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PvLOX2 silencing in common bean roots impairs arbuscular mycorrhiza-induced resistance without affecting symbiosis establishment

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Abstract. The arbuscular mycorrhizal (AM) symbiosis is an intimate association between specific soil-borne fungi and the roots of most land plants. AM colonisation elicits an enhanced defence resistance against pathogens, known as mycorrhizal-induced resistance (MIR). This mechanism locally and systemically sensitises plant tissues to boost their basal defence response. Although a role for oxylipins in MIR has been proposed, it has not yet been experimentally confirmed. In this study, when the common bean (*Phaseolus vulgaris* L.) lipoxygenase *PvLOX2* was silenced in roots of composite plants, leaves of silenced plants lost their capacity to exhibit MIR against the foliar pathogen *Sclerotinia sclerotiorum*, even though they were colonised normally. *PvLOX6*, a LOX gene family member, is involved in JA biosynthesis in the common bean. Downregulation of *PvLOX2* and *PvLOX6* in leaves of *PvLOX2* root-silenced plants coincides with the loss of MIR, suggesting that these genes could be involved in the onset and spreading of the mycorrhiza-induced defence response.

Additional keywords: fungi, RNA silencing, systemic resistance.

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Introduction

Plant roots are capable of forming beneficial interactions with soil micro-organisms, most commonly as a symbiotic arbuscular mycorrhiza (AM) association with fungi from the phylum Glomeromycota (Schüßler *et al*. 2001). The benefits of AM symbiosis on plant fitness are well known, and include improved nutrition and resistance to biotic and abiotic stresses (Pozo and Azcon-Aguilar 2007).

A reduction in the damage caused by soil-borne root pathogens to mycorrhiza-colonised plants has been widely documented for diverse plant species and pathogens (Whipps 2004; Hao *et al*. 2012; Vos *et al*. 2013), as well as shoot pathogens (Liu *et al*. 2007; Noval *et al*. 2007; Fiorilli *et al*. 2011; Campos-Soriano *et al*. 2012). Mycorrhizal-induced resistance (MIR) is suggested to be similar to induced systemic resistance (ISR), which is triggered by beneficial rhizobacteria colonisation (Pozo and Azcon-Aguilar 2007). Although plants are equipped with a basal defence system, both ISR and MIR manifest themselves by augmenting this defence response. ISR and MIR are preceded by the onset of a 'priming' state that allows colonised plants to respond faster and stronger to a pathogen attack. Priming is a systemic phenomenon, as ISR and MIR are expressed in both the roots (where colonisation occurs) and shoots (Conrath *et al*. 2006; Pozo and Azcon-Aguilar 2007; Van Wees *et al*. 2008; Ahmad *et al*. 2010; Conrath 2011). Priming takes place before the encounter with a pathogen, and it is not conspicuous until the plant tissue has been attacked. Defence priming has been observed as a result of

the interaction with such beneficial microorganisms as rhizobacteria and mycorrhiza fungi, as well as in response to pathogens, insects, and some chemical compounds (Kohler *et al*. 2002; Jung *et al*. 2009; Conrath 2011). Several mechanisms at the cellular level have been proposed to explain priming. For example, priming can be initiated by an enhanced accumulation of inactive defence metabolite conjugates that become active upon pathogen attack (Pastor *et al*. 2013). Priming can also be the result of an enhanced expression and accumulation of defence regulatory protein kinases. This would require a secondary post-translational modification to become active upon any subsequent treatment challenge (Beckers *et al*. 2009), as well as the accumulation of transcriptional factors (Van der Ent *et al*. 2009). Finally, it has been postulated that priming is related to sensitisation of the hormone-inducible defence response, possibly through an increased accumulation of signalling proteins that function in the response pathways of these hormones, which would require a secondary post-translational activation upon pathogen attack (Pastor *et al*. 2013).

At the molecular level, several reports have shown that some jasmonic acid (JA)-responsive genes show higher expression in leaves of mycorrhizal plants following a pathogen challenge, compared with non-colonised plants (Pozo *et al*. 2010; Gallou *et al*. 2011; Campos-Soriano *et al*. 2012). In addition, Campos-Soriano *et al*. (2012) have presented evidence suggesting that mycorrhiza colonisation induces JA biosynthesis and signalling pathways before a pathogen challenge. However, the molecular mechanisms controlling this phenomenon remain unknown.

MIR is elicited by mycorrhiza colonisation in the roots and is subsequently spread throughout the plant. This implies that a signal originates in mycorrhizal roots and translocates systemically, to sensitise distal tissues for an enhanced defence response. However, very little is known about this process. To address this concern, we have examined here the role of JA signalling in the onset of mycorrhiza-induced resistance in common bean roots and its spreading to the plant shoot, with a focus on the participation of the oxylipin pathway. Up to now, no molecular markers have been identified for the primed state or MIR that occurs before the pathogen attack. The only means of detection then is to perform post challenge defence response assays (Conrath *et al*. 2006).

Oxylipin compounds are produced from the oxidative metabolism of polyunsaturated fatty acids (PUFA), such as linolenic acid from cell membranes (Feussner and Wasternack 2002; Wasternack and Hause 2013). The first key enzyme during oxylipin biosynthesis is lipoxygenase (LOX; EC 1.13.11.12). LOX products can be formed by the cytosolic 9- and 13-LOXs, according to their region specificity. Chloroplasts contain only 13-LOXs (Porta *et al*. 2008). Types 1 and 2 13-LOXs have been described and only type 2 contains a plastid transit peptide and is associated with JA biosynthesis (Feussner and Wasternack 2002). 9-LOX, as well as type 1 13-LOX, give rise to other oxylipins besides JAs, such as divinyl ether PUFAS, alcohols, and aldehydes. LOX products can be used by at least seven metabolic branch points, yielding a diverse group of compounds (Feussner and Wasternack 2002; Wasternack and Hause 2013). Allene oxide synthase (AOS) plays a role in the JA biosynthetic branch (Wasternack and Hause 2013), catalysing the second step in JA biosynthesis after LOX (Feussner and Wasternack 2002). To date, six lipoxygenase gene isoforms (*PvLOX1* through *PvLOX6*) from the common bean have been isolated and partially characterised; their functions have thus been associated to tissue development, plant wounding and pathogen signalling pathways, and cell death induction (Meier *et al*. 1993; Eiben and Slusarenko 1994; Porta *et al*. 1999, 2008; Porta and Rocha-Sosa 2000, 2002). Although 9-LOXs have not yet been identified in *Phaseolus vulgaris*, their occurrence in this species cannot be ruled out (Porta and Rocha-Sosa 2000). We previously observed that mycorrhiza colonisation of *Phaseolus vulgaris* L. with *Rhizophagus irregularis* (syn. *Glomus intraradices*) induces systemic resistance against the shoot necrotrophic fungus pathogen *Sclerotinia sclerotiorum* (Mora-Romero 2008). However, the role of oxylipin biosynthetic genes in the systemic spreading and onset of MIR in shoots of this plant species is unknown. Here, we have investigated whether silencing of an oxylipin biosynthetic gene (such as *PvLOX2*) in roots of composite common bean plants can affect mycorrhiza establishment and MIR. Our results will contribute to the identification of molecular components and mechanisms involved in mycorrhiza-induced resistance.

Materials and methods

Construction of the pTDT-PvLOX2-RNAi plasmid

Reported sequences from cDNA libraries in the Common Bean Gene Index (http://compbio.dfci.harvard.edu/tgi/, accessed 12 October 2010) were used to design oligonucleotides for common bean genes, using the Primer 3 software (http://frodo.wi.mit.edu, accessed 12 October 2010). To reduce the chances of silencing genes other than *PvLOX2*, the DNA fragment used for the silencing construct was located on the 5′ UTR of the *PvLOX2* gene (see Fig. S1, available as Supplementary Material to this paper). For the *PvLOX2*RNAi construct, a 238 bp *PvLOX2* (accession number U76687.2) fragment, which includes 228 nucleotides from the 5′ UTR and 10 nucleotides of the coding sequence, was PCR amplified using the following oligonucleotide sets: *attPv*LOX2For 5′-GGGGACAAGTTTG TACAAAAAAGCAGGCTCAAATGATCAAGGAATGGTG AG-3′ and *attPv*LOX2Rev 5′-GGGGACCACTTTGTACA AGAAAGCTGGGTTTCCAAACATCTTTGCCAAT-3′. The oligonucleotides contained the *attB* universal cloning sites and the PCR product was cloned into pDNOR221, according to the BP reactions protocol (Invitrogen, Carlsbad, CA, USA). Proper insertion was confirmed by PCR amplifications, followed by the LR reaction protocol (Gateway Technology, Invitrogen). The binary vector pTDT-RNAi (Valdés-López *et al*. 2008) was then used to generate the pTDT-*PvLOX2*-RNAi construct. The correct orientation was analysed by PCR using the WRKY-5-Rev 5′-GCAGAGGAGGAGAAGCTTCTAG-3′ or WRKY-3-Fwd 5′-CTTCTCCAACCACAGGAATTCATC-3′ oligonucleotide set (Jang *et al*. 2007), and finally confirmed by sequencing. The resulting pTDT-*LOX2*-RNAi plasmid was introduced by electroporation into *Agrobacterium rhizogenes* K599, and then used for plant root transformation. An empty vector construct (pTDTRNAi) was prepared for use as an experimental control.

Composite plant material and growth conditions

Common bean seeds (*Phaseolus vulgaris* L. var. Negro Jamapa) were surface-disinfected by immersion in 20% commercial bleach solution (v:v) for five minutes, rinsed four times with sterile distilled water, immersed in 96% ethanol for 2 min, and rinsed four times with sterile distilled water. Seeds were subsequently germinated in sterile vermiculite. Composite root transformed plants were generated by inducing infections of *A. rhizogenes* (containing the corresponding gene-silencing or empty vector constructs) in cotyledon nodes, as described by Estrada-Navarrete *et al*. (2006). This procedure is schematised in Fig. 1. Putative transgenic hairy roots formed two weeks after bacterial infections. These were confirmed by inspecting for the presence of the red fluorescence resulting from the expression of the tdTomato (TDT) reporter gene by fluorescence stereomicroscopy. Non-fluorescent adventitious roots were eliminated and only red fluorescent roots were left on the plant. The normal non-fluorescent root system was then removed. Subsequently, composite plants (consisting of transformed roots and normal shoots) were transferred to 0.2 L rhizocones with vermiculite. Non-transformed (NT), composite silenced (*PvLOX2*RNAi), and composite empty vector (EV) plants were inoculated with 0.1 g of freshly minced *R. irregularis*-colonised transformed carrot roots for mycorrhiza colonisation (M). An equal number of plants per treatment were used for mock-inoculated (NM) controls, using minced non-mycorrhizal transformed carrot roots. To allow mycorrhiza colonisation to occur, plants were fertilised on a weekly basis by watering with Hoagland solution (Hoagland and Arnon 1950) containing one-tenth (20 µM) of the regular potassium phosphate content, and maintained in growth chambers (Binder, Tuttlingen, Germany, model KBW 400; 8 h light at 25°C/16 h dark at 18°C) for 4 more weeks.

Root analysis

At the end of the experiments, the root systems of plants were split into two parts. Half of the roots were frozen in liquid nitrogen and homogenised in a mortar for RNA extraction. The other half was fixed in 50% ethanol (v/v) for one hour, clarified in 20% KOH (w/v) for 3 days, and finally neutralised in 1% HCl (v/v) for 1 h. Subsequently, half of the fixed roots were stained in 2% trypan blue (w/v) and destained overnight with lacto glycerol (1 : 1 : 1 water, lactic acid, and glycerol; v/v/v) (Phillips and Hayman 1970) for determination of colonisation percentage by light microscopy, using the 'line intersection' method (Giovannetti and Mosse 1980). The other half of the fixed and clarified roots was stained with WGA-Alexa Fluor 488 (cat no. 411262, Life Technologies, Carlsbad, CA, USA), to analyse morphology of infection units and arbuscules by confocal microscopy (Fig. 1). Confocal images were obtained from merged acquisition channels using 497 and 554 nm excitation lasers; emission ranges of 502–548 and 570–640 nm were used for WGA Alexa Fluor 488 conjugate (green fluorescence) and vascular tissue endogenous fluorescence (red fluorescence), respectively, and obtained using a TCS SP5X microscope (Leica, Mannheim, Germany).

RNA extraction and cDNA synthesis

Total RNA was extracted from 50–100 mg of root or leaf tissues (previously frozen in liquid nitrogen) using TRIzol reagent (Ambion, Carlsbad, CA, USA, cat. no. 15596–026). Four plants were selected per treatment and considered as biological replicates. Reverse transcription was performed with the SuperScript III RT kit (Invitrogen cat. 18080–044), according to the manufacturer's instructions. Complementary DNAs were obtained from 1 µg of total DNase-treated RNA in a 20 µl reaction volume using TURBO DNAse (Invitrogen cat. no. AM2238).

Confirmation of transformation and gene silencing

End-point reverse transcription-PCR (RT–PCR) and quantitative RT–PCR (qRT–PCR) were used to estimate *PvLOX2* gene silencing, using the specific *PvLOX2* oligonucleotide set (*PvLOX2*-3′UTRF 5′-CGTCTGTGGTTTAATGTCTGTCC-3′ and *PvLOX2*-3′UTRR 5′-CACCCGACAATTATGCAGAG-3′) with cDNA obtained from root samples of each treatment replicate. The PCR end-point program consisted of an initial 5 min step at 95°C, followed by 20 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Subsequently, 3 µL of the PCR product were separated in a 2% agarose gel and visualised under UV light with ethidium bromide staining; qRT–PCR was performed using this set of primers using the methodology describe below.

Gene expression and AM colonisation by qRT–PCR

The PCR reaction mix contained 200 nM of each oligonucleotide, 10 µL of 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA, cat. no. 4364344), and 50 ng of cDNA in 20 µl total volume. Reactions were aliquoted on to 96-well plates in an Applied Biosystems 7500 Real-Time PCR System. Four independent biological replicates were used per treatment, and four technical replicates (per each biological replicate) were used for qRT–PCR gene expression analysis. The PCR program consisted of an initial 3 min step at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58−60°C, and 30 s at 72°C. Single-product amplification was confirmed by performing a dissociation curve after the final PCR cycle. The relative transcription levels were calculated by $2^{-\Delta Ct}$ using the *PvActin* gene for normalisation (PvActinF 5′-TGGTATTGC GGACAGAATGA-3′ and PvActinR 5′-AGCCAAGATAGA GCCACCAA-3′). The design of *PvLOX2* (*PvLOX2*-3′UTR F and *PvLOX2*-3′UTR R) and *PvLOX6* (PvLOX6 F 5′-TGG TTCAAATTCAGGTGCAA-3′ and PvLOX-6 R 5′-CATGG CTCACCCTTTTTAGC-3′) primers for qRT–PCR were based on published sequences (Porta *et al*. 1999, 2008). The *PvAOS* (PvAOS F 5′-ACCAATCTTCCGATCCG-3′ and PvAOS R 5′-CCTTGATTCTAGAAATAGTC-3′) and *PvCOI1* (PvCOI1 F 5′-TGGTTTTGTTCCCTTCAAATCT-3′ and PvCOI1 *R* 5′-AGGAAGCGAAGAACACAAGC-3′) primers for qPCR were based on the sequences TC39308 and TC44245, respectively, and designed with the Primer 3 program (http://frodo.wi.mit.edu, accessed 12 June 2012). These two sequences were previously obtained from the *Phaseolus vulgaris* Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=p_vulgaris, accessed 12 June 2012). *PvLOX2*, *PvLOX6*, *PvAOS*, *PvCOI1* and *PvActin* gene expression was measured in cDNA obtained from roots and leaf samples from all NT (both M and NM), EV and *PvLOX2*RNAi plants, as described in the previous section.

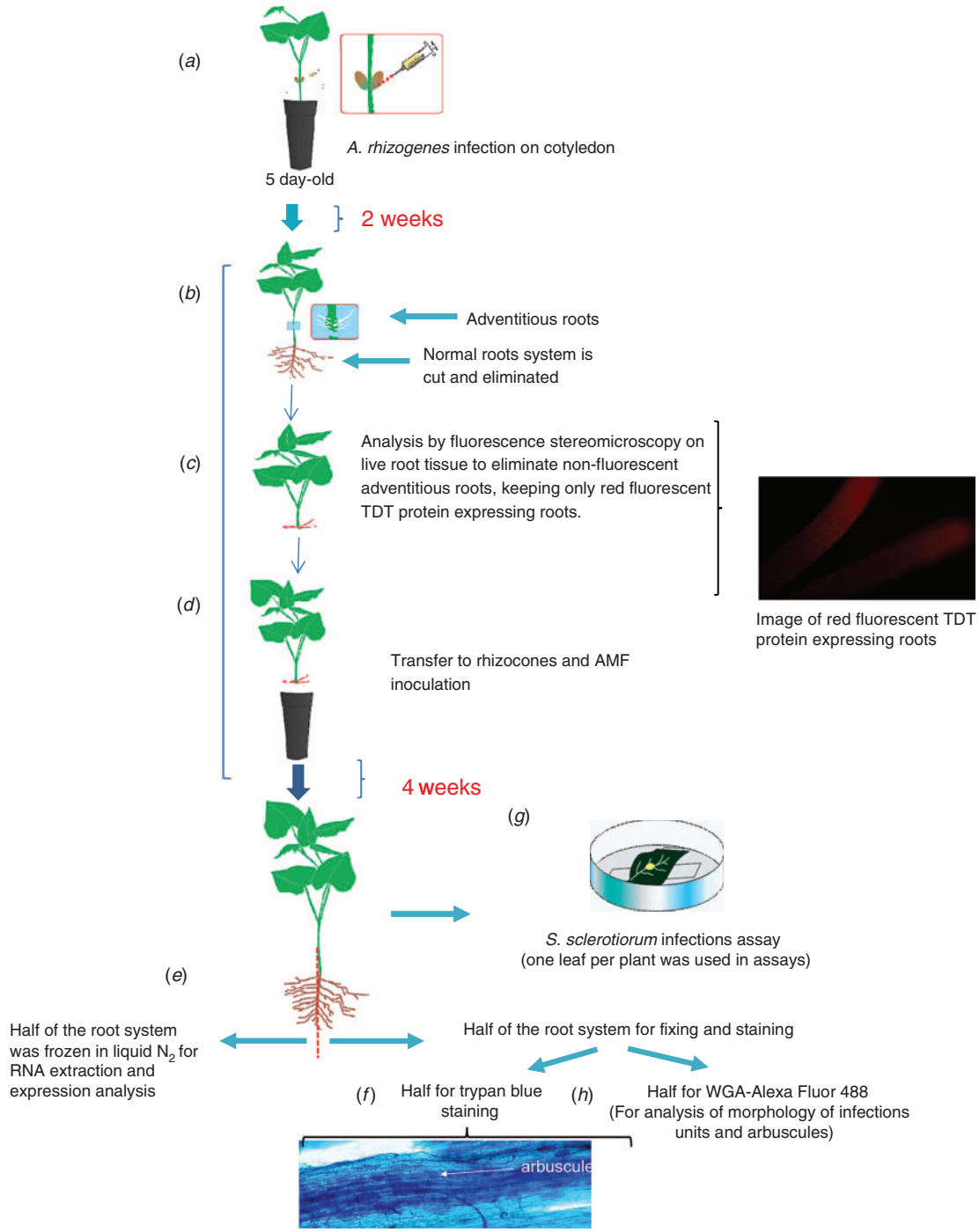

Fig. 1. Flow chart of composite plant induction and pathogen infection assay (*a–f*). Confirmation of transformation was performed by fluorescence microscopy on live adventitious roots (*c*). Gene silencing was analysed by molecular methods on transgenic roots frozen in liquid nitrogen (*e*), and mycorrhiza colonisation was determined by the intersection method on stained roots (*f*). Pathogen infection assay was performed using the detached leaflet assay (*g*). Arbuscule and infection unit morphology was analysed by confocal microscopy (*h*).

Additionally to the use of the line intersection method to corroborate arbuscular mycorrhiza colonisation, gene expression of the *R. irregularis* elongation factor was determined by qRT–PCR (Benabdellah *et al.* 2009). For normalisation, the plant *PvActin* gene was used.

S. sclerotiorum *infection assays in common bean leaves*

S. sclerotiorum infection assays were performed on M and NM NT, EV, and *PvLOX2*RNAi plants. The detached leaflet assay method (Steadman *et al.* 1997) was initially compared with a whole-plant leaflet assay, to evaluate the effect of leaflet excising

on *S. sclerotiorum* infection among several bean varieties. Since no trend differences were observed between experimental systems (G. A. Mora-Romero, R. Cervantes-Gámez, H. Galindo-Flores, A. González-Ortíz, R. Félix-Gastélum, I. E. Maldonado-Mendoza, R. Salinas Pérez, J. León-Félix and M. López-Meyer, unpubl. data), the detached leaflet assay method was used to evaluate *S. sclerotiorum* infection levels, due to its straightforwardness. Third node leaflets from four-week old plants were placed in a humid chamber, consisting of a Petri dish with water-soaked filter paper in the bottom, lined with two glass slides to prevent direct contact of plant tissue with the moist paper. A 5 mm-diameter agar plug containing *S. sclerotiorum* mycelium was placed in the middle of each leaflet. Petri dishes were sealed and incubated at 19°C. The progression of *S. sclerotiorum* infection was followed by measuring the diameter of the necrotic lesion surrounding the mycelium plug every 12 h. One leaflet per plant (biological replicate) was used in the *S. sclerotiorum* infection assays. Four biological replicates were used per treatment. Infection experiments were performed three times with similar results.

Experimental design and data analysis

A completely randomised design was used for *S. sclerotiorum* infection experiments. The extent of infected *S. sclerotiorum* tissue was analysed by one-way ANOVA for each time point. Mean separation was achieved using Tukey's test ($P < 0.05$). Before analysis, the data were subjected to $\sqrt{(x+1)}$ transformation.

For gene expression analyses, Levene's test was used to check the homogeneity of variances before performing ANOVA. DMS and Tukey's *post-hoc* multiple mean comparison test were used to assess significant differences between treatments ($P < 0.05$). A two-tailed test was used with $\alpha < 0.05$. All statistical analyses were performed with Minitab ver.16.

Sequence analysis

The MUSCLE program (Edgar 2004), embedded in the MEGA6 software (Tamura *et al*. 2013), was used to perform multiple sequence alignments with the *P. vulgaris PvLOX2* (AAB18970.2) amino acid sequence against lipoxygenases from four plants species: *P. vulgaris PvLOX6* (ABM88259.1), *Solanum lycopersicum* L. *LOXD* (AAB65767.1), *LOXA* (NP_001234856.1) and *LOXF* (ACM77790.1); *Glycine max* L. *GmLOX6* (AAA96817.1) and *GmLOX9* (ABS32275.1); *Solanum tuberosum* L. *LOX1* (P37831.1); and *Arabidopsis thaliana* (L. Heynh.) *AtLOX1* (Q06327.1), *AtLOX2* (P38418.1), *AtLOX3* (AAF79461.1) and *AtLOX4* (Q9FNX8.1). All sequences were downloaded from the NCBI GenBank database. Finally, we calculated a phylogenetic tree with the UPGMA method, and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is indicated next to the branches.

Results

PvLOX2 *silencing*

Plants with transformed (silenced) roots and a normal shoot system were generated from common bean composite plants. Comparison of the *PvLOX2* silencing DNA fragment against the GenBank database using the BLAST program revealed 100% homology to three sequences: the *PvLOX2* sequence reported by Porta *et al*. (1999) (corresponding to accession number U76687, which was used for the design of our silencing and expression primers), the *P. vulgaris* hypothetical protein (PHAVU_010G134900 g) mRNA (accession number XM_007135444.1), and the *P. vulgaris* clone BE819 lipoxygenase mRNA (accession number KF033486). Sequence analysis of these three accessions indicates that their sequences correspond to the same gene (data not shown). Apart from *PvLOX2*, the other five common bean lipoxygenase sequences in the GenBank database do not exhibit any homology to the silencing 5′ UTR DNA sequence of *PvLOX2*. Therefore, it is likely that silencing is specific to this gene (although cross-silencing of other unreported LOX-like sequences cannot be completely ruled out).

After infection with *A. rhizogenes*, induced adventitious roots were monitored by fluorescence microscopy to identify roots that do not express the red fluorescent protein–tdTomato–reporter gene (Fig. 1). Non-fluorescent adventitious roots were eliminated and the original normal root system was removed, leaving only fluorescent transformed roots as the root system of the plant.

Roots of each of four independent biological replicates of NT, EV and *PvLOX2*RNAi plants were analysed by end-point PCR for the expression of actin (as a constitutive gene), the tdTomato reporter gene (as a control for transformation), and *PvLOX2* (as a silencing control). As expected, all replicates in M and NM NT, EV and *PvLOX2*RNAi plant roots expressed the actin gene (Fig. 2*a*). Composite plants (EV and *PvLOX2*RNAi) expressed the tdTomato reporter gene, but not NT plants (Fig. 2*b*). Finally, NT and EV controls, but not silenced plants, expressed *PvLOX2* (Fig. 2*c*). *PvLOX2* expression in silenced plants was also analysed by qRT–PCR. The expression of *PvLOX2* in silenced plants relative to EV controls was only 1.1% (on average). Average fold change in expression of *PvLOX2* relative to actin for the four replicate silenced plants used in this work is presented in Fig. 2*d*.

AM colonisation is not affected in PvLOX2 *RNAi-silenced roots of composite plants*

NT common bean plants, as well as EV and *PvLOX2*RNAi composite plants, were inoculated with the AM fungus *R. irregularis*, to determine if RNAi-mediated disruption of *PvLOX2* in roots affects AM colonisation. Structures related to fungal colonisation were observed in roots of colonised plants 4 weeks after inoculation, confirming the establishment of an AM symbiotic association. Root colonisation ranged from 39 to 50% according to the line intersection method, and did not statistically differ among M-NT, M-EV, or M-*PvLOX2*RNAi composite plants (Fig. 3*a*). Transcript quantification (by qRT–PCR) of the *R. irregularis* elongation factor gene showed no difference in expression among roots of M-NT, M-*PvLOX2*RNAi and M-EV plants, indicating a similar colonisation level (Fig. 3*b*). No differences were observed in the morphology of fungal structures (e.g. arbuscules, internal hyphae or vesicles) by confocal microscopy, in composite

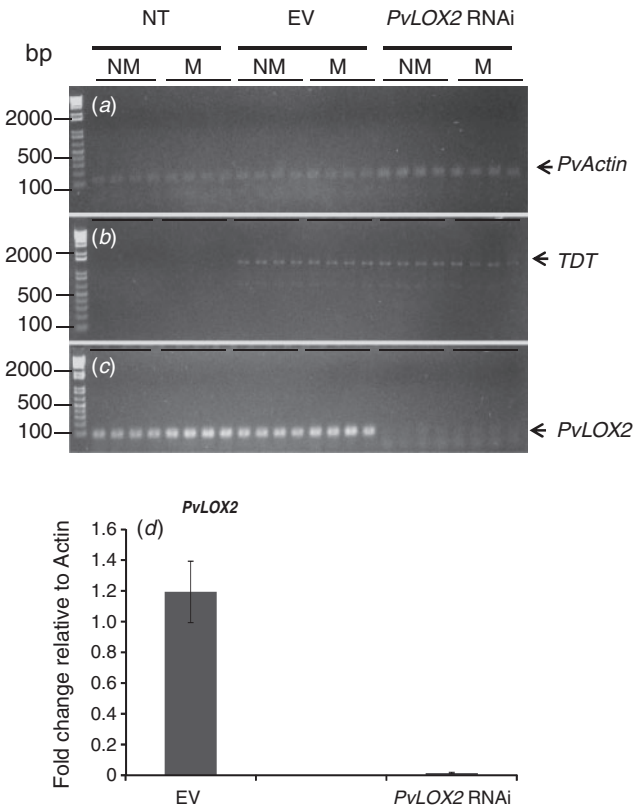

Fig. 2. *PvLOX2* is silenced in PvLOX2RNAi roots of composite plants. End-point RT–PCR using *PvActin* (*a*), *TDT* (*b*), and *PvLOX2* (*c*) primers in NT, EV and *PvLOX2*RNAi plants that were either mycorrhizal colonised (M) or non-colonised (NM). (*d*) Fold-change in *PvLOX2* expression relative to the actin gene. Solid bars correspond to the average of the four biological replicates of EV and *PvLOX2*RNAi NM plants used in this study. Values were calculated using $2^{-\Delta Ct}$. Error bars indicate s.d. ($n = 4$).

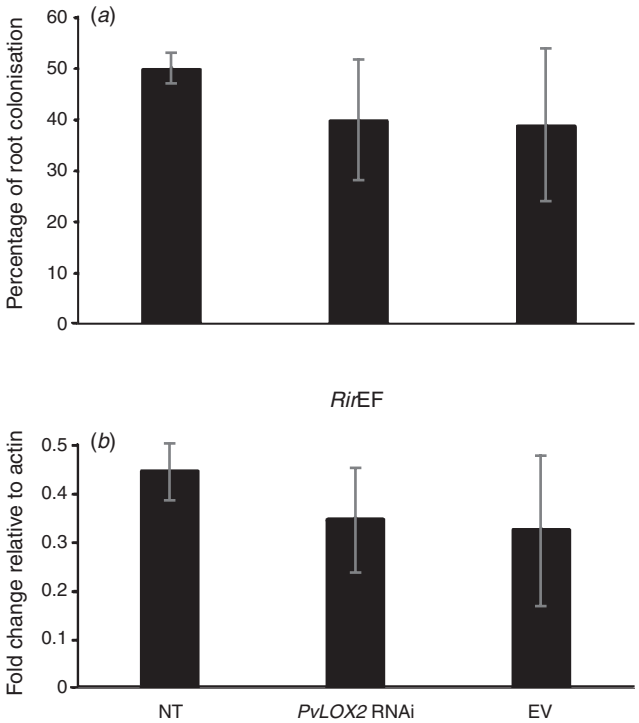

Fig. 3. AM colonisation is not affected in *PvLOX2*RNAi-silenced roots of common bean composite plants. Percentage of root colonisation in *Phaseolus vulgaris* NT and composite *PvLOX2*RNAi and EV plant roots (*a*). Relative expression of the *Rhizophagus irregularis* elongation factor gene (*RirEF*) in M NT, M *PvLOX2*RNAi, and M EV plant roots, as analysed by qRT–PCR (*b*). The *PvActin* gene was used for normalisation. Error bars indicate standard deviations ($n = 4$). No significant differences ($P < 0.05$) according to Tukey's mean test were found in (*a*) or (*b*).

control EV or silenced *PvLOX2*RNAi roots, compared with NT plants (Fig. 4). These results demonstrate that *PvLOX2* is not necessary for the establishment of mycorrhizal symbiosis.

Mycorrhiza-induced resistance is not observed in mycorrhizal PvLOX2*RNAi composite plants*

To investigate whether *PvLOX2* silencing in roots affects MIR in leaves, we challenged M-NT, NM-NT, M-EV, NM-EV, M-*PvLOX2*RNAi, and NM-*PvLOX2*RNAi plants with *S. sclerotiorum* in detached leaflet infection assays. Lesion diameter caused by the pathogen was significantly smaller in M-NT leaflets than in NM-NT ($P < 0.05$) 36 h after infection (Fig. 5). This confirms our previously observations regarding the protective effect of mycorrhiza colonisation against *S. sclerotiorum* in the common bean (Mora-Romero 2008). Similar to NT plants, M-EV plants were less susceptible to the pathogen than NM-EV plants (Fig. 5). This indicates that the observed induced resistance was not affected by the transformation procedure in composite plants. In contrast, the detached leaflet pathogen infection assay in *PvLOX2*RNAi plants demonstrates a failure in mycorrhizal induced resistance, since lesion diameter in M-*PvLOX2*RNAi tissues was not significantly different from NM-*PvLOX2*RNAi (Fig. 5). As early as 12 h after infection, lesions were clearly observed in leaflets of NM-NT, NM-*PvLOX2*RNAi, NM-EV, and M-*PvLOX2*RNAi plants, but not in M-NT and M-EV (Fig. 6). These results suggest that *PvLOX2* could be involved in defence via long-distance signalling from roots to aerial parts, and/or in the subsequent induced resistance response (MIR). We noted that lesion diameter in NM-*PvLOX2*RNAi leaves did not differ from NM-NT and NM-EV controls (Fig. 5), which indicates that *PvLOX2* silencing did not affect basal resistance. This experiment was performed three times with similar results (Fig. S2).

Effect of PvLOX2 *silencing in roots on* PvLOX2, PvLOX6, PvAOS *and* PvCOI1 *gene expression in roots and leaves of composite plants*

The fold change in gene expression relative to the actin gene was calculated for *PvLOX2* and *PvLOX6* genes by qRT–PCR in roots and leaves. In M-NT plants, *PvLOX2* was significantly upregulated in roots (4.4-fold) and leaves (2.8-fold) compared with NM-NT roots and leaves (Fig. 7*a*). A similar expression pattern trend was observed in M-EV roots (2-fold) and leaves (4-fold), compared with NM-EV. As expected, *PvLOX2* expression was very low in *PvLOX2*RNAi-silenced roots. We noted that *PvLOX2* expression in leaves of *PvLOX2*RNAi

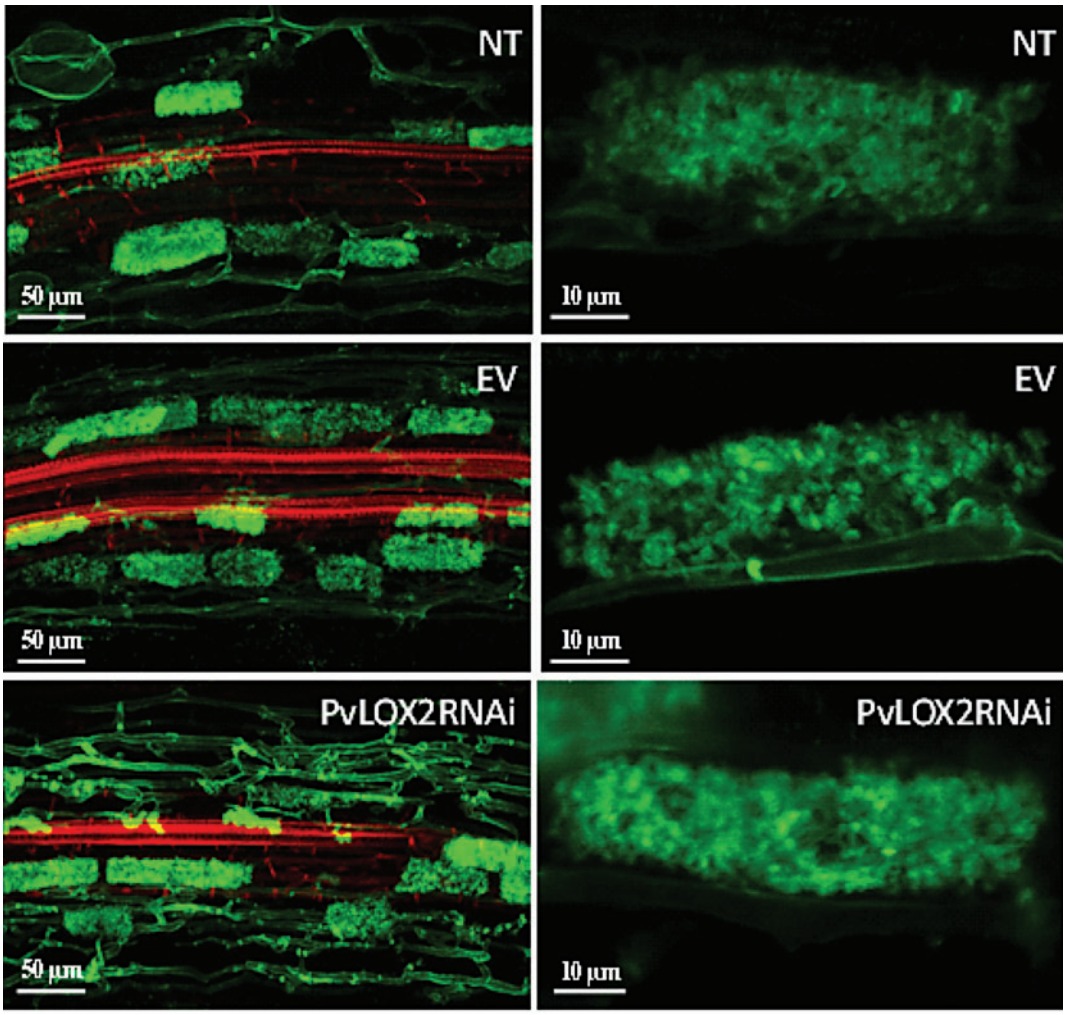

Fig. 4. Arbuscules and infection units morphology is not affected in *PvLOX2*RNAi-silenced roots of common bean composite plants. Confocal laser-scanning microscope images of common bean roots of NT, *PvLOX2*RNAi and EV plants colonised by *Rhizophagus irregularis*. Images were taken 4 weeks after *R. irregularis* inoculation. Roots were stained with WGA-Alexa Fluor 488 (green). Left column: portion of an infection unit. Right column: a single arbuscule from the same root. No red fluorescence from the TDT marker gene in composite plants was detected in these images, due to fixation of roots and carification in KOH.

composite plants was reduced in comparison to the non-silenced control (EV). In addition, mycorrhiza colonisation did not induce *PvLOX2* expression in leaves, as there was no significant difference in gene expression between M and NM *PvLOX2*RNAi (Fig. 7*a*). *PvLOX6* was not detected in root tissues of M or NM plants. However, it was expressed in leaves of NM-NT and NM-EV plants, where it was upregulated by mycorrhisation 2.8-fold in M-NT and almost 7-fold in M-EV leaves (Fig. 7*b*). *PvLOX6* expression, like *PvLOX2*, was strongly reduced in leaves of M-*PvLOX2*RNAi and NM-*PvLOX2*RNAi plants. In addition, mycorrhiza colonisation did not result in any detectable significant difference in expression for this gene, in leaves of silenced plants (Fig. 7*b*).

PvAOS (allene oxide synthase) and *PvCOI1* (coronatine-insensitive 1) gene expression was also measured. *PvAOS* was selected since its translated protein participates in the biosynthesis of jasmonates (Feussner and Wasternack 2002). The *PvCOI1* gene was chosen, as its product is an important element that is stimulated by JA-Ile to subsequently direct the ubiquitin-dependent degradation of jasmonate ZIM domain (JAZ) proteins, which are negative regulators of JA-responsive genes (Katsir *et al*. 2008). No significant changes in *PvAOS* expression were observed in roots of M plants, NT and EV controls, or in *PvLOX2*RNAi-silenced plants compared with NM plants (Fig. 7*c*). The *PvCOI1* expression level doubled in roots of M-*PvLOX2*RNAi plants compared with NM-*PvLOX2*RNAi (Fig. 7*d*). Otherwise, no significant difference in *PvCOI1* expression was observed between M and NM plants in the roots and leaves of NT and EV controls, or in leaves of *PvLOX2*RNAi plants (Fig. 7*d*).

Discussion

Mycorrhiza-induced resistance is an enhanced defence response against pathogen attack, distinct from the plant's basal defence

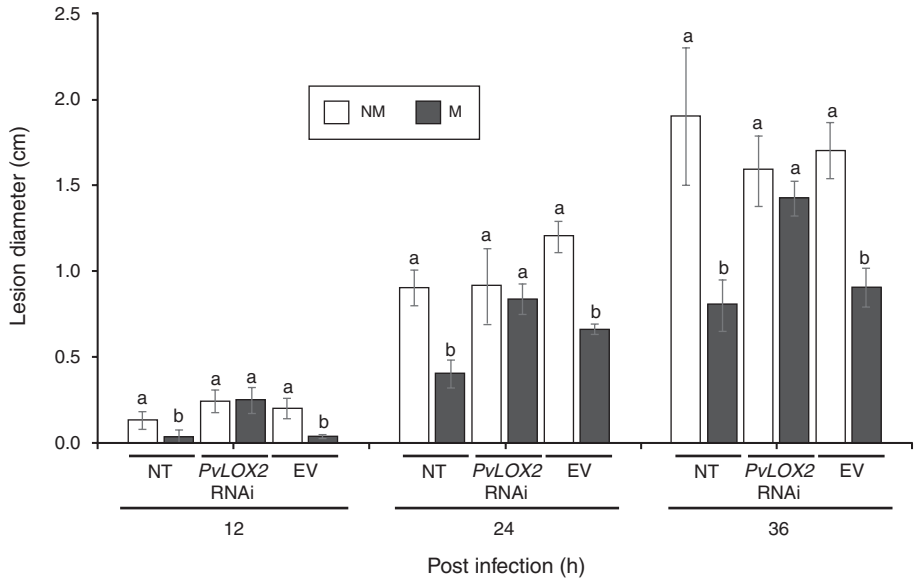

Fig. 5. *PvLOX2* silencing in roots of common bean composite plants impairs mycorrhiza induced resistance against *Sclerotinia sclerotiorum*. Diameter of lesions (cm) caused by *S. sclerotiorum* in leaflets 12, 24 and 36 h after pathogen infection. NM (white bars) and M (grey bars) were compared within each of the following conditions: NT, EV and *PvLOX*2RNAi silencing plants, for each time point. Data labelled 'a' and 'b' differ significantly ($P < 0.05$) according to Tukey's mean test, $n = 4$. Statistical comparisons were performed between M and NM conditions for each treatment. The experiment was repeated at least twice with similar results. Error bars indicate s.d.

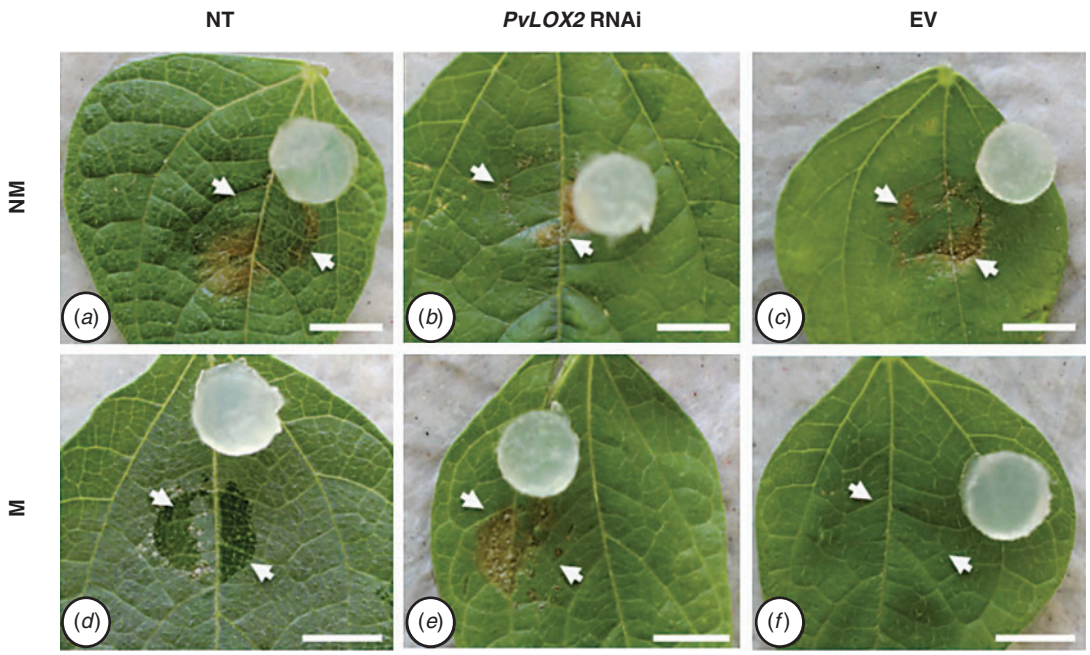

Fig. 6. Detached leaflet assay in the common bean, using the pathogen *Sclerotinia sclerotiorum*. Images were taken 12 h after infection by an agar plug containing *S. sclerotiorum* mycelium. (*a*) NM-NT; (*b*) NM-*PvLOX2*RNAi; (*c*) NM-EV; (*d*) M-NT, (*e*) M-*PvLOX2*RNAi, (*f*) M-EV. Scale bars = 5 mm. White arrows indicate infection site after removal of plugs.

responses. Oxylipins (including JA and its derivatives) and some of their responsive genes have been linked to defence responses, although their exact role in pathogen resistance and even mycorrhiza establishment is still controversial (Mosblech *et al.* 2009; León Morcillo *et al.* 2012; Wasternack and Hause 2013). To investigate the role of *PvLOX2*, an oxylipin biosynthesis

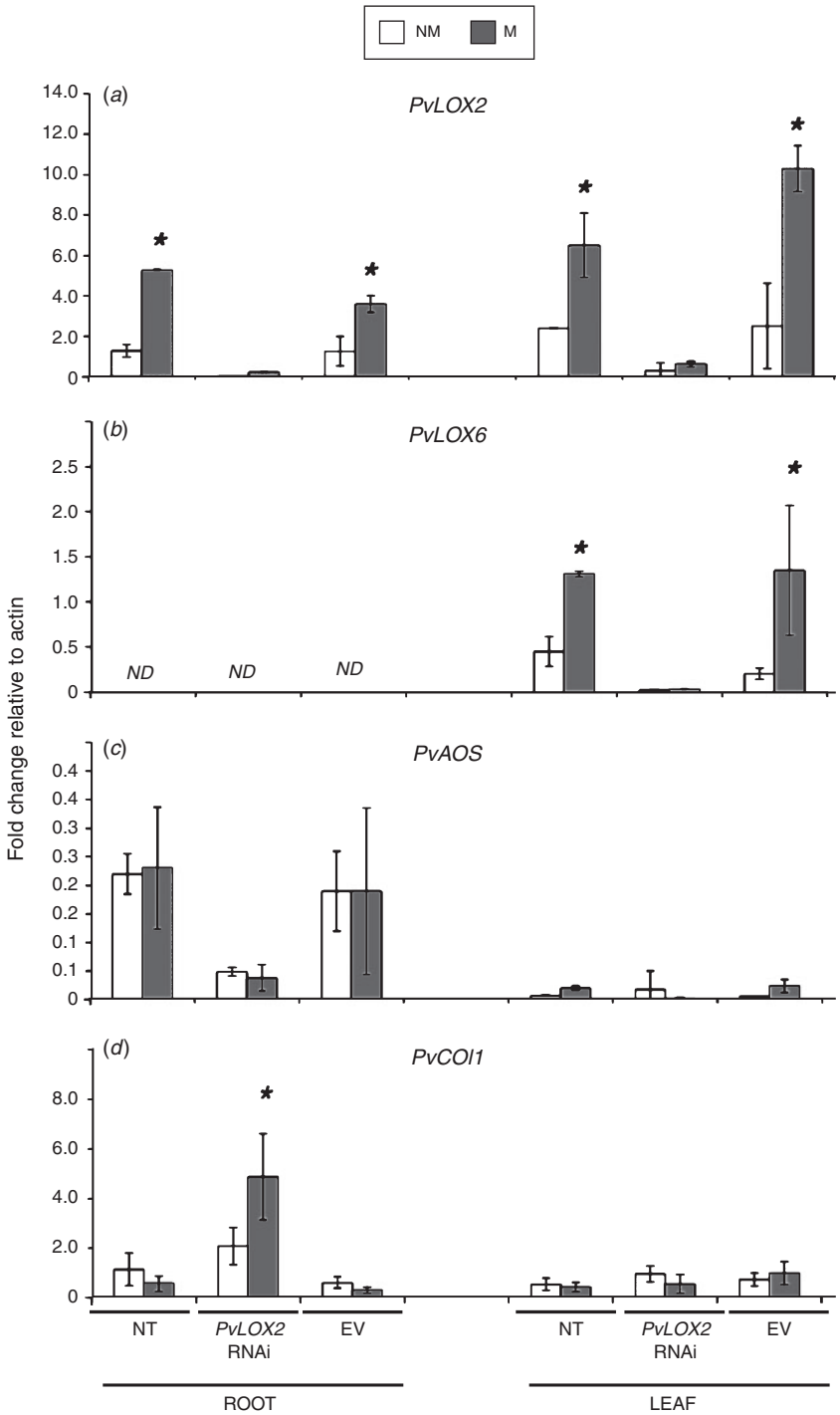

Fig. 7. *PvLOX2* silencing in roots of common bean composite plants downregulates *PvLOX2* and *PvLOX6* expression in leaves but not *PvAOS* and *PvCOI1*. Relative expression of (*a*) *PvLOX2*, (*b*) *PvLOX6*, (*c*) *PvAOS*, and (*d*) *PvCOI1* genes analysed by qRT–PCR. RNA was isolated from roots and shoots of NM (open bars) and M (grey bars) plants from the following conditions: NT, *PvLOX2*RNAi and EV plants ($n = 4$). Statistical comparisons were performed between M and NM conditions for each treatment. Significant differences are indicated: *, $P < 0.05$, according to Tukey's test. ND: transcript not detected. Error bars indicate s.d.

related gene, in MIR, we silenced expression of this gene in common bean roots and analysed whether this systemic enhanced resistance is maintained or lost during further pathogenesis assays.

PvLOX6 (a type 2 13-LOX), the only LOX gene currently known to have a role in the JA biosynthetic pathway in the common bean, is not expressed in roots (Porta *et al*. 2008) and is therefore not suitable for silencing. In contrast, our phylogenetic analysis of selected plant LOXs (Fig. 8) indicates that *PvLOX2* is a predicted type 1 13-LOX, since it groups with other LOXs of this type. *PvLOX2* could therefore be involved in the biosynthesis of several di-vinyl ether polyunsaturated fatty acids, as well as traumatin and certain aldehydes and alcohols, but not JA (Mosblech *et al*. 2009; Wasternack and Hause 2013). *PvLOX2* is expressed in common bean roots, nodules (Porta *et al*. 1999), and hypocotyls, and its expression is affected by ABA treatment, drought and cold conditions. Furthermore, wounding induces both local and systemic *PvLOX2* expression (Porta *et al*. 1999). These findings suggest that *PvLOX2* is likely to participate in systemic signalling and stress responses. *Glycine max LOX9* (*GmLOX9*) is a putative orthologue of *PvLOX2* due to its high protein sequence similarity (Fig. 8), as well as its similar tissue expression pattern in root vasculature, specifically in the phloem (Hayashi *et al*. 2008). This similar localisation pattern between *GmLOX9* and *PvLOX2*, together with its upregulated expression in roots and shoots of mycorrhizal colonised plants, suggests a role for *PvLOX2* in signalling translocation from mycorrhizal colonised roots to the leaves. Therefore, we chose to silence this gene in roots of common bean composite plants, in order to examine the involvement of *PvLOX2* in the systemic defence response triggered by mycorrhizal symbiosis.

Silencing *PvLOX2* had no effect on mycorrhiza colonisation, in terms of morphology of internal fungal structures or level of colonisation (Figs 3, 4). This demonstrates that *PvLOX2* does not play a role in the establishment of arbuscular mycorrhizal symbiosis. Although it is possible that non-transgenic roots develop in silenced composite plants (even following the excision of non-red fluorescence roots at the moment of AMF inoculation), we assert that a high proportion of the composite plant roots used in this work were silenced, since the average of *PvLOX2* relative expression in these roots was less than 5% of the average expression of this gene in roots of EV plants (Fig. 2*d*). In accordance with this result, antisense expression of *Nicotiana attenuata LOX3* (*NaLOX3*) did not cause any difference in mycorrhizal colonisation (Riedel *et al*. 2008). However, this

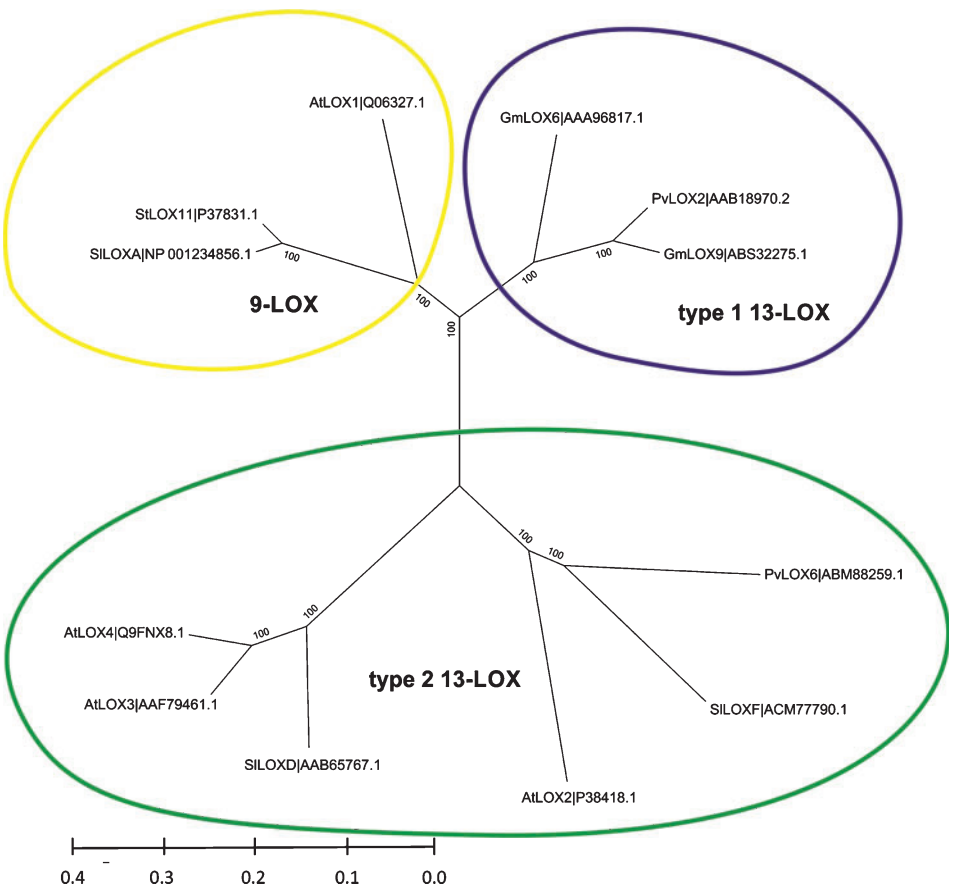

Fig. 8. Phylogenetic analysis of selected plant LOXs showing that *PvLOX2* sequence groups with type 1 13-LOX. The tree was generated by aligning full-length protein sequences of *Glycine max* (Gm), *Phaseolus vulgaris* (Pv), *Solanum lycopersicum* (Sl), *S. tuberosum* (St) and *Arabidopsis thaliana* (At). The 9-LOX, type 1 13-LOX and type 2 13-LOX groups are indicated by coloured circles.

does not rule out the possibility that other *PvLOX* gene family members could play a role in establishing this symbiosis, since differential expression among *PvLOX* genes has previously been described under different conditions (Meier *et al*. 1993; Eiben and Slusarenko 1994; Porta *et al*. 1999, 2008; Porta and Rocha-Sosa 2000, 2002). In contrast, silencing *PvLOX2* in roots appears to block the onset or spreading of systemic induced resistance, since no MIR was manifested in leaves of silenced plants when challenged with *S. sclerotiorum* (Figs 5, 6). These results indicate that mycorrhiza colonisation is necessary but not sufficient for the onset of MIR. We noted that silencing *PvLOX2* in roots did not alter basal resistance, since lesion diameter was similar in NM-*PvLOX2*RNAi, NM-NT and NM-EV plants (Fig. 5). In addition, silencing *PvLOX2* in roots resulted in the downregulation of *PvLOX6* in leaves, as well as its own downregulation (Fig. 7*a*, *b*). We therefore hypothesise that *PvLOX2* is involved in the production and/or translocation of a signal from the roots to the leaves, which induces *PvLOX6* and *PvLOX2* upregulation; this in turn prepares distal organs for an enhanced response to a future pathogen attack. Confirmation that *PvLOX2* localises to phloem in vascular tissues of roots, stems and leaves, as suggested by its similarity to its orthologue *GmLOX9* (Fig. 8), would support this hypothesis.

As stated above, *PvLOX6* is the only LOX with a role in the JA biosynthetic pathway (Porta *et al*. 2008), while the other LOXs are involved in the biosynthesis of other oxylipins. This inability to upregulate *PvLOX6* expression in leaves of *PvLOX2*RNAi plants (due to mycorrhisation) could possibly interfere with the production of JA and/or its derivatives, which will ultimately prevent the induced resistance to *S. sclerotiorum.* On the other hand, since a certain level of *PvLOX6* expression was detected in NM-NT and NM-EV leaves (Fig. 7) without a reduction in lesion diameter (in comparison to *PvLOX2*RNAi plants; Fig. 6), it is possible that a threshold level of *PvLOX6* expression (which is not reached in NM control plants) is required to induce this resistance. However, we cannot support this with evidence at this time, since JA accumulation was not determined in this study. The expression of other *PvLOX*s might also be affected by silencing *PvLOX2*, and their possible role in MIR is currently under investigation. It is clear, though, that *PvLOX2* silencing in roots was responsible for the failure of MIR onset in silenced plants driven by mycorrhiza colonisation, since it was not manifested when challenged with a pathogen (Fig. 5). This finding establishes a role for *PvLOX2* in the MIR process.

The gene expression profile of *PvAOS* differs somewhat from *PvLOX2* and *PvLOX6*. In the common bean, *PvAOS* does not appear to be directly regulated by mycorrhiza colonisation, since we did not observe any significant change in gene expression in the roots and leaves of M vs NM plants (Fig. 7*c*). In contrast, *AOS* expression was previously reported to be induced by mycorrhizal colonisation in roots of barley (Hause *et al*. 2002). Furthermore, *AOS1* and *AOS3* are upregulated in tomato roots by mycorrhizal colonisation (López-Ráez *et al*. 2010). However, the role of *AOS* in MIR has not yet been studied. We cannot definitively state if *PvAOS* plays a regulatory role in the onset of MIR in leaves, although our results indicate that it does not act at the transcriptional level. Nevertheless, other levels of regulation cannot be ruled out for this gene.

The *COI1* gene is an F-box protein that forms a complex with SCF in the presence of JA-Ile and determines its target specificity. SCF interacts with JAZ proteins and targets them for degradation by the 26S proteasome in response to JA signalling. JAZ proteins are negative regulators of transcription factors that affect JA-responsive genes. Once freed from JAZ, transcription factors can activate genes needed for a specific JA response (Wasternack and Hause 2013). Despite of its role in JA signalling, alteration of *PvCOI1* expression was not detected in leaves of any of the plants analysed in this work; only in roots of M-*PvLOX2*RNAi plants did *PvCOI*1 expression double in comparison to NM silenced plants (Fig. 7*d*). The slight *PvCOI1* upregulation in *PvLOX2*-silenced mycorrhizal roots and the observation that leaves of silenced plants do not exhibit induced resistance (MIR) against *S. sclerotiorum* suggest that *PvCOI1* could act as a negative regulator of the mechanisms that control MIR. On the other hand, upregulation of *PvCOI1* in roots of M-*PvLOX2*RNAi plants did not cause any change in mycorrhizal colonisation (Fig. 3*a*). This suggests that mycorrhizal establishment could be a *PvCOI1*-independent process, at least at the transcriptional level. This latter observation is consistent with previous work, in which silencing of *COI1* in *Nicotiana attenuata* did not alter mycorrhizal colonisation (Riedel *et al.* 2008). Gene function disruption experiments will be necessary to confirm the potential role of this gene in mycorrhiza establishment and systemic signalling in the common bean.

We have shown here that the genes *PvLOX2* and *PvLOX*6 are involved in the onset of systemic induced resistance that occurs through mycorrhiza colonisation. In addition, our results provide strong support that *PvLOX2* plays a role in the production and/or translocation of a mycorrhiza-derived signal from roots to shoots. We postulate that this signal would prepare leaf tissues for a defence response against pathogen attack, through activation of genes such as *PvLOX6*. It remains to be shown whether upregulation of *PvLOX6* expression in leaves of mycorrhizal plants promotes JA production as part of the defence response.

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