Research

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Nitric oxide and phytoglobin PHYTOGB1 are regulatory elements in the *Solanum lycopersicum–Rhizophagus irregularis* mycorrhizal symbiosis

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Summary

- The regulatory role of nitric oxide (NO) and phytoglobins in plant response to pathogenic and mutualistic microbes has been evidenced. However, little is known about their function in the arbuscular mycorrhizal (AM) symbiosis. We investigated whether NO and phytoglobin PHYTOGB1 are regulatory components in the AM symbiosis.
- *Rhizophagus irregularis in vitro*-grown cultures and tomato plants were used to monitor AM-associated NO-related root responses as compared to responses triggered by the pathogen *Fusarium oxysporum*. A genetic approach was conducted to understand the role of PHYTOGB1 on NO signaling during both interactions.
- After a common early peak in NO levels in response to both fungi, a specific NO accumulation pattern was triggered in tomato roots during the onset of the AM interaction. *PHYTOGB1* was upregulated by the AM interaction. By contrast, the pathogen triggered a continuous NO accumulation and a strong downregulation of *PHYTOGB1*. Manipulation of *PHYTOGB1* levels in overexpressing and silenced roots led to a deregulation of NO levels and altered mycorrhization and pathogen infection.
- We demonstrate that the onset of the AM symbiosis is associated with a specific NO-related signature in the host root. We propose that NO regulation by PHYTOGB1 is a regulatory component of the AM symbiosis.

Introduction

Plants encounter a myriad of microbes at the root–soil interface that can interact with roots with detrimental or beneficial outcomes for plant fitness. Prevalent beneficial associations between plants and microbes include the arbuscular mycorrhizal (AM) symbiosis. This symbiosis is estimated to be as old as land plants themselves, and plays a key role in terrestrial ecosystems regulating nutrient and carbon cycles, and influencing soil structure and ecosystem multifunctionality (Van der Heijden *et al.*, 2015). In the AM symbiosis the AM fungus inhabits the root cortical cells and provides the plant with an additional (fungal) pathway of mineral nutrient uptake from the soil (Smith *et al.*, 2011). Besides its nutritional aspects, the symbiosis may enhance plant resistance and tolerance to multiple stresses (Jung *et al.*, 2012; Barzana *et al.*, 2014). In return, the plant supplies the fungus with carbon in the form of photosynthesis-derived sugars and lipids (Pfeffer *et al.*, 1999; Jiang *et al.*, 2017). Accordingly, plants have evolved sophisticated mechanisms to accommodate these beneficial symbionts (Bonfante & Genre, 2010). While promoting these and other beneficial relationships, plants must restrict the establishment of pathogenic associations. Achieving this balance requires the perception of potential invading microorganisms, followed by a rapid and tight regulation of immune responses to promote or contain the microbial colonization of root tissues (Zamioudis & Pieterse, 2012; Plett & Martin, 2017; Zipfel & Oldroyd, 2017).

In the AM symbiosis, the plant actively accommodates the fungal partner, guiding it to the cortex where it forms the specialized, highly branched structures called arbuscules, where the exchange of nutrients takes place (Bonfante & Genre, 2010). The development of such intimate interaction relies on a continual signaling between the symbionts, and on the activation of an extensive genetic and developmental program in both partners (MacLean *et al.*, 2017). Multiple signaling components operate in the establishment and the maintenance of the AM symbiosis including calcium spiking, reactive oxygen species and plant hormones (Pozo *et al.*, 2015). The chemical communication between the host plant and the AM fungus is initiated in the rhizosphere, before the physical contact between the symbionts

(Buee *et al.*, 2000; Chabaud *et al.*, 2011). The perception of fungal diffusible signals by the plant is translated in a transcriptional response that prepares the plant for the subsequent fungal colonization (Maillet *et al.*, 2011; Genre *et al.*, 2013). In this route, fungal signals are interpreted into a signaling pathway that regulates the activation of essential symbiotic genes required to promote the symbiosis (Chabaud *et al.*, 2011; Genre *et al.*, 2013). A second generation of signaling during the root colonization triggers a transcriptional reprograming in epidermal and cortical cells, with differential expression of many genes associated with transcriptional regulation, cell wall modification and defense responses. This drives a strong cellular remodeling and the precise modulation of defense responses in the host root, which eventually leads to the establishment of the symbiosis (Liu *et al.*, 2003; Siciliano *et al.*, 2007; Genre *et al.*, 2008; Gaude *et al.*, 2012). For instance, it is proposed that the tight regulation of plant defense responses upon specific recognition of the fungal partner by the plant is essential for its active accommodation in the root tissues (Garcia-Garrido & Ocampo, 2002; Siciliano *et al.*, 2007). The degree of the symbiotic interaction is further regulated according to the plant needs and environmental conditions (Pozo *et al.*, 2015). This regulation, which is partially controlled by the host plant, aims to maintain the mutualistic character of the symbiosis, avoiding excessive root colonization (Vierheilig, 2004). Despite a significant progress over the last years, understanding the signaling hardware governing the AM symbiosis is an ongoing challenge. This is due mostly to the complex genetic make-up of the AM fungus, its obligate biotrophic nature and the asynchronous character of the fungal colonization (Sedzielewska-Toro & Delaux, 2016).

The highly reactive signal molecule nitric oxide (NO) is a key component of the signaling pathways regulating plant immunity (Delledonne *et al.*, 1998; Durner *et al.*, 1998; Bellin *et al.*, 2013). NO is produced rapidly in plant tissues during incompatible interactions with biotrophic pathogens as well as in compatible interactions with necrotrophic pathogens (van Baarlen *et al.*, 2004; Romero-Puertas *et al.*, 2004; Floryszak-Wieczorek *et al.*, 2007). NO also can be produced by microbial pathogens to promote the infection of plant tissues (Arasimowicz-Jelonek & Floryszak-Wieczorek, 2016), and participates in the proper establishment of the mutualistic association between legumes and rhizobia (Hichri *et al.*, 2015). In this symbiosis, NO is proposed to be involved in the activation of the developmental program required for nodule formation and development, and in the early repression of the plant defense reaction favoring symbiosis establishment (Ferrarini *et al.*, 2008; Boscari *et al.*, 2013). NO accumulation can be regulated by the activity of plant phytoglobins (previously known as nonsymbiotic hemoglobins; Perazzolli *et al.*, 2004; Qu *et al.*, 2006; Nagata *et al.*, 2008, 2009; Hill *et al.*, 2016), that may function as NO dioxygenases that catalytically metabolize NO to nitrate (Seregelyes *et al.*, 2004; Hill, 2012). Indeed, NO triggers the expression of the phytoglobin gene *PHYTOGB1* in a number of plant species (Perazzolli *et al.*, 2004; Bustos-Sanmamed *et al.*, 2011; Bai *et al.*, 2016); and the manipulation of the *PHYTOGB1* in transgenic lines evidenced its crucial role for NO bioactivity during plant–microbe interactions (Perazzolli *et al.*, 2004; Shimoda *et al.*, 2009; Mur *et al.*, 2012; Bai *et al.*, 2016; Fukudome *et al.*, 2016).

Increasing evidence is showing that NO also is produced during other symbiotic interactions including mycorrhizal and lichen symbioses (Weissman *et al.*, 2005; Calcagno *et al.*, 2012; Espinosa *et al.*, 2014). Moreover, genome-wide analysis of transcription patterns revealed *PHYTOGB1* as one of the mycorrhiza-early activated genes in the epidermal layer of *Medicago truncatula* roots interacting with different AM fungi (Siciliano *et al.*, 2007; Hogekamp & Küster, 2013). Together these observations suggest a potential role of NO and phytoglobins in AM symbiosis establishment. However, the role(s) of NO in the AM symbiosis remains elusive so far, and its regulation during the establishment and functioning of the symbiosis is still puzzling.

In the present contribution, we hypothesized that NO is a signaling component of the regulatory pathway that is activated in the host root during the onset of the AM symbiosis. We also hypothesized that the AM symbiosis-related signaling is associated with a specific NO signature, different to that associated with immunity-related signaling. We further explored the role of PHYTOGB1 in the regulation of NO bioactivity in mutualistic and pathogenic plant–microbe interactions. Our results demonstrate that the AM onset is associated with a specific NO-related signature and a specific regulation pattern of the *PHYTOGB1* gene in the host root. By using transgenic hairy roots silenced and overexpressing the *PHYTOGB1* gene, we demonstrated the role of PHYTOGB1 in the regulation of NO levels in tomato roots, and in the regulation of the AM establishment and pathogen infection.

Material and Methods

Plant and fungal material

Tomato (*Solanum lycopersicum* cv Moneymaker) seeds were surface-sterilized in 4% sodium hypochlorite and germinated in sterile water at 25°C in darkness. After 1 wk, seedlings were transferred to hydroponic conditions in 3-l tanks containing Long Ashton nutrient solution (Hewitt, 1966) with constant aeration. Plants were grown in the hydroponic tanks (six plants per tank) at 16 h : 8 h, light (24°C) : dark (16°C) cycle at 70% relative humidity for two weeks before use. The AM fungus *Rhizophagus irregularis* (Schenck and Smith DAOM 197198) was grown in monoxenic cultures, using Ri T-DNA (*Agrobacterium rhizogenes*)-transformed carrot (*Daucus carota* clone DC2) according to St-Arnaud *et al.* (1996). Cultures were established according to Chabot *et al.* (1992) in 100 × 20 mm Petri plates, placed in 150 × 25 mm Petri plates (Fig. 1a,b) to allow separating the root compartment from the hyphal compartment. Petri plates were incubated in the dark at 24°C until the hyphal plate, which contained M medium without sucrose, was profusely colonized by the fungus (*c.* 12 wk; Fig. 1c). The root plate was then removed, and plates were used for the experiments. *Fusarium oxysporum* f sp. *lycopersici* was grown on potato dextrose agar (PDA) at 28°C in dark conditions for 5 d.

Early interaction experiment set-up

A small orifice (3-mm diameter) was made in the side and the lid of the Petri dishes containing the *R. irregularis* or the *F. oxysporum* cultures. Two-week-old tomato plants, grown in the hydroponic tanks were transferred to the Petri plates, one plant per plate, placing the roots on the surface of the culture and the stem in the hole, letting the shoot expand outside the plate, in open air conditions (Fig. 1d) as described by Voets *et al.* (2005). Petri plates were closed and covered to keep the root system in the dark, and plants were kept in a growth chamber at 16 h : 8 h, light (24°C) : dark (16°C) cycle at 70% relative humidity. At 4, 8, 24, 48, 72 and 96 h after setting up the experiment, plants were harvested and root material was collected.

Fungal elicitors

Exudates were obtained from *c.* 1×10^8 germinating spores of *R. irregularis* and *F. oxysporum*. Sterile spores were germinated in 30 ml sterile distilled water for 1 wk at 24°C in dark (germination rate was *c.* 80%). The germinating spore suspensions were then collected and filtrated first through 0.45-μm and later through 0.22-μm Millipore filters. We denote the resulting filtrate as germinating spore exudates. Homogenates of *R. irregularis* and *F. oxysporum* cell wall were obtained from *R. irregularis* monoxenic cultures grown as described above, and *F. oxysporum* grown in potato dextrose broth media on a shaker for 5 d in dark conditions. The mycelium of the *R. irregularis* monoxenic culture was carefully removed with 10 mM sodium citrate to liquefy the culture media. The cell wall material from both fungi was prepared according to Ren & West (1992) and then ground to fine powder and lyophilized. Roots were treated for 3, 6 and 24 h with 3 ml germinating spore exudates from *R. irregularis* and *F. oxysporum*, or with 3 ml ground lyophilized cell walls resuspended in distilled sterile water at 0.1% (w/v).

Chemical treatments

The roots of 2-wk-old tomato plants grown in hydroponic tanks were treated with the nitric oxide (NO)-releasing compounds sodium nitroprusside (SNP; 200 μM; Sigma-Aldrich, St Louis, MI, USA), S-nitrosoglutathione (GSNO; 350 μM; Calbiochem,

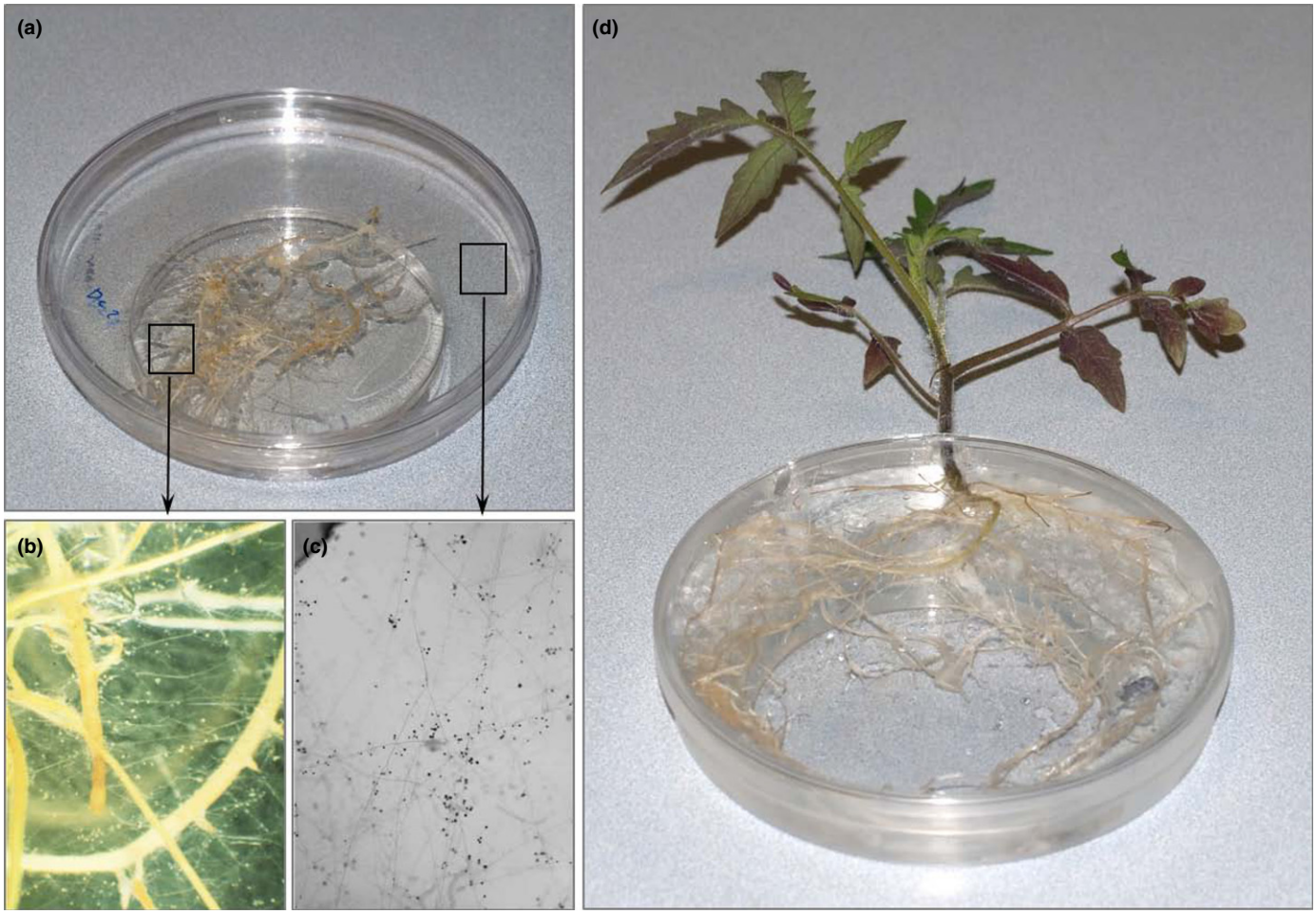

Fig. 1 Early interaction experiment set-up. (a) The arbuscular mycorrhizal fungus *Rhizophagus irregularis* was grown in monoaxenic cultures using Ri T-DNA-transformed carrots. The plate containing the root culture was placed in a bigger plate containing M medium without sucrose (b). When the M medium was profusely colonized by the fungus (c), the plate containing the root cultures was removed, and tomato plants were transferred to the plates, with the roots placed on the surface of the colonized medium and the shoot extending beyond the plate (d).

San Diego, CA, USA) and DETA-NONOate (500 μM; Cayman Chemicals, Ann Arbor, MI, USA), for 1 and 3 h. In the case of SNP, a control treatment with 200 μM of sodium ferricyanide was run in parallel (Bethke *et al.*, 2006).

NO detection and quantification

Quantitative NO determination was performed through spectrofluorometry as described previously (Nakatsubo *et al.*, 1998; Besson-Bard *et al.*, 2009). Briefly, 0.2 g of fresh root samples were ground in 0.8 ml extraction buffer (50 mM Tris-HCl, pH 7.8; 0.1 mM EDTA; 0.2% triton X-100; 10% glycerol; 2% PVPP) with a mortar. Homogenates were centrifuged at 11 300 ***g*** for 30 min. Aliquots of supernatants were immediately diluted 50-fold in HEPES buffer (50 mM, pH 7.5). DAF-2 (Merck Biosciences) was added at 2 μM final concentration and the reaction mixtures were incubated at 37°C in the dark for 2 h. Fluorescence was measured in a RF-540 spectrofluorophotometer (Shimadzu, Kyoto, Japan) at excitation and emission wavelengths of 485 and 515 nm, respectively. NO detection by microscopy was performed as described in Sandalio *et al.* (2008): segments of plant roots were incubated for 1 h in darkness with 10 μM 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA; Merck Biosciences, Darmstadt, Germany), prepared in 10 mM Tris-HCl (pH 7.4). As a negative control, roots segments were similarly incubated with the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Sigma) at a final concentration of 500 μM. The segments were washed three times for 15 min each in 10 mM Tris-HCl (pH 7.4) to remove dye excess. The fluorescence emitted by DAF-FM DA was detected by excitation at 495 nm and emission at 515 nm using a confocal microscope (Leica Microsystems, Wetzlar, Germany).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Extraction of total RNA from plant roots and synthesis of cDNA was performed according to Martínez-Medina *et al.* (2013). Real-time qRT-PCR reactions and relative quantification of specific mRNA levels were performed according to Martínez-Medina *et al.* (2013) and by using the gene-specific primers described in Table S1. The data were normalized using the housekeeping gene *SlEF* (X14449) encoding for the tomato translation elongation factor-1α, whose expression remained stable in the different lines and conditions. mRNA sequences of the tomato phytoglobin genes *PHYTOGB1* (AY026343), *PHYTOGB2* (AY026344) and *PHYTOGB3* (AW036344) were found in the online database NCBI. Gen structure information was obtained using the on-line database SOL Genomics Network (http://solgenomics.net/).

Generation of the *PHYTOGB1* RNAi and OE vectors and transformation by *Agrobacterium rhizogenes*

For the generation of the RNAi vector a PCR fragment of 201 bp including part of the 3′-UTR and coding region of the *PHYTOGB1* gene was amplified using tomato cDNA as template and the primers *RNAi-PHYTOGB1* Fw: 5′-CACCGGTT AGTGCTATCAAGACTGAGATGAAG-3′ and *RNAi-PHYTOGB1* Rv: 5′-GCACACAAATTAGATTATAAAATTTT GCAACG-3′. PCR was performed using Taq polymerase Poof-reading (Roche) according to manufacturer's instructions. The PCR product was purified by using the DNA clean and concentrator kit (Zymo Research, Irvine, CA, USA), and then cloned into pENTR-TOPO (Invitrogen, Carlsbad, CA, USA) according to manufacturer's indications. Subsequently a Gateway reaction was performed with destination expression vector *pRedRoot* (Limpens *et al.*, 2004). The inserts were verified by restriction digests and sequencing. The *pRedRoot* vector without insert (empty vector) was used for controls. The vectors were introduced into *A. rhizogenes* strain MSU440 by electroporation. *A. rhizogenes* was grown for 2 d at 28°C under spectinomycin selection (50 μg ml^{-1}). The integrity of the constructs was checked by sequencing. Tomato seeds were surface-sterilized in 4% sodium hypochlorite and germinated for 5 d in darkness in sterile conditions. The germinated seeds were transferred to a half strength Murashige and Skoog (MS) vitamin agar-solidified medium (pH 5.8) and grown for 5 d at 21°C with a photoperiod of 16 h : 8 h, light : dark. Tomato seedlings were transformed with *A. rhizogenes* containing the appropriate constructs according to Chabaud *et al.* (2006) with some modifications. Briefly, the roots of the seedlings were cut out and the seedlings were co-cultivated with MSU440 for 6 d at 21°C with a photoperiod of 16 h : 8 h, light : dark, in half-strength MS vitamin agar solidified medium. Seedlings were then transferred to MS agar-solidified medium supplemented with 500 μg ml^{-1} cefotaxime and 50 μg ml^{-1} kanamycin for 3 d at 25°C with a photoperiod of 16 h : 8 h, light : dark, to select positive transgenic individuals. Seedlings were then transferred to MS agar-solidified medium supplemented with 300 μg ml^{-1} cefotaxime and 50 μg ml^{-1} kanamycin and roots were cut out and then grown for 21 d at 25°C with a photoperiod of 16 h : 8 h, light : dark. Emerging roots were periodically screened for DsRED1 fluorescence. Red-fluorescent roots were retained, whereas all nonfluorescent roots were removed by excision.

For overexpression, the *PHYTOGB1* full-length open reading frame was amplified from tomato cDNA by using the specific primers *OE-PHYTOGB1* Fw: 5′- CACCATGAGTAGCT TTAGTGAAGAACAAGAAGC-3′ and *OE-PHYTOGB1* Rv: 5′- CTTCATCTCAGTCTTGATAGCACTAACC-3′, and cloned into pENTR-TOPO (Invitrogen) as described for the generation of the RNAi vector. Subsequently a Gateway reaction was performed with destination expression vector *pAtUbq10_DsRed* (Kryvoruchko *et al.*, 2016). The empty vector was used for controls. The verified construct was then introduced into *A. rhizogenes* strain MSU440 by electroporation and transformants were selected by resistance to streptomycin and spectinomycin. Generation of composite *S. lycopersicum* plants was performed according to Ho-Plágaro *et al.* (2018).

Colonization bioassays

Transformed plants were transferred to 100-ml pots containing a sterile sand : vermiculite mixture (1 : 1, v/v). Inoculation with *R. irregularis* was achieved according to Rivero *et al.* (2015). The

R. irregularis inoculum consisted of *R. irregularis* kept in a soil-sand mixture containing extraradical mycelium and spores, and mycorrhizal root fragments of *Trifolium repens* (Rivero *et al.*, 2015). Plants were placed in a completely randomized design in a growth chamber at 16 h : 8 h, light (24°C) : dark (16°C) cycle at 70% relative humidity. Plants were watered three times a week with nutrient solution (Hewitt, 1966) containing 25% of the standard phosphorus concentration. Six weeks after transplanting into pots, plants were harvested and root material was collected. Mycorrhizal structures were stained with trypan blue (Phillips & Hayman, 1970). Quantification of the different fungal structures within the roots was performed according to Trouvelot *et al.* (1986), using a Nikon Eclipse 50i microscope, Nikon, Tokyo, Japan. Molecular quantification of *R. irregularis* within the roots was performed by qRT-PCR using Ri-EF1α primers specific for the constitutively expressed *elongation factor 1α* from *R. irregularis* (Helgason *et al.*, 2003). The functionality of the mycorrhizal symbiosis was checked by analyzing the expression of the tomato *LePT4*, encoding a phosphate transporter specific for the AM symbiosis which is expressed in arbusculated cells (Balestrini *et al.*, 2007).

Results

NO levels oscillate in tomato roots differently during early steps of mycorrhizal and pathogenic interactions

Nitric oxide is involved in the plant responses to different microbes, including pathogens and rhizobial bacteria (Bellin *et al.*, 2013; Hichri *et al.*, 2015). To understand whether NO also is a signaling component of the AM symbiosis establishment, we first investigated NO levels in tomato roots during early stages of the AM interaction with *R. irregularis* by using a *R. irregularis in vitro*-grown culture (Fig. 1) and the fluorescent indicator for the detection of NO DAF-2. To further investigate the *in vivo* spatiotemporal fluctuation of NO accumulation in roots, we used the cell-permeable NO-specific probe DAF-FM DA and confocal laser microscopy. Moreover, to discern whether the AM symbiosis signaling is associated to specific patterns of NO accumulation, we studied in parallel the NO accumulation pattern in tomato roots during early stages of the pathogenic interaction with *F. oxysporum*. We detected a transient burst of NO in tomato roots 4 h after the contact with the AM fungus (Fig. 2a, b). After this first NO peak, NO production oscillated in time, showing two more peaks at 48 and 96 h. NO levels in *R. irregularis*-roots at 8, 24 and 72 h was similar to that observed in control plants. *R. irregularis*-induced NO accumulation was observed mainly in the outer cell layers (epidermal and cortical cells) and in root hairs (Fig. 3a,b). Incubation of roots with the NO scavenger cPTIO extinguished the fluorescence induced by *R. irregularis*, confirming that NO production was being detected (Fig. S1).

The interaction of the roots with the pathogen *F. oxysporum* also induced a strong and transient NO burst at 4 h (Fig. 2a,b). After 8 h, NO levels in *F. oxysporum*-roots returned to basal levels. However, 24 h after the contact with the pathogen, NO accumulation increased over time. It was remarkable that by contrast with the AM interaction, the pathogen-triggered NO accumulation was evenly distributed over the root fragments analyzed (Fig. 3b). Together our findings demonstrate that NO is accumulated in tomato roots during the early steps of both the mutualistic and pathogenic interactions. However, NO accumulation triggered by the AM interaction showed a specific spatiotemporal pattern, which differed significantly to that observed during interaction with the fungal pathogen.

Exudates from *R. irregularis* germinating spores induce NO accumulation in tomato roots

During the presymbiotic stages of the AM symbiosis diffusible molecules released by the AM fungus, the so-called MYC factors, activate early symbiotic responses in the roots (Maillet *et al.*, 2011). We reasoned that plant perception of MYC factors might trigger a NO-related response in the host root. To investigate this, we monitored NO accumulation in tomato roots after treatment with germinating spore exudates from *R. irregularis*. We found that the exudates triggered an early burst of NO, which occurred within the first 3 h post-treatment (Fig. 4a). The NO signal declined to basal levels after 6 h of treatment. A further burst of NO was observed 24 h after the application of the *R. irregularis* germinating spore exudates (Fig. 4a). It is remarkable that germinating spore exudates from the pathogenic fungus *F. oxysporum* did not significantly alter NO levels in the roots (Fig. 4a).

Fungal cell wall components also are known to elicit early plant defense responses, functioning as microbe-associated molecular patterns that can be recognized by the plant immune system (Zipfel & Robatzek, 2010). We analyzed whether cell wall components from *R. irregularis* or *F. oxysporum* elicit a NO-related response in the host roots. The application of a suspension of homogenized fungal cell walls from *R. irregularis* did not affect NO levels in tomato roots (Fig. 4b), whereas *F. oxysporum* cell walls induced a slight, although not significant, transient increase in NO accumulation after 6 h. These findings indicate that plant perception of bioactive molecules present in the AM fungal exudates triggers a NO-related signaling during the presymbiotic stage of the AM symbiosis.

The tomato *PHYTOGB1* gene is upregulated in tomato roots in response to NO

The tomato genome contains three genes encoding phytoglobins: one class 1 phytoglobin (PHYTOGB1), one class 2 phytoglobin (PHYTOGB2) and one truncated phytoglobin (PHYTOGB3) (Fig. S2). Previous studies provided compelling evidence that phytoglobin genes can be induced by NO, playing a major role in plant protection against nitrosative stress (Perazzolli *et al.*, 2004). We investigated whether tomato phytoglobin genes also are regulated by NO. To this end, we analyzed the transcriptional regulation of the set of tomato phytoglobin genes in roots after the treatment with the NO donors SNP, DNN and GSNO. Incubation with the different NO donors triggered the

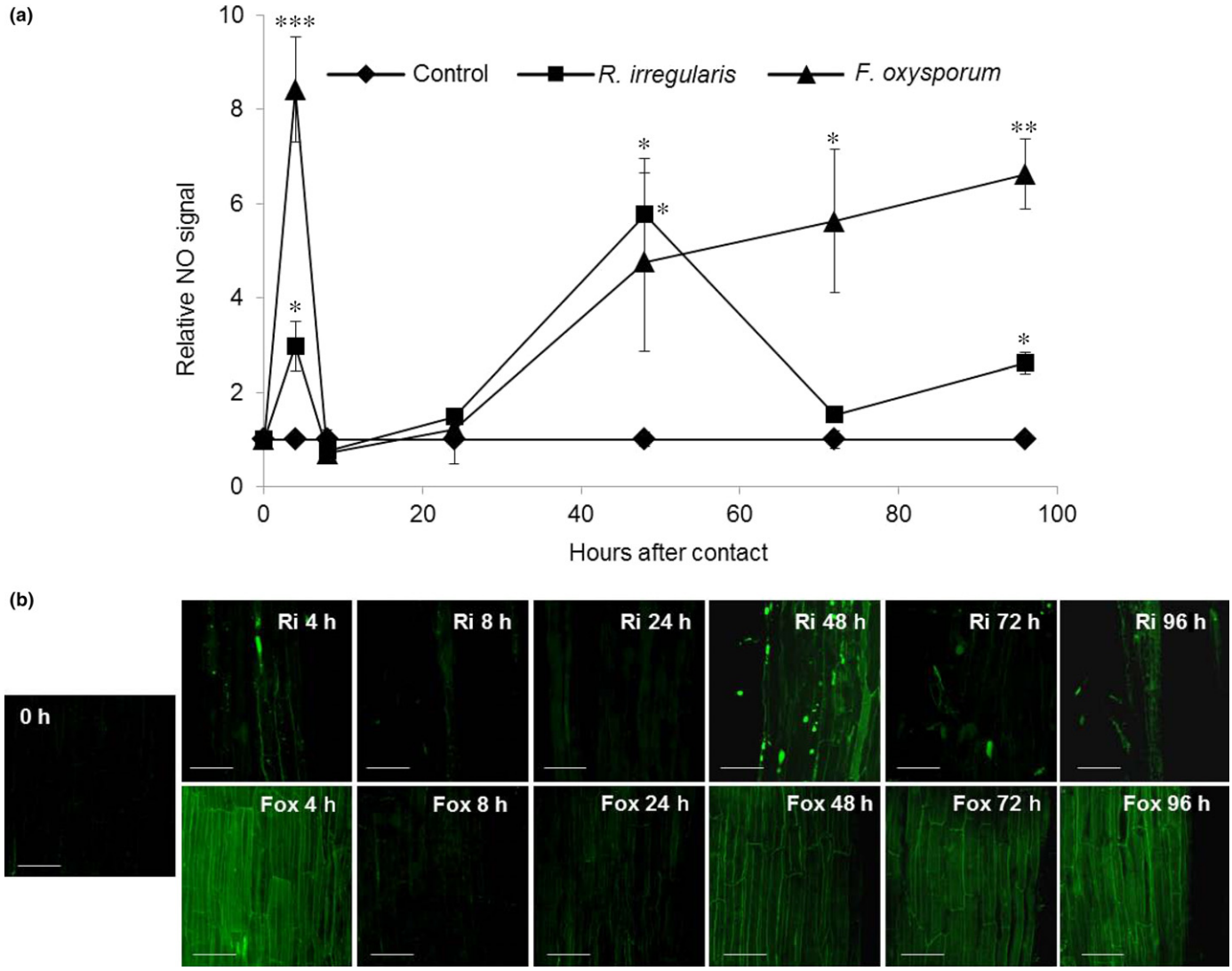

Fig. 2 Nitric oxide (NO) accumulation in tomato roots after contact with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* or the pathogenic fungus *Fusarium oxysporum*. (a) NO was detected by fluorimetry by using the specific NO detector DAF-2 at 4, 8, 24, 48, 72 and 96 h after the contact with the different fungi. *In vitro*-grown cultures of *R. irregularis* and *F. oxysporum* were used in the experiments. NO levels are reported as the fold increase relative to that of the control plants at each time point ± SE ($n = 4$ biological replicates). Asterisks indicate significant differences compared to control plants at ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$ according to Dunnett test. (b) Imaging of NO production in tomato roots by confocal laser microscopy. Images are projections of several optical sections collected by confocal microscopy showing the NO-dependent DAF-FM DA fluorescence (green; excitation at 495 nm, emission at 515 nm) from plants at 0, 4, 8, 24, 48, 72 and 96 h after contact with *R. irregularis* (Ri) or *F. oxysporum* (Fox). Bars, 50 μm. One independent representative of four biological replicates is shown. These results are representative for one of three independent experiments.

upregulation of the *PHYTOGB1* gene at 1 and 3 h post-treatment (Fig. 5a). NO donors did not significantly induce the expression of the other two phytoglobin genes (Fig. 5b,c). These results demonstrate that the tomato phytoglobin gene *PHYTOGB1* is consistently upregulated by NO, and suggest a potential role for PHYTOGB1 in NO metabolism in tomato roots.

The mycorrhizal and pathogenic interactions differentially regulate *PHYTOGB1* gene expression in tomato roots

Given the responsiveness of *PHYTOGB1* to NO and the impact of the AM and pathogenic interactions on NO accumulation, we reasoned that these interactions might elicit an early activation of *PHYTOGB1* in tomato roots. To investigate this, we analyzed the regulation of the tomato phytoglobin genes in roots during the early stages of the interaction with *R. irregularis* and with *F. oxysporum*. *PHYTOGB1* transcription was induced already 4 h after the contact with both fungi (Fig. 6a; Table S2). *R. irregularis*-triggered upregulation of *PHYTOGB1* was further observed at 8, 48 and 96 h after the interaction.

By contrast to the sustained induction of *PHYTOGB1* by the interaction with the AM fungus, the pathogen led to an initial increase of *PHYTOGB1* at 4 h after the interaction, but followed by a strong decrease later on (Fig. 6a). It is remarkable that *PHYTOGB1* upregulation induced by the pathogen at 4 h was

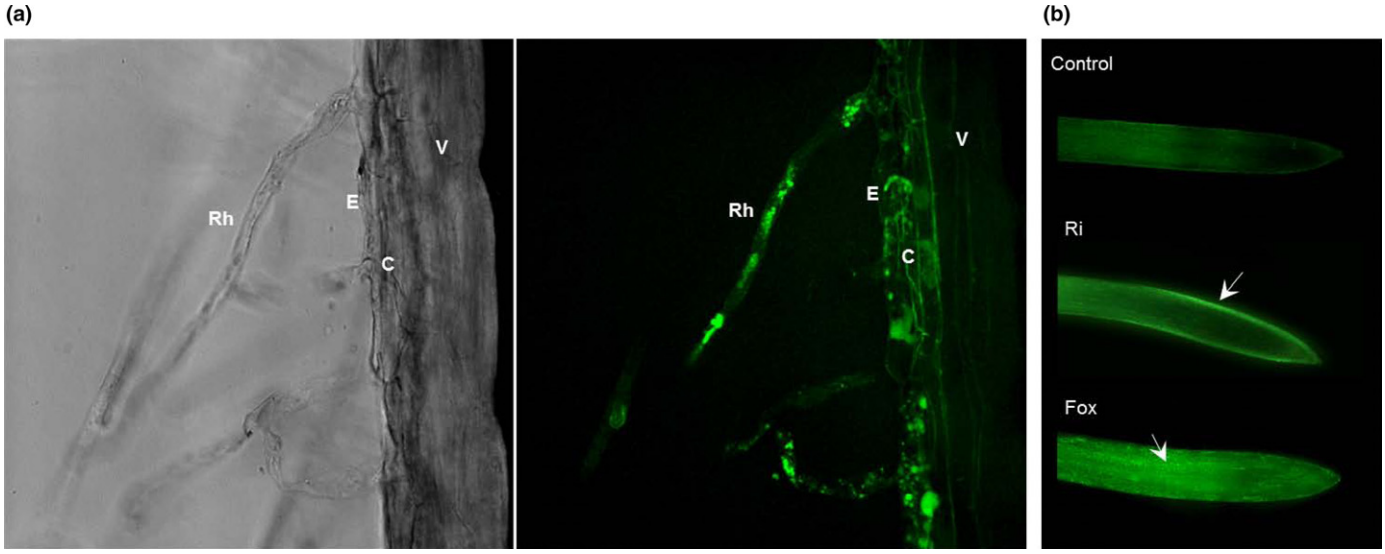

Fig. 3 Tissue-specific visualization of nitric oxide (NO) in tomato roots 48 h after contacting with *Rhizophagus irregularis* (a). Bright field (left panel) and fluorescence (right panel) images were taken by confocal microscopy. Green indicates NO-dependent DAF-FM DA fluorescence (excitation at 495 nm, emission at 515 nm). V, vascular bundle; E, epidermis; C, cortex; Rh, root hair. (b) NO also was visualized by fluorescence microscopy in roots of tomato plants mock inoculated (control) or 48 h after contacting with *R. irregularis* (Ri) or *Fusarium oxysporum* (Fox). Green indicates NO-dependent DAF-FM DA fluorescence (excitation at 495 nm, emission at 515 nm). The arrows point to NO-dependent DAF-FM DA signal confined mostly to the outer cell layers of *R. irregularis*-roots, and evenly distributed over the *F. oxysporum*-roots. Representative images are shown.

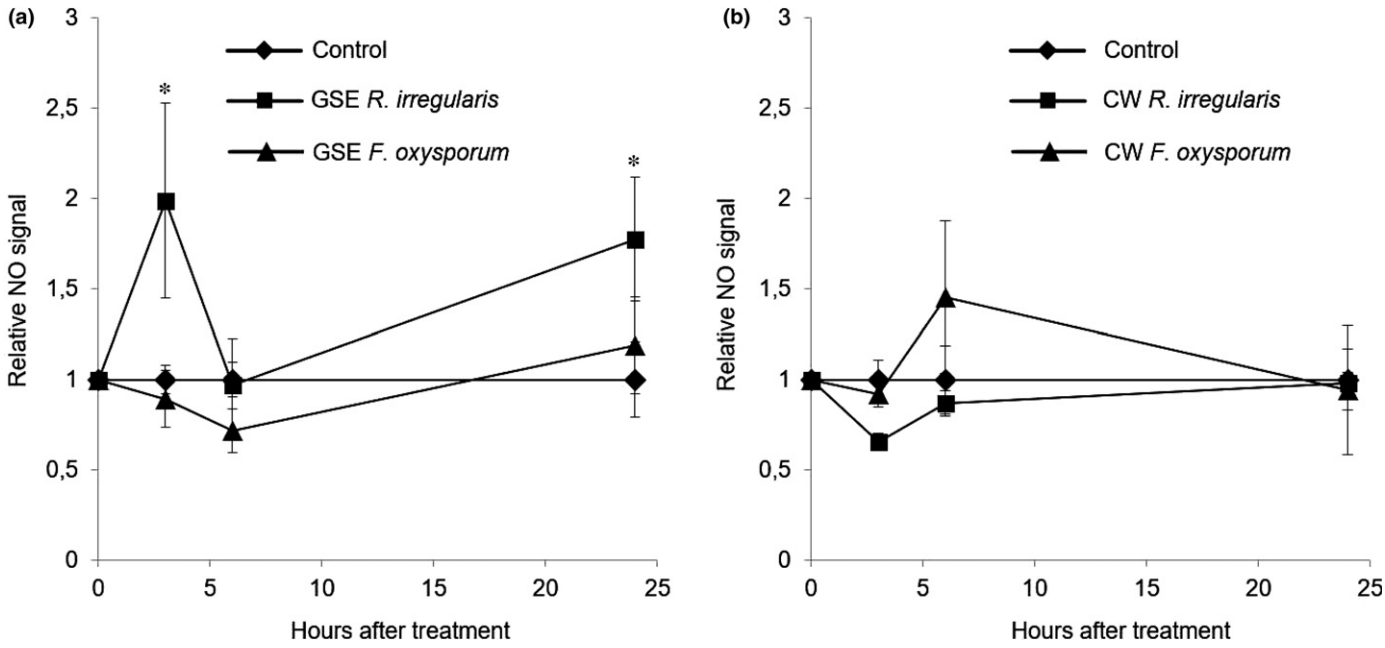

Fig. 4 Effect of exudates from germinating spores (GSE) and a suspension of cell walls (CW) from *Rhizophagus irregularis* and *Fusarium oxysporum* on endogenous nitric oxide (NO) root accumulation. NO was detected by fluorimetry by using the specific NO detector DAF-2 in tomato roots at 3, 6 and 24 h post-treatment with the GSE (a) or fungal CW (b). NO levels are showed as the fold increase relative to that of the control plants at each time point ± SE ($n = 4$ biological replicates). Asterisks indicate significant differences compared to control plants (Dunnett test, $P < 0.05$). These results are representative from one of two independent experiments.

c. 10 times higher than that seen in roots interacting with the AM fungus (Fig. 6a). The levels were still higher, although to a lesser extent, at 8 h. However, at later time points *PHYTOGB1* expression strongly decreased in the host roots, with transcripts barely detected by 72 or 96 h after contact (Fig. 6a). A similar inhibition of *PHYTOGB1* expression was found in tomato roots and shoots upon infection with the root and foliar pathogens *Phytophthora parasitica* and *Botrytis cinerea*, respectively (Fig. S3).

By contrast to *PHYTOGB1*, *R. irregularis* did not upregulate the expression of *PHYTOGB2* and *PHYTOGB3* throughout the monitored timespan (Fig. 6b,c). Indeed, *R. irregularis* reduced the expression levels of *PHYTOGB2* in tomato roots (Fig. 6b).

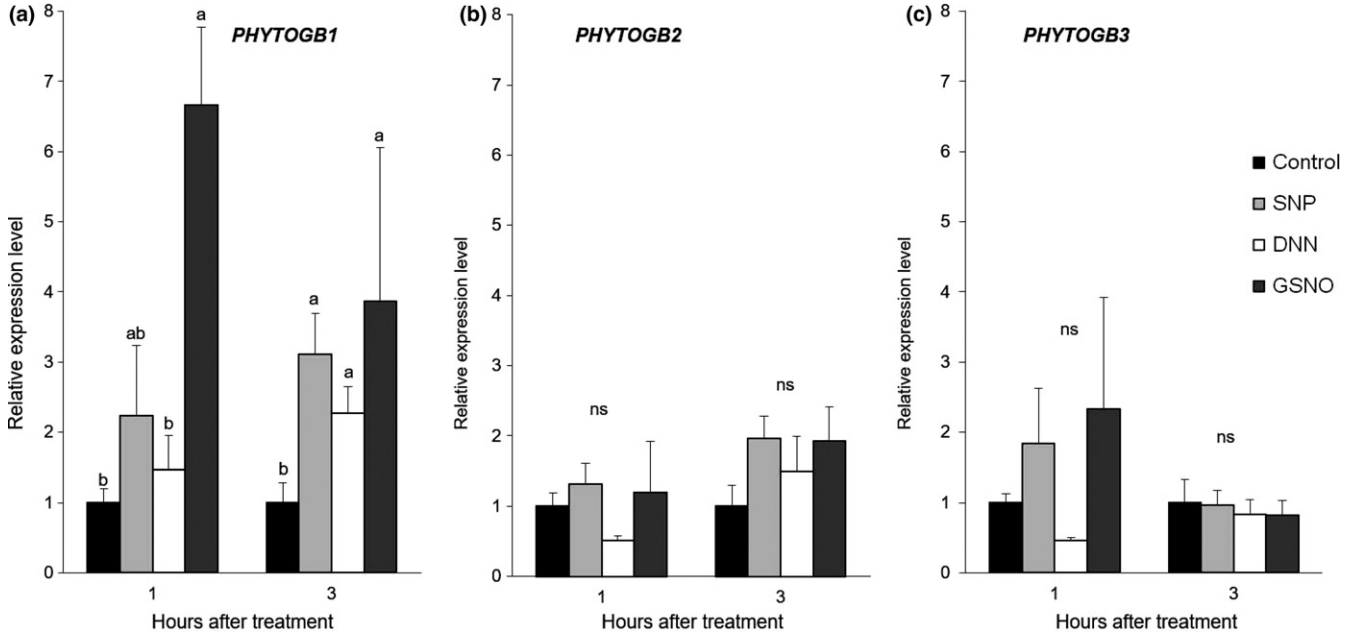

Fig. 5 Effect of nitric oxide (NO) on the regulation of tomato phytoglobin genes. Expression of (a) *PHYTOGB1*, (b) *PHYTOGB2* and (c) *PHYTOGB3* were analyzed in roots of tomato plants 1 and 3 h after treatment with the NO donors sodium nitroprusside (SNP), DETA-NONOate (DNN) and S-nitrosoglutathione (GSNO). Results were normalized by using the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the control plants not treated with the NO donors at each time point ± SE ($n = 3$ biological replicates). Data not sharing a letter in common at each time point differ significantly according to Tukey's honest significant difference test ($P < 0.05$). ns, not significant. These results are representative for one of two independent experiments.

We observed a similar reduction in *PHYTOGB2* in plants in contact with the pathogen, specifically from 48 h after the contact (Fig. 6b). These observations indicate that *PHYTOGB1* is specifically upregulated by the AM interaction, and may suggest a role for PHYTOGB1 during the onset of the AM symbiosis. Remarkably, in agreement with the NO accumulation pattern described earlier (Fig. 4), only germinating spore exudates from the mycorrhizal fungus, and not from *F. oxysporum*, significantly induced the expression of the *PHYTOGB1* gene in tomato roots (Fig. S4).

Altered *PHYTOGB1* levels in tomato roots leads to changes in NO and impacts mycorrhizal root colonization

In order to confirm whether *PHYTOGB1* is involved in NO metabolism in tomato, we generated composite plants with *PHYTOGB1* overexpressing (*PHYTOGB1*-OE) and the corresponding empty vector control roots. qRT-PCR analysis confirmed that the lines carrying the overexpressing construct had significantly increased *PHYTOGB1* expression levels compared with control roots carrying the empty vector, whereas the expression of the other phytoglobin genes remained unaltered (Fig. 7a). NO levels in *PHYTOGB1*-OE were lower compared to control roots transformed with the empty vector (Fig. 7b). As shown in Fig. 7c, a higher frequency (F%) and intensity (M%) of mycorrhizal colonization was found in the root system of *PHYTOGB1*-OE lines compared to plants transformed with the empty vector. Moreover, the intensity of the colonization (m%) within the colonized root fragments also was higher in *PHYTOGB1*-OE lines. It is remarkable that overexpressing *PHYTOGB1* did not affect the arbuscule abundance in the mycorrhizal parts (a%). The results from the histochemical analysis were further verified by molecular analysis. A higher accumulation of *Ri-EF1α* transcripts gene were detected in *PHYTOGB1*-OE roots compared to roots carrying the empty vector (Fig. 7d). Similarly, a stronger expression of *LePT4,* which encodes an AM-specific plant phosphate transporter, was found in the *PHYTOGB1*-OE roots (Fig. 7d).

We further investigated whether silencing of *PHYTOGB1* also had phenotypic effects on NO accumulation and mycorrhizal colonization patterns. With this aim a hairpin construct was created that targeted 201 bp of the tomato *PHYTOGB1* sequence. Composite plants were generated with *PHYTOGB1* silencing (*PHYTOGB1-RNAi*) and its corresponding empty vector (control) roots. qRT-PCR analysis showed that the RNAi construction significantly decreased the *PHYTOGB1* expression compared with control roots, but it did not alter significantly the expression of any of the other phytoglobin genes (Fig. 8a). A strong increase in NO accumulation was observed in *PHYTOGB1-RNAi* lines compared to control roots transformed with the empty vector (Fig. 8b), further demonstrating the role of PHYTOGB1 in regulating NO root metabolism. A higher frequency (F%) and intensity (M%) of mycorrhizal colonization was found in the *PHYTOGB1-RNAi* lines compared to plants transformed with the empty vector (Fig. 8c). Although not significant, *PHYTOGB1*-silenced lines also showed a slight increased in colonization intensity in the roots fragments (m%). As in the overexpressing lines, silencing *PHYTOGB1* did not affect arbuscule abundance in the mycorrhizal parts (a%). These results were further corroborated by the higher *Ri-EF1α* and *LePT4* transcript levels in *PHYTOGB1-RNAi* roots (Fig. 8d). Altogether our results demonstrate the importance of NO regulation by

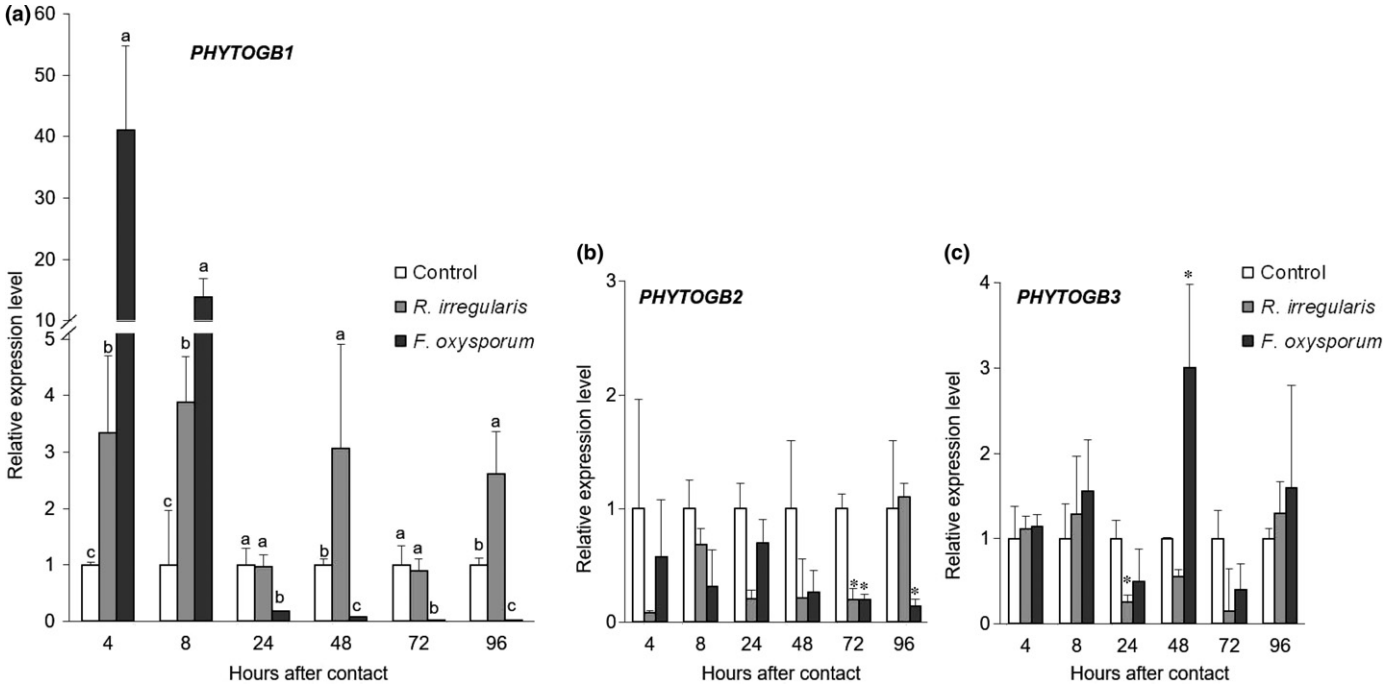

Fig. 6 Time course of expression of the tomato phytoglobin genes after contact with *Rhizophagus irregularis* or *Fusarium oxysporum*. The expression levels of (a) *PHYTOGB1*, (b) *PHYTOGB2* and (c) *PHYTOGB3* were analyzed in roots of tomato plants 4, 8, 24, 48, 72 and 96 h after contact with the arbuscular mycorrhizal fungus *R. irregularis* or the pathogen *F. oxysporum*. Results were normalized to *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the control plants at each time point ± SE ($n = 4$ biological replicates). In (a) data not sharing a letter in common at each time point differ significantly according to Tukey's honest significant difference test ($P < 0.05$). In (b) and (c) asterisks indicate significant differences in each time point compared to control plants (Dunnett test, $P < 0.05$). These results are representative for one of three independent experiments.

PHYTOGB1 specifically during the early stages of mycorrhizal establishment, related to root colonization, but not in the development of the arbuscules.

We investigated whether this alteration could be related to changes in plant defenses associated to the altered NO levels. With this aim, we tested whether alteration of NO levels by exogenous application of a NO donor (GSNO) and the NOS-like inhibitor aminoguanidine, or by altered levels of the *PHYTOGB1* lead to altered defense gene expression in tomato roots. The application of GSNO triggered a significant transient induction of several defense genes in tomato roots, whereas they were repressed by the application of aminoguanidine (Fig. S5). However, lower NO levels in the *PHYTOGB1-OE* lines were associated to higher basal levels of some defense-related genes, suggesting that NO can be a positive or negative regulator of defenses depending on its concentration and timing (Fig. S5). Remarkably, the increase of some defense genes triggered by the mycorrhizal colonization in control roots transformed with the empty vectors was not found in the *PHYTOGB1* overexpressing and silenced roots, supporting a release of the plant control over the fungus that may lead to higher mycorrhizal colonization (Fig. S5).

In analogy to the mycorrhizal interaction, deregulation of *PHYTOGB1* had an impact in the interaction with the root pathogen. We found an enhanced infection by *F. oxysporum* in the *PHYTOGB1-RNAi* roots displaying elevated NO levels (Fig. S6). By contrast, a lower incidence *of F. oxyporum* was found in *PHYTOGB1-OE* compared to plants transformed with the empty vector (Fig. S6).

Discussion

Nitric oxide (NO) accumulation in plant cells is an early component of the signaling pathways activated in plants during immune responses to pathogens, and also during rhizobial symbiosis establishment (Besson-Bard *et al.*, 2008; Hichri *et al.*, 2015). In several plant–microbe interactions NO bioactivity is regulated partially by the activity of class 1 phytoglobins (Qu *et al.*, 2006; Nagata *et al.*, 2008; Hill, 2012; Mur *et al.*, 2012). However, NO occurrence, function and regulation remain obscure in the case of the arbuscular mycorrhizal (AM) symbiosis. In the present study, we studied whether NO and its regulation by phytoglobins are regulatory components in the establishment and control of the AM symbiosis. We further addressed the specificity of the NO-related signature in the AM symbiosis by analyzing in parallel the NO accumulation pattern and NO-related responses triggered during the pathogenic interaction.

No oscillations and *PHYTOGB1* regulation are components of the signaling pathway regulating the onset of the AM symbiosis

Our study revealed the accumulation of NO during early stages of the interaction between tomato roots and the AM fungus

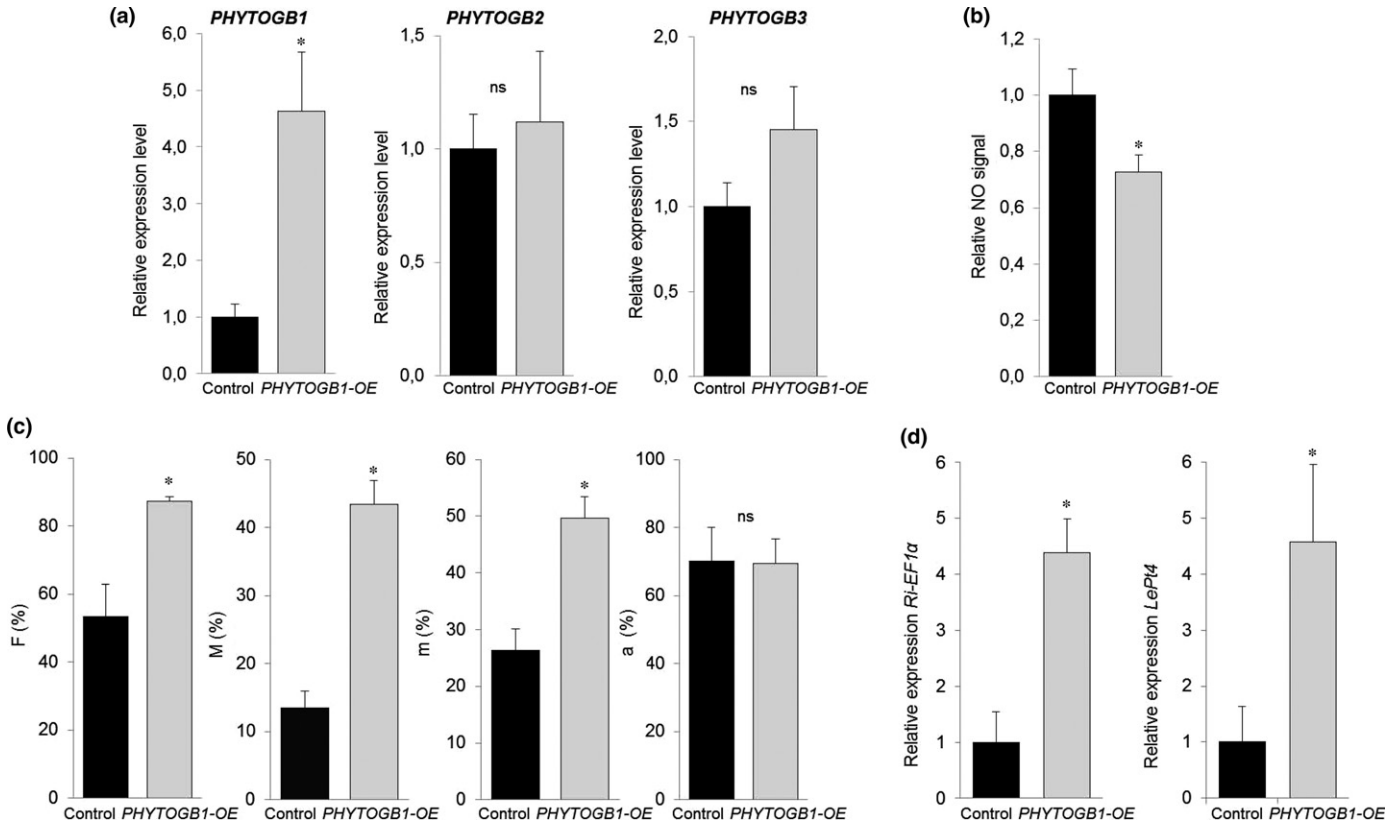

Fig. 7 Impact of overexpressing tomato *PHYTOGB1* on the expression of tomato phytoglobin genes, nitric oxide (NO) root accumulation and mycorrhizal colonization. Tomato plants were transformed with empty vectors (control) or *PHYTOGB1* overexpressing constructs (*PHYTOGB1*-OE). (a) Expression level of *PHYTOGB1* (left panel), *PHYTOGB2* (middle panel) and *PHYTOGB3* (right panel) was analyzed in empty vector controls and in *PHYTOGB1-OE* roots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the empty-vector control roots ± SE ($n = 6$ biological replicates). (b) NO accumulation was detected in empty vector controls and in *PHYTOGB1-OE* roots by fluorimetry using the specific NO detector DAF-2. NO levels are reported as the fold change relative to that of the empty-vector control roots ± SE ($n = 6$ biological replicates). (c) Arbuscular mycorrhiza fungal structures within the roots were analyzed in empty-vector controls and *PHYTOGB1-OE* roots 6 wk after inoculation with *Rhizophagus irregularis* in pots. F%, frequency of colonization in the root system; M%, intensity of colonization in the root system; m%, intensity of colonization within the mycorrhizal fragments; a%, arbuscule abundance in mycorrhizal parts. (d) Relative expression of the *R. irregularis* constitutive gen *Ri-EF1α* (left panel) and the mycorrhizal functionality marker gene *LePT4* (right panel) in empty-vector controls and *PHYTOGB1-OE* roots 6 wk after inoculation with *R. irregularis* in pots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the empty-vector control root ± SE ($n = 6$ biological replicates). The asterisks indicate a statistically significant difference in comparison to the empty-vector control root according to Student's *t*-test ($P < 0.05$). ns, not significant. These results are representative from one of two independent experiments.

Rhizophagus irregularis (Fig. 2). We found that NO accumulation oscillates in response to the AM fungus from the earliest time point monitored until the end of the study. These results suggest a potential role(s) for NO from the early host recognition to the transduction pathway leading to the symbiosis establishment upon contact with the AM fungal hyphae. Similarly, Espinosa *et al.* (2014) showed an increase in NO levels in roots of olive seedlings 1 h after contacting with *R. irregularis*. Although the authors did not monitor the temporal modulation of the NO signaling, these previous observations reinforce the idea that the early AM interaction is associated with NO-related signaling in the host roots.

Imaging of NO production further revealed that the AM fungus-triggered NO accumulation is located mainly in the outer cell layers of the root and in root hairs (Fig. 3). These specific root zones have been associated previously with a fast triggering of the calcium (Ca^{2+}) signaling in response to exudates from AM fungal germinating spores and AM fungal hyphopodia (Chabaud *et al.*, 2011; Genre *et al.*, 2013). This overlap between AM-triggered NO and Ca^{2+} signaling might suggest an interplay between both signaling components in the onset of the AM symbiosis. Indeed, NO has the capacity to act as a Ca^{2+} mobilizing intracellular messenger (Courtois *et al.*, 2008), and Ca^{2+} has been suggested to be linked to downstream NO generation through the action of calmodulin-like proteins (Ma *et al.*, 2008). Our results reveal that the AM interaction triggers an early NO-related response in root cell types that previously have been associated with early AM signaling.

Indeed, NO accumulation in the roots occurred within the few first hours after contact with the AM fungus. This could imply that the early NO signaling is triggered by diffusible fungal factors which activate the AM symbiosis pathway, and/

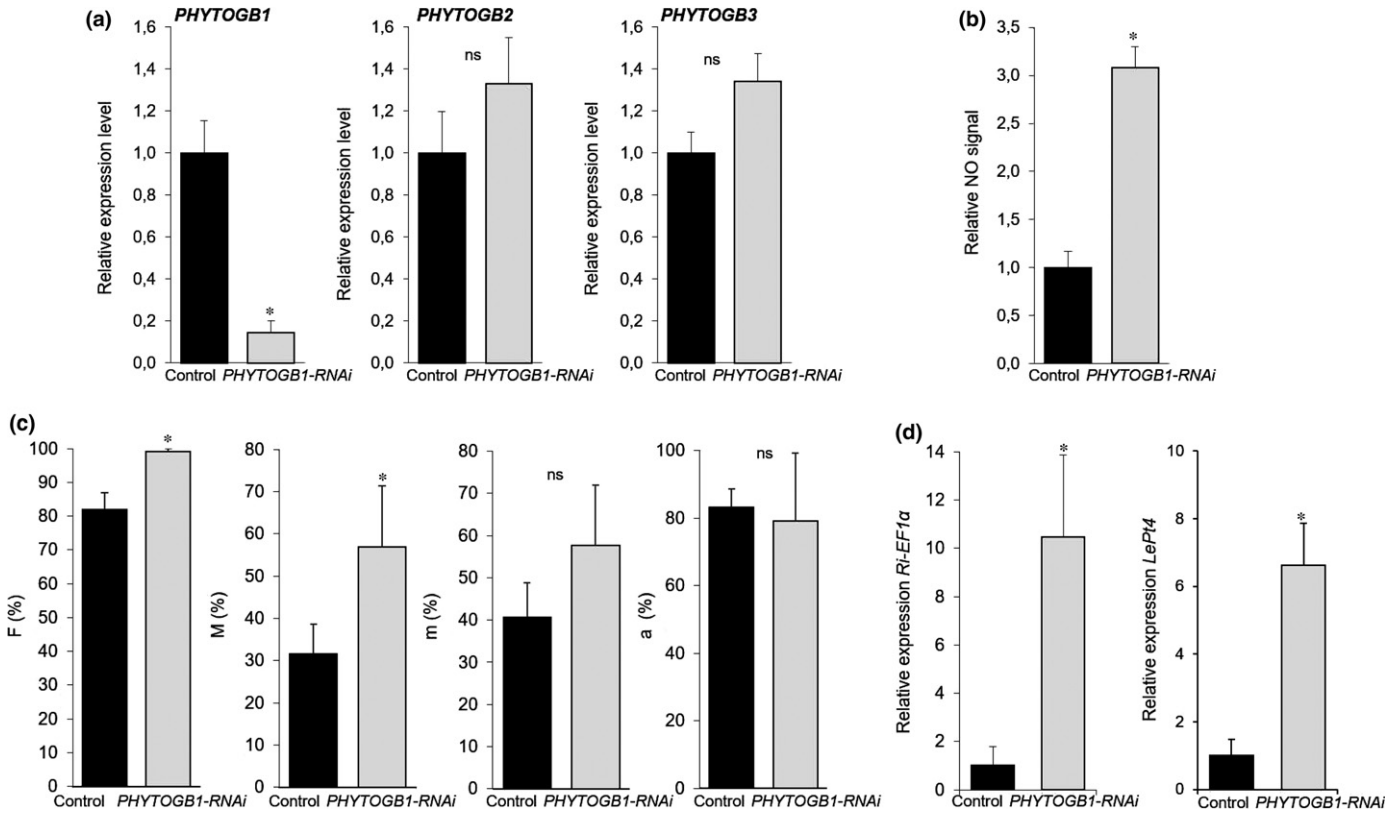

Fig. 8 Impact of silencing tomato *PHYTOGB1* on the expression of tomato phytoglobin genes, nitric oxide (NO) root accumulation and mycorrhizal colonization. Tomato plants were transformed with empty vectors (control) or *PHYTOGB1*-silenced constructs (*PHYTOGB1-RNAi*). (a) Expression level of *PHYTOGB1* (left panel), *PHYTOGB2* (middle panel) and *PHYTOGB3* (right panel) was analyzed in empty-vector controls and in *PHYTOGB1-RNAi* roots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold change relative to that of the empty-vector control roots ± SE ($n = 6$ biological replicates). (b) NO accumulation was detected in empty-vector controls and in *PHYTOGB1-RNAi* roots by fluorimetry using the specific NO detector DAF-2. NO levels are reported as the fold change relative to that of the empty-vector control roots ± SE ($n = 6$ biological replicates). (c) Arbuscular mycorrhiza fungal structures within the roots were analyzed in empty-vector controls and *PHYTOGB1-RNAi* roots 6 wk after inoculation with *Rhizophagus irregularis* in pots. F%, frequency of colonization in the root system; M%, intensity of colonization in the root system; m%, intensity of colonization within the mycorrhizal fragments; a%, arbuscule abundance in mycorrhizal parts. (d) Relative expression of the *R. irregularis* constitutive gen *Ri-EF1α* (left panel) and the mycorrhizal functionality marker gene *LePT4* (right panel) in empty-vector controls and *PHYTOGB1-RNAi* roots 6 wk after inoculation with *R. irregularis* in pots. Results were normalized to the *SlEF* gene expression in the same samples. The expression levels are reported as the fold increase relative to that of the empty-vector control root ± SE ($n = 6$ biological replicates). The asterisks indicate a statistically significant difference in comparison to the empty-vector control root according to Student's *t*-test ($P < 0.05$). ns, not significant. These results are representative for one of two independent experiments.

or by fungal cell wall components that could act as general microbe-associated molecular patterns activating a rapid and unspecific defense reaction (Boller & Felix, 2009). To clarify this, we treated tomato roots with exudates from *R. irregularis* germinating spores and with *R. irregularis* cell wall extracts. We found that NO signaling was specifically triggered by components in the exudates from the germinating spores, but not by the cell wall extracts (Fig. 4). These results indicate that the early NO signaling observed is triggered specifically by bioactive molecules present in the AM fungal exudates. This is in agreement with previous observations by Calcagno *et al.* (2012) revealing a transient accumulation of NO in *Medicago truncatula* root cultures in response to exudates from the AM fungus *Gigaspora margarita*. Our results reinforce the idea that NO signaling is a component of the early plant response to diffusible factors in the exudates from AM fungal germinating spores.

It is noteworthy that we found a temporal overlap between the AM fungus-triggered NO accumulation and the regulation of the specific tomato phytoglobin gene *PHYTOGB1* (Fig. 2). *PHYTOGB1* has been shown to be NO-inducible in other plant species (Ohwaki *et al.*, 2005; Bustos-Sanmamed *et al.*, 2011; Bai *et al.*, 2016), and here we confirm that *PHYTOGB1* was the only NO-inducible tomato phytoglobin gene (Fig. 5). This concomitant regulation of NO and *PHYTOGB1* suggests a role for PHYTOGB1 in regulating NO bioactivity during the onset of the AM symbiosis. Indeed, although the potential function(s) of PHYTOGB1 remained largely unknown, previous studies showed an upregulation of this gene in the model plant *M. truncatula* in response to the early interaction with the AM fungi *G. margarita* and *R. irregularis* (Siciliano *et al.*, 2007; Hogekamp & Küster, 2013). Altogether, our results point to a potential role of PHYTOGB1 and NO signaling in the signaling pathway activated during the AM symbiosis establishment.

The AM symbiosis displays a specific signature of NO accumulation in the host roots

We next investigated whether the NO-related response triggered by the AM fungus results from the specific plant recognition of its fungal symbiont or instead, it is part of a general immune response. To this end, we compared NO oscillations elicited by the AM interaction with those triggered by the pathogen *F. oxysporum*. The NO signatures elicited by the two interactions were significantly different (Fig. 2). For instance, the early (4 h) plant response to the pathogen was associated with a stronger accumulation of NO compared to that triggered by the fungal symbiont. In analogy to our observations, previous studies demonstrated that early NO-related responses elicited by mutualistic and pathogenic bacteria differ significantly (Nagata *et al.*, 2008; Espinosa *et al.*, 2014). It is noteworthy that the stronger NO burst triggered in the pathogenic interaction was accompanied by a stronger upregulation of the NO-inducible *PHYTOGB1* (Fig. 6). At later stages, the pathogen induced a continuous increase in NO, which was spread through the complete root (Fig. 2). This contrasts with the more regular oscillations of NO levels observed in the AM interaction, which was restricted to the outer cell layers. In the mycorrhizal system, *PHYTOGB1* expression followed an oscillatory pattern similar to that of NO levels. However, it is intriguing that during the pathogenic interaction, the increased NO accumulation triggered at later stages was accompanied by a strong downregulation of *PHYTOGB1*, despite the NO-inducible character of this gene (Fig. 5). These results suggest the ability of *F. oxysporum* for actively repressing *PHYTOGB1* expression, most likely to promote high levels of NO and create favorable conditions for the invasion (Arasimowicz-Jelonek & Floryszak-Wieczorek, 2016). In line with our observations, the symbiotic rhizobium *Mesorhizobium loti* and the pathogens *Ralstonia solanacearum* and *Pseudomonas syringe* triggered differential patterns of NO accumulation and regulation of the class 1 phytoglobin gene *LjHb1* in *Lotus japonicus,* being *LjHb1* transcriptional activation blocked by the pathogens (Nagata *et al.*, 2008). Interestingly, here we confirmed a similar repression pattern for tomato *PHYTOGB1* during other pathogenic interactions with the root oomycete *Phytophthora parasitica* and with the shoot necrotrophic fungus *Botrytis cinerea* (Fig. S3). These results pinpoint PHYTOGB1 as a key target for pathogenesis in different systems.

It also is interesting that by contrast to AM fungal diffusible signals, exudates from germinating spores of the pathogen did not trigger NO accumulation and *PHYTOGB1* upregulation in tomato roots (Figs 4, S4). This finding strongly suggests that the NO-related response triggered by the *R. irregularis* diffusible factor is not a general response to fungi, but most likely is specific to AM fungi. In general our observations indicate that although NO production is a common component of plant responses to the AM symbiont and the pathogen *F. oxysporum*, there are clear differences between the NO signatures elicited by both interactions. Such differences probably reflect different biological functions of NO and a differential regulation by PHYTOGB1 in both interactions.

PHYTOGB1 regulates NO levels in tomato and is involved in the regulation of mycorrhizal colonization

The role of class 1 phytoglobins as regulators of NO levels in plant–microbe interactions has been established in some legume plants and *Arabidopsis* (Shimoda *et al.*, 2009; Bustos-Sanmamed *et al.*, 2011; Fukudome *et al.*, 2016). To investigate whether *PHYTOGB1* also is involved in NO regulation in tomato, and if it is a regulator of the AM symbiosis, we generated tomato *PHYTOGB1* overexpressing and silenced hairy roots. We found that, indeed, the accumulation of NO was strongly reduced in the overexpressing lines and enhanced in the silenced ones when compared to their respective control roots (Figs 7, 8). These findings demonstrate that PHYTOGB1 control endogenous NO levels in tomato roots, consistently with its previously reported ability to catalytically metabolize NO to nitrate in other systems (Seregelyes *et al.*, 2004; Hill, 2012).

Our results further evidenced a stronger frequency and intensity of mycorrhizal colonization in the *PHYTOGB1* overexpressing roots compared to those carrying the empty vector (Fig. 7). Remarkably, overexpression of *PHYTOGB1* did not alter the abundance of arbuscules in the colonized areas, supporting a role of PHYTOGB1 in the regulation of the early events of the interaction leading to root colonization and its extension, but not in arbuscule formation. These results are in line with previous studies showing an upregulation of *PHYTOGB1* specifically in cells harboring the first mycorrhizal infection sites in *M. truncatula* roots (Siciliano *et al.*, 2007; Hogekamp & Küster, 2013). Intriguingly, a higher mycorrhizal colonization was also found in *PHYTOGB1*-silenced plants (Fig. 8). These findings support the hypothesis that precise fine-tuning of NO levels is required for the control of the AM symbiosis establishment and extension. Previous studies showed a similar contrasting role of NO in the control of nodulation in the rhizobial symbiosis: NO has been shown to promote nodule formation (Pii *et al.*, 2007), and to be deleterious to nodule production (Shimoda *et al.*, 2009). Our results evidenced that both, higher and lower NO accumulation in *PHYTOGB1-silenced* and overexpressing plants promoted mycorrhizal colonization. Taking into consideration the role of NO in the regulation of plant defenses (Fig. S5), we hypothesize that NO might be involved in the plant regulation of the degree of AM colonization by regulating plant defenses, however, the specific impact of NO on plan defenses during the mycorrhizal interaction remains unknown.

In analogy to the mycorrhizal interaction, deregulation of *PHYTOGB1* affected the interaction with the root pathogen (Fig. S6). An enhanced infection was found in the *PHYTOGB1*-silenced lines, whereas a lower incidence of the pathogen was observed in the overexpressing lines. These findings indicate that PHYTOGB1 bioactivity is required for the plant to restrict the pathogen infection, and reinforce the idea that blocking the transcriptional activation of *PHYTOGB1* can be a pathogen strategy to increase NO levels to favor infection (Nagata *et al.*, 2008). Our results reveal a major role of tomato PHYTOGB1 in regulating NO levels and root–fungi interactions, particularly in the establishment of the AM symbiosis. They also indicate that

PHYTOGB1 is involved in the control by the host plant of the extension of mycorrhizal colonization, most likely by regulating NO bioactivity in host roots.

Conclusion

We demonstrated that NO accumulation and *PHYTOGB1* transcriptional regulation are early components of the regulatory pathway that is activated in tomato roots during the onset of the AM symbiosis with *R. irregularis*. We further demonstrated that although NO-related signaling is a common regulatory component in mutualistic and pathogenic plant–microbe interactions, the NO-related signature and PHYTOGB1 regulation shows different patterns in both interactions. We propose that fine-tuned NO accumulation is required for proper AM establishment, and that PHYTOGB1 is triggered during the interaction to control NO levels in order to promote and control the AM symbiosis.

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Author contributions

AMM, MCRP and MJP planned and designed the research; AMM, LP, IF, MRS and JMG performed experiments; AMM, LP, IF, MRS, MCRP and MJP analyzed data; and AMM, MCRP and MJP wrote the manuscript with input of all the authors.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Imaging NO production in tomato roots interacting with *Rhizophagus irregularis* and treated with cPTIO.

Fig. S2 Exon–intron compositions of *Solanum lycopersicum* phytoglobin genes, and alignment of *Solanum lycopersicum* phytoglobins.

Fig. S3 *Solanum lycopersicum PHYTOGB1* gene expression in tomato plants after contact with the pathogens *Phytophthora parasitica* and *Botrytis cinerea*.

Fig. S4 Effect of exudates from germinating spores (GSE) from *Rhizophagus irregularis* and *Fusarium oxysporum* on the expression of the *PHYTOGB1* gene.

Fig. S5 Impact of NO on tomato plant defenses.

Fig. S6 Effect of altered *PHYTOGB1* levels on *Fusarium oxysporum* infection.

Table S1 List of primers used in the analyses.

Table S2 Basal expression levels of tomato phytoglobin genes in control (noninoculated plants) through the time course analysis.

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