

Transcriptomic and physiological responses of *Rhizobium* sp. IRBG74 to *Sesbania cannabina* and rice (*Oryza sativa* L) rhizosphere

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Abstract

Purpose

Rhizobium sp. IRBG74 (IRBG74), is a symbiont of *Sesbania cannabina* and a growth promoting endophyte of rice. Here, we compare the transcriptomic and physiological responses of IRBG74 in the rhizosphere of *S. cannabina* and rice.

Methods

We used RNA sequencing to determine transcriptomic changes at 12 and 72 hours post inoculation (hpi) in rhizosphere. Upregulation of key pathways was confirmed using β -glucuronidase (GUS) reporter strains and by histochemical and quantitative GUS activity.

Results

Significant changes in transcriptome with *S. cannabina* were detected at 12hpi but most gene expression changes with rice were observed at 72hpi. Many pathways including Nod factor synthesis, two component systems, ABC transporters, and synthesis of indole acetic acid (IAA) were upregulated whereas translation, RNA degradation, protein export and sulfur metabolism were downregulated with both plants. In contrast, motility and chemotaxis genes were induced specifically with *S. cannabina*. The upregulation of *nod* and IAA genes was confirmed using reporter strains. Nod factor synthesis provides competitive advantage for colonization of rice and chemotaxis is required for colonization of *S. cannabina* but not of rice.

Conclusion

IRBG74 responded to *S. cannabina* and rice using specific as well as common transcriptional changes. A mutant defective in Nod factor synthesis was outcompeted for rice colonization. Chemotaxis was required for colonization of *S. cannabina* but not for rice. IAA synthesis by IRBG74 could be a major mechanism of rice growth promotion. These results provide a foundation for further improvement of rhizobial interactions with rice and other cereals.

Introduction

Legume nodulating rhizobia (LNR) form nitrogen fixing symbiotic associations that play a critical role in agriculture and environmental sustainability (Graham and Vance 2003, Peoples et al. 2009; Gyaneshwar et al. 2011). The legume-rhizobia symbiosis involves extensive signal exchanges between rhizobia and the host legumes (Oldroyd and Downie, 2006; Stacey et al. 2006; Jones et al. 2007; Oldroyd 2013; Venkateshwaran et al. 2013).

Many strains of LNR can also colonize the internal tissues of cereals such as rice, maize and wheat forming endophytic associations (Reddy et al. 1997; Biswas et al. 2000a, b; Chaintreuil et al. 2000; Chi et al. 2005; Yanni and Dazzo, 2010; Mitra et al. 2016). These associations can lead to increased plant biomass and nutrient uptake (Biswas et al. 2000a, b; Yanni and Dazzo, 2010; Mitra et al. 2016). The association of LNR with cereals and their ability to promote cereal growth has led to studies examining the potential of these bacteria to form nitrogen-fixing symbiosis with cereals, similar to those with legumes (Ladha and Reddy, 2003; Betty and Good 2011, Mus et al. 2016; Pankiewicz et al. 2019; Bennet et al. 2020). Among cereals, rice has been a main target crop to characterize nitrogen fixation with cereals (Ladha and Reddy, 2000; Ladha and Reddy, 2003). Studies over the last decades revealed that many features of the rhizobial-legume interactions are also involved in rhizobial-rice interactions (Reddy et al. 1997, Reddy et al. 1998, Ladha and Reddy 2003). Based on these results, it has been suggested that rice contains many of the genetic determinants required for forming symbiosis with rhizobia. However, not much is known about the mechanisms that rhizobia and rice use to recognize each other and form beneficial associations.

We previously described *Rhizobium* sp. IRBG74, a nodulating and nitrogen fixing symbiont of aquatic legume *Sesbania cannabina* (Yellow Pea Bush) (Cummings et al. 2009). *Rhizobium* sp. IRBG74 can also promote rice growth (Biswas et al. 2000 a,b; Peng et al. 2002; Mitra et al. 2016). We determined the genome sequence of *Rhizobium* sp. IRBG74 (Crook et al. 2013) and showed that it colonized both the surface and internal tissues of rice roots and stems (Mitra et al. 2016) and thus can be further utilized as a model system to elucidate the mechanisms of rice-rhizobial interactions.

In this study we analyzed the transcriptomic responses of *Rhizobium* sp. IRBG74 after inoculation into *S. cannabina* and rice rhizosphere using RNA-sequencing (RNA-Seq). We used two different time frames to identify gene expression changes. We show that *Rhizobium* sp. IRBG74 responds to *S. cannabina* at both 12h and 72h post inoculation whereas the majority of transcriptional changes to rice occur at 72h. However, the majority of these responses are common to both host plants. Using reporter strains we demonstrate that genes required for the synthesis of Nod factor and of the phytohormone indole acetic acid are upregulated in the rhizosphere of both plants. We show that Nod factor synthesis is important not only for nodulation of *S. cannabina* but also for competitive colonization of rice. In contrast, chemotaxis is important for colonization of *S. cannabina* but not of rice.

Materials And Methods

Bacterial strains and growth conditions

The bacterial strains, plasmids and primers used in this study are listed in Table 1. The wild type and mutant *Rhizobium* sp. IRBG74 strains were maintained on Yeast Mannitol (YM) agar with appropriate antibiotics. The *E. coli* strains were maintained on LB agar with antibiotics as required.

Table 1
List of strains, plasmids and primers used in this study

Strains/ Plasmids	Description	Reference
Strains		
IRBG74	Wild type <i>Rhizobium</i> sp. IRBG74	(Tan et al. 2001)
IRBG74-GUS	IRBG74 marked with pCAM121 Spec ^r	Cummings et al. 2009)
IRBG74- <i>nodAGUS</i>	Wild type <i>Rhizobium</i> sp. IRBG74 with an insertion of pTH1703 in <i>nodA</i>	This study
IRBG74- <i>mcpEGUS</i>	Wild type <i>Rhizobium</i> sp. IRBG74 with an insertion of pTH1703 in <i>mcpE</i>	This study
<i>E. coli</i> TOP10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80/ <i>lacZ</i> Δ M15 Δ / <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galJ</i> <i>galK</i> <i>rpsL</i> (StrR) <i>endA1</i> <i>nupG</i>	Thermo Fischer Scientific USA
<i>E. coli</i> DH5 α	F ⁻ <i>endA1</i> <i>glnV44</i> <i>thi-1</i> <i>recA1</i> <i>relA1</i> <i>gyrA96</i> <i>deoR</i> <i>nupG</i> <i>purB20</i> ϕ 80d/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169, <i>hsdR17</i> (<i>r_K⁻m_K⁺</i>), λ ⁻	Thermo Fischer Scientific USA
Plasmids and Primers		
pENTR TM /D-TOPO ⁺ Vector	Entry vector for TOPO cloning Kan ^R	Life Technologies USA
pTH1703	Derivative of pTH1522 containing <i>gfp⁺</i> , <i>lacZ</i> , <i>gusA</i> , and <i>tdimer2</i> (12) with an expanded multiple cloning site Gen ^R	(Cowie et al. 2006)
<i>nodA</i> disrupt F	CCGCATGCTGAAGACGCGAGGTTGAATG	This study
<i>nodA</i> disrupt R	GGCTCGAGGTAAACATTGAATGACTAAG	This study
<i>nodA</i> disrupt C	CGAGCTGAAGTCCTTACGGC	This study
<i>nthB</i> disrupt F	CCGCATGCTTTCCTGTTCCCGTGTGGGTC	This study
<i>nthB</i> disrupt R	GGCTCGAGACGCAGTAGACAGGCATAGG	This study
<i>nthB</i> disrupt C	CCAACATCCTCGAAGAGCAC	This study
<i>mcpE</i> disrupt F	CCGGTACCGAGATGCAATCCGACTTCCTG	This study
<i>mcpE</i> disrupt R	GGCTCGAGTCTTGATCTCCCTTGCGGCATC	This study
<i>mcpE</i> disrupt C	GCTGGCGTTCTGAACCTTGG	This study
ML4876	ATAAGGGACTCCTCATTAAGATAAC	(Cowie et al. 2006)

Inoculation of *S. cannabina* and rice with *Rhizobium* sp. IRBG74 for transcriptomic analysis

Seeds of *S. cannabina* and rice were surface sterilized and germinated as described earlier (Mitra et al. 2016). The contamination free seedlings were transferred to Magenta boxes containing N-free plant growth media (Cummings et al. 2009). The seedlings were put on a mesh such that the roots grew into the growth media. *Rhizobium* sp. IRBG74 marked with β -glucuronidase (GUS) (Mitra et al. 2016) was grown in YM broth, harvested by centrifugation and resuspended in normal saline. The bacteria were then inoculated onto *S. cannabina* and rice seedlings to final O.D_{600nm} of 1.0. Each magenta box contained 20 seedlings and inoculations were done in triplicates. The inoculated plants were incubated in a growth chamber (14h light/10h dark cycle) at 27°C (day) and 25°C (night). Bacterial cells were collected by centrifugation before inoculation (0hrs) and at 12 and 72 hpi in 5% phenol in ethanol. The 12 hpi time point was chosen to determine early responses to plant rhizosphere, including signaling mechanisms, and 72 hpi was determined based on our earlier study showing increased colonization of *S. cannabina* and rice by *Rhizobium* sp. IRBG74 at that time point (Mitra et al. 2016).

RNA isolation and preparation

Total nucleic acids were extracted from each sample using phenol/chloroform extraction (Rigsbee et al. 2011). DNA was removed by treatment with RNase-free DNase I (New England Biolabs). Ribosomal RNA was depleted with Ribo-Zero rRNA Removal Bacteria Kit (Illumina) and samples were cleaned with ZR RNA Clean and Concentrator Kit (Zymo Research Corporation). The completeness of rRNA depletion was assessed on an Agilent Bioanalyzer with RNA Nano Chip (Agilent). An average of 647 ng of rRNA-depleted RNA was recovered in each sample with no observable 16S and 23S rRNA peaks. Samples were dehydrated and stored in RNAsable preservative (LD–Biomatrica). RNA samples were sequenced on Illumina HiSeq platform using standard Illumina RNA preparation protocol. An average of 9.21 million 2 x 150 nct paired reads were obtained per sample.

Gene expression analysis

All reads were quality filtered (Q = 18) in AfterQC (Chen et al. 2017); on average 96% of reads were retained. Rockhopper (McClure et al. 2013) was used to align and annotate reads to the circular and linear chromosomes and symbiotic plasmid of *Rhizobium* sp. IRBG74 genome. Any counts for non-protein coding RNAs were removed from analysis. While all experiments were originally performed in triplicates, 3 samples did not pass our control threshold for the minimum number of protein-coding annotated counts, and were removed from further analysis. Because depth of sequencing affects the probability of transcript identification, aligned sequence counts were then rarefied to the lowest count number among samples as described previously (Craig et al. 2020). This produced a full dataset of 5412 genes and 12 samples. Distributions of mRNA counts within each sample were quartile-normalized across all samples based on the assumption of a similar dispersal of mRNA expression values in all conditions.

To define differentially expressed genes, the genes were filtered based on four distinct criteria. Genes were not considered differentially expressed and were removed if they (1) did not fall into the top 90% percent of expression range in at least one sample type; (2) showed no variation in their expression level across samples (defined as coefficient of variance (CV) $\leq 31\%$, which constitutes 10th percentile of the CV range); (3) did not show at least 2-fold expression difference at least in one control vs. test sample type comparison; (4) showed no statistically significant ($q \leq 0.01$) expression difference in at least one comparison between different sample types. Statistical significance was calculated with moderated t-test incorporating Benjamini and Hochberg correction for multiple hypothesis testing as previously described (Withman et al. 2013). These filters produced a set of 1740 differentially expressed genes (DEGs) (Table S1).

Statistical and pathway analyses

Principal components analysis (PCA) was performed on full and differentially expressed genes (DEG) datasets utilizing in-house Matlab script (Shankar et al. 2017). DiVenn (Sun et al. 2019) was used to visualize the overlap of the differentially expressed genes among different conditions (in all cases compared to control samples). Hierarchical cluster analysis (average linkage) and K-means clustering ($K = 12$) were carried out in Genesis using Euclidean distance as we did previously (Gunasekera et al. 2008). PathView (Luo et al. 2017) was used to map DEGs to KEGG pathways and to define statistically significant differences in pathway expression among sample types utilizing the GAGE algorithm.

Construction of reporter and mutant strains of *Rhizobium* sp. IRBG74

To determine the role of *nod* genes and genes important for motility and chemotaxis in plant colonization, we constructed reporter mutants in *nodA*, *nthB* and *mcpE*, using pTH1703 (Cowie et al. 2006). pTH1703 contains promoter-less reporter genes GUS/RFP and β -gal/GFP and was used earlier to construct a reporter gene library in *Sinorhizobium melliloti* (Cowie et al. 2006). To generate the mutants, ~1Kb gene fragments were amplified by PCR using gene specific primers, purified and cloned into pENTER/D-TOPO vector (Life Technologies, USA). The gene fragments were then subcloned into pTH1703 using appropriate restriction enzymes to create a fusion to GUS-RFP. The recombinant PTH1703 plasmids were then mobilized into *Rhizobium* sp. IRBG74 by tri-parental mating using pRK2013 as helper plasmid. The transconjugants were selected on LB containing 125 μ g/ml gentamicin and 50 μ g/ml ampicillin. The gene insertion was confirmed by PCR using primers upstream of the cloned fragment and a vector specific primer, followed by sequencing.

Motility assays

The swimming motility phenotypic assay was as described (Mitra et al. 2016). The *mcpE* and wild type strains were inoculated onto LB plates containing 0.3% agar and motility was assessed by measuring the zone of swimming after incubation for 24 hours at 30°C.

Rice root colonization

The colonization of rice was determined as described earlier (Gyaneshwar et al. 2001). Briefly, seedlings were sampled at 3 days after inoculation (DAI) and the roots were then immersed in phosphate buffered saline (PBS) and vortexed at high for 30s. The resulting solution was serially diluted and placed on LB agar plates containing spectinomycin (100 µg/ml), incubated overnight at 30°C and colony forming units were counted.

Nodulation of *S. cannabina*

Nodulation of *S. cannabina* was determined as described earlier (Cummings et al. 2009; Mitra et al. 2016). *S. cannabina* plants, grown from surface-sterilized seeds, were grown in 100 ml paper cups containing sterile vermiculite and watered with nitrogen-free growth media. The plants were inoculated with the wild type and mutants strains of *Rhizobium sp.* IRBG74 and incubated in a growth chamber. The plants were harvested at 30 DAI and examined for nodulation.

β-glucuronidase (GUS) staining and activity

Histochemical GUS staining of roots using X-gluc was performed essentially as described earlier (Mitra et al. 2016). The GUS activity was quantified using 4-nitrophenyl β-D-glucuronide and expressed as Miller units (Miller 1972).

Data availability

Raw sequence datasets obtained in this project are available in the Sequence Read Archive repository (BioProject ID: PRJNA563912).

Results

Rhizobium sp. IRBG74 forms a nitrogen-fixing symbiosis with *S. cannabina* and is a growth promoting endophyte of rice. However, it is not known whether it utilizes similar or different mechanisms to sense and respond to its legume and cereal hosts. We have shown earlier that a *Rhizobium sp.* IRBG74 mutant defective in the O-antigen of lipopolysaccharide showed common as well as specific defects in colonization of *S. cannabina* and rice (Mitra et al. 2016). To further explore such mechanisms, we analyzed transcriptomic changes in *Rhizobium sp.* IRBG74 in the rhizosphere of *S. cannabina* and rice at 12 hpi and 72 hpi. The transcriptomes in the rhizosphere were compared with the transcriptome of bacterial cells cultured without the host plants.

Similarity of gene expression profiles

The transcriptomic dataset was quality filtered, annotated, trimmed to only contain protein-coding genes, rarefied, and normalized. This produced a dataset with 5412 genes. We used multivariate principal components analysis to assess the similarity of overall expression profiles among samples. As shown in Fig. 1A, control and rice 12 hpi samples formed tight sample groups that indicated very similar

expression profiles among independent biological replicates. Replicates collected at 72 hpi varied more within the same sample type. At 12 hpi, the overall pattern of gene expression in *Rhizobium* sp. IRBG74 exposed to *S. cannabina* roots changed extensively, which was in contrast to the samples exposed to rice roots (Fig. 1A). Numerically this can be shown by calculating the distribution of pair-wise distances between samples in three dimensional PCA space as we did previously (Labib et al. 2018) (Fig. 1B). While the distances between control and rice-12 hpi samples were small, distance magnitude was remarkably increased for the *S. cannabina*-12 hpi samples. At 72 hpi, expression profiles of *Rhizobium* sp. IRBG74 cells exposed to either *S. cannabina* or rice roots differed noticeably from that of the control samples (Fig. 1B). Similar trends were also observed when only the subset of differentially expressed genes was used for PCA analysis (data not shown).

Analysis of differentially expressed genes (DEGs).

A set of filter criteria (see Methods section) was used to define the subset of the transcriptome that responded to the presence of rice and/or *S. cannabina* rhizosphere. A total of 1740 genes were selected that showed differential expression in at least one of the comparisons (Table S1). Among these, 1025 genes were located on the circular chromosome, 548 genes were on the linear chromosome and 167 genes were present on the symbiotic plasmid (pSYM) (Table S1). Consistent with the findings of the PCA analysis described above, only 5 genes differed in expression between control and rice-12 hpi (Fig. 2). Interestingly, these genes (p0684-p0690) are present on pSYM and are annotated as being involved in the transport of dicarboxylates.

Significantly higher numbers of DEGs were revealed for other sample types in comparisons with the control samples. Among these, a set of 111 genes was differentially expressed in each of the remaining three comparisons, thus constituting a group of universally responsive genes (Table S2). Among these, 51 genes were upregulated and 60 were downregulated. The upregulated genes were located on the circular chromosome (12 genes), linear chromosome (33 genes), and on pSYM (6 genes). The genes showing higher transcription were annotated as encoding transporters for C4-dicarboxylates, dipeptides and sugars, and genes involved in sensing and regulating transcriptional responses. Of the downregulated genes, 45 were located on the circular chromosome, 13 were on the linear chromosome, and 2 were on pSYM (Table S2).

Host specific and common transcriptomic responses

Analysis of DE genes showed that at 12 hpi, *Rhizobium* sp. IRBG74 respond to *S. cannabina* rhizosphere in a substantially different manner than its response to the rice rhizosphere. In the rice rhizosphere, only 5 genes showed expression changes as compared to 540 genes in the rhizosphere of *S. cannabina* (Fig. 2). However, at 72 hpi, 569 DE genes were detected in the rice rhizosphere as compared to 510 DE genes in rhizosphere of *S. cannabina* (Fig. 2). Of these 206 and 136 genes were specific to rice and *S. cannabina*, respectively. These results indicate that *Rhizobium* sp. IRBG74 senses and responds to both its host plants and many of these responses are common.

Early responses of *Rhizobium* sp. IRBG74 to *S. cannabina* and rice

To better understand the physiology of *Rhizobium* sp. IRBG74 in the rhizosphere of a legume and cereal host, the 1740 DEGs were further mapped to functional pathways using the KEGG database. Statistically significant changes in many pathways were observed in each of the comparisons (Fig. 3, 4 and Table S3). Of the pathways that were significantly upregulated as compared to control at early time point (12hpi) but not at 72hpi, genes involved in chemotaxis and motility were highly expressed in the *S. cannabina* rhizosphere at 12hpi (Fig. 4 and Table S3). These included genes encoding methyl accepting chemotaxis proteins (MCPs) and those involved in further signal transduction and swimming motility. In contrast, genes involved in oxidative phosphorylation showed higher expression specifically in the rice rhizosphere at 12hpi (Fig. 4 and Table S3). These include genes coding for components of the electron transport chain such as succinate dehydrogenase, NADH- quinone oxidoreductase, cytochrome C, cytochrome D, high affinity cytochrome (*fixNOQP*) and ATPase.

Various physiologically important pathways showed increased expression in the rhizosphere of both plants. These included genes encoding two-component systems (TCS) important for sensing and responding to changes in the environment such as those important for nitrogen limitation (*ntrBC*, *glnB*, *glnD*, *rpoN*), phosphate limitation (*phoBR*), osmotic stress (*kdpDE*), acid stress (*chvIG*), polysaccharide biosynthesis (*ntrXY*), cell cycle regulation (*ctrA*), anaerobic respiration and denitrification (*regRS*) were upregulated (Fig. 4). In addition, genes annotated as peptidoglycan biosynthesis and those involved in metabolism of starch and sucrose were part of this early response. Genes involved in flagella synthesis were also upregulated.

Late responses of *Rhizobium* sp. IRBG74 to *S. cannabina* and rice

Bacterial growth in the plant rhizosphere is dependent upon utilization of nutrient sources in the root exudates. In accordance, genes annotated as ABC transporters were significantly upregulated in the rhizosphere of both *S. cannabina* and rice (Fig. 4). These contained transporters for sugars such as rhamnose, ribose, xylose, glucosides, maltose/trehalose and putative sugar transporters. In addition, transporters for branched chain amino acids, di and oligo peptides, nitrate, and ferric iron showed increased expression. Furthermore, genes involved in degradation of fatty acids, glyoxylate and dicarboxylate, inositol phosphate, branched chain amino acids and aromatic compounds showed increased expression. Other metabolic pathways of potential importance included genes involved in the metabolism of tryptophan and production of the plant hormone indole acetic acid (*nthAB*) (Fig. 4 and Table S3).

Pathways showing significant downregulation in the rhizosphere of *S. cannabina* and rice

In contrast to the upregulated genes for transport and degradation of likely nutrients present in the rhizosphere, genes involved in many central metabolic pathways were significantly down regulated as compared to controls, especially at 72hpi. These included genes involved in translation (ribosomal proteins, aminoacyl tRNA synthesis), RNA degradation, one carbon metabolism, fatty acid biosynthesis, and amino acid biosynthesis (Fig. 3, 4). In addition, genes involved in protein export were also downregulated. Interestingly the flagella assembly genes that showed upregulation at 12hpi were downregulated at 72hpi (Fig. 3, 4). We also observed that genes of sulfur assimilation were significantly downregulated (log ratio of -3 to -5) at 12hpi and 72 hpi. These included genes for transport and assimilation of sulfate into cysteine and methionine as well as those involved in utilization of organic sulfur (sulfonate) (Fig. 4 and Table S3).

Clustering genes by their expression patterns

The results of numerical and pathway analysis were also confirmed by K-means clustering (Gyaneshwar et al. 2005a,b; Sugawara et al. 2011) and were also used to delineate plant specific and general transcriptomic responses. In accordance with the overall observations of the pathway analysis, the gene expression patterns showed that transcriptomic changes of *Rhizobium* sp. IRBG74 to *S. cannabina* and rice could be separated as host-specific at 12 hpi but the responses were similar to both plant hosts at 72 hpi (Fig. 5). For example, cluster 5 contained genes that showed enhanced expression in the rhizosphere of *S. cannabina* at 12 hpi but not at 72 hpi and had no response to the rice rhizosphere (Fig. 5). This cluster contained genes involved in chemotaxis and motility.

In accordance with the numerical analysis, 572 genes in clusters 4, 7, 9 and 10 showed enhanced expression in the rhizosphere of both *S. cannabina* and rice at 72hpi (though at different levels) and thus were part of common responses of *Rhizobium* sp. IRBG74 to its legume and cereal hosts (Fig. 5). These genes consisted of those coding for functions likely important for growth in the plant rhizosphere such as environmental sensing, transporters for sugars, organic acids, fatty acids, amino acids and minerals, as well as microaerobic/anaerobic respiration and energy generation. In contrast to the clusters with upregulated genes, clusters 2, 6 and 12 together contained 656 genes that were downregulated in the rhizosphere of both plants (Fig. 5). The genes in these clusters comprised of those involved in translation (ribosomal proteins, initiation and elongation factors), one carbon metabolism, biosynthesis of amino acids and sulfur assimilation.

Role of nod genes in colonizing *S. cannabina* and rice

The rhizobial *nod* genes that lead to synthesis of Nod factors are induced by (iso)-flavonoids present in the host-legume root exudates. We found that *nod* genes were upregulated in both *S. cannabina* and rice rhizosphere. These included *nodAB* (synthesis of Nod factor backbone), *neoCP* (arabinosylation), *nodZ* (fucosylation), *nodIJ* (Nod factor secretion) and *nodD1* (transcriptional activator of *nod* genes). To confirm the expression of *nod* genes in association with *S. cannabina* and rice and to determine the role of Nod factor in plant colonization, we constructed a *Rhizobium* sp. IRBG74 strain expressing β -glucuronidase (GUS) under the control of *nod* promoter. In *Rhizobium* sp. IRBG74, the *nodABC* genes

form a single operon, which is expressed from the Nod box present upstream of *nodA* (Poinsot et al. 2016). The chromosomal integration of the *nodA*-GUS construct resulted in disruption of the *nod* operon and thus led to the lack of Nod factor production. The mutant strain was inoculated onto *S. cannabina* and rice seedlings and *nod* expression was determined by quantifying GUS activity. In accordance with the transcriptomic data, GUS activity was induced in cells growing in the rhizosphere of both *S. cannabina* and rice but at higher levels with *S. cannabina* as compared to rice. (Fig. 6). Additionally, GUS staining showed that *nodA* expression (blue color) was localized on the main root of *S. cannabina*, likely the site of infection and subsequent nodulation (Fig S1). In contrast, GUS staining on rice roots was more diffuse and was present both on the main and lateral roots (Fig S1). These results confirm that the *nod* genes of *Rhizobium* sp. IRBG74 are induced not only with *S. cannabina* but also with rice roots.

To further explore the role of Nod factor in plant colonization, the mutant was inoculated onto seedlings of *S. cannabina* and nodulation ability was observed 30 days after inoculation. Seedlings inoculated with the wild type and non-inoculated seedlings were used as positive and negative controls. As expected, the *nodA*-GUS mutant did not form nodules (Table 2) demonstrating that *Rhizobium* sp. IRBG74 requires Nod factor for nodulation of *S. cannabina*. To determine if Nod factor plays a role in rice colonization, the wild type (WT) and mutant strains were inoculated onto rice seedlings and colonization was determined by total cell counts of bacteria attached the root. In contrast to its interaction with *S. cannabina*, the *nodA* GUS mutant was not affected in colonizing rice roots as compared to the WT strain (Table 2). However, the mutant showed significantly less colonization when inoculated along with the wild type at 1:1 ratio (Table 2). These results show that Nod factor is not essential but provides a competitive advantage for colonization of rice by *Rhizobium* sp. IRBG74.

Table 2
Nodulation of *S. cannabina* and colonization of rice roots by *nodA*-GUS mutant of *Rhizobium* sp. IRBG74.

Strain	<i>S. cannabina</i> (No. nodules/plant)	Rice (cfu x 10 ³ /mg root fresh weight)
Wild type	38 ± 6	950 ± 30 ^a
<i>nodA</i> -GUS	0	780 ± 25 ^a
<i>nodA</i> -GUS + Wild type (1:1)	ND	145 ± 18 ^b
The results are expressed as Mean ± SD of three independent experiments. Values followed by different letter within a column are significantly different (P < 0.05) as determined by T-test. cfu: colony forming units.		

Expression of putative IAA synthesis genes in *S. cannabina* and rice rhizosphere

Transcriptomic analysis revealed an increased expression of tryptophan metabolic genes in the rhizosphere of both plants. Of these, nitrile hydratase (*nthB*, p0387)) and amidase (BN877_II1084) are

potentially involved in converting indole acetonitrile to IAA. An earlier report showed that IAA synthesis in *Rhizobium* sp. IRBG74 requires tryptophan supplementation (Biswas *et al.* 2000) indicating that IAA biosynthesis genes might be induced by tryptophan. To determine the expression of *nthB*, we constructed an *nthB*-GUS reporter strain and examined its expression with and without tryptophan. *nthB* expression significantly increased when the cells were grown with tryptophan as compared to cells without tryptophan (Fig S2). To confirm that *nthB* is upregulated in the rhizosphere, the reporter strain was inoculated on *S. cannabina* and rice and GUS activity was measured. In accordance with the transcriptomic data, *nthB* expression was significantly induced in the rhizosphere of both *S. cannabina* and rice (Fig S2). However, the mutant was not affected in its ability to nodulate *S. cannabina* or to colonize rice (data not shown).

Role of chemotaxis in plant colonization

As mentioned above, many genes involved in chemotaxis and motility showed increased expression in the rhizosphere of *S. cannabina* at 12hpi. To determine the role of chemotaxis in plant colonization, we constructed a *mcpE*-GUS insertion mutant of *Rhizobium* sp. IRBG74. *mcpE* (BN877_I0491) is the signaling protein in the main chemotaxis operon. The corresponding gene in *A. tumefaciens* (a close relative of *Rhizobium* sp. IRBG74) is important for chemotaxis towards many chemicals (Ye et al. 2021). Similar to *A. tumefaciens*, *Rhizobium* sp. IRBG74 *mcpE*-GUS strain showed significantly lower swimming motility on both YM and LB plates (Fig S3). The mutant was inoculated onto seedlings of *S. cannabina* and rice and the effect on symbiotic interactions was evaluated by determining nodulation (*S. cannabina*) or root colonization (rice) and comparing them to plants inoculated with the WT strain. Noninoculated plants served as controls. The *mcpE*-GUS mutant showed significant defect in the colonization (Fig S4) and nodulation of *S. cannabina* as compared to the WT strain (Table 3). However, no significant differences were observed in the colonization of rice roots as shown by GUS staining (Fig S4).

Table 3

A chemoreceptor mutant of *Rhizobium* sp. IRBG74 is defective in nodulation of *S. cannabina* but not in colonization of rice.

Strain	<i>S. cannabina</i> (No. nodules/plant)	Rice (cfu x 10 ⁻³ /mg root fresh weight)
Wild type	35 ± 12	926 ± 39 ^a
<i>mcpE</i> -GUS	0	845 ± 42 ^a
The results are mean ± SD of three independent experiments. Values followed by same letter within a column are not significantly different as determined by T-test		

Discussion

Rhizobium sp. IRBG74 forms endosymbiotic associations with a legume (*S. cannabina*) and cereal (rice) and has emerged as a model to compare the mechanism of rhizobial association with two different plant

hosts (Mitra et al. 2016, Poinso et al. 2016; Ryu et al. 2020). Analysis of transcriptomic changes in the rhizosphere of *S. cannabina* and rice revealed both host specific and common responses that could be important for signaling and metabolic coordination in the rhizosphere. In *S. cannabina* rhizosphere, significant transcriptomic changes were observed at 12hpi. In contrast, responses to the rice rhizosphere were detected mostly at 72hpi (Fig. 1, 2).

This difference in response timings could be due to more rapid signaling associated with rhizobial-legume interactions as compared to rice.

However, many of the transcriptional changes were detected in both rhizospheres (Fig. 2). Two-component systems (TCS) were a major functional group that showed altered expression (Fig. 3, 4). Rhizobial TCS important for utilization of nitrogen (*ntrBC*, *ntrXY*, *glnBD*) (Patriarca et al. 1993; Pawlowski et al. 1991; Amar et al. 1994), phosphorus (*phoBR*) (Bardin and Finan 1998), and dicarboxylates (*dctAB*), (Reid and Poole 1998) showed increased expression. In addition genes involved in degradation of branched chain and aromatic amino acids, oligo peptides and inositol phosphates were induced. Branched chain amino acids are a nutrient source for developing bacteroids in pea (Prell et al. 2009). Similarly, catabolism of inositol is important for competitive nodulation of alfalfa (Kohler et al. 2010). Similar induction of these genes was reported for *Rhizobium leguminosarum* in the rhizosphere of legume and the non-legume sugar beet (Ramachandran et al. 2011). Taken together, these results suggest that legume-nodulating rhizobia are likely exposed to similar nutrient sources in root exudates of legumes and non-legumes.

In contrast to upregulated genes, *Rhizobium* sp. IRBG74 responded to the plant rhizosphere by significantly downregulating genes important for growth such as those involved in translation and synthesis of amino acids, nucleic acids and vitamins. Similar downregulation of housekeeping genes was reported for *R. phaseoli* in bean and maize rhizosphere (Aguirre-Noyola et al. 2021). In the model bacterium *E. coli*, the translational machinery and other biosynthetic genes are downregulated during nutrient starvation (Gyaneshwar et al. 2005). However, we do not think that the downregulation of housekeeping genes is due to nutrient starvation as the transcriptional responses in the rhizosphere were compared with cells without plant exposure. Such a shift in transcriptional responses from growth related functions to functions that are specific for responding to the rhizosphere could be important for survival and competition for plant colonization by rhizobia. Similar transcriptional responses of *R. leguminosarum* and *R. phaseoli*, were observed in the rhizosphere of non-legumes such as sugar beet and maize (Ramachandran et al. 2011; Aguirre-Noyola et al. 2021). It is thus possible that these are general transcriptional responses of rhizobia in rhizosphere of legumes and non-legumes.

Apart from these housekeeping genes, sulfur assimilation pathway genes were significantly downregulated (Fig. 4 and Table S3). In *E. coli* and *Bradyrhizobium japonicum* cysteine represses sulfate and sulfonate assimilatory genes (Kertesz 2000, Gyaneshwar et al. 2005a; Sugawara et al. 2011). It is possible that the root exudates of both *S. cannabina* and rice contain cysteine or cysteine rich peptides (CRPs) that repress sulfur assimilation genes in *Rhizobium* sp. IRBG74. Many legumes contain CRPs that

are important for terminal bacteroid differentiation (Kondorosi et al. 2013; Alunni and Gourion 2016). Rice also contains several genes for CRPs (Silverstein et al. 2007) and further research could determine their roles in rice-rhizobial interactions.

Rhizobium sp. IRBG74 nod genes are important for colonizing both hosts

The rhizobial *nod* genes, involved in the synthesis of Nod factor are essential for initial signaling and subsequent nodulation of legumes. *nod* genes are induced by (iso)flavonoids present in the root exudates at early stages of colonization (Stacey et al. 2006; Jones et al. 2007; Oldroyd 2013). Our results show that that *nod* genes of *Rhizobium* sp. IRBG74 were induced in the rhizosphere of *S. cannabina* and of rice (Fig. 6 and Fig S1). These results are in contrast to an earlier study showing that rice root exudates lack the ability to induce *nod* genes in rhizobia (Reddy et al. 1997) but root exudates of transgenic rice modified to secrete isoflavones could induce *nodY-lacZ* fusion in *B. japonicum* (Sreevidya et al. 2006). However, the rhizobial strains used in these studies are not natural partners of rice. In contrast, our earlier studies demonstrated that *Rhizobium* sp. IRBG74 is a growth promoting endophyte of rice (Biswas et al. 2000; Mitra et al. 2016). Additionally, *nod* genes of *Rhizobium* sp. IRBG74 are induced by apigenin (Poinso et al. 2016), a flavone that can be synthesized by rice. Use of a *nodA*-GUS mutant revealed that Nod factor, though not essential, provides a competitive advantage for rice root colonization in addition to being essential for nodulation of *S. cannabina* (Table 2). An earlier report showed that *Azorhizobium caulinodans* ORS571 colonized *Arabidopsis thaliana* through a Nod factor independent mechanism (Stone et al. 2001). However, *A. thaliana* does not form symbiotic associations with microbes and lacks the common symbiotic pathway (CSP) and thus may not be an ideal model to determine the role of Nod factor. In contrast to *A. thaliana*, rice possesses CSP, forms mycorrhizal symbiosis and can respond to Nod factor (Reddy et al. 1997, 2000). In addition, Nod factor of *Bradyrhizobium japonicum* alleviated plant defense responses in non-legumes such as tomato and maize (Liang et al. 2013). It is however possible that the rice colonization defect of the *nod* mutant could be due to factors other than signaling such as formation of biofilms and surface attachment (Fujishige et al. 2016). It will be interesting to further determine if *Rhizobium* sp. IRBG74 utilizes Nod factor to overcome rice defenses for infection and subsequent endophytic colonization.

Chemotaxis is required for colonization of *S. cannabina* but not of rice

In natural environments, rhizobia need to recognize the plant host in the presence of many other microbes and chemotaxis towards the host root is likely to play an important role in plant colonization by rhizobia (Miller et al. 2006; Poole et al. 2018, Campton and Scharf 2021). We observed that genes involved in motility and chemotaxis were one of the major functional group that showed increased expression with *S. cannabina* but not with rice (Fig. 4). In accordance with the differences in gene expression, a mutant disrupted for *mcpE* (BN877_I0491) showed defects in colonization and subsequent nodulation of *S. cannabina* but not in colonization of rice (Table 3 and Fig S4). It is possible that *Rhizobium* sp. IRBG74

recognizes specific root exudate component(s) of *S. cannabina* but not of rice. Alternatively, *Rhizobium* sp. IRBG74 could utilize other MCPs for rice colonization as its genome contains 20 additional MCPs. An earlier report showed that motility and chemotaxis provides a competitive advantage in nodulation of pea by *R. leguminosarum* (Miller et al. 2006).

IAA biosynthesis by *Rhizobium* sp. IRBG74 in rhizosphere

Genes involved in metabolism of tryptophan, a precursor for synthesis of the phytohormone IAA, were induced in the rhizosphere of both plants (Table S3). The increased expression of a putative nitrilase and amidase operon was confirmed using a GUS reporter (Fig S2). Rhizobial synthesis of IAA plays a role in legume nodulation (Badenoch-Jones et al. 1982; Fukuhara et al. 1994). Additionally, production of IAA was suggested to be a major mechanism of rice growth promotion by *Rhizobium* sp. IRBG74 (Biswas et al. 2000) and IAA synthesis by rhizobia was implicated in the formation of short thick lateral roots on rice (Reddy et al. 1997). Further analysis of IAA production by *Rhizobium* sp. IRBG74 may result in better utilization of this bacterium for rice growth enhancement.

Conclusions

Rhizobium sp. IRBG74, showed host-specific and general transcriptional responses in the rhizosphere of *S. cannabina* and rice. Responses to *S. cannabina* were detected much earlier than responses to rice. Nod factor synthesis was not only important for nodulation but provided competitive advantage for rice colonization. In contrast, chemotaxis was required only for nodulation of *S. cannabina*. Additionally, IAA production is likely to be a major mechanism of rice growth promotion by *Rhizobium* sp. IRBG74. These transcriptomic data could serve as a base for further studies to identify mechanisms involved in rhizobial colonization of rice in order to enhance the growth promoting interactions.

Declarations

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

PG and OP conceptualized, designed, analyzed the data and wrote the manuscript. AG performed the RNA sequencing and processed transcriptomic data. LK constructed reporter and mutant strains. SS and SW conducted physiological experiments. All authors contributed to writing and editing the manuscript.

Data availability

Raw sequence datasets obtained in this project are available in the Sequence Read Archive repository (BioProject ID: PRJNA563912).

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Figures

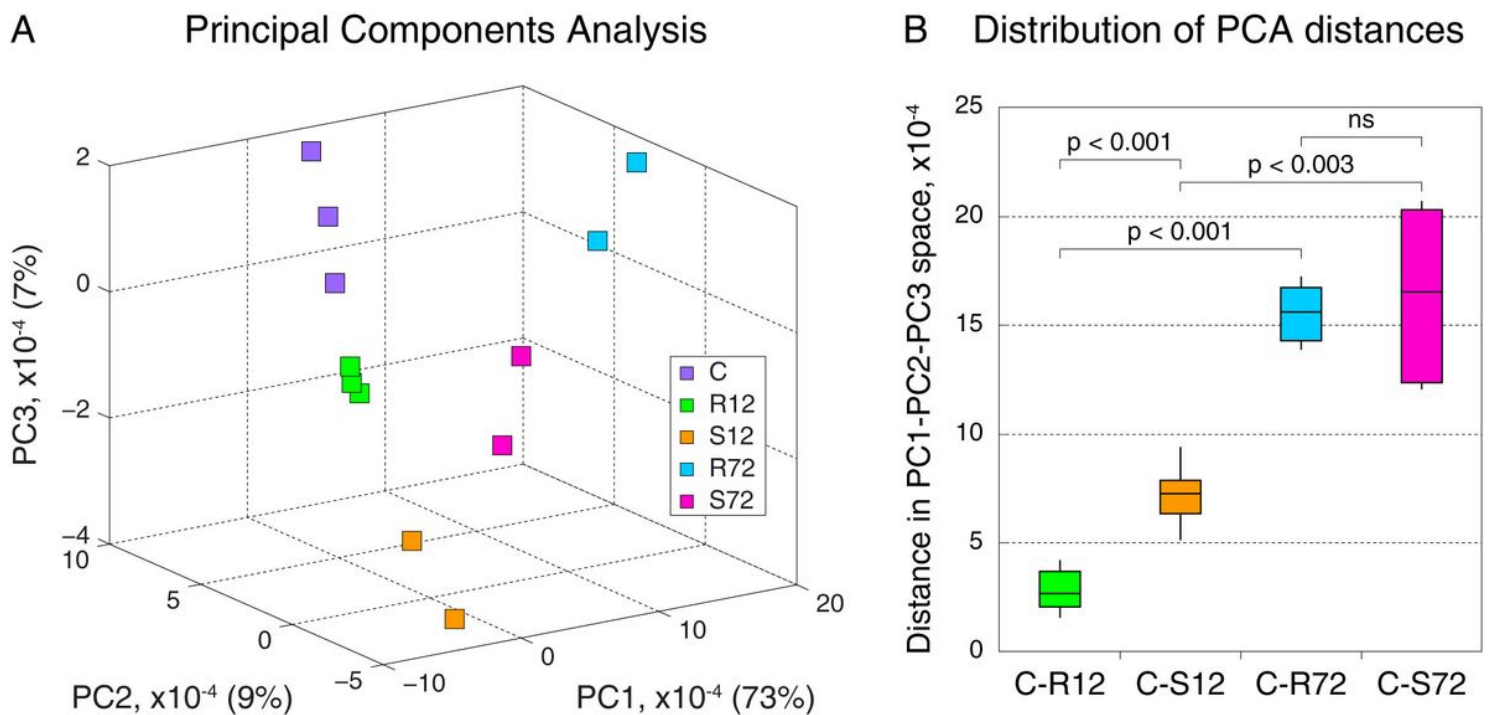


Figure 1

Similarity of global expression patterns among samples. Sample similarity was assessed by principal components analysis (PCA) using complete gene expression dataset. (A) Distribution of samples among the first three principal components (PC). Different groups are denoted by colors as shown in the legend. C – control samples, R12 – samples exposed to rice roots for 12 hours, R72 – rice roots for 72 hours; S12 – *Sesbania* roots for 12 hours, S72 – *Sesbania* roots for 72 hours. Percent of total variance captured by

each principal component is shown in parentheses. (B) Distribution of pair-wise distances in PC1-PC2-PC3 space between samples before and after supplementation. Boxplot whiskers represent minimum and maximum distances for each group. P values were calculated using two-tailed T-test; ns = not significant.

Figure 2

Overlap and regulation congruency of differentially expressed genes among comparisons. The set of 1740 DEGs was separated into subsets based on the comparisons in which the gene was differentially expressed. The up- or downregulation of each gene expression compared to the control samples is represented by yellow and purple color, respectively.

Figure 3

Hierarchical cluster ordination of the differences in pathway expression. Individual *Rhizobium* pathways as defined by Kyoto Encyclopedia of Genes and Genomes (KEGG) are shown in rows, comparisons are displayed as columns. Value of each pathway expression comparison is the cumulative $\log_2$ -transformed value of the expression ratios between each experimental sample type to the control samples. This value is represented by a color gradient as shown in the legend. Statistically significant differential expression (false discovery rate adjusted $p < 0.001$) is denoted by a star.

Sulfate / Thiosulfate	CysP Stop	CysU CysW	CysA
Alkanesulfonate	SsrA	SsrC	SsrB
Nitrate / Nitrite / Cyanate	NtrA	NtrB	NtrD
Phosphate	PstS	PstC PstA	PstB
Phosphonate	PhaD	PhaE	PhaC
Iron (III)	AftA	AftB	AftC
General L-Amino acid	AspJ	AspQ AspM	AspP
Branched-chain amino acid	LrvK	LrvH LrvM	LrvG LrvF
Glycine betaine / Proline	ProX	ProW	ProV
Osmoprotectant	OpuBC	OpuBB	OpuBA
Hydroxyproline	LhpP	LhpM LhpN	LhpO
D-Methionine	MetQ	MetI	MetH
Urea	UrtA	UrtB UrtC	UrtD UrtE
Raffinose/Stachyose/ Melibiose	MmsE	MmsF MmsG	MmsK
Lactose β -L-arabinose	LacE	LacF LacJ	LacK
Sorbitol / Mannitol	SmoE	SmoF SmoG	SmoK
α -Glucoside	AgtE	AgtF AgtG	AgtK
Oligogalacturonide	TsgB	TsgM TsgN	TsgA
α -1,4-Digalacturonate	AgaE	AgaF AgaG	?
Alduronate	LplA	LplB LplC	?
Trehalose / Maltose	ThrE	ThrF ThrG	ThrK
Glucose / Mannose	GtaA	GtaB GtaC	MalK
5 / Autoinducer 2 / D-Xylose	RbsB	RbsC	RbsA
Galactofuranose	YnfQ	YnfT	YnfR
D-Xylose	XylF	XylH	XylG
Fructose	FrcB	FrcC	FrcA
Rhamnose	RhaS	RhaP RhaQ	RhaT
myo-Inositol	IspA	IspP	IspA
Glycerol	GlnV	GlnP GlnQ	GlnS GlnT
sn-Glycerol 3-phosphate	UgpB	UgpA UgpF	UgpC

[illegible]

The diagram illustrates the regulatory network for the nitrogen fixation cycle. On the left, four environmental signals are shown: Nitrogen availability (low), K^+ limitation, Catabolite repression, and Redox signal. These signals regulate a central column of regulatory proteins. From top to bottom, these proteins are: NifC family (GlnD, NifB, NifX, NifA), Nitrogen assimilation (GlnA, GlnB, GlnC, GlnD, NifA), Potassium transport (KdpA, KdpB, KdpC, KdpE, KdpF), Tetracarboxylates transport (TetA, TetB, TetC, TetD), Electron transfer system (PetA, PetB, PetC, CycA/Y, CycN, CycO, CycX, CycP, CycD, CycB, CycE), and Aerobic respiration (CycA/Y, CycN, CycO, CycX, CycP, CycD, CycB, CycE). Arrows indicate the direction of regulation: '+' for activation and '-' for repression. For example, Nitrogen availability (low) activates NifC family, which in turn activates GlnD and NifB. K^+ limitation activates KdpA, which activates KdpB, KdpC, KdpE, and KdpF. Catabolite repression activates TetA, which activates TetB, TetC, and TetD. Redox signal activates RegB, which activates RegA, which in turn activates PetA, PetB, PetC, CycA/Y, CycN, CycO, CycX, CycP, CycD, CycB, and CycE.

Differences in gene expression of select pathways. Four groups of pathways were selected for visualization: ABC transporters, two-component systems, chemotaxis regulon, and flagellar assembly. For the first two sets, only operons with significant differential expression are shown. Each gene is colored based on the color gradient as shown in the legend; every gene box is split into four sections each corresponding to a separate comparison as shown. Gray color represents no difference in expression;

white color of gene box represents missing gene in the annotated *Rhizobium* genome as defined by KEGG.

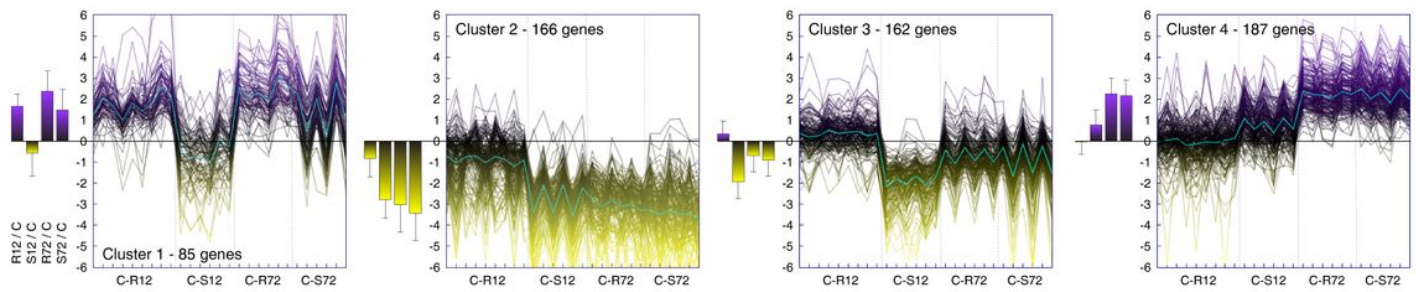


Figure 5

K-means clustering of the dataset of differentially expressed genes. Each box shows a transcriptional response of genes partitioned into a separate K-cluster. The pair-wise comparisons to the control samples are organized on the X axis into comparison groups as shown. Dotted line separates comparison sets. The log2-transformed expression ratios are indicated on the Y axis. Lines represent individual genes and are colored using the purple-to-yellow gradient matching those of Figures 2 and 3. The teal line designates the centroid. Average log2-transformed expression ratios of all genes in the cluster among each set of pair-wise comparisons are shown to the left of each cluster box; error bar indicates the standard deviation of values.

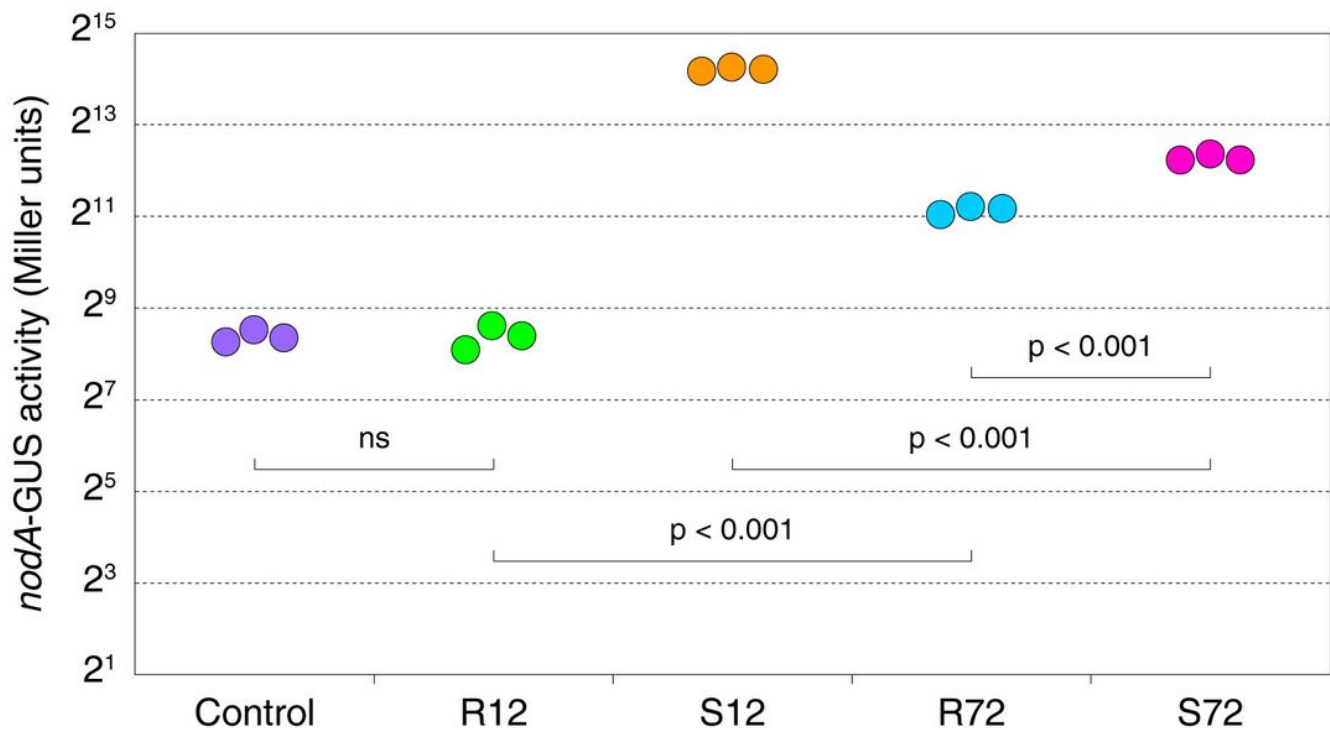


Figure 6

Expression of *nodA*-GUS in the rhizosphere of *S. cannabina* and rice. *Rhizobium* sp. IRBG74 strain containing *nodA*-GUS fusion was inoculated with *S. cannabina* or rice and GUS activity was quantified. S12 and S72- GUS activity in rhizosphere of *S. cannabina* at 12hpi and 72 hpi; R12 and R72- GUS activity in rice rhizosphere at 12 hpi and 72 hpi, respectively. The results are mean $\pm$ SD of three independent observations.

Supplementary Files

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- [TableS3.xlsx](#)