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

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19

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26

27 **Abstract**

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

45

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

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50 Introduction

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

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

88 Over the last decade key players regulating N- homeostasis and signaling have been identified
89 (Wang et al., 2018; Fredes et al., 2019). These include low- and high- affinity transporter protein
90 families in roots such as the NITRATE-TRANSPORTER 1/PEPTIDE-TRANSPORTER FAMILY
91 (NPF, also known as CHL1 and NRT1) and the NITRATE-TRANSPORTER 2 family (NRT2)
92 (Krapp et al., 2014; O'Brien et al., 2016). Members of both protein families are transcriptionally
93 regulated and functionally dependent on the plants' nutritional N-status. For example, the high-
94 affinity-transporting-systems *NRT2.1* and *NRT2.2* are induced after nitrate uptake (Cerezo et al.,
95 2001). Examples for root-expressed high nitrate transporters are NRT1.1 (NPF6.3) (dual-affinity
96 transporter) and NRT1.2 (NPF4.6) (Wang et al., 2018). Interestingly, it has been postulated that
97 NRT1.1 is also able to transport auxin and consequently regulates lateral root growth in
98 *Arabidopsis* (Krouk et al., 2010; Bouguyon et al., 2016; Maghiaoui et al., 2020). Its auxin
99 transporting activity depends on the availability of exogenous nitrate in a concentration-dependent
100 manner. In nitrate-rich patches, auxin accumulates in lateral roots and induces root emergence
101 and elongation, whereas auxin is depleted in sites with low nitrate availability resulting in reduced

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

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

127 pathways, as exemplified for AtNLP4 and AtNLP5 (Konishi and Yanagisawa, 2019; Hsin et al.,
128 2021).

129 Root nodule nitrogen-fixing symbiosis is evolutionary retained in different species of the Nitrogen-
130 Fixing- (NFN) clade (Fabales, Rosales, Cucurbitales, Fagales), where it is established between
131 several species and bacteria collectively named rhizobia (in Fabales and the Rosales species
132 *Parasponia andersonii*) or Frankia, which enables them to access atmospheric dinitrogen after its
133 fixation and conversion to ammonium by the symbiont (Werner et al., 2015; Griesmann et al.,
134 2018; van Velzen et al., 2018). In this process, one of the central transcriptional regulators is the
135 Nodule-INception protein (NIN), which was originally identified in *Lotus japonicus* (Schauser et al.,
136 1999). Different to other NLPs, the Nitrate-responsive domain at the N-terminal region of legume
137 NIN protein is only partially conserved, which explain the loss of direct nitrate responsiveness of
138 NIN and thus may represent an evolutionary adaptation of legumes to maintain this mutualistic
139 interaction (Suzuki et al., 2013; Mu and Luo, 2019). While the binding capacities of NIN and NLPs
140 to NRE sequences are similar (Suzuki et al., 2013), it should be noted that the functionality of root-
141 nodule symbiosis is strictly regulated by the availability of nitrate. Furthermore, this mechanism is
142 also regulated via NLP functions as exemplified by the Nitrate unResponsive SYmbiosis 1
143 (NRSYM1) and NRSYM2 in *Lotus japonicus* (Nishida et al., 2018; Nishida et al., 2021). Recently,
144 these two LjNLPs were shown to have distinct roles in nitrate signaling, and, moreover, influence
145 nitrate induced inhibition of nodulation on levels of symbiotic-gene regulation (Nishida et al., 2021).
146 The LjNLP4 (NRSYM1)- LjNIN interaction complex accumulates in the nucleus in response to high
147 nitrate conditions and regulates transcription of the CLE-RS2 peptide, which in turn negatively
148 controls nodule numbers. Remarkably, expression of the Arabidopsis *AtNLP6* and *AtNLP7*
149 rescued the nodulation phenotype of the *nrsym1* mutant, suggesting a functional conservation of
150 NLPs in legumes and non-legumes plants (Nishida et al., 2018). In *Medicago truncatula*
151 (*Medicago*), MtNLP1 was reported to mediate nitrate signaling and to re-localize from the cytosol
152 to the nucleus under high nitrate conditions. Subsequently, complex formation between MtNLP

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

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

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

175

176

177 Results

178 Rhizobia associate with the root surface of Arabidopsis seedlings

179 Following the findings that rhizobia and other diazotrophic bacteria are able to colonize the
180 endosphere of non-legume roots by entering via natural openings such as sites of lateral root
181 emergence (de Zelicourt et al., 2018; Schneijderberg et al., 2018), we assessed the colonization
182 patterns of rhizobia on Arabidopsis roots. For this, Arabidopsis plants were grown vertically *in vitro*
183 and inoculated with fluorescently labelled *M. loti* for three days. Bacterial biofilms formed along
184 the root epidermis (Fig. 1a) with enrichments at the root cap and the root elongation zone (Fig.
185 1d). Similar colonization patterns of bacteria were observed on plants growing on high-and low-
186 nitrate medium (Fig. 1b, c). Furthermore, rhizobia accumulated at sites of lateral root emergence
187 (Fig. 1e) and invaded the space of these natural epidermal openings (Fig. 1f). However, clear
188 signs of intercellular colonization in further tissues were not observed. Thus, rhizobia associated
189 with the non-legume Arabidopsis by establishing an interaction on the outside of the plant root,
190 independent of nitrate-availability conditions.

191

192 Rhizobia affect plant root development

193 As several soil-borne bacteria are known to alter the plant root architecture, thus enabling plants
194 to better access nutrients directly or indirectly, we tested whether *M. loti* and *S. meliloti* -effects
195 are influenced by the availability of exogenous nitrate. For this, we transferred 10-days old
196 Arabidopsis seedlings onto plant medium with either high nitrate (5mM KNO₃) or nitrate starvation
197 (0.1mM KNO₃) conditions and additionally inoculated these plants with either *S. meliloti* or *M. loti*
198 for 3 days. The number and length of lateral roots significantly increased when inoculating plants
199 grown under high nitrate conditions with rhizobia compared to the non-inoculated control (Fig. 2a-
200 e) while the primary root length was unaffected by this treatment (Fig. 2f). Nitrate deprivation fully
201 abolished the rhizobial effect on lateral root growth (Fig. 2d, e) and resulted in a moderate increase

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

205 Among other cells, root hairs can serve as primary contact sites and can be altered by
206 rhizobacteria (Poitout et al., 2017; de Zelicourt et al., 2018). Thus, we investigated root hair
207 development along young segments of the primary root. Here, we decided to inoculate rhizobia
208 as early as 7 days post germination, since root hairs develop faster than lateral roots. Furthermore,
209 we included a low-nitrate condition (0.25 mM) to further assess nutrient-limiting conditions rather
210 than nutrient stress responses. As expected, nitrate starvation led to a significant increase in root
211 hair length (Fig. 3a-b, h) and number (Fig. 3g) even in the absence of rhizobia, as published earlier
212 (Vatter et al., 2015). While the presence of *S. meliloti* moderately affected root hair patterns (Fig.
213 3c-d, g-h), a significant induction of root hair growth was observed upon inoculation with *M. loti*
214 under high (5 mM) and low (0.25 mM) nitrate conditions. No rhizobial effects on root hairs were
215 found under nitrate-starvation conditions (0.1mM nitrate) in comparison to non-treated plants (Fig.
216 3e-h). Therefore, we continued experiments on rhizobial effects on high-(5mM) and low-(0.25mM)
217 nitrate conditions rather than observing nutrient stress conditions at 0.1mM nitrate. To further test
218 the impact of rhizobia on root hair growth, we examined root hair-specific marker gene expression
219 in roots treated with rhizobia under different nitrate conditions. Indeed, rhizobia tended to induce
220 the morphogenetic root-hair genes *RHD6-LIKE4* (*RSL4*), and *EXTENSIN11* (*EXT11*), but not
221 *ROOT HAIR DEFECTIVE6* (*RHD6*) (Fig. 3). Overall, our results show that *M. loti* treatments were
222 particularly pronounced under high-nitrate conditions. These data demonstrate that Arabidopsis
223 root architecture and morphology are affected by rhizobia in a similar way as nitrate impacts root
224 modifications.

225

226 Rhizobia influence nitrate-related gene expression

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

236 Transcriptionally activated NIN-like proteins are known to play a role in nitrate signaling during
237 rhizobia-legume interactions. These proteins suppress gene expression by either directly binding
238 to NIN-proteins or by competitive binding to promoters of symbiosis-responsive genes (Lin et al.,
239 2018; Nishida et al., 2018). Large-scale phylogenetic analysis of the NLP family revealed their
240 conservation in land plants (Schauser et al., 2005). Furthermore, different duplication events led
241 to three NLP-clades that evolved prior to the divergence of seed plants (Fig. S2 and S3). As NLP7
242 (NIN –like protein 7) was identified as key regulator during the primary nitrate response in
243 *Arabidopsis* and accumulates as a heteromeric complex with NLP6 and TCP20 in the nucleus
244 during nitrate-starvation responses (Guan, 2017), we decided to analyze transcriptional activation
245 of NLPs in our experimental conditions (Fig. 4d-f), (Fig. S4). While NLPs are not transcriptionally
246 up-regulated during nutritional signaling *per se* (Chardin et al., 2014), we detected a significant
247 induction of *NLP4* and *NLP5* expression in roots inoculated with *M. loti* in high-nitrate and *S.*
248 *melliloti* in low-nitrate conditions (Fig. 4e, f). However, other NLPs were not influenced by the
249 presence of rhizobia (Fig. S4). Additionally, genes involved in nitrate metabolism such as *NIA1*
250 and *NIA2* (nitrate reductase), and *NIR1* (nitrite reductase), which are regulated during nitrate
251 responses, were also found to be induced upon rhizobial inoculation of plants grown under high-

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

255

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

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

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

294

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

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

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

319

320 Discussion

321 Gene regulatory networks control plant responses to different nutrient conditions, leading to
322 modifications of the root architecture. In natural soils, plant roots are additionally challenged by a
323 great diversity of beneficial and pathogenic microbes and the plants' overall nutrient status
324 determines the responsiveness to these microbes (Shahzad and Amtmann, 2017; Singh et al.,
325 2019). Among them are rhizobia, which have been mostly investigated in frame of the root-nodule
326 symbiosis, a process that ultimately results in the ability of these bacteria to fix atmospheric
327 dinitrogen gas. This mutualistic interaction strictly depends on the unavailability of nitrate and is
328 immediately terminated upon resupply of inorganic nitrate to the host plant (van Noorden et al.,
329 2016). While the root-nodule symbioses interaction mostly relies on a highly strain-specific

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

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

342 It is well documented that nitrate starvation is accompanied by stimulated lateral root emergence
343 and primary root elongation, while excess nitrate leads to root growth inhibition (Jia and Wiren,
344 2020). In line with this, the presence of PGPRs on *Arabidopsis* roots stimulates a low-nitrate
345 response. This can be exemplified by the PGPR *Phyllobacterium* STM196 that positively
346 influences root architecture under high-nitrate conditions. These rhizobacteria did not directly alter
347 the N-status in leaves or root nitrate uptake, but induced the expression of nitrate starvation
348 marker genes such as *AtNRT2.5* and *AtNRT2.6* (Mantelin et al., 2006) that are also postulated to
349 play a central role in bacterial-induced growth promotion (Kechid et al., 2013). This is in agreement
350 with our results on nitrate-influenced *M. loti*-induced root hair development (Fig. 3, 5, S7). Indeed,
351 one interesting aspect is the impact of rhizobia on nitrate starvation signaling (Fig. 4a-c), similarly
352 to nitrate-induced inhibition circuits in legume symbiosis. However, our data indicate a rather
353 specific rhizobium-induced high-nitrate signaling in plants, comprising *NRT1.1*, and nitrate
354 metabolic gene regulations (Fig. 4a, g-i). Furthermore, the effectiveness of *M. loti* influenced root
355 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

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

409

410 **Materials and Methods**

411 **Plant growth conditions and root observations**

412 *Arabidopsis thaliana* wild type ecotype Columbia (Col-0), Wassilewskija (WS) and knockout
413 mutant seeds were surface sterilized with 50% ethanol/0.5% Triton X-100 and briefly washed with
414 95% ethanol. Seeds for mutant plants *nlp4* (N563595), *nlp5* (N527488), and *nlp1* (N584809) were
415 provided by *The Nottingham Arabidopsis Stock Centre* (NASC) and homozygosity for T-DNA
416 insertion was validated by PCR with primers designed by SALK Institute Genomic Analysis
417 Laboratory. Seeds were germinated on half-strength Murashige-Skoog- medium (1/2 MS) after
418 stratification at 10°C for 3 days and grown horizontally for 7-days/10-days (as indicated) at 22°C
419 with 150 μ mol/m²/s light in a 12/12-h day/night photoperiod. After germination seedlings were
420 transferred and inoculated with bacteria on *nitrate-variable plant medium* containing
421 5mM/0.25mM/0.1mM (as indicated) KNO₃ or 5mM KCl (in low-nitrate conditions KCl was used to
422 buffer ion concentrations), 1mM KH₂PO₄, 0.5 mM CaSO₄, 0.5 mM MgCl₂, 3 mM MES, pH 5.8,
423 0.25% (w/v) sucrose and 0,1% micronutrients (Scheible et al., 2004) including 1% ferric citrate,
424 supplemented with 0.9% Phyto-Agar and grown vertically for 3 days. Root modifications were
425 quantified on images photographed with scale bar and analyzed on Image J Software. For root
426 hair quantifications pictures were taken using a stereomicroscope and root hairs were measured
427 and counted in 5 mm from the start of the differentiation zone (first root hair appearance). Data
428 were averaged from at least 7 root samples and experiments were performed at least 3 times
429 independently.

430

431 Bacteria growth conditions

432 *Sinorhizobium meliloti* strain Sm2011, tetracycline resistant with mCherry as fluorescent marker,
433 and *Mesorhizobium loti* strain MAFF, gentamycin resistant with dsRED as fluorophore, were used
434 for bacterial treatments. For each experiment bacteria were freshly plated on TY-medium and
435 incubated for 3 days at 28°C. Afterwards the inoculum culture was grown in 5 ml liquid TY-medium
436 for 48h. For inoculation, bacteria were pelleted, resuspended in sterile H₂O and diluted to a final
437 OD₆₀₀ of 0.1. Ten µl of bacteria solution per root were used to treat Arabidopsis seedling and
438 primary root length was marked after inoculation. For reproducibility the timing of bacterial pre-
439 culture growth is essential for an adequate-equal inoculum preparation.

440

441 Quantitative RT-PCR

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

454

455 Characterization of mutants and over-expression lines

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

465

466 Cloning method and selection of transgenic lines

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

504 **Accession numbers**

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

510

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521

522 **Author Contribution**

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

526

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- 784

785 **Figure Legends**

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

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

796
797 Figure 2.: Rhizobia-induced modifications on *Arabidopsis* root architecture depend
798 on nitrate availability.

799 Ten-days old seedlings were transferred on high-nitrate plant medium (5mM KNO₃) or nitrate-
800 starvation plant medium (0.1mM KNO₃) and roots were inoculated for 3 days with rhizobia (*S.*
801 *meliloti*) or (*M. loti*). (a) control seedlings grown for 3 days on high-nitrate plant medium. (b) *S.*
802 *meliloti* and (c) *M. loti* inoculated seedlings grown on high-nitrate plant medium scale bar presents
803 1 cm (starting root length, before inoculation, was marked with blue marker pen). (d)-(f)
804 Quantifications of number of lateral roots, lateral root length, and primary root length on different
805 nitrate conditions and indicated rhizobia treatment. Statistical significance was determined by
806 students *t*-test *=p<0.05; **=p<0.01.

807

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

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

819

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

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

826

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

828 Seven-days old seedlings of wild-type (Col-0), mutant plants (as indicated), or over-expression
829 lines were transferred on high-nitrate plant medium (5mM KNO_3) or low-nitrate plant medium
830 (0.25mM KNO_3) and roots were inoculated for 3 days with *M. loti*. Quantification of root hair
831 formation on primary root tip is presented. Down site representative close-up image of Col-0, and
832 root differentiation zone treated with *M. loti* for 3 days of Col-0, *nlp*-mutant (*nlp5*). and

833 *nlp5/pAtUBQ10:NLP5* line (OENLP5). Statistical significance was determined by one-factorial
834 ANOVA $\alpha=0.05$ followed by Scheffé post-hoc test.

835
836 **Figure 6.: *M. loti* impacts cell -proliferation and -elongation.**
837 Seven-days old seedlings of wild-type (Col-0) or indicated mutant plants were transferred on high-
838 nitrate plant medium or low-nitrate plant medium and roots were inoculated for 3 days with *M. loti*
839 (control = no inoculation). (a)-(j) FM4-64 staining of primary root tip and visualization using
840 confocal microscopy. (a) and (b) representative pictures of the first cells in the root elongation
841 zone (white arrowhead indicates first root hair cell), (c) and (d) pictures of primary root apical
842 meristem (white arrowhead indicates end of meristem), (a, c) control and (b, d) *M. loti* on high-
843 nitrate samples, white arrowheads showing cell size changes. Scale bar presents 100 μm . (e) -
844 (g) length of the first 5 elongated cells, of primary root samples with or without *M. loti* grown on
845 high- or low- nitrate plant medium. (h) – (j) quantification of meristem cortex cell number at the
846 primary root tip. (k) and (l) transcript levels of indicated genes quantified by qRT-PCR and
847 calculated relative to the housekeeping gene expression level of *UBQ4*. Statistical significance
848 was determined by students *t*-test **= $p<0.01$; ***= $p<0.001$.

849
850 **Figure 7.: Model of the rhizobium-induced NLP-pathway in *Arabidopsis thaliana***
851 **roots.**

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

858

859 **Supporting Information**860 **Fig. S1 Characterization of *NLP4* and *NLP5* mutations and over-expressions (OE)**861 Graphical presentation of *NLP4* and *NLP5* sequence with predicted T-DNA insertion sites (a).862 PCR-based validation of *NLP4* and *NLP5* mutation on genomic DNA using SALK-designed

863 primers, including t-DNA specific Primer LBb1.3 (b). Check for transcript levels by semi-

864 quantitative PCR using primers covering the exon of insertion (c) and qRT-PCR (d) on mutants

865 *nlp4* and *nlp5* and over-expression (OE) lines, as indicated.

866

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

868 Maximum Likelihood tree of the NLP family (model: GTR+F+R10; log-likelihood: -598245.4704).

869 Tree is rooted on the non-vascular plants. Clades are colored and named according the

870 *Arabidopsis thaliana* nomenclature. Only significant branch support values (>80%) are shown.

871

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

873 NLP sequences were retrieved using the tBALSTn algorithm v2.9.0+ by the NIN protein from the

874 model species *Medicago truncatula* as query. Reduced likelihood tree of the NLP family. Clades875 are colored and named according the *Arabidopsis thaliana* nomenclature. Only significant branch

876 support values (>80%) are shown.

877

878 **Fig. S4 Expression of other *NIN*-like proteins in response to rhizobia treatment**879 levels of indicated genes in 7-day old *Arabidopsis* root samples treated for 3-days with rhizobia *S.*880 *melliloti*, *M. loti* or in 10-day old control samples grown on high- (5mM) or low- (0.25mM) nitrate

881 plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping

882 gene expression level of *UBQ4*. Statistical significance was determined by students *t*-test

883 *=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 $\alpha=0.05$ 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 $\alpha=0.05$
899 followed by Scheffé post-hoc test

900

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

909
910 Fig. S8 Varying regulation of rhizobia-induced genes in *nlp4* and *nlp5* mutant roots.
911 Transcript levels of indicated genes in 7-day old Arabidopsis root samples treated for 3-days with
912 rhizobium *M. loti* or in 10-day old control samples grown on high- (5 mM) or low- (0.25 mM) nitrate
913 plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping
914 gene expression level of *UBQ4*. Statistical significance was determined by students *t*-test
915 $*=p<0.05$.

916
917

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	CTTCAGCTCGTTTTCTGTGCG
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	ATGAAGACTTTACGGGTCTCACACCA ^{tg} GTCCTATCACGACCTTCTGTTTG
GG-NLP4-rev1	ATGAAGACTTCAGAGGTCTCTCCTTGTGCCATAAACTGTATT ^g CAGTAGCT
Mut_NLP4_Bsal_fwd2	TAGAAGACAAaCCA ^{ACT} CAAGATACATCAGCTCT
Mut_NLP4_Bsal_rev2	TAGAAGACAATGGtGACCACATGAAGGAAGT
Mut_NLP4_Bpil_fwd3	TAGAAGACAAGAGACGAGCAGCGAAATCGAG
Mut_NLP4_Bpil_rev3	TAGAAGACAATCTcCTTCAATGCACCTCCTAC
NLP4_Bpil_fwd4	TAGAAGACAAaACAATGAATGGGTTTTGTTGACTTGC
NLP4_Bpil_rev4	TAGAAGACAATGTtTTCGTCTAAGTACTTTAGATC
GG-NLP5-fwd1	ATGAAGACTTTACGGGTCTCACACCA ^{tg} gaaaacaattctcttctatgg
GG-NLP5-rev1	ATGAAGACTTCAGAGGTCTCTCCTTt ^g aaagacatccactgacag
Mut_NLP5_Bsal_fwd2	TAGAAGACAAGccaacgcaagacacatc
Mut_NLP5_Bsal_rev2	TAGAAGACAAtggCgaccacatgaagg
Mut_NLP5_Bsal_fwd3	TAGAAGACAAGtcatgtctcaaca ^a agc

Mut_NLP5_Bsal_rev3 TAGAAGACAAtgaCacccatgtttgagc
NLP5_Bpil_fwd4 TAGAAGACAAAacaatgaatgggtttg
NLP5_Bpil_rev4 TAGAAGACAAtgtTtcatctaagtatttcagatcg

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

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

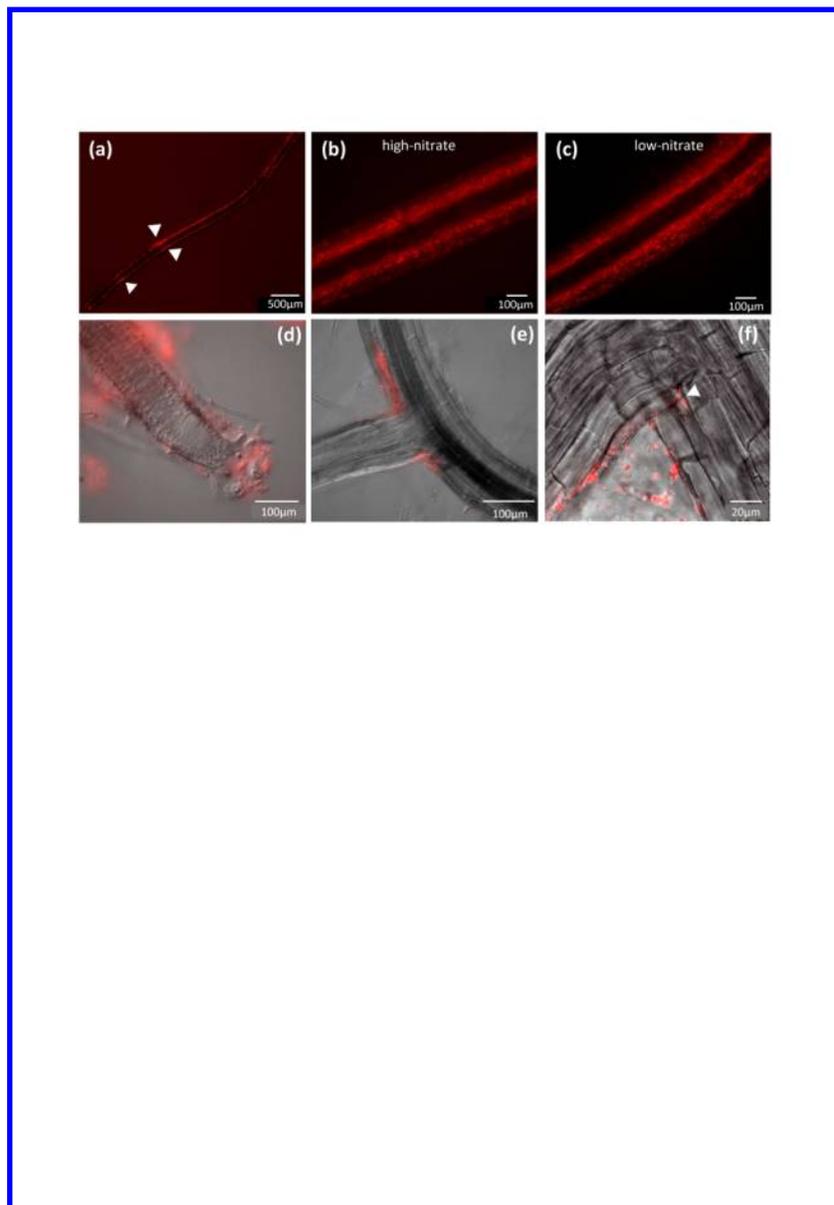


Figure 1.: Rhizobia attachment on roots of non-legume host plant *Arabidopsis thaliana*. Ten-days old *Arabidopsis* seedlings inoculated with transgenic rhizobia (*M. loti*) tagged with dsRed fluorophore grown on plant media with 5mM nitrate. (a-c) Bacterial biofilm formation on growing *Arabidopsis* 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).

190x275mm (600 x 600 DPI)

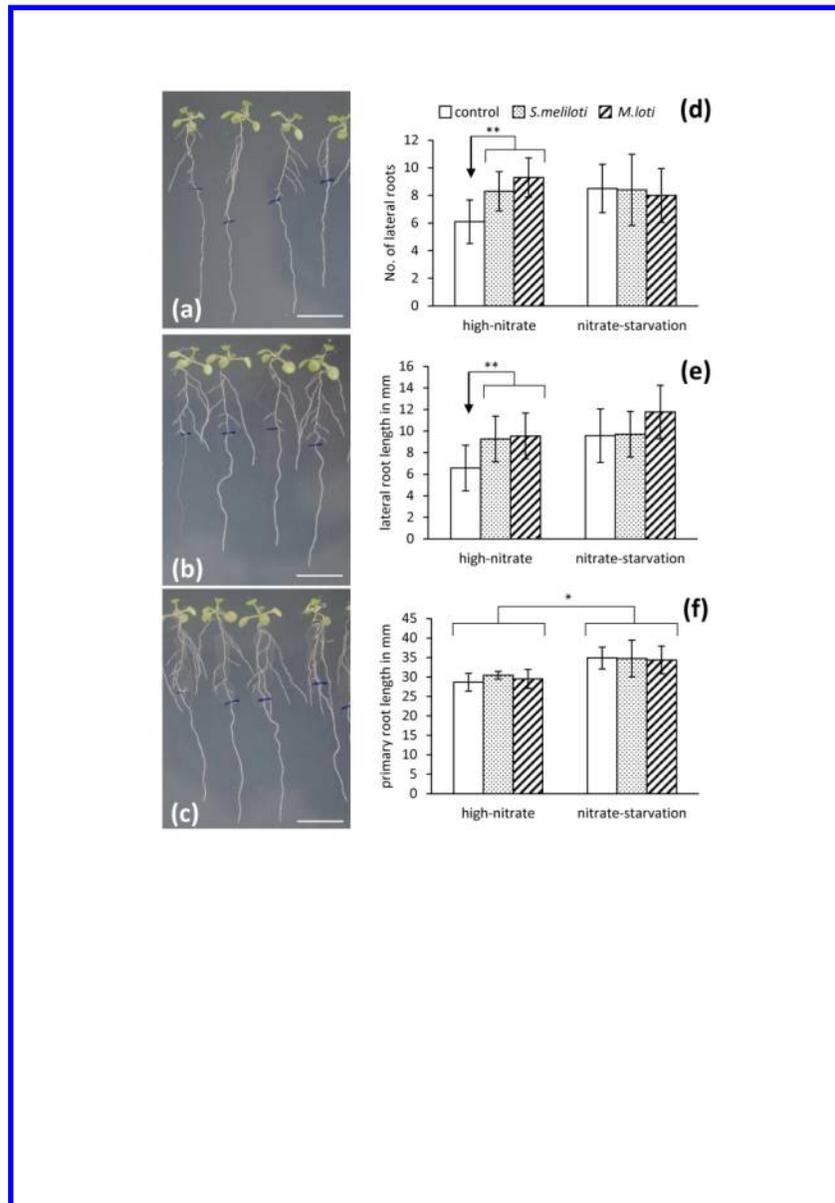


Figure 2.: Rhizobia-induced modifications on Arabidopsis root architecture depend on nitrate availability. Ten-days old seedlings were transferred on high-nitrate plant medium (5mM KNO₃) or nitrate-starvation plant medium (0.1mM KNO₃) and roots were inoculated for 3 days with rhizobia (*S. meliloti*) 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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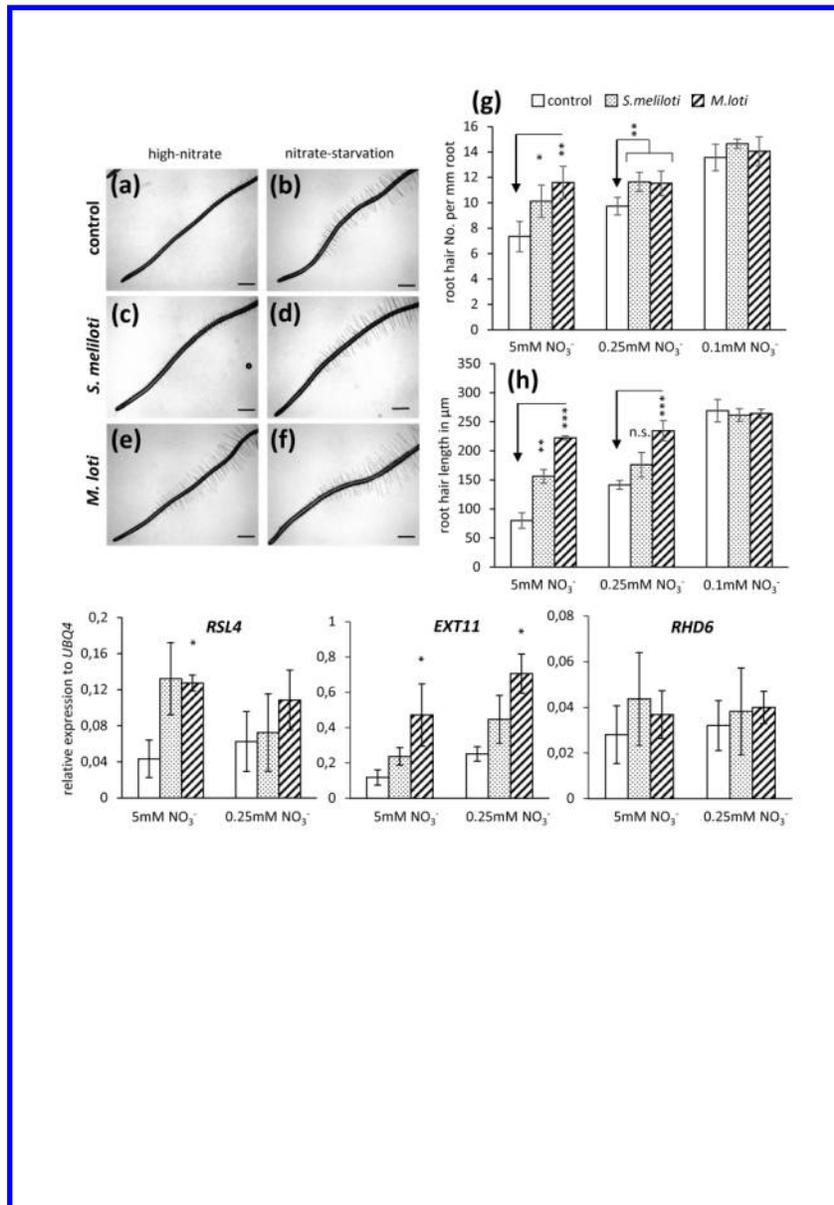


Figure 3.: Rhizobia-induced root hair formation depends on high-nitrate availability. Seven-days old seedlings were transferred on high-nitrate plant medium or nitrate-starvation plant medium and roots were inoculated for 3 days with rhizobia (*S. meliloti*) or (*M. loti*). Primary root tip of control 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.

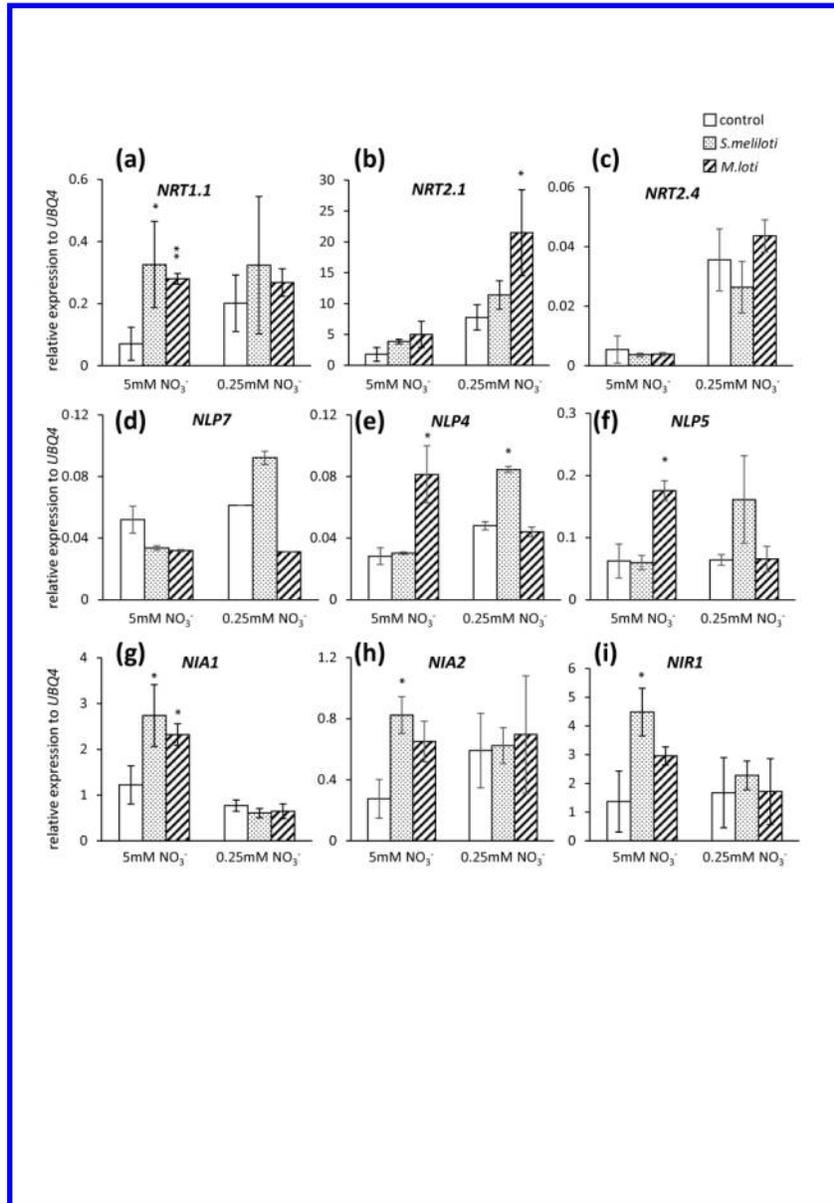


Figure 4.: Rhizobia-induced expression of nitrate-regulated genes. Transcript levels of indicated genes in 7-day old *Arabidopsis* root samples treated for 3-days with rhizobia *S. meliloti*, *M. loti* or in 10-day old control samples grown on high- (5mM) or low- (0.25mM) nitrate plant medium conditions. Quantified by qRT-PCR and calculated relative to the housekeeping gene expression level of UBQ4. Statistical significance was determined by students t-test *= $p < 0.05$; **= $p < 0.01$.

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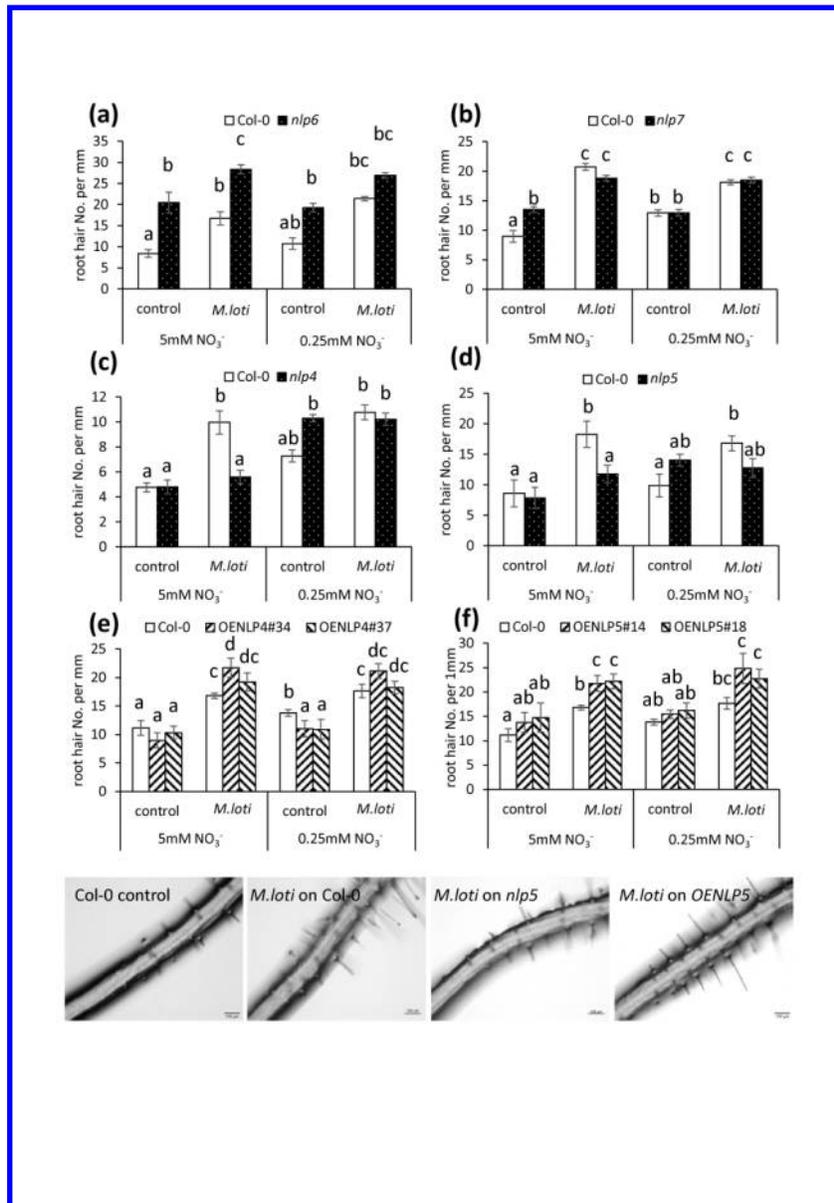


Figure 5.: Specific NIN-like Proteins are involved in *M. loti* induced root hair effect. Seven-days old seedlings of wild-type (Col-0), mutant plants (as indicated), or over-expression lines were transferred on high-nitrate plant medium (5mM KNO₃) or low-nitrate plant medium (0.25mM KNO₃) and roots 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 $\alpha=0.05$ followed by Scheffé post-hoc test.

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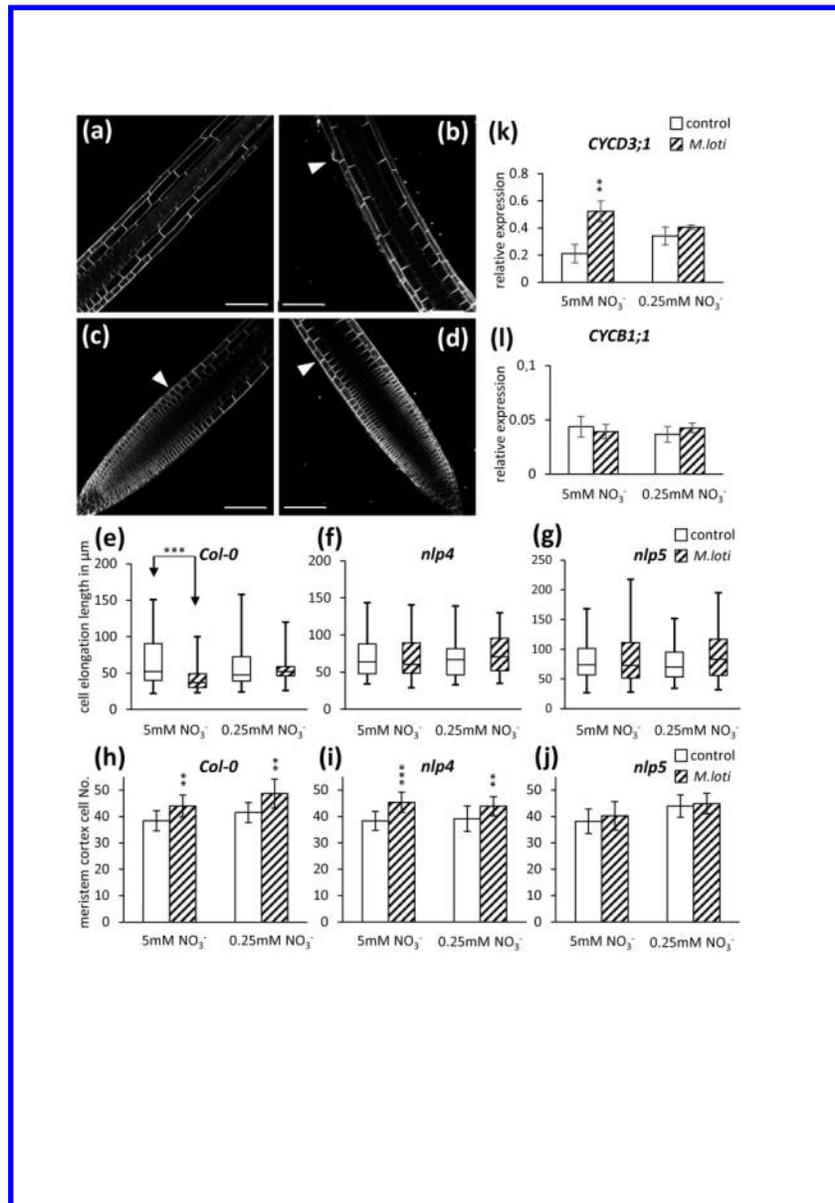


Figure 6.: *M. loti* impacts cell -proliferation and -elongation.

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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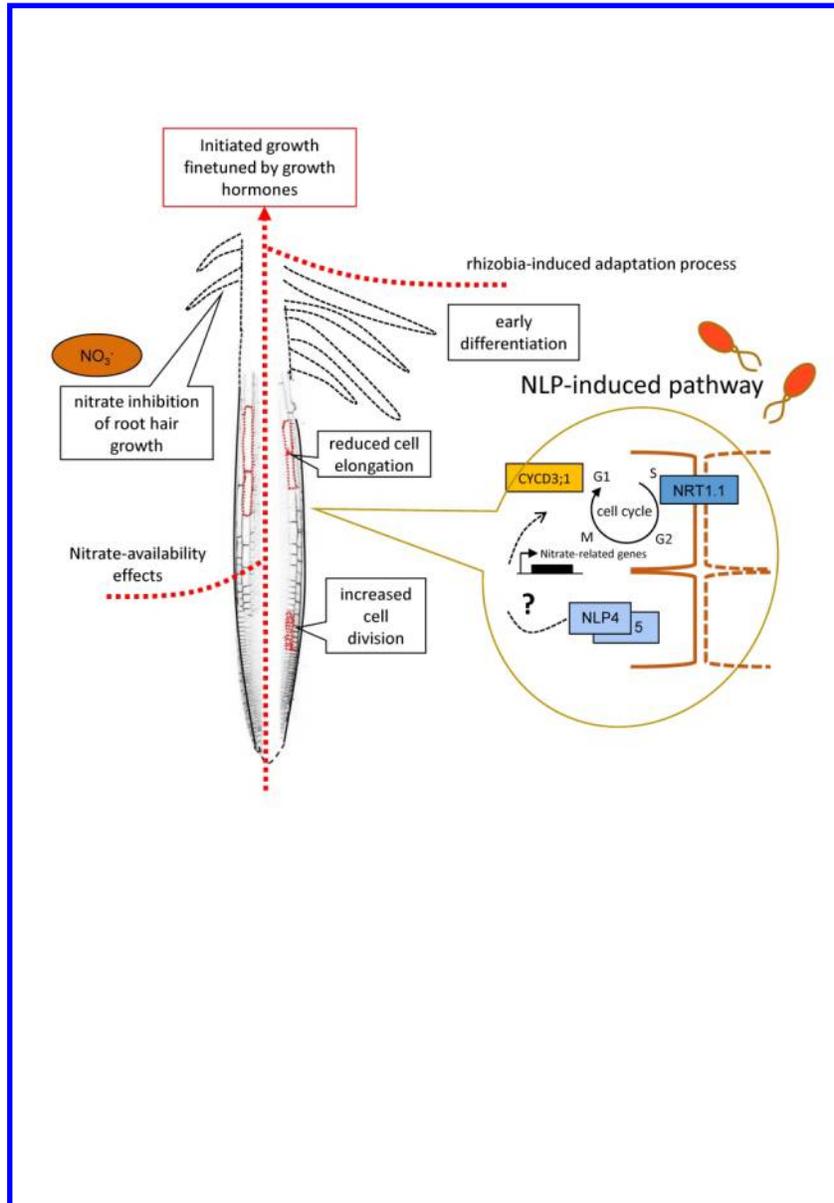


Figure 7.: Model of the rhizobium-induced NLP-pathway in *Arabidopsis thaliana* roots. Results obtained indicate rhizobia-induced local root developmental effects on the non-legume host *Arabidopsis thaliana*. We demonstrate an involvement of key NIN-like proteins and show an induction of nitrate signaling 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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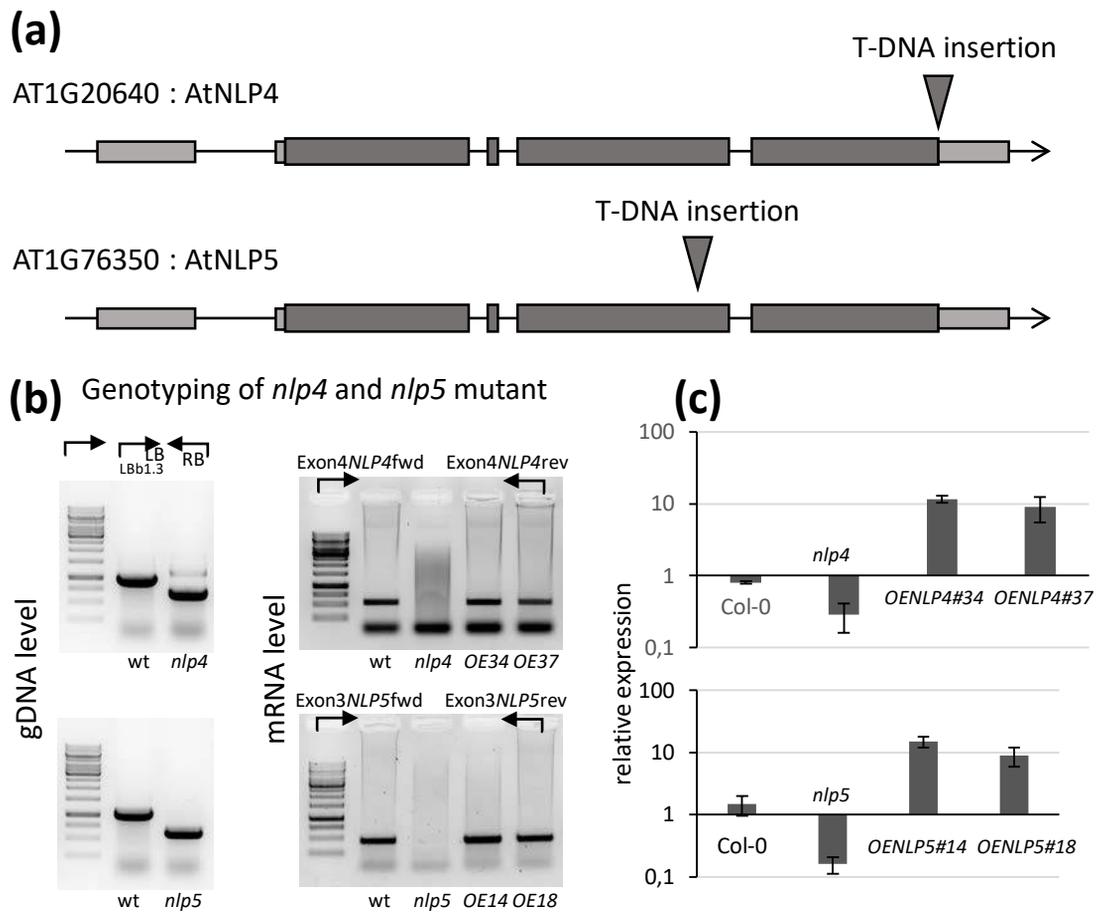


Fig. S1 Characterization of *NLP4* and *NLP5* mutations and over-expressions (OE)

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.

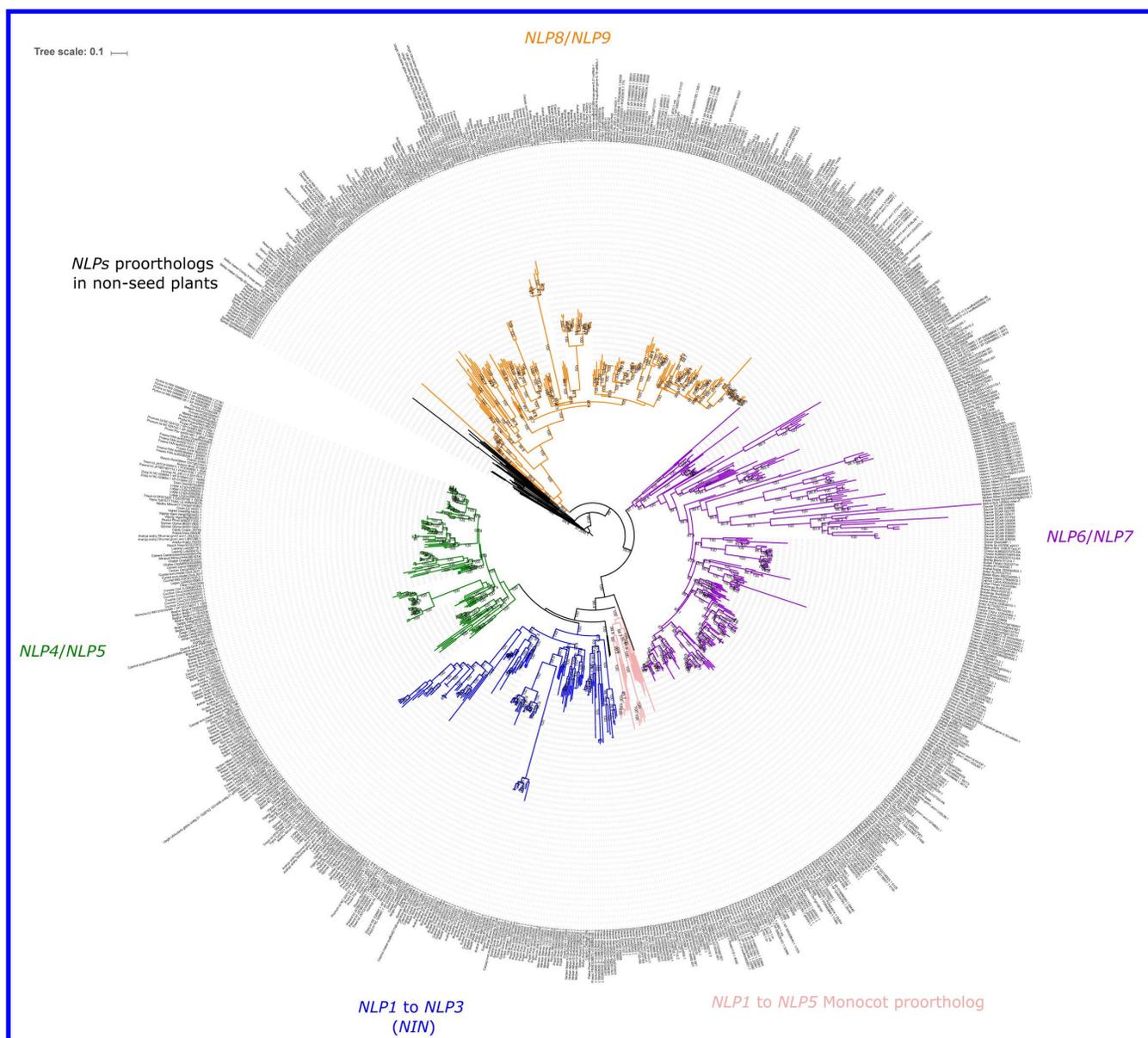


Fig. S2 Phylogenetic tree of NIN-like Proteins (main tree).

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 to the *Arabidopsis thaliana* nomenclature. Only significant branch support values (>80%) are shown.

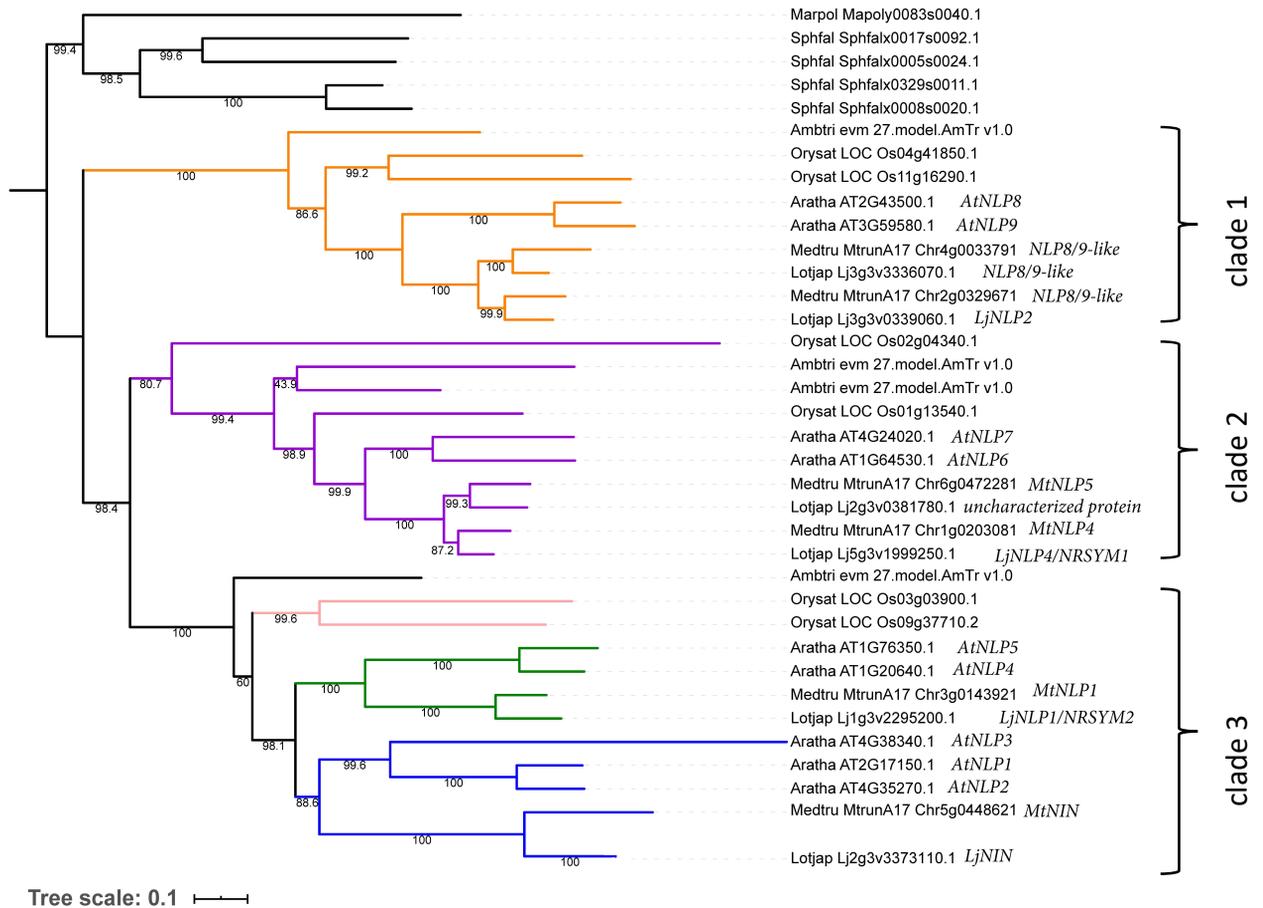


Fig. S3 Reduced phylogenetic tree of NIN-like Proteins.

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.

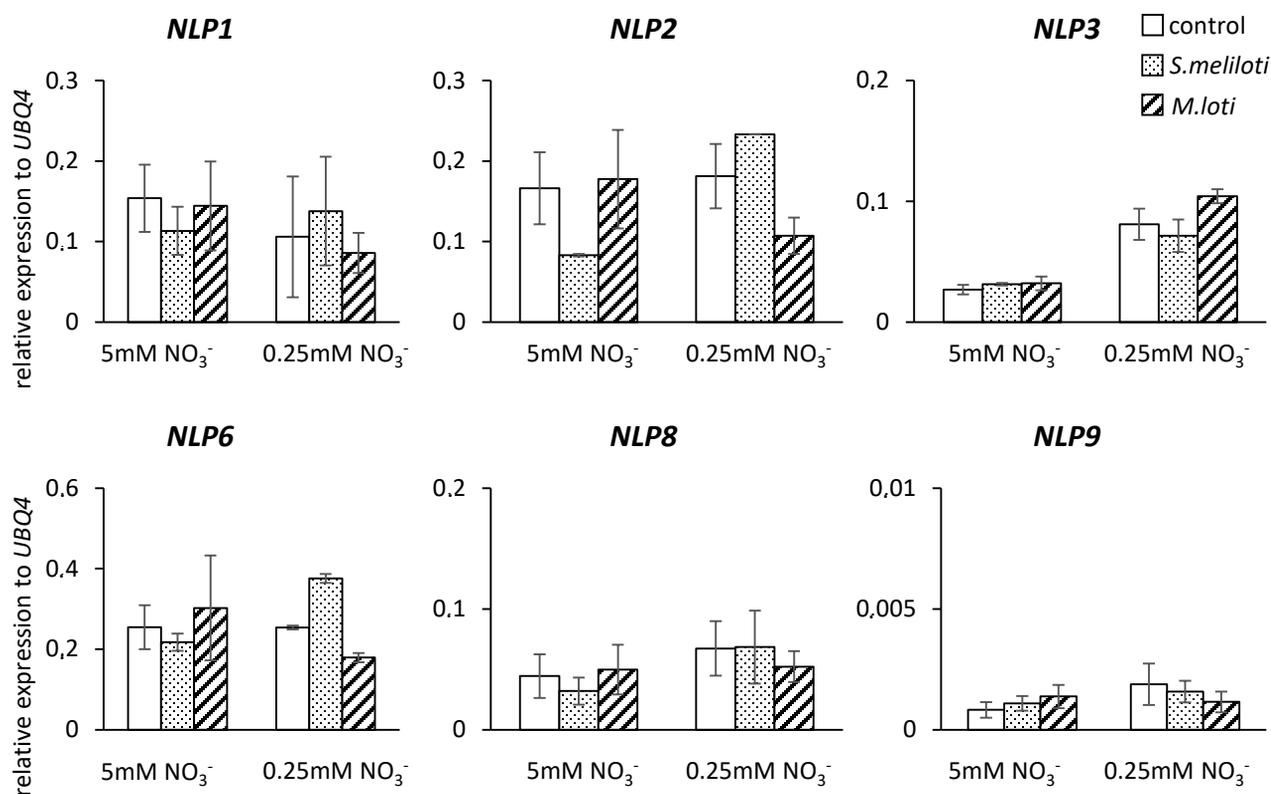


Fig. S4 Expression of other NIN-like proteins in response to rhizobia treatment.

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 *= $p < 0.05$; **= $p < 0.01$.

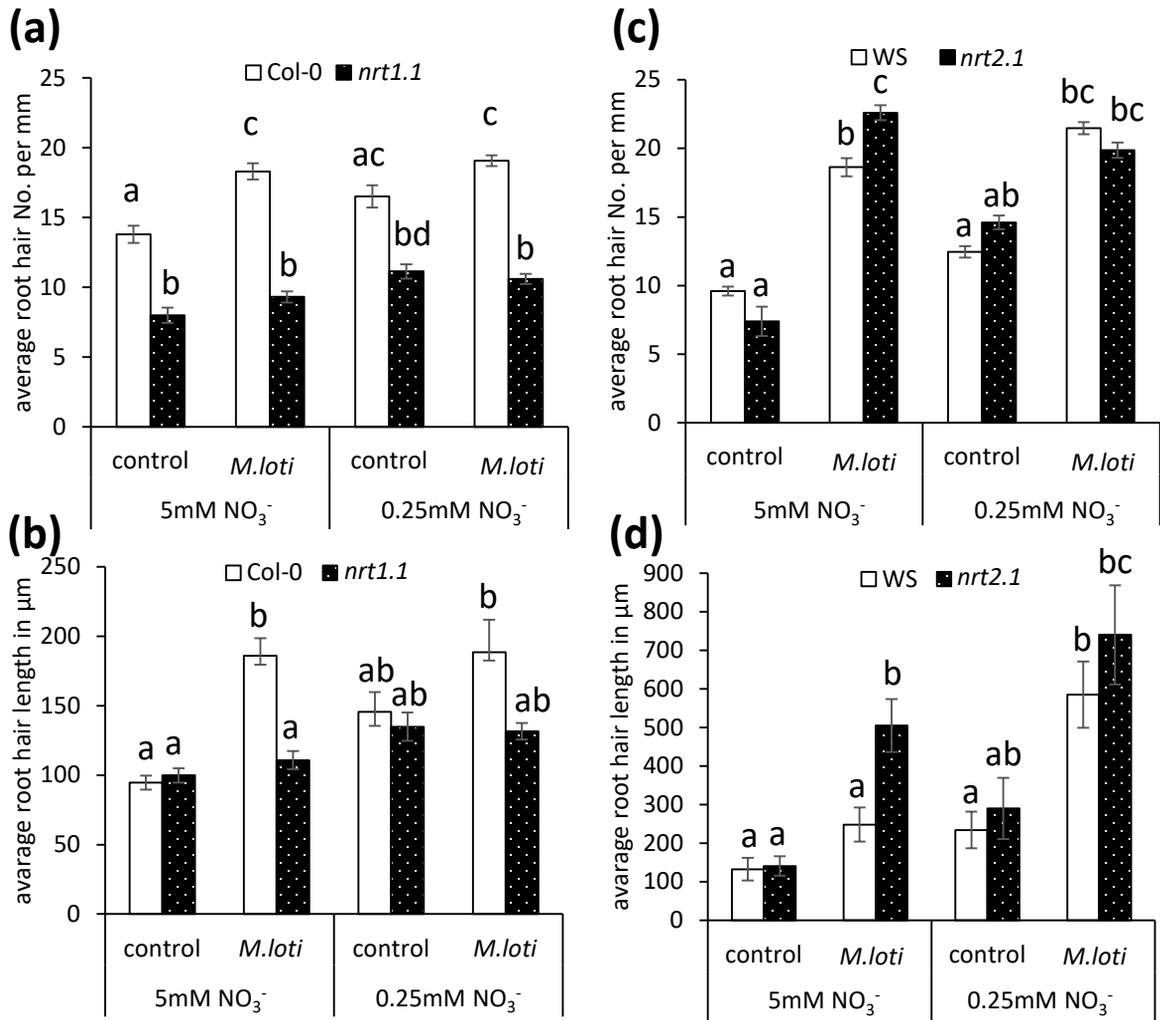


Fig. S5 Dual-affinity-transceptor NRT1.1 is needed for *M. loti*-induced impact on root morphology, but not NRT2.1.

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 $\alpha=0.05$ followed by Scheffé post-hoc test.

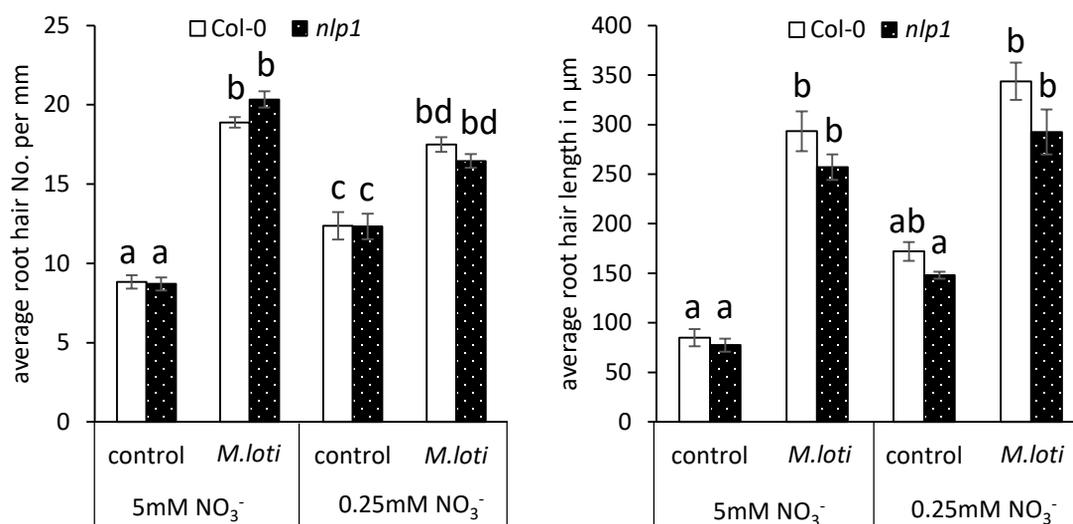


Fig. S6 A *NLP1* mutation is not influencing rhizobial effects on root hair development.

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 $\alpha=0.05$ followed by Scheffé post-hoc test

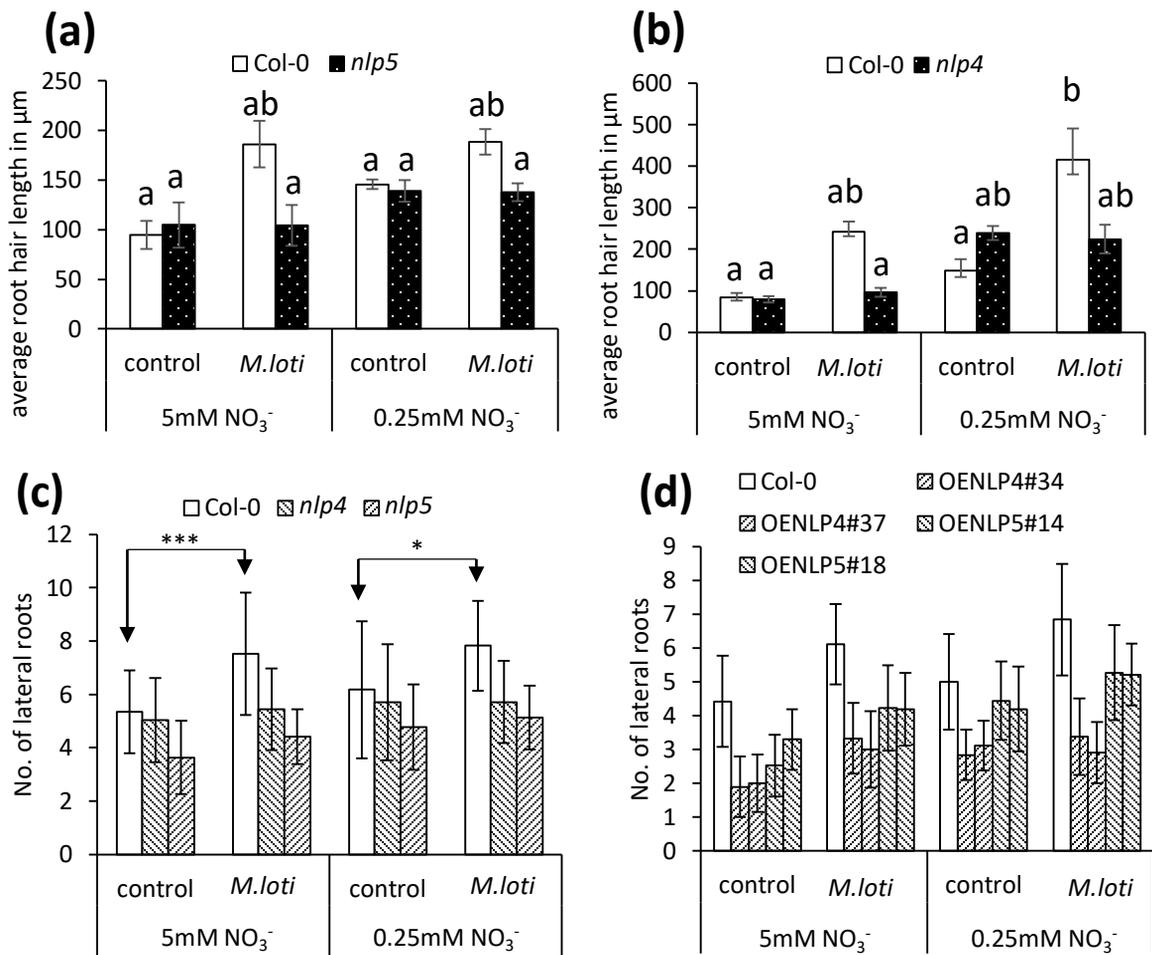


Fig. S7 AtNLP4 and AtNLP5 are involved in *M. loti* induced root modifications.

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. Statistical significance was determined by one-factorial ANOVA $\alpha=0.05$ 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 $*=p<0.05$; $***=p<0.001$.

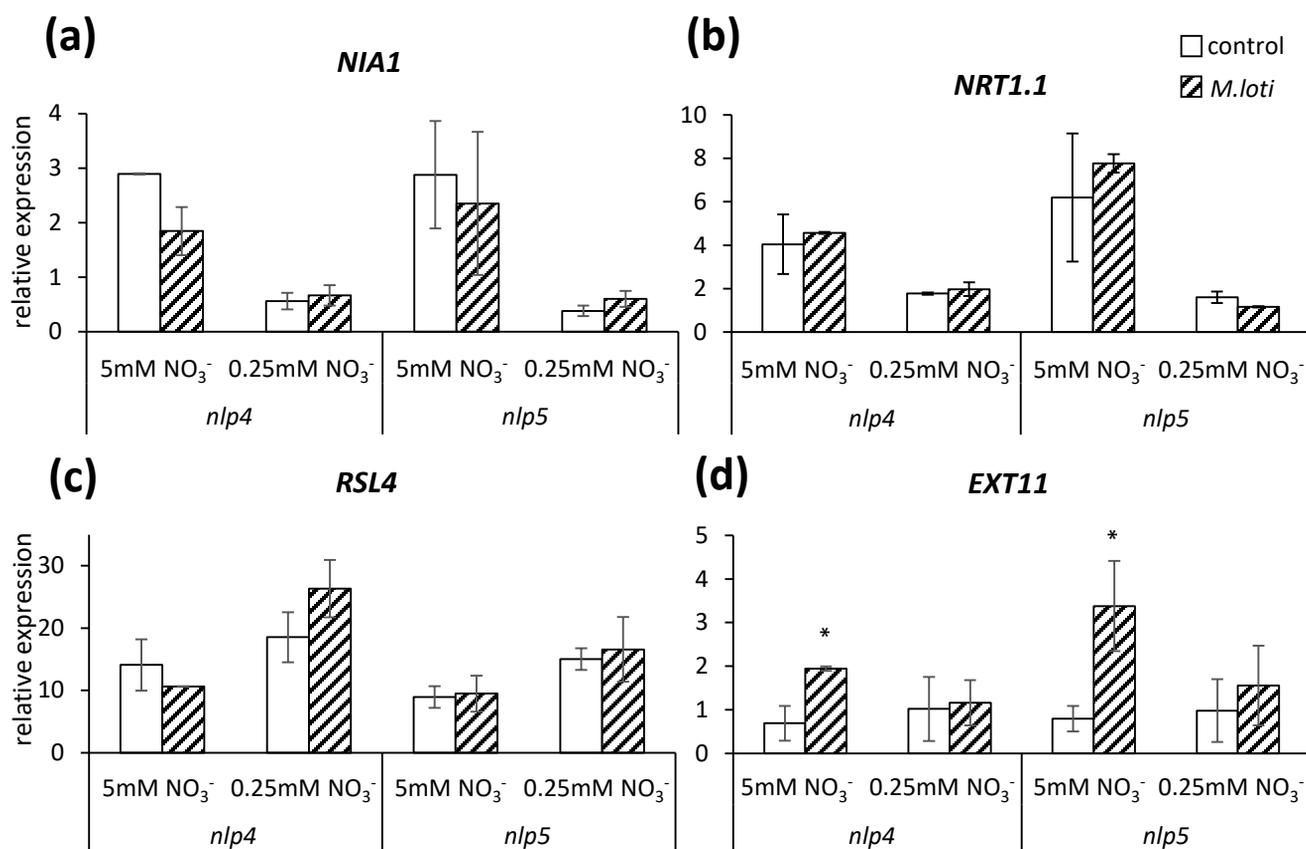


Fig. S8 Varying regulation of rhizobia-induced genes in *nlp4* and *nlp5* mutant roots.

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 $*=p<0.05$.