

An intergenic bidirectional promoter driven novel lncRNA (LjPLR) modulates the gene expression of a late nodulin in *Lotus japonicus*

Aniruddho Das

Government General Degree College, Narayangarh

Troyee Das

Bose Institute, Unified Academic Campus

Zhumur Ghosh

Bose Institute, Unified Academic Campus

Anirban Siddhanta

`asbioc@caluniv.ac.in`

University of Calcutta

Research Article

Keywords: *Lotus japonicus*, long non-coding RNA (lncRNA), SNF, LjPLP-IV, Bidirectional promoter

Posted Date: October 7th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7551253/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Root nodules are the only sites for symbiotic nitrogen fixation (SNF) in leguminous plants. The development and functioning of these nodules are governed by a cascade of gene expressions categorized as early and late nodulins. While early nodulins are rapidly induced by Nod factors and involved in infection and cortical cell division, late nodulins support mature nodule function. The regulation of these gene expressions involves several extra- and intracellular factors along with non-coding RNAs (ncRNAs). Despite extensive studies on ncRNAs in SNF, the role of long ncRNAs (lncRNAs) in it remains largely unexplored excepting the well-characterized early nodulin lncRNA ENOD40 and its natural antisense transcript DONE40. Here, we report the identification and characterization of a novel lncRNA, *Lotus japonicus* PLP-IV Long non-coding RNA (*LjPLR*), discovered through *in-silico* transcriptome analysis followed by *in-vivo* validation. *LjPLR* is an antisense transcript complementary to the *LjPLP-IV* gene, which encodes a phosphatidylinositol transfer protein-like protein implicated in membrane biogenesis. We have identified *LjPLP-IV* as the only putative target of *LjPLR*. The negatively correlated temporal gene expression patterns of *LjPLP-IV* and *LjPLR* during nodule biogenesis provide a new insight into the regulatory landscape of SNF in *Lotus japonicus*.

Key message

The study identifies and characterizes a novel long non-coding RNA, *LjPLR*, in *Lotus japonicus*, which is an antisense transcript of the *LjPLP-IV* gene presumably involved in membrane biogenesis. The inverse expression patterns of *LjPLP-IV* and *LjPLR* during nodule development offer new insights into the regulatory mechanisms governing symbiotic nitrogen fixation in *Lotus japonicus*.

Introduction

Symbiotic nitrogen fixation (SNF) involves cascade of gene expression which have been traditionally classified as early and late nodulins, reflecting the developmental time points of their expression (J. D. Murray 2011). Early nodulins are triggered within a few hours of perception of Nod factors and are responsible for some important morphogenetic processes such as pre-infection, infection, and cortical cell division etc. Some comprehensive reviews have extensively discussed about the early nodulins (Mylona et al. 1995; Schultze and Kondorosi 1998). After the successful induction of early nodulins, another set of genes are expressed which are known as late nodulins like carbonic anhydrase, sucrose synthase and Leghemoglobin (Stougaard 2000; Van De Sande and Bisseling 1997).

These early and late nodulin gene expressions are tightly regulated by both intra-cellular and extra-cellular factors. Recently, non-coding RNAs (ncRNA) are emerging as a potent candidate of intra-cellular regulators of gene expression in physiological processes. ncRNAs comprise of multiple subcategories in which long non-coding RNAs (lncRNA) that consist of more than 200 nucleotides (nts) in length, find a place in almost all eukaryotic life processes (St Laurent et al. 2015). Regulatory action of lncRNA in varied physiological processes in plants has been extensively reviewed by Chekanova J. A. (2015), Bhatia, G et al. (2017), Wang, H. V et al. (2017). Although the role of ncRNA in legume is extensively characterized, however the lncRNAs in SNF remains largely unexplored (Chand Jha et al, 2021). Notably, ENOD40, a well-studied early nodulin, was the only lncRNA reported to possess a role in the initiation of nodule biogenesis during SNF (Mylona et al. 1995; Charon et al. 1997; Charon et al. 1999). Recent report showed that ENOD40 is regulated by its novel natural antisense transcript DONE40 in *Medicago truncatula* (Ganguly, P. et al 2021).

Intriguingly, a high level of antisense RNA transcript was discovered by Kapranov et al. 2001 in the cDNA library *Lotus japonicus* nodules. However, the antisense RNA transcript was not fully characterized. While screening the cDNA library, they reported a novel gene family known as *LjPLPs* (isoforms I-IV) that code for *Lotus japonicus* PITP like Proteins (*LjPLP*) N-terminal of which shares considerable homology with mammalian PITP (Phosphatidyl Inositol Transfer Protein) and Sec14p of *Saccharomyces cerevisiae* proteins. Notably, the synthesis of the antisense RNA was described to be driven from an intergenic promoter in the DNA strand complementary to that of *LjPLP-IV* isoform. Plants like rice and *Arabidopsis thaliana* contain similar proteins supposedly involved in membrane biogenesis during organ development (Böhme et al. 2004).

Here, using *in-silico* approaches, we have identified the antisense RNA transcript mentioned above as a novel lncRNA (*Lotus japonicus* PLP-IV Long non-coding RNA; **LjPLR**). We also identified its putative target gene and its temporal expression pattern during nodule biogenesis.

Material and Methods

Transcriptome analysis and filtering out novel antisense long non-coding RNAs

Table 1: List of SRA files containing RNA transcriptome data from infected nodule samples (5 wpi) of *Lotus japonicus*.

Samples	Description
GSM6675108	root nodules, MG136 Infected, rep 2
GSM6675111	root nodules, MG79 Infected, rep 2
GSM6675114	root nodules, MG136 Infected, rep 5
GSM6675119	root nodules, MG79 Infected, rep 1
GSM6675121	root nodules, MG70 Infected, rep 6
GSM6675122	root nodules, MG70 Infected, rep 2
GSM6675123	root nodules, MG79 Infected, rep 6
GSM6675125	root nodules, MG136 Infected, rep 4
GSM6675126	root nodules, MG136 Infected, rep 1
GSM6675127	root nodules, MG70 Infected, rep 1
GSM6675128	root nodules, MG136 Infected, rep 3
GSM6675129	root nodules, MG79 Infected, rep 4
GSM6675133	root nodules, MG79 Infected, rep 3
GSM6675134	root nodules, MG70 Infected, rep 5
GSM6675137	root nodules, MG79 Infected, rep 5
GSM6675138	root nodules, MG70 Infected, rep 4
GSM6675140	root nodules, MG136 Infected, rep 6
GSM6675141	root nodules, MG70 Infected, rep 3

Lotus japonicus Gifu v1.2 assemblyfasta and genome annotation files have been downloaded from Lotus Base (<https://lotus.au.dk>). Moreover, previously published GEO (Gene Expression Omnibus) dataset of *Lotus japonicus* strand specific transcriptome (**Venado, R. E. et al. 2022**; <https://doi.org/10.1073/pnas.2206291119>) having accession id GSE216502 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE216502>) were utilized for analysis. Among 36 datasets of GEO data, we have selected 18 samples containing RNAseq data of infected nodules of 5 wpi (weeks post infection) (**Table 1**). Rest was not considered as those were from non-infected nodules. Qualities of the reads were checked with FastQC v0.11.7 (**Andrews, S. 2010**), Reads were aligned to the reference genome using STAR aligner tool (**Dobin, A., et al. 2013**). StringTie v1.3.4 (**Pertea, M., et al. 2016**) has been used to assemble the transcripts followed by the use of custom awk scripts to detect novel antisense transcripts (**Supplementary Table S1**).

Coding Potential of lncRNA

Coding potential score of the detected antisense lncRNA have been calculated using CPC v2.0 (**Kang et al., 2017**) (<http://cpc2.cbi.pku.edu.cn>). The non-coding transcript coming from the opposite strand of the *Lj*PLP-IV gene has been finally

selected for validation. This lncRNA has a length of 1330 nt having two exons and have been named as *Lotus japonicus* PLP-IV Long non-coding RNA (*LjPLR*).

In-silico* lncRNA target prediction of *LjPLR

The retrieved lncRNA sequence of *LjPLR* was analysed *in-silico* using LncTar tool (Li, J. et. al. 2015) (<https://doi.org/10.1093/bib/bbu048>) for its possible mRNA targets during nodulation (taking a normalized deltaG (ndG) cutoff = - 0.1). Various early and late nodulins were chosen as possible targets of *LjPLR* like *LjNFR1*, *LjNIN*, *LjENOD40*, *LjPLP-IV*, *LjNOD16* and all the variants of Leghemoglobins i.e. *Ljlb*, *Ljlb2* and *Ljlb3* mRNA. NCBI GenBank Accession ID of the targeted genes were provided in the **supplementary datasheet Table S1**.

Sequence alignments and primer design for lncRNA validation

For primer design, the nucleotide sequence of lncRNA *LjPLR* was subjected to sequence alignment with *LjPLP-IV* complete cds using NCBI blastn suite (Camacho, C. et al. 2009) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) and MultAlin (Corpet, F. 1988) (<http://multalin.toulouse.inra.fr/multalin/>) tool. The exon 1 and exon 2 of the lncRNA *LjPLR* aligns with the exonic region 5 to 6 and exonic region 8 to 10 of *LjPLP-IV* cds respectively (details of the sequence alignment have been stated in results section). Thus, the exon - intron boundary of *LjPLP-IV* was chosen for primer designing as the nucleotide sequence of these regions will be unique for *LjPLR* antisense transcript and not for *LjPLP-IV* mRNA. Therefore, three sets of primers were designed corresponding to intronic region - 5, 8 and 9 of *LjPLP-IV* and accordingly named as I-5, I-8 and I-9 respectively (as illustrated in the schematic diagram Fig.1).

Primers for PCR and Real Time-PCR

I-5_RT_Forward Primer: TGACAACTACCCAGAGGTGTG

I-5_RT_Reverse Primer: GTTCAAGGTCTGCAGTGGAGC

I-8_RT_Forward Primer: TACTTAGGGTGGGCCTTCCG

I-8_RT_Reverse Primer: GTGAGCACCATTCTGAACCATC

I-9_RT_Forward Primer: GCTTCTGTTTGAATTTGCCTCTGC

I-9_RT_Reverse Primer: CGTCAAACCTGGAAGTCTTGTCC

LjPLP-IV_RT_Forward Primer: AGGTCATGTCCGTTGAGATTG

LjPLP-IV_RT_Reverse Primer: GGGAGAAGATCGTCCGAAAT

LjPP2A_RT_Forward Primer: TGCTCCCTCTGGTTGTAAATG

LjPP2A_RT_Reverse Primer: ACAGGGACGGATGGTATTCT

Plant material and growth conditions

L. japonicus (Gifu B-129) seeds have been purchased from National Bioresource Project (*Lotus japonicus*, *Glycine max*) University of Miyazaki, Japan. The plants were grown in a controlled environment with a 16-h-day/8-h-night cycle, a 22°C-day/18°C-night temperature, and a relative humidity of 70% (Handberg and Stougaard 1992). Before germination, the seeds were pre-treated for 10 min in conc. H₂SO₄ for scarification and surface sterilization in 25% commercial bleach (1% hypochlorite) and 0.1% TritonX for 10 min, followed by washing with sterile water for 6 times respectively then soaked in water and kept overnight at room temperature. The soaked submerged seeds were then transferred to 4°C for 24h. After 24h the seeds are transferred to petri dishes, containing 1% solidified 1/4th B & D nutrient solution (Broughton and Dilworth 1971). The

inoculation is performed with *Mesorhizobium loti* (strain NZP2235), and the plants were grown in B & D nutrient solution (Broughton and Dilworth 1971). For nodulation, the plants were grown in Nitrogen free nutritive media.

Rhizobial strain, growth conditions and inoculation

The *Mesorhizobium loti* wild-type strain NZP2235 (Jarvis et al. 1982) was used for *Lotus japonicus* (Gifu B-129) nodulation. Rhizobia were grown in customized Yeast Mannitol Broth (containing Yeast Extract- 0.1g, K₂HPO₄- 0.05g, MgSO₄- 0.02g, NaCl- 0.01g, Mannitol- 1g and 10% 100 mM CaCl₂ for 100 ml broth with pH- 6.8) for 2 days in the dark at 28°C. Then the plants were flood inoculated with *Mesorhizobium loti* suspension at OD₆₀₀ 0.01-0.02.

Total RNA Isolation and cDNA Preparation

Total RNA was isolated from harvested root nodule of *Lotus japonicus* at different time points (i.e. 0, 9, 14, 21, 28 and 35 dpi) using Macherey-Nagel™ NucleoSpin™ RNA Plant Kit (Cat.No. 38220090). Following total RNA isolation, cDNA was prepared from 1µg RNA for each time point using random hexamer from Invitrogen™ SuperScript™ III First-Strand Synthesis System (Cat.No.18080051).

Real Time PCR

Real Time-PCR analysis of the cDNAs was done using 15ng cDNA with ThermoScientific™ DyNAmoColorFlash SYBR Green qPCR Kit (Cat. No. F-416L) in the presence of uniquely designed primers against specific regions for I-5, I-8, I-9 of *LjPLR* and *LjPLP-IV*, taking *LjPP2A* as an internal control. Experimental setup and execution were conducted using an Applied Biosystems ABI 7500 Fast Real-Time PCR system. Real Time PCR program: 1 cycle at 95°C for 5 mins, 40 cycles at 95°C for 15 secs, 62°C for 30 secs and 72°C for 30 sec. Expression data were obtained from three independent biological repetitions.

Relative expressions levels of the respective intronic regions of *LjPLR* and *LjPLP-IV* were determined by computing $2^{-\Delta\Delta C_t}$ from the output of the Real Time PCR. Comparative $2^{-\Delta\Delta C_t}$ values were used to create a histogram of relative transcript level of different intronic region of *LjPLR* and *LjPLP-IV* in the root nodules, harvested at different time points i.e. 0, 9, 14, 18, 21, 28 and 35 dpi from *Lotus japonicus* was represented.

Statistical analysis

Arithmetic means and standard deviations (SD) for the data obtained were calculated using Microsoft Excel. Statistical significance of the fold change between an infected sample of a given dpi and uninfected sample (0 dpi) was determined using paired t-tests using GraphPad. *, ** & *** denote p-values < 0.05, < 0.01 & < 0.001 respectively.

Results

Lotus japonicus root nodule (5 wpi) transcriptome contains a novel lncRNA (*LjPLR*)

It had been shown that high level of antisense transcripts was formed as a result of activation of a tissue-specific internal bidirectional promoter that is localized within intron 10 (Fig.2) of the late nodulin gene, *LjPLP-IV* (Kapranov et al. 2001) in *Lotus japonicus*.

However, the sequence and function of the antisense transcripts were not clearly elucidated. In an attempt to identify those antisense transcripts from the *Lotus japonicus* root nodule (5 wpi) transcriptome having strand specific paired end GEO dataset, *in-silico* analysis revealed more than 11,000 non-coding RNAs (Supplementary data sheet Table S2). Further processing of these results found out a novel RNA transcript sequence that is expressed from the loci of *LjPLP-IV* gene (Lotus Base Accession ID: LotjaGiC1v0000100.1 and NCBI GenBank Accession ID: AF367434.1) located in the chromosome number 2 (NCBI GenBank Accession ID: AP006643.1). This RNA was found to be transcribed in a direction opposite to that of *LjPLP-IV*. This antisense RNA is around 1330 nt long consisting of two exons-913 nt (exon 1) and 417 nt (exon 2) (supplementary data sheet Table S2). Then based on its length and coding potential it was identified as a putative lncRNA. The coding potential of

this lncRNA was determined using CPC v2 (Kang et al., 2017). To our satisfaction, its sequence got a Fickett score 0.32847 with a complete putative ORF 67 AA, a pI6.37542724609, which confirmed it as a non-coding sequence with coding probability 0.0390744 (Fig.3). We have named it *LjPLR* (*L*otus *j*aponicus *PLP*-IV *L*ong non-coding *R*NA) and the sequence was deposited in NCBI with the GenBank Accession ID: BK068989.

Sequence Alignment reveals that *LjPLR* is complementary to Ex5-Int5-Ex6 and Ex8-Int10 of *LjPLP*IV gene

Sequence alignments of *LjPLR* with *LjPLP*-IV gene using NCBI blastn suite (Fig.4; A) and MultAlin (supplementary Fig. S1) tools show 100 percent sequence identity between them. However, the exon1(913 nt) of *LjPLR* transcript aligns with the genic regions of *LjPLP*-IV from 2879 bp to 1966 bp which corresponds to Ex8-Int8-Ex9-Int9-Ex10-Int10 (total exonic sequence coverage 91.06%) while the exon 2 (417 nt) of *LjPLR* coincides from 1672 bp to 1255 bp corresponds to Ex5-Int5-Ex6 (total exonic sequence coverage 92.22%) of *LjPLP*-IV gene (Fig. 4; B).

LncTar analysis figures out that *LjPLR* transcript targets only *LjPLP*-IV mRNA

LjPLR being an antisense transcript of *LjPLP*-IV, it is highly likely that it might down regulate the expression level of *LjPLP*-IV gene. This is verified from LncTar tool analysis. While many other genes belonging to both early and late nodulins have been examined using this tool, but none of them were predicted to interact with *LjPLR* transcript. Only the *LjPLP*-IV mRNA was found out to be the possible target of *LjPLR* (Table 2). Moreover, none of the

Table 2: *In-silico* prediction by LncTar tool shows the only probable target of *LjPLR* transcript might be *LjPLP*-IV.

Query	Length Query (bp)	Targets		Length Target (bp)	dG	ndG	Start Position Query	End Position Query	Start Position Target	End Position Target
<i>LjPLR</i>	1330	1.	<i>LjPLP-IV</i>	4920	-931.23	-0.7158	1	1330	2042	3371
		2.	<i>LjPLP-I</i>	1653	Target genes from No. (2)- (22) were not predicted to be interacting with lncRNA <i>LjPLR</i> .					
		3.	<i>LjPLP-II</i>	1696						
		4.	<i>LjPLP-III</i>	1878						
		5.	Nlj16	426						
		6.	<i>Ljlb1</i>	441						
		7.	<i>Ljlb2</i>	441						
		8.	<i>Ljlb3</i>	443						
		9.	<i>LjCYCLOPS</i>	2224						
		10.	<i>Lotus japonicus</i> asparagine synthetase	2183						
		11.	<i>Lotus japonicus</i> POLLUX (ion channel)	3222						
		12.	<i>Lotus japonicus</i> sucrose synthase	2825						
		13.	<i>LjNFR 1a</i>	1866						
		14.	<i>LjNFR 1b</i>	1872						
		15.	<i>LjNFR 5</i>	1788						
		16.	<i>LjNIN</i>	2890						
		17.	<i>LjRLK 1</i>	1881						
		18.	<i>LjRLK2</i>	1875						
		19.	<i>LjENOD40</i>	702						
		20.	<i>LjNSP1</i> (nodulation signaling pathway 1)	1629						
		21.	<i>LjNSP2</i> (nodulation signaling pathway 2)	1500						
		22.	<i>LjCA1</i> (carbonic anhydrase)	792						

other members of the *LjPLP* family (i.e. *LjPLP-I*, *LjPLP-II* and *LjPLP-III*) came out to be a target of this *LjPLR* transcript.

Real-Time PCR validates the expression pattern of *LjPLR* transcript in *Lotus japonicus* root nodule

Our attempt to detect the presence of *LjPLR* in the cDNA of the nodule of *Lotus japonicus* failed since the *LjPLR* transcript (1330 nt) could not be amplified. Thus, we decided to amplify exon1 and exon 2 of *LjPLR* separately. Since exon 1 of *LjPLR* complements with exon 8 to intron 10 of *LjPLP-IV*, two sets of primers were designed corresponding to the exon-intron boundaries such as Ex8-Int8:Int8-Ex9 (I-8) and Ex9-Int9:Int9-Ex10 (I-9). For Exon 2, single set of primer was designed in the

exon-intron boundary such as Ex5-Int5:Int5-Ex6 (I-5). Specific amplicons corresponding to I-5, I-8 & I-9 were obtained (**supplementary Fig. S2**).

Then, to determine the quantitative expression pattern of those different regions of *LjPLR* transcript in the nodule at different dpi, we carried out Real-Time PCR from the cDNA using those primers. The results clearly indicate that all the three regions are maximally expressed at 14th dpi which corroborates with our PCR results. Notably, the expression of the region I-8 is significantly higher compared to the other two. However, the expression of I-8 region

remarkably went down after 14th dpi and became more stable from 21st dpi onward still 35th dpi (**Fig.5 and S2**). Thus, our results suggest that the 1st exon segment of *LjPLR* complementary to Ex8-Int8-Ex9 (i.e. I-8) of *LjPLP-IV* has significant stability as compared to the other regions (viz. I-5 & I-9) (**Figure 5 and S2**).

***LjPLR* transcript and *LjPLP-IV* mRNA reciprocally expressed during nodule biogenesis**

Now that we know the only target of *LjPLR* transcript is *LjPLP-IV* (**Table 1**), the expression of the latter was determined. The expression pattern of *LjPLP-IV* (**Fig.6A**) clearly shows that

it remained low during the early days of nodule development. However, its expression attained a maximum at 21st dpi. Our results demonstrate that expression of *LjPLR* reached a maximum at 14th dpi, after which it started to decline. Thus, the reciprocal expression patterns of *LjPLP-IV* and *LjPLR* clearly indicates that the latter downregulates the former (**Fig. 6B**).

Discussion

In the past few decades, major regulatory roles of ncRNAs in gene expressions across all eukaryotes have become increasingly evident (**St Laurent et al. 2015 and Panni, S et al.2020**). Seminal work unveiling the role of miRNA in gene-silencing in *Petunia hybrida* is one of the pioneers in the discovery of ncRNA (**Napoli et al. 1990; van der Krol et al. 1990**). Since then, numerous miRNAs and siRNAs have been found to regulate diverse developmental and physiological processes in plants. Some of the well documented micro-RNAs expressed during nodulation are Mt-miR166 (HD-ZIPIII), Gma-miR171 (NSP2) and Lja-miR2111 (TML) in *Medicago truncatula*, *Glycine max* and *Lotus japonicus* respectively (**Hoang, N. T. et al. 2020**). Interestingly, the diverse roles of lncRNAs have been well documented in other plants like ASCO-lncRNA (regulating in lateral root development) (**Bardou F et al. 2014**), COOLAIR (**Swiezewski S et al. 2009**) & COLDWRAP (**Kim D-H, Sung S 2017**) (both controlling vernalization and autonomous pathway during flowering) in *Arabidopsis thaliana* while LDMAR regulating photoperiod sensitive male sterility in rice (**Ding J. et al. 2012**).

Although the regulatory functions of miRNAs during nodulation have been extensively studied, the role of lncRNAs during SNF remains very limited barring ENOD40 and DONE40 (**Chand Jha et al. 2021**). ENOD40 is an early nodulin responsible for the initiation of cortical cell division in nodule primordial for the establishment of the symbiosomes (**Mylona et al. 1995; Charon et al. 1997; Charon et al. 1999**). Recently another novel lncRNA called DONE40, expressed as an early nodulin which regulates ENOD40 in *Medicago truncatula* has been reported (**Ganguly, P. et al. 2021**).

Furthermore, in the late pathway of nodulation, there is no report of any lncRNA so far. We for the first time identified and partially characterized a novel lncRNA, expressed as a late nodulin within a determinate nodule of the robinoid legume, *Lotus japonicus*. Although **Kapranov et al. 2001** reported the existence of a few antisense transcripts in the cDNA library of mature nodules of *L. japonicus*, the characterization and its biological functions remained undetermined. Consequently, our investigation of the late nodule transcriptome data conclusively identified those antisense transcripts (**Kapranov et al. 2001**), which being longer than 200nts (length *LjPLR* being 1,330 nt) in length and lacking coding potential (**Fig.2**) have been identified as a putative lncRNA in chromosome 2. Furthermore, *LjPLR* was found to be transcribed in a direction opposite to that of *LjPLP-IV* gene in the same loci. Intriguingly, **Kapranov et al. 2001** also reported that the antisense transcripts (*LjPLR*) are found to be driven by an intergenic promoter located in the Intron 10 of *LjPLP-IV* gene (**Fig.2**).

Interestingly, this promoter was characterized as a bi-directional promoter that drives the expression of another late nodulin, *LjNOD16* (**Fig. 2**), discovered by **Kapranov et al. 2001**. However, neither the sub-cellular localization nor the function of Nlj16 protein was known. Earlier results from our laboratory for the first time showed that Nlj16 interacts with Leghemoglobin in the root nodule of *Lotus japonicus* to enhance the ability to sequester oxygen (**Ghosh et al. 2019**). This interaction leads to membrane localization of Leghemoglobin in the infected cortical cells of the nodules (**Ghosh et al. 2022**).

Our sequence alignment results (**Fig. 4 and S1**) demonstrate that the two exons of *LjPLR* showed very good complementarity with the exon-intronic region of *LjPLP-IV* gene (i.e. Exon 5 to Exon 10). To our satisfaction, LncTar analysis also demonstrated that *LjPLP-IV* gene is the only target of *LjPLR*. *LjPLP-IV* (**Table 1**), late nodulin gene codes for *Lotus japonicus* PITP like Protein whose N-terminal has significant homology with mammalian PITP (Phosphatidyl Inositol Transfer Protein) and yeast Sec 14p which is crucial for the transfer of membrane lipids like phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) (**Kapranov et al. 2001**). It is to be noted that the region corresponding to Exon 5 to Exon 10 of *LjPLP-IV* gene codes for Sec14-like domain (**Fig.2**). Orthologs of this protein are found in many eukaryotes including AtSFH/COW1 in *Arabidopsis* and OsSNBP in rice. These orthologous proteins show activities in root hair tip and in pollen tube developments (**Böhme et al. 2004; Xu, W et al. 2024**). PITP (**Cleves et al. 1991; Wirtz 1991**) and Sec 14p (**Bankaitis et al., 1989, 1990**) are shown to be essential for the transfer of membrane lipids like phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho). Thus, it is highly likely that *LjPLP-IV* protein is responsible for mobilization of phospholipids during nodule biogenesis.

Nevertheless, the biological target of *LjPLR* was predicted to be *LjPLP-IV* only, none of the other isoforms of *LjPLP-IV* was found to be its probable target (**Table 1**). The primary reason for this could be the significant dissimilarities in their mRNA sequences (**Supplementary Fig. S3**). Furthermore, and most interestingly, the expression of *LjPLR* was observed to peak at 14 dpi (**Fig.5**), whereas *LjPLP-IV* reached its maximum at 21 dpi (**Fig.6A**). This strongly suggests that their expressions are negatively correlated (**Fig.6B**). Moreover, it has already been reported that *LjPLR* is exclusively expressed in the nodule and not in any other parts of the plant body (**Kapranov et al. 2001**). Taken together, *LjPLR*, a unique late nodulin lncRNA, has a probable function in down-regulating *LjPLP-IV*-induced phospholipid mobilization during SNF (**Fig. 8**). Further characterization of *LjPLR* will throw light into the regulatory events during late phase of nodule development.

Declarations

Author Contributions

AD and AS conceived the working hypothesis for the study. Transcriptomic analysis to identify the lncRNA was performed by TD and ZG. AD maintained the legume plant *L. japonicus* and the cognate *Rhizobium*, *M. loti*. Isolation of RNA from root nodules at different dpi and preparation of corresponding cDNA were done by AD. AD performed Real time PCR, data collection and analyses. The manuscript was written by AD and AS. TD and ZG reviewed and edited the manuscript. The work was supervised by AS & ZG. The work was supported by funding from ANRF, DST, Govt. of India to AS. All authors read and approved the final manuscript.

Funding

This work is funded by Grants from Govt. of India DST SERB (EMR/2017/004234) and DST ANRF (CRG/2023/005319) to AS. AD and TD were supported by PhD research fellowships from the University Grants Commission and Council of Scientific and Industrial Research, India & ZG's research support is funded by Department of Science and Technology, Govt. of India. This research is also supported by DST-FIST (Department of Biochemistry), DBT-Builder and DST-PURSE programs of University of Calcutta, India.

Data Availability

All the accession IDs of the genes are provided in the supplementary datasheet. The nucleotide sequence of *LjPLR* is deposited in NCBI with the GenBank Accession ID: BK068989.

Competing interests

References

1. **Andrews, S.**, *FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]*. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>, 2010.
2. **Bankaitis, V.A., Aitken, J.F., Cleves, A.E., and Dowhan, W.** (1990). An essential role for a phospholipid transfer protein in yeast Golgi function. *Nature* 347, 561–562. DOI: 10.1038/347561a0
3. **Bankaitis, V.A., Malehorn, D.E., Emr, S.D., and Greene, R.** (1989). The *Saccharomyces cerevisiae* SEC14 gene encodes a cytosolic factor that is required for transport of secretory proteins from the yeast Golgi complex. *J. Cell Biol.* 108, 1271–1281. DOI: 10.1083/jcb.108.4.1271
4. **Bardou, F., Ariel, F., Simpson, C. G., Romero-Barrios, N., Laporte, P., Balzergue, S., ... & Crespi, M.** (2014). Long noncoding RNA modulates alternative splicing regulators in *Arabidopsis*. *Developmental cell*, 30(2), 166-176. DOI: 10.1016/j.devcel.2014.06.017
5. **Bhatia, G., Goyal, N., Sharma, S., Upadhyay, S. K., & Singh, K.** (2017). Present Scenario of Long Non-Coding RNAs in Plants. *Non-coding RNA*, 3(2), 16. <https://doi.org/10.3390/ncrna3020016>
6. **Böhme, Karen, et al.** "The *Arabidopsis* COW1 gene encodes a phosphatidylinositol transfer protein essential for root hair tip growth." *The Plant Journal* 40.5 (2004): 686-698. DOI: 10.1111/j.1365-313X.2004.02245.x
7. **Broughton, W. J., and Dilworth, M. J.** "Control of leghaemoglobin synthesis in snake beans." *Biochemical journal* 125.4 (1971): 1075-1080. DOI: 10.1042/bj1251075
8. **Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L.** (2009). BLAST+: architecture and applications. *BMC bioinformatics*, 10(1), 421. DOI: 10.1186/1471-2105-10-421
9. **Chand Jha, U., Nayyar, H., Mantri, N., & Siddique, K. H.** (2021). Non-coding RNAs in legumes: their emerging roles in regulating biotic/abiotic stress responses and plant growth and development. *Cells*, 10(7), 1674. doi: 10.3390/cells10071674
10. **Charon, C., Johansson, C., Kondorosi, E., Kondorosi, A., and Crespi, M.** 1997. enod40 induces dedifferentiation and division of root cortical cells in legumes. *Proceedings of the National Academy of Sciences* 94:8901-8906. doi: 10.1073/pnas.94.16.8901
11. **Charon, C., Sousa, C., Crespi, M., and Kondorosi, A.J.T.P.C.** 1999. Alteration of enod40 expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti* 11:1953-1965. DOI: 10.1105/tpc.11.10.1953
12. **Chekanova J. A.** (2015). Long non-coding RNAs and their functions in plants. *Current opinion in plant biology*, 27, 207–216. <https://doi.org/10.1016/j.pbi.2015.08.003>
13. **Cleves, A.E., McGee, T.P., and Bankaitis, V.A.** (1991). Phospholipid transfer proteins: A biological debut. *Trends Cell Biol.* 1, 30–34. DOI: 10.1016/0962-8924(91)90067-j
14. **Corpet, F.** (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic acids research*, 16(22), 10881-10890. DOI: 10.1093/nar/16.22.10881
15. **Ding, J., Lu, Q., Ouyang, Y., Mao, H., Zhang, P., Yao, J., ... & Zhang, Q.** (2012). A long noncoding RNA regulates photoperiod-sensitive male sterility, an essential component of hybrid rice. *Proceedings of the National Academy of Sciences*, 109(7), 2654-2659. <https://doi.org/10.1073/pnas.1121374109>
16. **Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., ... & Gingeras, T. R.** (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 29(1), 15-21. DOI: 10.1093/bioinformatics/bts635
17. **Ganguly, P., Roy, D., Das, T., Kundu, A., Cartieaux, F., Ghosh, Z., & DasGupta, M.** (2021). The natural antisense transcript DONE40 derived from the lncRNA ENOD40 locus interacts with SET domain protein ASHR3 during inception of symbiosis in *Arachis hypogaea*. *Molecular Plant-Microbe Interactions*, 34(9), 1057-1070. DOI: 10.1094/MPMI-12-20-0357-R

18. Ghosh, A., Das, A., Ashrafi, M. A., Saha, S., Molla, F., DasGupta, M., & Siddhanta, A. (2022). Nodulin 16 of *Lotus japonicus* (Nlj16) regulates the recruitment of Leghemoglobin (LegH) to the infected nodule cell membrane during symbiotic nitrogen fixation. *bioRxiv*, 2022-07. doi: <https://doi.org/10.1101/2022.07.21.500945>
19. Ghosh, Amit, Kaushik Bhar, and Anirban Siddhanta. "Oxygen sequestration by Leghemoglobin is positively regulated via its interaction with another late nodulin, Nlj16 of *Lotus japonicus*." *Journal of Plant Biochemistry and Biotechnology* 28.4 (2019): 414-423. <https://doi.org/10.1007/s13562-019-00494-3>
20. Handberg, Kurt, and Jens Stougaard. "Lotus japonicus, an autogamous, diploid legume species for classical and molecular genetics." *The Plant Journal* 2.4 (1992): 487-496. <https://doi.org/10.1111/J.1365-313X.1992.00487.X>
21. Hoang, N. T., Tóth, K., & Stacey, G. (2020). The role of microRNAs in the legume–Rhizobium nitrogen-fixing symbiosis. *Journal of Experimental Botany*, 71(5), 1668-1680. DOI: 10.1093/jxb/eraa018
22. J. D. Murray, Invasion by invitation: Rhizobial infection in legumes. *Mol. Plant Microbe Interact.* 24, 631–639 (2011). DOI: 10.1094/MPMI-08-10-0181
23. Jarvis, B. D. W., C. E. Pankhurst, and J. J. Patel. "Rhizobium loti, a new species of legume root nodule bacteria." *International journal of systematic and evolutionary microbiology* 32.3 (1982): 378-380. <https://doi.org/10.1099/00207713-32-3-378>
24. Kang, Y.J., Yang, D.C., Kong, L., Hou, M., Meng, Y.Q., Wei, L., and Gao, G. 2017. CPC2: a fast and accurate coding potential calculator based on sequence intrinsic features. *Nucleic acids research* 45:W12-W16. DOI: 10.1093/nar/gkx428
25. Kapranov, Philipp, et al. "Nodule-specific regulation of phosphatidylinositol transferprotein expression in *Lotus japonicus*." *The Plant Cell* 13.6 (2001): 1369-1382. doi: 10.1105/tpc.13.6.1369
26. Kim, D. H., & Sung, S. (2017). Vernalization-triggered intragenic chromatin loop formation by long noncoding RNAs. *Developmental cell*, 40(3), 302-312. DOI: 10.1016/j.devcel.2016.12.021
27. Li, J., Ma, W., Zeng, P., Wang, J., Geng, B., Yang, J., & Cui, Q. (2015). LncTar: a tool for predicting the RNA targets of long noncoding RNAs. *Briefings in bioinformatics*, 16(5), 806–812. <https://doi.org/10.1093/bib/bbu048>
28. Mylona, P., Pawlowski, K., and Bisseling, T.J.T.P.C. 1995. Symbiotic nitrogen fixation 7:869. DOI: 10.1105/tpc.7.7.869
29. Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes in trans. *Plant Cell* 2: 279–289. DOI: 10.1105/tpc.2.4.279
30. Panni, S., Lovering, R. C., Porras, P., & Orchard, S. (2020). Non-coding RNA regulatory networks. *Biochimica et biophysica acta. Gene regulatory mechanisms*, 1863(6), 194417. <https://doi.org/10.1016/j.bbagr.2019.194417>
31. Perte, M., Kim, D., Perte, G. M., Leek, J. T., & Salzberg, S. L. (2016). Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. *Nature protocols*, 11(9), 1650-1667. DOI: 10.1038/nprot.2016.095
32. Schultze, M., & Kondorosi, A. (1998). Regulation of symbiotic root nodule development. *Annual review of genetics*, 32(1), 33-57. DOI: 10.1146/annurev.genet.32.1.33
33. St Laurent, G., Wahlestedt, C., & Kapranov, P. (2015). The Landscape of long noncoding RNA classification. *Trends in genetics: TIG*, 31(5), 239–251. <https://doi.org/10.1016/j.tig.2015.03.007>
34. Stougaard, Jens. "Regulators and regulation of legume root nodule development." *Plant physiology* 124.2 (2000): 531-540. DOI: 10.1104/pp.124.2.531
35. Swiezewski, S., Liu, F., Magusin, A., & Dean, C. (2009). Cold-induced silencing by long antisense transcripts of an Arabidopsis Polycomb target. *Nature*, 462(7274), 799-802. DOI: 10.1038/nature08618
36. Van de Sande, K., and T. Bisseling. "Signalling in symbiotic root nodule formation." *Essays in biochemistry* 32 (1997): 127-142. PMID: 9493016
37. Van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2: 291–299. DOI: 10.1105/tpc.2.4.291
38. Wang, H. V., & Chekanova, J. A. (2017). Long Noncoding RNAs in Plants. *Advances in experimental medicine and biology*, 1008, 133–154. https://doi.org/10.1007/978-981-10-5203-3_5
39. Wirtz, K.W.A. (1991). Phospholipid transfer proteins. *Annu. Rev. Biochem.* 60, 73–99. DOI: 10.1146/annurev.bi.60.070191.000445

Figures

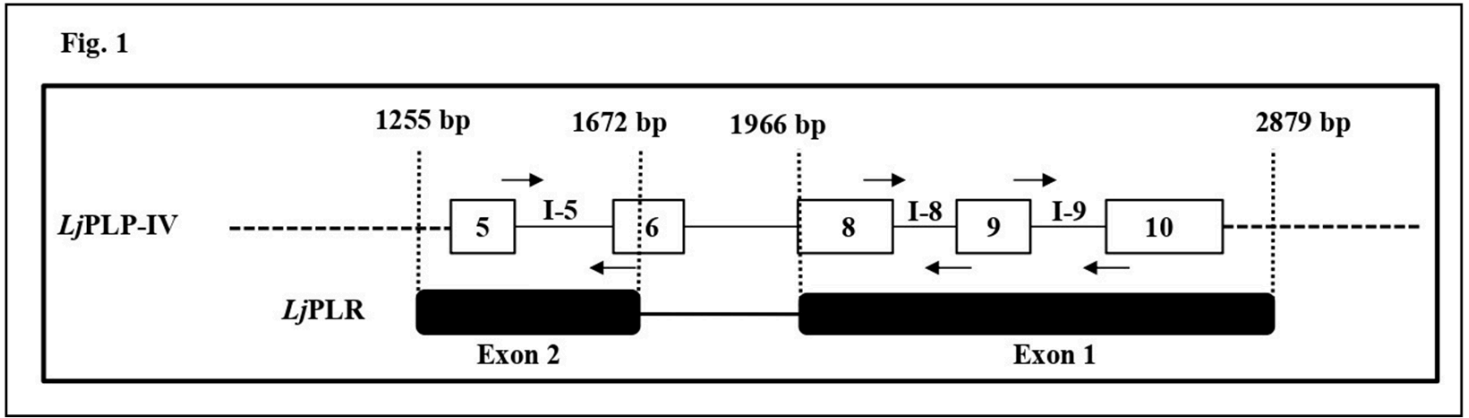


Figure 1

Illustration of the gene structure of *LjPLP-IV* showing the locations of Real-time PCR primers for different regions of *LjPLR*

The exon-intron boundaries of *LjPLP-IV* that corresponds to the exonic regions of *LjPLR* were chosen for primers designing as these regions will be unique for *LjPLR* antisense transcript but will not be present in *LjPLP-IV* mRNA. Therefore, three sets of primers were designed corresponding to intronic regions - 5, 8 and 9 of *LjPLP-IV* and accordingly named as I-5, I-8 and I-9 respectively. Arrows denote the positions of designing the forward and reverse primer.

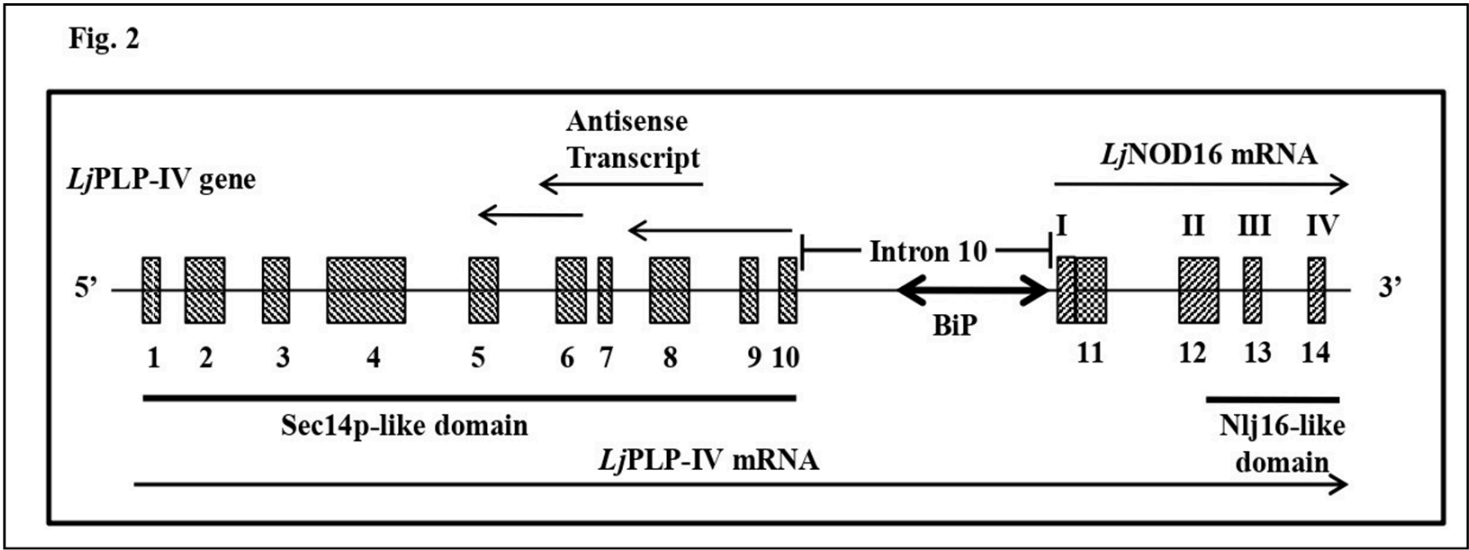


Figure 2

Scheme of the *LjPLP-IV* gene organization of *Lotus japonicus*

An intergenic bidirectional promoter (BiP) residing in intronic region 10 of *LjPLP-IV* gene plausibly drives the expression of both the antisense RNA transcript and *LjNOD16* in opposite direction during root nodule biogenesis. Numbers - 1 to 14 and I to IV denotes the exons of *LjPLP-IV* and *Nlj16* respectively. However, exon I to IV of *Nlj16* (partly coincides with 11th to 14th exon of *LjPLP-IV*) is expressed as a separated protein during nodule biogenesis after *Mesorhizobium sp* infection. (Adopted and modified from Kapranov et al 2001).

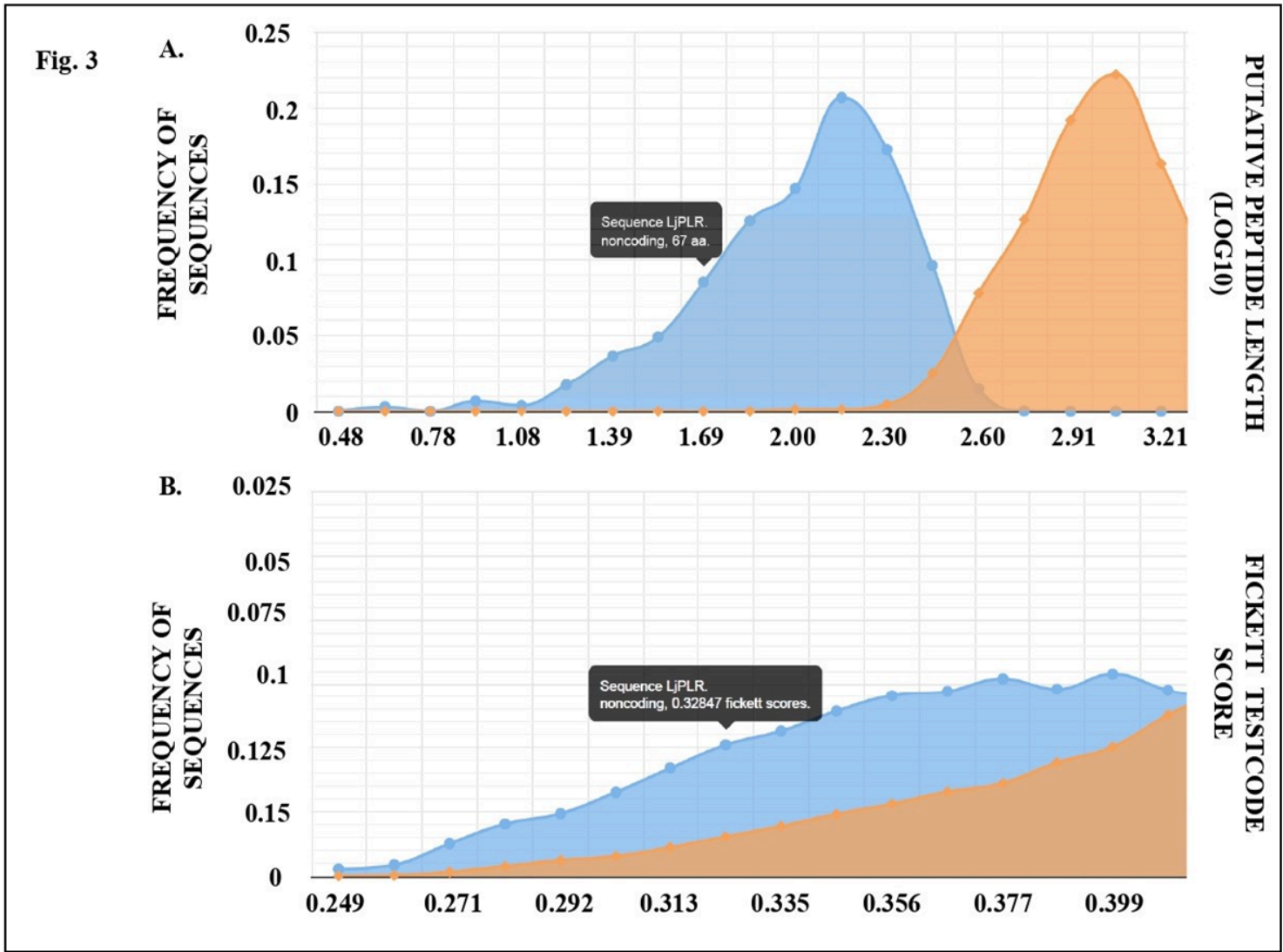


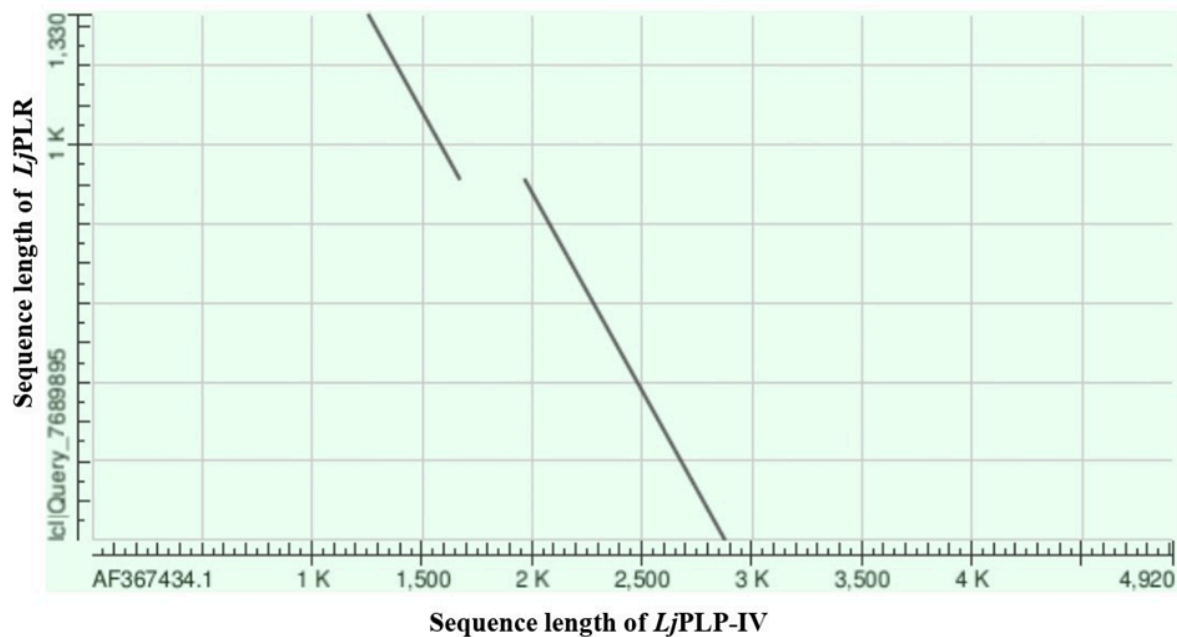
Figure 3

Determination of coding potential confirms that *LjPLR* as a non-coding RNA

Panel A: Coding Potential Calculator ver 2 was used to find out that the putative peptide length of the lncRNA region being 67 AA with a coding probability 0.0390744 and **Panel B:** the Fickett testcode score of 0.32847 which ascertains that the region produces an lncRNA transcript.

Fig. 4

A



B

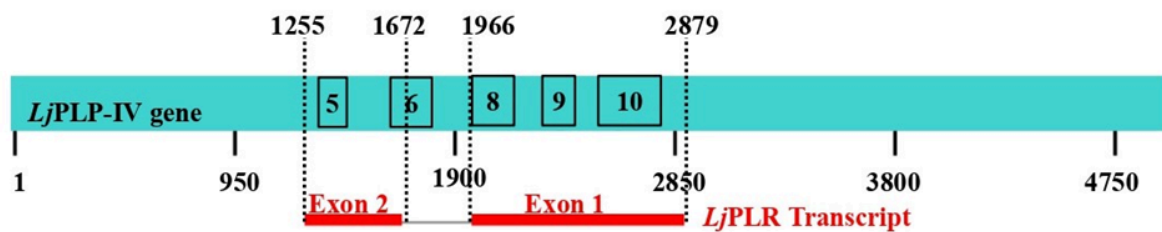


Figure 4

Sequence alignment of lncRNA, *LjPLR* and *LjPLP-IV* nucleotide sequences

A: Dot Plot and **B:** Graphical summary reveals two separate regions in *LjPLR* coinciding with *LjPLP-IV*, one from 1255 bp to 1672 bp (corresponding to exon 5, Intron 5 and 6; probable regions marked by square box) while the other from 1966 bp to 2879 bp (corresponding to exon 8, Intron 8, Exon 9, Intron 9 and exon 10; probable regions are marked by square box) of *LjPLP-IV*.

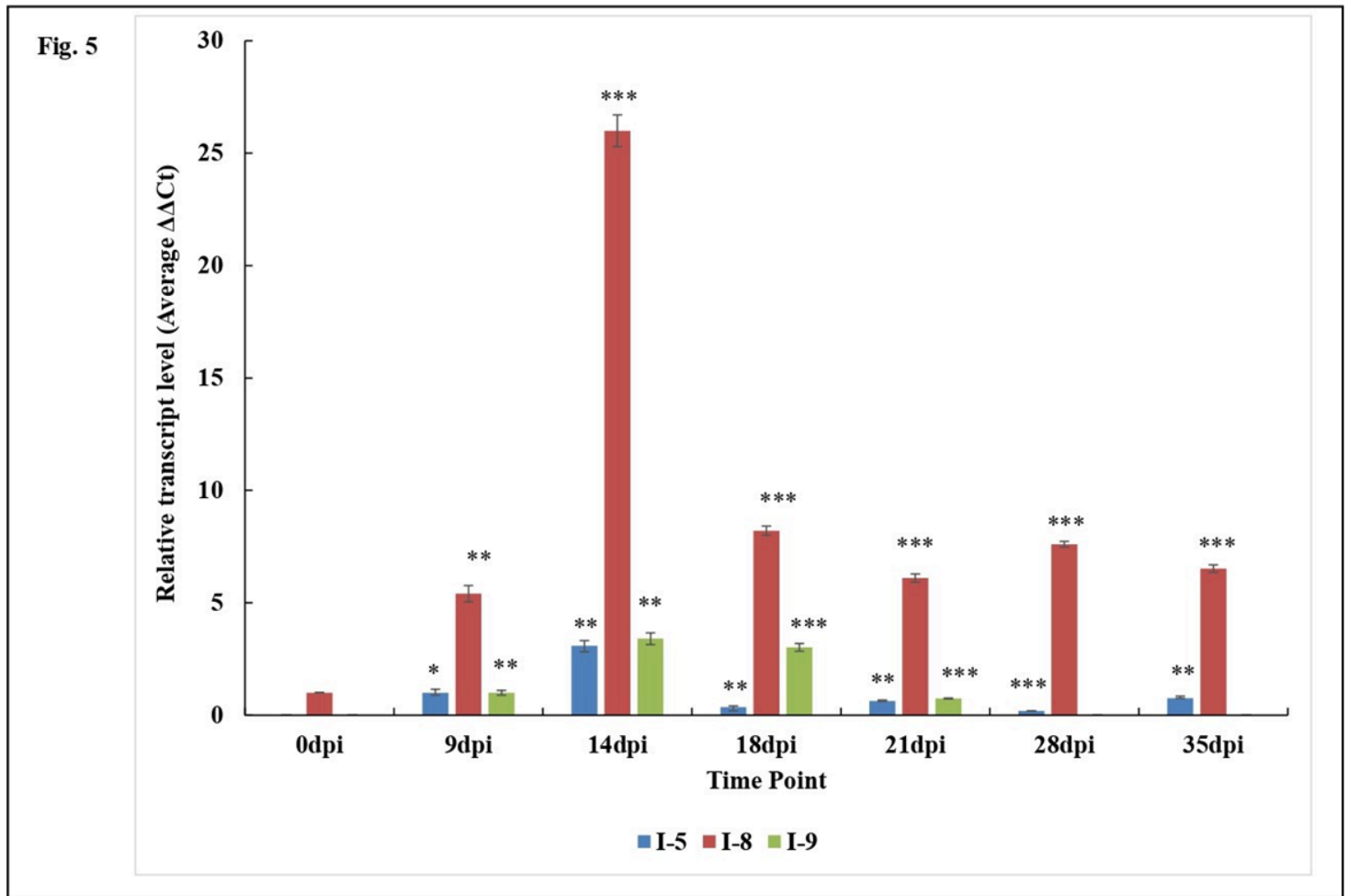


Figure 5

Real time PCR analysis demonstrates that the I-8 region of the lncRNA, *LjPLR* is maximally expressed at 14 dpi. Total RNA and corresponding cDNA from the root nodules of different dpi (as indicated) were prepared following the protocols mentioned in the 'Methods' section. Real time PCR was conducted to determine the gene expression profile of different regions (I-5 [blue], I-8 [red] & I-9 [green]) of *LjPLR* using respective primers described above (Fig. 1; Methods). The fold changes in expressions were obtained by computing respective $2^{-\Delta\Delta C_t}$ values using uninfected sample (0 dpi) and *LjPP2A* as internal control. Data are expressed as mean of triplicates $\pm$ SD. Statistical significance of the fold change between an infected sample of a given dpi and uninfected sample (0 dpi) was determined using paired t-tests. *, ** & *** denote p-values < 0.05, < 0.01 & < 0.001 respectively.

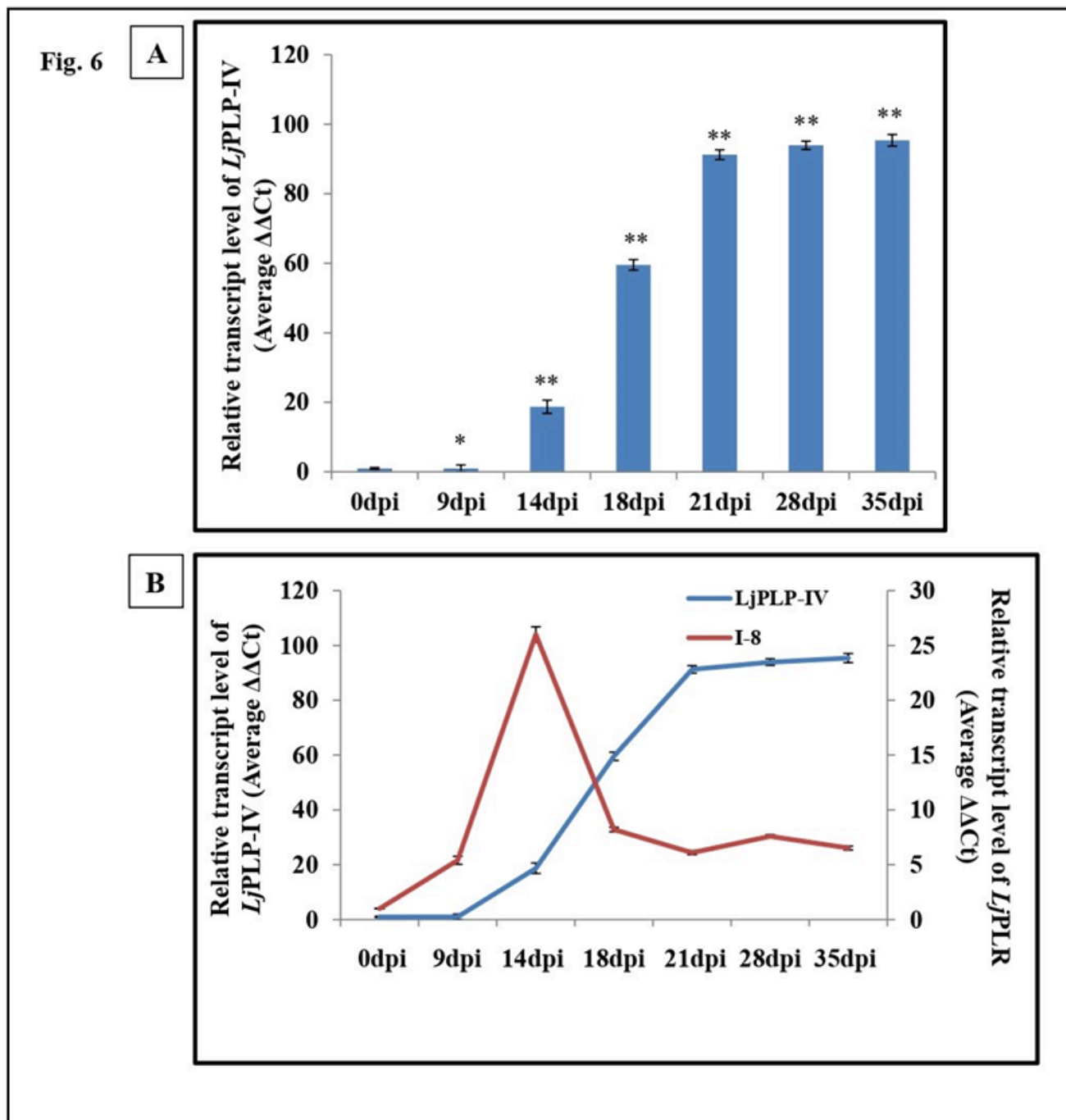


Figure 6

LjPLP-IV gene is maximally expressed at 21 dpi and thereafter

Panel A: Comparative expression profile of the *LjPLP-IV* in different dpi was determined following protocol similar that of Fig. 5. Data are expressed as mean of triplicates $\pm$ SD. Statistical significance of the fold change between an infected sample of a given dpi and uninfected sample (0 dpi) was determined using paired t-tests. * & ** denote p-values < 0.05 & < 0.01 respectively.

Panel B: Expression profiles of I-8 region of *LjPLR* (red) and *LjPLP-IV* (blue) transcripts show inverse relationship between them. The mean fold changes in expressions of I-8 region and *LjPLP-IV* transcripts were plotted. Data are expressed as mean of triplicates $\pm$ SD.

Image not available with this version

Figure 7

A scheme of the *LjPLP-IV* gene structure of *Lotus japonicus*

LjPLP-IV gene has 14 exons (sky blue) and 13 introns (dash) having an intragenic bidirectional promoter (BiP) located in the intron 10. *LjPLR* expression is driven by the BiP in a direction opposite to that of *LjPLP-IV*. *LjPLR* consists of two exons - one spanning over exon 5 to exon 6 of *LjPLP-IV* (417 bp) and another spanning over exon 8 to exon 10 of *LjPLP-IV* (913 bp). BiP also drives the expression of *LjNOD16* in the same direction as that of *LjPLP-IV* during later stage of root nodule biogenesis. (Adopted and modified from Kapranov .et. al 2001).

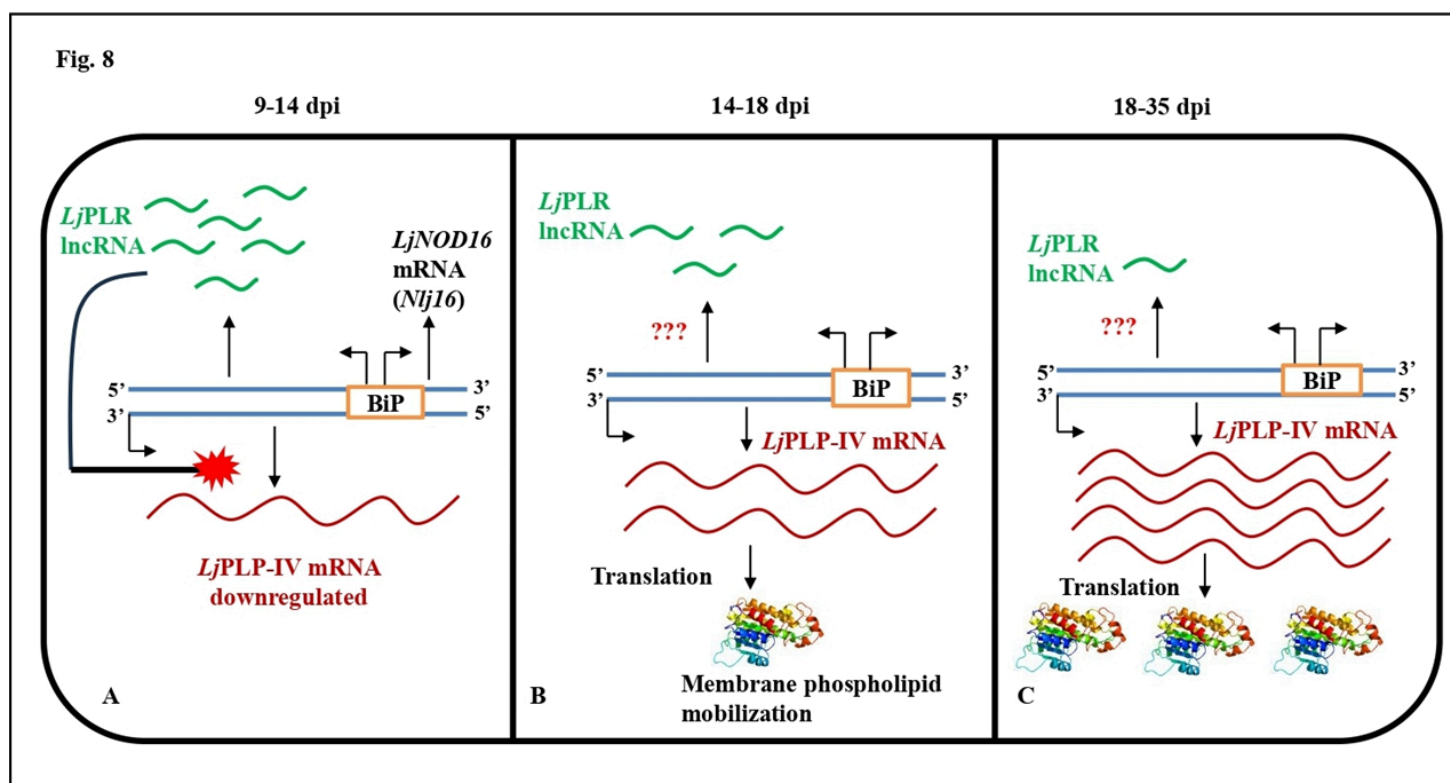


Figure 8

Predicted model for *LjPLR* function

Panel A: The intron 10 of *LjPLP-IV* gene has a bidirectional promoter (BiP). This promoter drives expression of a novel IncRNA, *LjPLR* (green) (this report) and a late nodulin, *Nlj16* (Kapranov et al. 2001, Ghosh et al. 2019 and Ghosh et al. 2022) in the opposite direction of each other. Root-nodule cells start producing the *LjPLR* from 9th dpi till 14th when it reaches maximum level. During this time, *LjPLR* down regulates *LjPLP-IV* mRNA (red), its sole target as predicted by IncTar. **Panel B & C:** From 14th

dpi onwards, *Lj*PLR (green) expression goes down by a yet-to-be-known mechanism (???) with a concomitant and obvious increase in the production of *Lj*PLP-IV mRNA (red) and the protein.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigures.pdf](#)
- [SupplementaryMaterialTableS1..docx](#)
- [SupplementaryDatasheetTableS2.xlsx](#)