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Hydrogen peroxide-regulated genes in the *Medicago truncatula–Sinorhizobium meliloti* symbiosis

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Summary

- Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), play an important role in signalling in various cellular processes. The involvement of H_2O_2 in the *Medicago truncatula–Sinorhizobium meliloti* symbiotic interaction raises questions about its effect on gene expression.

- A transcriptome analysis was performed on inoculated roots of *M. truncatula* in which ROS production was inhibited with diphenylene iodonium (DPI). In total, 301 genes potentially regulated by ROS content were identified 2 d after inoculation. These genes included *MtSpk1*, which encodes a putative protein kinase and is induced by exogenous H_2O_2 treatment.

- *MtSpk1* gene expression was also induced by nodulation factor treatment. *MtSpk1* transcription was observed in infected root hair cells, nodule primordia and the infection zone of mature nodules. Analysis with a fluorescent protein probe specific for H_2O_2 showed that *MtSpk1* expression and H_2O_2 were similarly distributed in the nodule infection zone. Finally, the establishment of symbiosis was impaired by *MtSpk1* downregulation with an artificial micro-RNA.

- Several genes regulated by H_2O_2 during the establishment of rhizobial symbiosis were identified. The involvement of *MtSpk1*in the establishment of the symbiosis is proposed.

Introduction

Plants are subjected to a continually changing environment, and must respond very quickly and efficiently to adjust to environmental fluctuations. It is now widely accepted that reactive oxygen species (ROS) are involved in signalling in response to biotic and abiotic stresses and in developmental processes (Apel & Hirt, 2004). The tight regulation of ROS homeostasis by a multifaceted network of ROS production and ROS scavenging enzymes creates a baseline from which ROS spikes may be generated and act as signals in various cellular processes (Mittler *et al*., 2004, 2011; Tognetti *et al*., 2012). ROS have been implicated in processes as diverse as root hair growth (Foreman *et al*., 2003; Jones *et al*., 2007; Monshausen *et al*., 2007), stomatal closure (Pei *et al*., 2000) and plant–microbe interactions (Apel & Hirt, 2004; Pauly *et al*., 2006; Nanda *et al*., 2010).

There is now compelling evidence that ROS play an important role in signalling processes during the establishment of legume–*Rhizobium*symbioses (Santos *et al*., 2001; Ramu *et al*., 2002; Rubio *et al*., 2004; Pauly *et al*., 2006; Jamet *et al*., 2007). This interaction involves a complex molecular dialogue between the host plant and the symbiont, leading to the root hair becoming infected with the bacterium and ultimately forming root nodules, in which nitrogen fixation takes place (Oldroyd & Downie, 2008). ROS, such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), have been detected at early stages of the symbiotic interaction (for a review, see Pauly *et al*., 2006; Nanda*et al*., 2010). O_2^- and H_2O_2 have been found in infection threads (ITs) (Santos *et al*., 2001; Jamet *et al*., 2007), which, in *c.* 75% of nodulating legumes (Sprent, 2007), are unique invasive invaginations of plant origin that allow the bacterium to invade the cortical cells (Oldroyd & Downie, 2008). The rate of continual ROS production in roots has been shown to decrease temporarily during the first few hours after Nod factor (NF) treatment (Shaw & Long, 2003; Lohar *et al*., 2007). Moreover, a *Sinorhizobium meliloti* nodC mutant with defective NF biosynthesis has

been shown to induce higher levels of H_2O_2 accumulation (Bueno *et al*., 2001). By contrast, no O_2^- production was observed when *Medicago truncatula* plants were inoculated with an *S. meliloti* nodD1ABC mutant unable to produce NFs (Ramu *et al*.,2002). Similarly, a transient increase in ROS levels has been detected at the tip of actively growing root hair cells within seconds of the addition of NF in *Phaseolus vulgaris* (Cardenas *et al*., 2008). Moreover, the inhibition of ROS production in *M. truncatula* roots inoculated with *S. meliloti* has been shown to prevent IT formation (Santos *et al*., 2001; Ramu *et al*., 2002; Peleg-Grossman *et al*., 2007). ROS thus seem to be critical for optimal symbiosis (Jamet *et al*., 2007; Marino *et al*., 2011).

NADPH oxidases, also known as respiratory burst oxidase homologues (RBOHs), appear to play an important role in ROS production during the symbiotic process (Marino *et al*., 2011). They have been identified as a major source of the oxidative burst observed during plant–pathogen interactions (Torres & Dangl, 2005; Sagi & Fluhr, 2006; Mittler *et al*., 2011; Marino *et al*., 2012). In the*S. meliloti*–*M. truncatula* symbiosis, diphenylene iodonium (DPI) – an inhibitor of flavoproteins, such as NADPH oxidases – abolishes ROS production early in the interaction (Peleg-Grossman *et al*., 2007; Cardenas *et al*., 2008). Seven NADPH oxidase-encoding genes have been identified in the *M. truncatula* genome (Marino *et al*., 2011) and the expression patterns of these genes have been characterized (Lohar *et al*., 2007; Marino *et al*., 2011). It has been suggested that the decrease in ROS efflux observed 1 h after the treatment of*M. truncatula* roots with NF is associated with a transient decrease in *MtRbohs* gene expression (Lohar *et al*., 2007). Furthermore, the downregulation of *MtRbohA* expression in the nodule by RNA interference methods leads to a decrease in biological nitrogen fixation, indicating a probable role in symbiosis (Marino *et al*., 2011). Similar results have been reported recently for *Phaseolus vulgaris,* in which a NADPH oxidase gene has been shown to be crucial for successful rhizobial colonization and, probably, for the maintenance of the correct growth and shape of ITs (Montiel *et al*., 2012).

Despite widespread acceptance of the role of ROS in signalling, it remains unclear how ROS signals are perceived, transmitted and induce a specific response (Mittler *et al*., 2011). Given the crucial role of these molecules in signalling in plant cells, efforts have recently been made to identify the genes regulated by ROS. Genome-wide transcriptome analyses have proved to be useful for the assessment of the specificity of ROS signalling, with the analysis of gene expression profiles after the modification of ROS levels (Gadjev *et al*., 2006; Inzé*et al*., 2011). These transcriptome analyses have made it possible to determine the effect, not only of different ROS, but also of the accumulation of ROS in various subcellular compartments on gene expression. They have shown that most of the genes responding to a stimulus (stress, oxidative stress-causing agents, etc.) are expressed in only one specific set of experimental conditions (i.e. in response to one particular ROS species). This strongly suggests that gene expression levels are a hallmark of specific oxidative signals characterized by the identity of the ROS concerned and/or its site of production (Gadjev *et al*., 2006; Grennan, 2008). Interestingly, a meta-analysis of H_2O_2-induced gene expression in eukaryotes (including *Arabidopsis*) has provided evidence to suggest that the regulation of gene expression by ROS is strongly conserved across kingdoms (Vandenbroucke *et al*., 2008).

In this context, the aim of this study was to identify the genes targeted by H_2O_2 during the establishment of the *M. truncatula–S. meliloti*symbiosis. The use of a combination of pharmacological and transcriptomic approaches led to the identification of several genes potentially regulated by H_2O_2 content. We then analysed the expression profile of an H_2O_2-induced gene encoding a putative serine/threonine protein kinase. Using an artificial micro-RNA (amiRNA) strategy, we showed that this gene plays an important role in the establishment of the rhizobial symbiosis.

Materials and Methods

Plant material, growth conditions, chemical treatments and rhizobial infection

Medicago truncatula cv Jemalong A17 and *dmi1* (Catoira *et al*., 2000) seeds were treated as described by Marino *et al*. (2011). Briefly, they were scarified in 1 M H_2SO_4 (6 min), sterilized in 6% sodium hypochlorite (3 min) and rinsed in sterile distilled water. They were then germinated on 0.4% agar plates incubated at 16°C for 2 d in the dark, and transferred to plates containing Fahraeus medium.

ROS levels were decreased by transferring 7-d-old plants to new plates containing 10 µM DPI (Alexis Biochemicals,http://www.enzolifesciences.com/alexis) or dimethyl sulfoxide (DMSO; mock treatment). These plates were incubated for 24 h and the plants were then inoculated with *S. meliloti* (200 µl, giving 0.05 OD_{600} units per root) and maintained in controlled conditions in a growth chamber (16-h photoperiod, 200 µmol m^{-2} s^{-1}, 25°C).

For NF treatment, the roots of 7-d-old plants were treated with a 10 nM solution of lipochitooligosaccharides (LCO-IV(C16:2, S); 200 µl per root) and the treated zone was collected at the time indicated. For paraquat treatment, the roots of 7-d-old plants were incubated with 50 µM methyl viologen (paraquat, Sigma-Aldrich).

An *S. meliloti* 2011 DsRed strain harbouring the pDG77 vector (Gage, 2002) was obtained by triparental conjugation for use in H_2O_2determinations *in vivo*. An *S. meliloti* 2011 lacZ strain was used in all the other experiments (Marino *et al*., 2011). *Sinorhizobium meliloti*strains were grown at 30°C on Luria–Bertani medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$.

Composite *M. truncatula* plants were obtained by transformation with *Agrobacterium rhizogenes* (Boisson-Dernier *et al*., 2001). Two weeks after transformation, transgenic roots were selected on the basis of green fluorescence protein (GFP) fluorescence (under a Leica MZFLIII binocular microscope, http://www.leica-microsystems.com; GFP Plus fluorescence filter set: 480/40-nm excitation filter; 510-nm barrier filter). For each plant, we retained a single root, which was transferred to nitrogen-free Fahraeus medium 3 d before inoculation with *S. meliloti*.

Medicago truncatula transgenic root cultures were initiated as described previously (Ramos & Bisseling, 2003). Briefly, transgenic root tips were excised from composite plants and transferred to Petri dishes containing SHB10 medium (Chabaud *et al*., 2003) supplemented with augmentin (200 µg ml^{-1}, Sigma, www.sigmaaldrich.com). Augmentin was maintained in the medium for three subculture cycles (2 wk each), but was not included in the medium for subsequent cycles.

Promoter and silencing constructs

Unless otherwise indicated, the plasmids used in this study were generated with Gateway technology, according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com). PCR products, flanked by *att*B sites, were inserted into the pDONR207 vector by the BP reaction (Invitrogen) and then into destination vectors by the LR reaction (Invitrogen).

A transcriptional fusion between the *MtSpk1* promoter and the β-glucuronidase (GUS) reporter gene was obtained by PCR amplification of the 1426-bp sequence upstream from the start codon from genomic DNA, with the appropriate primers (Supporting Information Table S1). The resulting fragment was then inserted into the pKGWFS7 vector (Karimi *et al*., 2007).

Plants with low levels of *MtSpk1* gene expression were generated by means of an amiRNA strategy (Schwab *et al*., 2006). The *MtSpk1*amiRNA construct was designed as described previously, with a web-based application (http://wmd3.weigelworld.org). The resulting primers (Table S1) were tested with the pRS300 matrix. The *MtSpk1* amiRNA construct was inserted into the pK7WG2D vector (Karimi*et al*., 2007). A control plasmid was generated in a similar manner, by the insertion of a 327-bp DNA fragment from the *LacZ* gene (encoding β-galactosidase) into the pK7WG2D vector.

The *MtSpk1* open reading frame was amplified by PCR with appropriate primers (Table S1) and the resulting fragment was inserted into the pK7FWG2 vector (Karimi *et al*., 2007), generating a translational C-terminal fusion of SPK1 and GFP. Two controls were used: GFP targeted to the cytosol (pK7WG2D control vector) and GFP targeted to the nucleus (Budding Uninhibited by Benzymidazol-related 1, BUBR1::GFP; Caillaud *et al*., 2009).

Colorimetric detection of H_2O_2 and O_2^-

H_2O_2 was detected by diaminobenzidine (DAB) staining (Sigma, www.sigmaaldrich.com). Plant roots were incubated in 0.1 M citrate buffer (pH 3.7) supplemented with 1 mg ml^{-1} DAB. The staining reaction was stopped by the addition of absolute ethanol. The roots were then cleared by incubation for 10 min in boiling lactic acid (10%; v/v).

O_2^- was detected by nitroblue tetrazolium (NBT) staining (Sigma, www.sigmaaldrich.com). Plant roots were infiltrated with 10 mM sodium phosphate buffer (pH 7.8) under vacuum at room temperature for 90 min. They were then incubated with the staining solution (1 mM NBT, 10 mM NaN_3, 50 μM NADPH, 10 mM sodium phosphate buffer, pH 7.8) for 20 min at 37°C. The staining reaction was stopped by boiling the roots three times, for 10 min each, in absolute ethanol. Finally, NBT- and DAB-stained samples were visualized with a Zeiss Axioplan 2 microscope under dark-field illumination (Zeiss, http://www.zeiss.com).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted as described previously (Marino *et al*., 2011). RT was carried out on 2 μg of RNA, with the Omniscript RT Kit (Qiagen, http://www.qiagen.com). We checked that the RNA was not contaminated with genomic DNA by carrying out PCR on the RT products, with intron-spanning primers directed against the *M. truncatula* glutathione synthetase gene (*MtGshs1*; Frendo *et al*., 2001). Real-time RT-qPCR was carried out with qPCR Mastermix Plus for the SYBR Green I reagent (Eurogentec, http://www.eurogentec.com), as described previously (Marino *et al*., 2011). GeNorm analysis was performed on five reference genes (Table S1) for selection of the two most stably expressed genes for each experiment (Vandesompele *et al*., 2002). Data were analysed with RqPCRBase, an R package running in the R computing environment for the analysis of real-time qPCR data (T. Tran & F. Hilliou, unpublished).

Histochemical localization of GUS and β-galactosidase activities

GUS and β-galactosidase activities were assayed as described previously (Marino *et al*., 2011). Slices of roots and nodules were visualized with a Zeiss Axioplan 2 imaging microscope under dark-field illumination (Zeiss).

***Medicago truncatula* transcriptome analysis**

Two independent biological replicates were analysed. For each replicate and each point, RNA samples were obtained by pooling the RNA isolated from 40 roots. Total RNA was extracted as described previously (Marino *et al*., 2011). The absence of genomic DNA contamination was checked by semi-quantitative RT-PCR with primers spanning the *MtGshs1* intron (Table S1). RNA sample integrity was checked with an Agilent 2100 bioanalyser (Waldbroon, http://www.agilent.com). Total RNA (1 μg) was used for the synthesis of biotin-labelled cRNAs with a one-cycle cDNA synthesis kit (Affymetrix, http://www.affymetrix.com). Superscript II reverse transcriptase and T7-oligo (dT) primers were used to synthesize the first-strand cDNA in a reaction carried out for 1 h at 42°C. The double-stranded cDNA was then produced by incubation with DNA ligase, DNA polymerase I and RNase H for 2 h at 16°C. The double-stranded cDNA was cleaned with the Sample Cleanup Module (Affymetrix) and then used for *in vitro* transcription in the presence of biotin-labelled UTP (GeneChip IVT labelling kit, Affymetrix). The resulting labelled cRNA was then cleaned up (Sample Cleanup Module, Affymetrix) and quantified (RiboGreen RNA Quantification Reagent, Invitrogen). The cRNA (10 μg) was fragmented by heating at 94°C for 35 min, and was then hybridized for 16 h at 45°C with the GeneChip *Medicago* genome array (Affymetrix). The arrays were then washed with two different buffers (stringent: 6 × SSPE, 0.01% Tween-20; nonstringent: 100 mM MES, 0.1 M Na^+, 0.01% Tween-20) and stained with a complex solution including streptavidin R–phycoerythrin conjugate (Invitrogen) and biotinylated anti-streptavidin antibody (Vector Laboratories, http://www.vectorlabs.com). The washing and staining steps were performed in a GeneChip Fluidics Station 450 (Affymetrix). Finally, the arrays were scanned with a GeneChip Scanner 3000 7G driven by GeneChip Operating Software (Affymetrix). The 'raw.CEL' files were imported into R software for data analysis. All the raw and normalized data are available from the CATdb database (AFFY_H2O2_medicago; Gagnot *et al*., 2008) and from the Gene Expression Omnibus Repository at the National Center for Biotechnology Information (GSE15866).

Data were normalized with the gcrma algorithm (Irizarry *et al*., 2003), available as part of the Bioconductor package (Gentleman & Carey,2002). Differentially expressed genes were identified in two-group *t*-tests, assuming equal variances for the groups tested. The variance of gene expression in each group was homoscedastic, and genes with extreme variances (too small or too large) were excluded. The raw *P*values were adjusted by the Bonferroni method, which controls for the family-wise error rate (Ge *et al*., 2003). A gene was considered to be differentially expressed if the Bonferroni *P* value was below 0.05. Finally, the robustness of the gene chip strategy was assessed. A set of 25 genes identified as differentially expressed in *M. truncatula* roots (after DPI treatment or *S. meliloti* inoculation) was randomly selected for validation by RT-qPCR analysis (Table 1).

Table 1 Validation of microarray experiments

		Microarray		RT-qPCR	
Probeset	Annotation	Symbiosis	DPI	Symbiosis	DPI
Mtr.8995.1.s1_at	NFXL1	−1.68	6.05		5.86
Mtr.48781.1.s1_at	GRP	−1.55	3.48		2.97
Mtr.13186.1.S1_at	Unknown	−1.51	5.30		2.86
Mtr.6889.1.S1_at	ABC transporter	−1.26	5.01		4.11
Mtr.43077.1.s1_at	TIR1	−1.25	3.50		3.12
Mtr.33133.1.S1_at	Protein kinase	−1.19	2.34		1.89
Mtr.17273.1.s1_s_at	ARR9	1.16	−4.19		−5.06
Mtr.39233.1.s1_at	ARF	1.25	−2.57	1.28	−2.56
Mtr.44789.1.S1_at	NSP2	1.39	−4.35		−5.64
Mtr.20281.1.s1_at	**ABIL**	1.42	−2.84	1.71	
Mtr.6956.1.S1_at	NSP1	1.54	−4.13	1.32	−3.47
Mtr.25211.1.S1_at	MtRIP1	1.85	−6.89	1.72	−4.10
Mtr.20266.1.s1_at	Protein kinase	2.05	−1.78	1.42	−3.64
Mtr.39654.1.s1_at	DUF26	2.33	−2.24	2.84	−2.00
Mtr.10993.1.S1_at	**MtbHLH1**	3.01	−4.93	2.07	−3.35
Mtr.35414.1.s1_at	Protein kinase	3.04	−2.91	1.88	−2.06
Mtr.24165.1.s1_at	**MtSPK2**	3.55	−4.81	2.38	−3.32
Mtr.16214.1.S1_at	**MtSPK1**	3.61	−6.32	3.98	−3.84
Mtr.28094.1.S1_at	MtNIN	4.96	−4.97	3.07	−5.06
Mtr.44069.1.S1_at	NCR247	5.71	−3.69	4.44	
Mtr.43750.1.S1_at	HAP2-1	6.60	−6.58	3.65	−5.06
Mtr.10626.1.s1_at	**MtSRL1**	6.70	−6.91	4.00	−3.64
Mtr.35511.1.s1_at	Proteinase inhibitor	8.31	−8.28	6.91	−6.64

For microarray experiments, 23 genes identified as differentially expressed were analysed in the same conditions (DMSO(i)/DMSO(ni); DMSO + DPI(i)/DMSO(ni)). A negative ratio (blue box) indicates that the gene is downregulated in the wild-type (WT); a positive ratio (yellow box) indicates that the gene is upregulated in the WT. The same colour scheme is used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A black box indicates that the expression of the corresponding gene is not significantly modified by diphenylene iodonium (DPI) treatment or symbiosis. Putative functions of probeset-associated proteins are given. Ratios are expressed on a $\log_2$ scale. Genes selected for further analyses are shown in bold typeface. DMSO, dimethyl sulfoxide; i, inoculated; ni, noninoculated.

Expression of the HyPer probe in *M. truncatula* and *in vivo* imaging of H_2O_2 in *M. truncatula*nodules

The gene encoding HyPer (an H_2O_2 sensor; pHyPer-Cyto vector, Evrogen, http://www.evrogen.com) was inserted between the *Nco*I and *Eco*RI restriction sites of the pENTR4 vector (Invitrogen), and then transferred to the binary plasmid pK2GW7 (Karimi *et al*., 2007) with the Gateway system (Invitrogen). As a control, another DNA sequence (327 bp of the GFP gene) was inserted into pK2GW7 in a similar manner. The control and HyPer vectors were used to generate composite plants of *M. truncatula*. The presence of the HyPer protein in transgenic roots was confirmed by incubation with a polyclonal antibody specifically directed against GFP (Interchim,http://www.interchim.com).

Nodules expressing the HyPer construct were embedded in 4% (w/v) agarose, and 100-µm sections were cut with a HM560V vibratome (Microm Microtech, http://mm-france.fr). HyPer fluorescence was analysed with a Leica TCS-SP2 confocal microscope, with a 20× dry objective (Leica). In addition to bright-field imaging, HyPer fluorescence in nodule sections was recorded on excitation at 405 nm and 488 nm. Ratiometric imaging was then performed with Leica Confocal Software for visual quantification of the relative concentration of H_2O_2 in the tissues imaged. The images shown are representative of 10 biological replicates.

Results

Transcriptional reprogramming of symbiotic *M. truncatula* roots in response to DPI

For the identification of the plant genes regulated by H_2O_2 during the establishment of the symbiotic interaction, we first defined the DPI treatment conditions inhibiting ROS production in *M. truncatula* roots. DPI has been shown to be effective at decreasing ROS production (Foreman *et al*., 2003; Rubio *et al*., 2004). The effect of various concentrations of this NADPH oxidase inhibitor on the production of $O_2^{\cdot-}$ and H_2O_2 was assessed in *M. truncatula* roots by staining with NBT and DAB. Concentrations of up to 0.1 µM DPI had no effect on the accumulation of $O_2^{\cdot-}$ and H_2O_2, which was observed mostly in the root tip region and in vascular tissues in control roots (data not shown). The use of DPI at a concentration of 1 µM appeared to

result in a slightly lower staining intensity (data not shown), but a concentration of 10 µM was required for the full inhibition of ROS production (Fig. 1).

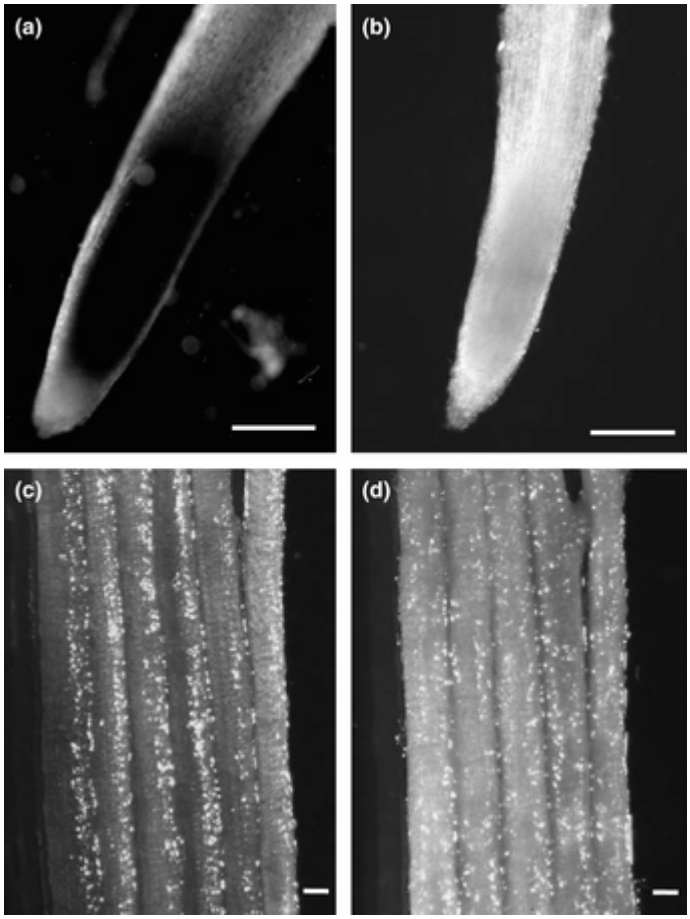

Figure 1. Changes to reactive oxygen species (ROS) production in diphenylene iodonium (DPI)-treated *Medicago truncatula* roots. Representative colorimetric detection of the superoxide anion (nitroblue tetrazolium, NBT; a, b) and hydrogen peroxide (H_2O_2) (diaminobenzidine, DAB; c, d) in control roots (a, c) and in roots treated with 10 µM DPI (b, d);*n* > 30. Bars, 200 µm.

A concentration of 10 µM DPI was therefore used in the microarray analysis for the identification of *M. truncatula* genes putatively regulated by ROS 2 d after infection (dai) with *S. meliloti* (Fig. 2). Following on from preliminary studies to determine the most suitable experimental conditions (data not shown), we confirmed that our chosen conditions yielded a robust plant response, with ROS production, in the shepherd's crook in particular (Fig. S1b,c). A two-fold change in expression level was used as the threshold value, with a significance level of $P < 0.05$. In these conditions, we identified 447 genes with expression modified by bacterial inoculation (Table S2). These results are consistent with published transcriptomic data (Benedito *et al*., 2008; Moreau *et al*., 2011). In these previous studies, 199 genes were found to have different patterns of expression in control roots (Root-0) and in inoculated roots (Nodule-4 dpi) (Benedito *et al*., 2008). In our study, 149 genes seemed to be regulated in a similar manner. Moreover, 12 genes previously identified as being expressed in the meristematic or infection zone of the nodule (Moreau *et al*., 2011) displayed a modulation of expression in this study (Table S3).

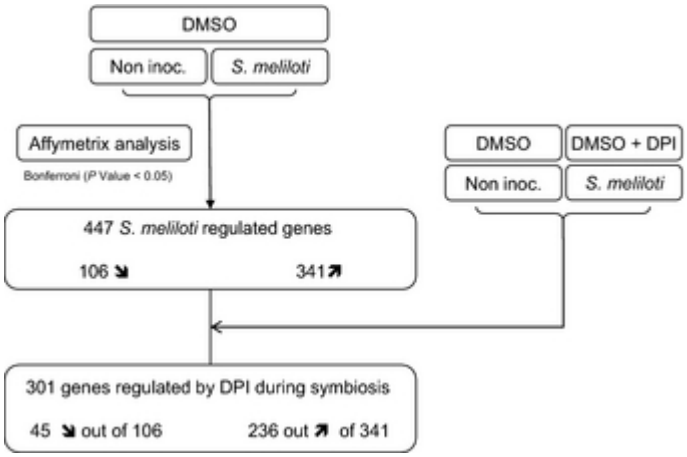

Figure 2. Identification of genes putatively regulated by reactive oxygen species (ROS) in the *Medicago truncatula–Sinorhizobium meliloti* symbiosis. Transcriptome analysis of*M. truncatula* roots 2 d after inoculation with *S. meliloti*identified 447 genes displaying significant deregulation (DMSO(i)/DMSO(ni)). A gene was considered to be differentially expressed if the Bonferroni *P* value was below 0.05. Of the 447 genes identified, 301 were affected by diphenylene iodonium (DPI) treatment (DPI(i)/DMSO(ni)). DMSO, dimethyl sulfoxide; i, inoculated; ni, noninoculated.

Of the 447 genes regulated by *S. meliloti* infection, 301 also appeared to be affected by DPI treatment in inoculated roots (Fig. 2), indicating that a major reprogramming of *M. truncatula* gene transcription was induced by DPI in symbiotic conditions.

The microarray data were validated by RT-qPCR on 23 selected genes for which upregulation by infection was strongly diminished, or even abolished, by DPI treatment. This RT-qPCR analysis validated *c*. 80% of the selected genes (Table 1, Fig. S2). DPI had a major effect on genes encoding early nodulins (*Enod8.1*, *Enod11*) and proteins involved in NF signalling, such as *MtNin1*, *MtNsp1* and *Hap2-1*(Table 1).

Regulation of *M. truncatula* symbiotic genes by H_2O_2

We hypothesized that symbiosis-regulated genes affected by DPI treatment would show a response to H_2O_2. We chose five genes (Table 1, Table S2) not previously known to be symbiosis related, each of which had a predicted function potentially related to the establishment of symbiosis. Whilst this study was underway, one of these five genes (Mtr.10993.1.s1_at; *MtbHlh1*) was shown to be upregulated during symbiosis and to be involved in the control of nodule vasculature patterning and nutrient exchanges between nodules and roots (Godiard*et al*., 2011).

The gene corresponding to Mtr.10626.1.S1_at encodes a protein with a sequence very similar to that of AtSRG1 (*Arabidopsis thaliana*SENESCENCE-RELATED GENE 1; Callard *et al*., 1996; 62% sequence identity) from the 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily. The genes of this family are induced by H_2O_2 (Gechev *et al*., 2005) and it has been suggested that they are involved in cellular homeostasis (Ozer & Bruick, 2007). We named this gene *MtSrl1* (Senescence Related-Like 1 gene). Another of the genes studied encoded an ABL-like protein (ABlL) serving as a subunit of the WAVE (WASP-family verprolin homologous protein) complex (Mtr.20281.1.S1_at). The WAVE complex is required for activation of the actin-related protein 2/3 (ARP2/3) complex, which is involved in actin microfilament nucleation and branching (Miyahara *et al*., 2010).

Protein kinases are involved in plant cell signalling in response to biotic and abiotic stresses. Two of the genes studied encode putative serine/threonine protein kinases (Mtr.16214.1.s1_at, Mtr.24165.1.s1_at;

Table 1). We named these protein kinases genes *MtSpk1* and*MtSpk2* (Symbiotic Protein Kinases 1 and 2), respectively.

For confirmation of the regulation of these genes by H_2O_2, gene expression was analysed by RT-qPCR after the exogenous application of H_2O_2 to *M. truncatula* roots. *MtRip1* gene expression, which has been shown to be regulated by H_2O_2 (Ramu *et al*., 2002), was analysed as a positive control. We first evaluated the relative expression level of the selected genes in roots before H_2O_2 treatment (Fig. S4). In our conditions, *MtRip1* was the most strongly expressed of the genes studied, with *MtbHlh1*, *MtSpk1*, *MtSpk2*, *MtNin* and *MtPrl* displaying levels of expression about one order of magnitude lower, and *MtAblL* and *MtSrl1* expressed to a level about two orders of magnitude lower, than that for *MtRip1* (Fig. S4). One hour after treatment with 1 mM H_2O_2, a *c*. four-fold induction of *MtRip1* was observed (Fig. 3). H_2O_2 treatment induced an accumulation of *MtSpk1*, *MtSrl1* and *MtAblL1* transcripts to about twice the levels observed in control roots. By contrast, expression of the *MtbHlh1* and *MtSpk2* genes was not significantly induced by H_2O_2 (Fig. 3). Increasing the incubation time (to 3 h) led to the induction of *MtSpk1* and *MtSpk2*, by factors of six and three, respectively (Fig. 3). As a negative control, the predicted lack of response to H_2O_2 was confirmed for two genes (*MtNin* and *MtPrl;* Fig. 3). Conversely, paraquat treatment, provoking the formation of $O_2^{\cdot-}$ in root mitochondria (Cocheme & Murphy, 2008), did not induce *MtSpk1* expression (data not shown). Thus, H_2O_2 regulates the expression of genes potentially important for the establishment of symbiosis. These results also suggest that a significant proportion of DPI-responsive genes may be under the control of H_2O_2.

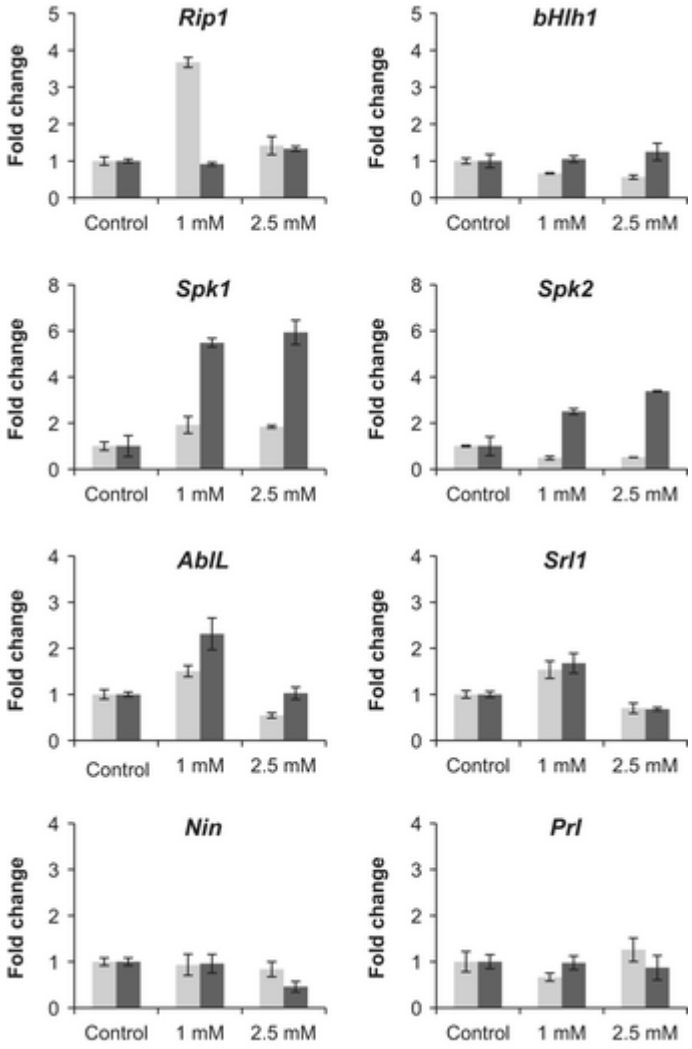

Figure 3. Identification of genes regulated by hydrogen peroxide (H_2O_2) in *Medicago truncatula*. Seven-day-old*M. truncatula* roots were treated with exogenous H_2O_2 (1 and 2.5 mM) and the relative expression levels of the corresponding candidate genes were analysed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), 1 h (light blue bars) and 3 h (dark blue bars) after treatment. The data shown are means ± SEM from two biological experiments.

MtSpk1 gene expression and H_2O_2 accumulation may be co-localized in root nodules

The possible regulation of *MtSpk1* expression by H_2O_2 *in vivo* was investigated further by the evaluation of the possible co-localization of this expression and H_2O_2 production. The spatiotemporal expression of the *MtSpk1* gene was studied by generating composite plants of*M. truncatula* expressing a transcriptional fusion of the *MtSpk1* promoter and the *GUS* reporter gene. In noninoculated plants, *MtSpk1*expression was restricted to the root meristem and vascular tissue (Fig. 4a,b). During the symbiotic interaction, a strong signal was observed in infected root hairs and nodule primordia (Fig. 4c,d). In older nodules, the expression of this gene appeared to be restricted to the infection zone (zone II, Fig. 4e). Thus, *MtSpk1* expression is associated with the bacterial infection process.

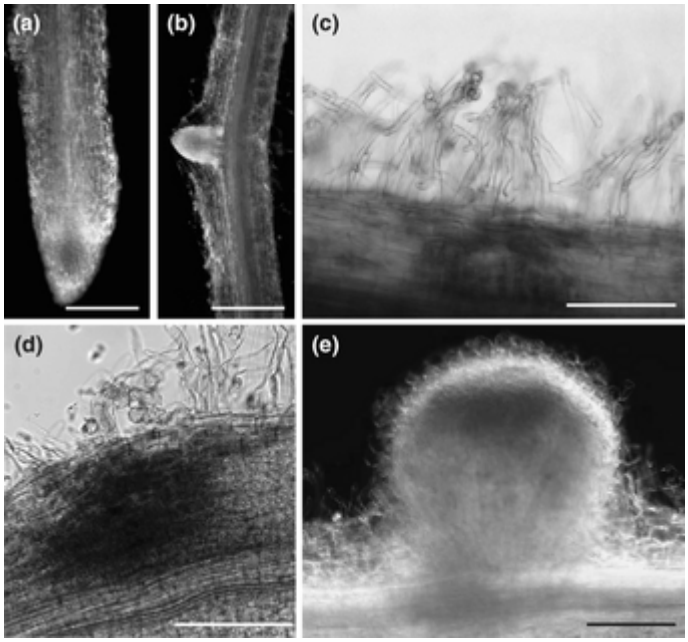

Figure 4. Histochemical analysis of *MtSpk1* expression in*Medicago truncatula* roots. *MtSpk1* is expressed in the main root tip (a) and the secondary apex (b) of noninoculated roots. On *Sinorhizobium meliloti* inoculation, *MtSpk1* expression is induced in root hairs and cortical cells (c; 2 d after infection (dai)), in young primordia (d; 3 dai) and in the nodule infection zone (e; 12 dai). Bars, 200 μm. $n > 10$.

H_2O_2 production has already been clearly demonstrated in infected root hairs and nodule primordia during the early steps of the *Medicago*sp.–*S. meliloti* interaction (Santos *et al*., 2001; Jamet *et al*., 2007; Fig. S1c). However, H_2O_2 production has not been clearly demonstrated previously in nodule zone II, in which *MtSpk1* is strongly expressed. In this study, levels of H_2O_2 *in vivo* in root nodules on transgenic roots expressing the fluorescent probe for H_2O_2 (HyPer, Belousov *et al*., 2006) were investigated by quantitative confocal microscopy. The HyPer probe, which consists of a circularly permuted yellow fluorescent protein inserted into the *Escherichia coli* OxyR regulatory domain, is specifically sensitive to H_2O_2. *M. truncatula* composite plants producing HyPer were inoculated with the *S. meliloti DsRed* strain and analysed by confocal microscopy (Fig. S3a,b). The emission spectra obtained after excitation at 405 and 488 nm were entirely consistent with those previously described for this probe (Belousov *et al*., 2006;

Fig. S3b). H_2O_2 production was detected in the inner cortex and nodule zone II (Fig. 5). These data do not demonstrate that H_2O_2 production and *MtSpk1* gene expression occur in exactly the same cells, but they do strongly suggest the co-localization of these two processes in nodule zone II. Overall, our data suggest that *MtSpk1* may be regulated by H_2O_2 during the symbiotic process *in vivo*.

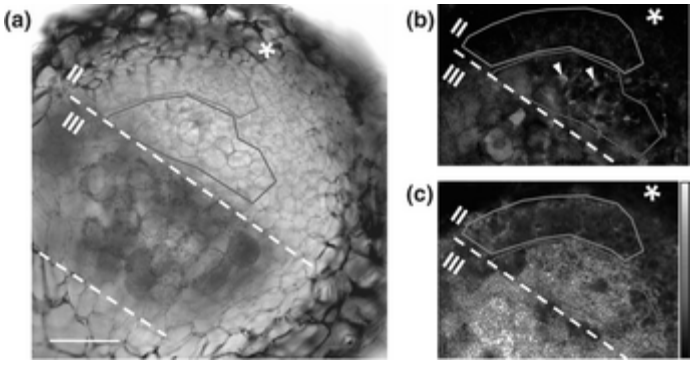

Figure 5. Hydrogen peroxide (H_2O_2) detection *in vivo* in root nodules. Composite plants of *Medicago truncatula* producing the HyPer protein probe were inoculated with *Sinorhizobium meliloti* Ds-Red, and 12-d-old nodules were analysed (100-µm vibroslices). (a) Transmission image of a 12-d-old nodule. Green and pink areas represent the two zones of H_2O_2quantification. (b) *Sinorhizobium meliloti* bacteria are localized in red. Arrowheads show infection threads. (c) Ratiometric H_2O_2 detection (488/405 nm) with the HyPer probe; 488 nm, excitation wavelength used for quantification of the fluorescence emission of the oxidized form of the HyPer probe; 405 nm, excitation wavelength used for quantification of the fluorescence emission of the reduced form of the HyPer probe. The pink zone corresponds to the inner cortex of the nodule. The green zone corresponds to zone II of the nodule, surrounding cells containing infection threads. A colour scale for the 488/405-nm ratio is shown on the right, with white corresponding to the highest ratio and black to the lowest ratio. Bar, 100 µm. *n* = 10. *, Meristematic zone; II, infection zone in which the infection threads release bacteria into plant cells; III, fixing zone in which bacteria differentiate into bacteroids.

MtSpk1 expression is regulated by NFs and is necessary for *M. truncatula–S. meliloti* symbiosis

The early induction of *MtSpk1* by *S. meliloti* inoculation (Table 1, Fig. 4) suggests that this gene may be regulated by NFs. *MtSpk1* gene expression was therefore analysed in *M. truncatula* roots treated with NF (Fig. 6a). An eight-fold induction of *MtSpk1* expression was observed after 12 h. For confirmation of the putative involvement of NF in the regulation of *MtSpk1* gene expression, we evaluated the expression of this gene at 2 dai in the *M. truncatula dmi1* mutant (which has no NF signalling cascade) inoculated with the *S. meliloti* wild-type (WT) strain. We also evaluated its expression in *M. truncatula* WT plants infected with various *S. meliloti* mutant strains with impaired symbiotic capacities (*nodD1D2D3*, defective in NF synthesis; *nodA*, absence of the fatty acid moiety of NF; *nodH*, absence of NF sulfatation;*exoA*, defective in exopolysaccharide biosynthesis). An upregulation of *MtSpk1* expression during infection with *S. meliloti* was observed only in the *exoA* mutant, which produced WT NF (Fig. 6b). With all the other mutants, producing only modified or no NF, no significant deregulation with respect to noninoculated plants was observed (Fig. 6b). Moreover, *MtSpk1* was not induced in the *M. truncatula dmi1*mutant (Fig. 6c). Thus, *MtSpk1* expression is regulated by NF, and our data suggest a possible role of the protein encoded by this gene in the NF signalling cascade.

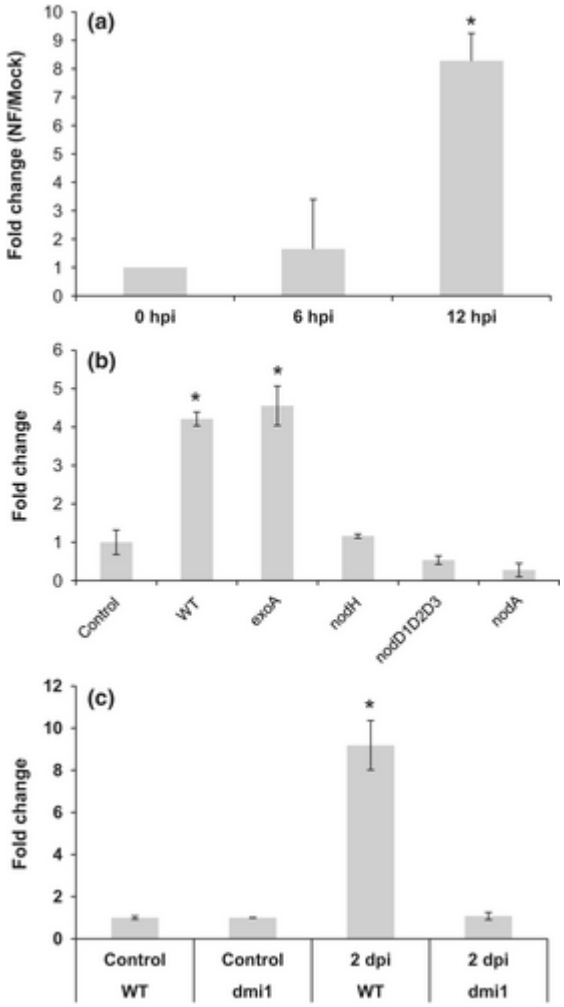

Figure 6. *MtSpk1* regulation by Nod factors (NFs). (a) Seven-day-old wild-type (WT) *Medicago truncatula* plants were treated with 10 nM NF (LCO-IV (C16:2, S)) and *MtSpk1* gene expression was analysed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), 1, 6 and 12 h after treatment (hpi). The values shown are the NF-treated/mock-treated *MtSpk1*gene expression ratios. *, $P < 0.05$. As a control, *MtNin* was induced by a factor of 15, 12 h after NF treatment. (b) Seven-day-old WT *M. truncatula*plants were inoculated with *Sinorhizobium meliloti* mutant strains with impaired symbiotic capacities: *nodD1D2D3* (defective NF synthesis), *nodA*(absence of the fatty acid moiety of NF), *nodH* (absence of NF sulfatation) and*exoA* (defective exopolysaccharide biosynthesis). *MtSpk1* gene expression was analysed by RT-qPCR, 2 d after infection (dai). The values shown are the inoculated/mock-treated *MtSpk1* gene expression ratios. *, $P < 0.05$. (c) Seven-day-old WT and *dmi1 M. truncatula* plants were inoculated with the WT*S. meliloti* strain and *MtSpk1* gene expression was analysed by RT-qPCR, at 2 dai. *, $P < 0.05$. Experiments were performed on two independent biological samples (20 plants each). The data shown are means ± SEM from three independent biological experiments.

An amiRNA strategy to decrease *MtSpk1* gene expression *in planta* (Schwab *et al*., 2006) was used to study the role of the *MtSpk1* gene in the *M. truncatula–S. meliloti* symbiosis. The efficiency of amiRNA-mediated silencing was confirmed by the analysis of *MtSpk1* gene expression by RT-qPCR (Fig. 7a). This approach decreased *MtSpk1* gene expression by *c*. 60%, and was therefore considered to be effective. Primary root growth was similar in *amiMtSpk1* and control plants (Fig. 7b). Interestingly, *amiMtSpk1* plants had significantly fewer nodules than plants expressing a control construct, from 7 to 21 dai (Fig. 7c), consistent with a role for *MtSpk1* in the establishment of symbiosis.

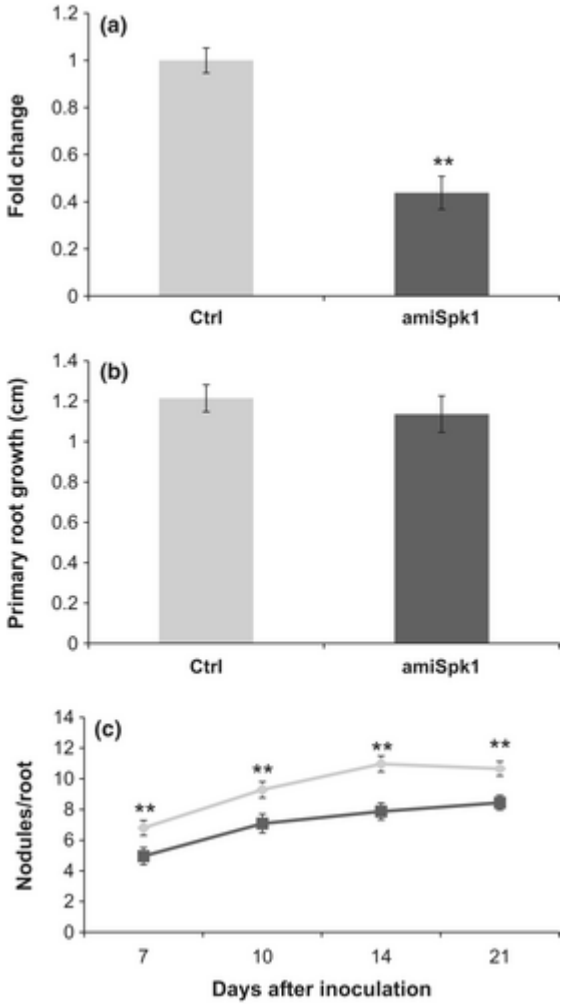

Figure 7. Artificial micro-RNA (amiRNA) *MtSpk1* phenotype. (a) *MtSpk1*expression was analysed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in composite *Medicago truncatula* plants expressing either an empty vector (control) or the amiRNA against *MtSpk1*. (b) Primary root growth of control and ami-*Spk1* composite plants. (c) Kinetics of nodule formation in control and ami-*Spk1* plants after inoculation with*Sinorhizobium meliloti*. **, $P < 0.01$ ($n > 100$ from three biological replicates). The data shown are means ± SEM from three independent biological experiments.

We investigated the putative role of *MtSpk1* by evaluating the morphology of IT in control and *amiMtSpk1* roots after infection with *S. meliloti*(Fig. 8). IT growth did not appear to be affected by a decrease in *MtSpk1* expression (Fig. S5).

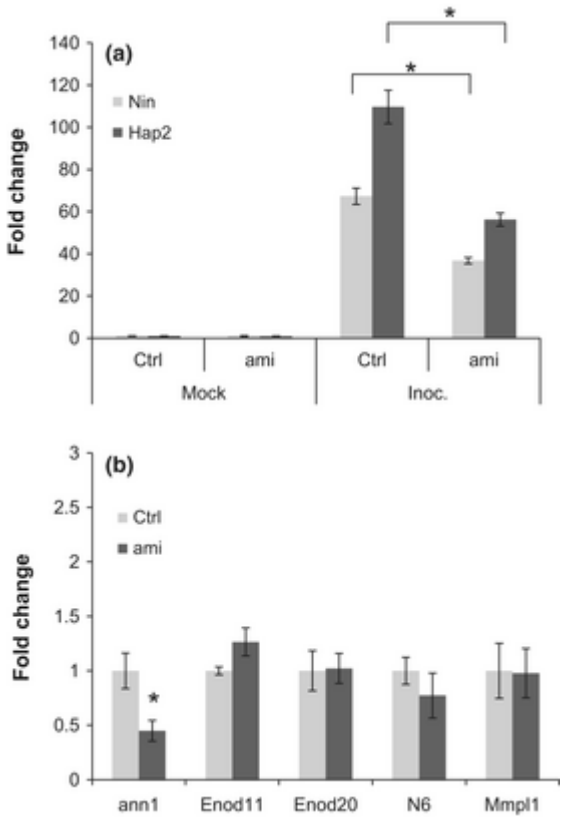

Figure 8. *Medicago truncatula* symbiotic gene expression in artificial micro-RNA (amiRNA) *MtSpk1* plants. *Medicago truncatula* composite plants were inoculated with the wild-type (WT) *Sinorhizobium meliloti* strain and the corresponding root infection zones were harvested 4 d after infection (dai). Relative expression levels of symbiotic genes were analysed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). (a) Relative expression (*amiSpk1*/ctrl) of *MtNin* and *MtHap2-1* in noninoculated plants (Mock) or composite plants inoculated (Inoc.) with the WT *S. meliloti* strain. (b) Relative expression (*amiSpk1*/ctrl) of selected *M. truncatula* genes at 4 dai. *,$P < 0.05$. The data shown are means ± SEM from three independent biological experiments.

We also determined the level of expression of genes already known to be important in nodule development, by RT-qPCR, in control and*MtSpk1* amiRNA roots. *MtSpk1* seemed to be involved in the regulation of the expression of genes (Fig. 8), such as *MtHap2-1* (Combier*et al*., 2006), *MtNin* (Marsh *et al*., 2007) and *MtAnn1* (de Carvalho Niebel *et al*., 1998). *MtHap2-1* encodes a transcription factor (TF) of the CCAAT-binding family which has been shown to play a key role in nodule development, possibly by controlling nodule meristem function (Combier *et al*., 2006). *MtNin* (nodule inception), encoding a TF, functions downstream from the early NF signalling pathway, coordinating and regulating the temporally and spatially correct formation of root nodules (Marsh *et al*., 2007). *MtAnn1* encodes a protein homologous to the calcium- and phospholipid-binding proteins of the annexin family, the production of which is induced by NF (de Carvalho Niebel *et al*.,1998). By contrast, the expression of the other genes investigated (*MtN6*, *MtEnod11*, *MtEnod20*) was not affected (Fig. 8b). Moreover, these results suggest that the ROS-driven modulation of *MtHap2-1* and *MtNin* expression may be mediated by the product of the *MtSpk1* gene. Overall, these results demonstrate the involvement of *MtSpk1* in the establishment of symbiosis.

Discussion

By using DPI to inhibit NADPH oxidases, we identified genes putatively regulated by H_2O_2 or other ROS (O_2^- or hydroxyl radical) during the establishment of the *M. truncatula–S. meliloti* symbiotic interaction. A large number of genes were found to be regulated in opposite ways by rhizobial infection and DPI (Tables 1, S2). Despite the probable side effects of DPI (indeed, flavoproteins, such as dehydrogenases, monooxygenases and other oxygenases may be DPI targets), this suggests an important role for H_2O_2 and/or other ROS in gene regulation early in the establishment of symbiosis. ROS are unlikely to regulate any particular gene directly: the changes in gene expression observed here probably result from signalling pathways involving ROS receptors, redox-sensitive TFs and Ca^{2+} signals (Mittler*et al*., 2011). In this framework, several possible H_2O_2 target genes can be identified. Some (18 genes) are involved in hormone synthesis or signalling (Table S2), consistent with the role of phytohormones in the establishment and functioning of symbiosis (Oldroyd & Downie,2008). This is the case, in particular, for genes involved in gibberellic acid (GA) (Mtr.156.1.s1_at; Mtr.25006.1.s1_at: ent-copalyl diphosphate synthase; Mtr.13370.1.S1_at: gibberellin 3-β-dioxygenase) or brassinosteroid (BR) (Mtr.25019.1.s1_at; Mtr.7754.1.s1_s_at: dihydroflavonol 4-reductase; Yuan *et al*., 2007) biosynthesis; these genes may be regulated by H_2O_2 during the establishment of symbiosis. Moreover, deregulation of the pea *Ramosus1* homologue (Mtr.37123.1.s1_s_at; Foo *et al*., 2005) may link H_2O_2 with auxin in nodule development, as suggested for root development (De Tullio *et al*., 2010).

Genes involved in cell wall synthesis (potentially related to IT progression) constitute a second group of possible targets (19 genes). Examples include genes encoding expansins (Mtr.22752.1.s1_at, Mtr.41561.1.s1_at), polygalacturonases (Mtr.31710.1.s1_at; Mtr.1427.1.s1_at), pectate lyases (Mtr.38613.1.s1_at, Mtr.26489.1.s1_at) and a cellulase (Mtr.1496.1.s1_at). Moreover, several*M. truncatula* subtilase-encoding genes (serine proteases; Mtr.5721.1.s1_s_at, Mtr.45771.1.s1_at, Mtr.45770.1.s1_at) were found to be induced. The upregulation of these genes seems to be maintained during rhizobial symbiosis (Benedito *et al*., 2008). Subtilase induction has also been demonstrated in plant–fungus interactions (Takeda *et al*., 2009) and in actinorhizal symbioses (Ribeiro *et al*., 1995; Laplaze*et al*., 2000). Indeed, a role for subtilases has been suggested in the exchange of signals between the two symbiotic partners, or in cell wall modification during IT growth (for a review, see Schaller *et al*., 2012). Similarly, modification of the cellular redox balance by glutathione depletion during nodulation has been shown to affect both hormonal balance (effects principally on auxin and ethylene) and cell wall formation (Pucciariello *et al*., 2009).

TFs are also possible targets. The *MtbHlh1* gene investigated here did not appear to be regulated by H_2O_2, but other bHLH-type TFs (Mtr.15416.1.s1_at, Mtr.24842.1.s1_at, Mtr.8357.1.s1_s_at) may display such regulation. Indeed, a bHLH (At1g10585) TF seems to be involved in the primary cellular stress responses mediated by high levels of H_2O_2 (Vanderauwera *et al*., 2005). Well-characterized TFs involved in the establishment of symbiosis are strongly affected by ROS depletion. This is the case, in particular, for NSP1/2 (Kalo *et al*.,2005; Smit *et al*., 2005), ERF (Middleton *et al*., 2007) and MtNIN (Marsh *et al*., 2007). Thus, genes encoding TFs regulated early in the interaction with *S. meliloti* may also be good candidates for regulation by H_2O_2.

Finally, 22 *M. truncatula* genes (of the 447 studied; Table S2) displayed sequence similarity to 19 *Arabidopsis* genes previously reported to be regulated by ROS (Table S4, Gadjev *et al*., 2006). These genes included those encoding jasmonic acid carboxyl methyltransferase (Mtr.407.1.S1_at), a TF (HAP2-1; Mtr.43750.1.S1_at) and a receptor kinase (Mtr.24165.1.S1_at), all of which may be of particular interest.

The *M. truncatula Hap2-1* gene encodes a CCAAT-binding TF involved in nodule development (Combier *et al*., 2006).

Moreover, the regulation of expression by H_2O_2 has been demonstrated for three genes. *MtSrl1* encodes a member of the 2OG and Fe(II)-dependent oxygenase superfamily (2-OG dioxygenases). Gechev *et al*. (2005) identified several 2-OG dioxygenases putatively regulated by H_2O_2. Van Damme *et al*. (2008) identified a gene encoding 2-OG dioxygenase (*Dmr6*; downy mildew resistant 6) as displaying an increase in expression in compatible and incompatible oomycete interactions, 1 d after inoculation. Genome-wide expression analysis of*Arabidopsis dmr6* mutants has revealed the enhanced expression of a subset of defence-associated genes, including *dmr6* itself, suggesting that DMR6-mediated resistance results from the activation of plant defence responses during interactions with plant oomycetes (Van Damme *et al*., 2008). Similarly, the early induction of *MtSrl1* expression by *S. meliloti* may downregulate plant defence reactions to facilitate the establishment of the symbiotic interaction.

ITs are produced during the *M. truncatula–S. meliloti* rhizobial symbiosis. These unique structures are associated with bacterial colonization in *c*. 75% of legume–*Rhizobium* symbioses (Sprent, 2007). They are invaginations of the plant cell membrane that grow towards the poles of the cell, with the cell wall matrix on the inside of the IT. Given the importance of the cytoskeleton in directing cell division and cell growth, IT development would be expected to require dynamic cytoskeleton rearrangements (Miyahara *et al*., 2010; Oldroyd *et al*., 2011). We found that a gene encoding an ABL-like protein (ABlL; Mtr.20281.1.S1_at; *MtAblL*) was regulated by *S. meliloti*infection and H_2O_2. The protein encoded by this gene is a subunit of the WAVE complex, which is involved in actin microfilament nucleation and branching (Szymanski, 2005). Loss-of-function mutations have recently been described in genes encoding proteins involved in actin rearrangements in leguminous plants (Yokota *et al*., 2009; Miyahara *et al*., 2010). Interestingly, the corresponding mutant plants have defects in the polar growth of root hairs and the formation and maintenance of ITs. The regulation of *MtAblL* by H_2O_2 may contribute to IT growth.

MtSpk1 appears to be induced by both NF and H_2O_2 treatment. Transcriptional fusion experiments confirmed that *MtSpk1* was expressed early in *S. meliloti* infection (Fig. 4c,d). Moreover, this gene was clearly expressed in the infection zone (Fig. 4e). We used the HyPer protein probe (Belousov *et al*., 2006) for the imaging of H_2O_2 accumulation in nodules. The genetic nature of the mechanism used to produce the HyPer probe means that this specific fluorescent probe can be expressed anywhere in the cell. It has been successfully targeted to plant peroxisomes, for example (Costa *et al*., 2010). With this ratiometric fluorescent probe, we detected a doubling of H_2O_2 levels in the infection zone (Fig. 5) in which *MtSpk1* was strongly expressed (Fig. 4e). This suggests that H_2O_2 may regulate *MtSpk1* expression *in vivo*. The*MtRbohB* gene is a good candidate for involvement in this H_2O_2 production, as it has been shown to be expressed in the same zone (Marino *et al*., 2011).

Inactivation of the *MtSpk1* gene resulted in significantly lower nodule numbers, with no effect on root development (Fig. 7), suggesting a role for *MtSpk1* in the establishment of the symbiotic interaction. Several receptor kinases participate in the NF signalling cascade (Oldroyd & Downie, 2004), but only a few reports have highlighted the involvement of soluble kinases in the *M. truncatula* symbiotic interaction (Gargantini *et al*., 2006). The presence of a putative calmodulin recruitment motif in the N-terminal region of MtSPK1 suggests that this protein may be involved in cross-talk with the well-known calcium signalling process occurring during rhizobial symbiosis (Oldroyd *et al*.,2011). Alternatively, *in silico* analysis of the MtSPK1 sequence suggests that this protein may be located in the nucleus (Chou & Shen,2010). Subcellular localization appeared to confirm this (Fig. S6), pointing to a role as a putative transcriptional regulator. This

situation is reminiscent of that for the *Arabidopsis* OXI1 gene, which encodes a serine/threonine kinase. This gene is induced in response to diverse H_2O_2-generating stimuli, including plant–microbe interactions (Rentel *et al*., 2004). OXI1 has been shown to be an essential part of the signal transduction pathway linking oxidative burst signals to diverse downstream responses (Rentel *et al*., 2004). Thus, overall, our results suggest that *MtSpk1* encodes a protein with an important role in the signalling processes associated with the establishment of rhizobial symbiosis. The results obtained here (Fig. 8) suggest that *MtSpk1* may be involved in the differentiation of nodule cells, via *MtHap2-1*(Combier *et al*., 2006), and in the correct formation of root nodules, via *MtNin* (Marsh *et al*., 2007), consistent with its localization in nodule primordia.

In conclusion, this work provides a first list of the genes putatively regulated by H_2O_2 during the establishment of symbiotic interactions. Moreover, to our knowledge, it also identifies the first gene involved in the early stages of symbiosis to be regulated by both H_2O_2 and NF. Further studies are required to determine the precise function of the protein encoded by this gene in the NF signalling cascade. Finally, the identification of molecular targets of MtSPK1 should make it possible to elucidate the role of this protein in plant–microsymbiont communication.

Acknowledgements

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