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NIN-Like Proteins; interesting Players in Rhizobia-Induced Nitrate Signaling Response during Interaction with Non-Legume Host Arabidopsis thaliana

Casandra Hernández-Reyes[1,3], Elisabeth Lichtenberg[1], Jean Keller[2], Pierre-Marc Delaux[2], and Thomas Ott[1,3], Sebastian T. Schenk[1]*

[1]Cell Biology, Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany

[2]Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, Centre National de la Recherche Scientifique, Université Paul Sabatier, INP Toulouse, 31326 Castanet Tolosan, France

[3]CIBSS – Centre of Integrative Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany

*Corresponding author: Sebastian T. Schenk,

Email: Sebastian.Schenk@biologie.uni-freiburg.de

Phone: +49 761 203 2855

Keywords: NIN-like Proteins (NLPs), NLP-pathway, nitrate-related signaling, Non-legume host Arabidopsis, rhizobia-interaction

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Abstract

Nitrogen (N) is an essential macronutrient and a key cellular messenger. Plants have evolved refined molecular systems to sense the cellular nitrogen status. This is exemplified by the root nodule symbiosis between legumes and symbiotic rhizobia, where nitrate availability inhibits this mutualistic interaction. Additionally, nitrate also functions as a metabolic messenger, resulting in nitrate signaling cascades which intensively cross-talk with other physiological pathways. (NODULE INCEPTION)-LIKE PROTEINS (NLPs) are key players in nitrate signaling and regulate nitrate-dependent transcription during legume-rhizobia interactions. Nevertheless, the coordinated interplay between nitrate signaling pathways and rhizobacteria-induced responses remains to be elucidated. In our study, we investigated rhizobia-induced changes in the root system architecture of the non-legume host Arabidopsis under different nitrate conditions. We demonstrate that rhizobium-induced lateral root growth and increased root hair length and density are regulated by a nitrate-related signaling pathway. Key players in this process are AtNLP4 and AtNLP5, since the corresponding mutants failed to respond to rhizobia. At the cellular level, *AtNLP4* and *AtNLP5* control a rhizobia-induced decrease in cell elongation rates, while additional cell divisions occurred independently of *AtNLP4*. In summary, our data suggest that root morphological responses to rhizobia are coordinated by a newly considered nitrate-related NLP-pathway that is evolutionary linked to regulatory circuits described in legumes.

Keywords: NIN-like Proteins (NLPs), NLP-pathway, nitrate-related signaling, Non-legume host Arabidopsis, rhizobia-interaction

Introduction

Nitrogen, as a plant macronutrient and limiting factor for plant growth, is highly demanded in agriculture. Thus, organic and synthetic nitrogen fertilizers are essential for productive farming (Vitousek et al., 2009). The advancement of fertilizer usage and the need for sustainable solutions drives research in two crucial perspectives (i) the molecular investigation of nitrate sensing/signaling of plants, to improve and understand nitrogen use -efficiency and -processing and (ii) the study of rhizospheric bacteria, that are important for the mineralization of nitrogen in the soil, but could also associate with plants and influence plant nitrogen consumption and uptake. In this context, nitrogen-fixing (diazotrophic) bacteria play a key role in making dinitrogen gas available for plants in the rhizosphere (Carvalho et al., 2014; Rosenblueth et al., 2018). Besides the endosymbiotic nitrogen fixation with rhizobia species and their specialized legume host, there are several free-living, associated, and/or endophytic rhizobacteria that interact with non-legume plant hosts and promote plant growth (Hardoim et al., 2008; Van Deynze et al., 2018). These mutualistic bacterial interactions facilitate biological nitrogen fixation (BNF), and further promote direct N uptake (Carvalho et al., 2014). Within the group of soil-borne beneficial bacteria, several isolates of bacteria are collectively called Plant-Growth-Promoting Rhizobacteria (PGPR). Over the last decade a great number of PGPRs have been identified and studied in detail not only for their potential to promote plant growth, but also for their induction of biotic and abiotic stress tolerance (Vacheron et al., 2013; Gopalakrishnan et al., 2015; Verbon and Liberman, 2016; Etesami and Maheshwari, 2018). The mode of action of these PGPRs are mechanistically diverse and far from being completely understood. However, plant growth hormone-signaling, and here specifically auxin and ethylene, are likely to be involved in driving rhizobacterial effects on plant physiology. Accordingly, plant hormones modulate root developmental changes, such as bacteria-induced inhibition of primary root length, positive effects on lateral root formation and root hair development, as well as inhibition of cell division and cell elongation within the root apical meristem and differentiation zone (Zamioudis et al., 2013; Wintermans et al., 2016; de Zelicourt

et al., 2018). These effects are often achieved by the secretion of plant hormone analogues directly produced by the rhizobacteria or by influencing endogenously plant growth hormone balance and signaling, causing plant developmental effects, as summarized previously (Tsukanova et al., 2017). Moreover, these processes are often co-regulated by nutritional availability and nutritional signaling. For example, the PGPR *Enterobacter radicincitans* induced growth promotion and colonization increased under high-nitrogen conditions in tomato. Reciprocally, reduced N fertilization led to altered plant hormone signaling and consequently to an inhibition of these PGPR-effects (Berger et al., 2013). In addition, inoculation of *Arabidopsis thaliana* (Arabidopsis) with a mixture of PGPR *Bacillus spp.* induced plant growth promotion effects by enhancing N uptake and chlorophyll content, which in turn influences nutritional signaling by a bacterial-induced transcriptional induction of nitrate and ammonium transporter genes (Calvo et al., 2019).

Over the last decade key players regulating N- homeostasis and signaling have been identified (Wang et al., 2018; Fredes et al., 2019). These include low- and high- affinity transporter protein families in roots such as the NITRATE-TRANSPORTER 1/PEPTIDE-TRANSPORTER FAMILY (NPF, also known as CHL1 and NRT1) and the NITRATE-TRANSPORTER 2 family (NRT2) (Krapp et al., 2014; O'Brien et al., 2016). Members of both protein families are transcriptionally regulated and functionally dependent on the plants' nutritional N-status. For example, the high-affinity-transporting-systems *NRT2.1* and *NRT2.2* are induced after nitrate uptake (Cerezo et al., 2001). Examples for root-expressed high nitrate transporters are NRT1.1 (NPF6.3) (dual-affinity transporter) and NRT1.2 (NPF4.6) (Wang et al., 2018). Interestingly, it has been postulated that NRT1.1 is also able to transport auxin and consequently regulates lateral root growth in Arabidopsis (Krouk et al., 2010; Bouguyon et al., 2016; Maghiaoui et al., 2020). Its auxin transporting activity depends on the availability of exogenous nitrate in a concentration-dependent manner. In nitrate-rich patches, auxin accumulates in lateral roots and induces root emergence and elongation, whereas auxin is depleted in sites with low nitrate availability resulting in reduced

overall root growth (Remans et al., 2006; Mounier et al., 2014). Notably, root system architecture, and here primarily lateral- and primary- root length, are stimulated during moderate but homogenous nitrate availability, but inhibited under excess conditions (Jia and Wiren, 2020). This indicates that different signaling cascades are activated in fluctuating nitrate conditions resulting in adaptive physiological and morphological processes.

The most studied nitrogen-signaling cascade is the so called Primary Nitrate Response (PNR), which describes the response to nitrate resupply after depletion (Medici and Krouk, 2014). This signaling pathway is rapidly activated upon nitrate perception and results in transcriptional changes within minutes. The PNR is directly regulated by transcription factors like the RWP-RK transcription factor NIN-like Protein7 (NLP7) (Bellegarde et al., 2017). The Arabidopsis genome contains nine NIN-like Proteins (NLPs), with all of them comprising the RWP-RK motif, enabling DNA binding, and an additional PB- domain (Protein Binding) (Chardin et al., 2014). Transcriptional activation of nitrate-related genes and binding capacity of RWP-RK motif to Nitrate-Responsive cis-Elements (NRE) have been shown for several NLPs of Arabidopsis (Konishi and Yanagisawa, 2013). In addition, NLPs comprise a highly conserved nitrate-responsive domain (NRD), which determines nitrate perception and signal transmission (Mu and Luo, 2019). In Arabidopsis, an NLP function is described for AtNLP8 and AtNLP6/AtNLP7 complex. AtNLP8 is a designated master regulator in nitrate-induced seed germination mediating catabolism of abscisic acid (Yan et al., 2016), while AtNLP7 functions as a key regulator in PNR (Castaings et al., 2009). Recently, AtNLP2 is postulated to have a redundant role in nitrate signaling and vegetative growth of Arabidopsis (Konishi et al., 2021). Interestingly, NLP6 and NLP7 form heterodimers via the PB domain and are suggested to interact in complex with the transcription factor TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1-20 (TCP20) in the nucleus and regulate meristem growth under nitrate starvation condition (Guan et al., 2017). Also, other NLPs can form heterodimers that are proposed to play a role in the complexity of nitrate-related signaling

pathways, as exemplified for AtNLP4 and AtNLP5 (Konishi and Yanagisawa, 2019; Hsin et al., 2021).

Root nodule nitrogen-fixing symbiosis is evolutionary retained in different species of the Nitrogen-Fixing- (NFN) clade (Fabales, Rosales, Cucurbitales, Fagales), where it is established between several species and bacteria collectively named rhizobia (in Fabales and the Rosales species *Parasponia andersonii*) or Frankia, which enables them to access atmospheric dinitrogen after its fixation and conversion to ammonium by the symbiont (Werner et al., 2015; Griesmann et al., 2018; van Velzen et al., 2018). In this process, one of the central transcriptional regulators is the Nodule-INception protein (NIN), which was originally identified in *Lotus japonicus* (Schauser et al., 1999). Different to other NLPs, the Nitrate-responsive domain at the N-terminal region of legume NIN protein is only partially conserved, which explain the loss of direct nitrate responsiveness of NIN and thus may represent an evolutionary adaptation of legumes to maintain this mutualistic interaction (Suzuki et al., 2013; Mu and Luo, 2019). While the binding capacities of NIN and NLPs to NRE sequences are similar (Suzuki et al., 2013), it should be noted that the functionality of root-nodule symbiosis is strictly regulated by the availability of nitrate. Furthermore, this mechanism is also regulated via NLP functions as exemplified by the Nitrate unResponsive SYMbiosis 1 (NRSYM1) and NRSYM2 in *Lotus japonicus* (Nishida et al., 2018; Nishida et al., 2021). Recently, these two LjNLPs were shown to have distinct roles in nitrate signaling, and, moreover, influence nitrate induced inhibition of nodulation on levels of symbiotic-gene regulation (Nishida et al., 2021). The LjNLP4 (NRSYM1)- LjNIN interaction complex accumulates in the nucleus in response to high nitrate conditions and regulates transcription of the CLE-RS2 peptide, which in turn negatively controls nodule numbers. Remarkably, expression of the Arabidopsis *AtNLP6* and *AtNLP7* rescued the nodulation phenotype of the *nrsym1* mutant, suggesting a functional conservation of NLPs in legumes and non-legumes plants (Nishida et al., 2018). In *Medicago truncatula* (Medicago), MtNLP1 was reported to mediate nitrate signaling and to re-localize from the cytosol to the nucleus under high nitrate conditions. Subsequently, complex formation between MtNLP

and MtNIN proteins in the nucleus suppresses the activation of the root-nodule symbiosis marker genes *CRE1* and *NF-YA* and thus induces the nitrate-dependent inhibition of nodulation (Lin et al., 2018).

Analyses of different natural soil samples indicates that the Arabidopsis rhizosphere also contains about 4% bacteria from the Rhizobiales order (Garrido-Oter et al., 2018) and effects of rhizobia on Arabidopsis root development have already been described. For example, the nodulating *Rhizobium sp. IRBG74* inhibits root development of Arabidopsis by impacting auxin signaling via a change in transcriptional activation of auxin responsive genes (Zhao et al., 2017). Similarly, *Mesorhizobium loti (M. loti),* the natural symbiont of *L. japonicus*, inhibits Arabidopsis primary root growth in an auxin-dependent manner, whereas an auxin-independent increase in root hair formation was observed (Poitout et al., 2017). Furthermore, the Medicago symbiont *Sinorhizobium meliloti (S. meliloti)* induces rapid transcriptional reprogramming of defense genes in Arabidopsis cortex cells followed by late responses of stress-related genes in the pericycle, a possible stress adaptation response resulting in an increased number of lateral roots (Walker et al., 2017).

Here, we investigated alterations of the Arabidopsis root system architecture upon inoculation with the widely used and well-characterized *M. loti* and *S. meliloti* with respect to nitrate availability and nitrate-related signaling. For our investigations we selected these two model rhizobia to adhere with the bacterial point of view as close as possible to symbiotic abilities on plant infection processes. We demonstrate that rhizobial effects on the Arabidopsis root architecture are initiated by a nitrate-related signaling pathway, specifically involving NIN-like proteins. Our study highlights the importance of nutrient-dependent signaling during bacterial-plant interactions and shed further light on the complex interplay between plant endogenous signaling and bacterial connectivity.

Results

Rhizobia associate with the root surface of Arabidopsis seedlings

Following the findings that rhizobia and other diazotrophic bacteria are able to colonize the endosphere of non-legume roots by entering via natural openings such as sites of lateral root emergence (de Zelicourt et al., 2018; Schneijderberg et al., 2018), we assessed the colonization patterns of rhizobia on Arabidopsis roots. For this, Arabidopsis plants were grown vertically *in vitro* and inoculated with fluorescently labelled *M. loti* for three days. Bacterial biofilms formed along the root epidermis (Fig. 1a) with enrichments at the root cap and the root elongation zone (Fig. 1d). Similar colonization patterns of bacteria were observed on plants growing on high-and low-nitrate medium (Fig. 1b, c). Furthermore, rhizobia accumulated at sites of lateral root emergence (Fig. 1e) and invaded the space of these natural epidermal openings (Fig. 1f). However, clear signs of intercellular colonization in further tissues were not observed. Thus, rhizobia associated with the non-legume Arabidopsis by establishing an interaction on the outside of the plant root, independent of nitrate-availability conditions.

Rhizobia affect plant root development

As several soil-borne bacteria are known to alter the plant root architecture, thus enabling plants to better access nutrients directly or indirectly, we tested whether *M. loti* and *S. meliloti* -effects are influenced by the availability of exogenous nitrate. For this, we transferred 10-days old Arabidopsis seedlings onto plant medium with either high nitrate (5mM KNO_3) or nitrate starvation (0.1mM KNO_3) conditions and additionally inoculated these plants with either *S. meliloti* or *M. loti* for 3 days. The number and length of lateral roots significantly increased when inoculating plants grown under high nitrate conditions with rhizobia compared to the non-inoculated control (Fig. 2a-e) while the primary root length was unaffected by this treatment (Fig. 2f). Nitrate deprivation fully abolished the rhizobial effect on lateral root growth (Fig. 2d, e) and resulted in a moderate increase

in the primary root length, which was independent of the presence of bacteria (Fig. 2f). These interaction responses are in contrast to observations in legumes, where a functional root-nodule symbiosis is strictly established only in limiting nitrate conditions.

Among other cells, root hairs can serve as primary contact sites and can be altered by rhizobacteria (Poitout et al., 2017; de Zelicourt et al., 2018). Thus, we investigated root hair development along young segments of the primary root. Here, we decided to inoculate rhizobia as early as 7 days post germination, since root hairs develop faster than lateral roots. Furthermore, we included a low-nitrate condition (0.25 mM) to further assess nutrient-limiting conditions rather than nutrient stress responses. As expected, nitrate starvation led to a significant increase in root hair length (Fig. 3a-b, h) and number (Fig. 3g) even in the absence of rhizobia, as published earlier (Vatter et al., 2015). While the presence of *S. meliloti* moderately affected root hair patterns (Fig. 3c-d, g-h), a significant induction of root hair growth was observed upon inoculation with *M. loti* under high (5 mM) and low (0.25 mM) nitrate conditions. No rhizobial effects on root hairs were found under nitrate-starvation conditions (0.1mM nitrate) in comparison to non-treated plants (Fig. 3e-h). Therefore, we continued experiments on rhizobial effects on high-(5mM) and low-(0.25mM) nitrate conditions rather than observing nutrient stress conditions at 0.1mM nitrate. To further test the impact of rhizobia on root hair growth, we examined root hair-specific marker gene expression in roots treated with rhizobia under different nitrate conditions. Indeed, rhizobia tended to induce the morphogenetic root-hair genes *RHD6-LIKE4* (*RSL4*), and *EXTENSIN11* (*EXT11*), but not *ROOT HAIR DEFECTIVE6* (*RHD6*) (Fig. 3). Overall, our results show that *M. loti* treatments were particularly pronounced under high-nitrate conditions. These data demonstrate that Arabidopsis root architecture and morphology are affected by rhizobia in a similar way as nitrate impacts root modifications.

Rhizobia influence nitrate-related gene expression

To continue analyzing rhizobia-induced root modifications and its impact on nitrate-dependent signaling responses, we investigated nitrate-related gene expression in roots treated with rhizobia. Due to the fact that nitrate conditions impact distinct set of genes, we studied *NRT* transceptors that have been reported to be up-regulated also in low-nitrate conditions and improve nitrate use efficiency (Bouguyon et al., 2016; Ye et al., 2019). Although *NRT2.1* and *NRT1.1* were induced under low-nitrate conditions, their expression was further increased in samples treated with rhizobia, for *NRT1.1* even in high-nitrate conditions (Fig 4a, b). By contrast, expression of the starvation marker gene *NRT2.4* that was reported to be transcriptionally induced in low-nitrate conditions (Kiba et al., 2012) was not modified by rhizobia (Fig. 4c).

Transcriptionally activated NIN-like proteins are known to play a role in nitrate signaling during rhizobia-legume interactions. These proteins suppress gene expression by either directly binding to NIN-proteins or by competitive binding to promotors of symbiosis-responsive genes (Lin et al., 2018; Nishida et al., 2018). Large-scale phylogenetic analysis of the NLP family revealed their conservation in land plants (Schauser et al., 2005). Furthermore, different duplication events led to three NLP-clades that evolved prior to the divergence of seed plants (Fig. S2 and S3). As NLP7 (NIN –like protein 7) was identified as key regulator during the primary nitrate response in Arabidopsis and accumulates as a heteromeric complex with NLP6 and TCP20 in the nucleus during nitrate-starvation responses (Guan, 2017), we decided to analyze transcriptional activation of NLPs in our experimental conditions (Fig. 4d-f), (Fig. S4). While NLPs are not transcriptionally up-regulated during nutritional signaling *per se* (Chardin et al., 2014), we detected a significant induction of *NLP4* and *NLP5* expression in roots inoculated with *M. loti* in high-nitrate and *S. meliloti* in low-nitrate conditions (Fig. 4e, f). However, other NLPs were not influenced by the presence of rhizobia (Fig. S4). Additionally, genes involved in nitrate metabolism such as *NIA1* and *NIA2* (nitrate reductase), and *NIR1* (nitrite reductase), which are regulated during nitrate responses, were also found to be induced upon rhizobial inoculation of plants grown under high-

nitrate, but not under low nitrate conditions (Fig. 4g-i). These results suggest that rhizobia intervene nitrate-dependent signaling by differentially regulating nitrate-related marker genes in distinct nitrate responses.

M. loti-induced root morphology changes are controlled by NLP4 and NLP5

As expression patterns of nitrate-related genes in rhizobia treated root samples indicate a distinct nitrate-dependent signaling during rhizobium-induced root modifications, we further tested the genetic dependency of our observations on root development using different nitrate signaling mutants. Here, the most obvious *M. loti* -induced phenotype, the impact on root hair development at the primary root, was selected as experimental readout. Due to the importance of transceptors (NRTs) during nitrate -transport, -sensing and -signaling in roots, we studied the impact of *M. loti* inoculation on *nrt1.1-5* (*chl1-5*) and *nrt2.1* mutant backgrounds (Fig. S5). The *nrt2.1* mutant exhibited wild-type responses towards the presence of rhizobia (Fig. S5c-d). This was different in the *nrt1-1* mutant that already showed a nitrate-independent reduced root hair formation phenotype under control conditions that was not rescued by the application of rhizobia (Fig. S5a-b). We then investigated downstream roles of the NLP master regulators during rhizobia-Arabidopsis interactions. For this, we obtained independent T-DNA insertion mutant alleles for NLP6 and NLP7 (Castaings et al., 2009) and analyzed root hair development in these mutant plants. Under control conditions (uninoculated, high-nitrate condition) *nlp6* and *nlp7* mutants already developed more root hairs compared to the wild-type, while mutants responded to the presence of rhizobia in a wild-type-like and nitrate-independent manner (Fig. 5a-b). Due to the striking importance of the Nodule-Inception Protein NIN during root nodule symbioses and in line with the *M. loti*-induced expression of *AtNLP4* and *AtNLP5* expression in Arabidopsis (Fig. 4), we investigated the NLP-clade NLP1-NLP5 (closely related to the clade containing NIN from legumes such as Medicago) (Fig. S3). Mutants of this NIN-related gene family in Arabidopsis, specifically *nlp1, nlp4* and *nlp5,* responded to nitrate limitation by increased root hair formation as observed

for the wild-type control (Fig. S6, Fig. 5c-d, Fig. S7a-b). However, and in contrast to the *nlp1* mutant, *nlp4* and *nlp5* mutants failed to induce pronounced root hair development upon application of *M. loti* (Fig. 5c-d, Fig. S7a-b). Furthermore, the rhizobial effects of induced lateral root emergence and overall lateral root length were also lost in *nlp4* and *nlp5* mutants (Fig. S7c-d). To further clarify the involvement of NLP4 and NLP5, we generated two constitutive over expression lines of genomic *NLP4* and *NLP5* into the respective mutant backgrounds. Two independent lines of genotype clearly showed rhizobia effects and were able to complement the *nlp4* and *nlp5* mutations (Fig. 5e, f, Fig. S7d). Following up on the significant phenotypes of *nlp4* and *nlp5* we further examined rhizobia-induced gene expression in these mutants (Fig. S8). Interestingly, the elevated transcription of nitrate-related genes in rhizobia treated samples as observed in wild-type plants (Fig. 4) was lost in *nlp4* and *nlp5* mutants (Fig. S8a-b). However, root hair development marker gene expression of *RSL4* and *EXT11* were still partially elevated in *nlp4* and *nlp5,* respectively (Fig. S8c-d). In summary, the root-development response to nitrate is not impaired in these mutants, while *nlp4* and *nlp5* fail to induce distinct rhizobia-induced nitrate-related signaling responses. Thus, these data suggest the presence of an NLP4/5-dependent signaling pathway in Rhizobia-Arabidopsis interactions, similar to nitrate-dependent regulation of symbioses.

M. loti alters cell proliferation and elongation patterns in the root tip

Besides nitrate being a signaling component during root development it also influences cell divisions at the root apical meristem and subsequent cell cycle progression (Guan et al., 2017; Gigli-Bisceglia et al., 2018). Similar effects were also described for treatments with PGPRs, which can influence root architecture via hormonal cross-talks (Verbon and Liberman, 2016). Since root hair development at the primary root is determined by the cell fate at the apical meristem (Vissenberg et al., 2020), we decided to test possible impacts of *M. loti* on Arabidopsis root cell patterning. For this, we transferred 7 days-old Arabidopsis seedlings on plant medium with high- or low- nitrate, inoculated them with *M. loti* for 3 days and stained root cell membranes with the

dye FM4-64 to visualize cell morphology and to quantify cell numbers (Fig. 6a-d). In wild-type Arabidopsis plants, the average length of the first five cortex cells above the root transition zone significantly decreased under high- but not under low- nitrate conditions in the presence of *M. loti* (Fig. 6e), whereas this effect was not observed in *nlp4* and *nlp5* mutant plants (Fig. 6f, g). This shorted elongation zone implies a bacteria-induced and NLP4/5-dependent early differentiation in high-nitrate conditions, and could explain the observed effects on root hair density on the primary root tip (Fig. 3h and 5c, d). To quantify the meristem size, we counted the cell number from the quiescent center until the transition zone (first doubling of cell size) in the cortex cell layer along a longitudinal axis. Cortical cell numbers were found to be increased by *M. loti* in both wild-type and *nlp4* mutant plants, while this rhizobial effect was lost in *nlp5* mutants (Fig. 6h-j). This was accompanied by increased transcription of the cell cycle-related cyclin D3 (*CYCD3;1*) (Fig. 6h), but not of *CYCB1;1* (Fig. 6i). Since *CYCD3;1* is expressed during the meiotic G1/S phase of the cell cycle, while *CYCB1;1* serves as a marker for a prolonged G2 phase, we conclude that cortical cell division rates are induced by *M. loti*. Overall, we demonstrate that rhizobium-induced signaling influences cell cycle progression and root cell elongation in a NLP5-dependent manner.

Discussion

Gene regulatory networks control plant responses to different nutrient conditions, leading to modifications of the root architecture. In natural soils, plant roots are additionally challenged by a great diversity of beneficial and pathogenic microbes and the plants' overall nutrient status determines the responsiveness to these microbes (Shahzad and Amtmann, 2017; Singh et al., 2019). Among them are rhizobia, which have been mostly investigated in frame of the root-nodule symbiosis, a process that ultimately results in the ability of these bacteria to fix atmospheric dinitrogen gas. This mutualistic interaction strictly depends on the unavailability of nitrate and is immediately terminated upon resupply of inorganic nitrate to the host plant (van Noorden et al., 2016). While the root-nodule symbioses interaction mostly relies on a highly strain-specific

interaction with a narrow host range, microbiome studies revealed the presence of rhizobia in the rhizosphere or as endophytes in roots of non-legume plants, as well as nitrate serving as a regulating factor mediating the microbial composition on Arabidopsis roots (Konishi et al., 2017; Garrido-Oter et al., 2018).

In this manuscript, we investigated rhizobium-induced root morphology changes and their dependence on nitrate availability and nitrate-related signaling cascades in Arabidopsis. We demonstrate that rhizobia affect lateral root emergence and -elongation (Fig. 2) and increase root hair density and length (Fig. 3, 5). Interestingly, these effects were found to be pronounced in high nitrate conditions and reduced or even abolished in low-nitrate conditions (Fig. 2, 3). In contrast to the inhibitory effects of nitrate on root-nodule symbiosis, our findings imply a supporting nitrate-dependent signaling and suggest an underlying complex crosstalk between nutrient-related signaling and rhizobia-induced responses on non-legume plants.

It is well documented that nitrate starvation is accompanied by stimulated lateral root emergence and primary root elongation, while excess nitrate leads to root growth inhibition (Jia and Wiren, 2020). In line with this, the presence of PGPRs on Arabidopsis roots stimulates a low-nitrate response. This can be exemplified by the PGPR *Phyllobacterium* STM196 that positively influences root architecture under high-nitrate conditions. These rhizobacteria did not directly alter the N-status in leaves or root nitrate uptake, but induced the expression of nitrate starvation marker genes such as *AtNRT2.5* and *AtNRT2.6* (Mantelin et al., 2006) that are also postulated to play a central role in bacterial-induced growth promotion (Kechid et al., 2013). This is in agreement with our results on nitrate-influenced *M. loti*-induced root hair development (Fig. 3, 5, S7). Indeed, one interesting aspect is the impact of rhizobia on nitrate starvation signaling (Fig. 4a-c), similarly to nitrate-induced inhibition circuits in legume symbiosis. However, our data indicate a rather specific rhizobium-induced high-nitrate signaling in plants, comprising *NRT1.1*, and nitrate metabolic gene regulations (Fig. 4a, g-i). Furthermore, the effectiveness of *M. loti* influenced root modifications (Fig. 3) can be explained by this specific rhizobium-induced high-nitrate

356 transcriptional response, which specifically involves differential expression of the transcription
357 factors *NLP4* and *NLP5* (Fig. 4e, f, Fig. 5e, f) and directs to a positive regulatory mechanism of an
358 NLP-dependent pathway (Fig. 7). In line with this, the corresponding *nlp4* and *nlp5* mutants failed
359 to morphologically respond to the presence of rhizobia at the primary root tip (Fig. 5, S7) and lose
360 rhizobia-induced nitrate-related gene expression (Fig. S8a, b). These results are of particular
361 interest, considering the evolutionary proximity of AtNLP4 and AtNLP5 to the legume gene NIN,
362 and their co-orthologs legume genes *MtNLP1*, *LjNLP4* and *LjNLP1* that are essential for controlling
363 nitrate-dependent root nodule symbiosis (Schauser et al., 2005; Lin et al., 2018; Nishida et al.,
364 2018; Mu and Luo, 2019; Nishida et al., 2021). Thus, the response to rhizobia could be trait shared
365 within the NLP clade (NLP1-5, angiosperms) and the Eudicots duplication leading to NLP4/5 and
366 NLP1/2/3 could have later allowed the possible neofunctionalization of NIN for root nodule
367 symbiosis (Fig. S2, S3). Additionally, we demonstrated a NLP4/NLP5-dependent and rhizobium-
368 induced stalling of cell elongation but increased cell division rates at the proximal part of the root
369 elongation zone (Fig. 6), which suggests an NLP-dependent (nitrate related) -signaling in the root
370 elongation zone and directs to an earlier differentiation of roots treated with rhizobia (Fig. 7). In
371 line with this hypothesis, cell wall integrity and meristem cell progression are postulated to depend
372 on nitrate reductase function and endogenous cytokinin homeostasis in roots tips (Gigli-Bisceglia
373 et al., 2018). In addition, morphological changes also coincided with an enhanced activation of the
374 cell-cycle marker gene *CYCB1;1* (Trinh et al., 2018). Reciprocally, the application of
375 *Pseudomonas putida WCS417* on Arabidopsis resulted in an inhibition of root growth, which was
376 likely caused by reduced cell elongation and driven by an observed activation of *CYCB1;1* in
377 inoculated samples (Zamioudis et al., 2013). While *M. loti* induced similar effects on cell
378 proliferation we observed an induced expression of *CYCD1;3* but not of *CYCB1;1* indicative for
379 an accelerated cell progression into the G1 phase (Fig. 6). Such induction of D-like cyclins (here:
380 CYCD1, D2 and D3) was also reported in rice seedlings when treated with *S. meliloti* (Wu et al.,
381 2018).

382 The importance of nitrate-regulated transcription factors during plant root development has been
383 extensively described. For example, the bZIP transcription factors TGA1 and TGA4 control nitrate-
384 induced lateral root formation (Alvarez et al., 2014) and root hair cell fate, latter by regulating the
385 root hair cell-specific gene *CAPRICE* (*CPC)* (Canales et al., 2017). Furthermore, a meta-analysis
386 indicates nitrate regulation of root hair growth by bZIP and G2- transcription factors coordinated
387 auxin signaling response (Canales et al., 2014). Interestingly, the NRT1.1-TGA1/4 nitrate
388 signaling complex additionally controls root hair density and trichoblast cell length (Canales et al.,
389 2017). Besides rhizobia-induced *NRT1.1* regulation (Fig. 4a), we also assume that NRT1.1
390 controls rhizobia-induced changes in trichoblast patterning (Fig.S5). However, mutation in the
391 *NRT1.1* locus affects already root hair development in high and low nitrate conditions (control
392 conditions) and may thus mask rhizobial responses. By contrast, *nlp4* and/or *nlp5* mutants are still
393 responsive to nitrate limitation and lose the rhizobial impact on root morphogenesis (Fig. 5 and
394 S7). Overall, these results reveal new promising NLP candidates and potential transcription factors
395 regulating rhizobia-induced nitrate-related signaling responses in Arabidopsis. Still, specific
396 functions of NLP4 and NLP5, including protein complex formation in the nucleus and the activation
397 of target genes, within the crosstalk signaling of nitrate availability adaptation processes and
398 rhizobia-induced responses need to be further investigated, however our results clearly point to a
399 so far uncovered regulatory circuit.

400 Taken together, we understand the investigated bacterial-plant interaction as a complex signaling
401 pathway between nutritional sensing followed by metabolic progressing in crosstalk with bacteria
402 interaction and stress-induced responses, which finally results in adaptive developmental
403 processes. Being fully aware of the well-documented crosstalk between nitrate signaling and
404 growth hormones (Takei et al., 2004; Vidal et al., 2010; Ma et al., 2014; Naulin et al., 2020), we
405 consider the observed rhizobia-induced nitrate responses as early signaling events after bacterial
406 perception, which finally lead to downstream fine-tuning of growth hormone effects (Fig. 7). Future

research in this line will contribute to the development of bacterial inoculums as effective and sustainable solutions in agriculture.

Materials and Methods

Plant growth conditions and root observations

Arabidopsis thaliana wild type ecotype Columbia (Col-0), Wassilewskija (WS) and knockout mutant seeds were surface sterilized with 50% ethanol/0.5% Tritron X-100 and briefly washed with 95% ethanol. Seeds for mutant plants *nlp4* (N563595), *nlp5* (N527488), and *nlp1* (N584809) were provided by *The Nottingham Arabidopsis Stock Centre* (NASC) and homozygosity for T-DNA insertion was validated by PCR with primers designed by SALK Institute Genomic Analysis Laboratory. Seeds were germinated on half-strength Murashige-Skoog- medium (1/2 MS) after stratification at 10°C for 3 days and grown horizontally for 7-days/10-days (as indicated) at 22°C with 150µmol/m^2/s light in a 12/12-h day/night photoperiod. After germination seedlings were transferred and inoculated with bacteria on *nitrate-variable plant medium* containing 5mM/0.25mM/0.1mM (as indicated) KNO_3 or 5mM KCl (in low-nitrate conditions KCl was used to buffer ion concentrations), 1mM KH_2PO_4, 0.5 mM $CaSO_4$, 0.5 mM $MgCl_2$, 3 mM MES, pH 5.8, 0.25% (w/v) sucrose and 0,1% micronutrients (Scheible et al., 2004) including 1% ferric citrate, supplemented with 0.9% Phyto-Agar and grown vertically for 3 days. Root modifications were quantified on images photographed with scale bar and analyzed on Image J Software. For root hair quantifications pictures were taken using a stereomicroscope and root hairs were measured and counted in 5 mm from the start of the differentiation zone (first root hair appearance). Data were averaged from at least 7 root samples and experiments were performed at least 3 times independently.

Bacteria growth conditions

Sinorhizobium meliloti strain Sm2011, tetracycline resistant with mCherry as fluorescent marker, and *Mesorhizobium loti* strain MAFF, gentamycin resistant with dsRED as fluorophore, were used for bacterial treatments. For each experiment bacteria were freshly plated on TY-medium and incubated for 3 days at 28°C. Afterwards the inoculum culture was grown in 5 ml liquid TY-medium for 48h. For inoculation, bacteria were pelleted, resuspended in sterile H_2O and diluted to a final OD_{600} of 0.1. Ten µl of bacteria solution per root were used to treat Arabidopsis seedling and primary root length was marked after inoculation. For reproducibility the timing of bacterial pre-culture growth is essential for an adequate-equal inoculum preparation.

Quantitative RT-PCR

All quantitative RT-PCR were performed with 7-day old seedlings grown for 3 days in high- (5mM) and low- (0.25mM) nitrate conditions. Total RNA was extracted from Arabidopsis root samples using an RNA extraction kit (Sigma Aldrich) and quality control was performed photometrically using a NanoDrop (Thermo Fisher Scientific) device and on agarose gel. To remove traces of genomic DNA, 1 µg of total RNA from each sample was treated with DNase-I and genomic-DNA contamination was excluded via quantitative RT-PCR. Reverse transcription was accomplished from purified RNA with SuperScript cDNA Synthesis Kit (Quanta BioScience). Efficiency of cDNA synthesis was verified by semi-quantitative PCR on housekeeping *actin2* transcripts. All primers used for qRT-PCR are listed in (Table S1). Quantitative RT-PCR was performed using an *Applied Biosystems* real time PCR system and a Sybr green Mix. Ct-values were normalized to the housekeeping gene *EF1α4 or UBQ4,* as indicated. Data were averaged from at least 3 independent experiments.

Characterization of mutants and over-expression lines

For genotyping of the T-DNA insertion mutants *nlp4* and *nlp5*, the recommended PCR set up from Salk Institute Genomic Analysis Laboratory (SALK) with left- and right border primers and the LBb1.3 primer was used. Genomic DNA was extracted from soil grown wild type Col-0 and mutant plants and subjected to PCR analysis. Homozygous plants were then selected for further experiments. To proof that the respective T-DNA is inserted in an exonic region of the gene of interest, semi-quantitative PCR was carried out on cDNA samples (as described in "Quantitative RT-PCR") using a primer pair covering the T-DNA flanking sequence. For transcript quantification qRT-PCR was performed on cDNA extracted from plant roots of non-treated samples (Fig. S1). All primers used for characterization are listed in (Table S1).

Cloning method and selection of transgenic lines

The cloning procedure used for constructing complementation vectors was the Golden Gate cloning method (Binder et al., 2014). Genomic DNA from Arabidopsis plants was extracted and the full-length genomic *NLP4* and *NLP5* was amplified with proofreading DNA polymerase PCR using corresponding Golden Gate primers. To keep Golden Gate compatibility silent mutations were performed on *BpiI* and *BsaI* restriction sites. All primers used for cloning are listed in Table S1. Level II constructs were assembled with vectors containing the promoter sequence of AtUBQ10, the fluorescent tag eGFP, and for transformant selection the herbicide resistance BASTA as well as a screenable marker FAST (Shimada et al., 2010). The resulting constructs were quality controlled by full-length sequencing. For plant transformation *Agrobacterium tumefaciens* (AGL1) was transformed with described construct and flower buds of soil grown 5-weeks old Arabidopsis mutant plants *nlp4* and *nlp5* mutant plants were infected with appropriate bacterial solution according the floral dip method (Clough and Bent, 1998). Infection was repeated after one week. T0 seeds were harvested and positive transformations were selected via FAST method (Shimada et al., 2010). Positive seeds were germinated on 10mM BASTA ½ MS plates

and plants were propagated until T3. For homozygosity of transformants segregation tests were proceed in T2 and resistant plant lines were selected in T3 for following experiments.

FM4-64 staining and image analyzes of root tip samples

Treated seedlings were incubated in 5 µM FM™4-64 dissolved in liquid *nitrate-variable plant medium* for 15 min. After 5 min washing in liquid plant medium primary root tips were mounted on a microscopy slide. Images of 3880x3880 resolution were taken with SP8-Leica confocal microscope using a 25x water objective and a white laser, emission of 554 to 591 nm.

Phylogenetic analysis

NLP sequences were retrieved by the tBLASTn algorithm v2.9.0+ (Camacho et al., 2009) using the NIN protein from the model species *Medicago truncatula* as query, a database containing 124 land plants genomes from the SymDB database (http://www.polebio.lrsv.ups-tlse.fr/symdb/web/) and no e-value greater than 1^{e-10}. Sequences were aligned using MAFFT v7.407 (Katoh and Standley, 2013) with default parameters. Alignments were cleaned using trimAl v1.4 rev22 (Capella-Gutierrez et al., 2009; Guindon et al., 2010) to remove positions with more than 50% of gaps. Cleaned alignments were subjected to Maximum Likelihood analysis using IQ-TREE v1.6.7 (Nguyen et al., 2015) after testing the best-fitting evolutionary model using ModelFinder (Kalyaanamoorthy et al., 2017). Branch support was tested with 10,000 SH-aLRT (Guindon et al., 2010). Trees were visualized and annotated using the iTOL platform v4.4.2 (Letunic and Bork, 2019). For readability reasons, presented phylogeny in (Fig. S3) has been reduced to 7 species with maintained topology of the 124 species- main tree (Fig. S2).

Accession numbers

Sequence data from this article can be found under the following Arabidopsis Genome Initiative accession numbers: NRT2.1 (AT1G08090), NRT1.1 (AT1G12110), NIA1 (AT1G77760), NIR1 (AT2G15620), RSL4 (AT1G27740), EXT11 (AT5G49080), RHD6 (AT1G66470), CYCD3;1 (AT4G34160), CYCB1;1 (AT4G37490), UBQ4 (AT5G25760), NLP1 (AT2G17150), NLP4 (AT1G20640), NLP5 (AT1G76350), NLP6 (AT1G64530), NLP7 (AT4G24020)

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Author Contribution

STS and TO conceived and designed the experiments; STS, CHR, EL, JK performed the experiments; STS, CHR, JK, P-MD, analyzed and interpreted the data; and STS, CHR and TO wrote the manuscript.

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Figure Legends

Figure 1.: Rhizobia attachment on roots of non-legume host plant *Arabidopsis thaliana.*

Ten-days old Arabidopsis seedlings inoculated with transgenic rhizobia (*M. loti*) tagged with dsRed fluorophore grown on plant media with 5mM nitrate. (a-c) Bacterial biofilm formation on growing Arabidopsis primary root (a) white arrows indicate bacterial enrichments (hot spots) (b) inoculated plants grown in high-nitrate conditions (5mM) and (c) in low-nitrate conditions (0.25mM). (d) Bacteria accumulation on root tip and root elongation/differentiation zone. (e) and (f) *M. loti* accumulation at lateral root emergence sites and intercellular space invasion, validated by z-stack evaluation. Samples were observed under Zeiss stereo fluorescence microscope (a-e) and a confocal-microscope (f).

Figure 2.: Rhizobia-induced modifications on Arabidopsis root architecture depend on nitrate availability.

Ten-days old seedlings were transferred on high-nitrate plant medium (5mM KNO_3) or nitrate-starvation plant medium (0.1mM KNO_3) and roots were inoculated for 3 days with rhizobia (*S. meliloti*) or (*M. loti*). (a) control seedlings grown for 3 days on high-nitrate plant medium. (b) *S. meliloti* and (c) *M. loti* inoculated seedlings grown on high-nitrate plant medium scale bar presents 1 cm (starting root length, before inoculation, was marked with blue marker pen). (d)-(f) Quantifications of number of lateral roots, lateral root length, and primary root length on different nitrate conditions and indicated rhizobia treatment. Statistical significance was determined by students *t*-test *=$p<0.05$; **=$p<0.01$.

Figure 3.: Rhizobia-induced root hair formation depends on high-nitrate availability.

Seven-days old seedlings were transferred on high-nitrate plant medium or nitrate-starvation plant medium and roots were inoculated for 3 days with rhizobia (*S. meliloti*) or (*M. loti*). Primary root tip of control samples after 3 days on plant medium (a) on high nitrate condition and (b) starvation condition (0.1 mM), (c), (d) *S. meliloti* treated samples, and (e), (f) *M. loti* treated samples, scale bar presents 100 µm. (g) and (h) quantification of root hair formation on primary root (average of first 5 mm) in nitrate conditions as indicated. (i)-(k) Transcript levels of indicated root hair-related genes, specifically *ROOT HAIR DEFECTIVE6* (*RHD6*), *RHD6-LIKE4* (*RSL4*), and *EXTENSIN11* (*EXT11*), quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4.* Statistical significance was determined by students *t*-test *=p<0.05; ***=p<0.001 and n.s. = not significant.

Figure 4.: Rhizobia-induced expression of nitrate-regulated genes.

Transcript levels of indicated genes in 7-day old Arabidopsis root samples treated for 3-days with rhizobia *S. meliloti*, *M. loti* or in 10-day old control samples grown on high- (5mM) or low- (0.25mM) nitrate plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4.* Statistical significance was determined by students *t*-test *=p<0.05; **=p<0.01.

Figure 5.: Specific NIN-like Proteins are involved in *M. loti* induced root hair effect.

Seven-days old seedlings of wild-type (Col-0), mutant plants (as indicated), or over-expression lines were transferred on high-nitrate plant medium (5mM KNO_3) or low-nitrate plant medium (0.25mM KNO_3) and roots were inoculated for 3 days with *M. loti*. Quantification of root hair formation on primary root tip is presented. Down site representative close-up image of Col-0, and root differentiation zone treated with *M. loti* for 3 days of Col-0, *nlp*-mutant (*nlp5*). and

nlp5/pAtUBQ10:NLP5 line (OENLP5). Statistical significance was determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test.

Figure 6.: *M. loti* impacts cell -proliferation and -elongation.

Seven-days old seedlings of wild-type (Col-0) or indicated mutant plants were transferred on high-nitrate plant medium or low-nitrate plant medium and roots were inoculated for 3 days with *M. loti* (control = no inoculation). (a)-(j) FM4-64 staining of primary root tip and visualization using confocal microscopy. (a) and (b) representative pictures of the first cells in the root elongation zone (white arrowhead indicates first root hair cell), (c) and (d) pictures of primary root apical meristem (white arrowhead indicates end of meristem), (a, c) control and (b, d) *M. loti* on high-nitrate samples, white arrowheads showing cell size changes. Scale bar presents 100 µm. (e) - (g) length of the first 5 elongated cells, of primary root samples with or without *M. loti* grown on high- or low- nitrate plant medium. (h) – (j) quantification of meristem cortex cell number at the primary root tip. (k) and (l) transcript levels of indicated genes quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4.* Statistical significance was determined by students *t*-test **=p<0.01; ***=p<0.001.

Figure 7.: Model of the rhizobium-induced NLP-pathway in *Arabidopsis thaliana* roots.

Results obtained indicate rhizobia-induced local root developmental effects on the non-legume host *Arabidopsis thaliana.* We demonstrate an involvement of key NIN-like proteins and show an induction of nitrate signaling during rhizobia responses, which finally impacts on cell cycle progression and morphological modifications. Furthermore, described effects are pronounced in high nitrate conditions and suggest a complex crosstalk between bacterial- and nitrate-related signaling pathways.

Supporting Information

Fig. S1 Characterization of *NLP4* and *NLP5* mutations and over-expressions (OE)

Graphical presentation of *NLP4* and *NLP5* sequence with predicted T-DNA insertion sites (a). PCR-based validation of *NLP4* and *NLP5* mutation on genomic DNA using SALK-designed primers, including t-DNA specific Primer LBb1.3 (b). Check for transcript levels by semi-quantitative PCR using primers covering the exon of insertion (c) and qRT-PCR (d) on mutants *nlp4* and *nlp5* and over-expression (OE) lines, as indicated.

Fig. S2 Phylogenetic tree of NIN-like Proteins (main tree).

Maximum Likelihood tree of the NLP family (model: GTR+F+R10; log-likelihood: -598245.4704). Tree is rooted on the non-vascular plants. Clades are colored and named according the *Arabidopsis thaliana* nomenclature. Only significant branch support values (>80%) are shown.

Fig. S3 Reduced phylogenetic tree of NIN-like Proteins.

NLP sequences were retrieved using the tBALSTn algorithm v2.9.0+ by the NIN protein from the model species *Medicago truncatula* as query. Reduced likelihood tree of the NLP family. Clades are colored and named according the *Arabidopsis thaliana* nomenclature. Only significant branch support values (>80%) are shown.

Fig. S4 Expression of other *NIN*-like proteins in response to rhizobia treatment

levels of indicated genes in 7-day old Arabidopsis root samples treated for 3-days with rhizobia *S. meliloti*, *M. loti* or in 10-day old control samples grown on high- (5mM) or low- (0.25mM) nitrate plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4.* Statistical significance was determined by students *t*-test *=p<0.05; **=p<0.01.

884

885 Fig. S5 Dual-affinity-transceptor NRT1.1 is needed for M. loti-induced impact on
886 root morphology, but not NRT2.1.

887 Seven-days old seedlings of wild-type (Col-0) and Wassilewskija (WS) or mutant plants (as
888 indicated) were transferred on high-nitrate plant medium (5mM) or low-nitrate plant medium
889 (0.25mM) and roots were inoculated for 3 days with *M. loti*. Quantification of root hair formation
890 and root hair length on primary root tip (first 5 mm) is presented. Statistical significance was
891 determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test.

892

893 Fig. S6 A *NLP1* mutation is not influencing rhizobial effects on root hair
894 development.

895 Seven-days old seedlings of wild-type (Col-0) or mutant plants (as indicated) were transferred on
896 high-nitrate plant medium (5mM) or low-nitrate plant medium (0.25mM) and roots were inoculated
897 for 3 days with *M. loti*. Quantification of root hair formation and root hair length on primary root tip
898 (first 5 mm) is presented. Statistic significance was determined by one-factorial ANOVA α=0.05
899 followed by Scheffé post-hoc test

900

Fig. S7 AtNLP4 and AtNLP5 are involved in *M. loti* induced root modifications. Seven-days old seedlings of wild-type (Col-0), mutant plants (as indicated) or over-expression lines were transferred on high-nitrate plant medium (5mM) or low-nitrate plant medium (0.25mM) and roots were inoculated for 3 days with *M. loti*. (a) and (b) quantification of root hair length on primary root tip (first 5 mm) is presented. Statistic significance was determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test. (c) and (d) quantifications of lateral root numbers and lateral root length are presented. Statistical significance was determined by students t-test *=p<0.05; ***=p<0.001.

Fig. S8 Varying regulation of rhizobia-induced genes in *nlp4* and *nlp5* mutant roots. Transcript levels of indicated genes in 7-day old Arabidopsis root samples treated for 3-days with rhizobium *M. loti* or in 10-day old control samples grown on high- (5 mM) or low- (0.25 mM) nitrate plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4.* Statistical significance was determined by students *t*-test *=p<0.05.

qPCR Primer sequence Hernandez-Reyes et al.

RSL4_F	AACCTTGTGCCAAACGGGAC
RSL4_R	CCAGGCCGTTGTAAGCCAAT
EXT11_F	GGCAGTTTTTGGTTTATGTCGTG
EXT11_R	GATGGCTGGGAAAATTGTATTGTG
RHD6_F	GCCCTAGATCCACCGAAACTCC
RHD6_R	TGGCTGCTAGGCTTTGTGG
NRT1.1_F	GCACATTGGCATTAGGCTTT
NRT1.1_R	CTCAATCCCCACCTCAGCTA
NRT2.1_F	AGTCGCTTGCACGTTACCTG
NRT2.1_R	ACCCTCTGACTTGGCGTTCTC
NIA1_R	ACGGAGCATGGATGAGTT
NIA1_F	ATCGTCAAAGAAACCGAAGTC
NIR1_F	TGCTGATGACGTTCTTCCACTCTGC
NIR1_R	CTGAGGGTTGACTCCGAAATAGTCTC
CYCB1;1_F	TCAGCTCATGGACTGTGCAA
CYCB1;1_R	GATCAAAGCCACAGCGAAGC
CYCD3;1_F	CTTCAGCTCGTTTCTGTCGC
CYCD3;1_R	TGTCTCCTCCACTTGAAAGTCT
AtNLP4_F	TCA CAA CAA GCC AAG ACC AC
AtNLP4_R	GGG TTT CAC TGG AGC AAC AT
AtNLP5_F	ATT CAC CAC CTG CAA AGT CC
AtNLP5_R	TAT CCG TGG GAT CTT CCT TG
AtNLP1_F	TCA AGG AGC CTC TTG TTC TTG
AtNLP1_R	ATG TCG CTT TCA CTT TGA TGG
AtNLP2_F	TGG TAC TGG CAC TTC CTT CA
AtNLP2_R	TGA TGG TGG TGA CTT TGG AG
AtNLP3_F	TGT TTG GAG ATT CGA TGC TG
AtNLP3_R	CGC TTT GCG ATC TCT CTT CT
AtNLP6_F	CTC ACC AGA GTC GGT AAG CA
AtNLP6_R	CGG AGA CCC ATT CTC TAT CG
AtNLP7_F	CGA ACC ATC AGG CTC AGA A
AtNLP7_R	CGT TCC TGC ATC AAC TTT CA
AtNLP8_F	CAG CGA GAG TGG ATC AAC AA
AtNLP8_R	CAA CTG ATG GCT CGA ACT TG
AtNLP9_F	AAT CGA GCG AGG TAA TGG AA
AtNLP9_R	GAA CTT CCC AGC AAA ACT GC

cloning Primers used in Hernandez-Reyes et al.

GG-NLP4-fwd1	ATGAAGACTTTACGGGTCTCACACCatgGTCCTATCACGACCTTCTGTTTG
GG-NLP4-rev1	ATGAAGACTTCAGAGGTCTCTCCTTGTGCCATAAACTGTATTCAGTAGCT
Mut_NLP4_BsaI_fwd2	TAGAAGACAAaCCAACTCAAGATACATCAGCTCT
Mut_NLP4_BsaI_rev2	TAGAAGACAATGGtGACCACATGAAGGAAGT
Mut_NLP4_BpiI_fwd3	TAGAAGACAAgAGACGAGCAGCGAAATCGAG
Mut_NLP4_BpiI_rev3	TAGAAGACAATCTcCTTCAATGCACCTCCTAC
NLP4_BpiI_fwd4	TAGAAGACAAaACAATGAATGGGTTTTGTTGACTTGC
NLP4_BpiI_rev4	TAGAAGACAATGTtTTCGTCTAAGTACTTTAGATC
GG-NLP5-fwd1	ATGAAGACTTTACGGGTCTCACACCatggaaaacaattctcttcctatgg
GG-NLP5-rev1	ATGAAGACTTCAGAGGTCTCTCCTTtgaaagacatccactgacag
Mut_NLP5_BsaI_fwd2	TAGAAGACAAGccaacgcaagacacatc
Mut_NLP5_BsaI_rev2	TAGAAGACAAtggCgaccacatgaagg
Mut_NLP5_BsaI_fwd3	TAGAAGACAAGtcatgtctcaaacaaagc

Mut_NLP5_BsaI_rev3	TAGAAGACAAtgaCacccatgtttgagc
NLP5_BpiI_fwd4	TAGAAGACAAAacaatgaatgggttttg
NLP5_BpiI_rev4	TAGAAGACAAtgtTttcatctaagtatttcagatcg

genotyping of *nlp4* and *nlp5* mutants used in Hernandez-Reyes et al.

LBb1.3-SALK	ATTTTGCCGATTTCGGAAC
NLP4-LP	GATCTCATGAAGCTCGAAACG
NLP4-RP	CTCTAGCCAATGTTGCTCCAG
NLP5-LP	AGCTCAAACATGGGTCTCATG
NLP5-RP	TTGTAGGGATGATGCTTCTGG
NLP4fwdEXON	GACGAGCAGCGAAATCGAGC
NLP4revEXON	GCCATAAACTGTATTCAGTAGC
NLP5fwdEXON	CACTGGCTCAGTTGATTTCG
NLP5revEXON	CTTGGCAGCATCTTTGAGAC

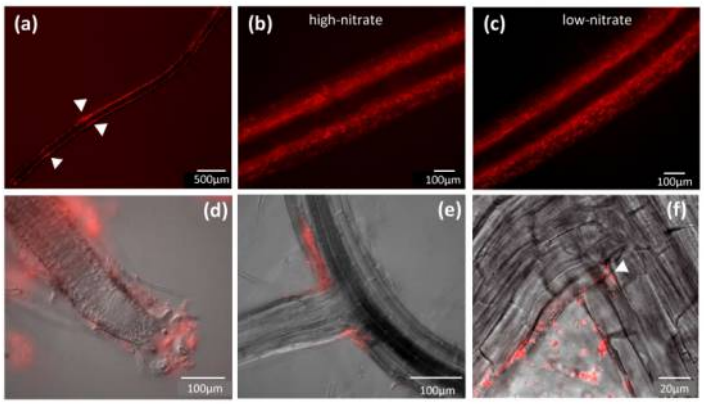


Figure 1.: Rhizobia attachment on roots of non-legume host plant Arabidopsis thaliana.Ten-days old Arabidopsis seedlings inoculated with transgenic rhizobia (M. loti) tagged with dsRed fluorophore grown on plant media with 5mM nitrate. (a-c) Bacterial biofilm formation on growing Arabidopsis primary root (a) white arrows indicate bacterial enrichments (hot spots) (b) inoculated plants grown in high-nitrate conditions (5mM) and (c) in low-nitrate conditions (0.25mM). (d) Bacteria accumulation on root tip and root elongation/differentiation zone. (e) and (f) M. loti accumulation at lateral root emergence sites and intercellular space invasion, validated by z-stack evaluation. Samples were observed under Zeiss stereo fluorescence microscope (a-e) and a confocal-microscope (f).

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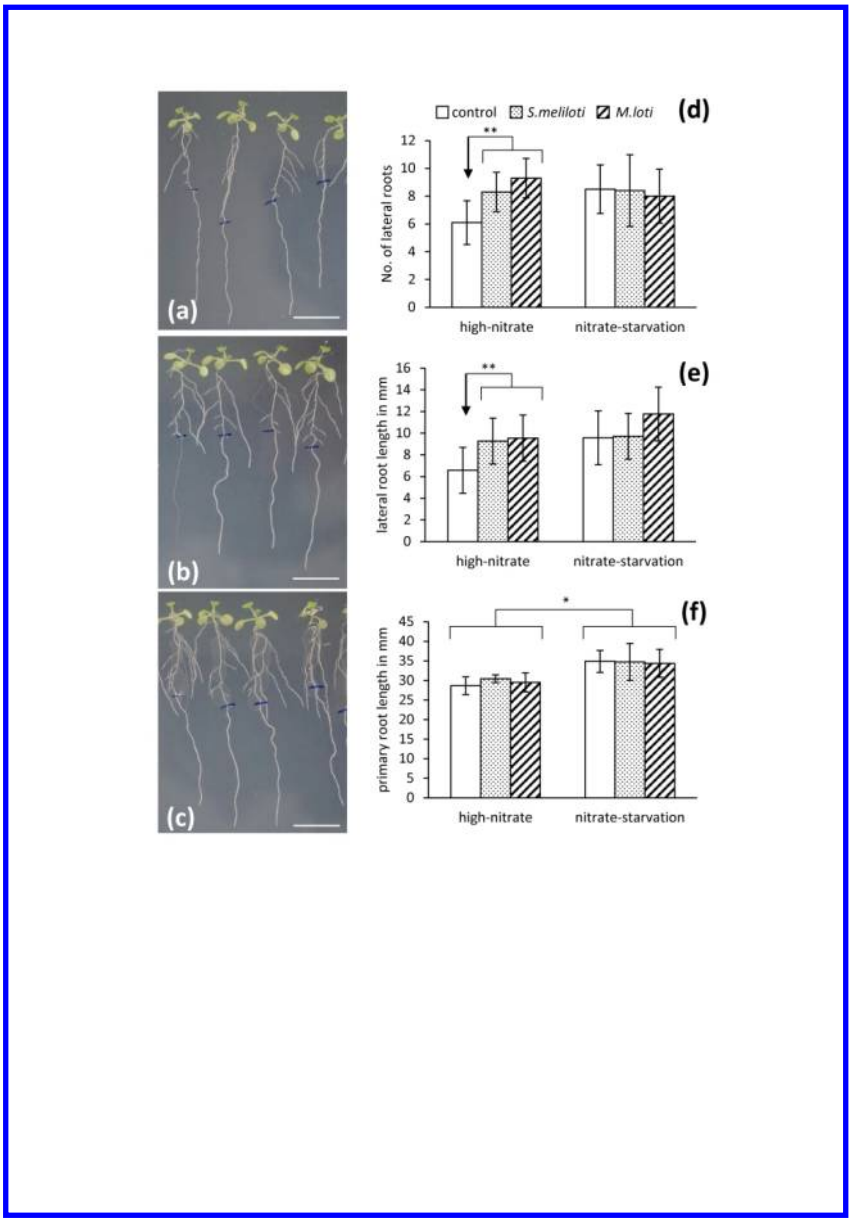


Figure 2.: Rhizobia-induced modifications on Arabidopsis root architecture depend on nitrate availability. Ten-days old seedlings were transferred on high-nitrate plant medium (5mM KNO3) or nitrate-starvation plant medium (0.1mM KNO3) and roots were inoculated for 3 days with rhizobia (S. meliloti) or (M. loti). (a) control seedlings grown for 3 days on high-nitrate plant medium. (b) S. meliloti and (c) M. loti inoculated seedlings grown on high-nitrate plant medium scale bar presents 1 cm (starting root length, before inoculation, was marked with blue marker pen). (d)-(f) Quantifications of number of lateral roots, lateral root length, and primary root length on different nitrate conditions and indicated rhizobia treatment. Statistical significance was determined by students t-test *=p<0.05; **=p<0.01.

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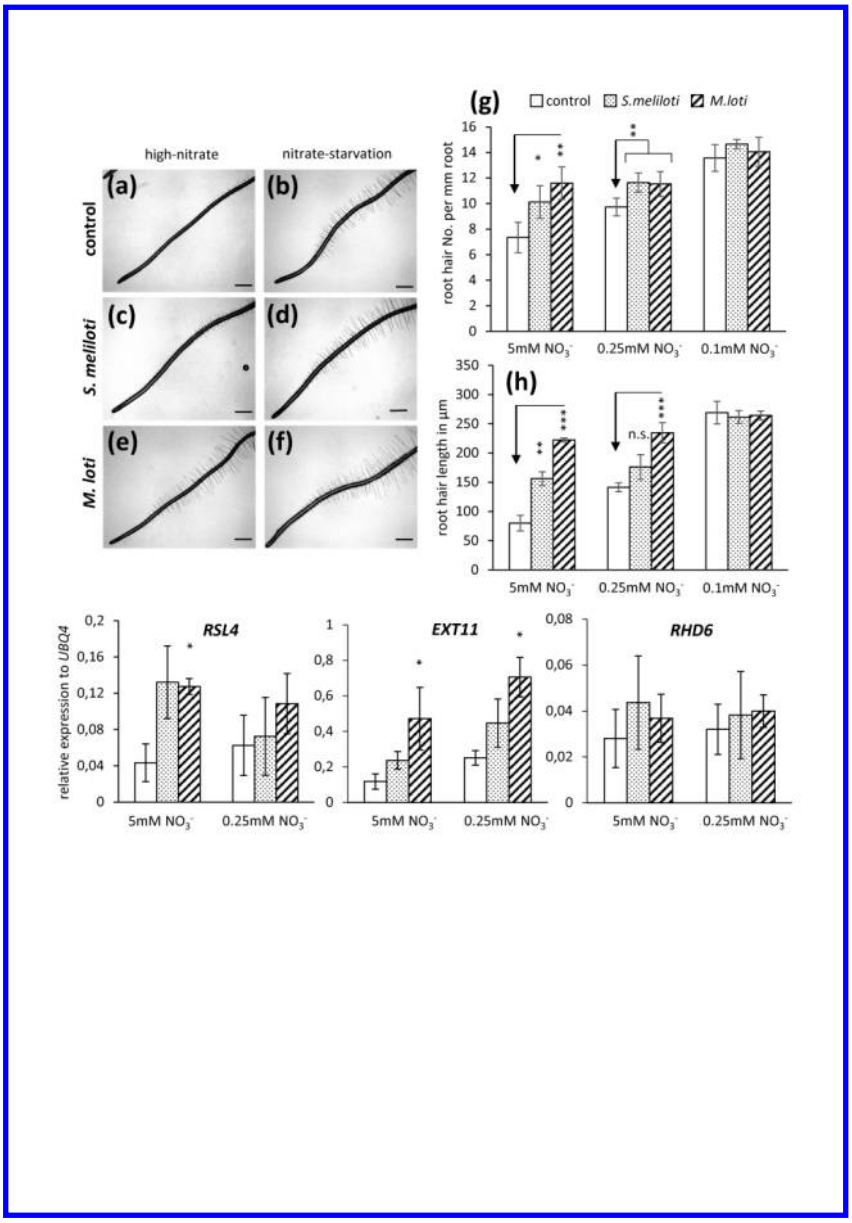


Figure 3.: Rhizobia-induced root hair formation depends on high-nitrate availability.
Seven-days old seedlings were transferred on high-nitrate plant medium or nitrate-starvation plant medium and roots were inoculated for 3 days with rhizobia (S. meliloti) or (M. loti). Primary root tip of control samples after 3 days on plant medium (a) on high nitrate condition and (b) starvation condition (0.1 mM), (c), (d) S. meliloti treated samples, and (e), (f) M. loti treated samples, scale bar presents 100 µm. (g) and (h) quantification of root hair formation on primary root (average of first 5 mm) in nitrate conditions as indicated. (i)-(k) Transcript levels of indicated root hair-related genes, specifically ROOT HAIR DEFECTIVE6 (RHD6), RHD6-LIKE4 (RSL4), and EXTENSIN11 (EXT11), quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of UBQ4. Statistical significance was determined by students t-test *=p<0.05; ***=p<0.001 and n.s. = not significant.

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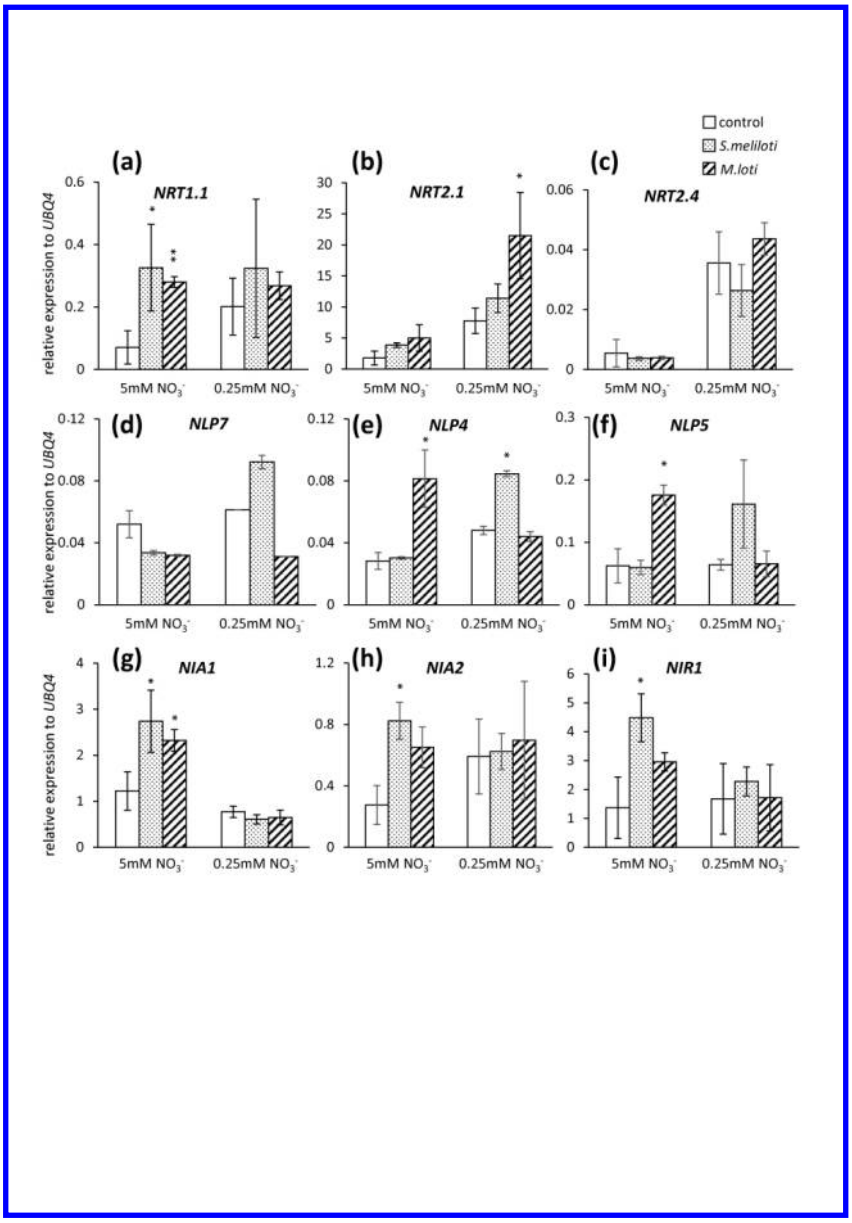


Figure 4.: Rhizobia-induced expression of nitrate-regulated genes.
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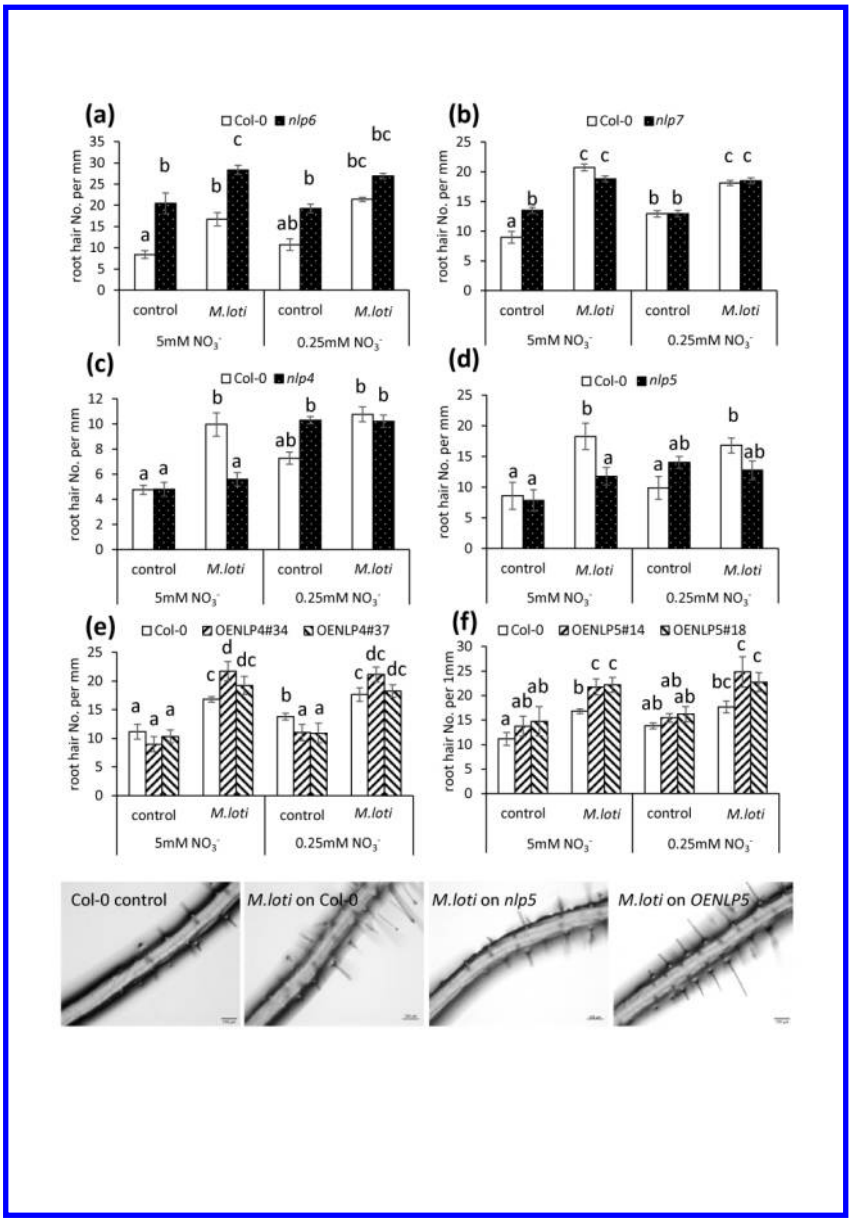


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Seven-days old seedlings of wild-type (Col-0), mutant plants (as indicated), or over-expression lines were transferred on high-nitrate plant medium (5mM KNO3) or low-nitrate plant medium (0.25mM KNO3) and roots were inoculated for 3 days with M. loti. Quantification of root hair formation on primary root tip is presented. Down site representative close-up image of Col-0, and root differentiation zone treated with M. loti for 3 days of Col-0, nlp-mutant (nlp5). and nlp5/pAtUBQ10:NLP5 line (OENLP5). Statistical significance was determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test.

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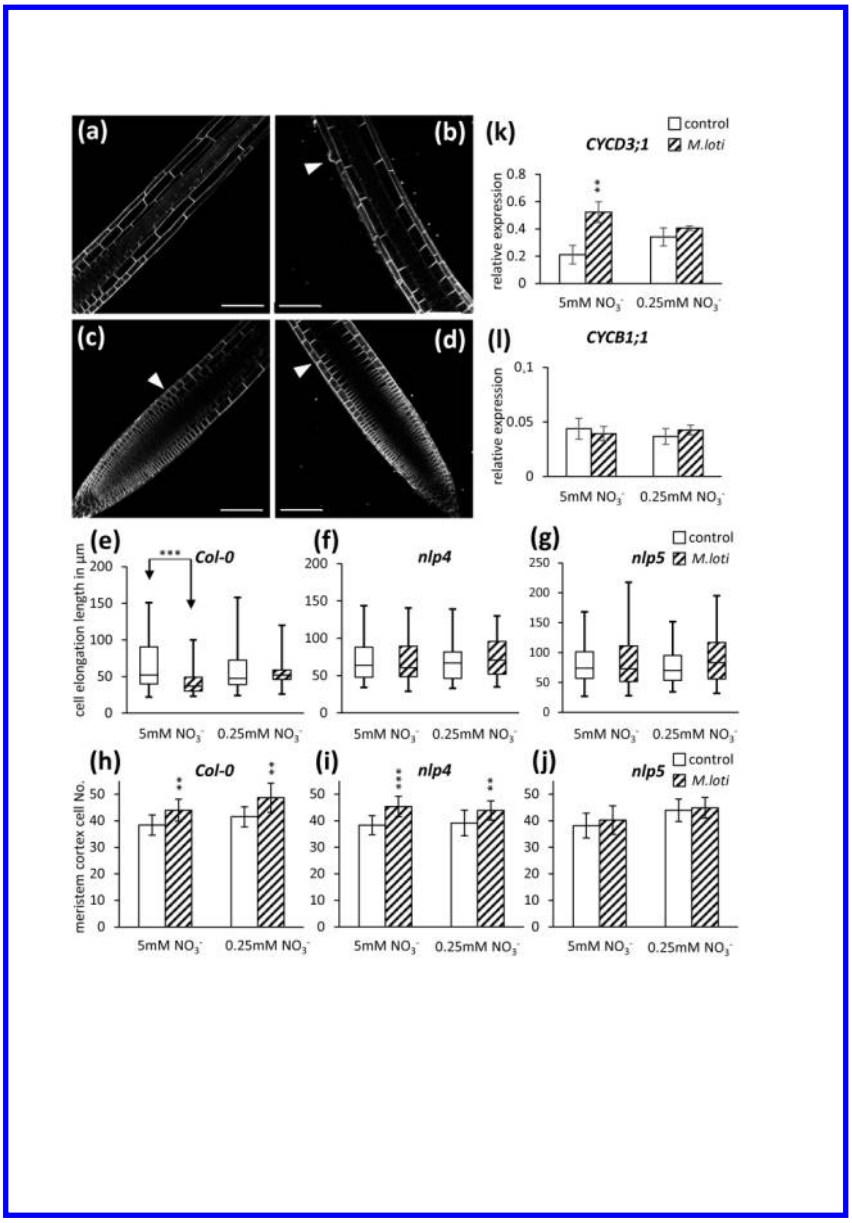


Figure 6.: M. loti impacts cell -proliferation and -elongation.
Seven-days old seedlings of wild-type (Col-0) or indicated mutant plants were transferred on high-nitrate plant medium or low-nitrate plant medium and roots were inoculated for 3 days with M. loti (control = no inoculation). (a)-(j) FM4-64 staining of primary root tip and visualization using confocal microscopy. (a) and (b) representative pictures of the first cells in the root elongation zone (white arrowhead indicates first root hair cell), (c) and (d) pictures of primary root apical meristem (white arrowhead indicates end of meristem), (a, c) control and (b, d) M. loti on high-nitrate samples, white arrowheads showing cell size changes. Scale bar presents 100 µm. (e) - (g) length of the first 5 elongated cells, of primary root samples with or without M. loti grown on high- or low- nitrate plant medium. (h) – (j) quantification of meristem cortex cell number at the primary root tip. (k) and (l) transcript levels of indicated genes quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of UBQ4. Statistical significance was determined by students t-test **=p<0.01; ***=p<0.001.

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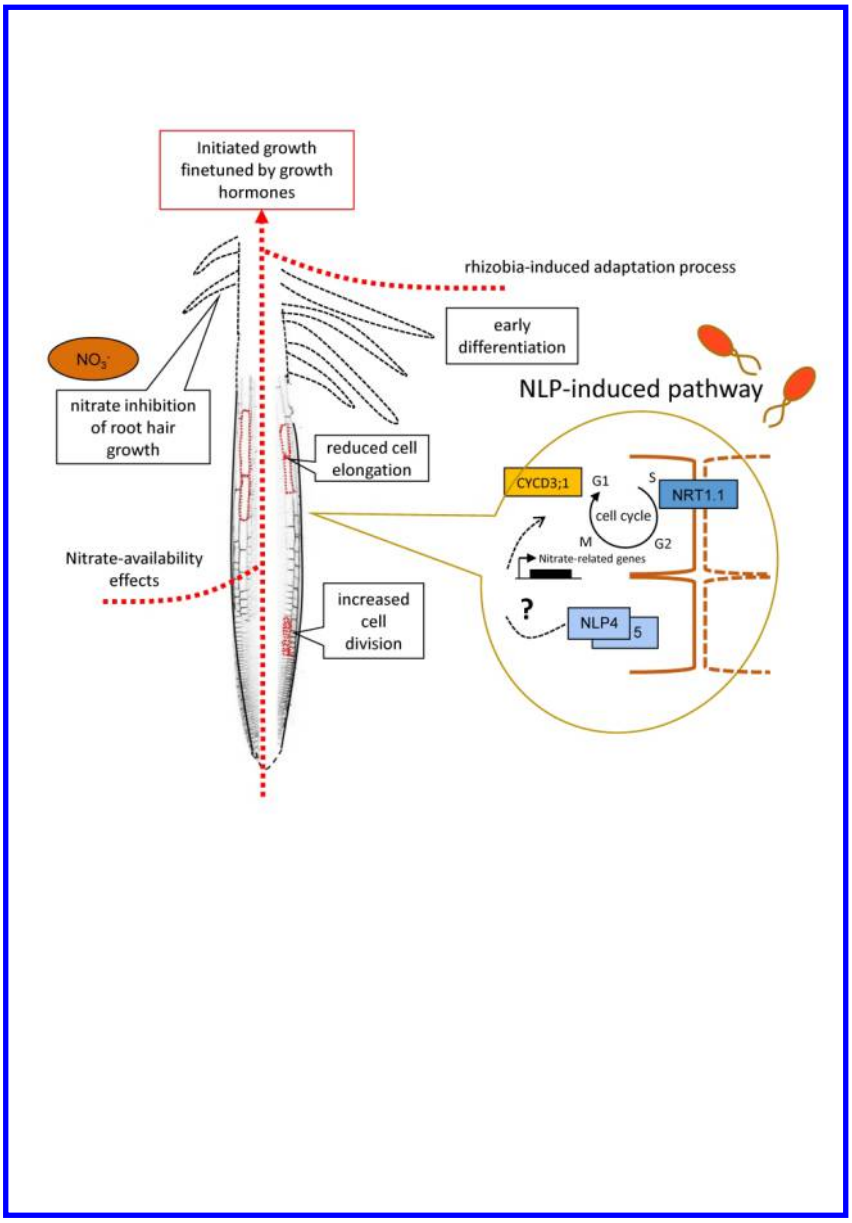


Figure 7.: Model of the rhizobium-induced NLP-pathway in Arabidopsis thaliana roots.
Results obtained indicate rhizobia-induced local root developmental effects on the non-legume host Arabidopsis thaliana. We demonstrate an involvement of key NIN-like proteins and show an induction of nitrate signaling during rhizobia responses, which finally impacts on cell cycle progression and morphological modifications. Furthermore, described effects are pronounced in high nitrate conditions and suggest a complex crosstalk between bacterial- and nitrate-related signaling pathways.

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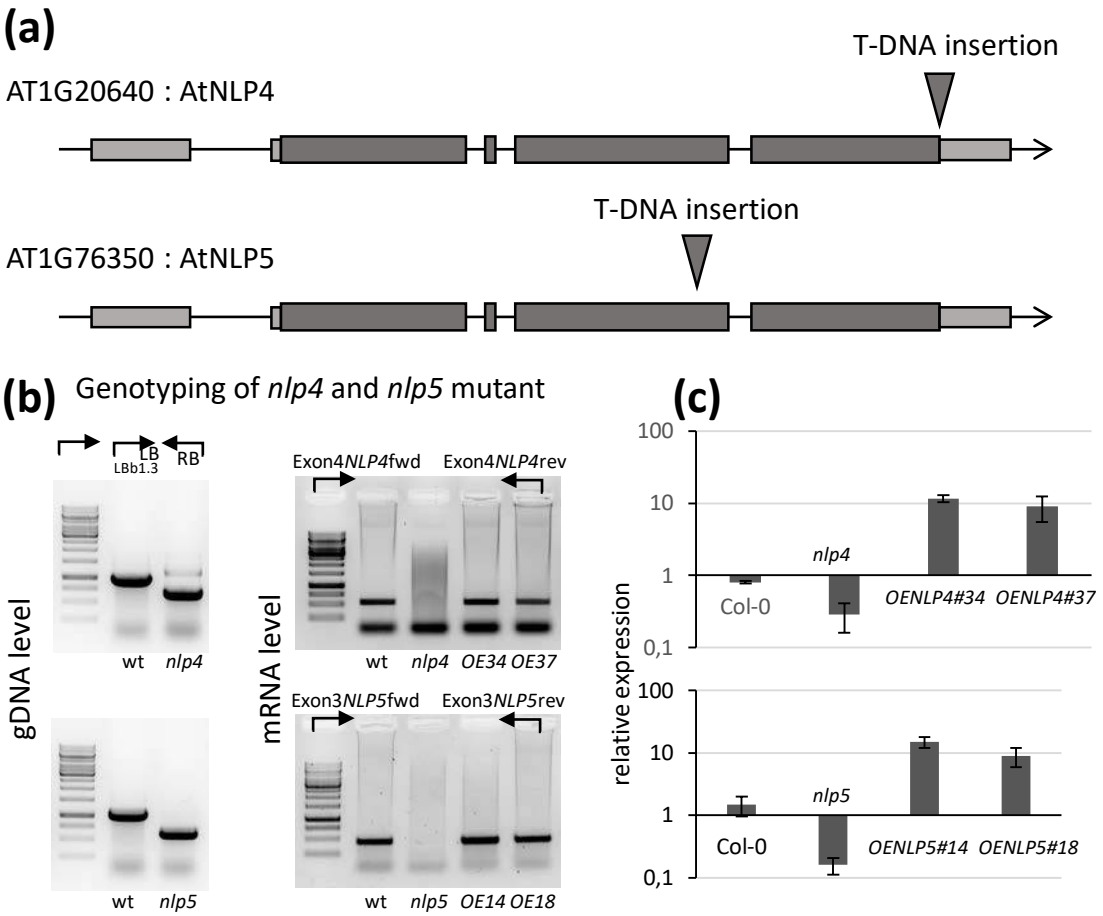


Fig. S1 Characterization of *NLP4* and *NLP5* mutations and over-expressions (OE) Graphical presentation of *NLP4* and *NLP5* sequence with predicted T-DNA insertion sites (a). PCR-based validation of *NLP4* and *NLP5* mutation on genomic DNA using SALK-designed primers, including t-DNA specific Primer LBb1.3 (b). Check for transcript levels by semi-quantitative PCR using primers covering the exon of insertion (c) and qRT-PCR (d) on mutants *nlp4* and *nlp5* and over-expression (OE) lines, as indicated.

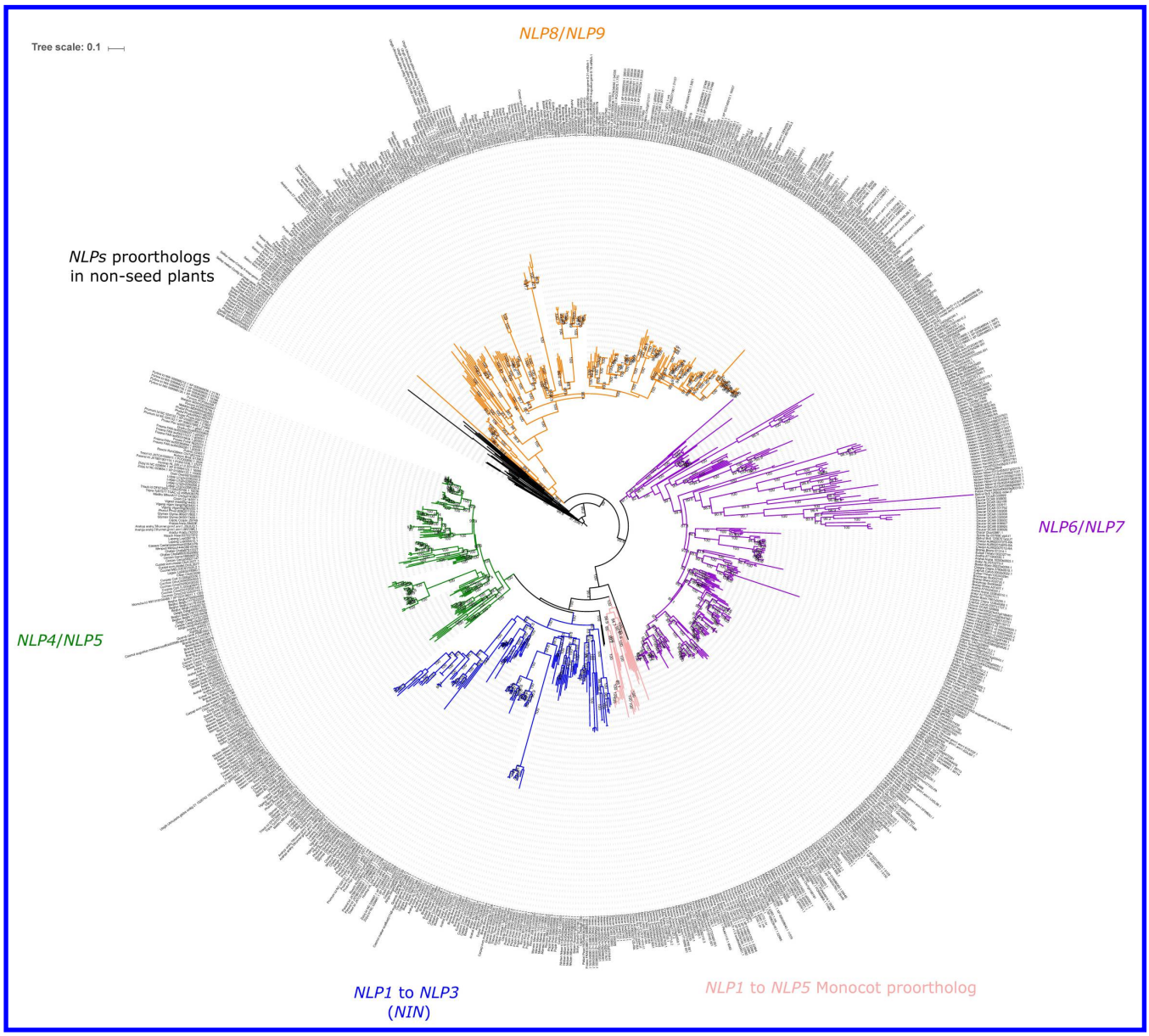


Fig. S2 Phylogenetic tree of NIN-like Proteins (main tree).
Maximum Likelihood tree of the NLP family (model: GTR+F+R10; log-likelihood: -598245.4704). Tree is rooted on the non-vascular plants. Clades are colored and named according the Arabidopsis thaliana nomenclature. Only significant branch support values (>80%) are shown.

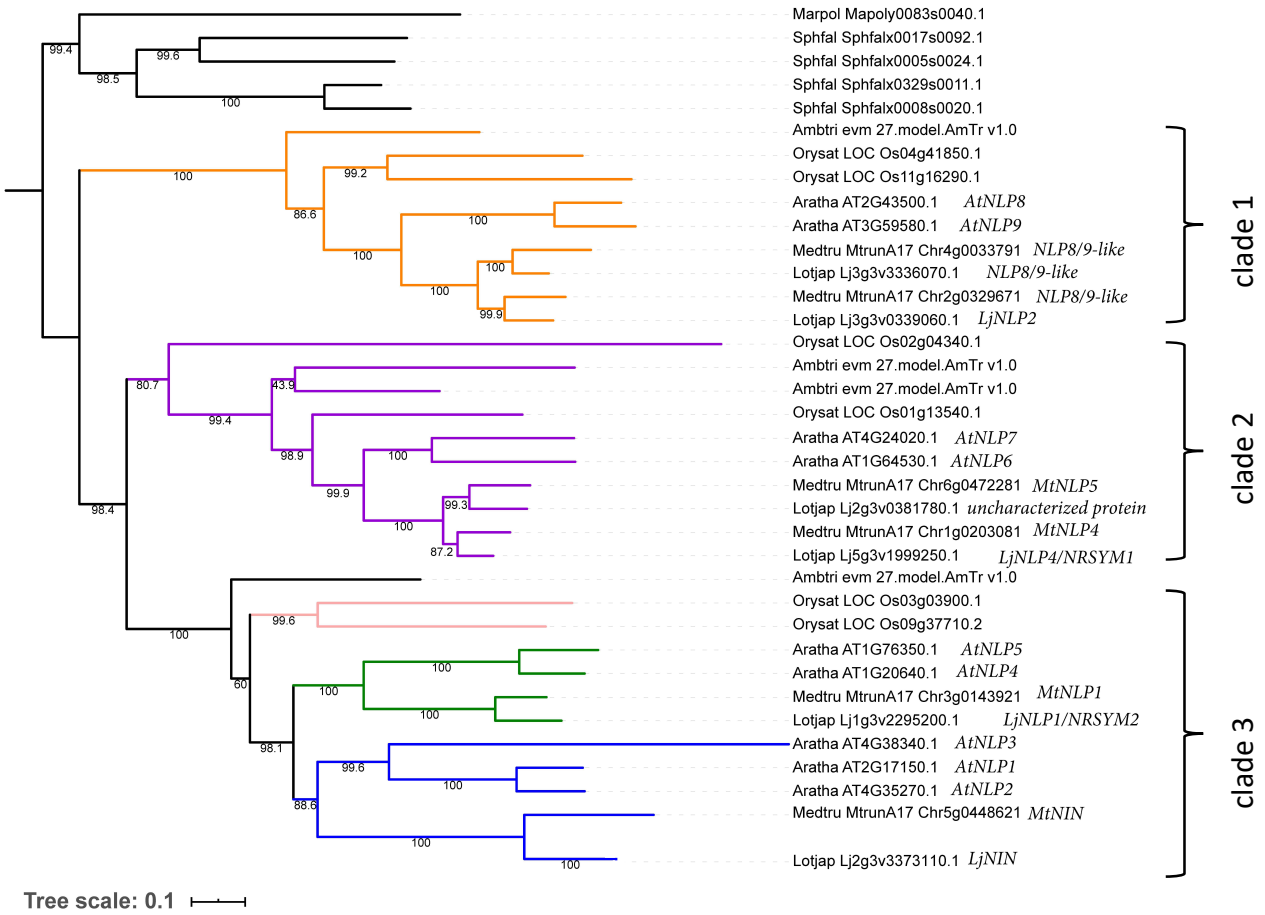


Fig. S3 Reduced phylogenetic tree of NIN-like Proteins.
NLP sequences were retrieved using the tBALSTn algorithm v2.9.0+ by the NIN protein from the model species Medicago truncatula as query. Reduced likelihood tree of the NLP family. Clades are colored and named according the Arabidopsis thaliana nomenclature. Only significant branch support values (>80%) are shown.

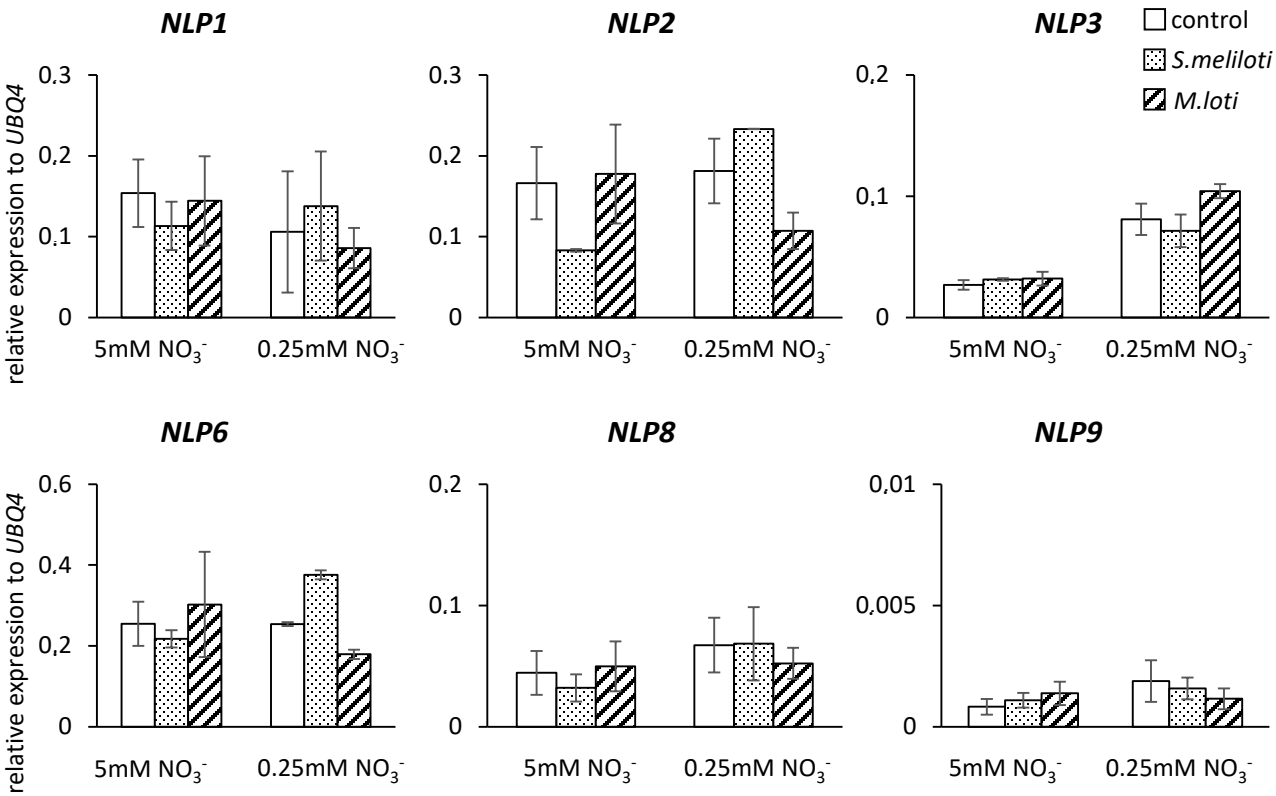


Fig. S4 Expression of other NIN-like proteins in response to rhizobia treatment.
levels of indicated genes in 7-day old Arabidopsis root samples treated for 3-days with rhizobia *S. meliloti, M. loti* or in 10-day old control samples grown on high- (5mM) or low- (0.25mM) nitrate plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4*. Statistical significance was determined by students t-test *=p<0.05; **=p<0.01.

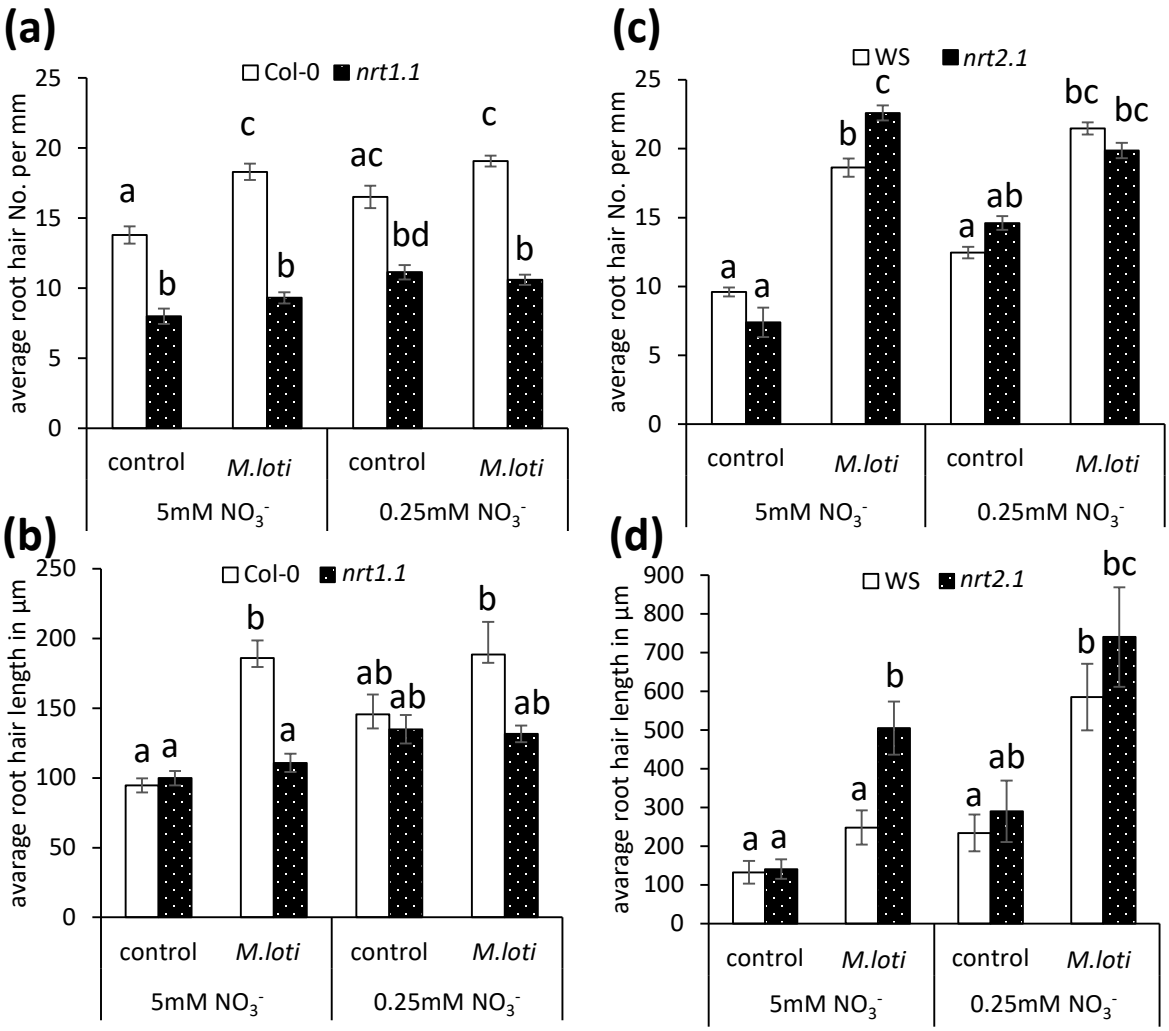


Fig. S5 Dual-affinity-transceptor NRT1.1 is needed for M. loti-induced impact on root morphology, but not NRT2.1.

Seven-days old seedlings of wild-type (Col-0) and Wassilewskija (WS) or mutant plants (as indicated) were transferred on high-nitrate plant medium (5mM) or low-nitrate plant medium (0.25mM) and roots were inoculated for 3 days with *M. loti.* Quantification of root hair formation and root hair length on primary root tip (first 5 mm) is presented. Statistical significance was determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test.

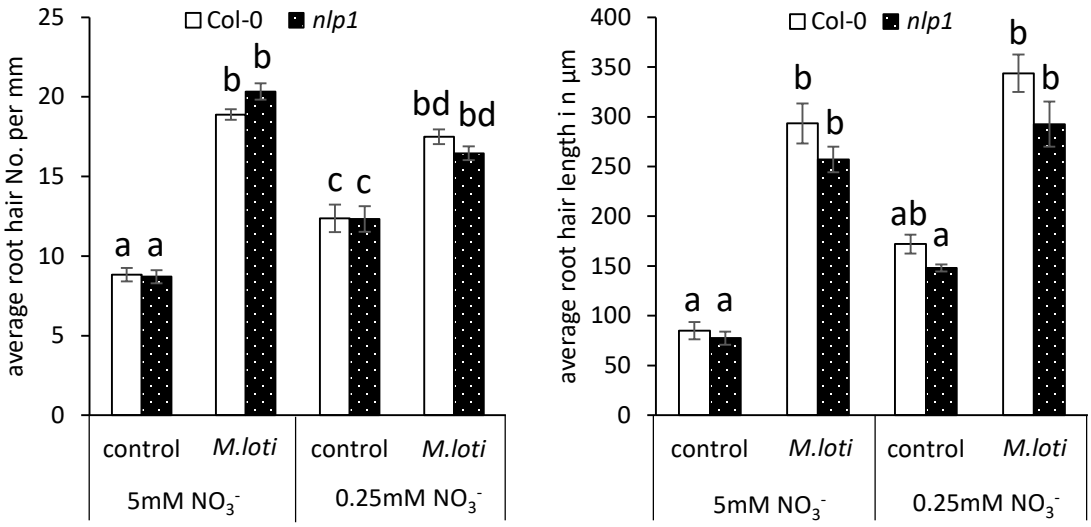


Fig. S6 A *NLP1* mutation is not influencing rhizobial effects on root hair development.

Seven-days old seedlings of wild-type (Col-0) or mutant plants (as indicated) were transferred on high-nitrate plant medium (5mM) or low-nitrate plant medium (0.25mM) and roots were inoculated for 3 days with *M. loti*. Quantification of root hair formation and root hair length on primary root tip (first 5 mm) is presented. Statistic significance was determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test

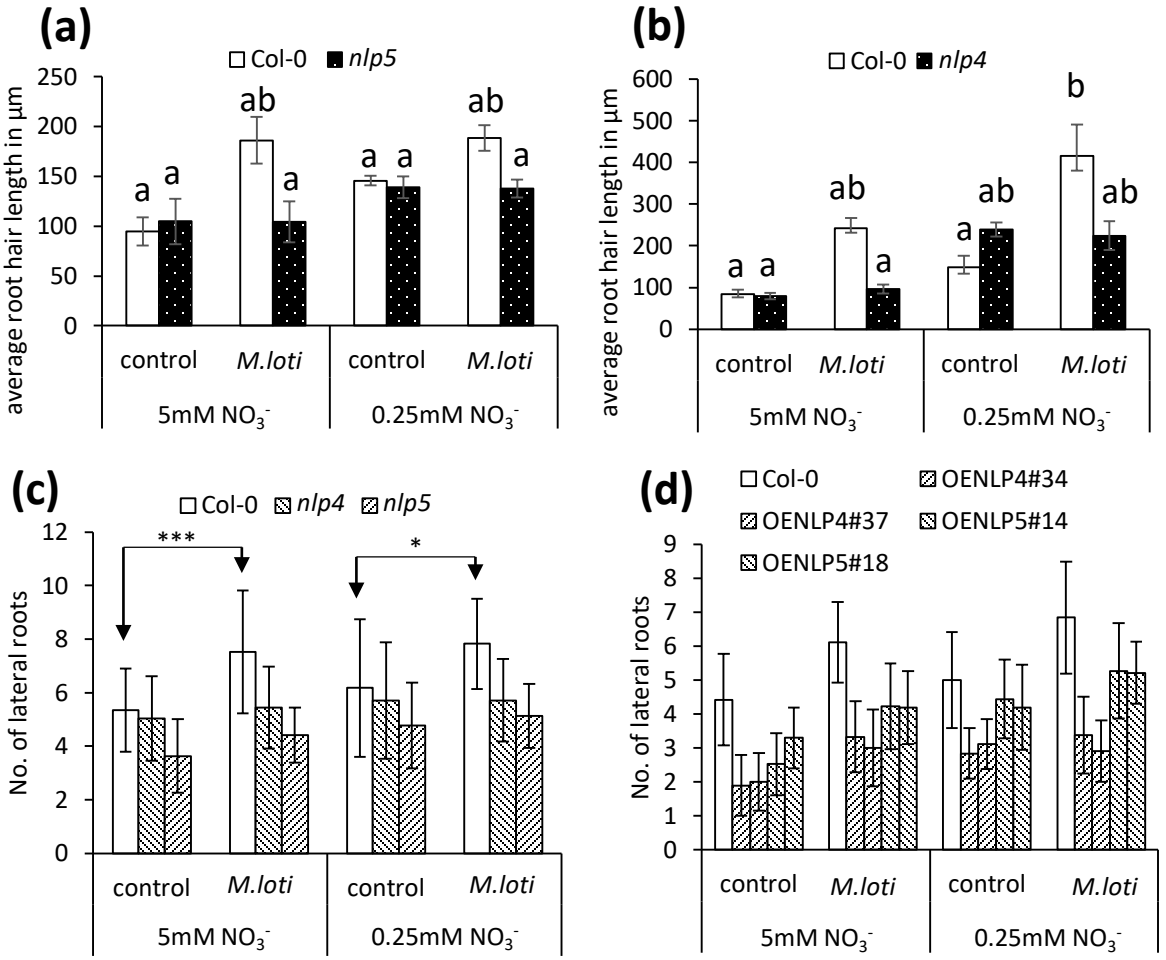


Fig. S7 AtNLP4 and AtNLP5 are involved in M. loti induced root modifications. Seven-days old seedlings of wild-type (Col-0), mutant plants (as indicated) or over-expression lines were transferred on high-nitrate plant medium (5mM) or low-nitrate plant medium (0.25mM) and roots were inoculated for 3 days with *M. loti.* (a) and (b) quantification of root hair length on primary root tip (first 5 mm) is presented. Statistic significance was determined by one-factorial ANOVA α=0.05 followed by Scheffé post-hoc test. (c) and (d) quantifications of lateral root numbers and lateral root length are presented. Statistical significance was determined by students t-test *=p<0.05; ***=p<0.001.

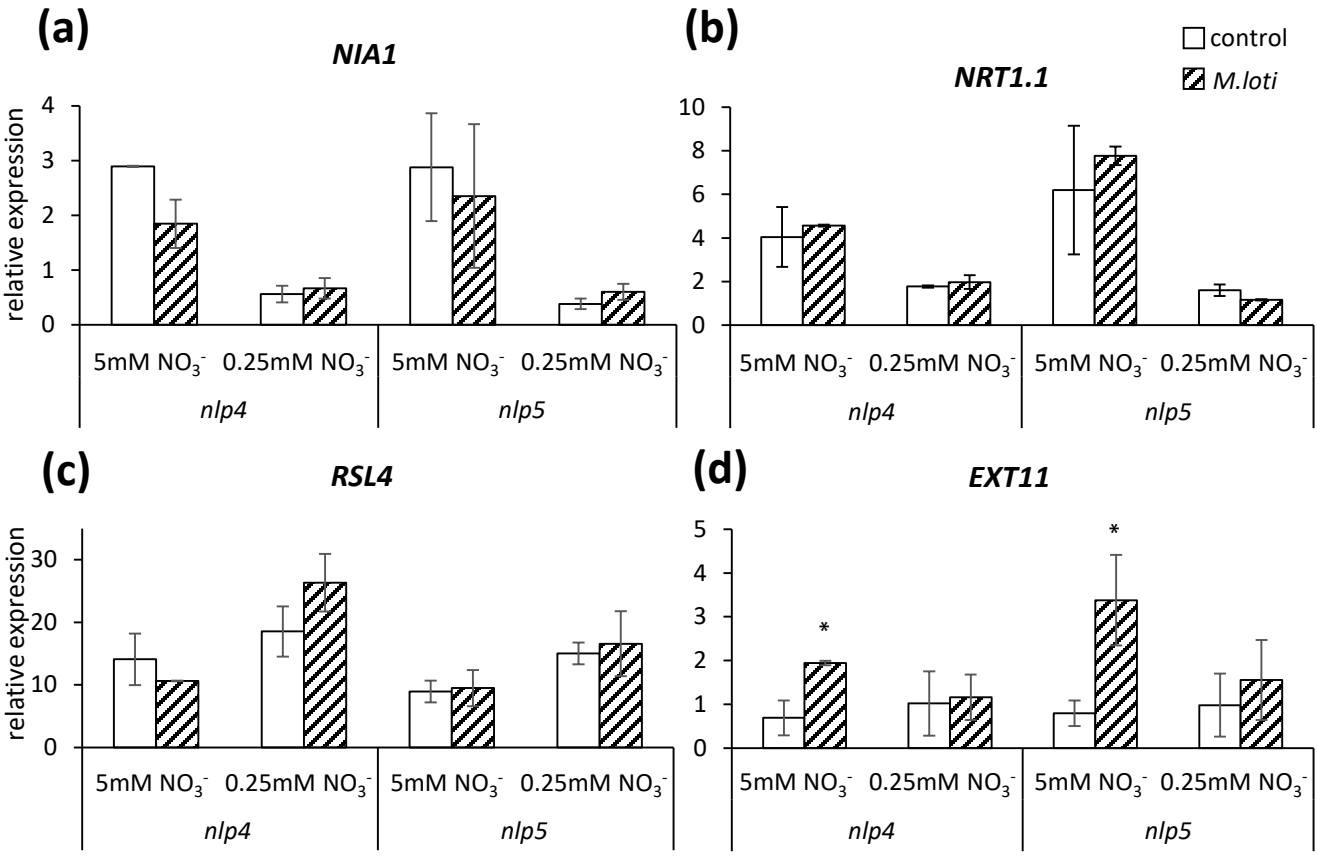


Fig. S8 Varying regulation of rhizobia-induced genes in *nlp4* and *nlp5* mutant roots.
Transcript levels of indicated genes in 7-day old Arabidopsis root samples treated for 3-days with rhizobium *M. loti* or in 10-day old control samples grown on high- (5mM) or low- (0.25mM) nitrate plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of *UBQ4*. Statistical significance was determined by students t-test *=p<0.05.