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

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One sentence summary:

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

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Abstract

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

Nitrate (NO$_3^-$) represents together with ammonium (NH$_4^+$) the main forms of inorganic nitrogen source for plant growth and metabolism with NO$_3^-$ being the largely dominant supply form in temperate climates (Miller & Cramer, 2005). Higher plants possess two NO$_3^-$ transport systems to cope with a wide range of external concentrations, the Low Affinity Transport System (LATS; > 0.5 mM) and the High Affinity Transport System (HATS; < 0.5 mM), both including constitutive and inducible types of transport (Tsay et al., 2007). In higher plants LATS proteins are mainly represented by the Nitrate Transporter 1/Peptide Transporter Family (NPF), which include a large number of genes (53 members in Arabidopsis, 80 in rice), divided in eight subfamilies and able to transport different substrates (Léran et al., 2014). To date, nitrate transport activity has been reported for 17 out of 53 NPF proteins in Arabidopsis thaliana (Corratgé-Faillie & Lacombe, 2017), with AtNPF6.3 being the only exception as it displays dual affinity for nitrate in the high and low concentration ranges (Liu et al. 1999). Dual affinity for NO$_3^-$ uptake in Xenopus oocytes has also been reported for the Medicago truncatula MtNPF6.8 (Morére-Le Paven et al., 2011) and MtNPF1.7 (previously named Numerous Infection and Polyphenolics/Lateral root-organ Defective; NIP-LATD) proteins (Bagchi et al. 2012). Moreover, MtNPF1.7 KO mutant plants display a more defective lateral root responses in planta at low KNO$_3$ concentrations than at higher concentrations indicating a high affinity transport physiological function (Bagchi et al. 2012). The specificity of spatio-temporal patterns of AtNPFs expression and of their regulatory profiles ensure nitrate uptake from soil, long-distance transport within the plant body and distribution from source to sink tissues (Krapp et al., 2014; Noguero & Lacombe, 2016).

It is well known that nitrate plays also a role as signalling molecule involved in the control of many physiological processes including gene regulation (Wang et al., 2004) and root development (Walch-Liu et al., 2006). A crucial role in the nitrate signalling pathway governing root system architecture and modulation of expression of many genes has been demonstrated for AtNPF6.3 that functions as a nitrate transceptor (Ho et al., 2009; Krouk et al., 2010). In particular, the control exerted by AtNPF6.3 on lateral root development, in response to different external nitrate concentrations, is mediated by its action as auxin transport facilitator (Krouk et al., 2010; Bouguyon et al., 2015). Plant NPFs members encompass proteins capable of transporting different substrates other than nitrate such as: di/tri-peptides, amino acids, glucosinolates, malate, auxin, abscisic acid (ABA), gibberellic acid and jasmonic acid (Frommer et al. 1994; Jeong et al. 2004; Waterworth & Bray, 2006; Krouk et al. 2010; Kanno et al. 2012; Nour-Eldin et al. 2012; Saito et al., 2015; Tal et
al., 2016). In particular, the multitransport feature recently reported for some NPF proteins displaying phytohormones transport capacities, may suggest additional roles played by these proteins on regulatory cross-talks linking different physiological signals (Krouk et al. 2010; Kanno et al. 2012; Saito et al., 2015; Chiba et al., 2015; Tal et al., 2016). However, the different transport capacities are distributed among the eight NPF subclades identified in plants (Leràn et al., 2014) as sequence homologies do not correlate with substrate specificity and the determination of the transported substrate cannot be determined from the sequences data alone.

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

In N-fixing nodules bacteria entered into root nodule cells are surrounded by a plant-derived membrane, the peri-bacteroidal membrane (PBM), which encloses the intracellular bacteria in a symbiosome (SM). Inside the SM bacteria differentiate into bacteroids with the ability to fix atmospheric N₂ via nitrogenase activity. A primary nutrient exchange across the PBM is the transport of a carbon energy as products of plant photosynthates to bacteroid in exchange for fixed-nitrogen. N-fixation is an energy intensive process that requires also O₂ for respiration to generate ATP and reducing equivalents for reduction of N₂ to NH₃. At the same time, as bacteroid nitrogenase is inactivated by O₂, a micro-aerophilic condition must be maintained in rhizobia-containing nodule cells. This is achieved by limiting the rate of O₂ influx through the outer uninfected cells layers of the nodule (Witty & Minchin, 1998) and by maintaining high rates of respiration in mitochondria and bacteroids of invaded cells (Bergensen 1996). High respiration rates are mainly ensured by the presence at millimolar concentrations of the high affinity O₂ binding...
protein leghemoglobin (Lb), which delivers O₂ efficiently to mitochondria and bacteroids for respiration while buffering free O₂ at the required level (Appleby 1984). This extremely high rate of respiration in the invaded nodule cells is the main reason of Reactive Oxygen Species (ROS) generation, whose steady-state concentrations must be strictly controlled as these represent not only toxic by-products of aerobic metabolism but also key signals for nodulation. Nodule specific metabolic pathways are completed by redox reactions involved in the control of concentrations of ROS generated in N₂-fixing nodules (Evans et al., 1999; Hernandez-Jimenez et al., 2002; Puppo et al., 2005; Becana et al., 2010; Matamoros et al., 2013). The distinct metabolic pathways of N₂-fixing nodules reflect changes in gene expression for related metabolic enzymes. Genome-wide transcriptomic analyses have allowed the classification of genes induced in nitrogen-fixing nodules and among these, a significant percentage of genes encoding for transporter proteins have been found (Colebatch et al., 2004; Hogslund et al., 2009; Takanashi et al., 2012). NPF proteins are largely represented in this category of transporters and at least eight members have been recently sub-classified as nodule induced (NI) genes in *L. japonicus* (Valkov & Chiurazzi, 2014).

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

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

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

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

Isolation of LORE1-insertion null mutants and phenotypic characterization

To determine the *in vivo* function of *LjNPF8.6*, three independent LORE1 insertion mutants have been isolated from the LORE1 lines collection (Fukai *et al*. 2012; Urbanski *et al*. 2012; 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.

second and third exon (Fig. 2), have been genotyped by PCR and plants homozygous for the insertion event into the *LjNPF8.6* gene, selected and transferred to the plant chamber for seeds
production. Endpoint RT-PCRs have revealed no detectable *LjNPF8.6* mRNA in mature nodules of homozygous plants screened from lines 53155, 49638 and 19899 and hence, these can be considered null mutants (Supplemental Fig. S1). Initially, two individual homozygous mutant plants from each line have been selected for analyses and because their growth phenotypes did not significantly differ, the data obtained with the selected individual mutants have been pooled in this study. The initial phenotyping of the three *LORE1* mutant lines included measurements of shoot lengths and fresh weights of 4-week-old plants with/out inoculation with *M. loti*. As shown in Fig. 3 in the absence of N sources (no N) or in the presence of KNO₃ concentrations (100μM; 1 mM) compatible with full nodulation capacity, the three lines did not present significant differences when compared to wild type plants, in terms of shoot biomass and nodules number (Fig. 3A-C). In order to test whether *LjNPF8.6*, which is also expressed at a significant level in the root tissue, could be involved in the nitrate dependent inhibitory pathways controlling the nodule formation process, nodulation capacity has been also tested in the presence of high external concentrations of KNO₃ (10 mM). As expected the number of nodules is strongly reduced in *L. japonicus* wild type plants (85%; Barbulova et al., 2007) and an identical inhibitory response is observed in the *Ljnpf8.6* mutants (Fig. 3C).

However, a careful analysis of phenotypes of the inoculated plants has allowed to detect visible accumulation of anthocyanin, conferring deep purple colour, in stems of mutant plants in symbiotic conditions when compared to wild type plants (Fig. 4A, B). The anthocyanin accumulation starts to be easily detectable in inoculated *Ljnpf8.6* plants at 17-20 days after inoculation and the spreading of pigments increases progressively up to the third internode (40-45 % of the stem length) at 4 wpi, whereas in wild type plants traces of pigmentation are observed only at the base of stem structure (Fig. 4A, B). A quantitative analysis performed through anthocyanin extraction from stem tissues at 4 wpi has revealed a content 210-250% higher in nodulated KO than wild type plants grown either under no N as well as 1 mM KNO₃ conditions (Fig. 4C). A significant systemic increase of

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**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.
Figure 3. Quantitative analysis of shoot biomass and nodulation capacity of *L. japonicus* wild type and *L2NPF8.6* null mutants, grown in the presence of different KNO₃ 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 KNO₃ 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 H₂O agar). Data in C have been scored 28 days after inoculation.

Anthocyanin content is also revealed in roots of plant mutants inoculated with *M. loti* (Supplemental...
Conversely, un-inoculated plant mutants did not display anthocyanin accumulation in stems and roots as no quantitative differences have been detected in wild type and mutants plants grown in the presence of 1 mM or 5 mM KNO$_3$ (Fig. 4C; Supplemental Fig. S2A). Line 53155 has been analyzed only for plants inoculated on 1 mM KNO$_3$ conditions and not further utilized for phenotypic characterization because of the segregation of the nod$^-$ phenotype due to the additional LORE1 insertion in the CERBERUS gene (Yano et al. 2009). However, the identical phenotype displayed by the 53155, 49638 and 19899 lines confirms that the LORE1 insertion in the LjNPF8.6 gene is the causal mutation of the increased anthocyanin content observed exclusively in symbiotic conditions. In addition, heterozygous plants for the LORE1 insertion in the LjNPF8.6 gene, isolated in the three lines, did not display high level of anthocyanin in the stem (data not shown).
Ljnf8.6 nodule mutants display nitrogenase activity deficiency under permissive low nitrate conditions, associated to a long-term shoot biomass reduction phenotype

Accumulation of anthocyanin is a clear marker of plant response to different stress conditions such as low N availability (Diaz et al. 2006). In order to investigate whether the anthocyanin accumulation detected in the Ljnf8.6 mutant in symbiotic conditions is correlated to a reduced nodule functionality, we have compared N-fixation activity in nodules of wild type and mutant plants at four weeks after inoculation. A significant 25% decrease of acetylene reduction activity (ARA) is detected in nodules of Ljnf8.6 mutants grown either on absence of N or in the presence of 1 mM KNO₃ (Fig. 5A). However, the reduction of N-fixation capacity detected in the Ljnf8.6 KO genetic background is not correlated to any evident shoot phenotype other than anthocyanin accumulation during in vitro growth, which must be limited to a short period of analysis (4 weeks post inoculation; Fig. 3A, B). In order to check whether a more severe shoot biomass phenotype could be displayed by the Ljnf8.6 mutants, 4 weeks old nodulated plants have been transferred on growth conditions compatible with long term phenotypic analyses. We have first tried to transfer the 4-weeks old nodulated plants to pots filled with inert material but the phenotyping analyses have been biased by a random, genotype-independent stress response due to bad adaptation to the new conditions of growth. Conversely, the transfer of nodulated plants to hydroponic conditions minimizes this unpredictable plant phenotype and all the plants could be scored for reliable shoot phenotypes after four more weeks. In Fig. 5B and C is shown the striking shoot biomass deficient phenotype displayed by the Ljnf8.6 mutants four weeks after the transfer in hydroponic conditions (8 wpi). The two main representative phenotypes observed in all the inoculated mutant plants are stunted shoots with pale green and/or abscissed leaves (Fig. 5B, C).

Ljnf8.6 mutants nodules display superoxide overproduction

In M. truncatula the NIP/LATD protein has been associated, through the characterization of the weak allelic mutant nip-3, to defects in bacteria release or proliferation within nodule infected cells where fewer bacteria are observed (Teillet et al., 2008). Therefore, we have tested whether the deficient N fixation activity observed in the Ljnf8.6 nodules (Fig. 5A) is associated to a reduced invasion capacity. Seedlings grown on N starvation conditions have been inoculated with a M. loti strain carrying a constitutively expressed hemA::lacZ reporter gene fusion for staining of young and mature nodules. As shown in Fig. 6 (A-D), no differences have been observed in the density of the invading M. loti strain in wild type and mutant nodules.
Reduction of N-fixation activity in mature nodules has been also associated to oxidative damages provoked by ROS overproduction that can be due to natural ageing or to exposure to different stress conditions (Becana et al., 2010). We have monitored superoxide (O$_2^-$) production at different stages of nodulation using the ROS-reactive dye NBT. The nascent nodule primordia are
strongly stained in both wild type and mutants (Fig. 6E, F) while as nodules mature and enlarge in size, the staining intensity strongly decreases in both genotypes but remains more intense in mutant nodules (Fig. 6G, H). In 60% of wild type big nodules the staining is not even detectable in whole mount samples, whereas in all the comparable mutant nodules this is still clearly visible. In whole
mount samples, NBT staining is confined to lenticel structure on the nodule surface (Fig. 6G, H). Longitudinal nodule sectioning has confirmed these differences of staining intensity in the parenchima region (Fig. 6I, J). The quantitative analysis conducted on mature nodules confirms a significant overall increase of $O_2^-$ content in the 49638 line compared to wild type plants (25%; Fig.
Superoxide content has been also quantitized on root and stem tissues of inoculated plants, where no significant differences are observed (Supplemental Fig. S2B). These results indicate a local $O_2^-$ increase into the nodule organ.

Figure 6. Phenotypic symbiotic characterization of Lmpg8.6 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 μm) 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).
**LjNPF8.6 is a nitrate transporter**

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

The uptake activity observed in *Xenopus* oocytes (Fig. 7) prompted us to test for possible roles of *LjNPF8.6* associated to nitrate transport function important for nodule activity. Therefore, we have checked whether *LjNPF8.6* could play a role in the inhibitory pathway responsible of the abrupt decrease of nodule activity described after exposure to external high nitrate concentrations (Schuller *et al.*, 1988; Vessey & Waterer, 1992). Wild type and mutant nodulated plants (4 wpi) have been transferred for three days in the presence of 10 mM KNO$_3$ and nodule activity analyzed by ARA. The nitrogenase activity is inhibited at the same level in both wild type and *Ljnpf8.6* plants ruling out the hypothesis of a *LjNPF8.6* involvement in the signalling pathway inhibiting nodule functioning at high external nitrate concentrations (Fig. 5A).

Jeong *et al.*, (2004) reported the identification of a *NPF* gene (*AgDCAT1*) expressed in nodules of *Alnus glutinosa* that encodes for a protein capable of malate transport in heterologous systems. Therefore, we have also tested the capacity of *LjNPF8.6* to transport malate, the carbon source supplied to bacteroids for metabolism and nitrogen fixation (Day & Copeland, 1991). *LjNPF8.6* has been cloned into the *E. coli* expression vector pKK223-3 under the control of the *tac* promoter (Brosius & Holy, 1984) and the resulting plasmid used for transforming the dicarboxylate transport mutant CBT315 strain (*dctA*; Lo *et al.* 1972). Functional complementation has been tested on M9 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 ± SE.

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

In order to check whether *LjNPF8.6* plays any role in the uptake of external nitrate and/or distribution of this nutrient to different plant tissues, we have compared the nitrate content of
different organs in wild type and \textit{Ljnpf8.6} mutant plants. The analyses have been conducted at 4
weeks after inoculation in plants grown in the presence of 1 mM KNO$_3$. The comparison of nitrate
content either in roots and leaves of wild type and mutant plants did not show significant
differences, confirming that \textit{LjNPF8.6} plays a role strictly related to the nodule functionality (Fig.
8A). In the the micro-aerophilic condition associated to nodule environment, the efficiency of N-
fixation is largely associated to nitrate dependent respiration pathways taking place in the nodule
infected cells (Kato \textit{et al.}, 2003; Meilhoc \textit{et al.}, 2010; Horchani \textit{et al.}, 2011). Therefore, we have

\begin{figure}
\centering
\includegraphics[width=\textwidth]{nitrate_acummulation.png}
\caption{A, Nitrate content of shoots and roots from wild type and 49638 plants grown on 1 mM KNO$_3$ and inoculated with \textit{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.}
\end{figure}
tested whether the reduced nitrogenase activity observed in the Ljnpf8.6 mutants in low nitrate permissive conditions (Fig. 5A) might be associated to a different nitrate content in nodules of wild type and mutants plants. The nitrate content has been quantified in detached wild type and mutant nodules of plants inoculated on no N and 1 mM KNO₃ conditions and displaying the anthocyanin accumulation phenotype. The results shown in Fig. 8B indicate that, independently by the presence of external nitrate supply, a significant content of this nutrient is detectable in nodule tissues and that no significant differences are observed on nitrate accumulation in nodules of wild type and mutant plants. However, since the nitrate dependent respiration pathway for maintenance of the nodules energy status becomes more important in hypoxic than normoxic conditions (Horchani et al. 2011; Hicri et al., 2015), we have also tested whether the stressful phenotype displayed by the Ljnpf8.6 null mutants at 4 wpi could qualitatively worsen in hypoxic conditions. Two-weeks old wild-type and 46938 plants with the same number of nodules that do not show yet any anthocyanin accumulation symptom, have been transferred in hydroponic cultures to reproduce water-logging conditions that provoke hypoxic stress. Interestingly, mutant plants grown for two more weeks in hydroponic conditions show a clear-cut increase of the anthocyanin accumulation phenotype that is clearly visible throughout the length of the stem tissue almost up to the shoot apex (80-85 % of the stem length vs 40-45 displayed in normoxic conditions; Fig. 4A, B), whereas wild type plants did not show such an evident stressful symptom (Supplemental Fig. S5).
Discussion

We report here the functional characterization of a member of the *L. japonicus* NPF family, LjNPF8.6 that plays a positive role on the symbiotic interaction through a nodule associated function. NPF proteins represent a significant number of the NI transporters, and the qRT-PCR analysis shown in Fig. 1 indicates that LjNPF8.6 is strongly induced in nodule tissue, although it cannot be considered a strict late nodulin gene as it is also expressed in root tissues. This result is consistent with the analysis reported by Hogslund *et al.*, (2009), indicating that most genes functioning in mature nodule are also expressed elsewhere in the plant. The induced profile of LjNPF8.6 expression in nodules is consistent with the specific symbiotic phenotypes displayed by independent knock out *Ljnpf8.6* LORE1 insertion mutants. In particular, a striking anthocyanin accumulation is observed in stems and roots of *Ljnfp8.6* mutants compared to wild type, only after *M. loti* inoculation as no increase of pigmentation is observed in un-inoculated plants grown in the presence of different nitrate concentrations (Fig. 4 and Supplemental Fig. S2A). This result clearly indicates that the role played by LjNPF8.6 is strictly associated to the symbiotic programme and it is not related to the N nutritional status of plants linked to the external nitrate availability. This is also confirmed by the analyses of nitrate content in roots and shoots that show no significant differences between wild type and mutant plants (Fig. 8A). Production of anthocyanin is considered a hallmark of the plant response to unfavourable growth conditions (Chalker-Scott, 1999) and N limitation has been reported to trigger different anthocyanin biosynthetic pathways and accumulation in various plant tissues (Diaz *et al.*, 2006; Rubin *et al.*, 2009; Kovinich *et al.*, 2014).

In the case of symbiotic nitrogen fixation, anthocyanin accumulation in the stem is a symptom normally exhibited by plant mutants showing an impaired N-fixation activity (Ott *et al.*, 2005; Krussell *et al.*, 2005; Bourcy *et al.*, 2013). The anthocyanin accumulation phenotype is displayed in the inoculated *Ljnfp8.6* mutants by 17-20 days post inoculation, after the observed induction of LjNPF8.6 expression in nodule tissue (Fig. 1B) and the onset of N-fixation, suggesting a role in the control of nodule functioning rather than development. Interestingly, further information about the spatial profile of LjNPF8.6 in mature *L. japonicus* nodules came from a tissue-specific profiling carried out by laser microdissection and microarray analysis, which revealed a specific expression of six N1 NPF members, including LjNPF8.6, in the central infection zone where N-fixation takes place (Takanashi *et al.* 2012). In particular, the LjNPF8.6 expression was not detected in the inner cortex and vascular bundle zones, suggesting for this NPF member a function specifically associated to N-fixation (Takanashi *et al.* 2012). Consistently, *Ljnfp8.6* mutants do not show any difference compared to wild type plants, in the nodule formation capacity at different KNO3 concentrations as well as in the early steps of nodule primordia invasion (Fig. 3C and 6A-D). The
direct involvement of LjNPF8.6 in the control of the nodule functionality is demonstrated by the
analysis reported in Fig. 5A where a significant reduced quote of N-fixation activity (25%) is
measured in mutants nodules. We also demonstrate that LjNPF8.6 does not play any role in the
quick transport of external high nitrate concentration to the nodule tissues and/or sensing, which
must be involved in the nitrate dependent N-fixation inhibitory pathway (Fig. 5A; Arrese-Igor et al.,
1998; Cabeza et al., 2014).

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

Reduction and loss of N-fixation activity is associated to oxidative stress during natural
senescence of nodules and similar correlated phenotypes can be observed in early senescence
induced by exposure to stress conditions (Puppo et al., 2005). Therefore, the N-fixation deficiency
and increased superoxide content phenotypes displayed in mature nodules of Ljnpf8.6 mutants (Fig.
5A, 6K) are strictly associated each other, although a cause-effect relationship can be difficult to
establish. The pattern of superoxide production during nodule growth and maturation, shown in the
time course experiment displayed in Fig. 6, is consistent with previous reports where NBT staining
revealed superoxide accumulation in nodule primordia of indeterminate as well as determinate
nodules (Fig. 6E, F; Santos et al., 2001; Montiel et al., 2016). To our knowledge, the pattern of O2−
accumulation reported in Fig. 6 (G-J) has never been described in mature determinate nodules. The
NBT staining indicates a predominant localization on the lenticels structures, characterized as the
choke points controlling the gaseous exchanges in determinate nodules (Frazer, 1942; Pankhurst &
Sprent, 1975; Jacobsen et al., 1998; Fig. 6G, H) and the parenchyma regions (Fig. 6I, J).

Biochemical and transcriptomic analyses indicate that in mature determinate nodules most of the
ROS generating processes, which occur during natural or early senescence induced by exposure to
stress conditions, originate in the central infected region and then spread outwards (Evans et al.,
1999; Puppo et al., 2005; Matamoros et al., 2013). Therefore, it is reasonable to predict the
superoxide diffusion and accumulation in lenticels structures and parenchymatic tissue, which are located a few cells apart from the infected zone. Furthermore, we demonstrate that the increased content of superoxide detected in the mutant plants is restricted to nodular tissue (Supplemental Fig. S2B) and therefore the systemic pattern of anthocyanin accumulation (Fig. 4 and Supplemental Fig. S2A) is likely not associated to a direct action of scavenging (Yamasaki et al., 1996).

Members of the NPF family may encompass different putative strategic roles associated to the control of nodule functioning as they can transport nitrate, amino acids, peptides, dicarboxylic acids and abscisic acid (ABA), (Frommer et al., 1994; Waterworth & Bray, 2006; Jeong et al. 2004; Kanno et al., 2012). Malate is the carbon source supplied to bacteroids for metabolism and nitrogen fixation (Day & Copeland, 1991) and in isolated soybean symbiosomes a carrier for monovalent dicarboxylate ions with a higher affinity for malate than for succinate has been identified (Udvardi and Day 1997). Jeong et al., (2004) also reported the identification of a NPF gene (AgDCAT1) expressed in nodules of Alnus glutinosa encoding for a protein capable of malate transport in heterologous systems, but no further functional characterization of AgDCAT1 has been provided.

The failure of LjNPF8.6 to complement the malate transport defect of the E. coli dctA mutant (Supplemental Fig. S4) does not allow to conclude that it is not involved in the C sources supply to bacteroids (Day & Copeland, 1991), as we cannot exclude a non functional expression of the Lotus transporter in E. coli. Another function that must be taken in account for explaining the critical role played by LjNPF8.6 on nodule activity is related to a peptide/amino acids transport capacity. The transport of these substrates may play a positive role for supplying branched-chain amino acids necessary for bacteroid development and persistence (Prell et al., 2009) and/or for supporting polyamines biosynthesis for Nitric Oxide (NO) production (Gupta et al., 2011). ABA signalling has been also involved in the control of nodule functionality. However, ABA has been reported to negatively regulate N-fixation in L. japonicus, as its reduced content obtained in low sensitive mutants as well as wild type plants treated with abamine (a specific inhibitor of 9-cis-epoxycarotenoid dioxygenase), resulted in increased N-fixation activity therefore making unlikely the involvement of LjNPF8.6 in the nodular ABA transport/signalling pathway (Tominaga et al., 2009).

The preliminary biochemical characterization of LjNPF8.6 carried out in our work, indicates that it is capable to achieve nitrate uptake in Xenopus oocytes at high (30 mM) and low (0.5 mM) external nitrate concentrations (Fig. 7). Importantly, the positive role played by LjNPF8.6 on nodule activity, indicated by the nitrogenase deficiency phenotype displayed by Ljnpf8.6 mutants (Fig. 5A), is observed in plants grown in chronically absence of N or in low permissive KNO3 conditions (≤ 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).

Conclusions

To our knowledge, LjNPF8.6 represents the first NPF protein playing a specific role on nodule functioning as demonstrated by the description of different N-deficiency associated phenotypes displayed by independent knock out mutants in symbiotic conditions. Further experiments will be...
necessary to interpret its mechanism of action, taking in account different substrates specificities and possible functional redundancy of these transporters in the nodule organ.

**Material and Methods**

**Plant material and growth conditions**

All experiments have been carried out with *Lotus japonicus* ecotype B-129 F14 GIFU (Handberg & Stougaard 1992; Jiang & Gresshoff 1997). Plants are cultivated in a growth chamber with a light intensity of 200 μmol m−2 sec−1 at 23°C with a 16 hr/8 hr day/night cycle. Solid growth media have the same composition as that of B5 medium (Gamborg, 1970), except that (NH₄)₂SO₄ and KNO₃ are omitted and/or substituted by different concentrations of KNO₃. KCl is added to the medium to replace the potassium source. The media containing vitamins (Duchefa catalogue G0415) are buffered with 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES; Duchefa, M1503.0250) and pH adjusted to 5.7 with KOH.

For hydroponic cultures, wild type and mutant nodulated plants are transferred in the same vessels with derivative B5 liquid medium with 0.5 mM KNO₃ or without N sources (6 plants per vessel). Media are renewed every four days, when also the pH is checked and maintained within close limits (5.6-5.8) during the whole growth.

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

**LORE1 lines isolation**

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

**Determination of Acetylene-Reduction Activity (ARA)**
Detached roots with comparable number of nodules are placed in glass vials. The vials are filled with an acetylene-air mixture (C2H2:air = 1:9 v/v). After 30 min of incubation at 25°C, the amount of ethylene in the gas phase is determined using gas chromatograph (PerkinElmer Clarus 580).

For the analysis of ARA activity after shift in high KNO3 conditions, 4 wpi nodulated plants are transferred on slanted Petri dishes where roots are placed in sandwich position between two filter papers wet with a Gamborg B5 liquid media containing no KNO3 or 10 mM KNO3. Plants are maintained for three days in these conditions with filter papers wet with 20 ml liquid media. After three days ARA activity is tested as described above.

**Estimation of anthocyanin**

Stem tissues from three plants per assay are weighed and then extracted with 99:1 methanol:HCl (v/v) at 4°C. The OD530 and OD657 for each sample is measured and relative anthocyanin levels determined with the equation OD530 – (0.25 X OD657) x extraction volume (ml) x 1/weight of tissue sample (g) = relative units of anthocyanin/g fresh weight of tissue.

**Determination of nitrate content**

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

**LacZ activity and histochemical localization**

Lotus roots isolated after *M. loti hemA::lacZ* infection are first gently rinsed in 50 mM KH2PO4 buffer pH 7.2 and then fixed for 1 hour with 1% paraformaldehyde, 0.3 M mannitol in 50 mM KH2PO4 buffer pH 7.2. The tissues are washed again with 5 mM KH2PO4 buffer pH 7.2 and the histochemical analysis performed according to Omrane *et al.* (2009). The stained nodulated roots are photographed with a Nikon microscope using bright-field and epipolarization optics.
Superoxide staining

For $O_2^-$ staining, Nitroblue Tetrazolium (NBT, Sigma Aldrich) was used. Nodulated roots are submerged in 1 mM NBT in 0.1 mM K-phosphate buffer (pH 7.5), vacuum infiltrated for 30 sec. and incubated 30 min. at R.T. NBT staining solution is removed and stained roots washed twice in 80% ethanol. NBT staining is repeated three times for a total of 10 nodules per experiment. For longitudinal sections 12 nodules from independent experiments have been sectioned in 100 μm thick slices. Samples are photographed with a Nikon microscope using bright-field and epipolarization optics. For $O_2^-$ quantification, NBT-stained nodules are first ground into a fine powder and dissolved in 2M KOH:DMSO (1:1.16, v/v) followed by centrifugation at 12,000g for 10 m. Absorbance at 630 nm is immediately measured and then compared with a standard curve plotted from known amount of NBT in the KOH:DMSO mix (Ramel et al. 2009). Experiments have been conducted on three biological replicates for a total of 40 nodules per genotype.

Quantitative Real-time RT-PCR

Real time PCRs have been performed with a DNA Engine Opticon 2 System, MJ Research (MA, USA) using SYBR to monitor dsDNA synthesis. The procedure is described in Rogato et al. (2008). The ubiquitin (UBI) gene (AW719589) has been used as an internal standard. The concentration of primers has been optimized for every PCR reaction and amplifications have been carried out in triplicate. The PCR program used was as follows: 95°C for 3 min and 39 cycles of 94°C for 15 sec., 60°C for 15 sec. and 72°C for 15 sec. Data have been analyzed using Opticon Monitor Analysis Software Version 2.01 (MJ Research). The qRT-PCR data have been analyzed using comparative Ct method. The relative level of expression is calculated with the following formula: relative expression ratio of the gene of interest is $2^{\Delta CT}$ with $\Delta CT = CT_{GENE} - CT_{UBI}$.

The efficiency of the $LjNPF8.6$ primers is assumed to be 2. Analysis of the melting curve of PCR product at the end of the PCR run has revealed a single narrow peak for each amplification product, and fragments amplified from total cDNA have been gel-purified and sequenced to assure accuracy and specificity. The oligonucleotides used for the qRT-PCR are listed in Table S1.

Plasmids preparation

The plasmid for expression in Xenopus laevis oocytes has been prepared in the following way: cDNA prepared from nodule RNA has been amplified with the forward primer containing the $Bam$HI site in combination with the reverse primer containing the $Eco$RI site (Table S1). The 1713 bp fragment has been double digested $Bam$HI-$Eco$RI and subcloned into the pGEMHE plasmid.
containing the 5′-UTR and 3′-UTR of the Xenopus β-GLOBIN gene (Liman et al., 1992), pre-
digested BamHI-EcoRI to obtain pGEMHE8.6. The correct coding sequence of LjNPF8.6 has been
verified by sequencing.

For expression in E. coli, pGEMH-8.6 has been double-digested BamHI-EcoRI and sub-cloned
in pKK223-3 plasmid (Brosius & Holy, 1984) to obtain pKK-8.6. Primers for the sub-cloning of the
A. thaliana At2G17470 gene used as positive control in the complementation test are indicated in
the Table S1.

Functional analysis of LjNPF8.6 in Xenopus laevis oocytes

pGEMHE-NPF8.6 has been linearized with NheI and capped mRNA transcribed in vitro using
the mMessage mMachine T7-ultra Kit (Life Technologies). Oocytes preparation has been described
in (Lacombe & Thibaud, 1998). Defolliculated oocytes are injected with 20 ng of complementary
RNA (cRNA) and stored in a modified ND96 medium (2 mM KCl, 96 mM NaCl, 1 mM MgCl2, 1.8
mM CaCl2, 5 mM HEPES, 2.5 mM sodium pyruvate, pH 7.5, supplemented with gentamycin
sulphate (50 μg mL⁻¹)). Two days after injection, batches of 10 injected oocytes are incubated in 1
mL of modified ND96 solution at pH 5.5 supplemented with 30 mM or 5 mM ¹⁵NO₃ supplied as
K¹⁵NO₃ for 2 hrs at 18°C. Oocytes are then rinsed five times in 15 mL cold modified ND96
solution. Batches of 2 oocytes are then analyzed for total N content and atom % ¹⁵N abundance by
Continuous-Flow Mass Spectrometry, using an Euro-EA Eurovector elementar analyzer coupled
with an IsoPrime mass spectrometer (GV Instruments, Crewe, UK). Oocytes injected with
AtNPF6.3 cRNA and water have been used as positive and negative controls, respectively. Results
are presented as NPF-dependent nitrate accumulation (total ¹⁵N in injected oocytes – ¹⁵N in water-
jected oocytes) normalized by the accumulation in 30 mM nitrate.

Complementation of Escherichia coli

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

Statistical analysis

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

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**Figure legends**

**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.

**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.

**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 KNO₃ 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 KNO₃ 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 H₂O agar). Data in C have been scored 28 days after inoculation.

**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 LORE1 lines. The different KNO₃ 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 C have been scored 28 days after inoculation. Asterisks indicate significant differences (p<0.001) with wild type levels.

**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).

**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 μm) of wild type (I) and 49638 (J) mature nodules stained with NBT. Arrows in panel I and J indicate staining in the parenchyma (p) and nodular vascular bundles (vb). K, Quantification of NBT staining in wild type and 48638 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).

**Figure 7**
Functional expression of *LjNPF8.6* in Xenopus oocytes in low (0.5 mM) and high (30 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 ± SE.

**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 four weeks old 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.

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

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

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

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

Supplemental Figure 5. Anthocyanin accumulation phenotype in hydroponic conditions.

Supplemental Table. Oligonucleotides sequences.

**Supplemental Figure S1.**
Homozygous plants for LORE1 insertions into the *LjNPF8.6* gene are null mutants. A, amplification of a 295 bp fragment of the *LjNPF8.6* coding sequence. B, ubiquitin amplification. The *LjNPF8.6* amplicon is undetectable after 35 cycles of RT-PCR. Primers for endpoints RT-PCR (listed in Supplemental Table S1) have been designed to bracket the LORE1 insertion site. M = molecular marker; H1, H2, H3 = three different homozygous plants of line 49638.

**Supplemental Figure S2.**
A, Analyses of anthocyanin content in roots of wild type and 49638 plants. N conditions and when performed, *M. loti* inoculation, are indicated. Asterisks indicate significant differences (p<0.005) with wild type levels. B, Quantification of NBT staining in roots and stems of wild type and 48638...
inoculated plants. N conditions are indicated. Data have been scored 28 days after inoculation. Data bars represent means and SE of measures from three experiments (8 plants per experiment per condition).

Supplemental Figure S3.
Effect of external nitrate concentration on $^{15}\text{N}$ accumulation in LjNPF8.6-expressing oocytes. Data are mean +/- SE (n=5-8 oocytes, batched by 2).

Supplemental Figure S4.
Complementation test of the *E. coli* dctA mutant.

Supplemental Figure S5.
Anthocyanin accumulation phenotype in hydroponic conditions. Two representative wild type and 49638 plants are shown on the right and left of the panel.

Supplemental Table S1.
Oligonucleotides sequences.

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