Group VII Ethylene Response Factors, MtERF74 and MtERF75, sustain nitrogen fixation in Medicago truncatula microoxic nodules

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Abstract

Group VII Ethylene Response Factors (ERF-VII) are plant-specific transcription factors (TFs) known for their role in the activation of hypoxia-responsive genes under low oxygen stress but also in plant endogenous hypoxic niches. However, their function in the microaerophilic nitrogen-fixing nodules of legumes has not yet been investigated. We investigated regulation and the function of the two *Medicago truncatula* ERF-VII TFs (*MtERF74* and *MtERF75*) in roots and nodules, *MtERF74* and *MtERF75* in response to hypoxia stress and during the nodulation process using an RNA interference strategy and targeted proteolysis of MtERF75. Knockdown of *MtERF74* and *MtERF75* partially blocked the induction of hypoxia-responsive genes in roots exposed to hypoxia stress. In addition, a significant reduction in nodulation capacity and nitrogen fixation activity was observed in mature nodules of double knockdown transgenic roots. Overall, the results indicate that MtERF74 and MtERF75 are involved in the induction of *MtNR1* and *Pgb1.1* expression for efficient Phytogb-NO respiration in the nodule.

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Summary

Group VII Ethylene Response Factors (ERF-VII) are plant-specific transcription factors (TFs) known for their role in the activation of hypoxia-responsive genes under low oxygen stress but also in plant endogenous hypoxic niches. However, their function in the microaerophilic nitrogen-fixing nodules of legumes has not yet been investigated. We investigated regulation and the function of the two*Medicago truncatula* ERF-VII TFs (*MtERF74* and*MtERF75*) in roots and nodules, *MtERF74* and *MtERF75*in response to hypoxia stress and during the nodulation process using an RNA interference strategy and targeted proteolysis of MtERF75.

Knockdown of *MtERF74* and *MtERF75* partially blocked the induction of hypoxia-responsive genes in roots exposed to hypoxia stress. In addition, a significant reduction in nodulation capacity and nitrogen fixation activity was observed in mature nodules of double knockdown transgenic roots. Overall, the results indicate that MtERF74 and MtERF75 are involved in the induction of *MtNR1* and*Pgb1.1* expression for efficient Phytogb-NO respiration in the nodule.

Keywords: Ethylene Response Factors Group VII family; hypoxia-responsive genes (HRGs); N-degron; nitrogen fixation; O_2-sensing; hypoxia stress; *Rhizobium* legumes symbiosis

Introduction

Legume crops belonging to the *Leguminosae* (*Fabaceae*), are known for their ability to form a symbiotic relationship with nitrogen-fixing rhizobia. This mutualism culminates in the formation of a new plant organ, the root nodule, in which bacterial nitrogenase converts atmospheric nitrogen (N_2) into ammonia (NH_3) that can be directly consumed by plants (Postgate, 1982). This interaction leads to a reduced need for N_2fertiliser, which is important for sustainable agriculture (Biswas and Gresshoff, 2014). In nodules, N_2 fixation is O_2 sensitive because nitrogenase is irreversibly inhibited by O_2 (Appleby, 1992). Therefore, it is important to keep O_2 levels low enough in the central nodule region, which is characterised by N_2-fixing symbiotic cells. In nodules of *Lotus japonicus* and *Medicago sativa* , a steep O_2 gradient from the surface to the innermost part was observed, ranging from 250 µM to 10-40 nM and characterised by high bacterial and mitochondrial respiration rates (Ott et al., 2009, 2005; Soupène et al., 1995). Legume nodules have evolved mechanisms to maintain low O_2 levels by differentiating an O_2 diffusion barrier and expressing symbiotic plant leghemoglobin (Lb), which regulates the rapid transport of O_2 to the site of respiration (Hunt and Layzell, 1993). Indeed, knockdown of *Lb* by RNA interference in *L. japonicus* increased the level of free O_2 in the nodule, dramatically reduced the amount of nitrogenase protein and suppress N_2-fixation (Ott et al., 2005). At the same time, nodules must maintain a high level of ATP for nitrogenase and N_2 fixation activities, which are very energy demanding. Thus, a balance must be achieved between stringent defence against O_2 and efficient energy production, referred to as the "O_2 paradox" of N_2-fixing legume nodules (Berger et al., 2019).

Molecular O_2 usually is the main substrate for energy production by mitochondrial respiration in aerobic organisms. When exposed to low O_2 environments plants rapidly adjust their metabolism to avoid energy crisis (Bailey-Serres et al., 2012; Loreti et al., 2016). The absence of O_2 as a terminal electron acceptor for the electron transport chain is associated to ATP production through glycolytic flux and subsequent regeneration of NAD^+ by the fermentation of pyruvate to ethanol via pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Perata and Alpi, 1993). In addition, a phytoglobin – nitric oxide respiration (Pgb-NO respiration) that allows ATP regenerative capacity even in the presence of low O_2 levels has been shown to occur in roots and nodules under hypoxia (Berger et al., 2021, 2019; Hichri et al., 2015;

Horchani et al., 2011). In this process, nitrite (NO_2^-) acts as the final electron acceptor instead of O_2 and is reduced to nitric oxide (NO) by the cytochrome oxidase of the mitochondrial electron transfer chain (mETC) (Igamberdiev and Hill, 2009). The changes caused by the switch from aerobic to hypoxic state have been extensively analysed in plants (for a review, see Voesenek and Bailey-Serres, (2015)). Considered together, all plant cells and organs exhibit a conserved response to low O_2 at the molecular level (Mustroph et al., 2009). This response involves the induction of a group of genes shortly after exposure to low O_2 stress, termed core anaerobic genes or hypoxia-responsive genes (HRG) (Mustroph et al., 2010, 2009), whose expression is coordinated by a family of transcription factors (TFs) belonging to the group VII Ethylene Response Factors (ERF-VII) (Gibbs et al., 2011; Licausi et al., 2011). In Arabidopsis, the ERF-VII family is composed of five elements:*RELATED TO APETALA2.2* (*RAP2.2*), *RAP2.3* , and*RAP2.12* are constitutively expressed, whereas *HYPOXIA RESPONSIVE1* (*HRE1*) and *HRE2* are induced by low oxygen (Gibbs et al., 2011; Licausi et al., 2011). Knockdown and knockout of*HRE1* and *HRE2* genes reduce plant resistance to anoxia stress (Hess et al., 2011; Yang et al., 2011), although there is evidence that HRE1 and HRE2 are not direct initial activators of*HRG* genes (Bui et al., 2015). The *rap2.2rap2.12* null double mutant shows a significant reduction in activating *HRG*genes under hypoxia, suggesting that RAP2.2 and RAP2.12 redundantly function as major triggers of most HRGs (Bui et al., 2015; Gasch et al., 2016; Hinz et al., 2010; Licausi et al., 2010; Xu et al., 2006).

Previous studies have shown that the conserved N-terminal motif (Met-Cys) of ERF-VII TFs is subject to targeted protein degradation by the N-degron-dependent ubiquitin-proteasome system in an O_2-dependent manner (Gibbs et al., 2014a, 2011; Licausi, 2011), with an enzymatic mechanism that is based on the activity of Plant Cysteine Oxydase (Weits et al., 2014; White et al., 2017). In addition to a function in the response to hypoxia stress, regulation by the N-degron pathway of ERF-VIIs is an important component of plant responses to various biotic and abiotic stresses (Papdi et al., 2015; Vicente et al., 2017). Recently, hypoxic niches have been shown to affect developmental processes, regulating leaf organogenesis in Arabidopsis shoot apical meristem (SAM, (Weits et al., 2019)) and lateral root development in lateral root primordia (LRP, (Shukla et al., 2019)). In LRP, ERF-VIIs control root architecture through the repression of key auxin-induced genes. There is evidence of local hypoxia establishment during plant-microbe interactions. In fact, Gravot et al. (2016) reported that ERF-VII TFs are necessary to support cell proliferation in crown galls and clubroots, most likely to promote metabolic adaptation to chronic hypoxia. The stabilisation of ERF-VIIs has also been suggested to be involved in Arabidopsis crown gall tumors formed upon *Agrobacterium tumefaciens* infection (Kerpen et al., 2019).

ERF-VII proteins have a characteristic N-terminus (N-degron) with a cysteine (Cys) residue at the second position that leads to specific degradation by the N-degron-dependent ubiquitin proteasome system (UPS, (Varshavsky, 2011)). In this pathway, methionine aminopeptidase (MetAP) activity removes terminal methionine, and the resulting terminal Cys is eventually oxidized to Cys-sulfenic acid by the action of plant cysteine oxidases (PCOs) in O_2-dependent (Weits et al., 2014; White et al., 2017). This triggers conjugation of the primary destabilizing Arg residue by Arg-tRNA transferase (ATE1/2) and subsequent ubiquitination by E3 ligase proteolysis 6 (PRT6), which targets the protein to the 26S proteasome (Garzón et al., 2007). Analysis of ERF-VII stability revealed that both NO and O_2 are required for destabilization of the protein through the N-degron pathway and that a decrease in NO is accompanied by stabilization of the ERF-VIIs (Gibbs et al., 2014b; Hu et al., 2005). However, the precise mechanism by which NO controls the stability of these TFs and the relationship between NO and O_2 during these processes remain to be elucidated.

We have identified four genes in the *Medicago truncatula* genome (*MtERF72, 73, 74* and *75*) that belong to the ERF-VII TF family and show strong similarity to ERF-VII from Arabidopsis and soybean. Using a double knockdown RNAi strategy, we investigated the role of two constitutively expressed genes, *MtERF74* and*MtERF75* , in *M. truncatula* in both roots exposed to low O_2 stress and nodules. Knockdown of *MtERF74* and*MtERF75* dampened the induction of hypoxia-responsive genes in roots exposed to low O_2 stress. In addition, a reduction in the number of nodulation and nitrogen fixation activity was observed in mature nodules, suggesting a crucial function of ERF-VIIs.

Materials and methods

Plants and growth conditions

Seeds of *M. truncatula* cv Jemalong A17 were surface-sterilized, germinated and transformed with binary vectors via *Agrobacterium rhizogenes* strain *Arqua1* (Quandt et al., 1993) according to Boisson-Dernier *et al.* (2001). Transgenic roots were selected under a Leica MZ FLIII fluorescence stereomicroscope (Leica, Wetzlar, Germany) based on the GFP signal two weeks after germination. After removing non-transgenic roots, composite plants were transferred to new plates with Fahräeus medium and 0.2 mM NH_4NO_3. One week after the transfer, plants were inoculated with *Sinorhizobium meliloti* at OD600 0.5 as described by Boisson-Dernier et al. (2001).

Hypoxia was performed by placing plants in hypoxic workstations, where the normal air was replaced with 100% N_2, and closed for 24 hours, in the dark. In control conditions, plants were placed in the dark for 24 hours.

For the experiment involving *A. thaliana* mesophyll protoplasts, Columbia-0 (Col-0) and *prt6* knockout Arabidopsis mutant seeds (Licausi *et al.* , 2011) were sown in a growing mixture containing soil (Spezial Substrate, Hawita Flor, Vechta, Germany) and perlite (3:1) (Agrilit, Perlite Italiana, srl) . Plants were grown at 25/23 degC day/night with a photoperiod of 12 h light (PAR 100 µmol $m^{-2}s^{-1}$) for 3 weeks.

Bacterial strains and growth conditions

Escherichia coli DH5α strains were grown at 37°C in Luria Bertani medium [LB medium: 30 g/L bacto-trypone, 15 g/L yeast extract and 30 g/L sodium chloride (NaCl) at pH 7.5] and supplemented with specific antibiotics: ampicillin 50 µg/ml, kanamycin 50 µg/ml. *A. rhizogenes* Arqua1 strains were grown at 28 °C in TY medium [5 g/L bacto-trypone, 3 g/L yeast extract and 6 mM of calcium chloride ($CaCl_2$)] supplemented with spectinomycin and streptomycin (100 µg/ml). *S. meliloti* RCR2011 were grown at 30°C in LB medium complemented with 2.5 mM $CaCl_2$, 2.5 mM magnesium sulphate ($MgSO_4$).

Plasmid constructions

The N-term sequence (first 240 bp) of the *MtERF75* gene was amplified by PCR reaction from *M. truncatula* cDNA template with specific primers (MC80F and MC80R, Table S1) using Phusion™ High Fidelity DNA-polymerase (New England Biolabs Inc. USA). A second forward primer (MA80F) was also used to introduce an Ala residue in place of the Cys residue in second position (MA80 construction). Both sequences were cloned in *Aat* II and*Nco* I restriction sites in 5' and 3' end respectively of pGEM®-T (Promega, USA) vector by ligation. The firefly luciferase (luc) reporter gene was obtained from gst1::luc pART7 vector (Grant *et al.* , 2000) and introduced between the *Nco* I and *Not* I restriction site of the previous constructs pGEMt-MA80 or pGEMt-MC80 by ligation. The resulting pGE-MC/MA::LUC vectors have been digested with*Bam* HI and *Not* I to transfer the constructions in a pENTR4 entry vector. Both constructs were then transferred in p2GW7 destination vector with Gateway® LR Clonase II Enzyme Mix (Invitrogen, USA) following the manufacturer's recommendations.

For C-terminal GFP fusion, a PCR reaction was used to amplify the full length ORF of *ERF75* from cDNA of *M. truncatula* with primers ERF75_MC_F and ERF75_GFP_R (lacking of stop codon). The PCR product was cloned in pDONR207 vector by BP recombination. For C-terminal GFP fusion, the resulting vector (pDO-ERF75Δstop) was recombined into pK7FWG2 destination vector (carrying a GFP gene) by an LR recombination reaction.

For the RNAi construct, a common region of 165 nt (Fig. S1), found in the two target genes *MtERF74* and *MtERF75* was amplified from the complete cDNA of *M. truncatula* by PCR using the primers RNAi74/75F and RNAi74/75R. This sequence was first introduced into the pJET1.2/blunt cloning vector (Thermo Fisher Scientific, USA), then in the pENTR4 vector by ligation between the *Xho* I and *Kpn* I sites, and finally in the pK7GWIW2D vector (Karimi *et al.* , 2002), able to produce the hairpin structure for the gene silencing,

by LR recombination. As control for the RNAi experiment, a pk7WΔCnt (Marino*et al.* , 2011) with a fragment of *lacZ* gene from *E. coli* in pK7GWIW2D vector was used.

All the primers sequences and vectors are provided in Table S1 and Table S2, respectively.

Gene expression analysis

Harvested nodules and roots were frozen in liquid nitrogen and ground. Total RNA extraction was performed using the RNAzol ® RT reagent following the manufacturer's recommendations (Sigma-Aldrich, USA). After the DNAse treatment with RQ1 DNAse (Promega, USA), 1 µg or total RNA was used for the cDNA synthesis (GoScript Reverse Transcription System, Promega). Quantitative real-time PCRs were performed in the Agilent AriaMx System using the GoTaq® qPCR Master Mix (Promega, USA). Two reference genes (MtC27 and a38) were used to normalize the transcript level. The complete list of primers is reported in Table S1. Data were quantified using the Aria Software and analyzed with RqPCRBase, an R package working on R computing environment for analysis of quantitative real-time PCR data (Rancurel et al., 2019).

Luciferase activity assay

Arabidopsis thaliana protoplasts were prepared according to the method described in Yoo et al. (2007) and transfected using 5 µg plasmid DNA of p2G-MC/MA80_75:LUC and p2GRenLuc (Licausi *et al.* , 2011). The luciferase activity assays were performed directly on protoplast suspension with Dual-Glo® Luciferase Assay System (Promega). The luminescent signal was measured on a Xenius XL luminometer (SAFAS, Monaco) and values normalized against the Renilla luminescence. The data are represented as the ratio of firefly to *Renilla* luciferase activity (Fluc/Rluc).

Localization of GFP in protoplasts

Protoplasts were prepared according to Yoo *et al* . (2007), with minor modifications. The digitate shape of Medicago's leaves and the high density in venation imposed to cut between venation. We were able to get a yield of about 2.10^6 protoplasts from 0.5 g of leaves. Transfection with a *35S:ERF75:GFP* construct were observed with a LSM 880 – confocal microscope (Zeiss, Germany) using laser wavelengths excitation for GFP (Laser Argon 458, 488 et 514 nm, 25 mW) and for chlorophyll autofluorescence excitation (Laser He-Ne 633nm, 5 mW). The *pAVA* vector (von Arnim et al., 1998) was used as a 35S:GFP control. Images were analyzed with ZEN 2 software (Zeiss, Germany).

Phylogenetic analysis

Phylogenetic relatedness of ERF-VII proteins was analysed using MEGA 7 (Kumar *et al.* , 2016). The full length of the coding regions (amino acid sequences) was aligned using Clustal Omega (Sievers *et al.* , 2014). A phylogenetic tree was constructed by the Neighbour-Joining method with Poisson correction and pairwise deletion of gaps. The reliability of the output phylogeny was estimated through bootstrap analysis with 1000 replicates.

Nitrogen-fixing capacity

The nitrogen-fixing capacity of nodules was determined *in vivo* by measuring the reduction of acetylene to ethylene, as previously described as acetylene-reducing activity (Hardy *et al.* , 1968). Nodulated roots were harvested and incubated at 30 degC for 1 h in rubber-capped tubes containing a 10% acetylene atmosphere. Two biological replicates have been performed with five technical replicates. Ethylene concentrations were determined by gas chromatography (Agilent GC 6890N, Agilent Technologies, Santa Clara, CA, USA) equipped with a GS-Alumina (30 m x 0.534 mm) separating capillary column.

Mass spectrometry

Two synthesized peptides, MR21 (MCGGAIISDFIPAAAAVGGSR) and CR20 (CGGAIISDFIPAAAAVGGSR) (Proteogenix, Schiltigheim, France) were treated with 0, 5, 10, 50, 100 and 500 µM of Diethylamine NONOate (Sigma-Aldrich, USA) for 5 min at RT. The sample is taken up in acetonitrile and formic acid (50% and 0.1% final respectively) and immediately injected into the ionization source of an ESI-Q-TOF

mass spectrometer (micrOTOF-Q II, Brucker Daltonics, Germany) precalibrated with a mixture of standard peptides (ESI-Low concentration Tunning Mix, Agilent Technologies, USA). Data were acquired in positive ionisation and in MS and MS/MS scan modes. Analysis parameters were optimized to maintain and to be able to detect a maximum of Cys modifications, particularly S-nitrosylation. The source temperature was 180°C, the spray voltage was 5 kV, nebuliser gas (nitrogen) was set at 2.9 bar, and dry gaz (nitrogen) was set at 10 L/min. Mass spectra acquisition corresponded to 5000 spectra/sec on a mass range of 50e3000 m/z. LC-MS raw data were processed using Data Analysis software (ESI Compass 1.3, Bruker Daltonics, Germany). The identification of peptides was performed using Biotools 3.2 and Sequence Editor Bruker's software. Collected spectrum data were compiled and presented with the SigmaPlot 10.0 software (Systat Software Inc, USA).

Statistical Analyses

All statistical analysis were generated using the Real Statistics Resource Pack software (Release 5.4) (*http://www.real-statistics.com/*).

Results

MtERF74/75 **are orthologues of RAP2.2 and RAP2.12 ERF-VII genes.**

Four genes were identified in *M. truncatula* as belonging to the ERF-VII TF family (Fig. 1a), all of which possess the conserved N-terminal MCGGAI(I/L) amino acid motif (Nakano *et al.* , 2006). A phylogenetic tree was constructed to show the evolutionary relationship in the ERF-VII family between *M. truncatula* , *A. thaliana* ,*Glycine max* and *Oryza sativa.* Phylogenetic analysis of the ERF-VII protein sequences from *M. truncatula* , soybean, Arabidopsis and rice was performed using the Neighbour-Joining method (Fig. 1b). The resulting phylogenetic tree showed a clear separation between rice ERF-VII (SUB1 or SK orthologs) and ERF-VII proteins from the three other species. ERF-VII members from *M. truncatula* and soybean were distributed into three distinct clades. In the first clade, the gene showing sequence similarity to *AtRAP2.3* was named*MtERF72* while the putative *Medicago* orthologous of*AtHRE2* was named *MtERF73* (Table S3). In both legume species, no sequences homologous to Arabidopsis *Hypoxia Responsive ERF1* (*HRE1*) (Tamang *et al.* , 2014) were identified (Fig. 1b). In the third clade, the two closest homologous sequences have been named *MtERF74* and *MtERF75* , as their orthologous in Arabidopsis, *AtRAP2.12* and *AtRAP2.2* (Gibbs et al., 2011; Licausi et al., 2011)(Table S3).

In silico gene expression analysis using MtExpress, a gene expression atlas that compiles an exhaustive set of published *M. truncatula* RNAseq data (*https://medicago.toulouse.inrae.fr/MtExpress*), revealed that the four *ERF-VII* genes were expressed in stems, leaves, seeds and roots, and also in nodules at different developmental stages (Fig. S2). *MtERF72* expression was higher in mature seeds, stems, and roots whereas *MtERF73* expression was strongly up-regulated during nodule development. *MtERF74* and*MtERF75* were found to be constitutively expressed in these different organs. However, *MtERF75* was expressed approximately 8-fold more strongly than *MtERF74* (Fig. S2). These observations were confirmed by a qPCR experiment performed in the roots and nodules of *M. truncatula* . *MtERF73* expression was increased 8-fold in nodules compared with non-inoculated roots (Fig. 2a) and was strongly induced (6-fold) in roots after 24 hours of hypoxia (Fig. 2b). Using the Symbimics database (*https://iant.toulouse.inra.fr/symbimics*(Roux *et al.* , 2014)) available on MtExpress database, we obtained also data on the expression of *ERF-VII* mRNAs in the different zones of *M. truncatula* nodules (Fig. S2). *MtERF72* showed higher expression in the zone I and II of the nodule, whereas*MtERF73* was found in the interzone II-III (IZ) and N_2-fixing zone III (Fig. S2). As previously observed for the different organs, *MtERF74* and *MtERF75* were found identically expressed in the different zones of the nodule.

MtERF74/75 **regulates hypoxia response genes in Medicago roots**

Constitutive expression of *M. truncatula MtERF74* and*ERF75* recalls the expression profile of *RAP2.2* and*RAP2.12* in Arabidopsis. Both constitutively synthesized TFs are described to play redundantly as main activators of core hypoxia-responsive genes in Arabidopsis (Gasch et al., 2016). To determine if MtERF74

and MtERF75 can also induce the expression of hypoxic stress in *M. truncatula* , *MtERF74/75* knock-down roots were generated by RNA interference, through targeting a specific region of their N-terminal part (Fig. S1). The construction of the double mutant was preferred due to the technical difficulty of performing the two single mutants in view of the level of identity between the two sequences (85% identities). We assessed the effectiveness and specificity of the *MtERF74/75* RNAi constructs by qPCR analysis. *MtERF74* and *MtERF75* transcript levels measured in independent non stressed *MtERF74/75* RNAi transgenic roots were 37 % and 47 % reduced when compared to control transgenic roots (Fig. S3). Furthermore, *MtERF72* and *MtERF73*expression were not affected in *MtERF74/75* RNAi construct showing the specificity of this construction for *MtERF74* and*MtERF75* . Concerning, *MtERF74/75* RNAi transgenic roots phenotype, root growth and secondary roots number showed respectively 29% and 18% reduction as compared to the control transgenic roots in aerobic condition (Fig. S4). Transgenic roots were then analyzed under hypoxic stress conditions. We exposed our transgenic roots knock-down for *MtERF74* and *MtERF75* to 24 hours of hypoxic stress in hypoxic chamber. The expression analysis of several *HRGs* , namely*ADH1* , *PDC1* , *Pgb1.1* , *AlaAT* , and *ERF73*(Mustroph et al., 2010) showed a reduction in transcript levels in the*MtERF74/75* RNAi transgenic roots, with a repression ranging from 50% (*ADH1*) to 24% (*AlaAT*) compared to the GUS RNAi control (Fig. 3 and S5). Thus, the results showed that ERF74 and/or ERF75 contribute to hypoxia-dependent gene activation in *M. truncatula* .

MtERF75/74 regulates nodule number and size and nitrogen fixation

MtERF74/75 RNAi transgenic roots were analyzed for their ability to establish a symbiotic interaction. Transformed composite plants were grown *in vitro* and nodulation efficiency was analyzed after inoculation with *S. meliloti* . At two weeks post-infection, the nodule number per plants in the *MtERF74/75* double knock-down roots was 30% reduced compared to the GUS RNAi control (Fig. 4a). At 3 weeks post-infection, furthermore, the N_2 fixation capacity of mature nodules from *MtERF74/75* RNAi transgenic roots was 52% reduced compared to the control (Fig. 4b), and a 17.6 % reduction in nodule size was observed in the *MtERF74/75* RNAi transgenic roots (Fig. 4c).

To get the respective role of *MtERF74* and *MtERF75* in symbiotic interaction, we looked at the Medicago *Tnt1* -insertion mutant population at the Samuel Roberts Noble Foundation. We identified one *Tnt1* -insertion line for the *MtERF74* . Unfortunately, no tnt1 insertion was available in *MtERF75* gene. After validation by qPCR of the invalidation of the gene (Fig. S4A), the mutant line*MtERF74-1^{ko}* was backcrossed 4 times to clean the genetic background of this mutant. However, this*MtERF74-1^{ko}* mutant did not show any phenotype on root growth, nodulation, biomass production or nitrogen fixation (Fig. S4BCD). This absence of phenotype could be explained by a functional redundancy between the MtERF74 and MtERF75, even if the higher level of *MtERF75* expression could also point for a more important role of MtERF75 in the process of nodulation (Fig. S2).

The strong impairment of N_2 fixation by the double knockdown of *MtERF74/75* (Fig. 4b) argues for a key role of these two TFs in the proper functioning of the nodule. In this context, the expression of different *HRG* genes was analyzed in 3 weeks post-inoculation control and *MtERF74/75* transgenic nodules (Fig. 5). As already observed in the hypoxic roots (Fig. 3), the expression of*ADH1* , *PDC1* , *Pgb1.1* , and *ERF73* was affected in the nodules of the double knockdown root with a reduction of 77%, 40%, 39%, and 35%, respectively, compared with the control. Expression of *VPE* and *CP6* , which are markers of nodule senescence (Pierre et al., 2014), and *leghemoglobin4*(*Lb4*), which is a marker of N_2 fixation (Berger et al., 2020b), was unaffected in the transgenic *MtERF74/75* RNAi nodules.

The expression of *NR1* , but not *NR2* , was greatly reduced in the double knockdown nodules, by almost 80%. Knowing the importance of the activity of NR for the production of NO (Berger et al., 2020a), we also examined the expression of marker genes that are regulated in function of NO level in *M. truncatula* . The expression of a glutathione S-transferase (*GST*), whose expression is associated with the defense response during nodule organogenesis (Boscari et al., 2013), was reduced by 40% in *MtERF74/75* RNAi transgenic nodules (Fig. 5). Analysis of NO production showed a 32% reduction in NO emission by transgenic nodules compared with the control (Fig. 6).

N-degron pathway of proteolysis conducts MtERF75 protein stability

ERF-VII proteins have a characteristic N-terminus (N-degron) with a cysteine (Cys) residue at the second position that leads to specific degradation by the N-degron-dependent ubiquitin proteasome system (UPS) in Arabidopsis. We tested whether the N-terminal part of MtERF conduct to targeted degradation by the N-degron pathway of proteolysis. Two constructions comprising the first 80 amino acids of MtERF75 were fused to the luciferase sequence (LUC). One version (MC80_ERF75) was characterized by the N-terminal Cys residue in MtERF75, whereas in MA80_ERF75 the Cys residue was replaced by an Ala that is insensitive to O_2. The measurement of the activity of LUC reveals the presence and thus the stability of the protein. The MC /MA80_ERF75: LUC constructs were tested in *A. thaliana* protoplasts of wild type (Col-0) (Fig. 7a) and a mutant line lacking the E3 ligase (*prt6*) involved in the N-degron pathway of proteolysis (Fig. 7b). Experiments with the MA:80_ERF75: LUC construct in Col-0 protoplasts showed a significant increase in luciferase activity compared with MC80_ERF75: LUC, indicating the destabilizing role of Cys. Similarly, the luciferase signal was also higher in protoplasts of *prt6*plants compared with Col-0, indicating that the stability of the MC80_MtERF75: LUC chimeric protein is regulated by the N-degron pathway.

Subcellular localization of MtERF75 in *M. truncatula*protoplast

Subcellular localization of MtERF75 was investigated in *M. truncatula* protoplasts using a MtERF75 protein fused to the green fluorescent protein (GFP). GFP signal of the pAVA control vector (von Arnim et al., 1998) was observed mainly in cytoplasm by confocal microscopy. In contrast, *M. truncatula* protoplasts transformed with MC_ERF75:GFP fusion protein present a clear fluorescent signal located in a single point in the cell (Fig. 8) suggesting the presence of the protein in the nucleus even in absence of hypoxic stress. A second construction, MA_ERF75:GFP fusion, where the Cys residue was replaced by an Ala, was used to determine the role of the N-terminal Cys residues in the subcellular localization of ERF75 fused to GFP. A similar subcellular localization of the fluorescence was observed with MA_ERF75:GFP fusion to that with the MC_-ERF75:GFP (Fig. S6A). However, we counted much more fluorescent protoplast after transformation with MA_ERF75:GFP construction (Fig. S6B). A statistical difference which could be due to a higher stability of the protein when the regulation by the N-end rule pathway is blocked.

Post-translational modification of N-terminal peptide of MtERF75

In *A. thaliana* , RAP2.2/RAP2.12 degradation has been shown to be dependent on the *in vivo* oxidation of its N-terminal cysteine, after the removal of the N-terminal methionine by a MetAP, and that this oxidation requires NO (Gibbs et al., 2014b; Hu et al., 2005). In addition, NO is produced significantly in N_2-fixing zone of mature nodules (Baudouin et al., 2006; Shimoda et al., 2009). The absence of proof of the S-nitrosylation of the Cys N-term of ERF-VII led us to investigate the direct post-translational modification of the N-terminal part of ERF75 by NO. The S-nitrosylation of Cys residue by NO has been analysed by mass spectrometry on synthetic peptides corresponding to the first 20 amino acids of MtERF75 protein (MR21) and without the first methionine (CR20). By this direct MS approach, we determined the level S-nitrosylation of these peptides after 5 min treatment with 0, 5, 10, 50, 100 and 500 µM of the NO donor DEA-NONOate (**Fig. 9**). Results showed a potential higher sensitivity of Cys residue to S-nitrosylation in CR20 (10 fold) compared to MR21. Indeed, at 50 µM of NO donor 25% of CR20 peptide were S-nitrosylated against only 5% for MR21 peptide. The percentage of S-nitrosylation rises to 60% when the peptides are incubated with 500 µM, but at this dose the same effect is observed on the 2 peptides. Dimerization, due to formation of disulfide linkages between monomers, was also enhanced in CR20 compared to MR21.

Discussion

Transcription factors of the ERF-VII family have been shown to be key regulators in plant responses to low oxygen stress (Gibbs *et al.* , 2011; Licausi *et al.* , 2011), and there is evidence that hypoxic niches are central in specific developmental programs (Shukla et al., 2019; Weits et al., 2021).

We identified four genes in the *M. truncatula* genome that belong to the ERF-VII TF family. Using a genome-wide analysis of the *M. truncatula* genome, Shu et al. (2016) identified 123 putative AP2/ERF genes, which

were designated *MtERF1-123* . Unfortunately,*MtERF73* (Medtr1g087920) was missing from this analysis, which led us to propose a different nomenclature based on phylogenetic analysis with well-characterized orthologs in Arabidopsis (Table S3). A phylogenetic analysis showed a clear separation between rice ERF-VII involved in submergence tolerance (SUB1 and SK orthologs) and ERF-VII proteins from *Glycine max* . Moreover, the separation between the different clades preceded the separation between *Fabales* and*Brassicales* which would be in favor of clade-specific specialization. Transcriptional analysis revealed that only*MtERF73* is up-regulated by hypoxia stress, such as HREs ortholog in Arabidopsis (Licausi et al., 2010), and during nodule development.*MtERF73* was found to be expressed mainly in the nodule zone III, which is microoxic (Appleby, 1992). The hypoxia-inducible ERF-VIIs*HRE1* and *HRE2* in Arabidopsis likely maintain the transcription of HRGs, while the ERF-VII RAPs initiate their up-regulation (Bui et al., 2015; Licausi et al., 2010), the presence of MtERF73 in microaerophilic nodule tissues suggests a role in long-term adaptation to microoxic conditions. Future work could clarify the precise role of MtERF73 in metabolic changes and developmental reprogramming under the conditions observed in microoxic nodules.

It should be emphasized that, constitutively expressed *MtERF74* and *MtERF75* , derived from recent duplications like their ortholog in different plant species analysed (van VEEN et al., 2014). Furthermore, their similarity to *RAP2.2* and *RAP2.12* in Arabidopsis suggests a possible similar function as major activators of hypoxia-responsive genes. Using *MtERF74/75* RNAi transgenic roots, we confirmed that TF are involved in expression of several *HRGs*(Mustroph et al., 2009), namely *ADH1* , *PDC1* , *Pgb1.1* ,*AlaAT* , and *ERF73* (Fig. 3). However, expression of*LDH* and *Susy* genes, belonging to the core anaerobic genes, was not affected in transgenic roots. Among the deregulated *HRG*genes, we identified *MtPgb1.1* , a gene coding for an enzyme involved in NO-scavenging (Berger et al., 2020b), as one of the genes mostly affected by silencing of *MtERF74/75* . Hartman et al. (2019) have recently shown that AtPGB1 from *A. thaliana* (ortholog of*MtPgb1.1*) plays a key role in low O_2 responses and links ethylene signalling to hypoxia tolerance through its ability to scavenge NO. In *M. truncatula* , the silencing of*Phytogb1.1* was found to conduct the increase in NO concentration, negatively impact the nodulation and mimics a situation of hypoxia by the overexpression of ADH and PDC (Berger et al., 2020b). Our results suggest that ERF74 and ERF75 control the expression of *MtPgb1.1*during hypoxic stress in *M. truncatula* .

Silencing of *MtERF74/75* demonstrates the importance of these TFs during the initial phase of nodule development, with a 30% reduction in nodulation efficiency (Fig. 4). In addition, we observed a phenotype, albeit very weak (18% reduction), in the number of lateral roots in*MtERF74/75* RNAi (Fig. S4). Previous reports described the presence of an hypoxic niche in LRM during lateral root formation that promote the stability of ERF-VII and thereby regulate the transition from LRP to LR (Shukla et al., 2019). It should be emphasized that LRP and nodule primordia (NP) share some common features. Schiessl et al. (2019) showed that there is overlap in the mechanisms of organogenesis and transcriptional regulation between nodule and lateral root initiation. The same authors showed that auxin and some auxin-responsive regulators are accumulated similarly in LRP and NP. In addition, NO is detected in dividing cortical cells of these two different developmental stages (Correa-Aragunde et al., 2004; del Giudice et al., 2011). Recent work showed that the production of NO, observed in nodule primordia 4 days post-inoculation in the *M. truncatula- S. meliloti* symbiosis is involved in the onset of nodule organogenesis (Berger et al., 2020b). NO scavenging by pharmacological (treatment with NO scavenger) or genetic approaches (overexpression of *Pgb1.1*) resulted in down-regulation of many genes related to cell division and growth (Berger et al., 2020b; Boscari et al., 2013; del Giudice et al., 2011). Thus, the 30% reduction in nodulation efficiency in *MtERF74/75*RNAi transgenic roots could be related to deregulation of *HRG*genes involved in this chronic hypoxia tissue at the NP stage.

In addition to the phenotypes in the early stages of nodule development, knock-down of *MtERF74/75* revealed an important role of these genes in mature nodule functioning with a marked decreased N_2 fixation activity (Fig. 4). Interestingly, although nodule development was impaired (Fig. 4c), the analysis of nodule ultrastructure showed no change (data not shown) and the unchanged level of *Lb4* expression (gene marker for the N_2fixation zone) in the transgenic knock-down *MtERF74/75* and control root nodules (Fig. 5) suggests that the reduction in N_2 fixation was not related to a failure in the development of the nodule N_2

fixation zone. Conclusion also confirmed by the absence of induction of *CP6* and *VPE*(Fig. 5), two markers of nodule senescence (Pierre et al., 2014).

N_2 fixation in the nodule is O_2sensitive since nitrogenase activity is irreversibly inhibited by O_2 (Appleby, 1992). Consequently, legume nodules have evolved mechanisms to reduce the level of O_2 by the presence of an O_2 diffusion barrier and by expressing leghemoglobin proteins (Appleby, 1992). Legume nodules work efficiently even if internal nodule O_2 concentration is between 10 and 50 nM of O_2 (Kuzma *et al.* , 1993), suggesting a peculiar and organ specific adaptation to innate low O_2 concentration. In normoxia, *M. truncatula*nodules exhibit ATP/ADP ratios of 6-8 (Horchani *et al.* , 2011), demonstrating a high energy state. It is generally assumed that in the O_2-limited environment of the nodule, glycolysis is shifted to malic acid synthesis, with further reductive synthesis to fumarate and succinate to feed the bacteroids (Vance and Gantt, 1992). Impairment of N_2 fixation activity in the transgenic nodules knockdown of *MtERF74/75* could be the consequence of the down-regulation of *HRG* genes necessary for maintaining optimal function in the microoxia prevailing in nodules. An observed exception, the *AlaAT* expression was not significantly affected in knockdown*MtERF74/75* nodules. As alanine has been involved in the adaptation to microoxia in roots (Ricoult et al., 2005) and nodules (Berger et al., 2020a), this suggests that *AlaAT* is regulated via an ERF74/75-independent pathway.

Interestingly, *NR1* and *Pgb1.1* , two important actors of the Pgb-NO respiration (Gupta and Igamberdiev, 2011), were severely impaired in the transgenic nodules (Fig. 8). NR enzymes are described as the major source of NO in nodules, enabling the reduction of NO_3^- to NO_2^-, which is subsequently reduced to NO via the mitochondrial electron transfer chain (Berger et al., 2020a; Hichri et al., 2015; Horchani et al., 2011). Recently, Berger et al. (2020a) observed that the decrease in NR activity in nodules either by a double RNAi::*NR1-2* or by the use of tungstate (NR inhibitor) was accompanied by a decrease in N_2 fixation activity. It was concluded that NRs in cooperation with Phytogb1.1 enables the maintenance of cell energy status in nodules and N_2-fixing metabolism through the functioning of the Phytogb-NO respiration. Therefore, it seems that one of the functions of MtERF74 and MtERF75 in long-term adaptation of nodule to microoxia is the induction of *MtNR1* and *Pgb1.1* expression for efficient Phytogb-NO respiration.

Furthermore, we investigated whether the stability of the ERF75 protein is controlled via the N-degron proteolytic pathway. We observed the highest stability of the chimeric protein after substitution of the terminal Cys by Ala (Fig. 6a) and in the *prt6* mutant in Arabidopsis protoplasts (Fig. 6b), indicating that the Cys residue at the N-terminal part of MtERF75 is involved in the stability of the protein. A result that was also observed with the GFP-fused MtERF75 protein (pMA-ERF75::GFP) in *M. truncatula* protoplasts. These results suggest that the turnover of the different members of the MtERF-VII family in *M. truncatula* may be regulated by the N-degron proteolysis pathway, as shown for RAP2.12 in Arabidopsis (Gibbs et al., 2011; Licausi et al., 2011). The investigation of the role of N-terminal amino acid residues in the subcellular localization of MtERF75 fused to GFP in *M. truncatula* protoplasts revealed that under aerobic conditions, the fusion protein may be localized in the nucleus (Licausi et al., 2011).

Both O_2 and NO are required to destabilise ERF-VII (Gibbs et al., 2015, 2014b). Regarding the involvement of NO, in animal cells it has been proposed that Cys nitrosylation precedes Cys oxidation (Hu et al., 2005). The high concentration of NO in nodules prompted us to investigate the role of NO in the targeted proteolysis of MtERF75. The internal O_2 concentration in nodules (10-50 nM (Kuzma et al., 1993)) should lead to stabilisation of ERF-VII. This implies that the O_2-sensitive regulation of the transcription factor MtERF-VII might be different in nodules than in root. An interesting hypothesis to test would be that high content of NO in nodules favour Cys oxidation at a low O_2concentration, degrading MtERF-VII under normal conditions. It has recently been demonstrated that enzymes from Plant Cystein Oxidase family (PCOs 1 and 4) are dioxygenases that catalyse the direct incorporation of O_2 into RAP2.2/RAP2.12 peptides to form Cys-sulfinic acid (White et al., 2017). However, a role for NO in the formation of a Cys-sulfonic acid product that is also a substrate for ATE1 has not been ruled out (White et al., 2017). In our work (Fig. 9), we show that S-nitrosylation, NO-dependent posttranslational modification (PTM) targeting specific Cys residues can be detected for NO concentrations on the order of a few micromoles (in the range of physiological values) in

peptides CR20 lacking the first methionine. Using this original and innovative approach, we demonstrated that S-nitrosylation, the first step in the oxidation of the Cys residue, could eventually occur. Whether this is altering the intervention of PCO1 and PCO2 enzymes, which catalyse the oxidation of the N-terminal Cys of the substrate with molecular O_2 as a co-substrate (Weits et al., 2014) is still to be defined.

In conclusion, MtERF74 and MtERF75 plays a key role in activating the anaerobic response in *M. truncatula* . Their impairment conduct to a significant reduction in nodulation capacity and nitrogen fixation activity in mature nodules that could be explain by a reduce efficiency of Pgb-NO respiration in nodules knock-down for both TF. Our results suggest that MtERF75 is target of the N-degron pathway and stable forms of the protein are localised in the nucleus. Moreover, S-nitrosylation of the Cys2 residue is promoted when the first Met is removed can occur. This evidence opens new questions regarding the existence of a specific function of NO in nodules related to the presence of an hypoxic niche. The NO regulation of MtERF-VII, and consequently of yet unknown target genes, for efficient N2 fixation and nodule organogenesis need to be explored in more depth.

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Author Contribution

MR, CP, and AB conceived and designed the research; MR, CP, CC, MF, MB, TAG and MLG carried out the experiments and analysis/interpretation of data; MR, CP, RB, PD and AB wrote the manuscript. All authors contributed to the discussion and approved the final manuscript.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Supplemental Figure 1: Localization of the specific region sequence targeted by the RNAi.

Supplemental Figure 2: *In silico* analysis of*MtERF-VII* gene expression in *M. truncatula* .

Supplemental Figure 3: Validation of effectiveness and specificity of the *MtERF74/75* RNAi constructs.

Supplemental Figure 4: Root growth and secondary roots phenotypes in composite knock-down roots transformed with the*MtERF74/75* :RNAi vector or with GUS vector.

Supplemental Figure 5: Effect of MtERF74/75 RNAi on transcripts level of some hypoxia-responsive genes in *M. truncatula* root submitted to 24 hours of hypoxia stress in hypoxic chamber.

Supplemental Figure 6: Identification of *Tnt1* mutant line NF1722 for *MtERF74* genes.

Supplemental Figure 7: Comparison of the level of expression of the ERFVII:: MA and MC constructs.

Supplemental Figure 8: Mass spectrometric analyses on CR20 peptide treated to identify S-nitrosylation post-translational modification.

Supplemental Table 1: Primer sequences for cloning and quantitative RT-PCR analysis.

Supplemental Table 2: All Plasmids used in this study.

Supplemental Table 3: Nomenclature of *M. truncatula*ERF-VII

Supplemental Table 4: Transcriptional profiles of *M. truncatula* genes in 3 weeks old nodules compared to the root.

Figure legends

Figure 1: Comparison of N-terminus and phylogenetic analysis of the ERF protein sequences of *M. truncatula, A. thaliana, Glycine max* and flooding related SK and SUB1 genes of*Oryza sativa.* (a) Multi-alignment with motives at the N-terminus of the ERF-VII protein sequences. The alignment quality and the consensus sequence were inferred using JalView (http://www.jalview.org/). The alignment file was obtained from Clustal Omega online tool (http://www.ebi.ac.uk). (b) A phylogenetic tree to highlight the evolutionary relationship in the ERF-VII families were inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates and the Evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA7 (https://www.megasoftware.net/).

Figure 2: Expression patterns of *M. truncatula* ERF-VII TFs performed by RTqPCR. (a) Expression analysis of *ERF72* ,*ERF73* , *ERF74* and *ERF75* in roots and nodules of 5 week-old plants. Nodules were collected 3 weeks post-inoculation. (b) Transcriptional regulation of *ERF73* in roots of 5 week-old plants growing in aerobic conditions and after 24 hours of hypoxia-treatment (mean ± SE, **p-value <0.01, ***p-value < 0.001, one-way ANOVA, n=4).

Figure 3: Effect of MtERF74/75 RNAi on transcripts level of some hypoxia-responsive genes in *M. truncatula* root. Relative expression measured by RTqPCR of transgenic hairy roots submitted to 24 hours of hypoxia stress in hypoxic chamber. Expression level of each gene in RNAi 74/75 was compared to control plants normalized to 100%. Results are the mean (± SE (*P<0.05, **P < 0.01, one-way ANOVA) of three independent experiments composed by, at least, 10 plants for each condition.

Figure 4: Effect of MtERF74/75 RNAi during the symbiotic interaction between *M. truncatula* and *S. meliloti* . (a) Nodules number per plant at 14 days post-inoculation (dpi) in RNAi composite plants (RNAi 74/75) compared to control (Ctrl). Three independent series of 100 plants were used to calculate the average nodule number for each condition. (b) The nitrogen fixing capacity was measured by the acetylene reduction assay (ARA) expressed in nmol of ethylene (C_2H_4) reduced per mg of nodules and per hour, from plant nodules at 5 weeks post-inoculation (wpi). Results are the mean ± SE of three independent experiments composed by 15 plants each. (c) Nodules were collected, photographed and measured in size using ImageJ imaging software (*https://imagej.nih.gov/ij/*). For each independent experiment, at least 100 nodules were collected. Results are mean ± SE (*** p-value < 0.001, ** p-value < 0.01, one-way ANOVA)

Figure 5: Effect of MtERF74/75 RNAi on transcripts level of some hypoxia-responsive genes in *M. truncatula* nodules. Relative expression measured by RTqPCR in transgenic nodules (RNAi 74/75) in comparison to control nodules at 5 weeks post-inoculation (wpi) (Ctrl). Expression level of each gene in RNAi 74/75 was compared to control condition normalized to 100%. The results shown are the mean ± SE

(*P<0.05, **P < 0.01, one-way ANOVA,) of five independent experiments with, at least, 10 plants for each condition.

Figure 6: Effect of *MtERF74/75* RNAi on NO production in3 wpi-old *M. truncatula* mature nodules. The fluorescence intensity of the NO production was measured using the DAF-2 fluorescent probe. The results are the mean ± SE (*P<0.05, **P < 0.01, one-way ANOVA,) of the five independent experiments.

Figure 7: Measurement of luciferase activity on *A. thaliana* protoplast with MC80-ERF75::LUC and MA80-ERF75::LUC construction. (a) Luciferase activity of MC80-MtERF75::LUC and MA80-MtERF75::LUC in protoplasts of *A. thaliana* (col-0); (b) Luciferase activity of MC80-MtERF75::LUC in control protoplast (col-0) and in protoplast obtained from transgenic lines lacking in E3 ligase enzyme (*prt6*) (mean ± SE, *p-value <0.05, **p-value < 0.01, t-student test, n=4)

Figure 8: Subcellular localization of MtERF75 transcription factor in *M. truncatula* leaf protoplasts. pAVA vector was used as a 35S:GFP control. pMC-ERF75::GFP vector, C-terminal GFP fusion with full length ERF75 protein, was used to follow the subcellular localisation of ERF75 protein. Autofluorescence of chloroplasts was visualized in red under TRITC filter. Merged is the superimposition of the Bright field, GFP and TRICT picture. Scale bar 5 µm.

Figure 9: Post-translational modifications (S-nitrosylation, dimerized peptide) of MR21 and CR20 peptides after DEA-NONOate treatment . Peptides were treated for 5 min with various concentrations of DEA-NONOate and subsequently analyzed by MS. NM : not modified, NO : nitric oxide. Dimer: dimerized peptide. N=3.

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