Title: PHOSPHATIDYLINOSITOL 3-KINASE AT THE VERY EARLY SYMBIONT PERCEPTION: A LOCAL NODULATION CONTROL UNDER STRESS CONDITIONS?

Running title: PI3K participates in the very early symbiont perception

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HIGHLIGHT

PI3K modulates the very early redox-host responses during symbiont perception, which also play a fundamental role for assessing environmental conditions for the nodulation process

ABSTRACT

Root hair curling is the early and essential morphological change for the success of the symbiotic interaction between legumes and rhizobia. At this stage rhizobia grow as infection thread within root hair and are internalized into the plant cells via endocytosis, where PI3K enzyme plays important roles. Former studies of our group showed that stress conditions affect previous stages of the symbiotic interaction, from 2 to 30 min post-inoculation, which we call as very early host-responses, and affect symbiosis establishment. Herein, we demonstrated the relevance of the very early host-responses for the symbiotic interaction, where PI3K and NADPH oxidase complex have key roles in the microsymbiont-recognition response modulating the apoplastic and intracellular/endosomal ROS induction in root hair. Interestingly, comparing 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 relevance of the very early host-responses on later stages of the symbiont interaction, but also postulate them as a mechanism for the local control of the nodulation capacity, prior to the abortion of infection thread, preventing the allocation of resources/energy for nodules formation under unfavorable environmental conditions.

KEY WORDS

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

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

INTRODUCTION

Regional climate changes affect natural and agricultural ecosystems around the world provoking changes in land use for agriculture. Moreover, the threats of erosion, loss of organic carbon and salinization of the soils are increasing. In this regard, soybean (*Glycine max* L.) has been classified as a salinity susceptible crop (Ashraf, 1994); being both the infection and nodulation processes by *Bradyrhizobium japonicum* severely affected by salinity (Singleton and Bohlool, 1984; Zahran and Sprent, 1986). The effects of abiotic stress in the soybean-*Bradyrhizonium 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 to 5 minutes after symbiont perception early specific responses are induced in 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 to 120 min post inoculation with the rhizobium have been described by our group (Muñoz *et al.*, 2012). Here, the study was focused within the first 2 to 30 minutes of the symbiotic interaction, a time period that we called as the "very early host-response".

Later, between 2 h and 48 h post inoculation, the early responses take place including root hair curling, which is the first early morphological response. Worth to mention that most of the studies on this issue have been 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 as host membrane-bound compartments, called symbiosomes (Roth *et al.*, 1988; Limpens *et al.*, 2009). During this last stage, phosphatidylinositol-3 kinase (PI3K) enzyme has been implicated in soybean nodule organogenesis and peribacteroid membrane development (Hong and Verma, 1994; Estrada-Navarrete *et al.*, 2016). By a pharmacological approach, it was also shown that PI3K was also implicated during early stages of legume-rhizobium interaction, between 16 h and 7 days, coordinating curling responses, the formation of the infection threads and ROS generation in roots (Peleg-Grossman *et al.*, 2007). Both in animal and plant systems, the participation of PI3K in the modulation of ROS production has been demonstrated (Ellson *et al.*, 2001; Park *et al.*, 2003; Hair *et al.*, 2008). In this regard, PI3K was 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 interaction (Peleg-Grossman *et al.*, 2007), a detailed analysis of subcellular ROS distribution has not been provided. Recently, the downregulation of the *PI3K* gene in *Phaseolus vulgaris*-hairy roots using an RNAi approach showed a notable decreased in root hair growth, as well as, root hair curling, infection thread growth, root-nodule number, and

symbiosome formation were severely affected (Estrada-Navarrete *et al*., 2016). Nevertheless, the role of PI3K during the very early host-responses at the root hair level, before the endocytotic internalization of the infection droplets, and the impact that they have on nodulation has 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 therefore impact on nodulation (Muñoz *et al.*, 2012, 2014; Robert *et al.*, 2014).

In the present work, we stand out the importance of the very early host-responses on the nodulation process in the soybean-*B. japonicum* interaction, highlighting the role of PI3K in the strict modulation of ROS in root hair required for the recognition of the microsymbiont by the plant. Moreover, under saline stress conditions, the very early recognition of the symbiont is affected and leads to root hair death, possibly triggering an immunity-like response that acts as a mechanism for assessing nodulation capacities 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 meanwhile pTdt-DC-RNAi has the red tomato fluorescent protein (Tdt, tandem dimer Tomato). The resulting constructs, PI3Ki-GFP and PI3Ki-Tdt, drives 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

A. rhizogenes strain K599 was used to infect cotyledon axes regions. *A. rhizogenes* K599 with PI3Ki-GFP, PI3Ki-Tdt, GUSi-GFP and pTdt-DC-RNAi constructors was grown in Luria-Bertani (LB) medium containing kanamycin (Km) at 50 µg mL^{-1} or Spectinomycin (Spc) 100 µg mL^{-1}. To get fresh cells, *A. rhizogenes* K599 was grown on LB plates containing Km or Spc and incubated 48 h at 28 ºC. 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.

A. 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 nutrient solution (B&D) solution (Broughton and Dilworth, 1971) and it was within a larger tube that function as moist chamber. Typically, soybean plants infected by *A. rhizogenes* started to show tumors approximately 5 days 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 *wildtype* 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 ºC during the first and second 24 h periods, respectively, to promote the growth of roots and root hair as it was previously reported (Muñoz *et al*., 2012). *Bradyrhizobium japonicum* USDA 138 was cultured in yeast extract mannitol (YEM) medium (Vincent, 1970) at 28 ºC with constant agitation for 5 days ($3x10^9$ 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 twelve days 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 two days, the nutrient solution was replaced by B&D with 2 mM KNO_3 and the inoculation with *B. japonicum* USDA138 was performed. The nodule number was assessed around 21 days post-inoculation.

RNA extraction and RT-qPCR

Samples were homogenized in a cold mortar with TRIzol Reagent (1:10 µg plant tissue: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 4ºC 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 4ºC for 15 min. The precipitate was washed with 70% ethanol and the samples were centrifuged again at 12,000 g, 4ºC for 15 min. The precipitate was dried and resuspended in DEPC 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.

RNA DNA-free (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 BioRad. 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 the reverse primer 5'-CCAACGATGGATAGTCTCAACC-3'. qPCR was performed in thermocycler iQ5 (BioRad) at 59ºC with iQ SYBR Green Supermix (BioRad), according to the manufacturer's instruction. Relative transcript abundance was calculated (Livak and Schmittgen, 2001).

Live imaging, treatment conditions and redox parameters

Intracellular ROS generation in hairy roots and colocalization analysis between the lipophilic dye FM4-64 for endosomes visualization, and the general ROS indicator chloromethyl dichlorofluorescin diacetate (CMH2DCFDA), were analyzed with an inverted confocal microscopy, Nikon Eclipse CZ1. The intracellular ROS generation in root hairs was observed with an Axiophot Zeiss 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 NADPH oxidase complex during the very early host-responses, two-day old soybean seedlings were pretreated 30 min with 30 µM LY294002 or 50 µM diphenyl iodonium (DPI), respectively, and DMSO as a control; then soybean seedlings were transferred to a clean tube with *B. japonicum* USDA138, and ROS production was evaluated as follow:

Intracellular reactive oxygen species (ROS): 10 µM chloromethyl dichlorofluorescin diacetate (CMH_2DCFDA) was added in the last 15 min of the pretreatments. Then, soybean seedlings were inoculated with *B. japonicum* USDA138 and images were taken with an Axiophot Zeiss microscope after 2-5 min post-inoculation.

For intracellular ROS generation analysis in hairy roots, controls K599-empty and 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 and the Tdt-fluorescence were evaluated by confocal microscopy with a 488-nm argon laser (BP 495-530 nm) and 543-nm (LP 560 nm), respectively.

Apoplastic superoxide radical: Superoxide levels in roots of two-day old soybean seedlings were determined with nitroblue tetrazolium (NBT), which reacts with superoxide radicals to produce a blue formazan precipitate, as previously done (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 presence of 0.01% (w/v) NBT in the dark for 30 min. The reaction was stopped with absolute ethanol.

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

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

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

Root hair death-inducing conditions

Two-day old soybean seedlings were treated 30 min with sterile water (control), *B. japonicum* (inoculated), 50 mM NaCl, *B. japonicum* in presence of 50 mM NaCl and 150 mM NaCl, according to previous reports (Muñoz *et al*., 2012; Muñoz *et al*., 2014; Robert *et al*., 2014). Root hair from roots subjected to different stress treatments were extracted by peeling the root zone containing young root hair, 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 root and pulling the epidermal

tissue containing the root hair using a fine-tipped clamp. Root hair of approximately 100 roots generate sufficient material for a sample.

Cell death evaluation was performed by Evans Blue staining as previously done (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 the membrane potential, the dye diffuses within the cell and it may visualized by conventional microscopy. The roots were incubated 10 min with Evans Blue 0.05% (w/v) in water or each NaCl levels assayed.

Statistical analyses

Data were analyzed by analysis of variance (ANOVA) followed by the DGC test model, using the InfoStat software (Di Rienzo et al., 2012).

RESULTS

PI3K gene-silencing in soybean composite plants. Effects on hairy root development and nodulation

G. max genome encodes for 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 (Fig. S1, 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 on legume-rhizobia symbiotic interaction, a highly conserved fragment of the coding region of both soybean *PI3K* genes was amplified to generate the constructs for the *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 and 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 protein, respectively (Fig. S2, upper panel), and presented very similar effects on the total *PI3K* transcripts levels in PI3Ki-hairy roots (Fig. 1A, 1B and 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 primers 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, high signal roots (+) and roots with poor or without fluorescence (-) (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 (Fig. S2).

PI3K gene-silencing soybean composite plants were inoculated with *B. japonicum* USDA138 and grown for 24 days, and then nodule formation was analyzed. Downregulation of *PI3K* affected the growth of hairy roots (Fig. S3) and root hair (Fig. 2A), and nodule formation (Fig. 2B, 2C, and Fig. S3). The transgenic hairy roots obtained by *Agrobacterium rhizogenes* infection have the particularity 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, Fig. S2 and Fig. S3). Given this result, we focused on analyzing hairy roots that showed high reporter-fluorescence. PI3Ki composite plants had drastically affected the nodulation capacity, showing a nodule number reduction about 80 % respect the controls wild type- and empty vector-composite plants (Fig. 2C). In this regard, it could be observed that meanwhile control pTdt-DC-RNAi composite plants presented nodules in fluorescent hairy roots, the nodulation in PI3Ki-Tdt composite plants were mainly located in 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 called the very early stages during symbiotic interaction, 2 to 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 Luis *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 compare to control hairy roots at the zone of root hair differentiation (Fig. 3A-D) and increased content of malondialdehyde (MDA) (Fig. 3E), which is an intermediary metabolite of lipid peroxidation used as oxidative stress marker. Due to this effect of the *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 very early stage of the symbiotic interaction and its impact on nodule formation capacity.

Initially, we performed 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 longer than 1 h induced root hair death assessed by blue evans staining (data not shown). Therefore, soybean seedlings were pretreated 30 min with 30 µM LY294002 or DMSO as a control; then roots were transferred to a clean tube and inoculated with *B. japonicum* USDA138 for 30 min the in 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 it 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), the intracellular ROS generation in root hair was markedly induced after 2-5 min post inoculation (Fig. 5). This response was strongly inhibited in root hair 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). Thus, the ROS generation provided by the apical region and the perinuclear area were 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 Luis *et al.*, 2008; Montiel *et al.*, 2012; Muñoz *et al.*, 2012). In order to investigate the participation of NADPH oxidase complex during the very early responses to the symbiont, pretreatments with 50 µM diphenyl iodonium (DPI), a well-known inhibitor of flavoprotein enzymes that is used extensively as an NAD(P)H oxidase inhibitor, were performed. The intracellular ROS production was evaluated in root hair 2-5 min post inoculation (Fig. 5B). In accordance with previous reports in *P. vulgaris* (Cárdenas Luis *et al.*, 2008), our results indicate the participation of the NADPH oxidase complex in the induction of intracellular ROS generation in soybean-root hair in response to the symbiont. Moreover, NADPH oxidase inhibition showed a very similar pattern of response compared 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 analyzing the 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, even 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 different responses that induces changes in young epidermal cells and, simultaneously, triggers molecular events that will lead to nodule organogenesis. Interestingly, these responses can occur independently one from the other (Oldroyd and Downie, 2008). The effects of the very early responses over the later stages were evaluated (Fig. S4). PI3K- and NADPH oxidase-inhibition pretreatments were performed for 30 min, and then roots were inoculated 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 previous to the inoculation in order to avoid any possible effect on the rhizobium (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 days, nodule number and its fresh weight were analyzed (Fig. 7). The inhibition of the very early responses, both by LY294002 or DPI, reduced the number of nodules 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 it 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 (if it were) a pathogen (Muñoz *et al.*, 2012, 2014). Therefore, we analyzed 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 hair under root hair death-inducing stress conditions: *B. japonicum* inoculation in 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 respect control root segments (Fig. 8A). On the other hand, the expression of *PI3K* in root hair showed differences respect to what was observed in root segments. *PI3K* expression levels under conditions of 50 mM NaCl 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 segment, the expression of *PI3K* showed a significant increase in root hair (Fig. 8B). Moreover, the downregulation of *PI3K* gene increased the susceptibility to saline stress (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 30 min with sterile water (control), *B. japonicum* (inoculated), 50 mM NaCl, and *B. japonicum* in presence of 50 mM NaCl (50 mM NaCl Inoc), and Evans Blue staining were performed in order to analyze root hair death (Fig. 9, Fig. S6) (Robert *et al*., 2014). No differences were observed between control and inoculated treatments in any of the genotypes (Fig. S6). As we have previously shown (Muñoz *et al*., 2012; Robert *et al*., 2014), the inoculation with *B. japonicum* under saline stress induced cell death compared to the 50 mM NaCl treatment in 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 presence of 50 mM NaCl (Fig. 9).

DISCUSSION

To make root hair curling happen, all the machinery involved in the root hair growth must function properly and in association with the responses generated by the perception of Nod factors. Phosphoinositide lipids and their metabolism enzymes are downstream elements of the Nod factor transduction pathway, although the biological role of these modifications is less understood. As shown previously and herein PI3K, and also phosphatidic acid (PA) production by activation of the phospholipase C (PLC), diacylglycerol kinase (DGK) and phospholipase D pathways, participate at the very early responses 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). (Gage, 2004; Peleg-Grossman *et al.*, 2007; Oldroyd and Downie, 2008; Blanco *et al.*, 2009; Muñoz *et al.*, 2012; Estrada-Navarrete *et al.*, 2016, incluir den Hartog). 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 soybean-*B. japonicum* interaction modulating the redox host-responses. In addition, and to the best of our knowledge, we showed for the first time the participation of PI3K during the very early redox-host responses, previous to the internalization of the infection droplets (Fig. 4, 5A), modulating the NADPH oxidase activity (Fig. 4 and 5B) (Muñoz *et al*., 2012); and the relevance of these very early responses on 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 *Phaseolus vulgaris* (Estrada-Navarrete *et al*., 2016) (Fig. 2A) as well as reduced nodule formation (Fig. 2B and 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 *Phaseolus vulgaris*-

Rhizobium tropici interaction (Estrada-Navarrete *et al.*, 2016). Furthermore, and in addition to the 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 treatments affects proteins involved in enzymatic antioxidant defense postulating a PI3K-dependent control over antioxidant enzyme machinery possibly linked to a decreased NADPH oxidase activity (Takáč *et al.*, 2013). In this regard, we have demonstrated in previous work a correlation between antioxidant enzyme activities and apoplastic superoxide level, suggesting the participation of NADPH oxidase complex in the modulation of 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) to evaluate the impact of the very early host responses on the nodulation process, a pharmacological approach using the PI3K-inhibitor LY294002 was used.

PI3K inhibition 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 *Sinorhizobium meliloti* infection (Peleg-Grossman *et al*., 2007). Here, *PI3K*-expression levels at very early stages of the symbiotic interaction in roots (Fig. 8A) and root hair (Fig. 8B) of soybean seedlings were analyzed. Interestingly, the total expression of *PI3K* was induced in roots but no differences were observed in root hair (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 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 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 endosomal level and modulated by the NADPH oxidase and PI3K activity. In this regard, it has been reported the induction of endosomal-ROS generation in response to saline stress in Arabidopsis root cells, and the participation of PI3K and 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 endosomes level (Fig. 6). In agreement with these observations, it was recently shown that clathrin- and microdomain-dependent endocytic pathways cooperatively regulate RbohD dynamics in *A. thaliana*, and that salt stress stimulates RbohD endocytosis via membrane microdomains (Hao *et al.*, 2014). Within 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, being endocytosis 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 despite the pretreatment with LY294002 (Fig. 6). This result is in agreement with previous reports that postulate that LY29400 not inhibit the early stages of endocytosis (Lee *et al*., 2008*a;* Takáč *et al.*, 2013), and suggests that PI3K-inhibition effect on the endosomal ROS generation could be more related to the modulation of the NADPH oxidase activity.

On the other hand, it has been suggested that the generation of apoplastic superoxide radical by NADPH oxidase activity maintains the curvature during the root hair curling (Muñoz *et al*., 2012). Our results showed that PI3K activity is also involved in the apoplastic ROS induction during 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 works, our group has reported the changes that take place in the very early redox-host responses concomitantly with the induction of an ordered-like 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 PR proteins, and root hair death (Muñoz *et al.*, 2012, 2014; Robert *et al.*, 2014), which is reminiscent to the response observed in root hair of *Phaseolus vulgaris* L. elicited with the fungal elicitor chitosan (Cárdenas *et al*. 2008; Muñoz *et al*. 2012). The increased expression of PI3K observed in root hair (Fig. 8B), but not in roots (Fig. 8A), under this combination of stimuli could be underling the increased and sustained intracellular ROS production above mentioned 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 (Fig. 4 and 5A), and ii) the increased *PI3K*-expression in root hair under 50 mM NaCl inoculated conditions (Fig. 8B), we hypothesized that inhibiting the very early symbiont perception by impairing the PI3K activity it will be able to 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 (Fig. S4).

Therefore, the induction of cell death in non-nodulated *nfr5* mutant-soybean plants were 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 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-responses as a 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.

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FIGURE LEGENDS

Figure 1: Post-transcriptional gene-silencing of *PI3K* genes in soybean hairy roots (PI3Ki-hairy roots). (A) Average of the total *PI3K* transcripts levels in PI3Ki-GFP and PI3Ki-Tdt hairy roots. (B) Variation in the levels of the total *PI3K* transcripts among PI3Ki-hairy roots. *PI3K* expression levels in PI3Ki-hairy roots were analyzed by RT-qPCR and expressed relative to control K599-empty hairy roots (value 1). Data are means ± SE of ten hairy roots. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 2: Effects of the *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. (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); meanwhile pTdt-DC-RNAi composite plants presented nodules in fluorescent hairy roots (arrows in left panel). (C) Nodule number in hairy roots of control composite plants (K599-empty and pTdt-DC-RNAi) and PI3Ki-silencing composite plants (PI3Ki-Tdt). Data are means ± SE of at least ten hairy roots. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 3: Effects of the *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 images A to C: bright field, fluorescence signal of CMH_2DCFDA, and fluorescence signal of the Tdt-reporter. Images were taken with a Nikon confocal microscope. (D) The fluorescence of CMH_2DCFDA and Tdt-reporter were measured and transformed into optical density (O.D.) 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 significant differences ($p<0.05$, DGC test).

Figure 4: Participation of PI3K at the very early redox-host responses: apoplastic ROS generation. (A) Apoplastic superoxide radical production at the zone of root hair differentiation (boxes). Two-day old soybean seedlings were pretreated 30 min with 30 µM LY294002 or DMSO as a control, and then inoculated with *B. japonicum* USDA138 for 30 min in 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 (O.D.) by the image processing software Optimas®. Results are the means of four independent experiments (three roots per treatment). Data are means ± SE. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 5: Participation of PI3K and NADPH oxidase complex at the very early redox-host responses: intracellular ROS generation. Two-day old soybean seedlings were pretreated 30 min with DMSO as a control or (A) 30 µM LY294002 or (B) 50 µM DPI, and then inoculated with *B. japonicum* USDA138 for 2 min. CMH2DCFDA was added to the pretreatment after 15 min. Upper panels showing intracellular ROS generation in root hair by using an Axiophot microscope (Zeiss, Germany) with excitation filter BP 450–490 and emission filter LP 520. Lower panels showing the

measurement of CMH_2DCFDA fluorescence transformed into optical density (O.D.) by the image processing software Optimas®. Fluorescence in root hair was separately quantified in apical and perinuclear regions. Results are the means of three independent experiments (two roots per treatment); approximately 20 root hair were quantified per treatment. Data are means ± SE. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 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 30 min with DMSO as a control (A and B) or 30 µM LY294002 (C), and then inoculated with *B. japonicum* USDA138 for 2 min (B and C). Intracellular ROS production and endosomes were analyzed by CMH_2DCFA and FM4-64 staining, respectively (bottom panels in A, B and C). Merged images of simultaneous emission of green and red filters were acquired by confocal microscopy. Inserted graphs in A, B, and C indicate relative fluorescence (gray value, y axis) of each filter along the transversal line of the root hair (x axis) quantified using ImageJ (upper panels in A, B and C).

Figure 7: Participation of the very early redox host responses on nodule formation. (A) Nodule number and (B) nodule weight were evaluated after 24 days 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 30 min with *B. japonicum* USDA138. Results are the means of at least 7 plants. Data are means ± SE. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 8: Total *PI3K* transcripts levels in two-day old soybean seedlings subjected 30 min to controls and different degrees of salt stress. (A) Total transcripts levels of *PI3K* in root segments and (B) in root hair after 30 min of incubation under control condition, inoculated with *B. japonicum* (inoculated), 50 mM NaCl, inoculated with *B. japonicum* in presence of 50 mM NaCl (50 mM NaCl Inoc), and 150 mM NaCl. The levels of *PI3K* expression are relative to control conditions (value 1). Data are means ± SE. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 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 30 min to 50 mM NaCl (as a control), and inoculated with *B. japonicum* in presence of 50 mM NaCl (50 mM NaCl Inoc). (B) Evans Blue stain at the zone of root hair differentiation (boxes in A) was measured and transformed into optical density (O.D.) by the image processing software Optimas®. Results are the means of three independent experiments (four roots per treatment). Data are means ± SE. Asterisks indicate significant differences ($p<0.05$, DGC test).

Figure 1.

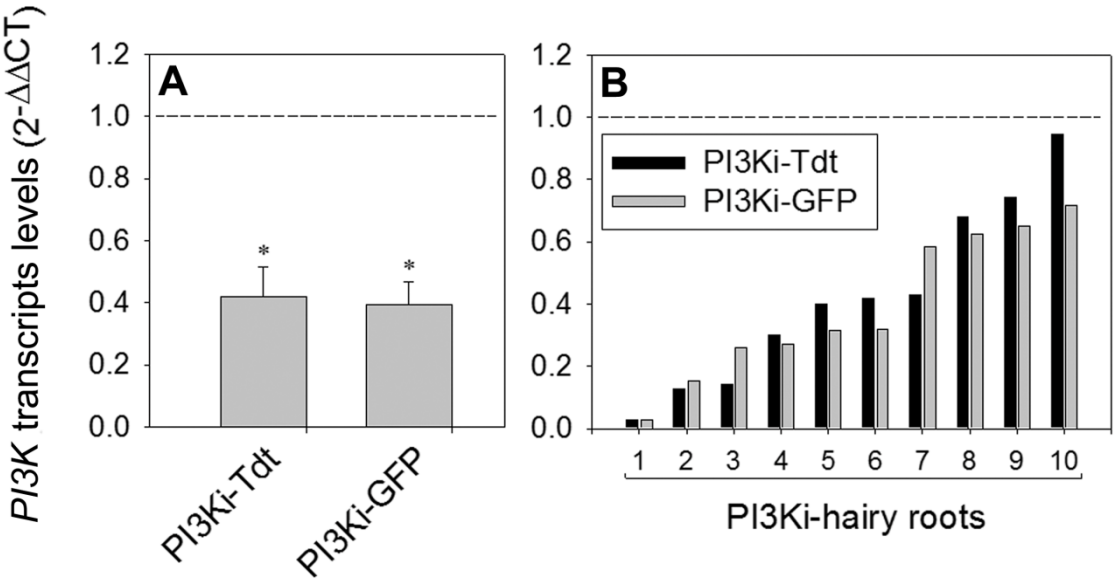
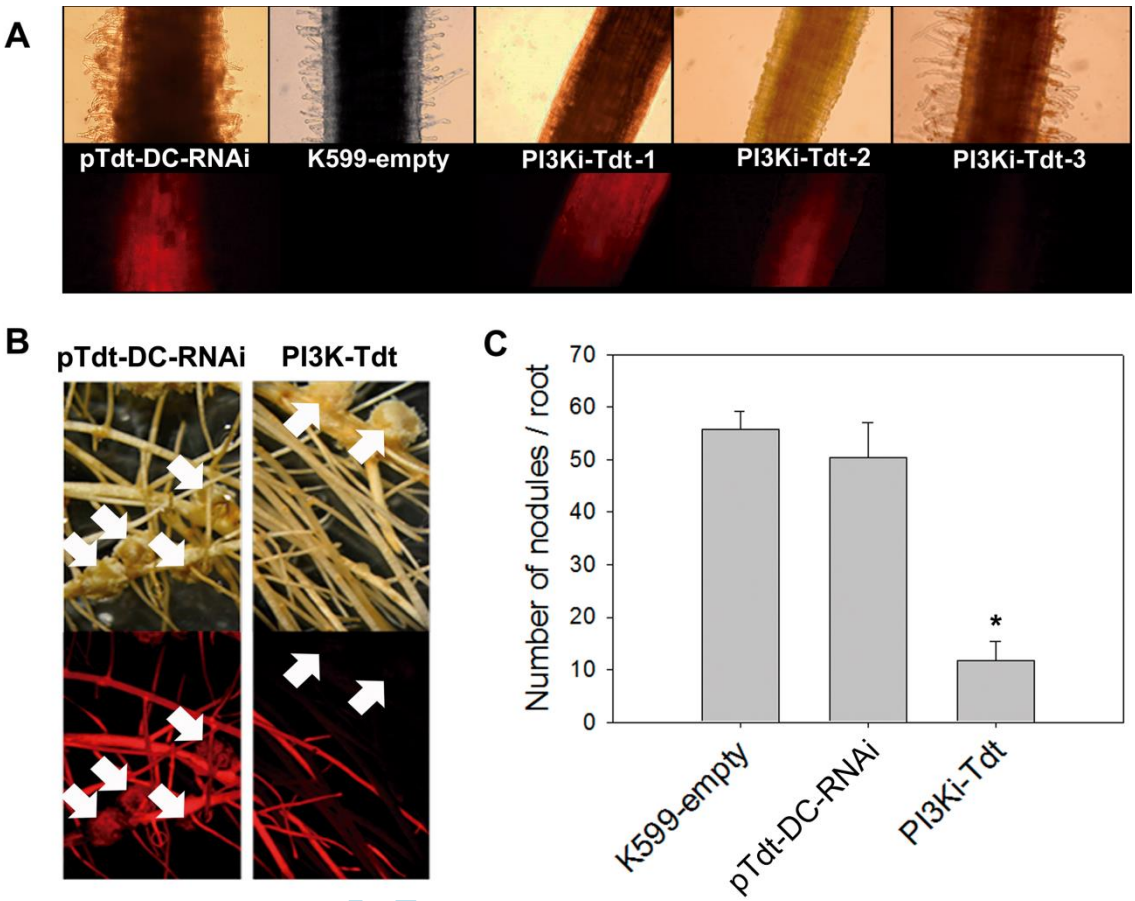


Figure 2.



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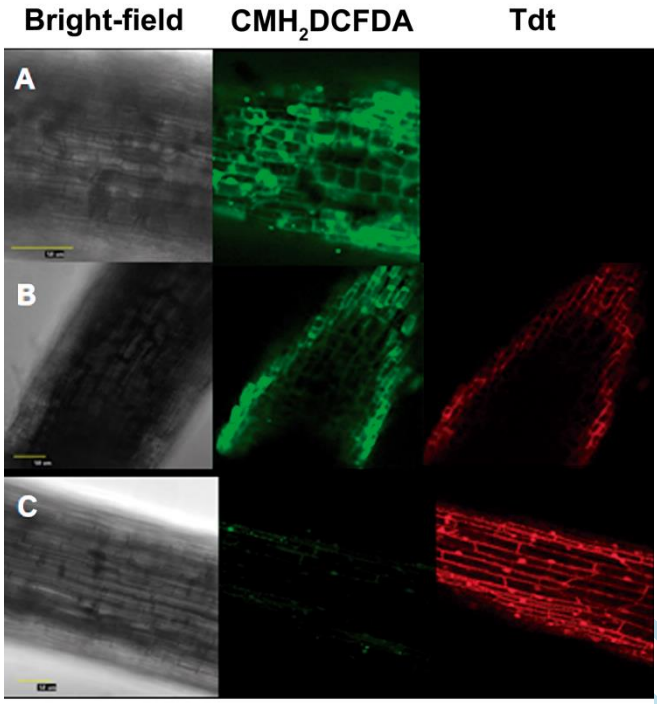
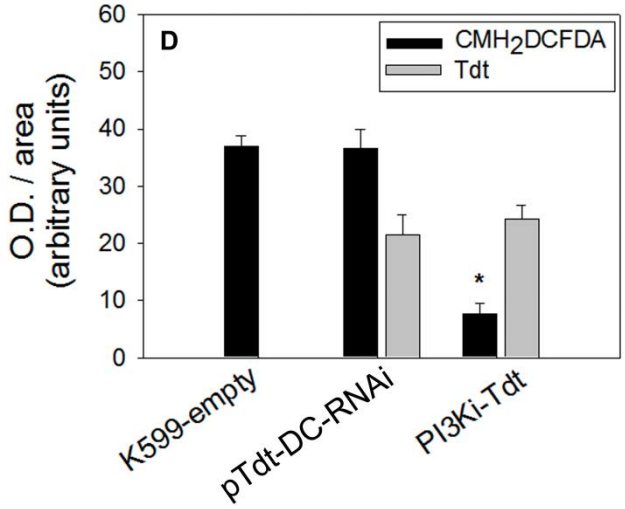




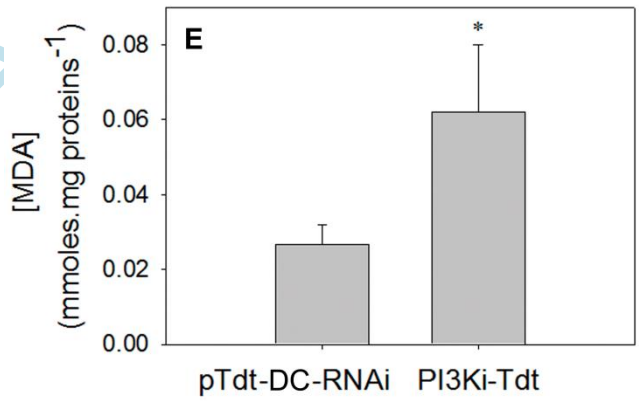


Figure 4.

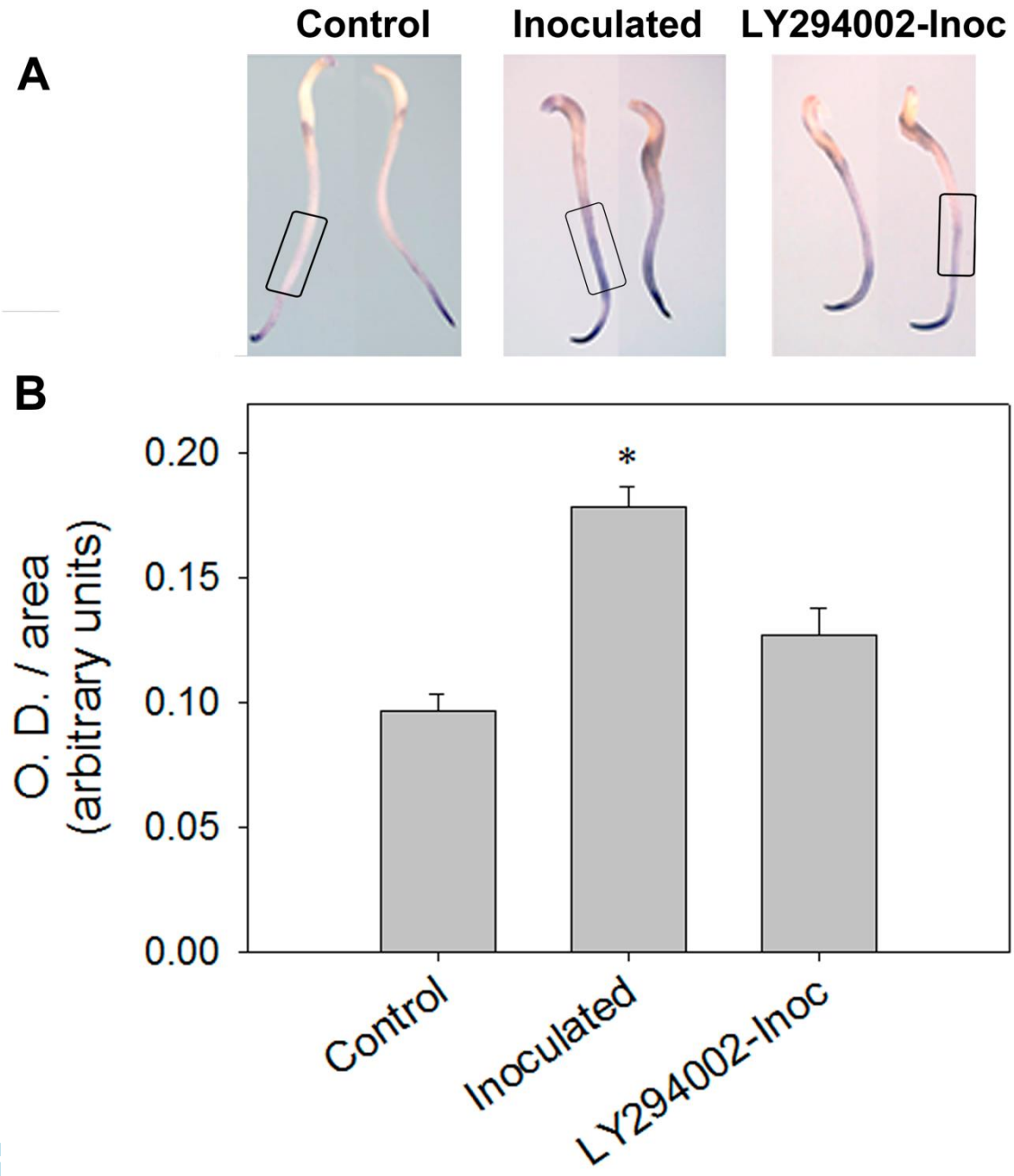
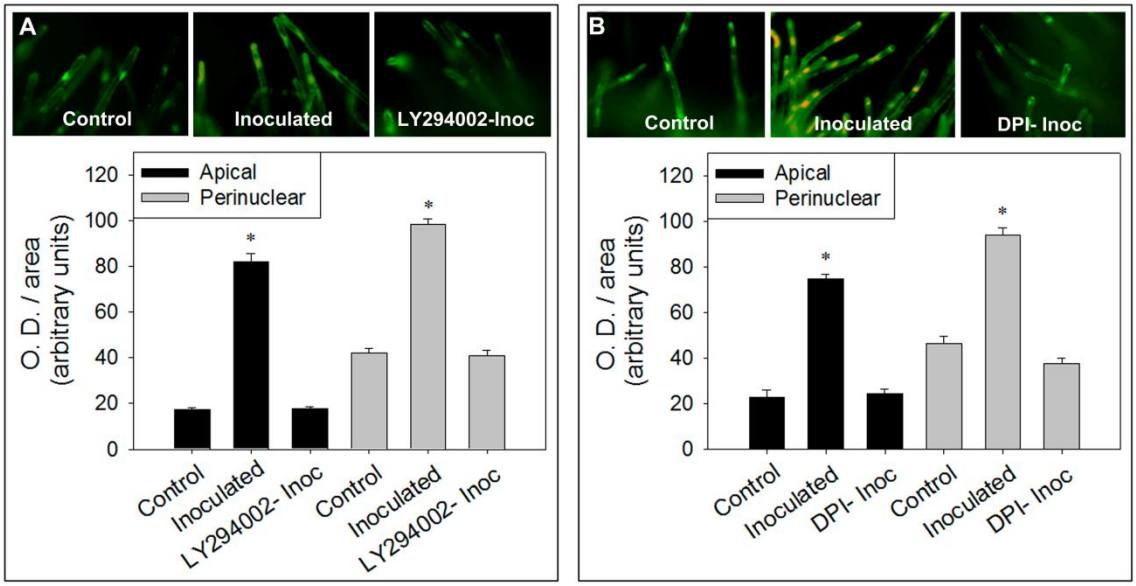


Figure 5.



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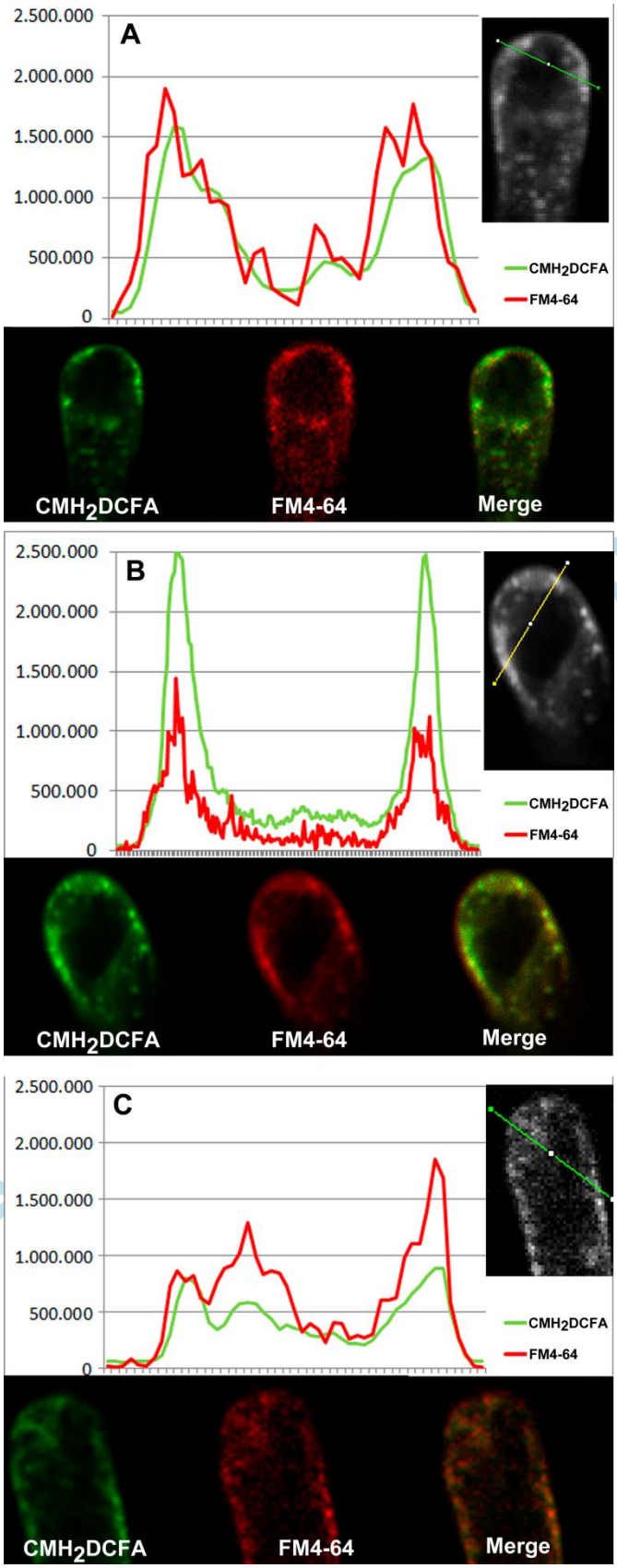

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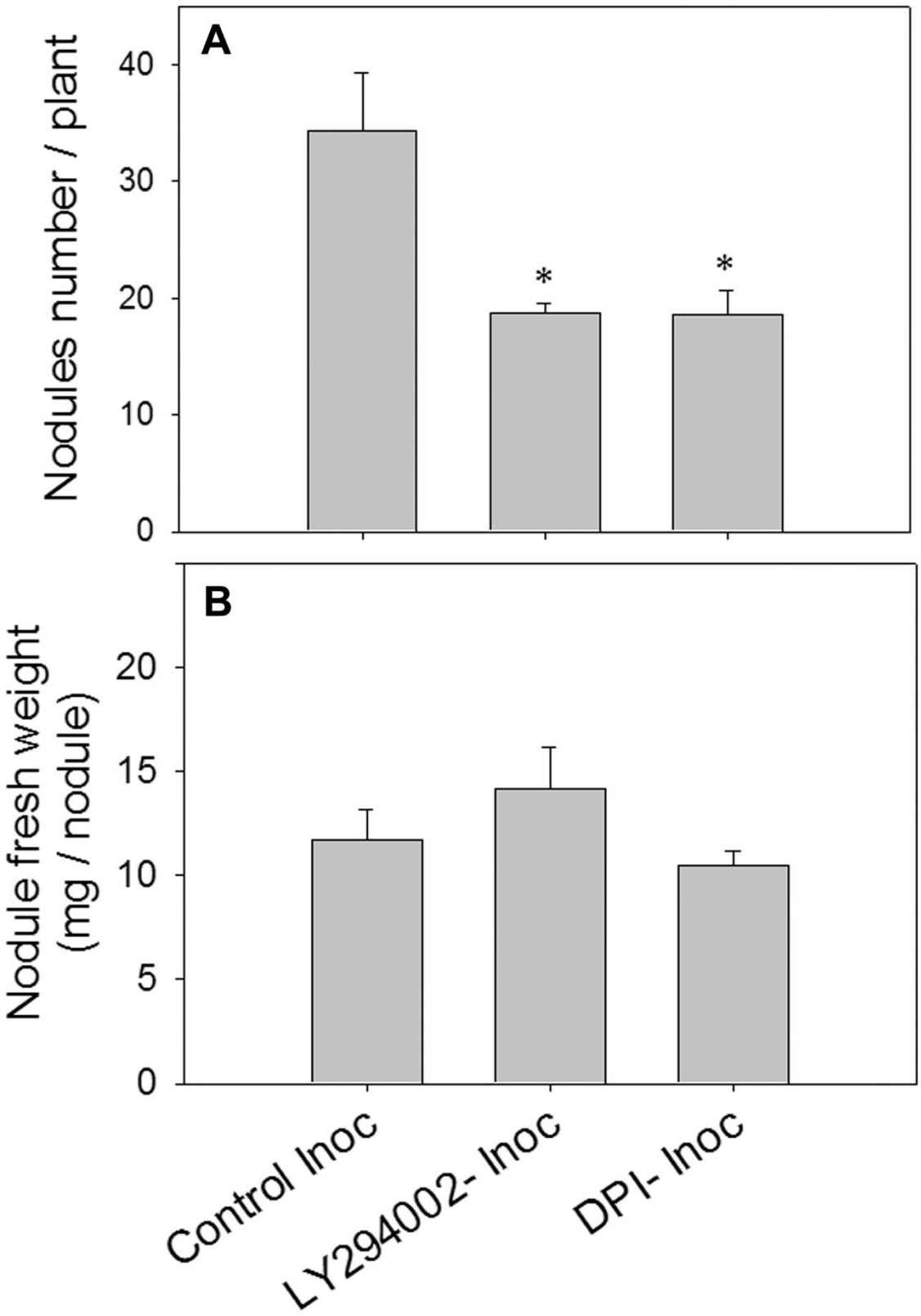

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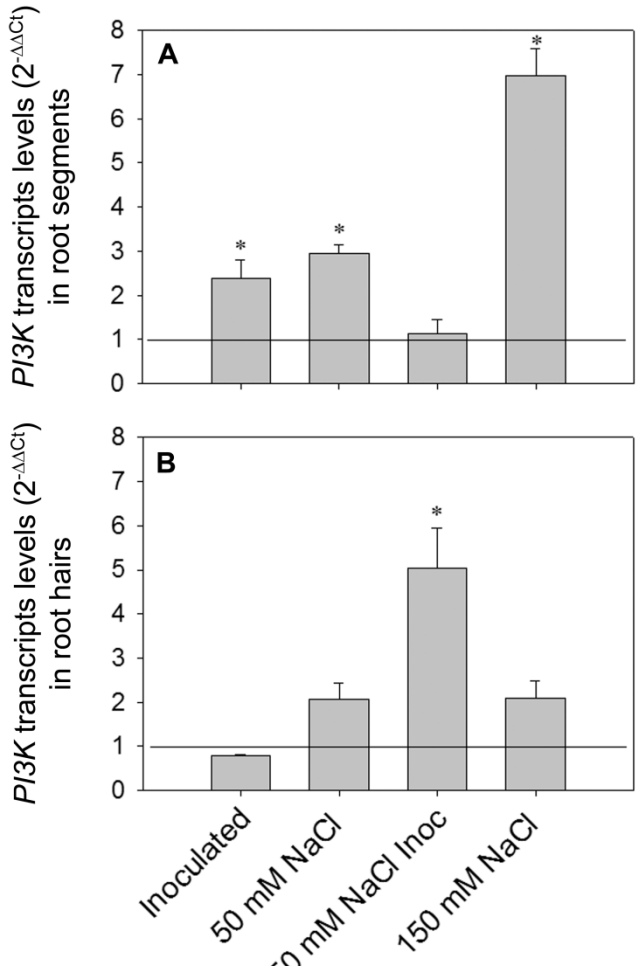

Figure 9.