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The expression of *LjROP4* is required for rhizobial infection in *Lotus japonicus*

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ABSTRACT

Increasing evidence suggests that ROP (Rho of plant) GTPases play important roles in the rhizobium-legume symbiotic nodulation, but the molecular mechanisms on their regulation in symbiosis remain poorly understood. In this study, we showed that LjROP4 (Rho of plant 4 in *Lotus japonicus*) is involved in the symbiotic interaction between *Lotus japonicus* and *Mesorhizobium loti.* Tissue expression analysis showed that *LjROP4* expressed highly in root. Histochemical staining analysis showed that after rhizobia inoculation, GUS reporter activity increased obviously in root vascular bundle, root tip and lateral root primordia. During nodule development, GUS activity was detected in the cortex of nodule primordia and young nodules. In the mature nodules, GUS activity was detected only in the vascular bundle of mature nodules. Compared with the control, overexpression of *ROP4* and *ROP4-CA* produced much more pronounced root hair swelling and curling induced by *M. loti*. The infection event and nodule number increased obviously, which was in consistent with this promotion of root hair deformation Moreover, RNA interference (RNAi) of *LjROP4* produced opposite phenotypes. These data suggest that *LjROP4* is required for root hair deformation during rhizobial infection. Thus, our study provides important information about root hair deformation responses induced by nod factors in the early stage of symbiotic interaction.

Keywords: Symbiotic nitrogen fixation; ROP GTPases; Rhizobial infection; Nodulation

Introduction

Leguminous plants can interact with nitrogen-fixing rhizobacteria to form a new organ in the root, the nodule, where rhizobia convert atmospheric nitrogen into ammonia to provide organic nitrogenous compounds to the plant. Legumes attract rhizobial strains by the production of flavonoids and then rhizobia release nod factors (NFs), activating the symbiotic signalling pathway. The root rhizobial infection process occurs by the infection thread (IT), which guides the bacteria from root hair to nodule primordium cells. Rhizobia are released from the ITs into the cytoplasm of developing nodule cells(Oldroyd and Downie 2008; Oldroyd et al. 2011; 2013).

Plant-specific ROP (Rho of plant) GTPases, cycling between the active GTP-bound form and the inactive GDP-bound form, act as molecular switches for numerous signal transduction events (Yang et al. 2007; Craddock et al. 2012). ROP GTPases activity is regulated by different regulatory proteins such as GTPase-activating proteins (RhoGAPs), guanine nucleotide exchange factors (RhoGEFs), and guanine nucleotide solutions inhibitor (RhoGDI), which is similar to other small GTPases in eukaryotes (Nibau et al. 2006).

A growing number of studies have shown that ROPs in legumes participate in the rhizobium and plant symbiotic nodulation through regulating polarization growth and deformation of root hair, as well as rhizobial infection (Liu et al. 2010; Kiirika et al. 2012; Wang et al. 2014). In *Medicago truncatula,* MtROP10 interacting with Nod factor receptor NFP has been confirmed in regulation of root hair deformation and continuous curling during rhizobial infection (Lei et al. 2015). In *Lotus japonicus*, an

LjROP6 GTPase interacting with Nod factor receptor NFR5 has been identified. Phenotypic analysis revealed that infection events were decreased in transgenic roots harboring the LjROP6 RNAi construct, concomitant with suppressed nodulation (Ke et al. 2012). Overexpression of LjROP6-CA promoted root hair curling, the infection events and nodule number were also increased, whereas overexpression of LjROP6-DN showed the opposite phenotype. These data suggested that LjROP6 acted as a positive regulator of infection thread formation and nodulation in *L. japonicus* (Ke et al. 2016).

More and more ROP GTPases of legumes have been found to participate in symbiotic nitrogen fixation process by regulating root hair polarized growth, root hair deformation, rhizobial infection and nodulation (Liu et al. 2010; Kiirika et al. 2012; Wang et al. 2014; Lei et al. 2015). Our previous study showed that the expression level of *LjROP4* increased rapidly in roots 5 h after inoculation with *M. loti*, peaked 3 days and then remained constant up to 20 days as compared to that in uninoculated control roots (Ke et al. 2016). However, whether *LjROP4* participate in the regulation of root hair deformation or rhizobial infection was not established. In this study, the spatiotemporal expression pattern of *LjROP4* during the nodulation process of *Lotus japonicus* was examined. To clarify whether *LjROP4* was differentially expressed in tissues and whether there was a dynamic change of *LjROP4* expression during rhizobium infection and nodulation of *L. japonicus*. More root hair curling, infection event and nodule number were observed in overexpressing *ROP4*, *ROP4-CA* transgenic hairy roots, consistent with high expression levels of early nodulation

genes such as *NIN* and *ENOD40s*. In addition, *LjROP4*-RNAi resulted in less root hair curling, infection event and nodule number, which was consistent with the lack of induction of early nodulation genes. Our results showed that *LjROP4* was required for the expression of early nodulation genes, and *LjROP4* was another key positive regulator for rhizobial infection in *L. japonicus*.

Materials and methods

Plant materials and bacterial strains

In this study, *L. japonicus* (MG-20) was used to conduct the experiments. *Mesorhizobium loti* MAFF303099 and PN28 were used as inoculated rhizobium. *Agrobacterium rhizogenes* LBA1334 strain was used for the hairy root transformation.

Plant growth conditions and sampling

After sterilized with 2% sodium hypochlorite solution, the seeds were placed in sterile wet filter paper at 22 ° C for 2-4 d in the dark. Then the seedlings were transferred into sterile sand basins and covered with fresh-keeping film for acclimatization, 16 / 8 h light /dark cycle. Then the seedlings were provided with nitrogen-free Fahraeus medium. *M. loti* MAFF303099 was inoculated to 7-day-old seedlings. Four weeks after inoculation, tissues including roots, stems, leaves, flowers and nodules were collected. Liquid nitrogen was employed to quickly frozen all these tissues and then they were stored at -70 °C for RNA extraction.

RNA preparation, cDNA synthesis and qRT-PCR analysis

The MiniBEST Plant RNA Extraction Kit (TaKaRa) was used to isolate the total

RNA. The NanoVue Plus (GE) was employed to determine the mass and concentration of purified total RNA. The FastQuant RT kit (TIANGEN) was used to synthesize First-strand cDNA from 0.5 ng total RNA. The quantitative real-time PCR reaction was performed on a Roche LightCycler apparatus using SuperReal PreMix Plus (TIANGEN) following the instruction. The thermal cycle was set as follows 95 °C for 10 s, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The *UBI* (*Polyubiquintin*, AW720576) was used as a reference gene. The experiment was repeated three times for each test condition. Primers were listed in Supplementary Table S1.

Cloning and sequencing of LjROP4 and its mutants

The full-length sequence of LjROP4 (Gene ID: Lj1g3v2185280) was obtained by RT-PCR with total RNA made from roots. The RT-PCR product was then cloned into the pMD18-T vector (TaKaRa). Two LjROP4 mutants, ROP4-CA and ROP4-DN, were created by substitution of specific amino acid residues. ROP4 was locked in the active GTP-binding state by the ROP4-CA (G15V, GGT→GTT) mutant, and was locked in the inactive GDP binding state by the ROP4-DN (D121N, GAT→AAT) mutant. PCR amplification products introduced point mutations of *LjROP4* (ROP4-CA and ROP4-DN) were inserted into plasmid pMD18-T (Takara). DNA sequencing was used to verify the correctness of the PCR products.

Plasmid construction

Approximately 2.1 kb upstream sequence of the initiation codon of LjROP4 was amplified by PCR using *L. japonicus* genomic DNA, and then inserted into the *BamH*

I / *Sma* I sites of pCAMBIA1391Z vector to generate a *ProLjROP4*:GUS recombinant plasmid. The full-length CDS of ROP4, ROP4-CA and ROP4-DN were inserted into the NcoI/SpeI site of pCAMBIA1302GUS (p1302G) vector respectively. p1302G is a modified vector that GUS gene has been used to replace the original hygromycin resistance gene in pCAMBIA1302.

For the construction of LjROP4 RNAi-1, a 190-bp fragment of the 5'-untranslated region (UTR) of LjROP4 was amplified by RT-PCR. For LjROP4 RNAi-2 construction, a 173-bp fragment of the 3'-UTR was amplified by RT-PCR. The forward primers were attached to a *Sma*I or *Pst*I site and the reverse primers to a *BamH*I or *Xba*I site. The PCR product was digested with *SmaI*/*BamH*I or PstI/*Xba*I and ligated into the pCAMBIA1301-35S-int-T7 vector. The sense and antisense *LjROP4* sequences were separated by an intron sequence from the Arabidopsis (*Arabidopsis thaliana*) *Actin11* gene. The RNAi construct was placed behind the cauliflower mosaic virus 35S promoter. The recombinant plasmids were transformed into *Agrobacterium rhizogenes* LBA1334 by freeze-thaw method.

Hairy root transformation and identification

According to the existing method, transgenic hairy roots of *L. japonicus* were produced (Chen et al. 2012). In Brief, seedling hypocotyls were infected by *Agrobacterium rhizogenes* LBA1334 suspension cells. Then they were transferred to MS (Sigma) plates for two weeks till the hairy roots grew from hypocotyl to 2-3 cm. After GUS staining, the positive hairy root was allowed to develop into a root system, and the negative roots were excised. RT-PCR was then applied to further detect

positive hairy roots. Total RNA from positive hairy roots was extracted and the *LjROP4* gene was amplified. *UBI* was used as an internal reference gene. The experiment was repeated three times.

Root hair deformation, infection events and nodulation assay

Twenty hairy roots were collected from each construct after transgenic plants inoculated with PN28 for 3 days. Hairy roots with PBS buffer were placed on microscope slides protected by coverslips. An Olympus microscope was used to take image of the root hair deformation. The microscope is also used to observe and count the root hair phenotype. 2-mm root segment about 1 cm from the apex was used for statistics of root hair deformation.

After inoculation with PN28 for 7 days, forty hairy roots were collected and stained for β-galactosidase activity. An Olympus BX51 microscope under bright field illumination was used to study the position of the infection threads in hairy roots. The number of nodules was counted 4 weeks after inoculation. The experiment was repeated three times.

Histochemical staining

The *ProLjROP4*:GUS construct was employed for hairy root transformation of *L. japonicus*. Samples were taken at different stages before and after inoculation of *M. loti*. The whole hairy roots were stained overnight in GUS staining buffer at 37 °C away from light. And then they would be observed by an Olympus stereomicroscope. The experiment was repeated three times.

Data analysis

For each experiment, data from qRT-PCR results were normalized. The *Polyubiquitin* (AW720576) transcript served as an internal control. Three independent biological replicates were performed for each tested condition. All data was statistically analyzed using Microsoft Excel 2007 to calculate the mean and SE. The Student's test in Microsoft Excel 2007 is used to finish the comparisons between groups.

Results

Expression of LjROP4 gene in roots and nodules

Our previous study showed that the expression level of *LjROP4* increased rapidly in roots 5 h after inoculation with *M. loti*, peaked 3 days and then remained constant up to 20 days as compared to that in uninoculated control roots (Ke et al. 2016). We further analyzed tissue-specific expression of *LjROP4* in different organs including root, stem, leaf, flower and nodule after inoculation with *M. loti* 4 weeks by qRT-PCR. The expression of reference gene *Polyubiquitin* was analyzed by RT-PCR (Supplementary Figure S1). The data showed that *LjROP4* was differentially expressed in all test organs while highly expressed in roots (Fig. 1A). GUS activity of LjROP4pro:GUS was only detected in root vascular bundles in uninoculated transgenic hairy roots (Fig. 1B and D). After inoculation with *M. loti*, the GUS activity increased significantly in root vascular bundles and the expression was extended to root tips of primary roots, lateral root primordia (Fig. 1C and E), cortex of

the nodule primordia and young nodules (Fig. 1F and G), and vascular bundle of mature nodules (Fig. 1H). These data suggested that *LjROP4* expression was up-regulated during the early symbiotic phase. *LjROP4* may play a part in rhizobium infection and nodulation of *L. japonicus*.

LjROP4 participates in rhizobium-induced early root hair deformation

The *LjROP4* gene, belonging to the ROP GTPase group I, contains 8 exons and 7 introns. LjROP4 encodes a protein with 197 amino acid residues and 7 conserved domains (Supplementary Figure S2). We got two LjROP4 mutants named ROP4-CA and ROP4-DN to study the function of LjROP4. These gain-of-function mutants were produced by replacing specific amino acid residues. The ROP4-CA (G15V) mutant may lock ROP4 in the active GTP-bound state, while the ROP4-DN (D121N) mutant locks ROP4 in the inactive GTP-bound state.

It is generally known that ROPs play an important role in regulating the polarized growth of the root hair tip (Molendijk et al. 2001; Jones et al. 2002). Therefore, the effect of LjROP4 on root hair development was examined. The tip of root hairs was straightly extended tubular and away from primary root axis in control transgenic hairy roots (Supplementary Figure S3 A). For transgenic roots overexpressing ROP4, ROP4-CA and ROP4-DN, they formed morphologically normal root hairs similar to the control (Supplementary Figure S3 B-D). The result suggested that LjROP4 did not directly affect the polar growth and development of root hair. However, after inoculation of *M. loti* for 5 h, transgenic hairy roots

overexpressing ROP4 produced more root hair swelling and curling than the control. Overexpression of ROP4-CA generated more prominent root hair curling than the ROP4-overexpression hairy roots. Overexpression of ROP4-DN produced the same phenotype as the control (Fig. 2D). At the same time, a RNA interference construct (RNAi-1, RNAi-2) was used to silence LjROP4 in transformed hairy roots. The results showed that knockdown of LjROP4 expression also altered root hair cell morphology after 5 h of inoculation with *M. loti*. The RNAi-1 and RNAi-2 transgenic hairy roots produced less root hair curling than the control transgenic plants (Fig. 2E). Transgenic positive hairy roots were detected by GUS staining and RT-PCR (Supplementary Figure S4). These results indicated that LjROP4 play a role in the early root hair deformation response induced by rhizobium.

Overexpression and gene silencing of LjROP4 promoted and inhibited the infection process, respectively

To further determine the role of *LjROP4* in the infection process, after inoculation with PN28 for 7 d, transgenic hairy roots were stained for β-galactosidase activity to observe the infection events. Firstly, the root hair curled to form a shepherd's crutch and the bacteria were entrapped (Fig. 3A), and then the infection thread formed in the root hair and reached the root epidermis (Fig. 3B). Subsequently, the infection thread penetrated into the root cortex and the bacteria released into the nodule primordium (Fig. 3C) and the developing nodules (Fig. 3D). The number of microcolonies in root tips (infected foci) and infection threads in root hairs, primordia

and developing nodules was quantitatively evaluated. Compared to the control roots transformed with empty vector, the infected foci and infection thread number was increased in ROP4- and ROP4-CA-overexpressing roots (Fig. 3E). These observations showed that the development of the infection thread was significantly promoted by overexpression of ROP4 or ROP4-CA. The total number of infection threads in the RNAi-1 and RNAi-2 transgenic roots was reduced remarkably (Fig. 3F). The result showed that knock-down of LjROP4 might prevent the formation of infection thread. Our results indicated that LjROP4 act as a positive regulator in the infection process.

Overexpression and gene silencing of LjROP4 promoted and suppressed nodulation respectively

We evaluated the number of nodules of transgenic hairy roots that overexpressed ROP4 or its mutants 4 weeks after inoculation. The morphology of the nodules formed on the roots overexpressing ROP4 or ROP4-CA, ROP4-DN was similar to that formed in the control roots (Supplementary Figure S5). However, the average nodule number per plant in plants overexpressing ROP4 (8.6 ± 1.47) or ROP4-CA (11.8 ± 3.15) was significantly more than control plants transformed with empty vector (5.7 ± 1.12) (Fig. 4A, Supplementary Table S2). There was no significant difference between ROP4-DN and the control. While the number of nodules in LjROP4 RNAi plants reduced significantly compared to the control plants (Fig. 4C, Supplementary Table S3). The morphology of the nodules formed on the LjROP4

RNAi roots was similar to that formed in the control roots (Supplementary Figure S6).

The qRT-PCR analysis of the three symbiotic marker genes (*NIN*, *ENOD40-1* and *ENOD40-2*) (Kang et al. 2011) showed that overexpressing ROP4 or ROP4-CA up-regulated the expression levels of the three genes while knock-down of *LjROP4* expression down-regulated them. The transgenes were overexpressed or knock-downed in the corresponding hairy roots was confirmed by real-time PCR analysis (Fig. 4B and 4D).

Discussion

ROPs in legumes may play a role in symbiotic signaling pathway. Based on this assumption, the expression profile of *LjROP4* gene during nodulation was examined. In the early stage of nodulation, the mRNA expression level of *LjROP4* was up-regulated in the inoculated roots. Tissue-specific expression analysis indicated that LjROP4 was highly expressed in root, followed by nodule, stem, leaf, and flower (Fig. 1A). GUS staining analysis was used to analyze the expression pattern of *LjROP4* promoter and the result indicated that after inoculation of *M. loti*, *LjROP4* was mainly expressed in root tips of primary roots, lateral root primordia, the cortex of nodule primordia and young nodules (Fig. 1B-H). From these data, it could be concluded that *LjROP4* expression was up-regulated during the early symbiotic stage. The high expression of *LjROP4* might be required for rhizobial infection and nodule initiation in *L. japonicus*.

Swelling or curling of root hair was a unique developmental process in response

to rhizobial inoculation (Esseling et al. 2003). The rhizobia aggregate into the infection thread and then reach the nodule primordia, release into mature nodules finally (Oldroyd et al. 2008; 2011; 2013). More curling root hairs can be observed on ROP4- and ROP4-CA-overexpression hairy roots inoculated with rhizobia in *L. japonicus* (Fig. 2). The infection event and nodule number increased obviously, which was in consistent with this promotion of root hair deformation (Fig. 3E and Fig. 4A). Thus, the increased infection event and nodule number in ROP4- and ROP4-CA-overexpression hairy roots might be caused by increased curling root hairs.

In *Oryza sativa*, the CERK1 receptor can phosphorylate RacGEF1 and the phosphorylated RacGEF1 functions as an activator of Rac1 (Akamatsu et al. 2013). In *M. truncatula*, overexpression of RopGEF2 showed short swollen root hairs, and RopGEF2 could interact with ROP10 in yeast cells (Riely et al. 2011). The GTP-bound ROP10 activated by GEF2 interacts with the NF receptors NFP and LYK3 to regulate the processing of key cellular signals. Overexpression of ROP10 or ROP10CA promotes a permanent interaction between activated ROP10 and the NF receptors, generating multiple polar growth sites at the PM and extensive root hair deformations (Lei et al. 2015). Therefore, we speculate that overexpression of ROP4-CA may promote a permanent interaction between activated ROP4 and downstream effectors, resulting in continue root hair deformation inoculated with rhizobia. This hypothesis also can explain the nodulation phenotype in the hairy roots of *LjROP4* RNAi reasonably. We concluded that less root hair curl was produced by knockdown of *LjROP4* expression and the total number of infection events and

nodules was significantly reduced (Fig. 3F and Fig. 4C). When RNAi-1 and RNAi-2 hairy roots were stimulated by rhizobia signals, 60-80% inhibition of *LjROP4* was not sufficient to continuously activate downstream effectors, resulting in nodule signaling pathways inhibition.

In addition, overexpression of ROP4-DN presents a similar phenotype compared with wild type and has no obvious defect in root hair deformation and subsequent nodulation, suggesting that the ROP GTPases are functionally redundant in *L. japonicus*. This hypothesis is supported by the similar result that overexpression of ROP3-CA, ROP5-CA, and ROP6-CA causes unobvious depolarization growth of root hairs in *M. truncatula* (Riely et al. 2011).What's interesting is that our previous studies showed that overexpression of ROP6-DN inhibited root hair deformation and nodulation. It can be possibly explained that different ROPs have different regulatory mechanisms in the nodule signaling pathway in *L. japonicus*.

Nodule initiation (NIN) is sufficient to promote cytokinin signaling in the root epidermis and nodular organogenesis in the inner root cortex. *NIN* activates the cortical process leading to nodular organogenesis, but inhibits further induction of NF responses in the root epidermis (Vernié et al. 2015). *ENOD40-1* was expressed in the early stages of bacterial infection and *ENOD40-2* was expressed in mature nodules (Kumagai et al. 2006). In association with the down-regulation of *LjROP4*, the transcription levels of *NIN*, *ENOD40-1* and *ENOD40-2* were reduced compared to control hairy roots (Fig. 4D). These observations indicate that down-regulated expression of *LjROP4* in the RNAi hairy roots has an inhibitory effect on the

expression of other nodulation-related genes, possibly leading to impaired IT initiation and nodule formation. *LjROP4* is upstream of these three marker genes in the nodule signaling pathway. The transcriptional level of *LjROP6* did not change significantly in control, LjROP4-RNAi or overexpressing ROP4-DN hairy roots (Supplementary Figure S7). The result showed that *LjROP6* was not regulated by *LjROP4*, and they might be independent regulators of nodulation.

In this report, we demonstrated that LjROP4 plays an important role in early symbiotic signal transduction and nodule organogenesis in *L. japonicus*. Thus, this work added a new interesting player to the establishment of Rhizobium-legume symbiosis and opened a new avenue for future research in the fields of ROP signaling and biological nitrogen fixation. In future studies, we will look for effector proteins of ROP4 and elucidate their mechanisms in the process of symbiotic signaling.

Conflict of interest

All authors confirm that there is no financial conflict of interest regarding this study

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References

Akamatsu, A., Wong, H.L., Fujiwara, M., Okuda, J., Nishide, K., Uno, K., Imai, K., Umemura, K., Kawasaki, T., Kawano, Y., and Shimamoto, K. 2013. An OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 module is an essential early component of chitin-induced rice immunity. Cell Host Microbe. **13**(4): 465-476.

Chen, T., Zhu, H., Ke, D., Cai, K., Wang, C., Gou, H., Hong, Z., and Zhang, Z. 2012. A MAP kinase kinase interacts with SymRK and regulates nodule organogenesis in Lotus japonicas. Plant Cell. **24**(2): 823-838.

Craddock, C., Lavagi, I., and Yang, Z. 2012. New insights into Rho signaling from plant ROP/Rac GTPases. Trends Cell Biol. **22**(9): 492-501.

Esseling, J.J., Lhuissier, F.G., and Emons, A.M. 2003. Nod factor-induced root hair curling: Continuous polar growth towards the point of nod factor application. Plant Physiol. **132**(4): 1982-1988.

Jones, M.A., Shen, J.J., Fu, Y., Li, H., Yang, Z., and Grierson, C.S. 2002. The Arabidopsis Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. Plant Cell. **14**(4): 763-776.

Kang, H., Zhu, H., Chu, X., Yang, Z., Yuan, S., Yu, D., Wang, C., Hong, Z., and Zhang, Z. 2011. A novel interaction between CCaMK and a protein containing the Scythe_N ubiquitin-like domain in Lotus japonicus, Plant Physiol. **155**(3): 1312-1324.

Ke, D., Fang, Q., Chen, C. Zhu, H., Chen, T., Chang, X., Yuan, S., Kang, H., Ma, L., Hong, Z., and Zhang, Z. 2012. The small GTPase ROP6 interacts with NFR5 and

is involved in nodule formation in Lotus japonicus. Plant Physiol. **159**(1): 131-143.

Ke, D., Li, X., Han, Y., Cheng, L., Yuan, H., and Wang, L. 2016. ROP6 is involved in root hair deformation induced by Nod factors in Lotus japonicus. Plant Physiol. Bioch. **108**: 488-498.

Kiirika, L.M., Bergmann, H.F., Schikowsky, C., Wimmer, D., Korte, J., Schmitz, U., Niehaus, K., and Colditz F. 2012. Silencing of the Rac1 GTPase MtROP9 in Medicago truncatula stimulates early mycorrhizal and oomycete root colonizations but negatively affects rhizobial infection, Plant Physiol. **159**(1): 501-516.

Kumagai, H., Kinoshita, E., Ridge, R.W., and Kouchi, H. 2006. RNAi knock-down of ENOD40s leads to significant suppression of nodule formation in Lotus japonicas. Plant Cell Physiol. **47**(8): 1102-1111.

Lei, M.J., Wang, Q., Li, X., Chen, A., Luo, L., Xie, Y., Li, G., Luo, D., Mysore, K.S., Wen, J., Xie, Z.P., Staehelin, C., and Wang, Y.Z. 2015. The small GTPase ROP10 of Medicago truncatula is required for both tip growth of root hairs and Nod factor-induced root hair deformation. Plant Cell. **27**(3): 806-822.

Liu, W., Chen, A.M., Luo, L., Sun, J., Cao, LP., Yu, G.Q., Zhu, J.B., and Wang, Y.Z. 2010. Characterization and expression analysis of Medicago truncatula ROP GTPase family during the early stage of symbiosis. J Integr Plant Biol. **52**(7): 639-652.

Molendijk, A.J., Bischoff, F., Rajendrakumar, C.S., Friml, J., Braun, M., Gilroy, S.,

and Palme, K. 2001. Arabidopsis thaliana Rop GTPases are localized to tips of root hairs and control polar growth. EMBO J. **20**(11): 2779-2788.

Nibau, C., Wu, H.M., and Cheung, A.Y. 2006. RAC/ROP GTPases: 'hubs' for signal integration and diversification in plants. Trends Plant Sci. **11**(6): 309-315.

Oldroyd, G.E., and Downie, J.A. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol. **59**: 519-546.

Oldroyd, G.E., Murray, J.D., Poole, P.S., and Downie, J.A. 2011.The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet. **45**: 119-144.

Oldroyd, G.E. 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol. **11**(4): 252-263.

Riely, B.K., He, H., Venkateshwaran, M., Sarma, B., Schraiber, J., Ané, J.M., and Cook, D.R. 2011. Identification of legume RopGEF gene families and characterization of a Medicago truncatula RopGEF medi-ating polar growth of root hairs. Plant J. **65**(2): 230-243.

Vernié, T., Kim, J., Frances, L., Ding, Y., Sun, J., Guan, D., Niebel, A., Gifford, M.L., de Carvalho-Niebel, F., and Oldroyd, G.E. 2015. The NIN Transcription Factor Coordinates Diverse Nodulation Programs in Different Tissues of the Medicago truncatula Root. Plant Cell. **27**(12): 3410-3424.

Wang, Q., Lei, M., Chen, A., Wang, R., Li, G., and Wang, Y. 2014. MtROP8 is involved in root hair development and the establishment of symbiotic interaction between Medicago truncatula and Sinorhizobium meliloti. Chin. Sci. Bull. **59**(32): 4289-4297.

Yang, Z., and Fu Y. 2007. ROP/RAC GTPase signaling. Curr Opin Plant Biol. **10**(5): 490-494.

Figure captions:

Figure 1. Expression pattern and histochemical localization of LjROP4 in *Lotus japonicus*.

(A) *LjROP4* expression in various plant tissues, including root, stem, leaf, flower and nodule. The *Polyubiquitin* (AW720576) transcript served as an internal control. Three independent biological replicates were performed for each tested condition. Error bars indicate SE. (B-H) Histochemical localization of ProROP4::GUS expression before and after inoculation with *M. loti*. ProROP4:GUS was weakly expressed in root tips (B) and lateral roots (D) without inoculation. After inoculation, ProROP4::GUS was highly expressed in roots including the root tips (C) and the apical region of the lateral root (E). GUS staining was intense in the cortex of nodule primordia (F) and developing nodules (G), and became very weak in mature nodules (H). n =20, Bars = 100 μm.

Figure 2. Root hair deformation phenotypes in transgenic hairy roots after inoculation with *M. loti*. (A), (B) and (C) Bright-field images of typical root hair deformation phenotypes. The positions of root hair deformation were indicated by arrows. Bars = 50 μm. (D) Root hair deformation events in transgenic hairy roots overexpressing ROP4 and its mutants (ROP4-CA and ROP4-DN) were observed and counted in 1cm root segments of twenty primary hairy roots at a 10× microscopic magnification after inoculation with *M. loti*. Empty vector (p1301U) served as control. (E) Effects of ROP4-RNAi on root hair deformation in transgenic hairy roots after inoculation with *M. loti*. Empty vector (pCAMBIA1301-35S-int-T7) served as control. Root hair

deformation events in ROP4-RNAi transgenic hairy roots were observed and counted in 1cm root segments of twenty primary hairy roots at a 10× microscopic magnification after inoculation with *M. loti*. Error bars indicate SE. Statistical significance (*P < 0.01, **p < 0.001)) was evaluated by Student's t test.

Figure 3. Rhizobial infection phenotypes in transgenic hairy roots after inoculation with *M. loti*. Transgenic hairy roots were inoculated with PN28. Rhizobial infection phenotypes were scored based the place where the IT tips located at: the curled root hairs (A), the root epidermal cells (B), the nodule primordia (C) and the nodule (D). Arrows indicate the location of the IT tips. Bars = 50 μm. (E) Frequencies of infection events in transgenic hairy roots expressing empty vector p1301U (Control), or overexpressing ROP4 and its mutants (ROP4-CA and ROP4-DN). n = 30. (F) Frequencies of infection events in transgenic hairy roots expressing empty vector pCAMBIA1301-35S-int-T7 (Control), or ROP4 RNAi-1 and RNAi-2. n = 30. Error bars indicate SE. Statistical significance (*P < 0.01, **p < 0.001)) was evaluated by Student's t test.

Figure 4. Effect of overexpression and RNAi of LjROP4 on nodulation in *L. japonicus*. (A) Mean numbers of nodules per plant expressing empty vector (Control), or overexpressing ROP4, ROP4-CA and ROP4-DN with standard deviation (SD) post inoculation with *M. loti* for 4 weeks. n = 30. (B) qRT-PCR analysis of transcript levels of *ROP4*, *NIN*, *Enod40-1* and *Enod40-2* in control, or overexpressing ROP4, ROP4-CA and ROP4-DN hairy roots. The *Polyubiquitin* (AW720576) transcript served as an internal control. Three independent biological replicates were performed

for each tested condition. (C) Mean numbers of nodules per plant expressing the empty vector (Control), or RNAi-1 and RNAi-2 post inoculation with *M. loti* for 4 weeks. n = 30. (D) qRT-PCR analysis of transcript levels of *ROP4*, *NIN*, *Enod40-1* and *Enod40-2* in control or RNAi-1 and RNAi-2 hairy roots. The *Polyubiquitin* (AW720576) transcript served as an internal control. Error bars indicate the mean of 3 biological replicates with SE. Statistical significance (*P < 0.01, **p < 0.001) was evaluated by Student's t test.

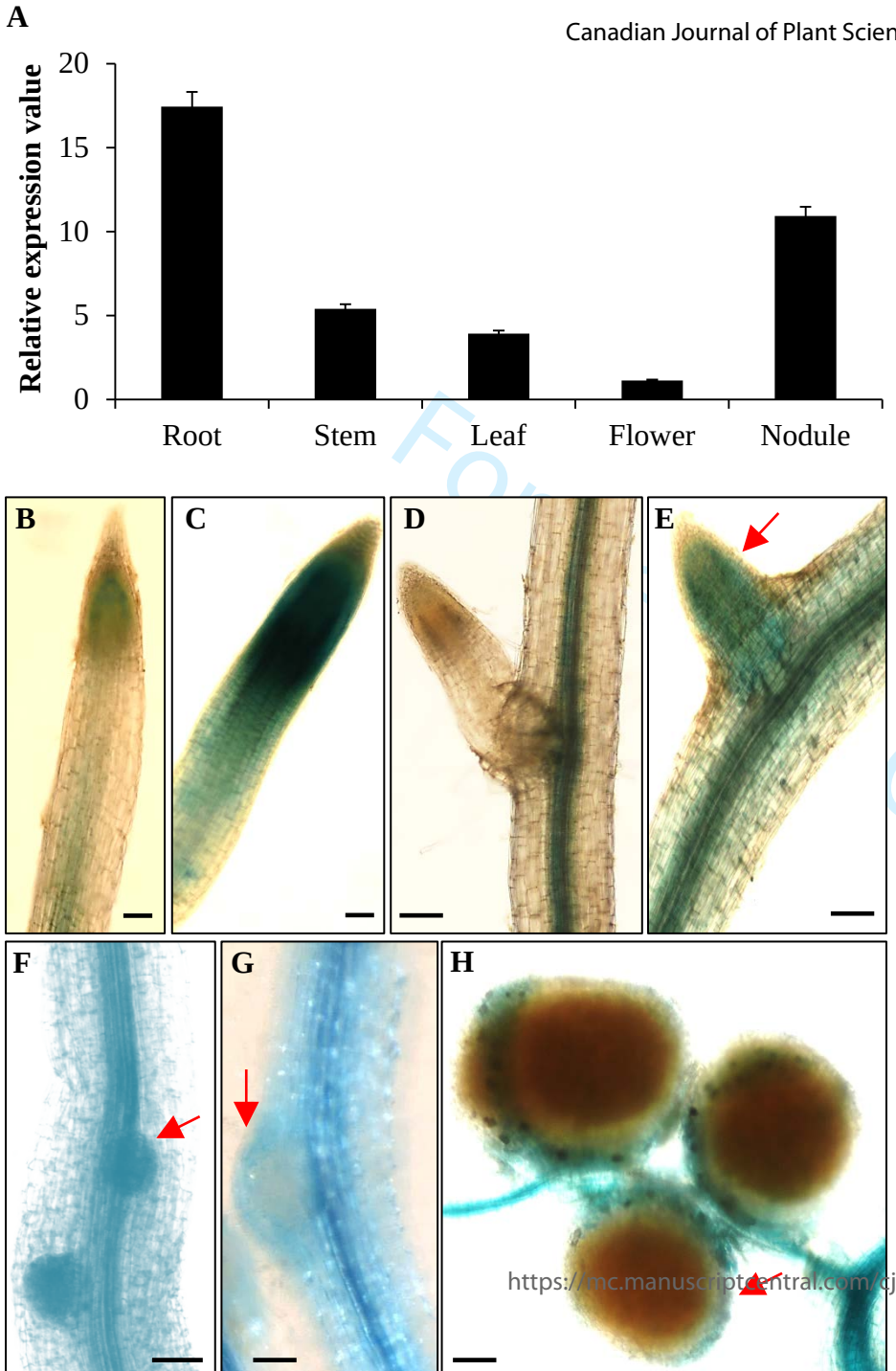
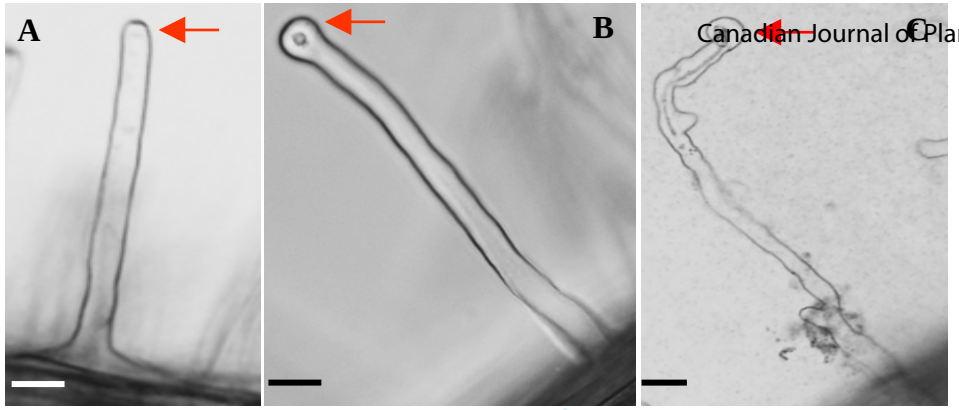
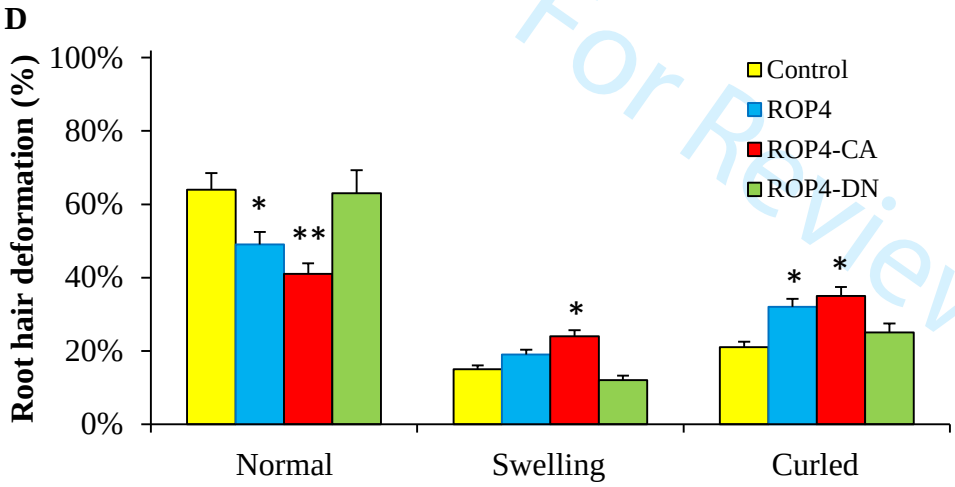


Figure 1





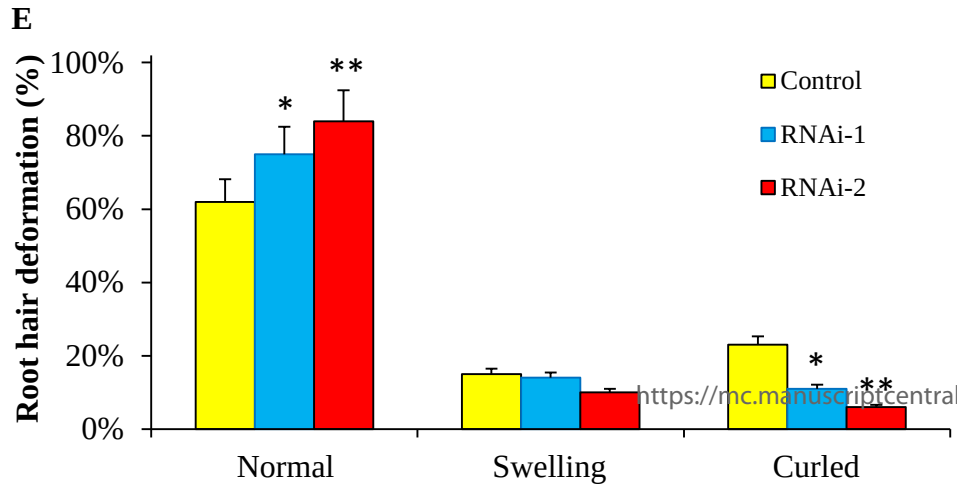
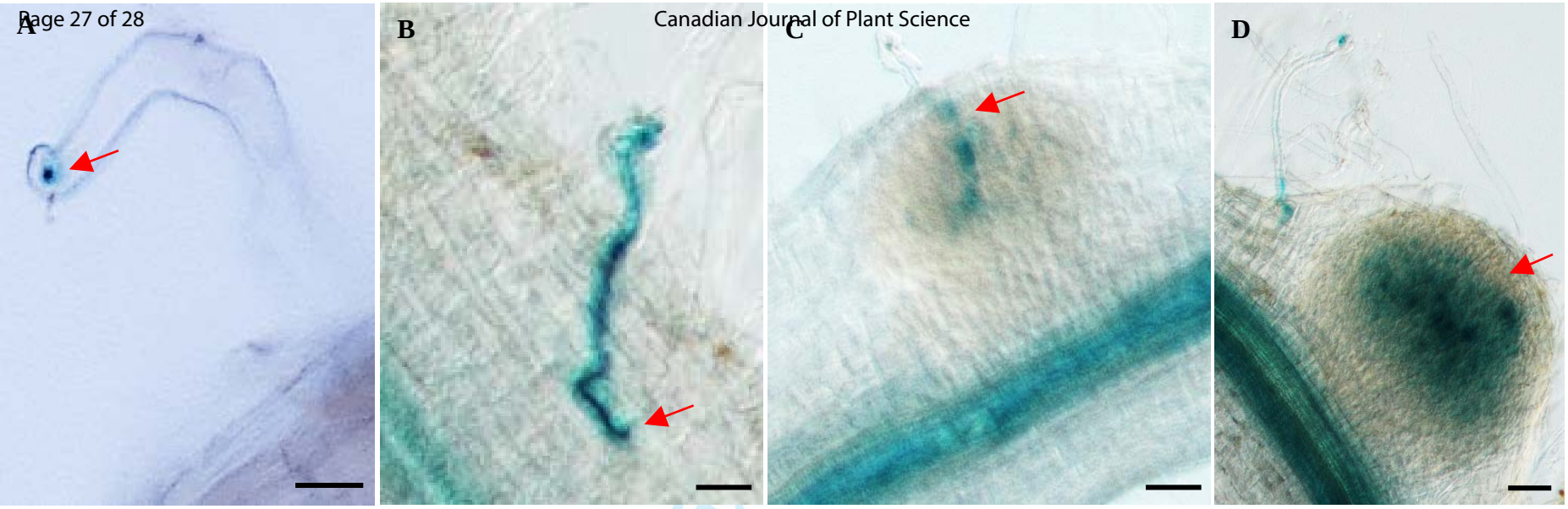
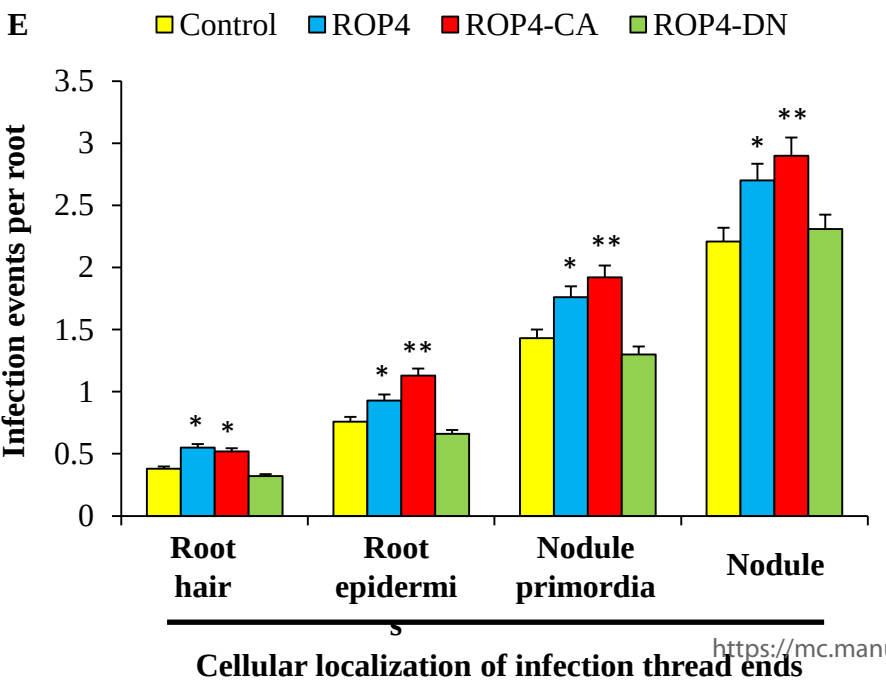


Figure 2





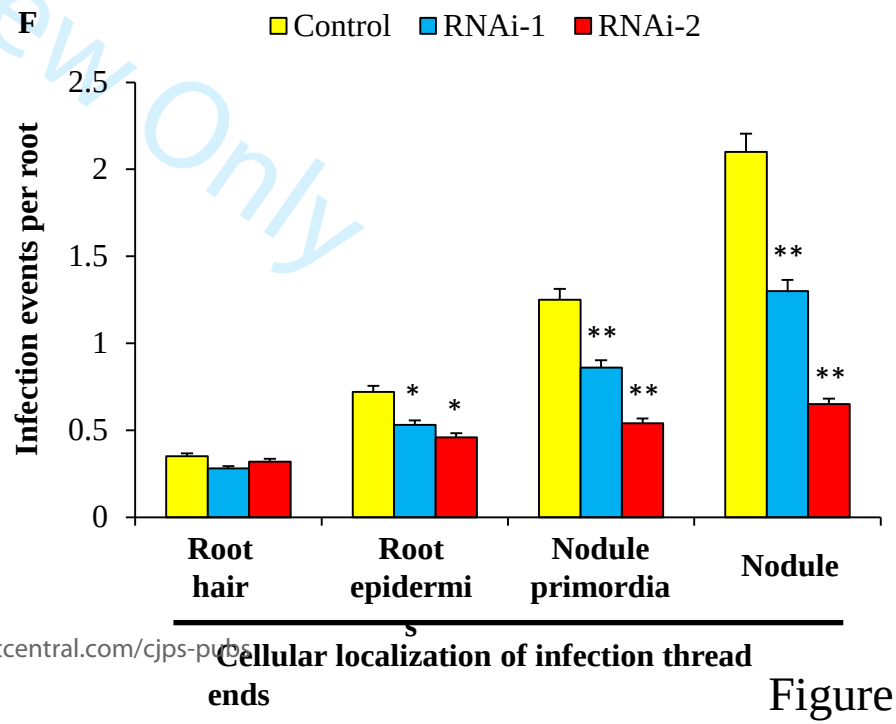


Figure 3

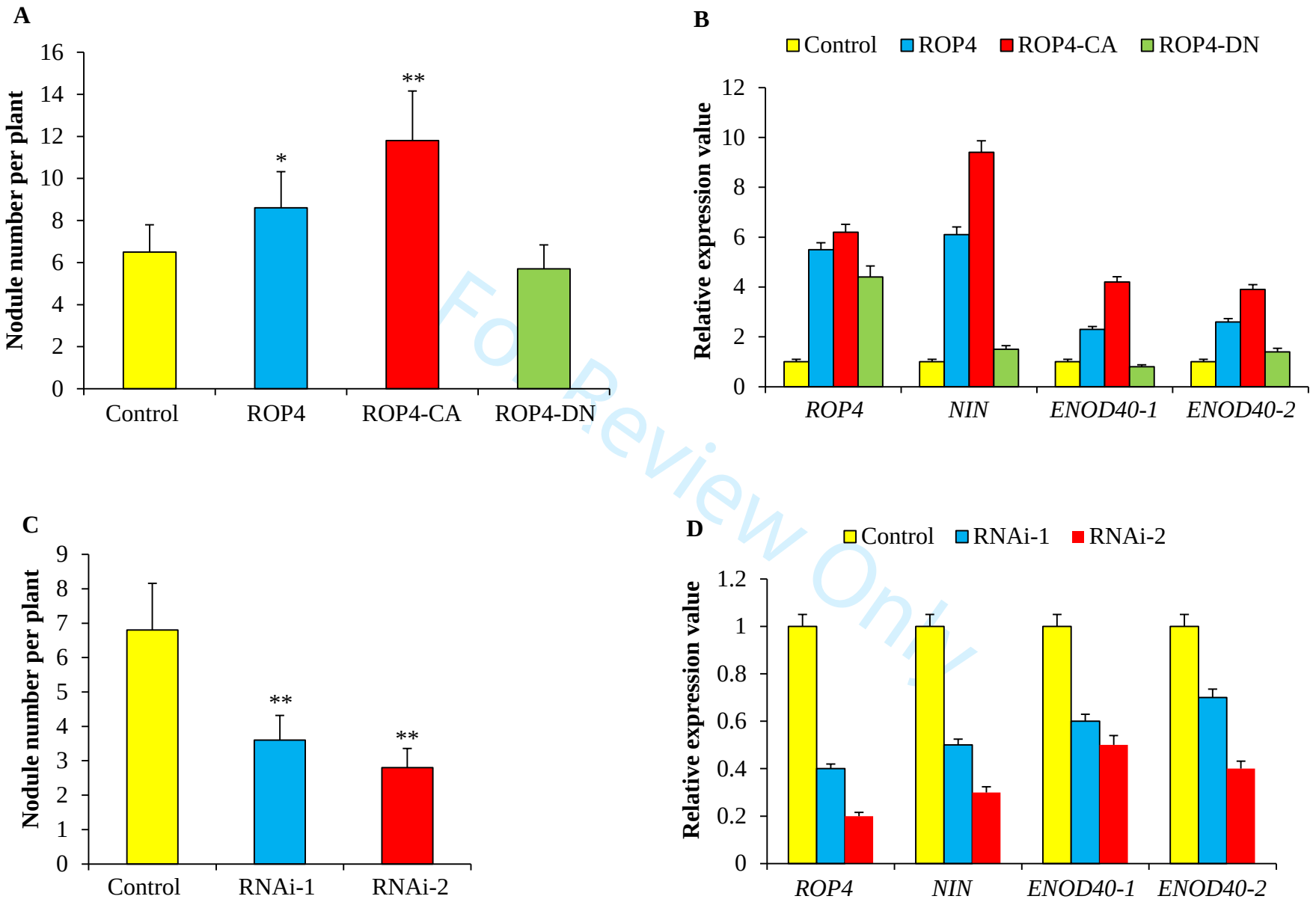


Figure 4