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REVIEW

Nitrate transporters: an overview in legumes

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Abstract

Main conclusion **The nitrate transporters, belonging to NPF and NRT2 families, play critical roles in nitrate signaling, root growth and nodule development in legumes.**

Nitrate plays an essential role during plant development as nutrient and also as signal molecule, in both cases working via the activity of nitrate transporters. To date, few studies on NRT2 or NPF nitrate transporters in legumes have been reported, and most of those concern *Lotus japonicus* and *Medicago truncatula*. A molecular characterization led to the identification of 4 putative *LjNRT2* and 37 putative *LjNPF* gene sequences in *L. japonicus*. In *M. truncatula*, the NRT2 family is composed of 3 putative members. Using the new genome annotation of *M. truncatula* (Mt4.0), we identified, for this review, 97 putative *MtNPF* sequences, including 32 new sequences relative to previous studies. Functional characterization has been published for only two *MtNPF* genes, encoding nitrate transporters of *M. truncatula*. Both transporters have a role in root system development via abscisic acid signaling: MtNPF6.8 acts as a nitrate sensor during the cell elongation of the primary root, while MtNPF1.7 contributes to the cellular organization of the root tip and nodule formation. An in silico expression study of *MtNPF* genes confirmed that *NPF* genes are expressed in nodules, as previously shown for *L. japonicus*, suggesting a role for the corresponding proteins in nitrate transport, or signal perception in nodules. This review summarizes our knowledge of legume nitrate transporters and discusses new roles for these proteins based on recent discoveries.

Keywords *Lotus japonicus* · *Medicago truncatula* · Nitrate signaling · NPF · NRT2

Introduction

World agricultural production has increased markedly since the 1950s, as part of an intensive production model to ensure food security. However, these intensive systems have serious negative consequences for the environment. In fact, it has been estimated that 85–90 million tons of nitrogen fertilizers are added to the soil around the world every year (Good et al. 2004). However, the sustainability of intensive production is increasingly challenged. In the context of plant nitrogen nutrition, the use of green manure, as cover crops, is increasing during crop rotation: species of the *Fabaceae* family, more commonly referred to as legumes, are sown only to be subsequently crushed and incorporated into the soil. Thus, legumes contribute to nitrogen soil enrichment, but they also represent a key source of protein for human and animal nutrition through forage legumes such as alfalfa and soybean.

Many minerals, such as nitrogen (N), have a considerable impact on plant development, especially of the root system development, right from the early stages after seed germination (López-Bucio et al. 2003). Plants can absorb both organic and inorganic nitrogen forms. However, there

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is no direct evidence that organic nitrogen contributes significantly to the nitrogen nutrition of the plant (Näsholm et al. 2009). Thus, most of the nitrogen taken-up by the plant is in an inorganic form, mainly nitrate (NO_3^-) and ammonium (NH_4^+). For most cultivated crops, nitrate is the major source of nitrogen for plant growth (Crawford and Glass 1998). However, plants grow under fluctuating environmental conditions and are consequently exposed to frequent changes in mineral nutrient availability. To respond to the availability of nitrate in the soil, plants adapt their root system architecture (Zhang and Forde 2000) and their absorption mechanisms.

In legumes, nitrate transporters have received little attention, perhaps because legumes have the ability to establish symbioses with *Rhizobium* bacteria for fixing atmospheric N_2. Effective symbiotic interaction is supported by a complex network of nutrient exchange between the two partners, plant and *Rhizobium*, and is controlled by a well-regulated carbon/nitrogen balance (Lodwig et al. 2003; Libault 2014). Indeed, photosynthate and other plant nutrients are transported to nodules to reduce optimally atmospheric N_2 (Lodwig et al. 2003; Libault 2014). However, nitrate uptake remains important for legumes, especially during early seedling growth when the ability to fix atmospheric nitrogen has not developed. Moreover, this nitrate absorption by the root system appears essential for legumes since some environmental factors have been reported to interfere with nodule establishment and functioning, and consequently with plant nutrition (Marino et al. 2007). Understanding nitrate uptake and its control of plant development are important challenges as we seek to optimize legume growth by controlling nitrogen input. This review aims to provide an update on current knowledge on nitrate transporters in legumes and their roles in nitrate signal transduction and plant development.

Molecular basis of nitrate transport in legumes

Our understanding of the molecular basis of nitrogen transport in higher plants has increased markedly since the identification of the first gene encoding a nitrate transporter initially named CHL1 (Chlorate resistant 1; Tsay et al. 1993). Many transporters are now characterized, transporting inorganic and organic nitrogen, both in some model species and plants of agronomic interest. In higher plants, nitrate transporters, or channels, belong to five families of proteins: nitrate transporter1/peptide transporter family (NPF), nitrate transporter2 (NRT2), chloride channel (CLC), aluminum-activated malate transporter (ALMT) and slow anion channel-associated 1 homolog 3 (SLAC1/SLAH3). Given that only members of the NPF and NRT2 families have been shown to be involved in nitrate uptake in roots (see review by Nacry et al. 2013), the present review focuses on these two families.

NPF family

Recently, a unified nomenclature, NPF, has been proposed for the nitrate transporter1/peptide transporter (NRT1/PTR) family (Léran et al. 2014). Previous studies have shown that a wide variety of molecules, such as nitrate, abscisic acid (ABA), auxin, dipeptide or glucosinolates, is transported by members of this NPF family, with some members able to transport two different substrates (see review by Léran et al. 2014).

In legumes, limited data are available on NPFs, unlike with *Arabidopsis thaliana* in which 18 of the 53 AtNPF members have been characterized as nitrate and/or dipeptide transporters (see review by Wang et al. 2012; Hsu and Tsay 2013). All those characterized as nitrate transporters are low-affinity nitrate transporters, except AtNPF6.8 (AtNRT1.1; CHL1) which is a dual-affinity nitrate transporter (Liu et al. 1999). In *Glycine max*, a cDNA encoding a putative NPF has been cloned, but functional characterization has not been published (Yokoyama et al. 2001). Except for this result, our knowledge comes from studies on two model legumes, *Lotus japonicus* and *Medicago truncatula*. A molecular characterization of the NPF family in *L. japonicus* led to the identification of 37 putative *LjNPF* sequences (Criscuolo et al. 2012). Up to now, no gene encoding an NPF has been isolated from *L. japonicus*. Concerning *M. truncatula*, the Mt3.5 annotation version of the sequenced genome enabled identification of 80 genes encoding putative NPFs, belonging to eight subfamilies (Léran et al. 2014). With the new genome annotation of *M. truncatula* (Mt4.0; Tang et al. 2014), we are able to identify 97 putative MtNPF sequences predicted to encode proteins ranging from 388 to 647 amino acids (Supplementary Table SI). Among the 80 sequences previously identified by Léran et al. (2014), 65 were retained in this study and 15 were deleted, either because the amino acid sequence was too small (less than 215 amino acids) or because the sequence no longer existed in the Mt4.0 version genome annotation. So, through this present study, 32 new sequences of putative MtNPFs have been identified. The 97 putative MtNPF sequences are distributed in eight clades (Fig. 1) as previously described by Léran et al. (2014) for the 80 MtNPF sequences. Such a phylogenetic study can be taken as an insightful clue to start an analysis of the protein family. For example, following a phylogenetic study on nitrate and peptide transporters, including several species of angiosperms, it has been proposed that each separate group of NPF protein sequences identified within the NPF family shares common function (von Wittgenstein et al. 2014). However, although

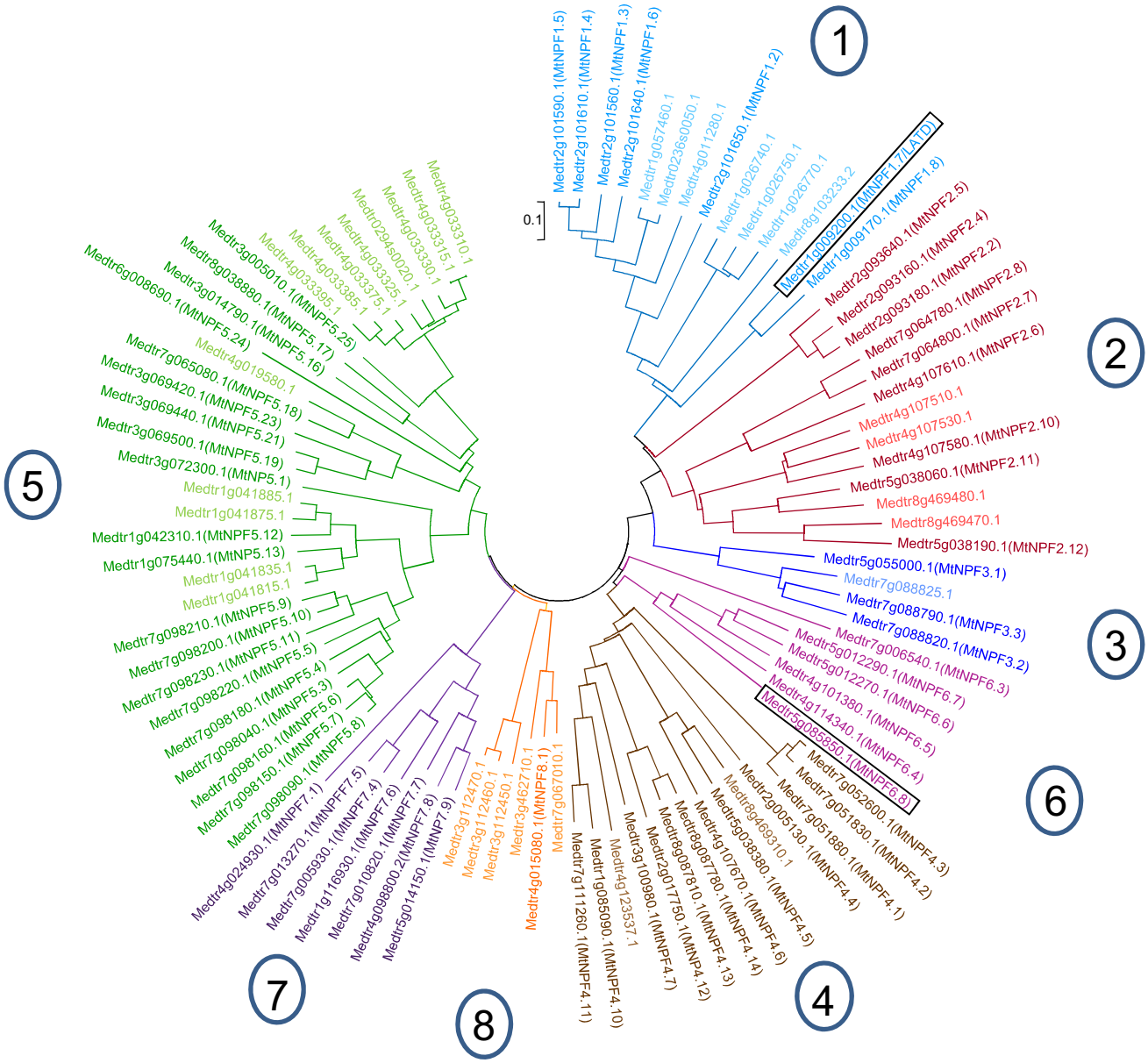

Fig. 1 Phylogenetic tree of MtNPF family of the model plant *M. truncatula*. Research in silico protein sequences was performed using the BLAST algorithm of the specific *Medicago* JCVI database (http://www.jcvi.org/medicago/) using the last version of the annotation genome (Mt4.0) (Tang et al. 2014). The phylogenetic tree was obtained through MEGA5 software. The analysis consists of 97 protein sequences identified as putative MtNPFs and grouped into eight clades numbered from 1 to 8 (Léran et al. 2014). The proteins belonging to a same clade are of the same color. The newly identified sequences, through the Mt4.0 genome annotation, are presented in light color. *Black boxes* indicated nitrate transporters already characterized

phylogenetically close, two genes may exhibit different patterns of expression. We investigated the in silico expression profiles of 44 *MtNPF,* available among the 97 *MtNPFs* genes of *M. truncatula,* by analysis of transcript microarray data (Fig. 2). Some genes were specifically expressed during nodule development (for example *MtNPF4.7*), seed development (for example *MtNPF4.12*), or in a particular plant tissue (for example *MtNPF6.8*). It can be noted that genes belonging to the same clade (same color in Fig. 2) are not necessary clustered. Two phylogenetically close proteins can also have different substrates. Indeed, AtNPF7.3 (AtNRT1.5) and OsNPF7.3 (OsPTR6), although belonging to the same clade (Léran et al. 2014), specifically transport nitrate (Lin et al. 2008) or dipeptides (Ouyang et al. 2010), respectively. So, it seems difficult to speculate on the nature of the substrate transported by these NPFs, or on their substrate affinity, without experimental characterization. Among genes encoding MtNPFs in *M. truncatula*, two genes have been extensively studied and characterized as being nitrate

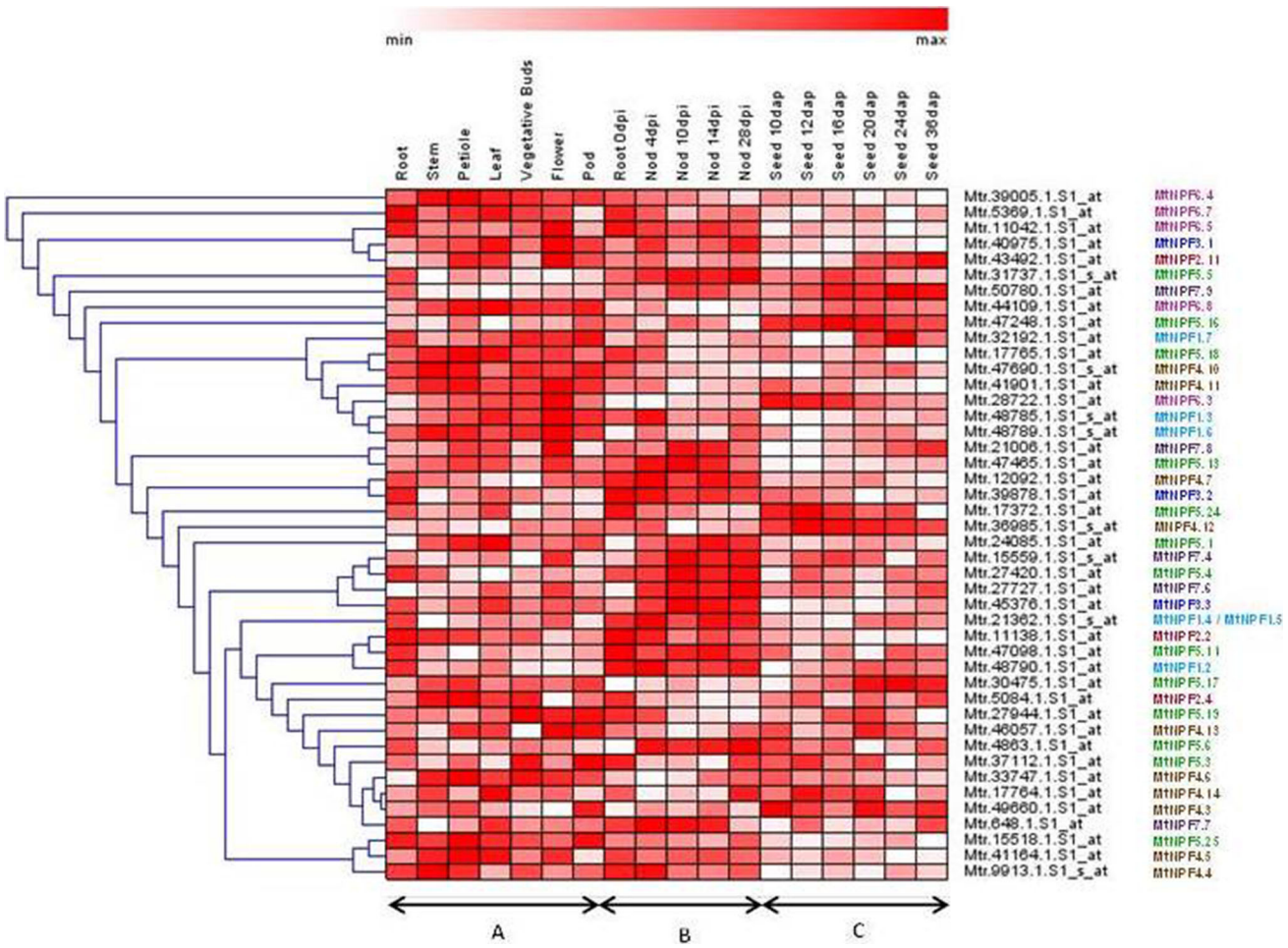

Fig. 2 Expression profile cluster analysis of *MtNPF* genes. The expression data were clustered using Genesis software and distributed according to the tissues (*A*), nodule development (*B*) and seed development (*C*). The gene names are of the *same color* as in Fig. 1. *DPI* days after inoculation, *DAP* days after pollination. Microarray data come from Benedito et al. (2008)

transporters. The first gene, *MtNPF1.7* (*LATD/NIP*), was cloned by Yendrek et al. (2010). The second gene, *MtNPF6.8* (*MtNRT1.3*), was isolated following a quantitative genetic study of post-germination stage seedlings (Morère-Le Paven et al. 2011). The potential roles of these two nitrate transporters in the plant are discussed in the following sections of this review.

NRT2 family

In legumes, a cDNA encoding a putative high-affinity nitrate transporter of *G. max* has been cloned (Amarasinghe et al. 1998). Subsequently, two studies were published describing the identification and molecular characterizations of the *NRT2* gene family; one using *L. japonicus*, the other *M. truncatula* (Criscuolo et al. 2012; Pellizzaro et al. 2015). In *L. japonicus*, interrogation of the genome database led to the identification of four putative *LjNRT2* named *LjNRT2.1*, *LjNRT2.2*, *CM0001.20* and *CM0161.180* (Criscuolo et al. 2012). Using similar approaches, Pellizzaro et al. (2015) revealed, for *M. truncatula,* a small NRT2 gene family composed of three *MtNRT2* genes (named *MtNRT2.1*, *MtNRT2.2* and *MtNRT2.3*), indicating a similar *NRT2* family size in legume or non-legume species. Indeed, seven *AtNRT2* genes were identified in *A. thaliana* (Orsel et al. 2002a, b; Okamoto et al. 2003) and four in rice (Araki and Hasegawa 2006; Cai et al. 2008; Feng et al. 2011). In these non-legume species, all characterized NRT2s have been reported to be strictly high-affinity nitrate transporters (Feng et al. 2011; Krapp et al. 2014). The function of almost all NRT2 of higher plants depends on a small protein of about 200 amino acids called NAR2 (NRT3), which interacts directly with NRT2 (see review by Krapp et al. 2014). In *M. truncatula*, in silico search revealed an *MtNAR2* gene family composed only of two genes (named *MtNAR2.1* and *MtNAR2.2*) (Pellizzaro et al. 2015), as in *A. thaliana* and *O. sativa* (Okamoto et al. 2003; Cai et al. 2008).

Function of nitrate transport in legumes

Role of transporters in HATS and LATS

To respond to soil nitrate availability, which can vary from μM to mM, plants adapt their absorption system. On the basis of their kinetics, two nitrate absorption systems have been described in many species: (a) a high-affinity transport system (HATS), and (b) a low-affinity transport system (LATS). The HATS is involved in nitrate uptake at low external nitrate concentrations (as low as 1 μM) while the LATS operates at higher concentration (>0.5 mM) (see review by Nacry et al. 2013). Both systems possess a constitutive component (cHATS; cLATS) and an inducible component (iHATS; iLATS) (Siddiqi et al. 1989, 1990; Faure-Rabasse et al. 2002).

Among the nine *LjNPF* genes studied in *L. japonicus*, expression of only *CM0608.1210* was induced by nitrate. However, without biochemical information and genetic characterizations, the authors were not able to define the functional role in LATS (Criscuolo et al. 2012). In *M. truncatula*, both MtNPFs studied were expressed and characterized in *Xenopus* oocytes. The first studied protein, MtNPF1.7, was demonstrated to be a high-affinity nitrate transporter (Bagchi et al. 2012). This result indicates that the classification made until now (indicating that low-affinity nitrate transporters belong to the NPF family and high-affinity transporters belong to the NRT2 family) is too simplistic. MtNPF1.7 can restore chlorate sensitivity in resistant mutants of *A. thaliana,* but is not involved in the nitrate uptake in *M. truncatula* (Bagchi et al. 2012; Salehin et al. 2013). The second studied protein, MtNPF6.8, has the proprieties of a dual-affinity nitrate transporter when expressed in *Xenopus* oocytes (Morère-Le Paven et al. 2011). However, MtNPF6.8 does not contribute to HATS *in planta* (neither the constitutive, nor the inducible component) (Pellizzaro et al. 2014). Indeed, MtNPF6.8 is specifically involved in the inducible component of the LATS. The knockdown of *MtNPF6.8* affected the entire inducible component of the LATS, suggesting that MtNPF6.8 is an essential actor of the inducible LATS (Pellizzaro et al. 2014). So, to date, both studied nitrate transporters from *M. truncatula*, MtNPF6.8 and MtNPF1.7, can transport nitrate at low concentrations when expressed in heterologous transport system, without having a HATS activity *in planta* (Morère-Le Paven et al. 2011; Bagchi et al. 2012; Pellizzaro et al. 2014). Interestingly, AtNPF6.3 of *A. thaliana*, known to be a dual-affinity nitrate transporter in oocytes, has been shown to be involved in HATS *in planta* (Wang et al. 1998; Liu et al. 1999; Ho et al. 2009), although this is a matter of debate (Glass and Kotur 2013).

Concerning *NRT2*, at least in post-germinative *M. truncatula* seedlings, *MtNRT2.1* is expressed predominantly in roots and is induced by nitrate (Pellizzaro et al. 2015). Based on the observation that, in *A. thaliana*, a strong correlation exists between induction of gene expression in response to nitrate and HATS activity (Krouk et al. 2006), it was suggested that MtNRT2.1 could be a nitrate transporter with a major contribution to HATS activity in *M. truncatula* (Pellizzaro et al. 2015). In addition, co-expression of *MtNRT2.1* and *MtNAR2* suggests interaction of these two proteins for nitrate uptake (Pellizzaro et al. 2015). In *L. japonicus*, two of the four *NRT2* genes, *LjNRT2.1* and CM0161.180, showed a major increase of transcript abundance in the roots of plants grown under low nitrate concentrations (Criscuolo et al. 2012), suggesting involvement of both putative nitrate transporters in the HATS activity.

Long-distance nitrate transport

It is now accepted that the role of nitrate transporters extends beyond their function in the active uptake of nitrate from the environment (Wang et al. 2012). Indeed, nitrate transporters are expressed in all plant organs, allowing both long-distance transport of nitrate, and tailored delivery to cell types. Provision of nitrate to aerial organs first requires xylem loading by nitrate transporters (Lin et al. 2008; Li et al. 2010; Wang and Tsay 2011) and, therefore, identification of the transporters responsible may be assisted by knowledge of the tissue patterning of gene expression. *MtNPF6.8* is expressed in shoots as well as in the pericycle region in roots (Pellizzaro et al. 2014). In addition, study of *MtNPF6.8* expression in 28 day-old plants (Benedito et al. 2008; Fig. 2) or in 2-month-old plants (Morère-Le Paven, personal data) showed the presence of transcripts in all plant organs and mainly in the leaves. While analyses suggest that MtNPF6.8 does not participate significantly in nitrate translocation at the seedling stage (10 day-old plants), it is possible the functional contribution changes in more advanced developed stages. Concerning the NRT2 family, *MtNRT2.3* is expressed in both roots and shoots, a pattern similar to that observed for *OsNRT2.3* in *O. sativa*, or *AtNRT2.4* in *A. thaliana*, suggesting a role in the transport of nitrate inside the plant from the root to shoot (Feng et al. 2011; Kiba et al. 2012; Pellizzaro et al. 2015). Furthermore, the tissue-wide expression pattern of *MtNAR2.2* suggests that this protein may interact with MtNRT2.3 for this function (Pellizzaro et al. 2015).

Nitrate transporters and nitrate signal perception

Nitrate is not only a nutrient; it is also a molecular signal governing root development and massive reprogramming of gene expression (see review by Nacry et al. 2013). In

this context, it is appropriate to discuss these signaling aspects and to highlight the molecular and physiological responses, the molecular players and the signaling pathways likely involved in nitrate signaling in legumes.

Primary nitrate response

It is well established that nitrate is a signal molecule inducing massive and rapid change in gene expression (Wang et al. 2003). Following a period of nitrate deficiency, new nitrate supply, even at low concentrations (250 µM nitrate), induces rapid (within 30 min) changes in the expression of over 1000 genes. This response is named the Primary Nitrate Response (PNR) (Wang et al. 2003; Krapp et al. 2014; Medici and Krouk 2014). In *M. truncatula*, the PNR has been studied (summarized on Fig. 3), especially in the nodule after 4 h or 8 h of nitrate supply (Cabeza et al. 2014) where massive regulation of gene expression, affecting 20% of all genes expressed in the treatments and/or control, was observed. The expression of some genes involved in nitrate transport (encoding low- and high-affinity nitrate transporters) or nitrate assimilation (encoding the nitrate reductase (NR) for example) is up-regulated (Fig. 3). Cabeza et al. (2014) have also identified modulation of gene expression induced by nitrate that impacts the activity of the nodules. For example, all nine of the expressed genes for leghemoglobin, the symbiotic hemoglobin involved in the scavenging of O_2 and its transport for symbiotic nitrogen fixation, were down-regulated. Furthermore, strong evidences suggesting that the PNR in *M. truncatula* is mediated by MtNPF6.8 are provided (Fig. 3) (Pellizzaro et al. 2014). Indeed, nitrate induction of *NR* genes is impaired in *npf6.8* mutants at 5 mM but also at 250 µM of nitrate supply. Since MtNPF6.8 does not influence the nitrate uptake at 250 µM of nitrate supply nor the global nitrogen status of the plant, it is possible that the role of MtNPF6.8 in response to nitrate is independent of its nitrate transport function. So, MtNPF6.8 could be a nitrate transceptor, having a nitrate-transporter and a nitrate-sensor function (Pellizzaro et al. 2014), as found for AtNPF6.3 (Ho et al. 2009; Ho and Tsay 2010). In addition, Pellizzaro et al. (2014) noticed that the induction of *NR1* and *NR2* was reduced in *npf6.8* mutants by 60%, suggesting the existence of other nitrate sensors in *M. truncatula* (Fig. 3). Finally, the capacity of MtNPF6.8 to transport ABA, and its involvement in the PNR as nitrate-sensor, suggest a possible role of ABA in the PNR signaling response (Fig. 3). This hypothesis is supported by recent results showing that, in *A. thaliana*, ABI2 (ABA-insensitive 2; a protein phosphatase 2C) enhanced AtNPF6.3-dependent nitrate transport, nitrate sensing and nitrate signaling (Léran et al. 2015).

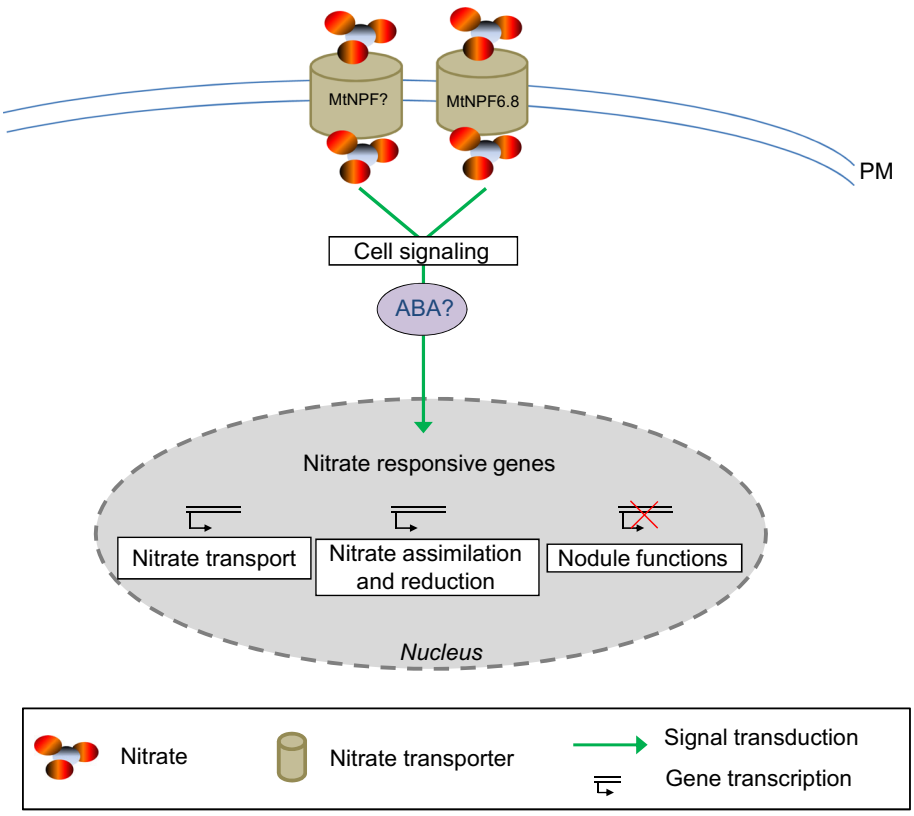

Fig. 3 Schematic representation of primary nitrate response (PNR) in *M. truncatula*. Results obtained in roots indicated that MtNPF6.8 is a receptor of the PNR; MtNPF6.8 has a role in nitrate signaling inducing changes in gene expression involved in nitrate assimilation (Pellizzaro et al. 2014). Other nitrate sensors may also exist. The involvement of ABA in the signaling pathway remains a hypothesis. Results obtained in nodules after nitrate supply (4 or 8 h) show some genes involved in nitrate transport or assimilation/reduction are up-regulated while other genes, involved in the activity of the nodules, are down-regulated (Cabeza et al. 2014). *PM* plasma membrane

Regulation of root system architecture

Developmental plasticity enables plants to respond to nutrient availability by changing the architecture of their root system (Zhang and Forde 2000; Linkohr et al. 2002). Nitrate is one of the major nutrients known to act as a signal molecule in the regulation of root architecture through the regulation of primary root growth and by influencing the emergence and development of lateral roots (LR) (Zhang and Forde 1998, 2000; Zhang et al. 1999; Tian et al. 2008; Vidal et al. 2010; Celis-Arámburo et al. 2011). This effect is complicated due to the changes in root architecture caused by changes in root environment and their influence on plant physiology. For example, hormones coordinate the responses modulated by external environment, regulating both primary root growth and LR initiation and development. Auxin (indole-3-acetic acid, IAA) and ABA have both been proposed to play a role in mediating nitrate effects on LR development (Signora et al. 2001; De Smet et al. 2006; Walch-Liu et al. 2006; Zhang et al. 2007).

In *M. truncatula*, studies (summarized on Fig. 4) have shown that both characterized nitrate transporters, MtNPF1.7 and MtNPF6.8, play a role in root development (Harris and Dickstein 2010; Bagchi et al. 2012; Pellizzaro et al. 2014). *MtNPF1.7* is expressed in the tips of primary and lateral roots (including the root cap, meristem and proximal portion of the elongation zone) as well as in the nodule meristem (Fig. 4) (Yendrek et al. 2010). Accordingly, MtNPF1.7 plays a role in perception of ABA (Yendrek et al. 2010) and contributes to LR formation, nodule meristem development and primary root growth organization of the root tip (Veereshlingam et al. 2004; Liang et al. 2007). *MtNPF1.7* expression is controlled by several hormones, in particular by ABA, but is not regulated by nitrate (Yendrek et al. 2010). Interestingly, root development defect of mutants affected in *MtNPF1.7* (noted *latd*) can be rescued by ABA addition (Liang et al. 2007), indicating a positive role of ABA in the establishment, or the maintenance, of root meristem function (Fig. 4) (Harris 2015). Since ABA levels in *latd* mutants are indistinguishable from those in wild type (Liang et al. 2007), the defect in *latd* mutants is likely to be in ABA transport or signaling. However, to date, MtNPF1.7 has not been shown to be an ABA transporter. Recently, Zhang et al. (2014) demonstrated that ABA restores root length in the *latd* mutant by reducing ROS accumulation levels and by increasing cell length demonstrating an important role of MtRbohC (encoding a NADPH oxidase) as a negative regulator of root elongation. Zhang et al. (2014) propose that one hypothesis to explain this finding could be that MtNPF1.7 and ABA act in parallel to regulate ROS levels and root elongation (Fig. 4).

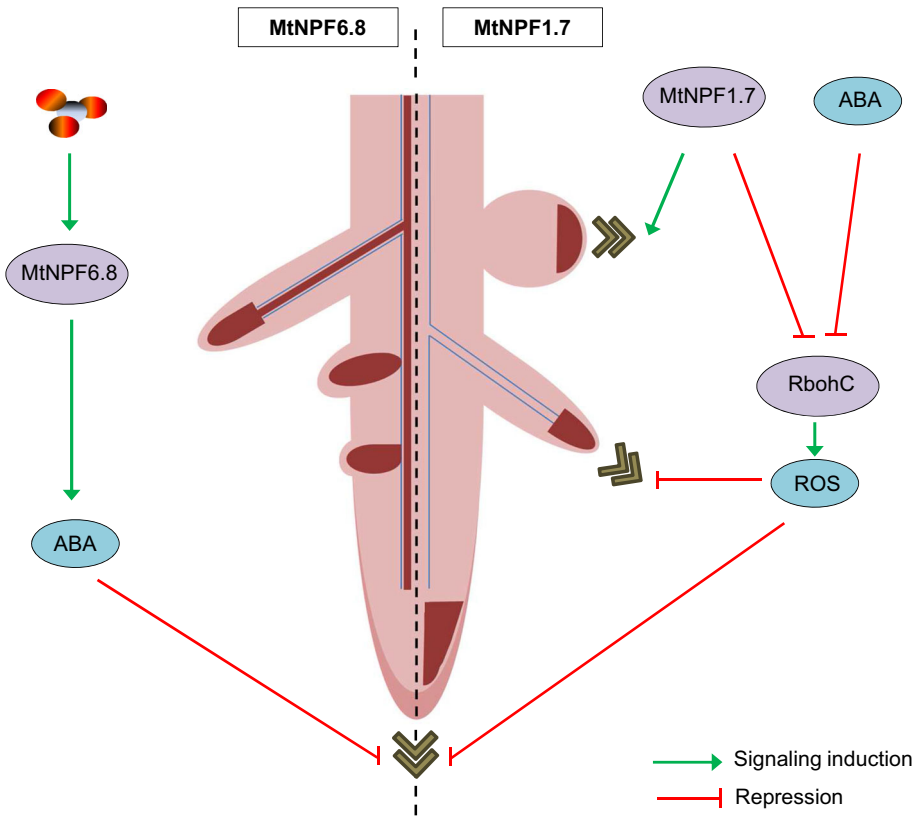

Fig. 4 Role of both characterized nitrate transporters of *M. truncatula*, MtNPF1.7 and MtNPF6.8, on root system development. The data concerning MtNPF6.8 and MtNPF1.7 are indicated on the left and on the right, respectively. Regions, where genes are expressed, are indicated in red. MtNPF6.8 is a nitrate sensor acting on cell elongation of the primary root. ABA acts downstream of MtNPF6.8 in the signaling pathway (Pellizzaro et al. 2014). MtNPF1.7 contributes to nodule meristem development, LR formation and primary root growth organization of the root tip (see review by Harris 2015). Zhang et al. (2014) propose that one hypothesis is that ABA and MtNPF1.7 could function in parallel to modulate root elongation via reactive oxygen species (ROS) generated by MtRbohC NADPH oxidase

Nitrate perceived by a NPF transporter and nitrate impact on root architecture have been studied in *M. truncatula*. In this species, nitrate has an inhibitory effect on primary root growth at low and high concentrations in two different ecotypes, A17 and R108 (Yendrek et al. 2010; Morère-Le Paven et al. 2011; Pellizzaro et al. 2014). A quantitative genetic approach allowed us to identify a major QTL involved in the control of *M. truncatula* radicle elongation in post-germination phase in both N-free medium and medium supplied with 5 mM nitrate. The *MtNPF6.8* gene, which coincided with the peak of the QTL, appeared to be a significant candidate involved in the control of primary root growth and nitrate-sensing (Morère-Le Paven et al. 2011). Further experiments using *npf6.8* mutants indicated that MtNPF6.8 has the hallmarks of a nitrate sensor (Fig. 4), which can regulate primary root growth via control of primary root cell elongation (Pellizzaro et al. 2014). The fact that *MtNPF6.8* expression was stimulated by ABA, which also restored the inhibitory effect of nitrate on primary root growth in *npf6.8* mutants, allowed us to hypothesize that ABA acts downstream MtNPF6.8 in this nitrate-signaling pathway. MtNPF6.8 was also demonstrated to be an ABA facilitator/transporter when expressed in *Xenopus* oocytes, confirming the ability of a single protein to transport both nitrate and ABA as previously shown for the AtNPF4.6 transporter of *A. thaliana* (Huang et al. 1999; Kanno et al. 2012). However, no direct link between a nitrate signal mediated by MtNPF6.8 acting on the primary root growth and ABA transport has been demonstrated.

In summary, in *M. truncatula*, two nitrate transporters, MtNPF1.7 and MtNPF6.8, are implicated in primary root growth by regulating root cell length, both in connection with ABA signaling. As noted by Harris (2015), it is surprising that 10 µM ABA rescues the wild type phenotype either by increasing (for *latd* mutants) or decreasing (for nitrate-treated *npf6.8* mutants) root length, suggesting contradictory results (Fig. 4). However, the genotypes used in both cases were different. It is also important to note that studies on MtNPF6.8 concern nitrate effect on primary root length, since MtNPF6.8 was shown to function as a nitrate sensor. The role of MtNPF1.7 in nitrate sensing is still unknown. Finally, whether MtNPF6.8 and MtNPF1.7 contribute to the same signaling pathway in root growth control is still to be unravelled.

Nitrate transporters and their possible roles in rhizobial symbiosis

In legumes, it has been determined that nitrate is involved in the regulation of nodulation (Heath et al. 2010; Saito et al. 2014). Nitrate transporters are thus likely to have important roles to play during symbiosis establishment. Indeed, in a recent study, Damiani et al. (2016) analysed the root hair transcriptome from roots of control *M. truncatula* or following treatment with nod-factors (NF). NF are signaling molecules produced by rhizobia, inducing many of the plant responses during the early stages of plant-bacteria interaction. The authors paid particular attention to proteins likely to play a role in nutrient ion uptake. *Medtr5g012290* (also named *MtNPF6.7*; Léran et al. 2014), a homolog of AtNPF6.3, was the most highly expressed NPF member in *M. truncatula* root hairs, while *MtNPF1.7* expression was 6 times lower than that of *MtNPF6.7* and *MtNPF6.8* was not expressed. Upon NF treatment, the expression of *MtNPF6.7* was up-regulated (Damiani et al. 2016). In the NRT2 family, *MtNRT2.1* and *MtNRT2.2* are also expressed in root hairs and up-regulated upon NF treatment (Damiani et al. 2016). In the present study, the microarray data analysis showed that many *M. truncatula NPF* genes are expressed in nodules (Fig. 2). For some genes (*NPF4.7*, *NPF3.2*, *NPF7.4*, *NPF5.4*, *NPF7.6*, *NPF3.3*, *NPF1.4*, *NPF5.11*, *NPF1.2* and *NPF5.6*), the expression in nodules (Fig. 2B) is higher than in other plant tissues (Fig. 2A), or during seed development (Fig. 2C). Furthermore in *M. truncatula*, MtNRT2s and MtNAR2s are expressed in mature nodules (Criscuolo et al. 2012; Pellizzaro et al. 2015). This expression of *NPF* and *NRT2* genes in nodules raise the question of a possible role of the corresponding transporters in nitrate transport inside the nodule. However, direct involvement of nitrate transporters in the exchange of nutrients between roots and nodules has never been reported.

One might ask, what are the roles attributed to these transporters in nodules, if they are not involved in transporting nitrate? In *M. truncatula*, it has been shown that MtNPF1.7 is essential in the formation and maintenance of nodule meristems and in rhizobial invasion during nodulation (Veereshlingam et al. 2004; Harris and Dickstein 2010). Using different mutants of MtNPF1.7, Bagchi et al. (2012) proposed that the ability of MtNPF1.7 to transport nitrate is correlated with the abilities of mutants to form and maintain nodules. However, these same authors also proposed that MtNPF1.7 should have another unknown activity, besides nitrate transport, in nodule development and in root architecture. MtNPF1.7 may be important for nitrate redistribution within the plant or could transport nitrate that would be useful as a precursor for the synthesis of nitric oxide (NO), a potent signaling molecule shown to be an important regulatory player in the *Rhizobium*-legume symbiotic interaction (Hichri et al. 2015). Otherwise, since new nodule formation is partially dependent on the suppression of lateral root emergence, *MtNPF1.7*, which is expressed in both of these organs, could play a role in coordinating development of lateral roots and nodules

(Harris and Dickstein 2010). Alternatively, the role of MtNPF1.7 in ABA signaling in root meristem function, as shown by Liang et al. (2007), suggests that nodule formation, controlled by MtNPF1.7, may require ABA (Harris and Dickstein 2010). High expression of *MtNRT2.1* and *MtNRT2.3* in *M. truncatula* nodules indicates that nitrate transporters of the MtNRT2 family could also be involved in signaling pathways governing nodule formation, development and/or function (Pellizzaro et al. 2015). The involvement of nitrate transporters in the establishment of the symbiotic interaction has also been proposed after studying some members of the NPF and NRT2 families in another species of legume, *L. japonicus* (Criscuolo et al. 2012). These authors suggested the possible involvement of LjNRT2 and LjNPF in the high and/or low nitrate-signaling pathways affecting nodule formation, but also a possible role for a LjNPF, named CM0826.370, in mature nodules. All these data provide a basis for future experiments that will help to understand the possible roles of these nitrate transporters in the nodule.

Conclusion

Fundamental studies to understand mechanisms that govern nitrate transport and signaling offer prospects to better control nitrogen use efficiency in plants. Mining of extant datasets enables identification of candidate genes and consideration of their possible roles *in planta*. To date, few studies of legume nitrate transporters have been undertaken, and thus much remains undiscovered, in particular the role of these transporters in nodules.

Experimental characterization of nitrate transporters is usually performed under optimal or at least unchallenging conditions. However, plants are continuously subjected to ever changing environmental conditions. Their sessile lifestyle exposes them to unavoidable frequent and severe climatic changes and attacks by herbivores and microorganisms. It is well known that legume plants adapt their root architecture and their metabolism to abiotic stress through signaling pathways involving ABA and NO (Planchet et al. 2011, 2014). Given that studies on both characterized nitrate transporters, MtNPF1.7 and MtNPF6.8, connect these NPFs with root architecture and ABA, it will be valuable to fully investigate the role of legume NPFs in response to challenging environmental conditions.

Author contribution statement AP designed the outline of the article. BA investigated the in silico gene expression profiles. AP and BA composed figures and the supplementary table. AP, EP, AL and MCML wrote the manuscript.

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