimizes this unpredictable plant phenotype and all the plants could be scored for reliable shoot
 281 phenotypes after four more weeks. In Fig. 5B and C is shown the striking shoot biomass deficient
 282 phenotype displayed by the *Ljnpf8.6* mutants four weeks after the transfer in hydroponic conditions
 283 (8 wpi). The two main representative phenotypes observed in all the inoculated mutant plants are
 284 stunted shoots with pale green and/or abscised leaves (Fig. 5B, C).

285

286 ***Ljnpf8.6* mutants nodules display superoxide overproduction**

287

288 In *M. truncatula* the NIP/LATD protein has been associated, through the characterization of the
 289 weak allelic mutant *nip-3*, to defects in bacteria release or proliferation within nodule infected cells
 290 where fewer bacteria are observed (Teillet *et al.*, 2008). Therefore, we have tested whether the
 291 deficient N fixation activity observed in the *Ljnpf8.6* nodules (Fig. 5A) is associated to a reduced
 292 invasion capacity. Seedlings grown on N starvation conditions have been inoculated with a *M. loti*
 293 strain carrying a constitutively expressed *hema::lacZ* reporter gene fusion for staining of young and
 294 mature nodules. As shown in Fig. 6 (A-D), no differences have been observed in the density of the
 295 invading *M. loti* strain in wild type and mutant nodules.

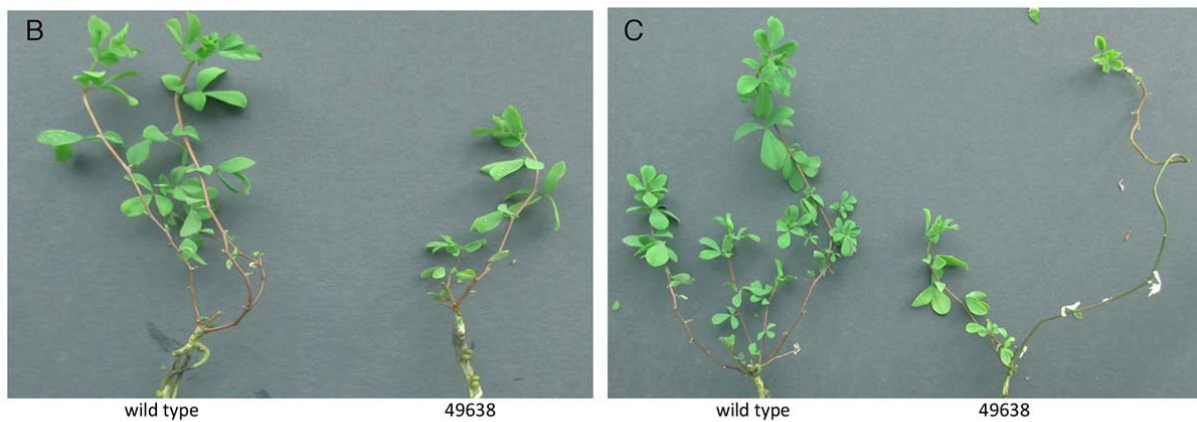
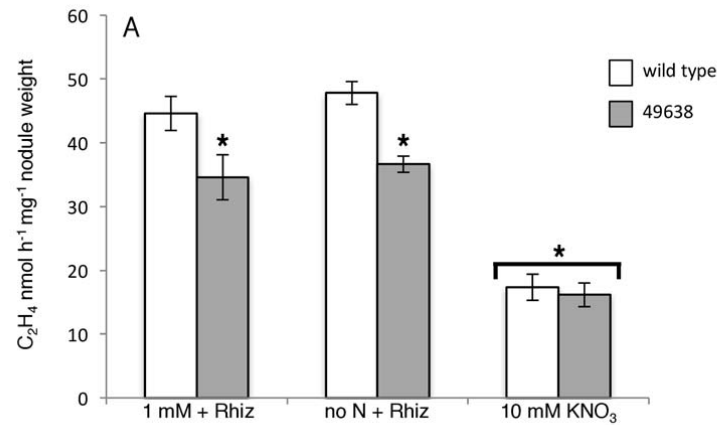


Figure 5. A, Acetylene Reduction Activity (ARA) per nodule weight of wild type and 49638 plants. The different KNO₃ conditions are indicated. Data bars indicate the mean and SE of three independent experiments (n=8 plants per exp.). Asterisks indicate significant differences (p<0.005) between wild type and 49638 nodules in no N and 1 mM KNO₃ conditions. The asterisk over the bar across the 10 mM condition indicates a significant reduction of ARA activity in nodules of both plant genotypes shifted on high nitrate conditions compared to nodules of plants maintained on low permissive conditions (p<0.001). B, C, Representative shoot phenotypes of eight weeks post inoculation wt and 49638 plants, transferred on hydroponic conditions in the presence of 0.5 mM KNO₃ at 4 wpi. Wild type and mutant plants have been maintained in the same vessels (4 vessels, 16 plants).

296 Reduction of N-fixation activity in mature nodules has been also associated to oxidative
 297 damages provoked by ROS overproduction that can be due to natural ageing or to exposure to
 298 different stress conditions (Becana *et al.*, 2010). We have monitored superoxide (O₂⁻) production at
 299 different stages of nodulation using the ROS-reactive dye NBT. The nascent nodule primordia are

300 strongly stained in both wild type and mutants (Fig. 6E, F) while as nodules mature and enlarge in
301 size, the staining intensity strongly decreases in both genotypes but remains more intense in mutant
302 nodules (Fig. 6G, H). In 60% of wild type big nodules the staining is not even detectable in whole
303 mount samples, whereas in all the comparable mutant nodules this is still clearly visible. In whole

304 mount samples, NBT staining is confined to lenticel structure on the nodule surface (Fig. 6G, H).
305 Longitudinal nodule sectioning has confirmed these differences of staining intensity in the
306 parenchima region (Fig. 6I, J). The quantitative analysis conducted on mature nodules confirms a
307 significant overall increase of O_2^- content in the 49638 line compared to wild type plants (25%; Fig.

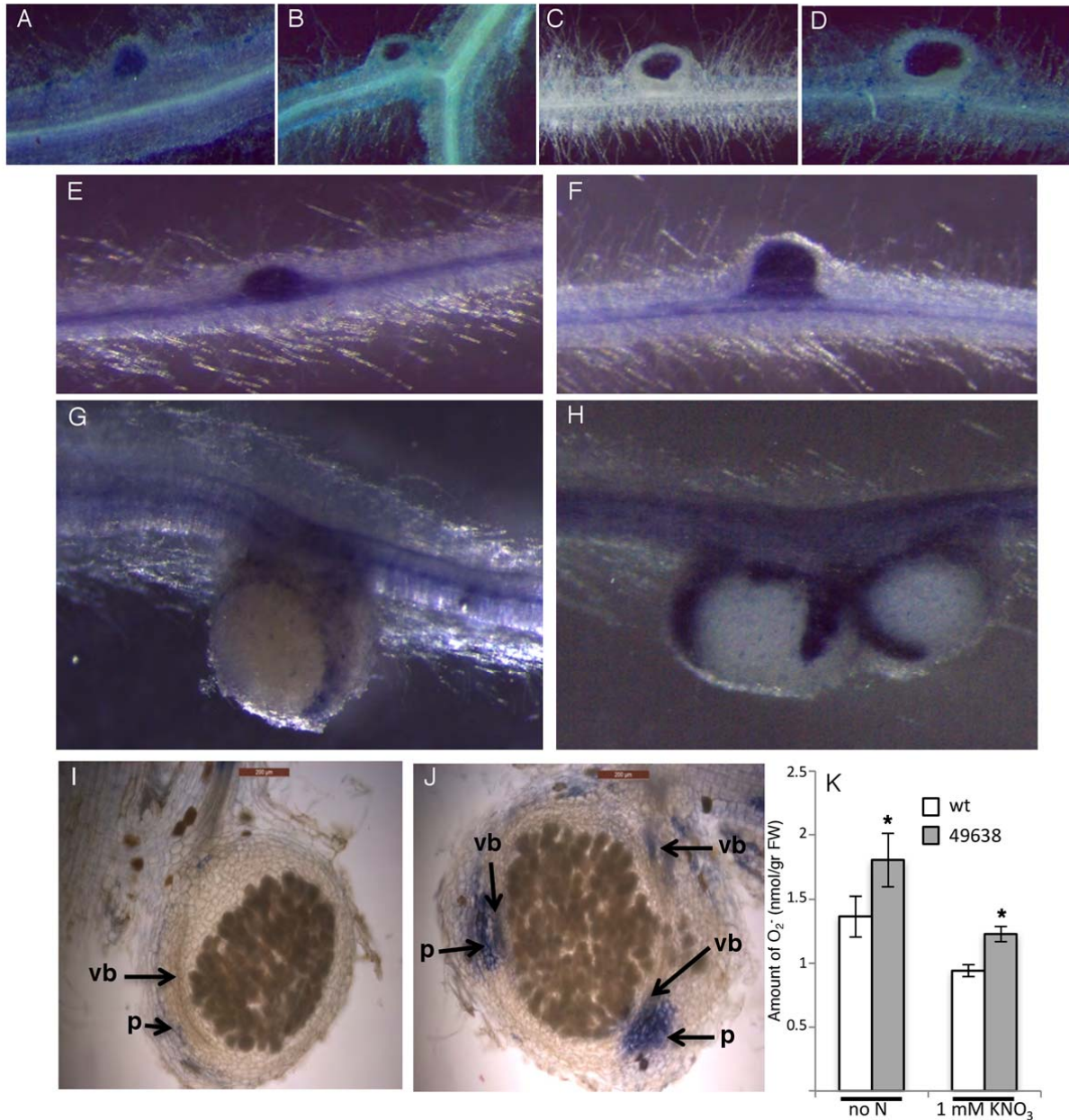


Figure 6. Phenotypic symbiotic characterization of *Ljnpf8.6* mutants. A-D, Histochemical detection of β -galactosidase activity to test *M. loti* (carrying the *hemA::lacZ* expressing plasmid) density in young and mature nodules of wild type (A, C) and 49638 plants (B, D). E-H, Whole mount NBT staining for superoxide anions detection of wild type (E, G) and 49638 (F, H) nodule primordia and mature nodules. I, J, Sections (100 μ m) of wild type (I) and 49638 (J) mature nodules stained with NBT. Arrows indicate staining in the parenchyma (p) and nodular vascular bundles (vb). K, Quantification of NBT staining in wild type and 49638 mutant line. Data bars represent means and SE of nodules from three independent samples (8 plants per experiment). Asterisks indicate significant differences with wild type values ($p < 0.05$).

308 6K). Superoxide content has been also quantized on root and stem tissues of inoculated plants,
 309 where no significant differences are observed (Supplemental Fig. S2B). These results indicate a
 310 local O_2^- increase into the nodule organ.
 311

312 **LjNPF8.6 is a nitrate transporter**

313

314 Nitrate transport activity has been reported for 17 out of 53 NPF proteins in *Arabidopsis thaliana*
 315 (Corratgé-Faillie & Lacombe, 2017), which have been characterized as low-affinity transporters
 316 with the exceptions of AtNPF6.3/NRT1.1 that is a dual-affinity nitrate transporter (Ho *et al.* 2009;
 317 Krouk *et al.* 2010). In the clade 8 of the plant NPF family (Léran *et al.* 2014), the only NPF
 318 member up to now characterized as a nitrate transporter when expressed in *Xenopus* oocytes, is the
 319 *Oryza sativa* OsNPF8.9 protein, which shares 45% amino acid identity with LjNPF8.6 (Lin *et al.*
 320 2000). In order to assess whether *LjNPF8.6* encodes a nitrate transporter, *in vitro*-synthesized
 321 *LjNPF8.6* complementary RNA (cRNA) has been injected into *Xenopus* oocytes for functional
 322 assay. Two days after injections oocytes have been tested for nitrate $^{15}\text{NO}_3$ uptake activity at two
 323 different nitrate concentrations at pH 5.5: low (0.5 mM) and high (30 mM). *LjNPF8.6* cRNA-
 324 injected *Xenopus laevis* oocytes have been compared to the *AtNPF6.3* injected ones. Both batches
 325 of oocytes display NPF-dependent $^{15}\text{NO}_3$ accumulation in 30 mM as well as low 0.5 mM of
 326 external nitrate (Fig. 7). Within this range of concentrations, a Michaelis-Menten fit leads to a Km
 327 of 7.8 mM indicating a LjNPF8.6 low-affinity transport capacity (Supplemental Fig. S3) while
 328 high-affinity capacity (low NO_3^- concentrations) range has not been tested.

329 The uptake activity observed in *Xenopus* oocytes (Fig. 7) prompted us to test for possible roles
 330 of LjNPF8.6 associated to nitrate transport function important for nodule activity. Therefore, we
 331 have checked whether LjNPF8.6 could play a role in the inhibitory pathway responsible of the
 332 abrupt decrease of nodule activity described after exposure to external high nitrate concentrations
 333 (Schuller *et al.*, 1988; Vessey & Waterer, 1992). Wild type and mutant nodulated plants (4 wpi)
 334 have been transferred for three days in the presence of 10 mM KNO_3 and nodule activity analyzed
 335 by ARA. The nitrogenase activity is inhibited at the same level in both wild type and *Ljnpf8.6*
 336 plants ruling out the hypothesis of a LjNPF8.6 involvement in the signalling pathway inhibiting
 337 nodule functioning at high external nitrate concentrations (Fig. 5A).

338 Jeong *et al.*, (2004) reported the identification of a NPF gene (*AgDCAT1*) expressed in nodules
 339 of *Alnus glutinosa* that encodes for a protein capable of malate transport in heterologous systems.
 340 Therefore, we have also tested the capacity of LjNPF8.6 to transport malate, the carbon source
 341 supplied to bacteroids for metabolism and nitrogen fixation (Day & Copeland, 1991). *LjNPF8.6* has
 342 been cloned into the *E. coli* expression vector pKK223-3 under the control of the *tac* promoter
 343 (Brosius & Holy, 1984) and the resulting plasmid used for transforming the dicarboxylate transport
 344 mutant CBT315 strain (*dctA*; Lo *et al.* 1972). Functional complementation has been tested on M9
 345 medium with 10 mM malate as the sole carbon source with/out isopropylthio- β -D-thiogalactoside

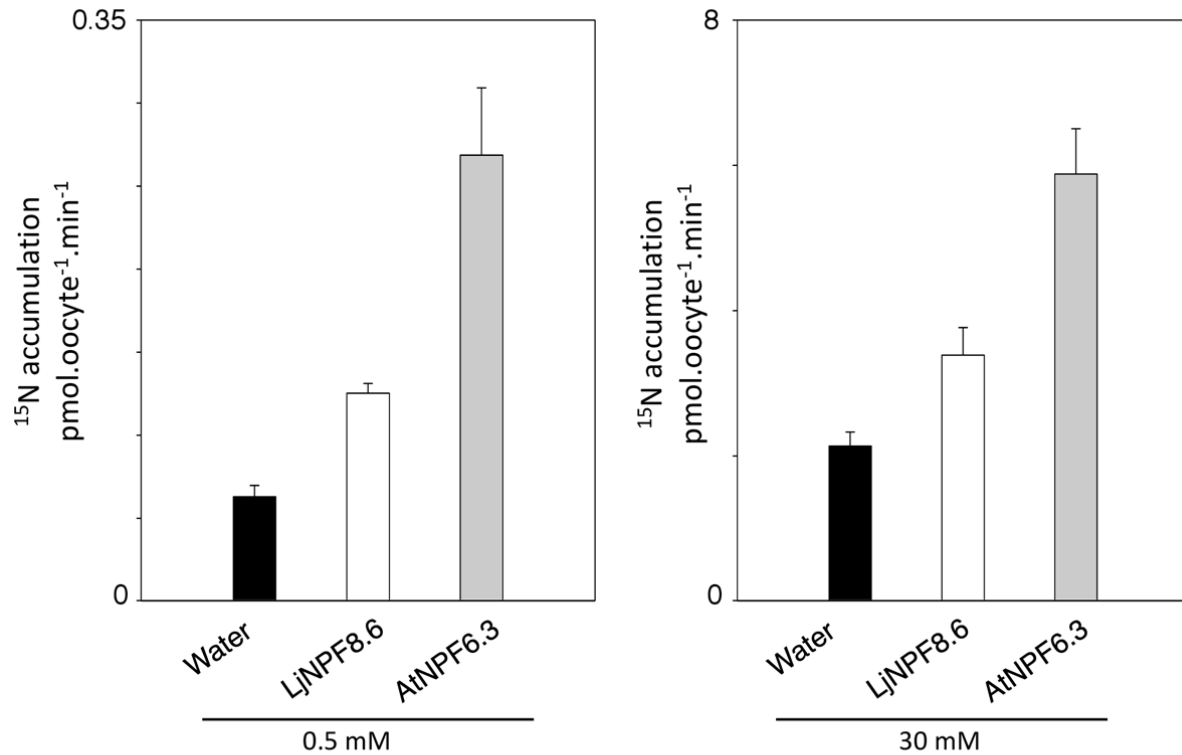


Figure 7.

Functional expression of *LjNPF8.6* in *Xenopus* oocytes in low (0.5 mM) and high (10 mM) external nitrate concentration. Nitrate uptake in control oocytes (black bars), injected with complementary RNAs (cRNAs) expressing *LjNPF8.6* (white bars) or *AtNPF6.3* (grey bars) (n = 5-8). Values are means \pm SE.

346 as inducer of the *tac* promoter and the *A. thaliana* gene *AtALMT6* (At2G17470), encoding a
 347 member of the alumin-activated malate transporter family, has been used as a positive control
 348 (Meyer *et al.*, 2011). We did not observe any growth of the CBT315 strain transformed with the
 349 *LjNPF8.6* expressing plasmid while *At2G17470* was able to complement the malate transport
 350 defect (Supplemental Fig. S4). This negative result suggests that *LjNPF8.6* doesn't encode for a
 351 malate transporter although we cannot exclude that the Lotus protein is not correctly expressed
 352 and/or addressed to the plasma membrane in *E. coli*.

353

354

355 ***Ljnpf8.6* mutants have unaltered nitrate content in different plant organs**

356

357 In order to check whether *LjNPF8.6* plays any role in the uptake of external nitrate and/or
 358 distribution of this nutrient to different plant tissues, we have compared the nitrate content of

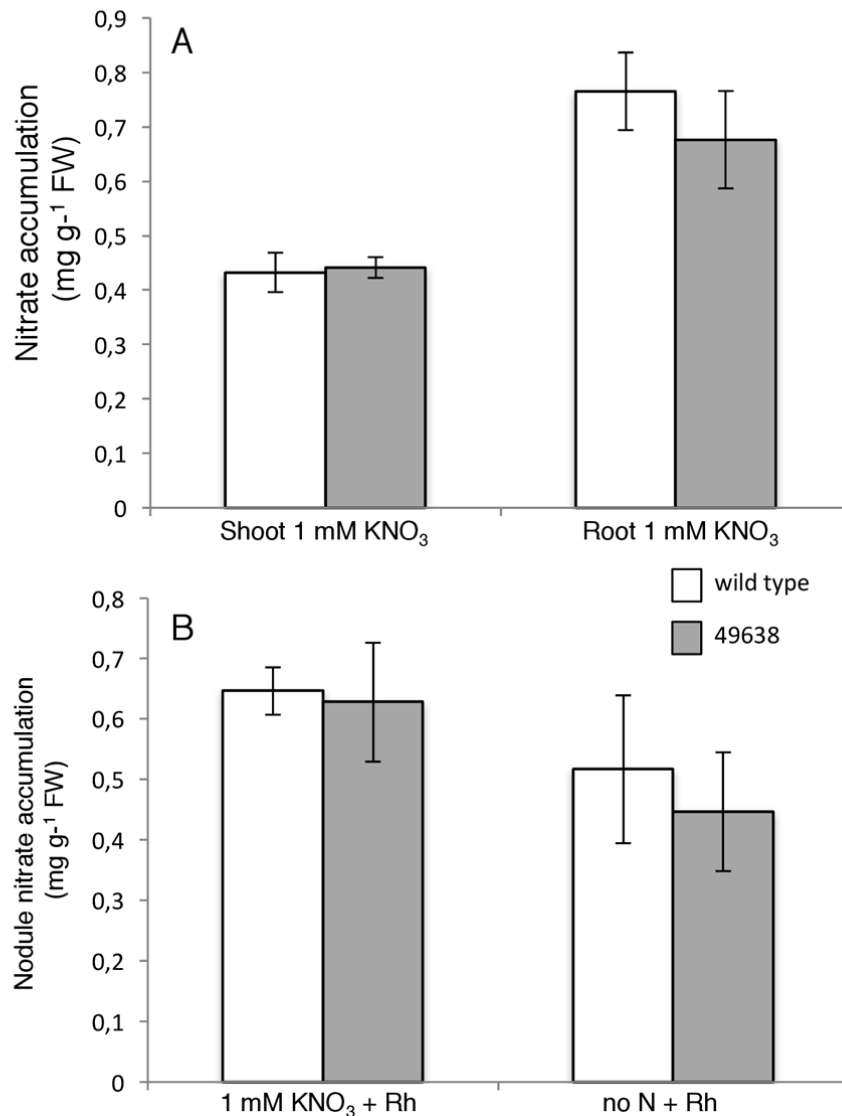


Figure 8. A, Nitrate content of shoots and roots from wild type and 49638 plants grown on 1 mM KNO₃ and inoculated with *M. loti*. Data bars represent means and SE from three independent samples (10 plants per sample). B, Nitrate content in wild type and 49638 nodules. Data bars represent means and SE of nodules from three independent samples (10 plants per sample). Bars corresponding to wild type and 49638 plants are indicated. Bars corresponding to wild type and 49638 plants are indicated.

359 different organs in wild type and *Ljnpf8.6* mutant plants. The analyses have been conducted at 4
 360 weeks after inoculation in plants grown in the presence of 1 mM KNO₃. The comparison of nitrate
 361 content either in roots and leaves of wild type and mutant plants did not show significant
 362 differences, confirming that LjNPF8.6 plays a role strictly related to the nodule functionality (Fig.
 363 8A). In the the micro-aerophilic condition associated to nodule environment, the efficiency of N-
 364 fixation is largely associated to nitrate dependent respiration pathways taking place in the nodule
 365 infected cells (Kato *et al.*, 2003; Meilhoc *et al.*, 2010; Horchani *et al.*, 2011). Therefore, we have

366 tested whether the reduced nitrogenase activity observed in the *Ljnpf8.6* mutants in low nitrate
367 permissive conditions (Fig. 5A) might be associated to a different nitrate content in nodules of wild
368 type and mutants plants. The nitrate content has been quantified in detached wild type and mutant
369 nodules of plants inoculated on no N and 1 mM KNO₃ conditions and displaying the anthocyanin
370 accumulation phenotype. The results shown in Fig. 8B indicate that, independently by the presence
371 of external nitrate supply, a significant content of this nutrient is detectable in nodule tissues and
372 that no significant differences are observed on nitrate accumulation in nodules of wild type and
373 mutant plants. However, since the nitrate dependent respiration pathway for maintenance of the
374 nodules energy status becomes more important in hypoxic than normoxic conditions (Horchani et
375 al. 2011; Hicri et al., 2015), we have also tested whether the stressful phenotype displayed by the
376 *Ljnpf8.6* null mutants at 4 wpi could qualitatively worsen in hypoxic conditions. Two-weeks old
377 wild-type and 46938 plants with the same number of nodules that do not show yet any anthocyanin
378 accumulation symptom, have been transferred in hydroponic cultures to reproduce water-logging
379 conditions that provoke hypoxic stress. Interestingly, mutant plants grown for two more weeks in
380 hydroponic conditions show a clear-cut increase of the anthocyanin accumulation phenotype that is
381 clearly visible throughout the length of the stem tissue almost up to the shoot apex (80-85 % of the
382 stem length vs 40-45 displayed in normoxic conditions; Fig. 4A, B), whereas wild type plants did
383 not show such an evident stressful symptom (Supplemental Fig. S5).

384

385

386 Discussion

387 We report here the functional characterization of a member of the *L. japonicus* NPF family,
388 LjNPF8.6 that plays a positive role on the symbiotic interaction through a nodule associated
389 function. NPF proteins represent a significant number of the NI transporters, and the qRT-PCR
390 analysis shown in Fig. 1 indicates that *LjNPF8.6* is strongly induced in nodule tissue, although it
391 cannot be considered a strict late nodulin gene as it is also expressed in root tissues. This result is
392 consistent with the analysis reported by Hogslund *et al.*, (2009), indicating that most genes
393 functioning in mature nodule are also expressed elsewhere in the plant. The induced profile of
394 *LjNPF8.6* expression in nodules is consistent with the specific symbiotic phenotypes displayed by
395 independent knock out *Ljnpf8.6* LORE1 insertion mutants. In particular, a striking anthocyanin
396 accumulation is observed in stems and roots of *Ljnpf8.6* mutants compared to wild type, only after
397 *M. loti* inoculation as no increase of pigmentation is observed in un-inoculated plants grown in the
398 presence of different nitrate concentrations (Fig. 4 and Supplemental Fig. S2A). This result clearly
399 indicates that the role played by LjNPF8.6 is strictly associated to the symbiotic programme and it
400 is not related to the N nutritional status of plants linked to the external nitrate availability. This is
401 also confirmed by the analyses of nitrate content in roots and shoots that show no significant
402 differences between wild type and mutant plants (Fig. 8A). Production of anthocyanin is considered
403 a hallmark of the plant response to unfavourable growth conditions (Chalker-Scott, 1999) and N
404 limitation has been reported to trigger different anthocyanin biosynthetic pathways and
405 accumulation in various plant tissues (Diaz *et al.*, 2006; Rubin *et al.*, 2009; Kovinich *et al.*, 2014).
406 In the case of symbiotic nitrogen fixation, anthocyanin accumulation in the stem is a symptom
407 normally exhibited by plant mutants showing an impaired N-fixation activity (Ott *et al.*, 2005;
408 Krussell *et al.*, 2005; Bourcy *et al.*, 2013). The anthocyanin accumulation phenotype is displayed in
409 the inoculated *Ljnpf8.6* mutants by 17-20 days post inoculation, after the observed induction of
410 *LjNPF8.6* expression in nodule tissue (Fig. 1B) and the onset of N-fixation, suggesting a role in the
411 control of nodule functioning rather than development. Interestingly, further information about the
412 spatial profile of *LjNPF8.6* in mature *L. japonicus* nodules came from a tissue-specific profiling
413 carried out by laser microdissection and microarray analysis, which revealed a specific expression
414 of six NI NPF members, including *LjNPF8.6*, in the central infection zone where N-fixation takes
415 place (Takanashi *et al.* 2012). In particular, the *LjNPF8.6* expression was not detected in the inner
416 cortex and vascular bundle zones, suggesting for this NPF member a function specifically
417 associated to N-fixation (Takanashi *et al.* 2012). Consistently, *Ljnpf8.6* mutants do not show any
418 difference compared to wild type plants, in the nodule formation capacity at different KNO₃
419 concentrations as well as in the early steps of nodule primordia invasion (Fig. 3C and 6A-D). The

420 direct involvement of LjNPF8.6 in the control of the nodule functionality is demonstrated by the
421 analysis reported in Fig. 5A where a significant reduced quote of N-fixation activity (25%) is
422 measured in mutants nodules. We also demonstrate that LjNPF8.6 does not play any role in the
423 quick transport of external high nitrate concentration to the nodule tissues and/or sensing, which
424 must be involved in the nitrate dependent N-fixation inhibitory pathway (Fig. 5A; Arrese-Igor *et al.*,
425 1998; Cabeza *et al.*, 2014).

426 Nitrogen-fixation is an extremely expensive process for legume plants as root nodules are
427 optional C sink organs that exploit large amount of photosynthate resources. The partially
428 compromised N-fixation activity displayed by the *Ljnpf8.6* mutants in the presence of low
429 concentration of N sources (Fig. 5A), determines certainly a stressful condition, which is
430 responsible of the anthocyanin accumulation (Fig. 4 and Supplemental Fig. S2A and S4), but it is
431 still sufficient at 4 wpi, to sustain a normal shoot biomass phenotype (Fig. 3A, B). However, when
432 plants are maintained for longer time in symbiotic conditions, clear-cut N deficiency symptoms
433 such as stunted shoots with pale green and/or abscised leaves are clearly displayed by the *Ljnpf8.6*
434 mutants (Fig. 5B, C). These phenotypes have been classified as $\text{Fix}^+/\text{Fix}^-$, associated to plant
435 mutants with a less efficient N-fixation activity, which display N-deficiency phenotypes not as
436 severe as in the *fix*⁻ mutants (Pislariu *et al.*, 2012).

437 Reduction and loss of N-fixation activity is associated to oxidative stress during natural
438 senescence of nodules and similar correlated phenotypes can be observed in early senescence
439 induced by exposure to stress conditions (Puppo *et al.*, 2005). Therefore, the N-fixation deficiency
440 and increased superoxide content phenotypes displayed in mature nodules of *Ljnpf8.6* mutants (Fig.
441 5A, 6K) are strictly associated each other, although a cause-effect relationship can be difficult to
442 establish. The pattern of superoxide production during nodule growth and maturation, shown in the
443 time course experiment displayed in Fig. 6, is consistent with previous reports where NBT staining
444 revealed superoxide accumulation in nodule primordia of indeterminate as well as determinate
445 nodules (Fig. 6E, F; Santos *et al.*, 2001; Montiel *et al.*, 2016). To our knowledge, the pattern of O_2^-
446 accumulation reported in Fig. 6 (G-J) has never been described in mature determinate nodules. The
447 NBT staining indicates a predominant localization on the lenticels structures, characterized as the
448 choke points controlling the gaseous exchanges in determinate nodules (Frazer, 1942; Pankhurst &
449 Sprent, 1975; Jacobsen *et al.*, 1998; Fig. 6G, H) and the parenchyma regions (Fig. 6I, J).
450 Biochemical and transcriptomic analyses indicate that in mature determinate nodules most of the
451 ROS generating processes, which occur during natural or early senescence induced by exposure to
452 stress conditions, originate in the central infected region and then spread outwards (Evans *et al.*,
453 1999; Puppo *et al.*, 2005; Matamoros *et al.*, 2013). Therefore, it is reasonable to predict the

454 superoxide diffusion and accumulation in lenticels structures and parenchymatic tissue, which are
455 located a few cells apart from the infected zone. Furthermore, we demonstrate that the increased
456 content of superoxide detected in the mutant plants is restricted to nodular tissue (Supplemental Fig.
457 S2B) and therefore the systemic pattern of anthocyanin accumulation (Fig. 4 and Supplemental Fig.
458 S2A) is likely not associated to a direct action of scavenging (Yamasaki *et al.*, 1996).

459 Members of the NPF family may encompass different putative strategic roles associated to the
460 control of nodule functioning as they can transport nitrate, amino acids, peptides, dicarboxylic acids
461 and abscisic acid (ABA), (Frommer *et al.*, 1994; Waterworth & Bray, 2006; Jeong *et al.* 2004;
462 Kanno *et al.*, 2012). Malate is the carbon source supplied to bacteroids for metabolism and nitrogen
463 fixation (Day & Copeland, 1991) and in isolated soybean symbiosomes a carrier for monovalent
464 dicarboxylate ions with a higher affinity for malate than for succinate has been identified (Udvardi
465 and Day 1997). Jeong *et al.*, (2004) also reported the identification of a *NPF* gene (*AgDCAT1*)
466 expressed in nodules of *Alnus glutinosa* encoding for a protein capable of malate transport in
467 heterologous systems, but no further functional characterization of *AgDCAT1* has been provided.
468 The failure of *LjNPF8.6* to complement the malate transport defect of the *E. coli dctA* mutant
469 (Supplemental Fig. S4) does not allow to conclude that it is not involved in the C sources supply to
470 bacteroids (Day & Copeland, 1991), as we cannot exclude a non functional expression of the Lotus
471 transporter in *E. coli*. Another function that must be taken in account for explaining the critical role
472 played by *LjNPF8.6* on nodule activity is related to a peptide/amino acids transport capacity. The
473 transport of these substrates may play a positive role for supplying branched-chain amino acids
474 necessary for bacteroid development and persistence (Prell *et al.*, 2009) and/or for supporting
475 polyamines biosynthesis for Nitric Oxide (NO) production (Gupta *et al.*, 2011). ABA signalling has
476 been also involved in the control of nodule functionality. However, ABA has been reported to
477 negatively regulate N-fixation in *L. japonicus*, as its reduced content obtained in low sensitive
478 mutants as well as wild type plants treated with abamine (a specific inhibitor of 9-cis-
479 epoxy-carotenoid dioxygenase), resulted in increased N-fixation activity therefore making unlikely
480 the involvement of *LjNPF8.6* in the nodular ABA transport/signalling pathway (Tominaga *et al.*,
481 2009).

482 The preliminary biochemical characterization of *LjNPF8.6* carried out in our work, indicates that
483 it is capable to achieve nitrate uptake in *Xenopus* oocytes at high (30 mM) and low (0.5 mM)
484 external nitrate concentrations (Fig. 7). Importantly, the positive role played by *LjNPF8.6* on nodule
485 activity, indicated by the nitrogenase deficiency phenotype displayed by *Ljnpf8.6* mutants (Fig.
486 5A), is observed in plants grown in chronically absence of N or in low permissive KNO_3 conditions
487 (≤ 1 mM), which is consistent with the reported capacity of enhancing nitrate uptake in *Xenopus*

oocytes at 0.5 mM external nitrate concentration (Fig. 7). NPF members are significantly represented in the protein fraction associated to the PBM and characterized either in *L. japonicus* and soybean nodules (Wienkoop & Saalbach., 2003; Clarke *et al.*, 2015). In particular, the recent comprehensive sampling reported for PBM proteins in soybean has allowed the identification of five NPF members associated to PBM. Interestingly, the retrieved GmNPF8.6 protein (Glyma02g38970.1) is also a member of the clade 8 and shares 69% amino acid identity with LjNPF8.6 (Clarke *et al.*, 2015). A nitrate flux through the PBM, critical for nodule activity, has been previously proposed. Anion transporters, members of the Major Facilitator Superfamily, with selectivity preference for nitrate, have been identified in the PBM of soybean (*Glycine max*) and *L. japonicus* nodules (GmN70 and LjN70) and their role for the control of ion and symbiosome membrane potential homeostasis has been postulated (Udvardi *et al.*, 1991; Vincill *et al.*, 2005). In addition, nitrate transport through the PBM has been proposed to be crucial for the nitrate-Nitric Oxide (NO) respiration process reported in nodules microaerobic conditions, which is important for the maintenance of the energy status required for nitrogen fixation in normoxic and hypoxic conditions (Igamberdiev & Hill, 2009; Kato *et al.*, 2010; Sanchez *et al.*, 2010; Horchani *et al.*, 2011). Nitrate in the cytosol and symbiosomes of invaded plant cells is produced at elevated rates (Herold & Puppo, 2005; Meilhoc *et al.*, 2010; Horchani *et al.*, 2011) and our analysis of nitrate content in nodule tissues confirms the presence of an active nitrate biosynthetic pathway within nodules independently by external supply (Fig. 8B), which may justify the symbiotic phenotypes detected in the *Ljnpf8.6* mutants even in the absence of external N sources (Fig. 4, 5A). Therefore, LjNPF8.6 might play an active role in the control of nodule activity by participating to the nitrate flux through the PBM (Udvardi *et al.*, 1991; Horchani *et al.*, 2011). In this context, the un-changed nitrate content observed in whole detached nodules of wild type and mutant inoculated plants (Fig. 8B) can be expected as it would be the distribution of nitrate between different compartment of N-fixing nodule cells to be rather affected in the *Ljnpf8.6* background. Interestingly, we have observed an increased stressful phenotype associated to anthocyanin accumulation in the *Ljnpf8.6* mutants grown under hydroponic hypoxic conditions, where the achieving of nitrate reduction into nitrite constitute the main route for NO biosynthesis (Supplemental Fig. S5; Horchani *et al.*, 2011; Hicri *et al.*, 2015).

517

518 **Conclusions**

519 To our knowledge, LjNPF8.6 represents the first NPF protein playing a specific role on nodule
520 functioning as demonstrated by the description of different N-deficiency associated phenotypes
521 displayed by independent knock out mutants in symbiotic conditions. Further experiments will be

522 necessary to interpret its mechanism of action, taking in account different substrates specificities
523 and possible functional redundancy of these transporters in the nodule organ.

524

525

526 **Material and Methods**

527 *Plant material and growth conditions*

528

529 All experiments have been carried out with *Lotus japonicus* ecotype B-129 F14 GIFU (Handberg
530 & Stougaard 1992; Jiang & Gresshoff 1997). Plants are cultivated in a growth chamber with a light
531 intensity of 200 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ at 23°C with a 16 hr/8 hr day/night cycle. Solid growth media have
532 the same composition as that of B5 medium (Gamborg, 1970), except that $(\text{NH}_4)_2\text{SO}_4$ and KNO_3
533 are omitted and/or substituted by different concentrations of KNO_3 . KCl is added to the medium to
534 replace the potassium source. The media containing vitamins (Duchefa catalogue G0415) are
535 buffered with 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES; Duchefa, M1503.0250) and pH
536 adjusted to 5.7 with KOH.

537 For hydroponic cultures, wild type and mutant nodulated plants are transferred in the same
538 vessels with derivative B5 liquid medium with 0.5 mM KNO_3 or without N sources (6 plants per
539 vessel). Media are renewed every four days, when also the pH is checked and maintained within
540 close limits (5.6-5.8) during the whole growth.

541 *M. loti* inoculation is performed as described in Barbulova *et al.*, (2005). For phenotypic
542 comparisons, after germination unsynchronized seedlings are discarded. The strain R7A is used for
543 the inoculation experiments and is grown in liquid TYR-medium supplemented with rifampicin (20
544 mg/L). The *M. loti hemA::lacZ* strain was kindly provided by Dr. Jens Stougaard (University of
545 Aarhus, Denmark) and is grown in the same medium supplemented with rifampicin and tetracycline
546 (20 mg L⁻¹).

547

548 *LORE1 lines isolation*

549 LORE1 lines 30053155, 30049638 and 30019899 (hereafter abbreviated as 53155, 49638 and
550 19899, respectively) have been obtained from the *LORE1* collection (Fukai *et al.*, 2012; Urbanski *et al.*,
551 2012; Malolepszy *et al.* 2016). The plants in the segregating populations have been genotyped
552 and expression of homozygous plants tested with primers listed in Table S1.

553

554 *Determination of Acetylene-Reduction Activity (ARA)*

555 Detached roots with comparable number of nodules are placed in glass vials. The vials are filled
556 with an acetylene-air mixture (C_2H_2 :air = 1:9 v/v). After 30 min of incubation at 25°C, the amount
557 of ethylene in the gas phase is determined using gas chromatograph (PerkinElmer Clarus 580).

558 For the analysis of ARA activity after shift in high KNO_3 conditions, 4 wpi nodulated plants are
559 transferred on slanted Petri dishes where roots are placed in sandwich position between two filter
560 papers wet with a Gamborg B5 liquid media containing no KNO_3 or 10 mM KNO_3 . Plants are
561 maintained for three days in these conditions with filter papers wet with 20 ml liquid media. After
562 three days ARA activity is tested as described above.

563

564 *Estimation of anthocyanin*

565 Stem tissues from three plants per assay are weighed and then extracted with 99:1 methanol:HCl
566 (v/v) at 4°C. The OD_{530} and OD_{657} for each sample is measured and relative anthocyanin levels
567 determined with the equation $OD_{530} - (0.25 \times OD_{657}) \times \text{extraction volume (ml)} \times 1/\text{weight of tissue}$
568 $\text{sample (g)} = \text{relative units of anthocyanin/g fresh weight of tissue}$.

569

570 *Determination of nitrate content*

571 Colorimetric determination of nitrate content in nodules extracts has followed the procedure
572 described by Pajuelo *et al.*, (2002). 200 μL of 5% (w/v) salicylic acid in concentrated sulfuric acid
573 is added to aliquots of 50 μL from the crude extracts and left to react for 20 min at room
574 temperature. NaOH (4.75 ml of 2N) is added to the reaction mixtures and the absorbance read at
575 405 nm after cooling. A calibration curve of known amount of nitrate dissolved in the standard
576 extraction buffer is used for analytical determinations. Controls are set up without salicylic acid.

577

578 *LacZ activity and histochemical localization*

579 Lotus roots isolated after *M. loti hema::lacZ* infection are first gently rinsed in 50 mM KH_2PO_4
580 buffer pH 7.2 and then fixed for 1 hour with 1% paraformaldehyde, 0.3 M mannitol in 50 mM
581 KH_2PO_4 buffer pH 7.2. The tissues are washed again with 5 mM KH_2PO_4 buffer pH 7.2 and the
582 histochemical analysis performed according to Omrane *et al.* (2009). The stained nodulated roots
583 are photographed with a Nikon microscope using bright-field and epipolarization optics.

584

585 *Superoxide staining*

586 For O₂⁻ staining, Nitroblue Tetrazolium (NBT, Sigma Aldrich) was used. Nodulated roots are
 587 submerged in 1 mM NBT in 0.1 mM K-phosphate buffer (pH 7.5), vacuum infiltrated for 30 sec.
 588 and incubated 30 min. at R.T. NBT staining solution is removed and stained roots washed twice in
 589 80% ethanol. NBT staining is repeated three times for a total of 10 nodules per experiment. For
 590 longitudinal sections 12 nodules from independent experiments have been sectioned in 100 µm
 591 thick slices. Samples are photographed with a Nikon microscope using bright-field and
 592 epipolarization optics. For O₂⁻ quantification, NBT-stained nodules are first ground into a fine
 593 powder and dissolved in 2M KOH:DMSO (1:1.16, v/v) followed by centrifugation at 12,000g for
 594 10 m. Absorbance at 630 nm is immediately measured and then compared with a standard curve
 595 plotted from known amount of NBT in the KOH:DMSO mix (Ramel *et al.* 2009). Experiments have
 596 been conducted on three biological replicates for a total of 40 nodules per genotype.

597

598 *Quantitative Real-time RT-PCR*

599 Real time PCRs have been performed with a DNA Engine Opticon 2 System, MJ Research (MA,
 600 USA) using SYBR to monitor dsDNA synthesis. The procedure is described in Rogato *et al.*
 601 (2008). The ubiquitin (*UBI*) gene (AW719589) has been used as an internal standard. The
 602 concentration of primers has been optimized for every PCR reaction and amplifications have been
 603 carried out in triplicate. The PCR program used was as follows: 95°C for 3 min and 39 cycles of
 604 94°C for 15 sec., 60°C for 15 sec. and 72°C for 15 sec. Data have been analyzed using Opticon
 605 Monitor Analysis Software Version 2.01 (MJ Research). The qRT-PCR data have been analyzed
 606 using comparative Ct method. The relative level of expression is calculated with the following
 607 formula: relative expression ratio of the gene of interest is $2^{-\Delta CT}$ with $\Delta CT = Ct_{\text{GENE}}$ minus Ct_{UBI} .
 608 The efficiency of the *LjNPF8.6* primers is assumed to be 2. Analysis of the melting curve of PCR
 609 product at the end of the PCR run has revealed a single narrow peak for each amplification product,
 610 and fragments amplified from total cDNA have been gel-purified and sequenced to assure accuracy
 611 and specificity. The oligonucleotides used for the qRT-PCR are listed in Table S1.

612

613 *Plasmids preparation*

614 The plasmid for expression in *Xenopus laevis* oocytes has been prepared in the following way:
 615 cDNA prepared from nodule RNA has been amplified with the forward primer containing the
 616 *Bam*HI site in combination with the reverse primer containing the *Eco*RI site (Table S1). The 1713
 617 bp fragment has been double digested *Bam*HI-*Eco*RI and subcloned into the pGEMHE plasmid

618 containing the 5'-UTR and 3'-UTR of the *Xenopus* β -*GLOBIN* gene (Liman *et al.*, 1992), pre-
619 digested *Bam*HI-*Eco*RI to obtain pGEMHE8.6. The correct coding sequence of *LjNPF8.6* has been
620 verified by sequencing.

621 For expression in *E. coli*, pGEMHE-8.6 has been double-digested *Bam*HI-*Eco*RI and sub-cloned
622 in pKK223-3 plasmid (Brosius & Holy, 1984) to obtain pKK-8.6. Primers for the sub-cloning of the
623 *A. thaliana* At2G17470 gene used as positive control in the complementation test are indicated in
624 the Table S1.

625

626 *Functional analysis of LjNPF8.6 in Xenopus laevis oocytes*

627 pGEMHE-NPF8.6 has been linearized with *Nhe*I and capped mRNA transcribed *in vitro* using
628 the mMessage mMachine T7-ultra Kit (Life Technologies). Oocytes preparation has been described
629 in (Lacombe & Thibaud, 1998). Defolliculated oocytes are injected with 20 ng of complementary
630 RNA (cRNA) and stored in a modified ND96 medium (2 mM KCl, 96 mM NaCl, 1 mM MgCl₂, 1.8
631 mM CaCl₂, 5 mM HEPES, 2.5 mM sodium pyruvate, pH 7.5, supplemented with gentamycin
632 sulphate (50 μ g mL⁻¹). Two days after injection, batches of 10 injected oocytes are incubated in 1
633 mL of modified ND96 solution at pH 5.5 supplemented with 30 mM or 5 mM ¹⁵N₃ supplied as
634 K¹⁵N₃ for 2 hrs at 18°C. Oocytes are then rinsed five times in 15 mL cold modified ND96
635 solution. Batches of 2 oocytes are then analyzed for total N content and atom % ¹⁵N abundance by
636 Continuous-Flow Mass Spectrometry, using an Euro-EA Eurovector elemental analyzer coupled
637 with an IsoPrime mass spectrometer (GV Instruments, Crewe, UK). Oocytes injected with
638 *AtNPF6.3* cRNA and water have been used as positive and negative controls, respectively. Results
639 are presented as NPF-dependent nitrate accumulation (total ¹⁵N in injected oocytes – ¹⁵N in water-
640 injected oocytes) normalized by the accumulation in 30 mM nitrate.

641

642 *Complementation of Escherichia coli*

643 *E. coli* K-12 (DCT) and its dicarboxylate transport mutant strain CBR315 (CGSC5269) have
644 been obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). The
645 phenotypes of the transformed strains have been compared on M9 medium with malate or glucose
646 as the sole carbon sources.

647

648 *Statistical analysis*

649 Statistical analyses have been performed using the VassarStats analysis of variance program.

650

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653 supporting the microscopy analysis and Stefano Rubino, Sara Salvia and Danilo Maiello for
654 technical assistance.

655

656 **Figure legends**

657

658 **Figure 1**

659 *LjNPF8.6* transcriptional regulation. A, Expression in different organs. RNAs have been extracted
660 by wild type plants grown on Gamborg-B5 derivative medium without N source at four weeks after
661 inoculation. Mature flowers have been obtained from Lotus plants propagated in the growth
662 chamber. B, Time course of expression in root and nodule tissues after *M. loti* inoculation. RNAs
663 have been extracted from roots of wild type seedlings grown in N starvation conditions at different
664 times after inoculation (R0, 24 hrs, 72 hrs) and from young (10 dpi) and mature nodules (28 dpi).
665 Expression levels are normalized with respect to the internal control ubiquitin (*UBI*) gene and
666 plotted as relative to the expression of flowers (A) and T0 (B). White bars, *LjNPF8.6*; grey bars,
667 *LjNIN*. Data bars represent the mean and standard deviations of data obtained with RNA extracted
668 from three different sets of plants and 3 real-time PCR experiments.

669

670 **Figure 2**

671 Exon/intron organization of the *LjNPF8.6* gene. Insertion sites and relative orientations of the
672 LORE1 retrotransposon element in the 53155, 19899 and 49638 lines are indicated.

673

674 **Figure 3**

675 Quantitative analysis of shoot biomass and nodulation capacity of *L. japonicus* wild type and
676 *LjNPF8.6* null mutants, grown in the presence of different KNO₃ concentrations, in symbiotic and
677 non-symbiotic conditions. A, Shoot length per plant. B, Fresh shoot weight per plant. C, Nodule
678 numbers per plant. The different KNO₃ concentrations and when performed, *M. loti* inoculations are
679 indicated. Bars corresponding to wild type and different LORE1 plants are indicated. Data bars
680 represent means and SE of measures from three experiments (12 plants per experiment per
681 condition). Data in A and B have been scored 25 days after sowing (21 days after transferring the
682 plants from H₂O agar). Data in C have been scored 28 days after inoculation.

683

684 **Figure 4**

685 Analysis of anthocyanin content. A, Three representative wild type and 49638 plants are shown on
 686 the right and left of the Petri dish, respectively. B, Higher magnification showing intense purple
 687 colours in the stems of mutant plants (on the left). C, Anthocyanin content in stems of wild type and
 688 LORE1 lines. The different KNO₃ concentrations and when performed, *M. loti* inoculations are
 689 indicated. Bars corresponding to wild type and different LORE1 plants are indicated. Data bars
 690 represent means and SE of measures from three experiments (12 plants per experiment per
 691 condition). Data in C have been scored 28 days after inoculation. Asterisks indicate significant
 692 differences (p<0.001) with wild type levels.

693

694 **Figure 5**

695 A, Acetylene Reduction Activity (ARA) per nodule weight of wild type and 49638 plants. The
 696 different KNO₃ conditions are indicated. Data bars indicate the mean and SE of three independent
 697 experiments (n=8 plants per exp.). Asterisks indicate significant differences (p<0.005) between wild
 698 type and 49638 nodules in no N and 1 mM KNO₃ conditions. The asterisk over the bar across the
 699 10 mM condition indicates a significant reduction of ARA activity in nodules of both plant
 700 genotypes shifted on high nitrate conditions compared to nodules of plants maintained on low
 701 permissive conditions (p<0.001). B, C, Representative shoot phenotypes of eight weeks post
 702 inoculation wt and 49638 plants, transferred on hydroponic conditions in the presence of 0.5 mM
 703 KNO₃ at 4 wpi. Wild type and mutant plants have been maintained in the same vessels (4 vessels,
 704 16 plants).

705

706 **Figure 6**

707 Phenotypic symbiotic characterization of *Ljnpf8.6* mutants. A-D, Histochemical detection of β -
 708 galactosidase activity to test *M. loti* (carrying the *hemA::lacZ* expressing plasmid) density in young
 709 and mature nodules of wild type (A, C) and 49638 plants (B, D). E-H, Whole mount NBT staining
 710 for superoxide anions detection of wild type (E, G) and 49638 (F, H) nodule primordia and mature
 711 nodules. I, J, Sections (100 μ m) of wild type (I) and 49638 (J) mature nodules stained with NBT.
 712 Arrows in panel I and J indicate staining in the parenchyma (p) and nodular vascular bundles (vb).
 713 K, Quantification of NBT staining in wild type and 48638 mutant line. Data bars represent means
 714 and SE of nodules from three independent samples (8 plants per experiment). Asterisks indicate
 715 significant differences with wild type values (p<0.05).

716

717 **Figure 7**

718 Functional expression of *LjNPF8.6* in *Xenopus* oocytes in low (0.5 mM) and high (30 mM) external
 719 nitrate concentration. Nitrate uptake in control oocytes (black bars), injected with complementary
 720 RNAs (cRNAs) expressing *LjNPF8.6* (white bars) or *AtNPF6.3* (grey bars) (n = 5-8). Values are
 721 means \pm SE.

722

723 **Figure 8**

724 A, Nitrate content of shoots and roots from wild type and 49638 plants grown on 1 mM KNO₃ and
 725 inoculated with *M. loti*. Data bars represent means and SE from three independent samples (10
 726 plants per sample). B, Nitrate content in wild type and 49638 four weeks old nodules. Data bars
 727 represent means and SE of nodules from three independent samples (10 plants per sample). Bars
 728 corresponding to wild type and 49638 plants are indicated.

729

730 **Supplemental Data**

731 Supplemental Figure 1. Homozygous plants for LORE1 insertions into the *LjNPF8.6* gene are null
 732 mutants.

733 Supplemental Figure 2. Analyses of anthocyanin content in roots of wild type and 49638 plants and
 734 quantification of NBT staining in roots and stems of wild type and 48638 inoculated plants.

735 Supplemental Figure 3. Effect of external nitrate concentration on ¹⁵N accumulation in *LjNPF8.6*-
 736 expressing oocytes.

737 Supplemental Figure 4. Complementation test of the *E. coli dctA* mutant.

738 Supplemental Figure 5. Anthocyanin accumulation phenotype in hydroponic conditions.

739 Supplemental Table. Oligonucleotides sequences.

740

741 **Supplemental Figure S1.**

742 Homozygous plants for LORE1 insertions into the *LjNPF8.6* gene are null mutants. A,
 743 amplification of a 295 bp fragment of the *LjNPF8.6* coding sequence. B, ubiquitin amplification.

744 The *LjNPF8.6* amplicon is undetectable after 35 cycles of RT-PCR. Primers for endpoints RT-PCR
 745 (listed in Supplemental Table S1) have been designed to bracket the LORE1 insertion site. M =

746 molecular marker; H1, H2, H3 = three different homozygous plants of line 49638.

747 **Supplemental Figure S2.**

748 A, Analyses of anthocyanin content in roots of wild type and 49638 plants. N conditions and when
 749 performed, *M. loti* inoculation, are indicated. Asterisks indicate significant differences (p<0.005)

750 with wild type levels. B, Quantification of NBT staining in roots and stems of wild type and 48638

751 inoculated plants. N conditions are indicated. Data have been scored 28 days after inoculation. Data
752 bars represent means and SE of measures from three experiments (8 plants per experiment per
753 condition).

754

755 **Supplemental Figure S3.**

756 Effect of external nitrate concentration on ^{15}N accumulation in LjNPF8.6-expressing oocytes. Data
757 are mean +/- SE (n=5-8 oocytes, batched by 2).

758

759 **Supplemental Figure S4.**

760 Complementation test of the *E. coli dctA* mutant.

761

762 **Supplemental Figure S5.**

763 Anthocyanin accumulation phenotype in hydroponic conditions. Two representative wild type and
764 49638 plants are shown on the right and left of the panel.

765 **Supplemental Table S1.**

766 Oligonucleotides sequences.

767

768

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