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; Vanderwaera 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 (N_2) 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 (H_2O_2) 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 (Santos et al., 2001; Rubio et al., 2004). In the early stages, superoxide anion (O_2^-) is detected in infection threads (Santos et al., 2001). More recently, the generation of ROS...
in the cortical cells of *Medicago truncatula* roots after inoculation with *Sinorhizobium meliloti* has been observed in vivo, using the 2′,7′-dichlorofluorescein probe (Peleg-Grossman et al., 2007). The importance of ROS production was confirmed using a catalase-overexpressing S. meliloti strain, acting as an \( \text{H}_2\text{O}_2 \) 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 gp91phox 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 (gp91phox) 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 \( \text{O}_2 \) electron acceptor, which is reduced to \( \text{O}_2^- \) 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, *AtRbohAG* and *AtRbohAI*, 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 (Kwik 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 (atRpm1) and the physopathogenic oomycete *Hyaloperonospora parasitica* (Torres et al., 2002); *NtRbohD* is involved in ROS production in cryptogen-elicited tobacco cells (Simon-Plas et al., 2002). Moreover, during the tobacco response to *Phytophthora infestans* oomycete, *NtRbohA* and *NtRbohB* have been shown to be required for ROS accumulation (Yoshioka 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* 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 \( \mu \)l of *S. meliloti* 2011 suspension (\( \text{OD}_{600} = 0.05 \)) 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 \( \mu \)E 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 1021 *bacA* (Glazebrook et al., 1993) mutants were used.

For hypoxia treatment, 4-wk-old inoculated or control plants were waterlogged with \( \text{O}_2 \)-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 pK7GWIWG2D(II) vector (Karimi et al., 2002) was replaced by the nodule-specific MtNCR001 promoter (E. Boncompagni, IBSV, Sophia-Antipolis, France, unpublished). The fragment for MtRbohA inactivation was amplified with 5'-GTG GTACCACTGAGCCAATGGATTG-3' and 5'-CAG CTCCAGGACAAAAACCCGTTGGCTTTACAG-3' as a forward and reverse primers, respectively, and cloned by restriction (Kpn I and Xhol) 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 pK7GWIWG2D(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-indoly-β-p-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 Axioplan 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). Root hairs were obtained as described previously (Sauviac et al., 2005). The integrity of total RNA was checked on agarose gel and its quantity, as well as purity, was determined spectrophotometrically. Two micrograms of RNA were used as a template for RT reaction in a reaction volume of 20 µl using the Omniscript RT Kit (Qiagen, http://www.qiagen.com) with oligo(dT) or random primers (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 to two endogenous controls: 40S Ribosomal Protein S8 (TC137982) and Mtc27 (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: 2−ΔΔCT, with ΔCT = CTgene of interest−CThousekeeping gene. For each experiment, 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.,
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 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 (PF08030)]. In 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, ca. 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 non-long 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name IMGAGa</th>
<th>Genbankb accession number</th>
<th>ORF length (bp)</th>
<th>Number of ESTsc</th>
<th>TC number (TIGR)c</th>
<th>Affymetrix Probesetd</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtRbohA</td>
<td>Medtr1g099800</td>
<td>–</td>
<td>2658</td>
<td>7</td>
<td>TC112710</td>
<td>Mtr.39812.1.S1_s_at</td>
</tr>
<tr>
<td>MtRbohB</td>
<td>Medtr3g151540</td>
<td>–</td>
<td>2772</td>
<td>6</td>
<td>TC123192, TC123112</td>
<td>Mtr.32104.1.S1_s_at</td>
</tr>
<tr>
<td>MtRbohC</td>
<td>Medtr3g151570</td>
<td>–</td>
<td>2754</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MtRbohD</td>
<td>Medtr3g151600</td>
<td>–</td>
<td>2109</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MtRbohE</td>
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<td>AY821801</td>
<td>2799</td>
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<td>TC126164</td>
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</tr>
<tr>
<td>MtRbohF</td>
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<td>–</td>
<td>2550</td>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>MtRbohG</td>
<td>Medtr7g138940</td>
<td>AY821802</td>
<td>2688</td>
<td>47</td>
<td>TC112621</td>
<td>Mtr.32307.1.S1_s_at</td>
</tr>
</tbody>
</table>

a The International Medicago Genome Annotation Group (IMGAG) (http://www.medicago.org).


c Designation of tentative consensus (TC) from TIGR (http://www.tigr.org). ORF, open reading frame; EST, expressed sequence tag.
d 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).
To localize more specifically root tissue expression, histochemical staining was performed using a promoter GUS transcriptional fusion approach. An approximately 2 kb promoter region was chosen (Table S2). As a result of their very low expression levels, MtRbohA 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,1,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 Rhob gene expression during M. truncatula interaction with its symbiont. 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 ca. 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

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**Fig. 2** MtRboh gene expression analysis in different plant 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 represent standard errors.
gain the capacity to fix N₂ (Gage, 2004). Therefore, these results show a strong link between the nodule functionality, in terms of N₂ 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⁻ 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. In 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

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**Fig. 4** Histochemical analysis of *MtRboh* expression in nodules of *M. truncatula* inoculated with *S. meliloti*. Nodules at 12 d post-inoculation (a, c, e, g, i) and 5–7 wk post-inoculation (b, d, f, h, j). *MtRbohA* (a, b), *MtRbohB* (c, d), *MtRbohE* (e, f), *MtRbohF* (g, h), *MtRbohG* (i, j). Scale bars represent 200 μm. n > 20.
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 synthase 1a (Medtr6g080780.1), phosphoenol pyruvate

Fig. 5 MtRbohA expression during nodule development. Time course of MtRbohA expression in nodules from 1 to 14 wk post-inoculation (a), and MtRbohA and MtRbohB expression in roots (without nodules) and nodules from 3-wk-old plants inoculated with Sinorhizobium meliloti nifH and bacA mutants (b). MtRbohA and MtRbohB relative expression levels in roots and nodules from control or hypoxic conditions (c): 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 represent standard errors.

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). 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. Asterisk (*) represents significant differences compared with control plants for P < 0.05. Error bars represent standard errors.
carboxylase (Medtr2g092340.1) and phosphoenol pyruvate carboxylase kinase (Medtr1g093530.1), which play major roles in nodule functioning (Carvalho et al., 2003; Nomura et al., 2006; Bai et al., 2007; Xu et al., 2007), did not appear to show modulated expression (data not shown). In 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. truncatula genes 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; Liszky 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 (Monshausen 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 O$_2^-$ and H$_2$O$_2$ 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 paralleled 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 ca. 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 MtRBOHA and nodule functionality. The down-regulation of the microsymbiont nitfD 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 MtRBOHA 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 isoform as 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 nitrogenase. 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


### 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 *S. 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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