MtNOA1/RIF1 modulates Medicago truncatula–Sinorhizobium meliloti nodule development without affecting its nitric oxide content

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Abstract

AtNoa1/Rif1 (formerly referred to as AtNos1) has been shown to modulate nitric oxide (NO) content in Arabidopsis. As NO generation in the legume–rhizobium symbiosis has been shown, the involvement of an MtNoa1/Rif1 orthologue from Medicago truncatula (MtNoa1/Rif1) during its symbiotic interaction with Sinorhizobium meliloti has been studied. The expression of MtNoa1/Rif1 appeared to occur mainly in nodule vascular bundles and the meristematic zone. Using an RNA interference strategy, transgenic roots exhibiting a significantly decreased level of MtNoa1/Rif1 expression were analysed. NO production was assessed using a fluorescent probe, and the symbiotic capacities of the composite plants upon infection with Sinorhizobium meliloti were determined. The decrease in MtNoa1/Rif1 expression level resulted in a decrease in NO production in roots, but not in symbiotic nodules, indicating a different regulation of NO synthesis in these organs. However, it significantly lowered the nodule number and the nitrogen fixation capacity of the functional nodules. Although having no influence on NO production in nodules, MtNOA1/RIF1 significantly affected the establishment and the functioning of the symbiotic interaction. The impairment of plastid functioning may explain this phenotype.

Key words: cpGTPase, Medicago truncatula–Sinorhizobium meliloti symbiosis, nitric oxide, NOA1/RIF1 protein, nodule development and functioning.

Introduction

In recent years nitric oxide (NO) has emerged as an important endogenous signalling molecule in plants orchestrating a wide range of developmental and physiological processes. Indeed, the role of NO has been highlighted during disease resistance (Delledonne et al., 1998; Durner et al., 1998), stomatal closure (Neill et al., 2002), flowering (He et al., 2004), seed dormancy (Bethke et al., 2006), pollen tube growth (Prado et al., 2004), and root organogenesis (Pagnussat et al., 2002). Furthermore, there is now evidence for a role for this molecule in plant growth and development (del Rio et al., 2004; Delledonne, 2005). It appears that NO may act through the modulation of gene expression (Parani et al., 2004; Ferrarini et al., 2008) as well as through post-translational modification (Lindermayr et al., 2005; Leitner et al., 2009).

In this framework, there is increasing evidence that NO plays important roles in the legume-rhizobia symbiosis (Pauly et al., 2006). Using the NO-specific fluorescent
probe 4,5-diaminofluorescein diacetate (DAF2-DA), NO production was detected in functional *Medicago truncatula–Sinorhizobium meliloti* indeterminate nodules. In this model, NO detection was restricted to the bacteroid-containing cells of the nodule fixation zone (Baudouin et al., 2006). Production of NO in the very early steps of the *Lotus japonicus–Mesorhizobium loti* symbiosis has also been reported (Shimoda et al., 2005, 2009; Nagata et al., 2008), and NO, together with auxin, has been shown to control indeterminate nodule formation (Pii et al., 2007).

In spite of evidence demonstrating the involvement of NO in different aspects of plant metabolism, physiology, and defence (Besson-Bard et al., 2008), the mechanism(s) responsible for the synthesis of this reactive nitrogen species in plants have not yet been deciphered. Three main routes able to yield NO in plants have been described: non-enzymatic conversion of nitrite to NO in the apoplast (Bethke et al., 2004), nitrate reductase (NR)-dependent NO formation (Desikan et al., 2002), and NO synthase (NOS)-like activity converting arginine to citrulline and NO (del Rio et al., 2004; Corpas et al., 2009). Although numerous studies using pharmacological tools suggested the occurrence of NOS-like activities in plants, the molecular identity of the enzyme(s) remains unknown (Moreau et al., 2008). Based on homology to a hypothetical snail NOS, Crawford and co-workers (Guo et al., 2003) identified a potential NOS in *Arabidopsis*, AtNOS1. *AtNOS1* T-DNA knockout plants (*atnos1*) exhibited an altered growth phenotype that can be rescued by the application of NO donors (Guo et al., 2003). Moreover, chemical probes sensitive to NO indicated reduced NO levels in *atnos1* compared with wild-type plants (Guo et al., 2003; Zeidler et al., 2004; Guo and Crawford, 2005; Bright et al., 2006). However, the true function of AtNOS1 was called into question (Crawford et al., 2006; Zemojtel et al., 2006), leading to its renaming as NO-associated protein 1 (AtNOA1) (Crawford et al., 2006). AtNOA1 is a 561 amino acid protein that has no sequence homology with the animal NOSs. It belongs to the circularly permuted GTPase (cpGTPase) family (Anand et al., 2006). The central domain of AtNOA1 contains guanine-binding motifs (G motifs) characteristic of small GTPases (Bourne et al., 1991), but in an unusual arrangement; G4–G5 are N-terminal of G1–G2–G3. Among the four subfamilies of cpGTPase represented by YlqF (in *Bacillus subtilis*), YjeQ (in *Escherichia coli*), YawG (in *Saccharomyces pombe*), and YqfH (in *B. subtilis*), the latter is the closest homologue of AtNOA1 (Moreau et al., 2008; Sudhamsu et al., 2008). The biological function of the protein has been highlighted first through biochemical and structural analyses as a functional cpGTPase (Moreau et al., 2008; Sudhamsu et al., 2008). These reports clearly demonstrated that AtNOA1 does not have NO synthase activity. In bacteria and in some eukaryotes, this GTP-binding protein family is associated with RNA/ribosome binding function, and bacterial cpGTPases are also essential for cell growth (Moreau et al., 2008; Sudhamsu et al., 2008). Furthermore, it has been shown that the AtRIF1 protein, initially reported as AtNOS1, is required for proper ribosome function in chloroplasts (Flores-Perez et al., 2008). Very recently, an MtNOA1/RIF1 functional homologue has been characterized in *Oryza sativa* and it has been proposed that a highly conserved nuclear-encoded cpGTPase is essential for proper chloroplast ribosome assembly and/or translation in plants (Liu et al., 2010).

The aim of the present work was to evaluate the involvement of the AtNOA1/RIF1 orthologue from *M. truncatula* in the symbiotic interaction with *S. meliloti*. For this purpose, the *MtNOA1/RIF1* gene was cloned and the effect of modifying its expression on the NO production and symbiotic capacities of *M. truncatula* composite plants was analysed.

### Materials and methods

**Plant, growth conditions, and rhizobial strain**

*M. truncatula* cv. Jemalong seeds scarified in 1 M H₂SO₄ (8 min) were sterilized successively in 2% HgCl₂ (10 min) and 6% bleach solution (4 min), and then rinsed with sterile distilled water. Germination was carried out for 2 d on 0.4% agar plates in the dark. For short-term experiments (up to 10 d), seedlings were grown under sterile conditions in 10 cm square Petri dishes containing modified Fahreus medium supplemented or not with 0.5 mM NH₄NO₃ (Boisson-Dernier et al., 2001). For long-term experiments, seedlings were transferred to sterile sand and were watered with nutrient solution in the absence of nitrogen supply. Plantlets were grown in a climatic chamber as described (Frendo et al., 1999) and were inoculated when 7 d old with wild-type *Sinorhizobium meliloti* 1021 or 2011 strains.

**MtNOA1/RIF1 cDNA identification and cloning**

cDNA obtained from several *M. truncatula* tissues (leaves, roots, and nodules) were used in order to amplify a partial *MtNOA1/RIF1* cDNA by PCR overlapping the two expressed sequence tags (ESTs; BF640001 and BE317063) sharing strong homologies with the *AtNOA1/RIF1* gene (Guo et al., 2003). Rapid amplification of cDNA ends (RACE) was carried out using a 5′/3′ RACE kit, 2nd generation, (Roche Diagnostics) according to the manufacturer’s recommendations. The resulting *MtNOA1/RIF1* full-length cDNA sequence is accessible in the GenBank database (accession no. HM448907)

**Sequence and phylogenetic tree analysis of NOA1 proteins**

The accession numbers and/or gene identification of the NOA1 proteins are listed in Supplementary Table S1 available at *JXB* online. The analysis was performed on the www.phylogeny.fr platform (Dereeper et al., 2008) and comprised the following steps: after alignment of NOA1/RIF1 protein sequences with T-Coffee (v6.85), ambiguous regions (i.e. containing gaps and/or poorly aligned) were removed with Gblocks (v0.91b) using standard parameters. The phylogenetic tree was reconstructed using the Bayesian inference method implemented in the MrBayes program (v3.1.2). The number of substitution types was fixed to 6. The Poisson model was used for amino acid substitution, while rates variation across sites was fixed to ‘invgamma’. Four Markov Chain Monte Carlo (MCMC) chains were run for 10 000 generations, sampling every 10 generations, with the first 250 sampled trees discarded as ‘burn-in’. Finally, a 50% majority rule consensus tree was constructed. Graphical representation and edition of the phylogenetic tree were performed with TreeDyn (v198.3).
Gene expression analysis

Total RNAs were isolated from *M. truncatula* tissues using Trizol reagent (Invitrogen, Cergy Pontoise, France), according to the manufacturer’s recommendations, and then used as templates for real-time RT-PCR analysis. A 2 μg aliquot of total RNA was reverse transcribed (Omniscript; Promega, Charbonnières-les-Bains, France) using an oligo(dT) primer. cDNAs were used as templates for quantitative real-time RT-PCR (qRT-PCR) carried out using the qPCR MasterMix Plus for SYBR Green I reagent (Eurogentec, Angers, France), with 1 μl of a 1/10 (v/v) dilution of the first-strand cDNA reaction. Forward (5'-CTT GAG GCC TCT ACC GGA AA) and reverse (5'-GCT CAG CTT TCT TTC CA) primers were used to amplify a 149 bp region of *MtNoa1/Rif1*. Reactions were run on the Chromo4 Real-Time PCR Detection System (Bio-Rad, Marnes-la-Coquette, France) and quantification was performed with the Opticon Monitor analysis software v. 3.1 (Bio-Rad, Marnes-la-Coquette, France). The PCR program used was as follows: polymerase activation (95 °C for 5 min), amplification and quantification cycles repeated 40 times (94 °C for 15 s, 60 °C for 1 min), and melting curve (40–95 °C with one fluorescence read every 0.5 °C). The plant mRNA levels were normalized against two endogenous controls, 40S ribosomal protein S8 (TC137982) and MtCc27 (TC132510) (Van de Velde et al., 2006). The following formula was used for the relative expression ratio calculation: 2^-ΔΔCt, with ΔΔCt=CTgene of interest - CThousekeeping gene.

Nitric oxide detection

NO was observed as previously described (Baudouin et al., 2006). Briefly, *M. truncatula* transgenic fresh root apexes and fresh nodule slices (150 μm) obtained with a 1000 Plus vibratome (Labonord, Templemars, France) were incubated for 30 min in the dark with detection buffer (DB; 10 mM TRIS-HCl, pH 7.4, 10 mM KCl) containing 10 μM DAF-2DA (Sigma-Aldrich, Lyon, France). Plant tissues were subsequently washed for 30 min with water, air-dried in the dark, and visualized using a Zeiss LSM 500 confocal laser microscope (Carl Zeiss SA, Le Pecq, France) upon excitation at 488 nm with an Argon 2 laser. Dye emission was recorded using a 505–530 nm band-pass filter coupled with a 515 nm long-pass filter. Images were processed and analysed using the Zeiss LSM510 Meta software (http://www.zeiss.de) and Adobe Photoshop (www.adobe.com), respectively.

NO production rate by nodules

The measurement of NO production by nodules was adapted from Planchet and Kaiser (2006). A 10–20 mg aliquot of detached nodules was incubated in the dark, at 23 °C, in Eppendorf tubes containing 500 μl of detection medium (10 mM TRIS-HCl pH 7.4, 10 mM KCl) in the presence of 10 μM DAF-2DA (Sigma-Aldrich, Lyon, France). Plant tissues were subsequently washed for 30 min with DB and were mounted in DB for observation.

The formation of the highly fluorescent triazolofluorescein compound (DAF-2T) following NO reaction with DAF-2DA was visualized using a Zeiss LSM 500 confocal laser microscope (Carl Zeiss SA, Le Pecq, France) upon excitation at 488 nm with an Argon 2 laser. Dye emission was recorded using a 505–530 nm band-pass filter coupled with a 515 nm long-pass filter. Images were processed and analysed using the Zeiss LSM510 Meta software (http://www.zeiss.de) and Adobe Photoshop (www.adobe.com), respectively.

RNA interference (RNAi) and *MtNoa1/Rif1* promoter cloning procedures

A 295 bp cDNA fragment of *MtNoa1/Rif1* was amplified by PCR using the following primers: forward 5'-CTT AAC CCC TCT TTC TCT CC-3' and reverse 5'-AGG AGC GTC ATT ATC AGA AG-3', and cloned into the pGEMT-Easy vector (Promega, Charbonnières-les-Bains, France). The Neo-Gus insert was subsequently introduced into the pENTR4 vector (Invitrogen) and the resulting vector was recombined with pK7GWIG2 (Karimi et al., 2005) using Gateway technology (Invitrogen), according to the manufacturer’s recommendations.

The *MtNoa1/Rif1* promoter (~1.3 kb upstream the start codon) was amplified by PCR using the following primers (forward, 5'-TTC TTG TTC GCT CCC AAG TG-3'; reverse, 5'-TGC TTG CGC ACT AAT TTC AG-3'). Each PCR fragment was recombined into the pDONR207 vector (Invitrogen) and then in the plant expression vector pKGWSF7 (Karimi et al., 2002) using Gateway technology (Invitrogen).

Agrobacterium rhizogenes root transformation and inoculation

The pK7GWIG2-MtNoa1/Rif1 (RNAi::*MtNoa1/Rif1*) and PKGWSF7-pMtNoa1/Rif1 (pMtNoa1/Rif1::GUS) plasmids were introduced into *A. rhizogenes* Arq1al (Quandt et al., 1993) or A4RS strain (Tepfer, 1990). *Medicago truncatula* plants were transformed with *A. rhizogenes* in either axenic (Boisson-Dernier et al., 2001) or non-axenic conditions (Vieweg et al., 2005; Floss et al., 2008), using the Arq1al and A4RS strains, respectively. Control plants were transformed with *A. rhizogenes* containing the pK7GWIG2 or PKGWSF7 empty vector. For axenic conditions, healthy composite plants were transferred onto agar plates containing modified Fahraeus medium without nitrogen or kanamycin. Plants were inoculated with *S. meliloti* 3 d after transfer as described above. For non-axenic conditions, 4 weeks after *A. rhizogenes* transformation, the main roots of the composite plants were removed and were then inoculated with *S. meliloti*.

Determination of acetylene reduction activity

Whole root system from composite plants was placed in glass bottles (30 ml) containing wet filter paper. The bottles were filled with 1.9 acetylene:air. After 1 h of incubation at 25 °C, the amount of ethylene in the gas phase was determined using a GS-Alumina 30 m×0.534 mm column (Agilent Technology, Massy, France).

Results

An AtNOA1/RIF1 orthologue occurs in the *M. truncatula* genome

A tBLASTN (Altschul et al., 1997) analysis was used to search for AtNOA1/RIF1 (Guo et al., 2003) homologues in the *M. truncatula* genome index (MtGI), and two ESTs (BF640001 and BE317063) were found. Subsequently, 5′- and 3′-RACE were carried out to identify the 5′ and 3′ ends of the *MtNoa1/Rif1* transcript. 5′-RACE identified a very short 5′ end corresponding to a 5′-untranslated region of 21 bases upstream of the predicted translational start codon. 3′-RACE yielded a single 3′ end of 194 nucleotides. The *MtNoa1/Rif1* cDNA of 1859 nucleotides corresponding to the longest transcript identified in this work is available in the GenBank database (accession no. HM448907; Medtr02g071140.1).

In silico analyses of the available *M. truncatula* DNA databases indicated that *MtNoa1/Rif1* probably exists as a single copy in the *M. truncatula* genome as observed in the available sequenced genomes (*Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera*, *Populus trichocarpa*, and *Sorghum bicolor*) including another model legume (*L. japonicus*).
However, data mining in the Glycine max database reveals two putative genes sharing a high level of homology (91%; Gm09g35850.1 and Gm12g01500.1). The presence of two genes in soybean is more likely to be a consequence of the tetraploidy of this genome (Schmutz et al., 2010).

The deduced amino acid sequence of MtNOA1/RIF1 shares 72% identity with Arabidopsis AtNOA1/RIF1. Based on the latest data published on AtNOA1/RIF1 (Moreau et al., 2008; Sudhamsu et al., 2008), three characteristic domains could be identified (Supplementary Fig. S1A, S2 at JXB online): the zinc-binding domain (ZBD, N-terminus) with the conserved cysteines, the cpGTPase domain which has been crystallized in the YqeH protein from Geobacillus stearothermophilus (Sudhamsu et al., 2008), and a C-terminal domain (CTD) of unknown function. A sequence analysis has been performed using other NOA proteins available in plant databases (Solanum tuberosum, Nicotiana benthamiana, Ricinus communis, Brassica juncea, Zea mays, Picea sitchensis, Mimulus guttatus, Manihot esculenta, Cucumis sativus, and Brachypodium distachyon; Supplementary Table S1). These proteins share 60–97% homology, indicating that they are highly conserved in the plant kingdom (Supplementary Table S2 and Fig. S2). Phylogenetic analysis (Fig. 1) confirmed that dicot and monocot sequences form separate clades (Liu et al., 2010). Moreover, it pointed to a clustering of legume NOA1/RIF1 proteins.

A bacterial artificial chromosome (BAC) sequence from M. truncatula (AC195570) gave additional information on MtNoa1/Rif1 genomic organization. Indeed, as in the A. thaliana (Flores-Perez et al., 2008) and O. sativa genomes (Liu et al., 2010), MtNOA1/RIF1 protein is encoded by 13 exons and 12 introns (Supplementary Fig. S1B).

**MtNoa1/Rif1 is expressed in all plant organs**

To study MtNoa1/Rif1 expression, qRT-PCR was carried out on total RNA extracted from different organs of *M. truncatula* wild-type plants (roots, nodules, leaves, stems, pods, and flowers). MtNoa1/Rif1 transcripts were detected in all considered parts of the plant (Fig. 2) as is also observed in Arabidopsis (www.geneinvestigator.com). However, MtNoa1/Rif1 is expressed more in *M. truncatula* leaves compared with the other plant organs. This result is in agreement with the available transcriptome data from the Noble foundation (Benedito et al., 2008; Supplementary Fig. S3 at JXB online). On the other hand, no significant difference in expression levels was observed during the symbiotic interaction (Supplementary Fig. S3).

To perform a more specific analysis of MtNoa1/Rif1 expression in root and nodule, a histochemical staining through a promoter–β-glucuronidase (GUS) transcriptional fusion approach was used. In root tissues, *MtNoa1/Rif1*...
expression appeared to occur in principal and secondary root apexes and vascular tissues (Fig. 3A, B). Based on microarray expression profiles of a high-resolution set of nearly all *Arabidopsis* root cell types (Brady et al., 2007), it may be noted that the *MtNoa1/Rif1* root expression profile is similar to the one obtained in *Arabidopsis* (Fig. 3D). To investigate the cellular localization of *MtNoa1/Rif1* promoter activity during the symbiotic process, composite transgenic plants were inoculated with an *S. meliloti* strain and longitudinal sections of nodules were assayed. In 12-week-old root nodules, *MtNoa1/Rif1* promoter activity was detected mainly in vascular bundles, which are continuing the root central cylinder already shown to exhibit *MtNoa1/Rif1* expression (Fig. 3C). Interestingly, no staining was observed in the nitrogen-fixing zone where NO production was evidenced previously (Baudouin et al., 2006); staining was restricted to the apical region corresponding mainly to the permanent meristem, which is characteristic for indeterminate nodules.

**RNAi::MtNoa1/Rif1 M. truncatula composite plants exhibit a decreased NO production in the root apex but not in nodules**

Based on reports about AtNOA1/RIF1 participation in plant development (Guo et al., 2003; He et al., 2004) as well as in plant–pathogen interactions (Zeidler et al., 2004; Asai et al., 2008; Kato et al., 2008), the involvement of MtNOA1/RIF1 during the *M. truncatula–S. meliloti* symbiosis was assayed. For this purpose, a 295 nucleotide sequence from *MtNoa1/Rif1* was used for RNAi experiments. In order to avoid unexpected silencing effects on similar genes (such as those encoding cpGTPases), an analysis has been performed using the small interfering RNA (siRNA) scan web application (Xu et al., 2006) on the latest available *Medicago* database. This analysis clearly showed that the RNAi construct specifically targeted the *MtNoa1/Rif1* gene (data not shown).

The RNAi construct (RNAi::*MtNoa1/Rif1*) was introduced into *A. rhizogenes* and the resulting strain was used to obtain composite plants in axenic (Boisson-Dernier et al., 2001), as well as in non-axenic conditions (Vieweg et al., 2005). The overall phenotype of the RNAi::*MtNoa1/Rif1* composite plants appeared similar to that of the control plants (Fig. 4A). A qRT-PCR analysis of *MtNoa1/Rif1* gene expression using pooled root samples from composite plants showed that the expression level was decreased by 90% (Fig. 4B), indicating that the RNAi::*MtNoa1/Rif1* construct used was efficient in the present conditions. It must be noted that only the root tissues were affected by the RNAi construct, which may explain the difference from the affected phenotype in the knock-out *atnoa1* mutant (Guo et al., 2003; Lozano-Juste and Leon, 2010). Moreover, no chlorotic phenotype is observed in leaves as in *Arabidopsis* (Flores-Perez et al., 2008) or rice (Liu et al., 2010), indicating no systemic incidence of the RNAi transgenic roots (Fig. 4A).

The NO accumulation in transgenic root apexes was analysed using the NO-specific permeant probe DAF2-DA. The RNAi::*MtNoa1/Rif1* lines exhibited a strong decrease in NO accumulation (Fig. 5A), as already observed in the *atnoa1* mutant (Guo et al., 2003). This reinforces that the
MtNoa1/Rif1 gene is a functional orthologue of the Arabidopsis AtNoa1/Rif1 gene.

In contrast, no significant difference in NO accumulation was observed in 4-week-old nodules formed on transgenic roots (Fig. 5B). To confirm this result further, the NO synthesis rate in root nodules was also determined. In the present experimental conditions, the control and RNAi::MtNoa1/Rif1 nodules showed a similar NO production (Fig. 5C). Taken together, these results indicate that the MtNoa1/Rif1 gene is not participating in the NO generation evidenced in M. truncatula–S. meliloti nodules (Baudouin et al., 2006) (Fig. 5B).

The ability of the MtNOA1/RIF1 protein to produce NO was evaluated. As previously described for AtNOA1/RIF1 (Zemojtel et al., 2006; Moreau et al., 2008), neither the full-length nor the signal peptide-deleted MtNOA1/RIF1 proteins produced in E. coli were able to generate NO and $[^3]$H]-citruline from $[^3]$H]-arginine (data not shown).

MtNOA1/RIF1 plays an important role in the M. truncatula–S. meliloti symbiotic process

Even if no difference was observed in terms of NO accumulation in nodules, the symbiotic interaction of RNAi::MtNoa1/Rif1 plants with S. meliloti appeared to be significantly affected. A strong decrease in nodule primordia (Fig. 6A) as well as nodule number (Fig. 6B) was observed in composite plants. Furthermore, a strong decrease (~50%, n=40) of the nitrogen fixation capacity (as determined by the acetylene reduction assay) was observed in RNAi::MtNoa1/Rif1 plants (Fig. 6C). This is paralleled by a strong decrease in the size of the nitrogen-fixing zone (data not shown). Taken together, these results show that MtNoa1/Rif1 plays an important role in the establishment and the functioning of the M. truncatula–S. meliloti symbiosis.

Discussion

In this work, the AtNoa1/Rif1 orthologue gene was cloned in M. truncatula. The availability of genomic resources from several plants made it possible to compare NOA1/RIF1 sequences. With the exception of the G. max genome, plant genomes sequenced so far present a single copy of the Noa1/Rif1 gene. The presence of two putative Noa1/Rif1 genes in the soybean genome could be explained by the tetraploidization of its genome (Shoemaker et al., 1996). The MtNOA1/RIF1 protein shows all the motifs already described for AtNOA1/RIF1 (Moreau et al., 2008): a cpGTPase domain, a zinc-binding domain, a C-terminal domain, and an N-terminal signal peptide (Supplementary Figs S1A, S2 at JXB online).
No impact on NO production was observed in RNAi nodules; it must be underlined here that NO has been shown to be produced in the nodule nitrogen-fixing zone in which MtNoa1/Rif1 was not expressed. However, the NO production was modulated in RNAi root apices, indicating that MtNoa1/Rif1 is a functional orthologue of AtNoa1/Rif1. Furthermore, it has been proposed that the elevated amount of reactive oxygen species (ROS) observed in the Atnoa1/rif1 mutant is responsible for the reduced NO accumulation, since NO can react very quickly with superoxide anion and lipid radicals and thus reduce the amount of detectable NO (Moreau et al., 2008). The strong antioxidant defence occurring in nodules (Pauly et al., 2006) may prevent this ROS accumulation, thus avoiding any effect on NO concentration. As is the case for plants in general, the origin of the NO synthesis in root nodules remains to be identified. Although several reports point to the possible involvement of NOS-like systems (Cueto et al., 1996; Baudouin et al., 2006), the possible role of a nitrate/nitrite-dependent pathway, involving cytosolic NR (Yamasaki and Sakihama, 2000) and a root-specific plasma membrane nitrite-NO reductase (Ni-NOR) (Stohr et al., 2001) or of polyamine oxidases (Yamasaki and Cohen, 2006) cannot be ruled out. In any case, data presented here suggest that NO generation pathways are differently regulated in roots and nodules, even if the influence of the organ/tissue oxidative state cannot be ruled out.

Medicago truncatula RNAi::MtNoa1/Rif1 transgenic roots did not show any root phenotype, in contrast to the Arabidopsis atnoa1 knockout mutant phenotype (Guo et al., 2003; Lozano-Juste and Leon, 2010). However, it must be noted that no root phenotype was observed recently in RNAi::OsNoa1/Rif1 rice plants (Liu et al., 2010). The results obtained here might be explained by the remaining MtNoa1/Rif1 expression in transgenic roots as well as the possible influence of the non-transgenic shoot. However, a clear symbiotic phenotype (in terms of both nodule formation and efficiency) was observed in RNAi::MtNoa1/Rif1 composite plants where a decrease in infection events, nodule number, and nitrogen fixation was evidenced. This clearly shows that MtNOA1/RIF1 modulates nodule development and metabolism.

Recent works have shown that NOA1/RIF1 proteins are targeted to the chloroplast in Arabidopsis and rice (Flores-Perez et al., 2008; Liu et al., 2010). Moreover, using a quantitative proteomic approach, Liu et al. (2010) reported that the OsNOA1/RIF1 mutant showed a significantly decreased expression level of chloroplast-encoded proteins as well as nuclear-encoded components of chloroplast enzyme complexes. They proposed that a highly conserved nuclear-encoded cpGTPase is essential for proper chloroplast assembly and/or translation in plants (Liu et al., 2010). Based on these reports, it can be suggested that MtNOA1/RIF1 is also targeted to root and nodule plastids. The impairment of plastidial functioning of the meristematic zone of the nodule is likely to affect nodule formation and differentiation, as observed. This may be linked to the role of plastids in antioxidation protection and in control of thiol synthesis (Frendo et al., 1999; Moran et al., 2000). In this framework, it should be mentioned that the glutathione synthesis pathway is regulated by NO in plants (Innocenti et al., 2007). Furthermore, it has been shown that the expression of plastidial glutathione peroxidases is modulated by NO (Ramos et al., 2009). The observed effects may also occur through the modification of the plastid retrograde signalling to the nucleus (Woodson and Chory, 2008). On the other hand, the impairment of plastidial functioning in vascular bundles may disturb the carbon supply to the nodule and contribute to explaining the observed decreased in the nitrogen fixation activity. Moreover, it must be noted that a gene encoding a zinc finger protein involved in nodule organogenesis has already been shown to be expressed in vascular bundles (Frugier et al., 2000). Moreover, it must be underlined that, although they have also been detected in the nuclear envelope (Charpentier et al., 2008), the plastidial CASTOR and POLLUX proteins have been shown to be crucial for symbiotic fungal and bacterial entry into plant roots (Imaizumi-Anraku et al., 2005). In any case, this points to an important role for

Fig. 6. Symbiotic capacities of RNAi::MtNoa1/Rif1 composite plants. Nodule primordia number in each root from axenic composite plants (A). Total nodule number in non-axenic composite plants (B). Acetylene reduction activity in non-axenic composite plants (C). Values are representative of three independent biological replicates ±SE (n > 40). An asterisk represents a significant difference from RNAi::MtNoa1/Rif1 compared with control for P < 0.05.
plastids in the legume–rhizobium symbiosis and to the pleiotropic effects of Noa1/Rif1 gene silencing. On the other hand, the impairment of NO production in roots by MtNoa1/Rif1 silencing may also affect the early steps of the nodulation process. In this regard it should be mentioned that reducing the NO level using 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl imidazole-1-oxyl-3-oxide (cPTIO), an NO scavenger, led to a delayed nodulation (A. Boscari, personal communication).

Taken together, the data presented here show that MtNoa1/Rif1 is involved in the M. truncatula–S. meliloti symbiosis in both nodule development and functioning. Future additional studies will help in elucidating its role in these processes.

Supplementary data
Supplementary data are available at JXB online.

Table S1. NOA1/RIF1 protein sequences used for the phylogenetic analysis.
Table S2. NOA1 protein homology.
Figure S1. MtNoa1/Rif1 primary structure and MtNoa1/Rif1 genomic organization.
Figure S2. Multiple alignment of plant NOA1/RIF1s.
Figure S3. MtNoa1/Rif1 expression profile using the Medicago truncatula Gene Expression Atlas.

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References
Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.
Anand B, Verma SK, Prakash B. 2006. Structural stabilization of GTP-binding domains in circularly permuted GTPases: implications for RNA binding. Nucleic Acids Research 34, 2196–2205.
Asai S, Ohta K, Yoshioha K. 2008. MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in Nicotiana benthamiana. The Plant Cell 20, 1390–1406.
Baudouin E, Pieuchot L, Engler G, Pauly N, Puppo A. 2006. Nitric oxide is formed in Medicago truncatula–Sinorhizobium melloti functional nodules. Molecular Plant-Microbe Interactions 19, 970–975.
Benedito VA, Torres-Jerez I, Murray JD, et al. 2008. A gene expression atlas of the model legume Medicago truncatula. The Plant Journal 55, 504–513.
Besson-Bard A, Pugin A, Wendehenne D. 2008. New insights into nitric oxide signaling in plants. Annual Review of Plant Biology 59, 21–39.
Bethke PC, Badger MR, Jones RL. 2004. Apoplastic synthesis of nitric oxide by plant tissues. The Plant Cell 16, 332–341.
Bethke PC, Libourel IG, Jones RL. 2006. Nitric oxide reduces seed dormancy in Arabidopsis. Journal of Experimental Botany 57, 517–526.
Boisson-Dernier A, Chabaud M, Garcia F, Becard G, Rosenberg C, Barker DG. 2001. Agrobacterium rhizogenes-transformed roots of Medicago truncatula for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. Molecular Plant-Microbe Interactions 14, 695–700.
Bourne HR, Sanders DA, McCormick F. 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature 349, 117–127.
Brady SM, Orlando DA, Lee JY, Wang JY, Koch J, Dinnyen JR, Mace D, Ohler U, Benfey PN. 2007. A high-resolution root spatiotemporal map reveals dominant expression patterns. Science 318, 801–806.
Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H2O2 synthesis. The Plant Journal 45, 113–122.
Charpentier M, Bredemeier R, Wanner G, Takeda N, Schleiff E, Parniske M. 2008. Lotus japonicus CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. The Plant Cell 20, 3467–3479.
Corpas FJ, Palma JM, del Rio LA, Barroso JB. 2009. Evidence supporting the existence of l-arginine-dependent nitric oxide synthase activity in plants. New Phytologist 184, 9–14.
Crawford NM, Galli M, Tischner R, Heimer YM, Okamoto M, Mack A. 2006. Response to Žemojtel et al: Plant nitric oxide synthase: back to square one. Trends in Plant Science 11, 526–527.
Cueto M, Hernandez-Perera O, Martin R, Bentura ML, Rodrigo J, Lamas S, Golvano MP. 1996. Presence of nitric oxide synthase activity in roots and nodules of Lupinus albus. FEBS Letters 398, 159–164.
Delledonne M. 2005. NO news is good news for plants. Current Opinion in Plant Biology 8, 390–396.
Delledonne M, Xia Y, Dixon RA, Lamb C. 1998. Nitric oxide functions as a signal in plant disease. Nature 394, 585–588.
del Rio LA, Corpas FJ, Barroso JB. 2004. Nitric oxide and nitric oxide synthase activity in plants. Phytochemistry 65, 783–792.
Dereeper A, Guignon V, Blanc G, et al. 2008. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Research 36, W465–W469.
Desikan R, Griffiths R, Hancock J, Neill S. 2002. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 99, 16314–16318.
Durner J, Wendehenne D, Kissel DF. 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proceedings of the National Academy of Sciences, USA 95, 10328–10333.
Ferrarini A, De Stefano M, Baudouin E, Pucciariello C, Polverari A, Puppo A, Delledonne M. 2008. Expression of *Medicago truncatula* genes responsive to nitric oxide in pathogenic and symbiotic conditions. *Molecular Plant-Microbe Interactions* 21, 781–790.

Flores-Perez U, Sauret-Gueto S, Gas E, Jarvis P, Rodriguez-Concepcion M. 2008. A mutant impaired in the production of plastome-encoded proteins uncovers a mechanism for the homeostasis of isoprenoid biosynthetic enzymes in Arabidopsis plastids. *The Plant Cell* 20, 1303–1315.

Floss DS, Hause B, Lange PR, Kuster H, Strack D, Walter MH. 2008. Knock-down of the MEP pathway isoenzyme 1-deoxy-d-xylulose 5-phosphate synthase 2 inhibits formation of arbuscular mycorrhiza-induced apocarotenoids, and abolishes normal expression of mycorrhiza-specific plant marker genes. *The Plant Journal* 56, 86–100.

Frendo P, Gallesi D, Turnbull R, Van de Sype G, Hérouart D, Puppo A. 1999. Localisation of glutathione and homoglutathione in *Medicago truncatula* is correlated to a differential expression of genes involved in their synthesis. *The Plant Journal* 17, 215–219.

Frugier F, Poirier S, Satiat-Jeunemaitre B, Kondorosi A, Crespi M. 2000. A Kruppel-like zinc finger protein is involved in nitrogen-fixing root nodule organogenesis. *Genes and Development* 14, 475–482.

Guo FQ, Crawford NM. 2005. Arabidopsis nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *The Plant Cell* 17, 3436–3450.

Guo FQ, Okamoto M, Crawford NM. 2003. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* 302, 100–103.

He Y, Tang RH, Hao Y, et al. 2004. Nitric oxide represses the Arabidopsis floral transition. *Science* 305, 1968–1971.

Imaizumi-Anraku H, Takeda N, Charpentier M, et al. 2005. Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots. *Nature* 433, 527–531.

Innocenti G, Pucciariello C, Le Gleuher M, Hopkins J, de Stefano M, Delledonne M, Puppo A, Baudouin E, Frendo P. 2007. Glutathione synthesis is regulated by nitric oxide in *Medicago truncatula* roots. *Planta* 225, 1597–1602.

Karimi M, De Meyer B, Hilson P. 2005. Modular cloning in plant cells. *Trends in Plant Science* 10, 103–105.

Karimi M, Inze D, Depicker A. 2002. GATEWAY vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science* 7, 193–195.

Kato H, Asai S, Yamamoto-Katou A, Yoshioka H, Doke N, Kawakita K. 2008. Involvement of NbNOA1 in NO production and defense responses in *N. benthamiana* treated with Brassica napus. *Journal of General Plant Pathology* 74, 15–23.

Leitner M, Vandel E, Gaupe F, Bellin D, Delledonne M. 2009. NO signals in the haze: nitric oxide signalling in plant defence. *Current Opinion in Plant Biology* 12, 451–458.

Lindermayr C, Saalbach G, Durner J. 2005. Proteomic identification of S-nitrosylated proteins in Arabidopsis. *Plant Physiology* 137, 921–930.

Liu H, Lau E, Lam M, Chu H, Li S, Huang G, Guo P, Wang J, Jiang L, Chu IK, Lo C, Yao T. 2010. OsNOA1/RIF1 is a functional homolog of AtNOA1/RIF1: implication for a highly conserved plant cGTPase essential for chloroplast function. *New Phytologist* 187, 83–105.

Lozano-Juste J, Leon J. 2010. Enhanced abscisic acid-mediated responses in *nia1nia2noa1-2* triple mutant impaired in NIA/NR- and AtNOA1-dependent nitric oxide biosynthesis in Arabidopsis. *Plant Physiology* 152, 891–903.

Moran JF, Iturbe-Ormaetxe I, Matamoros MA, Rubio MC, Clemente MR, Brewin NJ, Becana M. 2000. Glutathione and homoglutathione synthetases of legumes nodules. Cloning, expression, and subcellular localization. *Plant Physiology* 124, 879–888.

Moreau M, Lee GI, Wang Y, Crane BR, Klessig DF. 2008. AtNOS/AthA1 is a functional Arabidopsis *thaliana* cGTPase and not a nitric-oxide synthase. *Journal of Biological Chemistry* 283, 32957–32967.

Nagata M, Murakami E, Shimoya Y, Shimoda-Sasakura F, Kucho K, Suzuki A, Abe M, Higashi S, Uchiumi T. 2008. Expression of a class I hemoglobin gene and production of nitric oxide in response to symbiotic and pathogenic bacteria in *Lotus japonicus*. *Molecular Plant-Microbe Interactions* 21, 1175–1183.

Neilj SJ, Desikan R, Clarke A, Hancock JT. 2002. Nitric oxide is a novel component of abscissic acid signaling in stomatal guard cells. *Plant Physiology* 128, 13–16.

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L. 2002. Nitric oxide is required for root organogenesis. *Plant Physiology* 129, 954–956.

Parani M, Rudrabhatla S, Myers R, Weirich H, Smith B, Leaman DW, Goldman SL. 2004. Microarray analysis of nitric oxide responsive transcripts in Arabidopsis. *Plant Biotechnology Journal* 2, 359–366.

Pauly N, Pucciariello C, Mandon K, Innocenti G, Jamet A, Baudouin E, Herouart D, Frendo P, Puppo A. 2006. Reactive oxygen and nitrogen species and glutathione: key players in the legume-rhizobium symbiosis. *Journal of Experimental Botany* 57, 1769–1776.

Pii Y, Crimi M, Cremonese G, Spena A, Pandolfini T. 2007. Auxin and nitric oxide control indeterminate nodule formation. *BMC Plant Biology* 7, 21.

Planchet E, Kaiser WM. 2006. Nitric oxide (NO) detection by DAF-2 fluorescence and chemiluminescence: a comparison using abiotic and biotic NO sources. *Journal of Experimental Botany* 57, 3043–3055.

Prado AM, Porterfield DM, Feijo JA. 2004. Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. *Development* 131, 2707–2714.

Quandt H-J, Pühler A, Broer I. 1993. Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. *Molecular Plant-Microbe Interactions* 6, 699–706.

Ramos J, Matamoros MA, Naya L, James EK, Rouhier N, Sato T, Tabata S, Becana M. 2009. The glutathione peroxidase gene family of *Lotus japonicus*: characterization of genomic clones,
expression analyses and immunolocalization in legumes. *New Phytologist* **181**, 103–114.

Schmutz J, Cannon SB, Schlueter J, *et al*. 2010. Genome sequence of the palaeopolyploid soybean. *Nature* **463**, 178–183.

Shimoda Y, Nagata M, Suzuki A, Abe M, Sato S, Kato T, Tabata S, Higashi S, Uchiumi T. 2005. Symbiotic rhizobium and nitric oxide induce gene expression of non-symbiotic hemoglobin in Lotus japonicus. *Plant and Cell Physiology* **46**, 99–107.

Shimoda Y, Shimoda-Sasakura F, Kucho K, Kanamori N, Nagata M, Suzuki A, Abe M, Higashi S, Uchiumi T. 2009. Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between *Mesorhizobium loti* and Lotus japonicus. *The Plant Journal* **57**, 254–263.

Shoemaker RC, Polzin K, Labate J, *et al*. 1996. Genome duplication in soybean (Glycine subgenus soja). *Genetics* **144**, 329–338.

Stohr C, Strube F, Marx G, Ulrich WR, Rockel P. 2001. A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* **212**, 835–841.

Sudhamsu J, Lee GI, Klessig DF, Crane BR. 2008. The structure of YqeH. An AtNOS1/AtNOA1 ortholog that couples GTP hydrolysis to molecular recognition. *Journal of Biological Chemistry* **283**, 32968–32976.

Teepfer D. 1990. Genetic transformation using Agrobacterium rhizogenes. *Physiologia Plantarum* **79**, 140–146.

Van de Velde W, Guerra JC, De Keyser A, De Ruycke R, Rombaurs S, Maunoury N, Mergaert P, Kondorosi E, Holsters M, Goormachtig S. 2006. Aging in legume symbiosis. A molecular view on nodule senescence in *Medicago truncatula*. *Plant Physiology* **141**, 711–720.

Vieweg MF, Hohnjec N, Kuster H. 2005. Two genes encoding different truncated hemoglobins are regulated during root nodule and arbuscular mycorrhiza symbioses of *Medicago truncatula*. *Planta* **220**, 757–766.

Woodson JD, Chory J. 2008. Coordination of gene expression between organellar and nuclear genomes. *Nature Reviews Genetics* **9**, 383–395.

Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS. 2006. Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. *Plant Physiology* **142**, 429–440.

Yamasaki H, Cohen MF. 2006. NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants? *Trends in Plant Science* **11**, 522–524.

Yamasaki H, Sakihama Y. 2000. Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NRI-dependent formation of active nitrogen species. *FEBS Letters* **468**, 89–92.

Zeidler D, Zahringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J. 2004. Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proceedings of the National Academy of Sciences, USA* **101**, 15811–15816.

Zemojtel T, Fröhlich A, Palmieri MC, *et al*. 2006. Plant nitric oxide synthase: a never-ending story? *Trends in Plant Science* **11**, 524–525.