**A Medicago truncatula NADPH oxidase is involved in symbiotic nodule functioning**

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**Summary**

- The plant plasma membrane-localized NADPH oxidases, known as respiratory burst oxidase homologues (RBOHs), appear to play crucial roles in plant growth and development. They are involved in important processes, such as root hair growth, plant defence reactions and abscisic acid signalling.
- Using sequence similarity searches, we identified seven putative RBOH-encoding genes in the *Medicago truncatula* genome. A phylogenetic reconstruction showed that Rboh gene duplications occurred in legume species. We analysed the expression of these MtRboh genes in different *M. truncatula* tissues: one of them, MtRbohA, was significantly up-regulated in *Sinorhizobium meliloti*-induced symbiotic nodules.
- MtRbohA expression appeared to be restricted to the nitrogen-fixing zone of the functional nodule. Moreover, using *S. meliloti* bacA and nifH mutants unable to form efficient nodules, a strong link between nodule nitrogen fixation and MtRbohA up-regulation was shown. MtRbohA expression was largely enhanced under hypoxic conditions. Specific RNA interference for MtRbohA provoked a decrease in the nodule nitrogen fixation activity and the modulation of genes encoding the microsymbiont nitrogenase.
- These results suggest that hypoxia, prevailing in the nodule-fixing zone, may drive the stimulation of MtRbohA expression, which would, in turn, lead to the regulation of nodule functioning.

**Introduction**

It is now well established that plants generate reactive oxygen species (ROS) as signalling molecules to control various cellular mechanisms (Neill *et al.*, 2002). Indeed, accumulating experimental evidence shows that ROS are key players in fundamental processes, such as cellular growth (Foreman *et al.*, 2003), stomatal closure (Pei *et al.*, 2000) and plant defence against pathogens (Apel & Hirt, 2004). Moreover, ROS are known to orchestrate plant gene expression (Neill *et al.*, 2002; Vandrauwera *et al.*, 2005), as well as to modulate the activity of key signalling components, such as mitogen-activated protein (MAP) kinases (Rentel *et al.*, 2004).

The involvement of ROS in the legume–rhizobia symbiotic interaction has also been highlighted (Pauly *et al.*, 2006). Legumes are the only plant family with the ability to establish a symbiotic interaction with soil bacteria, commonly named rhizobia, leading to the formation of a new organ, the root nodule, whose primary function is dinitrogen (N₂) fixation. Nodule formation implicates extensive recognition by both partners in order to allow both an organized journey of the bacteria through the plant, and cell division and differentiation processes leading to the development of the nodule meristem. Finally, nodules will be colonized by bacteria released from infection threads formed on infection (Long, 2001; Oldroyd & Downie, 2008).

The production of ROS has been evidenced in both functional nodules and during the early steps of the interaction. Hydrogen peroxide (H₂O₂) has been detected in mature 6-wk-old nodules, mainly in the cell walls of infected cells and also in some infection threads around bacteria.
S. meliloti strain, acting as an H$_2$O$_2$ sink, which showed production was confirmed using a catalase-overexpressing observed in vivo (Peleg-Grossman et al., 2007). The importance of ROS production was confirmed using a catalase-overexpressing strain, acting as an H$_2$O$_2$ sink, which showed delayed nodulation (Jamet et al., 2007). Thus, it appears that ROS are essential for optimal symbiosis establishment, and that they are produced as a specific response to infection associated with the nodule developmental programme, rather than as an oxidative burst similar to that encountered in pathogenic systems (Pauly et al., 2006). Moreover, the use of diphenylene iodonium (DPI), which inhibits flavoproteins, such as the gp91$^{\text{phox}}$ catalytic subunit of NADPH oxidases (NOXs), abolished ROS production and also suppressed root hair curling and infection thread formation (Lohar et al., 2007; Peleg-Grossman et al., 2007). This strongly suggests the involvement of M. truncatula NOX(s) in such ROS production (Peleg-Grossman et al., 2007).

The plant plasma membrane-localized NOXs are homologous to the catalytic subunit (gp91$^{\text{phox}}$) of mammalian phagocyte NOXs (Sagi & Fluhr, 2001). Plant NOXs are known as respiratory burst oxidase homologues (RBOHs). RBOHs are transmembrane proteins composed of six transmembrane domains supporting two haem groups, FAD and NADPH hydrophilic domains in the C-terminal region and two calcium-binding domains (EF-hand) in the N-terminal region. NADPH acts as a cytosolic electron donor to the extracellular O$_2$ electron acceptor, which is reduced to O$_2^-$ via FAD and two independent haems (Sagi & Fluhr, 2001). Arabidopsis contains 10 RBOH homologues (Sagi & Fluhr, 2006). Microarray data compiled in Genevestigator showed their distribution into three classes: AtRbohD and AtRbohF, which are expressed in all plant parts, AtRbohA-G and AtRbohI, which are expressed in the roots, and AtRbohH and AtRbohJ, specifically expressed in pollen (https://www.genevestigator.com).

Plant RBOHs play crucial roles in plant health and metabolism. AtRbohD and AtRbohF are involved in ROS-dependent abscisic acid (ABA) signalling and guard cell ABA signal transduction (Kwak et al., 2003). The Arabidopsis thaliana rhd2 mutant lacking a functional AtRBOHC is root hair defective, thus underlining the role of these proteins in ROS-mediated plant cell growth (Foreman et al., 2003). RBOHs also appear to play important roles in pathogenic plant–microbe interactions. AtRbohD and AtRbohF are required for full ROS production observed during incompatible interactions with the bacterial pathogen Pseudomonas syringae pv tomato DC3000 (avrRpm1) and the phytopathogenic oomycete Hyaloperonospora parasitica (Torres et al., 2002). NiRbohD is involved in ROS production in cryptogein-elicited tobacco cells (Simon-Plas et al., 2002). Moreover, during the tobacco response to Phytophthora infestans oomycete, NiRbohA and NiRbohB have been shown to be required for ROS accumulation (Yoshio et al., 2003). Moreover, a role of NOXs in oxygen-sensing processes has been suggested (Jones et al., 2000; Bailey-Serres & Chang, 2005).

In this framework, there is a need to characterize the roles of RBOHs in the legume–rhizobia symbiotic interaction and, to our knowledge, this is the first analysis on Rboh genes in legume nodules. In this work, we describe the phylogenetic analysis and expression profiles of M. truncatula Rboh genes, and point out the importance of one RBOH for nodule functioning.

**Materials and Methods**

**Plant growth and bacterial strains**

Medicago truncatula Gaertn. cv Jemalong J6 was used throughout the experiments. Surface-sterilized seeds were placed on 0.4% agar plates in the dark for 1 d at 4°C and then for 3 d at 14°C. Germinated seeds were transferred into either nitrogen-free modified Fahraeus agar plates (root hair isolation) or 1 : 2 sand : vermiculite pots. One week after transfer, axenic plants were inoculated with 200 μl of S. meliloti 2011 suspension (OD$_{600}$ = 0.05) per root and nonaxenic plants with 10 ml per pot. Plants in pots were irrigated twice a week with a nitrogen-free nutrient solution (Rigaud & Puppo, 1975). The chamber conditions were 25°C : 22°C day : night, 75% hygrometry, 200 μmol m$^{-2}$ s$^{-1}$ light intensity and a 16 h : 8 h light : dark photoperiod.

For MtRbohA expression analysis in nonfixing nodules, S. meliloti 1021 nifH (Ruvkun et al., 1982) and 1021bacA (Glazebrook et al., 1993) mutants were used.

For hypoxia treatment, 4-wk-old inoculated or control plants were waterlogged with O$_2$-deprived nutrient solution for 24 h. Nodules and root tissues were harvested and MtRboh gene expression analysis was performed. The efficiency of hypoxia treatment was evaluated by analysing the up-regulation of pyruvate decarboxylase Medtr2g019000 gene expression (data not shown).

**Identification and phylogenetic analysis of Rboh sequences**

Rboh sequences were retrieved via a similarity search using BlastP (Altschul et al., 1997) with Arabidopsis Rboh sequences as queries against different plant protein sequence databases: http://www.medicago.org/ for M. truncatula, http://www.kazusa.or.jp/lotus/ for Lotus japonicus, rice.plantbiology.msu.edu/ for rice and http://www.phytozome.com/ for all the other already sequenced plant genomes (Supporting Information, Table S1). Multiple sequence alignment was performed using the Muscle program (Edgar, 2004) with...
standard parameters. The alignment was visually examined and edited for the elimination of sequences that were too short and the removal of alignment columns with too many gaps. Phylogenetic analyses were performed using a maximum likelihood (ML) approach with PhyML (Guindon & Gascuel, 2003) and a Bayesian approach with MrBayes (Ronquist & Huelsenbeck, 2003). ML phylogeny was performed with the LG model of evolution; a gamma distribution of variable substitution rates and a proportion of invariable sites were evaluated from the data by the software, and an approximate likelihood ratio test (aLRT) was launched to evaluate the robustness of the nodes. Bayesian phylogenetic reconstruction was performed with a mixed model of evolution and an evaluation of the gamma distribution and proportion of invariable sites. Congruence was reached with a total of 100 000 generations. Phylogenetic trees were visualized and annotated using FigTree (tree.bio.ed.ac.uk/software/figtree/).

Construction of a binary vector for hairy root transformation

For MtRboh promoter transcriptional fusions, fragments of c. 2000 bp upstream of the start codon were amplified by PCR using the primers indicated in Table S2. Each PCR fragment was first cloned into the pDONR207 donor vector and then into the plant expression vector pKGWFS7 (Karimi et al., 2002) using Gateway technology (Invitrogen, http://www.invitrogen.com).

For the RNA interference (RNAi) construct, the constitutive cauliflower mosaic virus (CaMV) 35S promoter in the MtNCR001 promoter (E. Boncompagni, unpublished). The fragment for MtRbohA inactivation was amplified with 5′-GTGGTACCACTGTGGAGCACATG and 5′-CAGCTCGAGCAAAAAGCCTTGG TCTTACAG-3’ and 5′-CAGCTCGAGCAAAAAGCCTTGG TCTTACAG-3’ as forward and reverse primers, respectively, and cloned by restriction (KpnI and XhoI) into pENTR4 (Invitrogen, http://www.invitrogen.com). This fragment, corresponding to the 3′-untranslated region (3′-UTR) of the MtRbohA gene, was then cloned into pK7GWTWG2D(II) containing the MtNCR001 promoter using Gateway technology (Invitrogen, http://www.invitrogen.com). The constructs were checked by DNA sequencing, introduced by electroporation into Agrobacterium rhizogenes strain ARqua1 and used for M. truncatula root transformation as described previously (Boisson-Dernier et al., 2001).

Histochemical localization of β-glucuronidase (GUS) activity

GUS activity was assayed histochemically from the nodulated roots of composite plants fixed at −20°C in 90% acetone for 60 min and incubated overnight in 0.5 mM K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆, 0.8 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, Eurogentec, http://www.eurogentec.com), 0.1 M potassium phosphate buffer, pH 7. Eighty-micrometre-thick vibroslices, obtained with a HM560V Vibratome (Microm, http://www.microm.de) after embedding plant material in 4.5% low-melting-point agarose, were visualized with a Zeiss Axiosplan 2 microscope (Carl Zeiss, http://www.zeiss.com) using dark-field optics.

Total RNA isolation, reverse transcription (RT) and gene expression analysis

Two hundred milligrams of plant material (roots, root hairs, nodules, flowers, pods, shoots, leaves) were ground in liquid nitrogen and total RNA was isolated using Trizol Reagent (Invitrogen, http://www.invitrogen.com) for plant and bacterial genes, respectively. Quantitative real-time RT-PCR was carried out using the qPCR Mastermix Plus for SYBR Green I reagent (Eurogentec, http://www.eurogentec.com). Reactions were run on the Chromo4 Real-Time PCR Detection System (Bio-Rad, http://www.bio-rad.com), and quantification was performed with Opticon Monitor analysis software v. 3.1 (Bio-Rad, http://www.bio-rad.com). Every reaction was set up in three technical replicates. The PCR programme 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 to 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 Mtx27 (TC132510) (Van de Velde et al., 2006). The Smc00324 housekeeping gene was used to normalize the bacterial mRNA levels (Becker et al., 2004). NifH/D primers were designed as described previously (Naya et al., 2007). The following formula was used for the relative expression ratio calculation:

\[ \frac{\Delta CT}{\Delta CT} = \frac{C_{\text{gene of interest}} - C_{\text{reference}}}{C_{\text{reference}} - C_{\text{reference}}} \]

The stability of the reference genes across samples was tested using geNorm software (Vandesompele et al., 2002). The absence of contamination with genomic DNA was tested by quantitative RT-PCR in all RNA samples, before RT. The gene-specific primers used are listed in Table S2.

Determination of acetylene reduction activity

Nitrogen fixation was determined using the acetylene reduction assay as described previously (Hardy et al., 2007).

\[ \frac{\text{Nitrogen fixing units}}{\text{Leaf area}} = \frac{\text{Acetylene reduction activity}}{\text{Leaf area}} \times \text{Nitrogen concentration} \]

This was calculated from the acetylene reduction rate (A), the leaf area (L), and the nitrogen concentration (N):

\[ \text{Nitrogen fixing units} = \frac{A}{L} \times N \]

The acetylene reduction rate (A) was calculated from the concentration of the accumulated ethylene (E) and the specific activity (S):

\[ \frac{\text{Acetylene reduction rate}}{\text{Leaf area}} = \frac{\text{Ethylene production}}{\text{Leaf area}} \times \text{Specific activity} \]

This was calculated from the ethylene concentration (E) and the specific activity (S):

\[ \text{Acetylene reduction rate} = \frac{E}{L} \times S \]

The specific activity (S) was calculated from the acetylene reduction rate (A) and the leaf area (L):

\[ \text{Specific activity} = \frac{A}{L} \]

The leaf area (L) was calculated from the number of leaves (N) and the leaf area per leaf (Lp):

\[ \text{Leaf area} = \frac{N \times Lp}{L} \]

The nitrogen concentration (N) was calculated from the nitrogen fixation (Nf) and the leaf area (L):

\[ \text{Nitrogen concentration} = \frac{Nf}{L} \]
1968). Nodulated roots from each composite plant (control or RNAi) were placed in 30 ml glass flasks filled with an acetylene–air mixture (C2H2 : air = 1 : 10 v/v). After 1 h of incubation at 25°C, the amount of ethylene in the gas phase was determined by gas chromatography using a 6890N Network GC system (Agilent Technologies, http://www.agilent.com).

### Results

Identification, annotation and phylogenetic analysis of *M. truncatula* Rboh genes

Ten Rboh genes are present in the *A. thaliana* genome (Sagi & Fluhr, 2006). Using protein sequence similarity search tools with Arabidopsis sequences as queries, we found seven RBOH-encoding genes in the *M. truncatula* genome (http://www.medicago.org). According to their localization in the *M. truncatula* genome and the widely used nomenclature (Torres & Dangl, 2005), we named these genes *MtRbohA–G*. For five of them (*MtRbohA, B, E–G*), expressed sequence tags (ESTs) are available in the ‘TIGR *M. truncatula* Gene Index’, and, for four isoforms (*MtRbohA, B, E and G*), tentative consensus sequences have been proposed from EST contigs (Table 1).

Six of the Rboh genes (*MtRbohA–C, E–G*) have open reading frames (ORFs) of between 2550 and 2790 bp. Analysis of the domain composition of the corresponding encoded proteins (PFAM; Bateman et al., 2004) showed the presence of five typical domains of plant NOXs [respiratory burst NOX domain (PF08414); EF hand (PF00036); ferric reductase-like transmembrane component (PF01794); FAD-binding domain (PF08022); and a ferric reductase NAD-binding domain (PF0830)]. By contrast, the *MtRbohD* sequence has a shorter ORF of 2109 bp, corresponding to a truncated protein lacking the C-terminal ferric reductase NAD-binding domain. However, c. 5 kb downstream of the *MtRbohD* stop codon, there is a predicted sequence (TC130541) which corresponds to the lacking ferric reductase NAD-binding domain. Within this 5 kb region, we found a sequence encoding a ‘putative nonlong terminal repeat retroelement reverse transcriptase’, suggesting a retrotransposon insertion within the theoretical *MtRbohD* ancestral sequence. The available data do not provide any evidence to allow the confirmation that *MtRbohD* is able to encode the five-domain full-length protein.

The seven MtRBOH protein sequences exhibit 47–69% similarity (Table S3) and were used to build phylogenetic trees (Figs 1, S1). There are nine complete Rboh genes in the rice (*Oryza sativa*) genome (Wong et al., 2007), five in the not yet fully sequenced genome of *L. japonicus* and 18 in soybean (*Glycine max*). Phylogenetic reconstructions of these plant RBOH proteins, together with those of *A. thaliana* and *M. truncatula*, converged in producing a highly supported topology with both Bayesian and ML approaches, as illustrated by posterior probability and aLRT values. A total of five groups of orthologues can be defined from the phylogenetic tree (Fig. 1). Groups 1, 2 and 5 contain representatives from all the selected species. Interestingly, no *M. truncatula* orthologues are found in groups 3 and 4, which are closely related on the phylogenetic tree. This may reflect incompleteness or gaps in the current version of the *M. truncatula* genome. Group 3 contains representatives of all the other species and group 4 contains all others apart from *L. japonicus* (Fig. 1).

### MtRboh expression exhibits specific localization profiles

To characterize the expression profile of *M. truncatula* Rboh genes, we analysed their transcript abundance by

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**Table 1** *Medicago truncatula* respiratory burst oxidase homologue (Rboh) genes

| Gene   | Name IMGAG<sup>a</sup> | Genbank<sup>b</sup> accession number | ORF length (bp) | Number of ESTs | TC number (TIGR)<sup>c</sup> | Affymetrix Probeset<sup>d</sup> |
|--------|------------------------|----------------------------------------|-----------------|----------------|-------------------------------|----------------------------------|
| *MtRbohA* | Medtr1g099800          | –                                      | 2658            | 7              | TC112710                      | Mtr.39812.1.S1_s_at               |
| *MtRbohB* | Medtr3g151540          | –                                      | 2772            | 6              | TC123192, TC123112             | Mtr.2439.1.S1_at, Mtr.45354.1.S1_at |
| *MtRbohC* | Medtr3g151570          | –                                      | 2754            | –              | –                             | –                                |
| *MtRbohD* | Medtr3g151600          | –                                      | 2109            | –              | –                             | –                                |
| *MtRbohE* | Medtr4g144710          | AY821801                               | 2799            | 33             | TC126164                      | Mtr.17607.1.S1_at                 |
| *MtRbohF* | Medtr7g067680          | –                                      | 2550            | 3              | –                             | –                                |
| *MtRbohG* | Medtr7g138940          | AY821802                               | 2688            | 47             | TC112621                      | Mtr.32307.1.S1_at                 |

<sup>a</sup> The International Medicago Genome Annotation Group (IMGAG) (http://www.medicago.org).
<sup>b</sup> Genbank (http://www.ncbi.nlm.nih.gov).
<sup>c</sup> Designation of tentative consensus (TC) from TIGR (http://www.tigr.org). ORF, open reading frame; EST, expressed sequence tag.
<sup>d</sup> Affymetrix Probeset (http://bioinfo.noble.org/gene-atlas/v2/).
quantitative real-time PCR in different plant tissues (leaves, roots, root hairs, nodules, stems, flowers and pods). *MtRbohB* is notably expressed in all the analysed tissues, *MtRbohE* and *G* exhibit low expression levels in all tested tissues and *MtRbohF* is significantly up-regulated in roots and root hairs (Fig. 2). *MtRbohC* and *MtRbohD*, which are highly similar at the amino acid level (c. 69%, Table S3) and are grouped in the same phylogenetic cluster (Fig. 1), presented a very low expression level in all tissues examined, which is in agreement with the lack of ESTs in the databases for both genes. The most striking result lies in the remarkable up-regulation of *MtRbohA* expression in nodules. Indeed, *MtRbohA* showed a six-fold higher expression level in nodules than in roots, and four-fold higher than *MtRbohB*, the second most expressed *MtRboh* in nodules (Fig. 2). Our expression profiles are mostly consistent with the already available transcriptome analysis (Benedito et al., 2008) (Fig. S2). The only difference concerned *MtRbohE*, which is detected at the same level in roots and leaves in our conditions, although it was weakly expressed in leaves (Fig. S2).
Gene expression analysis in different *Medicago truncatula* tissues. RNA from root hairs (10-d-old seedlings), leaves, roots, nodules, stems (5-wk-old plants), flowers and pods (7-wk-old plants) were used for quantitative real-time RT-PCR analysis. Values were normalized against 40S Ribosomal Protein S8 and *MtC27* gene expression, which were used as housekeeping genes. Samples were obtained by pooling tissues of 10 plants, and the values are representative of three independent biological replicates. Error bars, ±SE.

To localize more specifically root tissue expression, histochemical staining was performed using a promoter GUS transcriptional fusion approach. An approx. 2 kb promoter region was chosen (Table S2). As a result of their very low expression levels, *MtRbohC* and *D* were not included in this study. Main root tip (division and elongation zones) and central cylinder staining was observed in the root systems for *MtRbohB, E–G* (Fig. 3d,g,j,m). Secondary root meristems were also stained for these genes (Fig. 3e,h,k,n). *MtRbohB, E* and *F* root GUS staining was very strong compared with that of *MtRbohG*. All of these *MtRboh* genes (*B, E, F, G*) showed the same localization. These staining conditions did not reveal *MtRbohA* expression in roots (Fig. 3a–c), although it was detected in the quantitative real-time PCR experiments (Fig. 2). However, when the staining time was increased to 16 h, slight *MtRbohA* expression was observed in vascular tissues (data not shown). An examination of semi-thin transverse sections revealed that the promoter fusion of *MtRbohB, E* and *F* directed strong GUS expression, which was restricted to the phloem and the surrounding parenchyma (Fig. 3f,i,l,o). Considering that several authors have found endogenous GUS-like activity in different plant tissues, including *M. truncatula* cv Jemalong (Journet *et al.*, 2001), we assayed either transformed control plant (empty vector) or nontransformed plants, and no background staining was detected for the considered incubation time (data not shown). *MtRbohF* shows a very high expression level in roots, more than 200-fold higher than in any other tissue. Moreover, its expression in root hairs was four-fold higher than in the rest of the roots (Fig. 2). We also detected *MtRbohF* expression in root hairs (Fig. 3j, inset).

We analysed *Rboh* gene expression during *M. truncatula* interaction with its microsymbiont. To investigate the cellular localization of *MtRboh* promoter activity during this process, composite transgenic plants were inoculated with *S. meliloti* and longitudinal sections of the nodules were assayed. In 12-d-old root nodules, *MtRbohE–G* GUS staining was detected in vascular bundles, which are a continuation of the root central cylinder already shown to be coloured (Fig. 4e,g,i). GUS coloration was also apparent in the apical region corresponding to the permanent meristem, which is characteristic for indeterminate nodules; no expression was observed in any other zone of the nodule. *MtRbohB* promoter activity, in good agreement with the quantitative real-time PCR results, showed a ubiquitous expression in the nodule (Fig. 4c).

Interestingly, *MtRbohA* expression appears to be restricted to the central tissue of the root nodule (Fig. 4a,b). The limitation of its expression to the infection zone was confirmed by the use of an *S. meliloti* 2011 strain expressing a constitutive *hemA::lacZ* construct (Leong *et al.*, 1985), which allowed the colocalization of *MtRbohA* GUS expression with lacZ staining (Fig. S3). In older nodules (5–7 wk post-inoculation), in which indeterminate nodule zonation is evident, none of the analysed *MtRboh* promoters generated GUS staining in the senescence zone (Fig. 4b,d,f,h,j). In addition, the GUS staining of 12-d-old nodules confirmed the restricted *MtRbohA* expression to the nodule nitrogen-fixing zone where cells are infected and the nitrogen fixation process takes place (Fig. 4a). Taken together, the results obtained point to a possible role of *MtRbohA* in nodule functioning. Thus, we focused our work on studying further its involvement in nodule performance.

*MtRbohA* is linked to nodule nitrogen fixation activity

The expression of *MtRbohA* in nodules 7 d post-inoculation was found to be at the same level as in the roots. However, from 2 to 14 wk post-inoculation, *MtRbohA* expression was c. 10-fold higher in nodules compared with roots (Fig. 5a). The 7-d-old nodules are small and white, the cells are starting to be infected, but are still ineffective, unable to fix N₂. Later, the nodules become pink, because of the presence of leghaemoglobin, an essential cytosolic oxygen transporter to the microsymbionts, and thus the nodules gain the capacity...
to fix N\textsubscript{2} (Gage, 2004). Therefore, these results show a strong link between the nodule functionality, in terms of N\textsubscript{2} fixation, and the expression of MtRbohA.

To further confirm this relationship, we inoculated M. truncatula roots with S. meliloti mutants unable to form functional nodules. S. meliloti nifH mutants are known to form fix\textsuperscript{−} nodules and are described as being early senescent (Hirsch et al., 1983). The nodules formed by nifH mutants are similar in structure to the wild-type, except that nifH bacteroids accumulate a compact, electron-dense body (Hirsch et al., 1983). In contrast with S. meliloti nifH mutants, S. meliloti bacA mutants form nodules with a disrupted structure compared with the wild-type; these nodules lack the nitrogen fixation zone, as, during the infection process,
bacteria are released from the infection thread, but then undergo senescence without infecting plant cells (Glazebrook et al., 1993).

The 3-wk-old nodules formed with either S. meliloti nifH or bacA mutants showed an MtRbohA expression level not significantly different from that of the roots, in contrast with nodules formed with S. meliloti wild-type bacteria which showed the already described enhanced expression (Fig. 5b). These results indicate that a nonfunctional nodule, caused either by the absence of infected cells or the inability of bacteria to fix N₂ (as their nitrogenase complex is nonfunctional), do not show an increase in MtRbohA expression. By contrast, MtRbohB expression was not modulated in these fix⁻ phenotypes (Fig. 5b). These data further support the link between nodule functionality and MtRbohA expression, and point to a potential role for MtRbohA in nodule performance.

As the nodule fixation zone is characterized by a low oxygen tension, and as it has been proposed that NOXs may act as oxygen sensors under hypoxic conditions (Jones et al., 2000; Bailey-Serres & Chang, 2005), the effect of hypoxia on MtRbohA expression was tested. The results (Fig. 5c) clearly show that MtRbohA expression is enhanced significantly under hypoxic conditions in both roots and nodules. It must be highlighted here that MtRbohB expression was not modulated by hypoxia, indicating that hypoxia does not result in a generalized effect on the expression of all MtRboh genes (Fig. 5c).

Thus, we used an RNAi approach to study the effect of a reduction in MtRbohA transcript levels on the ability of the
nodule to fix N₂. For this purpose, we used the MtNCR001 promoter (Mergaert et al., 2003), which expresses constitutively in the nitrogen-fixing zone, in order to drive the expression of an RNAi construct targeting the 3'-UTR of MtRbohA. This targeted approach to nodule functionality avoids any other collateral effect that could affect root or nodule development. An empty vector was used as a control.

The RNAi construct led to a reduction of > 60% in the MtRbohA mRNA level in nodules (compared with control transgenic nodules), whereas no effect was detected on other MtRboh gene expression (Fig. 6a). The decrease in mRNA level provoked a 25% reduction in nodule nitrogen fixation activity (Fig. 6b), which was not related to nodule fresh weight as that did not vary (12.3 ± 1.2 and 12.2 ± 0.9 mg of nodule fresh weight per plant for controls and RNAi lines, respectively). In addition, no difference was observed either macroscopically or by light microscopy in nodule structure that could explain the depletion of nitrogen fixation in RNAi lines (Fig. S4). Thus, this phenotype may be caused by an effect on nodule metabolism rather than to a disruption of nodule structure. It should be noted that similar results were obtained with the constitutive 35S promoter (Fig. S5); no other phenotype was observed in these plants.

The expression levels of plant and bacterial genes known to be involved in nodule functioning were tested in nodules formed on roots transformed with the MtRbohA RNAi construct or with the control vector. Plant genes encoding sucrose synthase (MtSucS1; Medtr8g133160.1), glutamine

Fig. 5 MtRbohA expression during nodule development. (a) Time course of MtRbohA expression in nodules from 1 to 14 wk post-inoculation (root, light grey bars; nodule, dark grey bars). (b) MtRbohA (closed bars) and MtRbohB (chequered bars) expression in roots (light grey bars) and nodules (dark grey bars) from 3-wk-old plants inoculated with Sinorhizobium meliloti nifH and bacA mutants. (c) MtRbohA and MtRbohB relative expression levels in roots and nodules from control (open bars) or hypoxic conditions (closed bars). The value for the control condition is set to unity as reference. Values were normalized as in Fig. 2. Samples were obtained from the pooling of nodules of 20 plants and are a mean of three independent biological replicates. Asterisks (*) represent significant differences for nodules compared with roots for P < 0.05 (a, b) and significant differences between control and hypoxia-treated plants for P < 0.05 (c). Error bars, ± SE.

Fig. 6 MtRbohA RNA interference (RNAi) phenotype. Relative MtRboh gene expression (a), nitrogen fixation activity (b) and relative Sinorhizobium meliloti nitrogenase gene expression (c) were obtained from the pooling of controls (empty vector) or MtRbohA RNAi composite plants (n > 40). Control, light grey bars; RNAi, black bars. Values are representative of three independent biological replicates. Gene expression values were normalized against 40S Ribosomal Protein S8, Mtc27 (Medicago truncatula) and smc00324 (S. meliloti) genes. ARA, acetylene reduction assay; NFW, nodule fresh weight. Asterisk (*) represents significant differences compared with control plants for P < 0.05. Error bars, ± SE.
involved in osmotic protection (Boscari et al.) expression of other genes – for example BetS NifD did not appear to show modulated expression (data not shown). By contrast, the expression of the microsymbiont NifD and NifH genes was decreased significantly in nodules formed on MtRbohA transgenic roots (Fig. 6c), whereas the expression of other genes – for example BetS, which is involved in osmotic protection (Boscari et al., 2006) – was not affected (Fig. 6c).

Discussion

Rboh genes in the M. truncatula genome

The aim of our work was to evaluate the involvement of M. truncatula RBOH proteins during its symbiotic interaction with S. meliloti. Using a sequence similarity search, we were able to identify seven genes encoding MtRBOH proteins in the incomplete M. truncatula genome with identity levels ranging from 47% to 69%. Based on data from already sequenced higher plant genomes and ESTs, other Rboh genes should be present in the M. truncatula genome. Indeed, the presence of four ESTs (EST642356, EST396294, EST317358, TC116307) matching Rboh sequences distinct from MtRbohA–G clearly suggests a larger number of Rboh genes in the M. truncatula genome. Interestingly, legume-specific duplications of Rboh genes, encompassing those of M. truncatula, can be deduced from the phylogeny in groups 1 and 2. In group 2, the duplications preceded the separation of the different legume species and continued independently in M. truncatula and G. max, but apparently not in L. japonicus. This tendency is particularly exemplified by the MtRbohB–D genes, which are all co-orthologous to Glyma04g38040 and Glyma06g17030 genes. These three M. truncatulagenes are localized in chromosome 3 within 40 kbp, suggesting tandem duplications. In group 1, duplications also appear to have preceded the speciation of the different legume species, but apparently pursued only in L. japonicus and G. max (Schmutz et al., 2010). Overall, these legume-specific duplications may have allowed functional divergence or the emergence of new function. Interestingly, all Arabidopsis genes have orthologues in legumes, except AtRbohD, which was either lost in legumes or has not yet been identified in these plants.

MtRboh genes during root and nodule development

The connection between ROS formation, root development and physiological processes has already been highlighted (Joo et al., 2001; Liszkay et al., 2004; Su et al., 2006; Li et al., 2009). Nevertheless, little information is available on ROS generation related to root growth. The very high MtRboh expression level in the meristematic and elongation zones of the root, shown in Fig. 3, suggests the involvement of NOXs during root growth, where they may also play a role in cell wall expansion (Monsma et al., 2007; Macpherson et al., 2008). Furthermore, the high MtRbohF expression in developing root hairs is in line with previous results showing that ROS accumulate in growing root hairs (Foreman et al., 2003), and that blocking of the activity of NOXs with DPI inhibits ROS formation and affects root hair growth (Foreman et al., 2003; Cardenas et al., 2008). Thus, one can suggest that MtRbohF could play a role in M. truncatula root hair development. This would be in agreement with the concept of ROS production by plasma membrane RBOHs being a general mechanism in the control of the polarized growth of plant cells (Liu et al., 2009).

The expression of MtRboh genes in the meristematic zone and vascular tissues of the root nodule, on symbiotic interaction with S. meliloti (Fig. 4), is in agreement with the detection of O2− and H2O2 in the nodule cortex and meristematic cells (Groten et al., 2005; Rubio et al., 2009). This points to a role for NOXs in nodule development. It must be underlined here that ROS produced by a fungal NOX (NoxA) regulate hyphal growth in the mutualistic interaction between a fungal endophyte and its grass host (Tanaka et al., 2006). Moreover, a regulator of NoxA is essential in planta for the symbiotic interaction (Takemoto et al., 2006). Taken together, these data suggest that RBOHs are required for the optimal establishment of fungal (Scott & Eaton, 2008) and rhizobial symbioses.

Interestingly, none of the analysed MtRboh promoters yielded GUS staining in the senescence zone (Fig. 4). Nodule senescence is an active process programmed in development, in which ROS, antioxidants, hormones and proteinases have a key role (Puppo et al., 2005). On the other hand, several reports have described ROS and RBOH involvement during programmed cell death during plant–pathogen interactions (Torres et al., 2002; Torres & Dangl, 2005). Our results suggest that these MtRboh genes do not appear to be involved in nodule senescence. Therefore, these results are in agreement with the hypothesis proposed by Puppo et al. (2005) involving ROS in nodule senescence related to a progressive decline in antioxidant content (ascorbate and glutathione), rather than to an increase in ROS production itself.

MtRbohA and nodule functioning

Our results indicate that MtRbohA is involved in nodule functioning. Indeed, the MtRbohA expression level appeared to be concomitant with the establishment of a functioning nodule, as from 2 wk post-inoculation with S. meliloti, MtRbohA expression was c. 10-fold higher in nodules than in roots, whereas, in very young nodules, its expression was at the same level as in the roots (Fig. 5a).
Moreover, this was not observed when the inoculation was performed with rhizobial mutants unable to form functional nodules (Fig. 5b). In the same way, the decrease in MtRbohA expression via the RNAi approach led to a reduction in the nitrogen fixation capacity (Fig. 6b), again showing the link between MrRBOHA and nodule functionality. The down-regulation of the microsymbiont nifD and nifH genes may contribute to an explanation of the decrease in nitrogen fixation activity. Indeed, these genes encode the Mo–Fe and Fe proteins of the nitrogenase complex, respectively, which is responsible for dinitrogen reduction into ammonia. This may indicate that MrRBOHA activity contributes to the communication between the plant and the endosymbiont. Again, this is reminiscent of the important role played by NOXs in the establishment of some beneficial plant–microbe interactions (Takemoto et al., 2006; Tanaka et al., 2006).

However, the absence of any effect of the 35S construct on either the kinetics or intensity of the nodulation process indicates that MtRbohA does not play a role in the early steps of the symbiotic interaction, thus excluding this isoflavanone from being a candidate for ROS production at this stage (Santos et al., 2001; Ramu et al., 2002).

Moreover, MtRbohA expression appears to be largely increased under hypoxic conditions (Fig. 5c). Similarly, the nitrogen-fixing zone has a microaerobic environment, allowing the functioning of the microsymbiont nitrogens. Although the possible role of NOXs in the oxygen-sensing processes has been suggested (Jones et al., 2000; Bailey-Serres & Chang, 2005), they do not appear to have redox centres that are oxidized/reduced in response to oxygen. Thus, the cascade of events could be as follows: the hypoxia-driven stimulation of MtRbohA expression would, in turn, lead to the regulation of the expression of genes and/or to post-translational modifications involved in nodule functioning.

In conclusion, the results presented in this report shed new light on the role(s) of RBOHs in plant–microbe interactions. Until now, their roles have been essentially, if not exclusively, linked to plant defence reactions against invading microbes in incompatible reactions. We have shown, in particular, that at least one RBOH may be necessary for optimal functioning of the M. truncatula–S. meliloti nodule. Future work will aim at studying the involvement of other(s) MtRboh gene(s) in the symbiotic process. Moreover, the identification of MtRbohA molecular targets in both partners will help to elucidate its role in plant–microsymbiont communication.

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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.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* 55: 537–593.

Baier MC, Barsch A, Kuster H, Hofnjec N. 2007. Antisense repression of the *Medicago truncatula* nodule-enhanced sucrose synthase leads to a handicapped nitrogen fixation mirrored by specific alterations in the symbiotic transcriptome and metabolome. *Plant Physiology* 145: 1600–1618.

Bailey-Serres J, Chang R. 2005. Sensing and signalling in response to oxygen deprivation in plants and other organisms. *Annals of Botany* 96: 507–518.

Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A, Marshall M, Moxon S, Sonnhammer EL et al. 2004. The Pfam protein families database. *Nucleic Acids Research* 32 D138–D141.

Becker A, Berges H, Kroel E, Bruand C, Ruberg S, Capela D, Lauber E, Meilhoc E, Ampe F, de Bruijn FJ et al. 2004. Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. *Molecular Plant–Microbe Interactions* 17: 292–303.

Benedito VA, Torres-Jerez I, Murray JD, Andrianjakia A, Allen S, Kakar K, Wandrey M, Verdier J, Zuber H, Ott T et al. 2008. A gene expression atlas of the model legume *Medicago truncatula*. *Plant Journal* 55: 504–513.

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.

Boscari A, Van de Sype G, Le Rudulier D, Mandon K. 2006. Overexpression of BetC, a *Sinorhizobium meliloti* high-affinity betaine transporter, in bacteroids from *Medicago sativa* nodules sustains nitrogen fixation during early salt stress adaptation. *Molecular Plant–Microbe Interactions* 19: 896–903.

Cardenas L, Martinez A, Sanchez F, Quinto C. 2008. Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFS). *Plant Journal* 56: 802–813.

Carvalho HG, Lopes-Cardoso IA, Lima LM, Melo PM, Cullimore JV. 2006. Nodule-specific modulation of glutamine synthetase in transgenic *Medicago truncatula* leads to inverse alterations in asparagine synthetase expression. *Plant Physiology* 133: 243–252.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD et al. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422: 442–446.

Gage DJ. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews* 68: 280–300.
Glazebrook J, Ichige A, Walker GC. 1993. A Rhizobium meliloti homolog of the Escherichia coli peptide-antibiotic transport protein Bram is essential for bacteroid development. Genes and Development 7: 1485–1497.

Grotten K, Vanacker H, Dutilleul C, Bastian F, Bernard S, Carzaniga R, Foyer CH. 2005. The roles of redox processes in pea nodule development and senescence. Plant, Cell & Environment 28: 1293–1304.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.

Hardy RW, Holsten RD, Jackson EK, Burns RC. 1968. The acetylene–ethylene assay for N2 fixation: laboratory and field evaluation. Plant Physiology 43: 1185–1207.

Hirsch AM, Bang M, Ausubel FM. 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by nif::Tn5 mutants of Rhizobium meliloti. Journal of Bacteriology 155: 367–380.

Jameet A, Mandon K, Puppo A, Herouart D. 2007. H2O2 is required for optimal establishment of the Medicago sativa/Sinorhizobium meliloti symbiosis. Journal of Bacteriology 189: 8741–8745.

Jones RD, Hancock JT, Morandi D, Barker DG, Gianinazzi-Pearson V. 2001. Medicago truncatula ENOD11: a novel RPP-encoded early nodulin gene expressed during mycorrhization in arbuscule-containing cells. Molecular Plant–Microbe Interactions 14: 737–748.

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

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JD, Schroeder JI. 2003. NADPH oxidase ArbohD and ArbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO Journal 22: 2623–2633.

Leong SA, Williams PH, Ditta GS. 1985. Analysis of the 5′ regulatory region of the gene for δ-aminolevulinic acid synthetase of Rhizobium meliloti. Nucleic Acids Research 13: 5965–5976.

Li SW, Xue LG, Xu SJ, Feng HY, An LZ. 2009. Hydrogen peroxide acts as a signal molecule in the adventitious root formation of mung bean seedlings. Environmental and Experimental Botany 65: 63–71.

Liszkay A, van der Zalm E, Schopfer P. 2004. Production of reactive oxygen intermediates O2−, H2O2, and OH by maize roots and their role in wall loosening and elongation growth. Plant Physiology 136: 3114–3123.

Liu P, Li RL, Zhang L, Wang QL, Niehaus K, Baluska F, Samaj J, Lin JX. 2009. Lipid microdomain polarization is required for NADPH oxidase-dependent ROS signaling in Pisum sativum pollen tube tip growth. Plant Journal 60: 303–313.

Lohar DP, Haridas S, Gantt JS, VandenBosch KA. 2007. A transient decrease in reactive oxygen species in root gravitropism. Plant Physiology 143: 522–528.

Monselvas GB, Bibikova TN, Messerli MA, Shi C, Gilroy S. 2007. Oscillations in extracellular pH and reactive oxygen species modulate tip growth of Arabidopsis root hairs. Proceedings of the National Academy of Sciences, USA 104: 20996–21001.

Naya L, Ladra R, Ramos J, Gonzalez EM, Arrese-Igor C, Minchin FR, Becana M. 2007. The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to the subsequent recovery of plants. Plant Physiology 144: 1104–1114.

Nell S, Desikan R, Hancock J. 2002. Hydrogen peroxide signalling. Current Opinion in Plant Biology 5: 388–395.

Nomura M, Mai HT, Fujii M, Hata S, Iizu K, Tajima S. 2006. Phosphonopruvate carboxylase plays a crucial role in limiting nitrogen fixation in Lotus japonicus nodules. Plant and Cell Physiology 47: 613–621.

Oldroyd GE, Downie JA. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. Annual Review of Plant Biology 59: 519–546.

Pauly N, Pucciariello C, Mandon K, Innocenti G, Jamet A, Baudouin E, Herouart D, Frenod 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.

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature 406: 731–734.

Pelec-Grossman S, Volpin H, Levine A. 2007. Root hair curling and Rhizobium infection in Medicago truncatula are mediated by phosphatidylinositolisolate-regulated endocytosis and reactive oxygen species. Journal of Experimental Botany 58: 1637–1649.

Puppo A, Grotten K, Bastian F, Carzaniga R, Soussi M, Lucas MM, de Felipe MR, Harrison J, Vanacker H, Foyer CH. 2005. Legume nodule senescence: roles for redox and hormone signalling in the orchestration of the natural aging process. New Phytologist 165: 683–701.

Ramu SK, Peng H-M, Cook DR. 2002. Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene rip1 in Medicago truncatula. Molecular Plant–Microbe Interactions 15: 522–528.

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grieron CS, Hirst H et al. 2004. OX11 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. Nature 427: 858–861.

Rigaud J, Puppo A. 1975. Indole-3-acetic acid catabolism by soybean bacteria. Journal of General Microbiology 88: 223–228.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rubio MC, Becana M, Kanematsu S, Ushimaru T, James EK et al. 2004. Ox11 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. New Phytologist 165: 395–407.

Rubio MC, James EK, Clemente MR, Buccinelli B, Fedorova M, Vance CP, Becana M. 2004. Localization of superoxide dismutases and hydrogen peroxide in legume root nodules. Molecular Plant–Microbe Interactions 17: 1294–1305.

Ruvkun GB, Sundaresan V, Ausubel FM. 1982. Directed transposon Tn5 mutagenesis and complementation analysis of Rhizobium meliloti symbiotic nitrogen fixation genes. Cell 29: 551–559.

Sagi M, Fluur R. 2001. Superoxide production by plant homologues of the gox1− phenotype NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiology 126: 1281–1290.

Sagi M, Fluur R. 2006. Production of reactive oxygen species by plant NADPH oxidases. Plant Physiology 141: 336–340.

Santos R, Herouart D, Siguat S, Touati D, Puppo A. 2001. Oxidative burst in alfalfa–Sinorhizobium meliloti symbiotic interaction. Molecular Plant–Microbe Interactions 14: 86–89.
Sauviac L, Niebel A, Boisson-Dernier A, Barker DG, de Carvalho-Niebel F. 2005. Transcript enrichment of Nod factor-elicited early nodulin genes in purified root hair fractions of the model legume Medicago truncatula. Journal of Experimental Botany 56: 2507–2513.

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J et al. 2010. Genome sequence of the palaeopolyploid soybean. Nature 463: 178–183.

Scott B, Eaton CJ. 2008. Role of reactive oxygen species in fungal cellular differentiations. Current Opinion in Microbiology 11: 488–493.

Simon-Plas F, Elmayan T, Blein JP. 2002. The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells. Plant Journal 31: 137–147.

Su GX, Zhang WH, Liu YL. 2006. Involvement of hydrogen peroxide generated by polyamine oxidative degradation in the development of lateral roots in soybean. Journal of Integrative Plant Biology 48: 426–432.

Takemoto D, Tanaka A, Scott B. 2006. A p67Phox-like regulator is recruited to control hyphal branching in a fungal–grass mutualistic interaction. Plant Cell 18: 2807–2821.

Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B. 2006. Reactive oxygen species play a role in regulating a fungus–perennial ryegrass mutualistic interaction. Plant Cell 18: 1052–1066.

Torres MA, Dangl JL. 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Current Opinion in Plant Biology 8: 397–403.

Torres MA, Dangl JL, Jones JD. 2002. Arabidopsis gp91phox homologues AtRbohD and AtRbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proceedings of the National Academy of Sciences, USA 99: 517–522.

Van de Velde W, Guerra JC, De Keyser A, De Rycke R, Rombauds S, Mauourney 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.

Vanderauwera S, Zimmermann P, Rombauds S, Vandenabeele S, Langebartels C, Gruissem W, Inze D, Van Breusegem F. 2005. Genome-wide analysis of hydrogen peroxide-regulated gene expression in Arabidopsis reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiology 139: 806–821.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3: http://genomewebiology.com/content/3/5/RESEARCH0034.

Wong HL, Pinontoon R, Hayashi K, Tabata R, Yaeo T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T et al. 2007. Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell 19: 4022–4034.

Xu W, Sato SJ, Clemente TE, Chollet R. 2007. The PEP-carboxylase kinase gene family in Glycine max (GmPpK1–4): an in-depth molecular analysis with nodulated, non-transgenic and transgenic plants. Plant Journal 49: 910–923.

Yoshio H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JDG, Doke N. 2003. Nicotiana benthamiana gp91phox homologs NbrbohA and NbrbohB participate in H2O2 accumulation and resistance to Phytophthora infestans. Plant Cell 15: 706–718.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Bayesian phylogenetic tree of respiratory burst oxidase homologue (RBOH) amino acid sequences in Viridiplantae.

Fig. S2 MtRboh gene expression analysis in different plant tissues.

Fig. S3 Simultaneous MtRbohA expression and Sinorhizobium meliloti localization in nodules.

Fig. S4 Morphological analysis of control and MtRbohA RNAi nodules.

Fig. S5 Nitrogen fixation activity in 3SS::MtRbohA RNAi nodules.

Table S1 Respiratory burst oxidase homologue (RBOH) protein sequences used for the phylogenetic analysis

Table S2 Primers used for quantitative real-time PCR analysis and for MtRboh promoter cloning

Table S3 MtRBOH protein sequence similarities

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