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RESEARCH PAPER

Phosphatidylinositol 3-kinase function at very early symbiont perception: a local nodulation control under stress conditions?

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Abstract

Root hair curling is an early and essential morphological change required for the success of the symbiotic interaction between legumes and rhizobia. At this stage rhizobia grow as an infection thread within root hairs and are internalized into the plant cells by endocytosis, where the PI3K enzyme plays important roles. Previous observations show that stress conditions affect early stages of the symbiotic interaction, from 2 to 30 min post-inoculation, which we term as very early host responses, and affect symbiosis establishment. Herein, we demonstrated the relevance of the very early host responses for the symbiotic interaction. PI3K and the NADPH oxidase complex are found to have key roles in the microsymbiont recognition response, modulating the apoplastic and intracellular/endosomal ROS induction in root hairs. Interestingly, compared with soybean mutant plants that do not perceive the symbiont, we demonstrated that the very early symbiont perception under sublethal saline stress conditions induced root hair death. Together, these results highlight not only the importance of the very early host-responses on later stages of the symbiont interaction, but also suggest that they act as a mechanism for local control of nodulation capacity, prior to the abortion of the infection thread, preventing the allocation of resources/energy for nodule formation under unfavorable environmental conditions.

Keywords: Abiotic stress, nodulation, phosphatidylinositol 3-kinase, PI3K, reactive oxygen species, ROS, symbiont perception, symbiotic interaction.

Abbreviations: GFP, green fluorescent protein; PI3K, phosphatidylinositol 3-kinase; qPCR, quantitative real-time PCR; ROS, reactive oxygen species; RT, reverse transcription; Tdt, tandem dimer tomato fluorescent protein.

Introduction

Regional climate changes affect natural and agricultural ecosystems around the world, provoking changes in agricultural land use. Moreover, the threat of erosion, loss of organic carbon and salinization of the soil is increasing. In this regard, soybean (*Glycine max* L.) has been classified as a salinity-susceptible crop (Ashraf, 1994) as the processes of both infection and nodulation by *Bradyrhizobium japonicum* are severely affected by salinity (Singleton and Bohlool, 1984; Zahran and Sprent, 1986). The effects of abiotic stress in the soybean–*B. japonicum* interaction have been mainly studied in the late events of this symbiosis, focusing on carbon and nitrogen metabolism in nodules, and consequently the effects of stress on early symbiotic events, previous to nodule formation, have been less explored.

It is well known that 2–5 min after symbiont perception, early specific responses are induced in the growing root hair, involving plasma membrane depolarization, calcium spiking, phospholipid signaling, intracellular alkalization, and the generation of reactive oxygen species (ROS) (Oldroyd and Downie, 2008). In this respect, Cárdenas *et al.* (2008) identified the NADPH oxidase complex as a source of intracellular ROS during this stage of the symbiotic interaction. Besides, apoplastic ROS production and its relationship with intracellular ROS generation after 2–120 min post-inoculation with the rhizobium have been described by our group (Muñoz *et al.*, 2012). Here, the study focused on the first 2–30 min of the symbiotic interaction, a time period that we call the very early host response.

Later, between 2 and 48 h post-inoculation, the early responses take place, including root hair curling, which is the first early morphological response. It is worth mentioning that most of the studies on this topic have focused on this stage of the symbiotic interaction (Cárdenas *et al.*, 2000; Felle *et al.*, 2000; Peleg-Grossman *et al.*, 2007; Muñoz *et al.*, 2012). Finally, the bacteria enter nodule cells in the root cortex through an endocytosis-like process and are maintained in host membrane-bound compartments called symbiosomes (Roth *et al.*, 1988; Limpens *et al.*, 2009). During this last stage, the enzyme phosphatidylinositol 3-kinase (PI3K) has been implicated in soybean nodule organogenesis and peribacteroid membrane development (Hong and Verma, 1994; Estrada-Navarrete *et al.*, 2016). With a pharmacological approach, it was shown that PI3K was also implicated during early stages of legume–rhizobium interaction, between 16 h and 7 d, coordinating curling responses, the formation of the infection threads, and ROS generation in roots (Peleg-Grossman *et al.*, 2007). The participation of PI3K in the modulation of ROS production has been demonstrated in both animal and plant systems (Ellson *et al.*, 2001; Park *et al.*, 2003; Hair *et al.*, 2008). In this regard, PI3K was found to be involved in the regulation of plasma membrane internalization and ROS production within endosomes in root cells in response to salt stress (Leshem *et al.*, 2007). Moreover, it has been postulated that PI3K modulates the ROS production during seed germination by regulating NADPH oxidase activity through recruiting Rac-1 to the plasma membrane (Liu *et al.*, 2012). However, although PI3K inhibitors suppressed ROS production in root cells during symbiotic interactions (Peleg-Grossman *et al.*, 2007), a detailed analysis of subcellular ROS distribution has not been provided. Recently, the down-regulation of the *PI3K* gene in *Phaseolus vulgaris* hairy roots using an RNAi approach showed a notable decrease in root hair growth, as well as root hair curling, infection thread growth, and root nodule number, and symbiosome formation was severely affected (Estrada-Navarrete *et al.*, 2016). Nevertheless, the role of PI3K during the very early host response at the root hair level, before the endocytotic internalization of the infection droplets, and the impact that the very early responses have on nodulation have not been evaluated. Furthermore, previous reports from our group have shown that stress conditions affect the very early redox responses during the symbiotic interaction, from 2 to 30 min, and also induce root hair death and thereby impact nodulation (Muñoz *et al.*, 2012, 2014; Robert *et al.*, 2014).

In the present work, we set out the importance of the very early host response in the nodulation process in the soybean–*B. japonicum* interaction, highlighting the role of PI3K in the strict modulation of ROS in the root hair required for the recognition of the microsymbiont by the plant. Moreover, the very early recognition of the symbiont is affected by saline stress conditions, leading to root hair death, possibly triggering an immunity-like response that acts as a mechanism for assessing nodulation capacity when environmental conditions are not favorable.

Materials and methods

Vector construction

In order to silence the expression of both Gm*PI3K* genes in soybean (Glyma.04G094500 and Glyma.06G096300), a highly conserved fragment of the coding region was amplified by PCR from the cDNA obtained from soybean roots by using the forward primer 5′-CACCCCGAGAATCGTAGCATCATAAGC-3′ and the reverse primer 5′-CCATCTGAGGGAATAATGCACTC-3′. The PCR product was cloned into the pENTR/D-TOPO vector according to the manufacturer's instructions (Invitrogen), confirmed by sequencing, and recombined into the compatible recombination sites of the Gateway-based hairpin pK7GWIWG2D(II) (Karimi *et al.*, 2002) and pTdt-DC-RNAi vectors (Valdés-López *et al.*, 2008). pK7GWIWG2D(II) contains green fluorescent protein (GFP) as reporter and pTdt-DC-RNAi has the red tomato fluorescent protein (Tdt, tandem dimer Tomato). The resulting constructs, PI3Ki-GFP and PI3Ki-Tdt, drive the transcription of a hairpin loop Gm*PI3K*-RNAi under control of the 35S promoter. pK7GWIWG2D(II) was also recombined with pENTR-GUS (Invitrogen) to generate GUSi-GFP as a control. pTdt-DC-RNAi was used as a control of the PI3Ki-Tdt construction.

Agrobacterium rhizogenes-*mediated root transformation*

Agrobacterium rhizogenes strain K599 was used to infect cotyledon axis regions. *Agrobacterium rhizogenes* K599 with PI3Ki-GFP, PI3Ki-Tdt, GUSi-GFP and pTdt-DC-RNAi constructs was grown in Luria–Bertani (LB) medium containing kanamycin at 50 μg ml^{-1} or Spectinomycin 100 μg ml^{-1}. To obtain fresh cells, *A. rhizogenes* K599 was grown on LB plates containing kanamycin or Spectinomycin and incubated for 48 h at 28 °C. Cells were collected from these plates and diluted into 0.25 ml of sterile water. For control hairy roots (K599-empty), a fresh culture of *A. rhizogenes* K599 lacking the binary vector was grown in LB medium without antibiotics.

Agrobacterium rhizogenes-mediated root transformation was performed as previously by Robert and coworkers (2014), modified from Estrada-Navarrete *et al.* (2006). Briefly, after germination, sprouts were inoculated by injection directly into the cotyledonary nodes with a syringe and transferred to a hydroponic double tube system and incubated in a growth chamber under 16 h photoperiod (300 μmol m^{-2} s^{-1}) at 26 ± 2 °C. The smaller tube contained the sprout watered with Broughton and Dilworth (B&D) nutrient solution (Broughton and Dilworth, 1971) and the larger tube functioned as a moist chamber. Typically, soybean plants infected by *A. rhizogenes* started to show tumors approximately 5 d after inoculation. Twelve days after *A. rhizogenes* infection, plantlets exhibited numerous induced hairy roots per wound site.

Bacterial strain and plant material

Soybean seeds (*Glycine max* L. Bragg wild type or Bragg nod139) were disinfected with sodium hypochlorite 5% (v/v) for 5 min and germinated in the dark for 48 h on filter paper moistened with distilled water. The seeds were incubated at 28 and 37 °C during the first and second 24 h periods, respectively, to promote the growth of roots and root hairs as previously reported (Muñoz *et al.*, 2012). *Bradyrhizobium japonicum* USDA 138 was cultured in yeast extract mannitol (YEM) medium (Vincent, 1970) at 28 °C with constant agitation for 5 d (3 × 10^9 cells ml^{-1}). The bacteria were washed and resuspended in sterile water.

Evaluation of nodulation in hairy roots

The primary soybean root was removed around 12 d post-infection with *A. rhizogenes* by cutting approximately 1 cm below the cotyledon nodes, and the composite plants were placed in aerated plastic trays with B&D solution with 5 mM KNO_3 in a growth chamber under 16 h photoperiod (300 mmol m^{-2} s^{-1}) at 26 ± 2 °C. After 2 d, the nutrient solution was replaced by B&D solution with 2 mM KNO_3 and the inoculation with *B. japonicum* USDA138 was performed. The nodule number was assessed around 21 d post-inoculation.

RNA extraction and RT-qPCR

Samples were homogenized in a cold mortar with TRIzol Reagent (1 μg plant tissue: 10 ml reagent), mixed for 1 min and incubated at room temperature for 5 min. Then, 0.2 ml chloroform per ml of TRIzol Reagent was added and incubated at room temperature for 3 min. After incubation, the samples were centrifuged at 12 000 g at 4 °C for 15 min and the aqueous phases were transferred to clean tubes. RNA was precipitated by adding 1 vol of isopropanol, incubated at room temperature for 10 min and centrifuged at 12 000 g at 4 °C for 15 min. The precipitate was washed with 70% ethanol and the samples were centrifuged again at 12 000 g, 4 °C for 15 min. The precipitate was dried and resuspended in diethyl pyrocarbonate-treated water and its concentration was quantified using a NanoDrop 3300 spectrometer (Thermo Scientific). Purified RNA was treated with DNase I (Invitrogen) to remove genomic DNA, according to the manufacturer's instructions.

DNA-free RNA (1 to 2.5 μg) was used with oligo(dT) for first strand cDNA synthesis using the Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega) according to the manufacturer's instructions. For each primer pair, the presence of a unique product of the expected size was checked on ethidium bromide-stained agarose gels after PCR reactions. Absence of contaminant genomic DNA was confirmed in reactions with DNase-treated RNA as template. The qPCR reaction was performed using iQ Universal SYBR Green Supermix (Bio-Rad). Amplification of actin with the forward primer 5′-AACGACCTTAATCTTCATGCTGC-3′ and the reverse primer 5′- GGTAACATTGTGCTCAGTGGTGG-3′, and elongation factor-1α (EF1α) with the forward primer 5′-GGTCATTGGTCATGTCGACTCTGG-3′ and the reverse primer 5′-GCACCCAGGCATACTTGAATGACC-3′ was used to normalize the amount of template cDNA. The specific primers pair employed for the detection of both transcripts of *PI3K* (Glyma.04G094500 and Glyma.06G096300) was forward primer 5′-CCTGAAAAGGGGATTCTTAAGC-3′ and reverse primer 5′-CCAACGATGGATAGTCTCAACC-3′. qPCR was performed in thermocycler iQ5 (Bio-Rad) at 59 °C with iQ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instruction. Relative transcript abundance was calculated (Livak and Schmittgen, 2001).

Live imaging, treatment conditions and redox parameters

Analysis of intracellular ROS generation in hairy roots and colocalization analysis with the lipophilic dye FM4-64 for endosome visualization and the general ROS indicator chloromethyl dichlorofluorescin diacetate (CMH2DCFDA) was performed with an inverted confocal microscope (Nikon Eclipse CZ1). The intracellular ROS generation in root hairs was observed with a Zeiss Axiophot microscope. Images were taken with a Nikon DS camera (DS Camera Control Unit DS-L1, DS Camera Head DS-5M, and DS Cooled Camera Head DS-5Mc). The images were quantified by the image processing software Optimas®.

In order to evaluate the participation of PI3K and the NADPH oxidase complex during the very early host responses, 2-day-old soybean seedlings were pretreated for 30 min with 30 μM LY294002 or 50 μM diphenyl iodonium (DPI), respectively, with dimethyl sulfoxide (DMSO) as a control; then soybean seedlings were transferred to a clean tube with *B. japonicum* USDA138, and ROS production was evaluated as follows.

Intracellular ROS

Chloromethyl dichlorofluorescin diacetate (CMH_2DCFDA), 10 μM, was added in the last 15 min of the pretreatments. Then, soybean seedlings were inoculated with *B. japonicum* USDA138 and images were taken with a Zeiss Axiophot microscope after 2–5 min post-inoculation.

For intracellular ROS generation analysis in hairy roots, control K599-empty, pTdt-DC-RNAi hairy roots and PI3Ki-Tdt hairy roots were excised when they reached approximately 2.5 cm in length and incubated with 10 μM CMH_2DCFDA for 15 min. The intracellular ROS production was evaluated by confocal microscopy with a 488 nm argon laser (BP 495–530 nm), and the Tdt fluorescence was evaluated with a 543 nm He–Ne laser (LP 560 nm).

Apoplastic superoxide radical

Superoxide levels in roots of 2-day-old soybean seedlings were determined with nitroblue tetrazolium (NBT), which reacts with superoxide radicals to produce a blue formazan precipitate, as previously (Muñoz *et al.*, 2012). As the staining of apoplastic superoxide depends on the concentration of NBT and the time of incubation (Jones *et al.*, 2007), the roots were inoculated in the presence of 0.01% (w/v) NBT in the dark for 30 min. The reaction was stopped with absolute ethanol.

Malondialdehyde content of hairy roots

The samples were homogenized using a mortar and pestle under liquid nitrogen and thawed in 3% (v/v) trichloroacetic acid (TCA) and then centrifugation was carried out at 13 000 g, 4 °C for 15 min. Malondialdehyde (MDA) levels were quantified (Heath and Packer, 1968). Briefly, 100 μl of sample was mixed with 100 μl of 20% TCA+0.5% thiobarbituric acid, incubated at 90 °C for 20 min and ice-chilled rapidly. The mix was centrifuged at 13 000 g for 10 min. The absorbance of the supernatant was read at 532 and 600 nm.

In order to evaluate the effects of the very early responses on nodule formation, 2-day-old soybean seedlings were pretreated with 30 μM LY294002 or 50 μM DPI, with DMSO as a control, for 30 min. Then, roots were inoculated for 30 min with *B. japonicum* USDA138 and washed with sterile distilled water. After that, the inoculated seedlings were grown in hydroponic medium for 24 d and nodulation was assayed.

To observe the intracellular ROS generation and endosomes, 10 μM CMH_2DCFDA and 5 μg ml^{-1} FM4-64 (Molecular Probes/Invitrogen, Carlsbad, CA, USA) were loaded in 2-day-old soybean seedlings 15 and 3 min before the inoculation, respectively. Samples were then washed twice to remove excess dye and fluorescence emissions were collected by confocal microscopy.

Root hair death-inducing conditions

Two-day-old soybean seedlings were treated for 30 min with sterile water (control), *B. japonicum* (inoculated), 50 mM NaCl, *B. japonicum* with 50 mM NaCl and 150 mM NaCl, according to previous reports (Muñoz *et al.*, 2012, 2014; Robert *et al.*, 2014). Root hairs from roots subjected to different stress treatments were extracted by peeling the root zone containing young root hairs, which were immediately frozen in liquid air for subsequent RT-qPCR analysis. Peeling was performed under a magnifying glass by making an incision with a scalpel in the root and pulling the epidermal tissue containing the root hair using a fine-tipped clamp. Root hairs of approximately 100 roots generated sufficient material for a sample.

Cell death evaluation was performed by Evans Blue staining as done previously (Muñoz *et al.*, 2012; Robert *et al.*, 2014). Evans Blue is a dye used for the determination of cell viability due to its inability to permeate intact cell membranes. When cells lose their membrane potential, the dye diffuses into the cell and may be visualized by conventional microscopy. The roots were incubated for 10 min with Evans Blue 0.05% (w/v) in water or each NaCl level assayed.

Statistical analyses

Data were analysed by analysis of variance (ANOVA) followed by the Di Rienzo–Guzman–Casanoves (DGC) test model, a cluster-based method for identifying groups of non-homogeneous means, using InfoStat software (Di Rienzo *et al.*, 2012).

Results

PI3K *gene silencing in soybean composite plants: effects on hairy root development and nodulation*

The *G. max* genome has two *PI3K* genes whose protein products belong to the class III PI3K, the only class of PI3K present in plants as observed for plant members whose genomes have been sequenced (Supplementary Fig. S1 at JXB online; Estrada-Navarrete *et al.*, 2016). Hong and Verma (1994) reported the differential expression of the two soybean *PI3K* genes (Glyma.04G094500 and Glyma.06G096300) in roots and mature nodules, and young nodules, respectively. In order to evaluate the function of PI3K in the legume–rhizobium symbiotic interaction, a highly conserved fragment of the coding region of both soybean *PI3K* genes was amplified to generate the constructs for *PI3K* post-transcriptional gene silencing. Soybean composite plants with *PI3K* post-transcriptional gene silencing mediated by RNA interference (RNAi) were obtained by *Agrobacterium rhizogenes* infection (Fig. 1; Supplementary Fig. S2) (Estrada-Navarrete *et al.*, 2006; Robert *et al.*, 2014). Two different plasmids were assessed to obtain hairy roots with post-transcriptional *PI3K* silencing, pTdt-DC-RNAi (Valdés-López *et al.*, 2008) and pK7GWIWG2D(II) (Karimi *et al.*, 2002). The plasmids have different reporter genes, Tdt and GFP, respectively (Supplementary Fig. S2, upper panel) and presented very similar effects on the total *PI3K* transcript level in PI3Ki hairy roots (Fig. 1A, B; Supplementary Fig. S2). It is important to mention that due to the highly conserved sequence between the *PI3K* genes, and because a highly conserved fragment of the coding region of both soybean *PI3K* genes was used for the *PI3K* post-transcriptional gene silencing, the primer pair for *PI3K* qPCR analysis was designed for the detection of both transcripts (total *PI3K* transcripts levels). We grouped the hairy roots according to the fluorescence intensity given by the reporter, with high signal roots and roots with poor or no fluorescence (Supplementary Fig. S2), and the efficiency of the *PI3K* silencing was evaluated. The RT-qPCR analysis showed a positive correlation between the intensity levels of the reporter and *PI3K* silencing levels (Supplementary Fig. S2).

PI3K gene-silenced composite soybean plants were inoculated with *B. japonicum* USDA138 and grown for 24 d, and nodule formation was analysed. Down-regulation of *PI3K* affected the growth of hairy roots (Supplementary Fig. S3) and root hair (Fig. 2A), and nodule formation (Figs 2B, C; Supplementary Fig. S3). The transgenic hairy roots obtained by *A. rhizogenes* infection have the particular feature that each hairy root is an independent transgenic event (Robert *et al.*, 2014), having each of them different levels of gene silencing, and therefore they showed variations in the phenotype (Fig. 1B; Fig. 2A; Supplementary Figs S2 and S3). Given this result, we focused on analysing hairy roots that showed high reporter fluorescence. PI3Ki composite plants had drastically affected nodulation capacity, showing a reduction in nodule

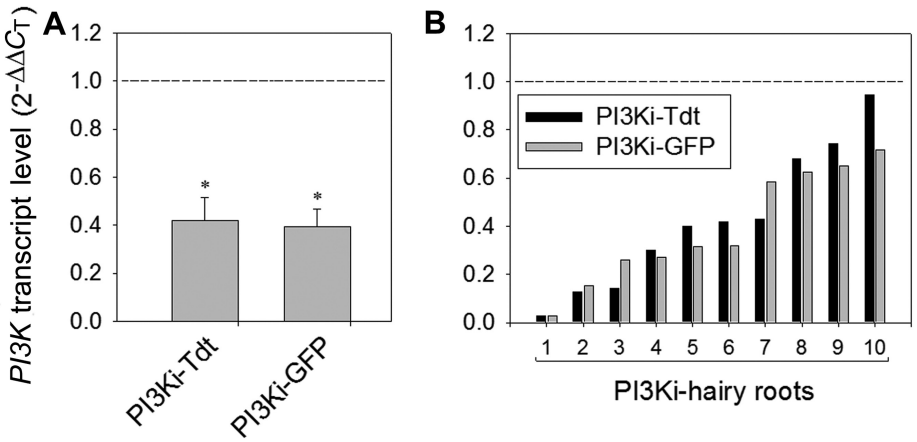

Fig. 1. Post-transcriptional gene silencing of *PI3K* genes in soybean hairy roots (PI3Ki hairy roots). (A) Mean total *PI3K* transcript level in PI3Ki-GFP and PI3Ki-Tdt hairy roots. (B) Variation in the levels of the total *PI3K* transcripts among 10 individual PI3Ki hairy roots. *PI3K* expression levels in PI3Ki hairy roots were analysed by RT-qPCR and expressed relative to control K599-empty hairy roots (assigned a value of 1). Data are means ±SE of 10 hairy roots. Asterisks indicate a significant difference ($P<0.05$, DGC test).

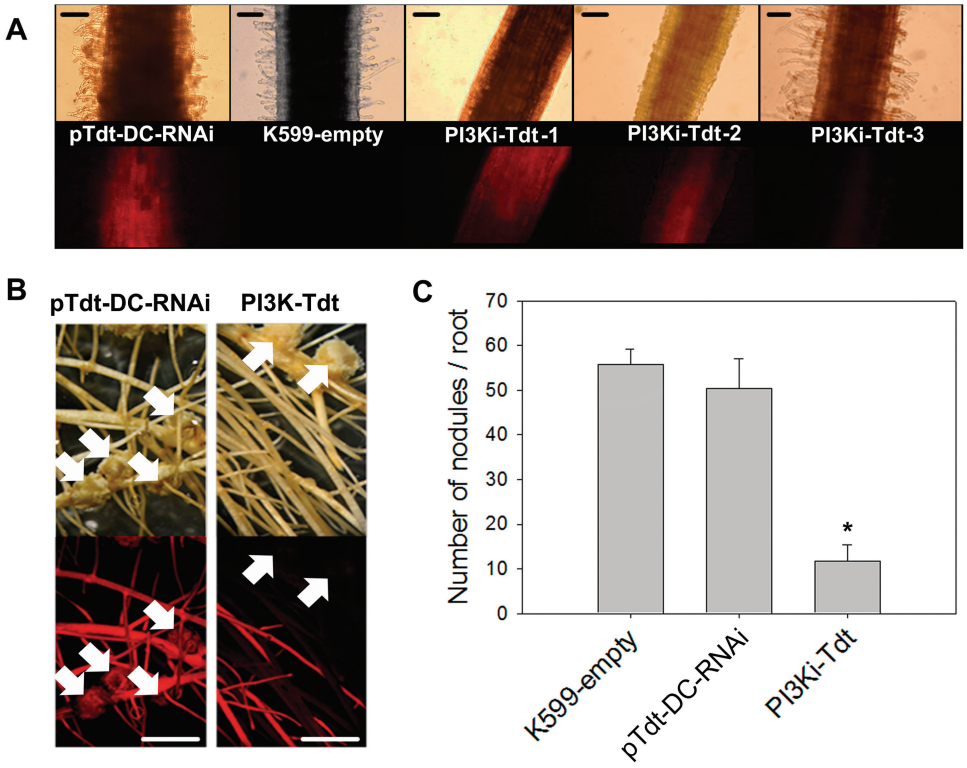

Fig. 2. Effect of *PI3K* silencing on root hair growth and nodule formation. (A) Root hair growth in control (pTdt-DC-RNAi and K599-empty hairy roots) and in three PI3Ki-Tdt hairy roots with different *PI3K* silencing levels. Upper panel, bright-field images; lower panel, Tdt fluorescence. Scale bars: 100 μm. (B) Nodule formation in control pTdt-DC-RNAi and PI3Ki-Tdt hairy roots (arrows). Upper panel, bright-field images; lower panel, Tdt fluorescence. Note that nodules in PI3Ki-Tdt composite plants were mainly located in non-fluorescent hairy roots (arrows in right panel); pTdt-DC-RNAi composite plants presented nodules in fluorescent hairy roots (arrows in left panel). Scales bars: 5 mm. (C) Nodule number in hairy roots of control composite plants (K599-empty and pTdt-DC-RNAi) and PI3Ki silenced composite plants (PI3Ki-Tdt). Data are means ±SE of at least 10 hairy roots. Asterisk indicate a significant difference ($P<0.05$, DGC test).

number of about 80% with respect to the control wild type and empty vector composite plants (Fig. 2C). In this regard, it could be observed that control pTdt-DC-RNAi composite plants had nodules on fluorescent hairy roots and the nodules in PI3Ki-Tdt composite plants were mainly located on non-fluorescent hairy roots (Fig. 2B).

PI3K and redox changes during the very early stages of the symbiotic interaction

To study in-depth the PI3K function during the legume–rhizobium interaction and its relationship with redox changes, we focused our attention on what we call the very early stages during symbiotic interaction, 2–30 min post-inoculation, where we previously characterized marked changes on root hair redox homeostasis (Muñoz *et al.*, 2012). These redox changes induced by rhizobia involve a peak of intracellular ROS production in the root hair between 2 and 5 min post-inoculation (Cárdenas *et al.*, 2008; Muñoz *et al.*, 2012) and a sustained apoplastic ROS generation (Muñoz *et al.*, 2012).

PI3Ki-Tdt hairy roots showed a marked decrease in intracellular ROS levels compared with control hairy roots at the zone of root hair differentiation (Fig. 3A–D) and increased content of MDA (Fig. 3E), which is an intermediary metabolite of lipid peroxidation used as an oxidative stress marker. Due to this effect of *PI3K* silencing on the hairy root redox homeostasis, in addition to the negative effects on root hair growth and nodulation (Fig. 2), pharmacological approaches were used to study the involvement of PI3K in the redox homeostasis at the very early stage of the symbiotic interaction and its impact on nodule formation capacity.

Initially, we used different times of pretreatment with 30 μM LY294002, a widely used PI3K inhibitor (Leprince *et al.*, 2015), to rule out negative effects on root hair viability. Incubations with 30 μM LY294002 for longer than 1 h induced root hair death assessed by Evans Blue staining (data not shown). Therefore, soybean seedlings were pretreated for 30 min with 30 μM LY294002 or DMSO as a control and roots were then transferred to a clean tube and inoculated with *B. japonicum* USDA138 for 30 min in the presence of 0.01% (w/v) nitroblue tetrazolium (NBT), which reacts with the apoplastic superoxide radicals to produce a blue formazan precipitate (Fig. 4A). As was previously demonstrated by Muñoz and coworkers (2012), the apoplastic ROS production during the symbiotic interaction was increased after 30 min *B. japonicum* inoculation (Fig. 4). Interestingly, LY294002 pretreatment inhibited the apoplastic ROS induction in response to the symbiont (Fig. 4).

The intracellular ROS production was assayed by using the ROS-sensitive fluorescent dye CMH_2DCFA and imaging by fluorescence microscopy (Fig. 5). Similarly to the results reported by Cárdenas *et al.* (2008) and Muñoz *et al.* (2012), intracellular ROS generation in root hairs was markedly

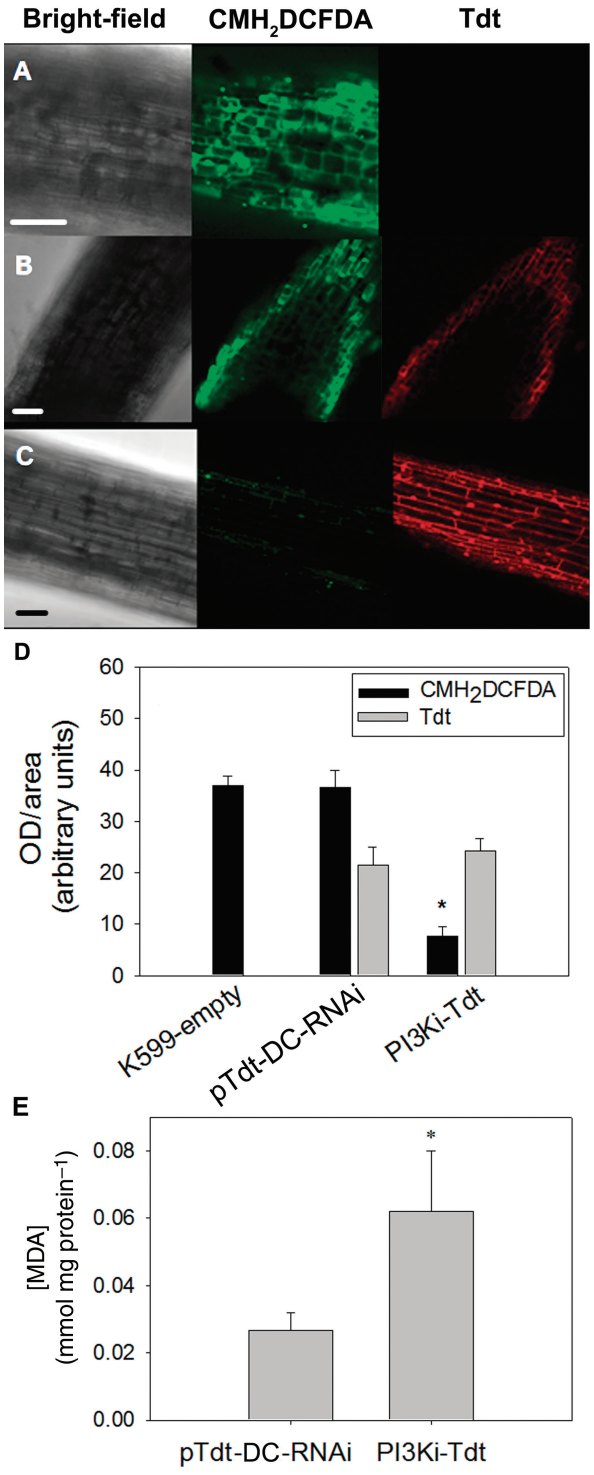

Fig. 3. Effect of *PI3K* silencing on the redox homeostasis of hairy roots. Analysis of intracellular ROS generation at the zone of root hair differentiation by using the fluorescent dye CMH_2DCFDA (10 µM) and laser scanning confocal microscopy in (A) control K599-empty hairy roots, (B) control pTdt-DC-RNAi hairy roots, and (C) PI3Ki-Tdt hairy roots. The panels in (A–C) are bright field, fluorescence signal of CMH_2DCFDA, and fluorescence signal of the Tdt reporter. Images were taken with a Nikon confocal microscope. Scale bars: 50 µm. (D) The fluorescence of CMH_2DCFDA and Tdt reporter were measured and transformed into optical density (OD) by the image processing software Optimas®. Data are means ±SE of five hairy roots. (E) MDA content in pTdt-DC-RNAi and PI3Ki-Tdt hairy roots. Results are the means of four independent experiments (at least two roots per treatment). Data are means ±SE. Asterisks indicate a significant difference (P<0.05, DGC test).

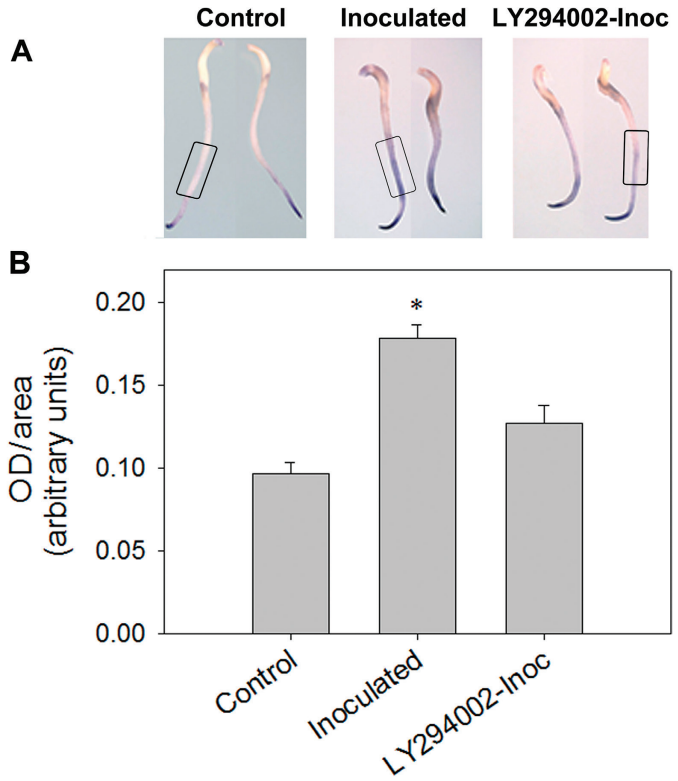

Fig. 4. Participation of PI3K in the very early host redox response: apoplastic ROS generation. (A) Apoplastic superoxide radical production at the zone of root hair differentiation (boxes). Two-day-old soybean seedlings were pretreated for 30 min with 30 µM LY294002 or DMSO as a control, and then inoculated with *B. japonicum* USDA138 for 30 min in the presence of 0.01% (w/v) NBT. (B) NBT precipitated at the zone of root hair differentiation (boxes in A) was measured and transformed into optical density (OD) by the image processing software Optimas®. Results are the means of four independent experiments (three roots per treatment). Data are means ±SE. Asterisk indicates significant difference (P<0.05, DGC test). (This figure is available in color at *JXB* online.)

induced after 2–5 min post-inoculation (Fig. 5). This response was strongly inhibited in root hairs from LY294002-pretreated roots, suggesting that PI3K activity is necessary for ROS production (Fig. 5A). Previously, our group reported that this intracellular ROS production in response to the symbiont could be discriminated into two zones, perinuclear and at the tip of the root hair (Muñoz *et al.*, 2012). Therefore, the ROS generation provided by the apical region and the perinuclear area was independently quantified (Fig. 5A). The specific symbiont-perception response was inhibited in LY294002-pretreated roots, affecting equally the apical and perinuclear ROS generation (Fig. 5A).

On the other hand, several lines of evidence have indicated the involvement of the NADPH oxidase complex during the symbiotic interaction (Peleg-Grossman *et al.*, 2007; Cárdenas *et al.*, 2008; Montiel *et al.*, 2012; Muñoz *et al.*, 2012). In order to investigate the participation of the NADPH oxidase complex during the very early responses to the symbiont, pretreatments were performed with 50 µM DPI, a well-known inhibitor of flavoprotein enzymes that is used extensively as an NAD(P)H oxidase inhibitor. The intracellular ROS production was evaluated in root hairs 2–5 min post-inoculation (Fig. 5B). In accordance with previous reports in *P. vulgaris* (Cárdenas *et al.*, 2008),

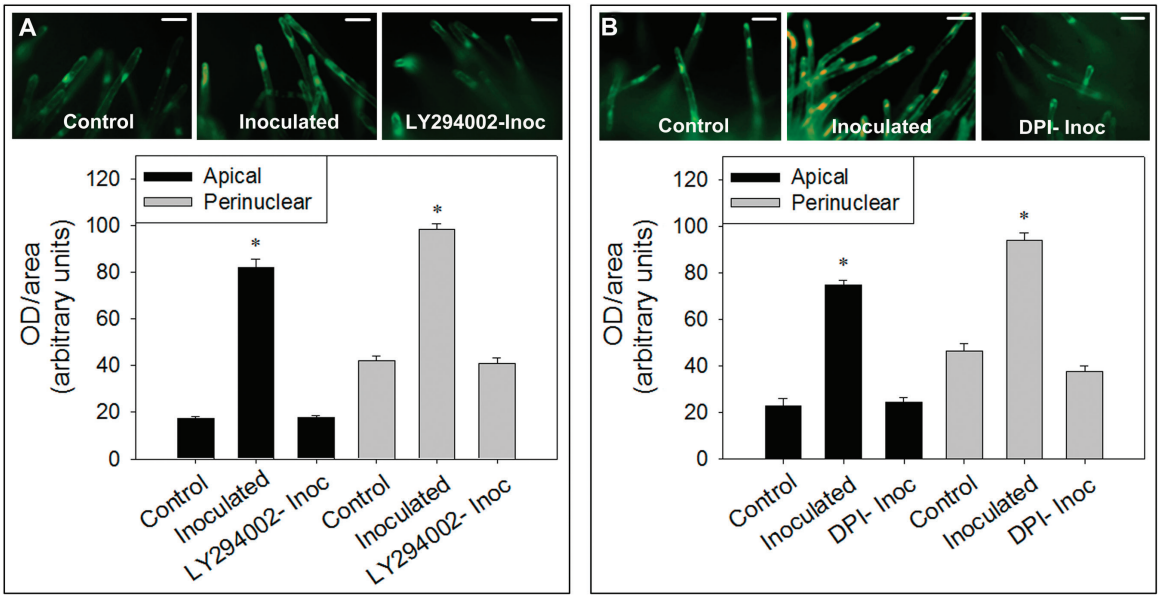

Fig. 5. Participation of PI3K and the NADPH oxidase complex in the very early host redox responses: intracellular ROS generation. Two-day-old soybean seedlings were pretreated for 30 min with DMSO as a control or with 30 μM LY294002 (A) or 50 μM DPI (B), and then inoculated with *B. japonicum* USDA138 for 2 min. CMH2DCFDA was added to the pretreatment after 15 min. Upper panels show intracellular ROS generation in root hairs by using a Zeiss Axiophot microscope with excitation filter BP 450–490 and emission filter LP 520. Scale bars: 35 μm. Lower panels show the measurement of CMH_2DCFDA fluorescence transformed into optical density (OD) by the image processing software Optimas®. Fluorescence in root hairs was separately quantified in apical and perinuclear regions. Results are the means of three independent experiments (two roots per treatment); approximately 20 root hairs were quantified per treatment. Data are means ±SE. Asterisks indicate a significant differences ($P<0.05$, DGC test). (This figure is available in color at *JXB* online.)

our results indicate the participation of the NADPH oxidase complex in the induction of intracellular ROS generation in soybean root hairs in response to the symbiont. Moreover, NADPH oxidase inhibition showed a very similar pattern of response to the PI3K inhibition experiments, affecting in a similar fashion ROS production and location (Fig. 5). Finally, we investigated the localization of intracellular ROS production at the endosomes level, analysing CMH_2DCFA and FM4-64 staining by confocal microscopy. The results suggested that intracellular ROS induction was located at least in part in endosomes in root hair (Fig. 6). Besides, FM4-64 staining did not show differences between the treatments at this very early time, in spite of the pretreatment with LY294002 (Fig. 6).

The impact of the very early responses on later stages: nodule formation

The perception of the rhizobium by the plant triggers responses that induce changes in young epidermal cells and, simultaneously, triggers molecular events that will lead to nodule organogenesis. Interestingly, these responses can occur independently of one another (Oldroyd and Downie, 2008). The effects of the very early responses over the later stages were evaluated (Supplementary Fig. S4). PI3K- and NADPH oxidase-inhibition pretreatments were performed for 30 min, and then roots were inoculated for 30 min with *B. japonicum* USDA138 and washed with sterile distilled water. It is important to mention that the pretreatments with the inhibitors were carried out only in the seedlings prior to the inoculation in order to avoid any possible effect on the rhizobium (Supplementary Fig. S4). In this regard, colony forming unit (CFU) analysis conducted to evaluate the effect of pretreatments on the viability of *B. japonicum* showed no significant differences (data not shown; Muñoz *et al.*, 2012). After 24 d, nodule number and fresh weight were analysed (Fig. 7). The inhibition of the very early responses, both by LY294002 and DPI, reduced the number of nodules by around 45% (Fig. 7A), although the nodule fresh weight was not significantly affected (Fig. 7B).

*Induced root hair death during soybean–*B. japonicum *interaction under sublethal saline stress: a problem of perception?*

As was mentioned above, previous research led us to hypothesize that the changes observed in the very early redox responses during symbiont perception under saline stress lead to a perception of the symbiont as (or as if it were) a pathogen (Muñoz *et al.*, 2012, 2014). Therefore, we analysed the relevance of the very early host responses to the symbiont in the induction of root hair death under saline stress conditions.

PI3K expression levels were measured in both roots and root hairs under root hair death-inducing stress conditions: *B. japonicum* inoculation in the presence of sublethal 50 mM NaCl (50 mM NaCl Inoc) and severe 150 mM NaCl salt stress (Fig. 8A) (Muñoz *et al.*, 2012; Robert *et al.*, 2014). Under 50 and 150 mM NaCl, there was an induction in the expression of *PI3K* in root segments dependent on NaCl concentration (Fig. 8A). Interestingly, inoculation in the presence of 50 mM NaCl repressed the salt-stimulated *PI3K* expression, showing no significant differences with respect to control root segments (Fig. 8A). On the other hand,

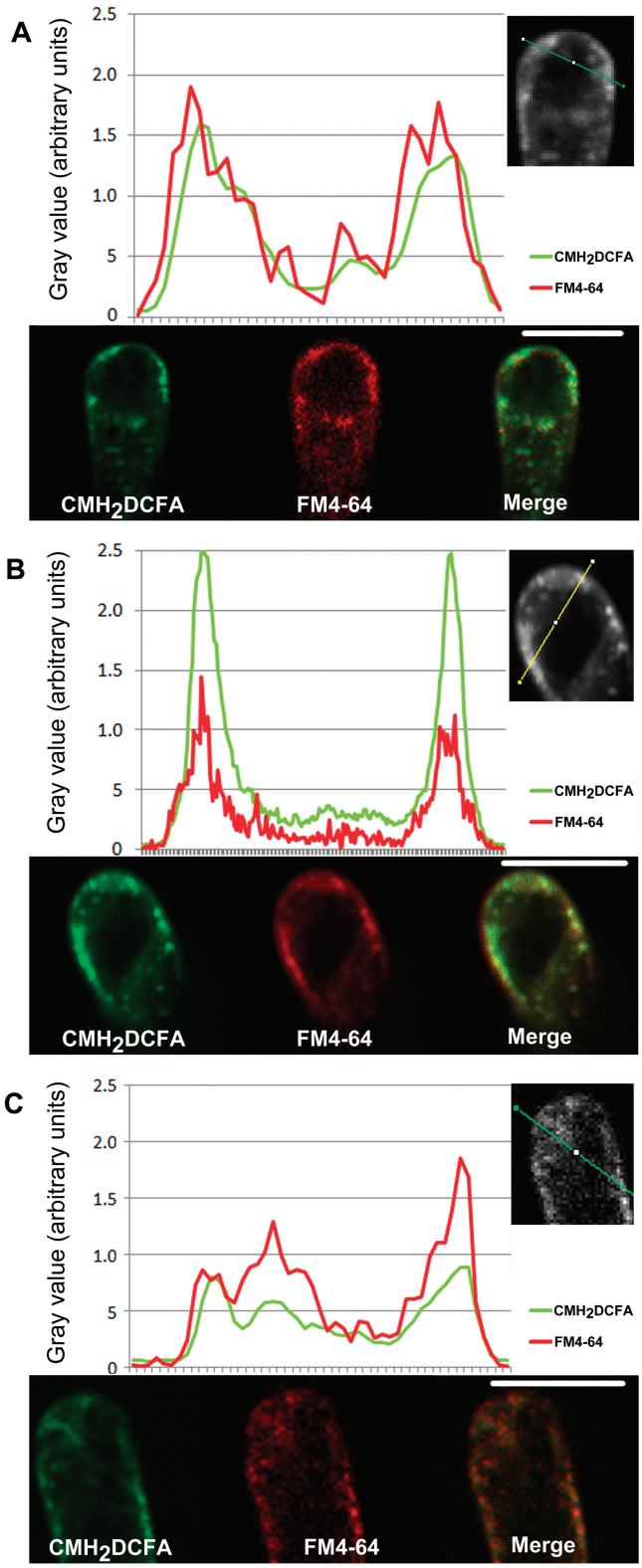

Fig. 6. Intracellular ROS production at the endosomes level. (A–C) Intracellular ROS production in root hair after 2–5 min post-inoculation with *B. japonicum*. Two-day-old soybean seedlings were pretreated for 30 min with DMSO as a control (A, B) or 30 µM LY294002 (C), and then inoculated with *B. japonicum* USDA138 for 2 min (B, C). Intracellular ROS production and endosomes were analysed by CMH_2DCFA and FM4-64 staining, respectively (bottom panels). Merged images of simultaneous emission of green and red filters were acquired by confocal microscopy and quantified using ImageJ. Graphs in A–C, indicate relative fluorescence (gray value) of each filter (green and red) along the line in the inset images. Scale bars: 15 µm.

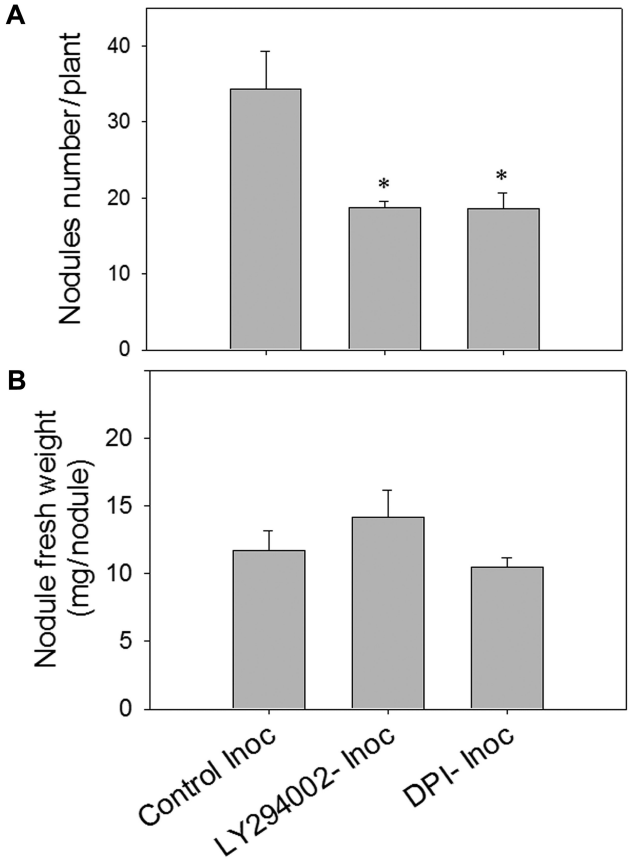

Fig. 7. Participation of the very early host redox responses in nodule formation. Nodule number (A) and nodule weight (B) were evaluated after 24 d post-inoculation. Roots of two-day-old soybean seedlings were pretreated with DMSO as a control, and 30 µM LY294002 or 50 µM DPI for 30 min, and then roots were inoculated for 30 min with *B. japonicum* USDA138. Results are the means of at least seven plants. Data are means ±SE. Asterisks indicate a significant difference ($P<0.05$, DGC test).

the expression of *PI3K* in root hairs showed differences in respect to what was observed in root segments. *PI3K* expression levels under conditions of 50 and 150 mM NaCl did not differ significantly from the control (Fig. 8B). However, under inoculation treatment in the presence of saline stress, unlike root segments, the expression of *PI3K* showed a significant increase in root hairs (Fig. 8B). Moreover, the down-regulation of the *PI3K* gene increased the susceptibility to saline stress (Supplementary Fig. S5).

To further evaluate the participation of the very early symbiont perception on the induced root hair death under saline stress conditions, we investigated the induction of root hair death in non-nodulated *nfr5* mutant soybean plants (Bragg nod139), which carry a mutated version of the Nod factor receptor 5 (*NFR5*) gene (Carroll *et al*., 1986). Two-day-old Bragg WT and Bragg nod139 soybean seedlings were treated for 30 min with sterile water (control), *B. japonicum* (inoculated), 50 mM NaCl, and *B. japonicum* in the presence of 50 mM NaCl (50 mM NaCl Inoc), and Evans Blue staining was performed in order to analyse root hair death (Fig. 9; Supplementary Fig. S6; Robert *et al*., 2014). No differences were observed between control and inoculated treatments in any of the genotypes (Supplementary Fig. S6). As we have previously shown (Muñoz *et al*., 2012; Robert *et al*., 2014), the

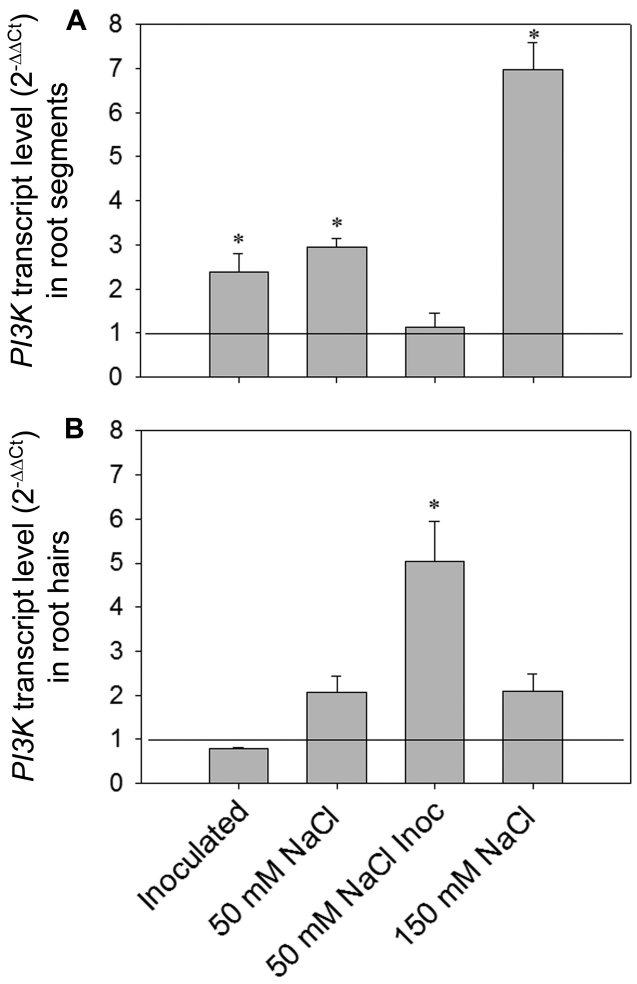

Fig. 8. Total *PI3K* transcript level in 2-day-old soybean seedlings subjected for 30 min to control and different degrees of salt stress. Total transcript level of *PI3K* in root segments (A) and in root hairs (B) after 30 min of incubation under control condition, inoculated with *B. japonicum* (inoculated), in 50 mM NaCl, inoculated with *B. japonicum* in presence of 50 mM NaCl (50 mM NaCl Inoc), and in 150 mM NaCl. The levels of *PI3K* expression are relative to control conditions (assigned a value of 1). Data are means ±SE. Asterisks indicate a significant difference ($P<0.05$, DGC test).

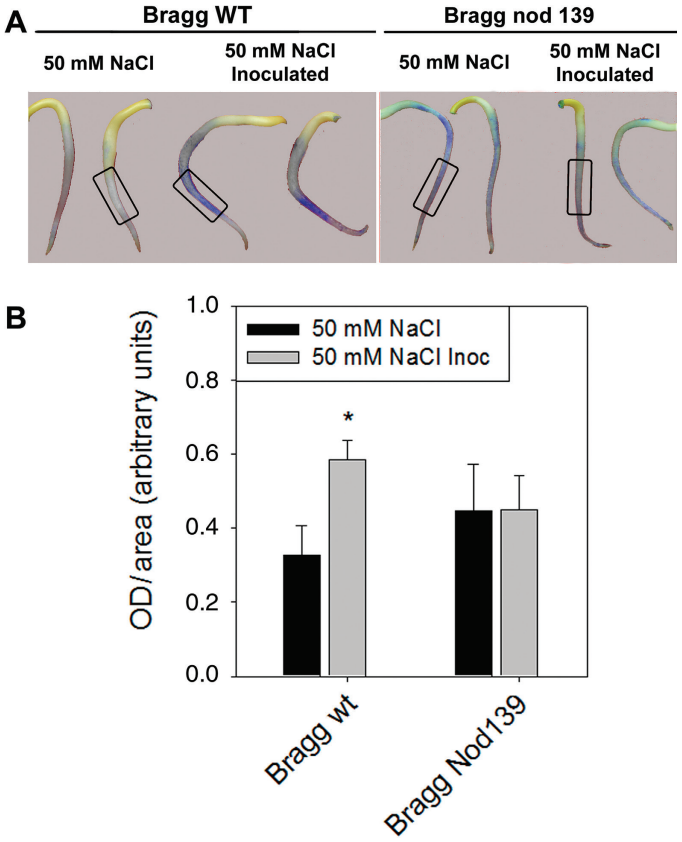

Fig. 9. Symbiont perception under sublethal saline stress induced root hair death. (A) Evans Blue staining of roots showing loss of membrane integrity at the root hair differentiation zone (boxes). Two-day-old soybean Bragg wild type (Bragg WT) or non-nodulated *nfr5* mutant (Bragg nod139) seedlings were subjected for 30 min to 50 mM NaCl (as a control), and inoculated with *B. japonicum* in the presence of 50 mM NaCl (50 mM NaCl Inoc). (B) Evans Blue staining at the zone of root hair differentiation (boxes in A) was measured and transformed into optical density (OD) by the image processing software Optimas®. Results are the means of three independent experiments (four roots per treatment). Data are means ±SE. Asterisk indicates a significant differences ($P<0.05$, DGC test). (This figure is available in color at *JXB* online.)

inoculation with *B. japonicum* under saline stress induced cell death compared with the 50 mM NaCl treatment in the Bragg WT genotype (Fig. 9). Interestingly, there were no significant differences between root hair death in roots of *nfr5* mutant plants treated with 50 mM NaCl and roots inoculated in the presence of 50 mM NaCl (Fig. 9).

Discussion

To make root hair curling happen, all the machinery involved in root hair growth must function properly and in association with the responses generated by the perception of Nod factors. Phosphoinositide lipids and their metabolic enzymes are downstream elements of the Nod factor transduction pathway, although their biological role is less understood. As shown previously and herein, PI3K, and also phosphatidic acid production by activation of the phospholipase C (PLC), diacylglycerol kinase, and phospholipase D pathways, participates in the very early response of the symbiotic interaction and during the development of the infection thread (Den Hartog *et al.*, 2001; Gage, 2004; Peleg-Grossman *et al.*, 2007; Oldroyd and Downie, 2008; Blanco *et al.*, 2009; Muñoz *et al.*, 2012; Estrada-Navarrete *et al.*, 2016). It was also reported that at later stages when the nodule is developed, the *DNF2* gene encoding a PLC-like protein is required for the symbiotic repression of plant defenses at the stage of the bacterial internalization in the interaction between *Medicago truncatula* and *Sinorhizobium meliloti* (Berrabah *et al.*, 2014).

Here, we demonstrated by genetic and pharmacological approaches the participation of PI3K during the soybean–*B. japonicum* interaction modulating the host redox responses. In addition, to the best of our knowledge, we showed for the first time the participation of PI3K during the very early host redox responses, previous to the internalization of the infection droplets (Figs 4 and 5A), modulating the NADPH oxidase activity (Figs 4 and 5B) (Muñoz *et al.*, 2012), and the relevance of these very early responses for later stages such as nodule formation (Fig. 7).

PI3Ki soybean hairy roots had reduced root hair growth, similar to what was observed in Arabidopsis (Lee *et al.*, 2008*a*) and *P. vulgaris* (Estrada-Navarrete *et al.*, 2016; Fig. 2A) as well as reduced nodule formation (Fig. 2B, 2C). Indeed, the negative effects of *PI3K* silencing on root hair growth as well as in early stages of the symbiotic interaction, such as root hair curling, infection thread growth, and nodulation, were carefully described recently studying the *P. vulgaris–R. tropici* interaction (Estrada-Navarrete *et al.*, 2016). Furthermore, and in addition to the above-mentioned *PI3K* silencing effects, we also showed here that PI3Ki hairy roots have altered redox homeostasis such as reduced intracellular ROS generation and increased levels of MDA (Fig. 3). The increased MDA levels in PI3Ki hairy roots could be due to the altered redox homeostasis, and a possible role of PI3K in the maintenance of membrane homeostasis should not be ruled out (Gary *et al.*, 1998). Moreover, and as we also suggest here, it has been postulated that PI3K modulates mainly the intracellular ROS production involved in signal transduction (Leshem *et al.*, 2007; Takáč *et al.*, 2013). In addition, it has been previously shown that LY294002 treatment affects proteins involved in enzymatic antioxidant defense, suggesting a PI3K-dependent control over antioxidant enzyme machinery possibly linked to decreased NADPH oxidase activity (Takáč *et al.*, 2013). In this regard, we have demonstrated in previous work a correlation between antioxidant enzyme activity and apoplastic superoxide level, suggesting the participation of the NADPH oxidase complex in the modulation of the antioxidant defense response (Robert *et al.*, 2009).

Thus, given these effects of the *PI3K* silencing, and with the aim of (i) investigating the PI3K function related to the redox changes that take place during the very early host responses in the legume–rhizobium interaction, and (ii) evaluating the impact of the very early host responses on the nodulation process, a pharmacological approach using the PI3K inhibitor LY294002 was used.

Inhibition of PI3K abolished the redox-specific very early responses to *B. japonicum* in soybean root hair, such as the intracellular (Fig. 5A) and apoplastic ROS induction (Fig. 4), indicating its participation at this stage of the symbiotic interaction. In this sense, it has been shown that vesicle trafficking genes, including *PI3K*, are highly expressed in *M. truncatula* roots after 24 h of *S. meliloti* infection (Peleg-Grossman *et al.*, 2007). Here, *PI3K* expression levels at the very early stages of the symbiotic interaction in roots (Fig. 8A) and root hairs (Fig. 8B) of soybean seedlings were analysed. Interestingly, the total expression of *PI3K* was induced in roots but no differences were observed in root hairs (Fig. 8). The product of PI3K activity is a signaling phosphoinositide, phosphatidylinositol 3-phosphate (PI3P), and as such provides a spatial and temporal signal whose levels are tightly regulated within short periods of time. The result observed in root hair suggests that PI3K activity is probably modulated by post-translational modifications and/or by changing its localization during the very early responses to the rhizobia in the root hair.

Besides, the apoplastic (Muñoz *et al.*, 2012) and intracellular ROS induction in response to the symbiont was also NADPH oxidase dependent (Fig. 5B). In this sense, the apical and perinuclear intracellular ROS generation were equally abolished in LY294002- and in DPI-pretreated roots (Fig. 5). These results are very interesting since the NADPH oxidase complex is mainly located at the plasma membrane but its activity affects the induction of intracellular ROS generation in response to the symbiont. Thus, it is possible that intracellular ROS induction in response to the rhizobia also occurs at the endosomal level and is modulated by the NADPH oxidase and PI3K activity. In this regard, it has been reported that induction of endosomal ROS generation occurs in response to saline stress in Arabidopsis root cells, and the participation of PI3K and the NADPH oxidase complex have been postulated (Leshem *et al.*, 2007; Hao *et al.*, 2014). Similarly, the induction of PI3K- and NADPH oxidase-dependent intracellular ROS generation in response to the symbiont was also partially located at the endosomal level (Fig. 6). In agreement with these observations, it was recently shown that clathrin- and microdomain-dependent endocytic pathways cooperatively regulate RbohD dynamics in Arabidopsis, and that salt stress stimulates RbohD endocytosis via membrane microdomains (Hao *et al.*, 2014). In this regard, a strong connection between PI3P species (PI3P and $PI(3,5)P_2$) and their effector proteins (proteins carrying FYVE or PHOX domains) as key regulators of processes at intracellular membranes, endocytosis being one of them, has been previously established (Lee *et al.*, 2008*a*,*b*; Hirano *et al.*, 2015, 2017). No clear differences were observed in the endocytosis process among the treatments, at least at this very early time, even with pretreatment with LY294002 (Fig. 6). This result is in agreement with previous reports that LY29400 does not inhibit the early stages of endocytosis (Lee *et al.*, 2008*a*; Takáč *et al.*, 2013), and suggests that the PI3K inhibition effect on endosomal ROS generation could be more related to the modulation of NADPH oxidase activity.

On the other hand, it has been suggested that the generation of apoplastic superoxide radicals by NADPH oxidase activity maintains the curvature of the root hair curling (Muñoz *et al.*, 2012). Our results showed that PI3K activity is also involved in the apoplastic ROS induction during the symbiotic interaction (Fig. 4), and explain, at least in part, the inhibition of the root hair curling at later stages observed by Peleg-Grossman *et al.* (2007).

In previous work, our group has reported the changes that take place in the very early host redox response concomitantly with the induction of an ordered root hair death when roots were inoculated under both abiotic (Muñoz *et al.*, 2012; Robert *et al.*, 2014), and biotic stress (López *et al.*, 2017). In this regard, we have shown that the inoculation of roots under sublethal saline stress conditions induced a sustained intracellular ROS production, increased expression of pathogenesis-related proteins, and led to root hair death (Muñoz *et al.*, 2012, 2014; Robert *et al.*, 2014), which is reminiscent of the response observed in root hair of *P. vulgaris* elicited with the fungal elicitor chitosan (Cárdenas *et al.* 2008; Muñoz *et al.* 2012). The increased expression of PI3K observed in root hairs (Fig. 8B), but not in roots (Fig. 8A), under this combination of stimuli could underlie the increased and sustained intracellular ROS production mentioned above leading to the root hair death. Thus, based on the results indicating (i) the participation of PI3K in the very early host responses during the symbiotic interaction (Figs 4 and 5A), and (ii) the increased *PI3K*

expression in root hair under 50 mM NaCl inoculation conditions (Fig. 8B), we hypothesized that inhibiting the very early symbiont perception by impairing PI3K activity will reduce the root hair death in inoculated roots under saline stress. However, it is important to note that PI3K activity also participates in the salt tolerance responses through the activation of the endocytosis process and intracellular NADPH oxidase-dependent ROS generation (Leshem *et al.*, 2007) and proline biosynthesis (Leprince *et al.*, 2015). Accordingly, cell death was increased in PI3Ki hairy roots under saline stress (Supplementary Fig. S5).

Therefore, the induction of cell death in non-nodulated *nfr5* mutant soybean plants was investigated to further evaluate the participation of the very early symbiont perception in inducing root hair death under sublethal saline stress conditions (Fig. 9). NFR5 receptors mediate the perception of the bacterial Nod factor and hence the symbiont is not perceived by *nfr5* mutant plants. The results showed no significant differences between saline stress and the inoculation in the presence of NaCl, demonstrating that cell death induction depends on the very early symbiont perception (Fig. 9).

In brief, we demonstrated that induced root hair death occurring under sublethal saline stress in combination with *B. japonicum* depends on symbiont perception. We emphasize the relevance of the very early host response as the first control of the nodulation capacity, prior to the well-reported abortion of infection threads, as a mechanism for assessing environmental conditions for the nodulation process, inducing root hair death when conditions are not favorable, inhibiting nodulation and therefore the formation of a new organ.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Phylogenetic tree based on reported *PI3K* class III amino acid sequences from *G. max* and other plants.

Fig. S2. Correlation between the reporter fluorescence intensity and *PI3K* silencing in PI3Ki hary roots.

Fig. S3. Correlation between the reporter fluorescence intensity and the phenotype of PI3Ki hairy roots.

Fig. S4. Scheme of the experiment to investigate the impact of the very early responses on nodule formation.

Fig. S5. Cell death induction in control pTdt-DC-RNAi and PI3Ki-Tdt hairy roots treated with 150 mM NaCl.

Fig. S5. Cell death induction in pTdt-RNAi and PI3Ki-Tdt hairy roots subjected to control and saline stress.

Fig. S6. Root hair death in 2-day-old soybean seedlings incubated for 30 min under control or inoculated conditions.

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References

Ashraf M. 1994. Breeding for salinity tolerance in plants. Critical Reviews in Plant Science **13,** 37–42.

Berrabah F, Bourcy M, Cayrel A, Eschstruth A, Mondy S, Ratet P, Gourion B. 2014. Growth conditions determine the DNF2 requirement for symbiosis. PLoS One **9,** e91866.

Blanco FA, Meschini EP, Zanetti ME, Aguilar OM. 2009. A small GTPase of the Rab family is required for root hair formation and preinfection stages of the common bean–*Rhizobium* symbiotic association. The Plant Cell **21,** 2797–2810.

Broughton WJ, Dilworth MJ. 1971. Control of leghaemoglobin synthesis in snake beans. The Biochemical Journal **125,** 1075–1080.

Cárdenas L, Holdaway-Clarke TL, Sánchez F, Quinto C, Feijó JA, Kunkel JG, Hepler PK. 2000. Ion changes in legume root hairs responding to Nod factors. Plant Physiology **123,** 443–452.

Cárdenas L, Martínez A, Sánchez F, Quinto C. 2008. Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). The Plant Journal **56,** 802–813.

Carroll BJ, Mcneil DL, Gresshoff PM. 1986. Mutagenesis of soybean (*Glycine max* (L.) Merr.) and the isolation of non-nodulation mutants. Plant Science **47,** 109–114.

Den Hartog M, Musgrave A, Munnik T. 2001. Nod factor-induced phosphatidic acid and diacylglycerol pyrophosphate formation: a role for phospholipase C and D in root hair deformation. The Plant Journal **25,** 55–65.

Di Rienzo JA, Guzman AW, Casanoves F. 2002. A multiple-comparisons method based on the distribution of the root node distance of a binary tree. JABES **7**, 129–142.

Ellson CD, Gobert-Gosse S, Anderson KE, *et al*. 2001. PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of $p40^{phox}$. Nature Cell Biology **3,** 679–682.

Estrada-Navarrete G, Alvarado-Affantranger X, Olivares JE, *et al*. 2006. *Agrobacterium rhizogenes* transformation of the *Phaseolus* spp.: a tool for functional genomics. Molecular Plant-Microbe Interactions **19,** 1385–1393.

Estrada-Navarrete G, Cruz-Mireles N, Lascano R, *et al*. 2016. An autophagy-related kinase is essential for the symbiotic relationship between *Phaseolus vulgaris* and both rhizobia and arbuscular mycorrhizal fungi. The Plant Cell **28,** 2326–2341.

Felle HH, Kondorosi E, Kondorosi A, Schultze M. 2000. How alfalfa root hairs discriminate between Nod factors and oligochitin elicitors. Plant Physiology **124,** 1373–1380.

Gage DJ. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiology and Molecular Biology Reviews **68,** 280–300.

Gary JD, Wurmser AE, Bonangelino CJ, Weisman LS, Emr SD. 1998. Fab1p is essential for PtdIns(3)P 5-kinase activity and the maintenance of vacuolar size and membrane homeostasis. The Journal of Cell Biology **143,** 65–79.

Hair R, Oa G, Lee Y, Bak G, Choi Y, Chuang W, Cho H, Lee Y. 2008. Roles of Phosphatidylinositol 3-kinase in root hair growth. Plant Pathology **147,** 624–635.

Hao H, Fan L, Chen T, Li R, Li X, He Q, Botella MA, Lin J. 2014. Clathrin and membrane microdomains cooperatively regulate RbohD dynamics and activity in Arabidopsis. The Plant Cell **26,** 1729–1745.

Heath RL, Packer L. 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics **125,** 189–198.

Hirano T, Munnik T, Sato MH. 2015. Phosphatidylinositol 3-phosphate 5-kinase, FAB1/PIKfyve kinase mediates endosome maturation to establish endosome-cortical microtubule interaction in Arabidopsis. Plant Physiology **169,** 1961–1974.

Hirano T, Munnik T, Sato MH. 2017. Inhibition of phosphatidylinositol 3,5-bisphosphate production has pleiotropic effects on various membrane trafficking routes in Arabidopsis. Plant & Cell Physiology **58,** 120–129.

Hong Z, Verma DP. 1994. A phosphatidylinositol 3-kinase is induced during soybean nodule organogenesis and is associated with membrane proliferation. Proceedings of the National Academy of Sciences, USA **91,** 9617–21.

Jones MA, Raymond MJ, Yang Z, Smirnoff N. 2007. NADPH oxidase-dependent reactive oxygen species formation required for root hair growth depends on ROP GTPase. Journal of Experimental Botany **58,** 1261–1270.

Karimi M, Inzé D, Depicker A. 2002. GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. Trends in Plant Science **7,** 193–195.

Lee Y, Bak G, Choi Y, Chuang WI, Cho HT, Lee Y. 2008*a*. Roles of phosphatidylinositol 3-kinase in root hair growth. Plant Physiology **147,** 624–635.

Lee Y, Kim ES, Choi Y, Hwang I, Staiger CJ, Chung YY, Lee Y. 2008*b*. The Arabidopsis phosphatidylinositol 3-kinase is important for pollen development. Plant Physiology **147,** 1886–1897.

Leprince AS, Magalhaes N, De Vos D, Bordenave M, Crilat E, Clément G, Meyer C, Munnik T, Savouré A. 2015. Involvement of phosphatidylinositol 3-kinase in the regulation of proline catabolism in *Arabidopsis thaliana*. Frontiers in Plant Science **5,** 772.

Leshem Y, Seri L, Levine A. 2007. Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. The Plant Journal **51,** 185–197.

Limpens E, Ivanov S, van Esse W, Voets G, Fedorova E, Bisseling T. 2009. *Medicago* N_2-fixing symbiosomes acquire the endocytic identity marker Rab7 but delay the acquisition of vacuolar identity. The Plant Cell **21,** 2811–2828.

Liu J, Zhou J, Xing D. 2012. Phosphatidylinositol 3-kinase plays a vital role in regulation of rice seed vigor via altering NADPH oxidase activity. PloS One **7,** e33817.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. Methods **25,** 402–408.

López M, Muñoz N, Lascano HR, Izaguirre-Mayoral ML. 2017. The seed-borne *Southern bean mosaic virus* hinders the early events of nodulation and growth in *Rhizobium*-inoculated *Phaseolus vulgaris* L. Functional Plant Biology **44,** 208–218.

Montiel J, Nava N, Cárdenas L, Sánchez-López R, Arthikala MK, Santana O, Sánchez F, Quinto C. 2012. A *Phaseolus vulgaris* NADPH oxidase gene is required for root infection by Rhizobia. Plant & Cell Physiology **53,** 1751–1767.

Muñoz N, Robert G, Melchiorre M, Racca R, Lascano R. 2012. Saline and osmotic stress differentially affects apoplastic and intracellular reactive oxygen species production, curling and death of root hair during *Glycine max* L.–*Bradyrhizobium japonicum* interaction. Environmental and Experimental Botany **78,** 76–83.

Muñoz N, Rodriguez M, Robert G, Lascano R. 2014. Negative short-term salt effects on the soybean–*Bradyrhizobium japonicum* interaction and partial reversion by calcium addition. Functional Plant Biology **41,** 96–105.

Oldroyd GE, Downie JA. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. Annual Review of Plant Biology **59,** 519–546.

Park KY, Jung JY, Park J, Hwang JU, Kim YW, Hwang I, Lee Y. 2003. A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells. Plant Physiology **132,** 92–98.

Peleg-Grossman S, Volpin H, Levine A. 2007. Root hair curling and *Rhizobium* infection in *Medicago truncatula* are mediated by phosphatidylinositide-regulated endocytosis and reactive oxygen species. Journal of Experimental Botany **58,** 1637–1649.

Robert G, Melchiorre M, Racca R, Trippi V, Lascano HR. 2009. Apoplastic superoxide level in wheat protoplast under photooxidative stress is regulated by chloroplast redox signals: effects on the antioxidant system. Plant Science **177,** 168–174.

Robert G, Muñoz N, Melchiorre M, Sánchez F, Lascano R. 2014. Expression of animal anti-apoptotic gene Ced-9 enhances tolerance during *Glycine max* L.–*Bradyrhizobium japonicum* interaction under saline stress but reduces nodule formation. PloS One **9,** e101747.

Roth E, Jeon K, Stacey G. 1988. Homology in endosymbiotic systems: the term "symbiosome". In: Palacios R, Verma DPS, eds. Molecular genetics of plant microbe interactions. St Paul: American Phytopathological Society, 220–229

Singleton PW, Bohlool BB. 1984. Effect of salinity on nodule formation by soybean. Plant Physiology **74,** 72–76.

Takáč T, Pechan T, Šamajová O, Šamaj J. 2013. Vesicular trafficking and stress response coupled to PI3K inhibition by LY294002 as revealed by proteomic and cell biological analysis. Journal of Proteome Research **12,** 4435–4448.

Valdés-López O, Arenas-Huertero C, Ramírez M, Girard L, Sánchez F, Vance CP, Luis Reyes J, Hernández G. 2008. Essential role of MYB transcription factor: PvPHR1 and microRNA: PvmiR399 in phosphorus-deficiency signalling in common bean roots. Plant, Cell & Environment **31,** 1834–1843.

Vincent JM. 1970. A Manual for the practical study of the root-nodule bacteria. Oxford: Blackwell Scientific Publications, 164.

Zahran HH, Sprent JI. 1986. Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta **167,** 303–309.