

1 **A transcriptomic analysis of the effect of genistein on *Sinorhizobium fredii* HH103 reveals novel rhizobial genes**  
2 **putatively involved in symbiosis.**

3

4 **Authors:** Pérez-Montaña F, Jiménez-Guerrero I, Acosta-Jurado S, Navarro-Gómez P, Ollero FJ, Ruiz-Sainz JE, López-Baena FJ, Vinardell JM\*.

5

6 Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Sevilla, Spain.

7

8 **Supplementary Information:**

9

10 **- Supplementary Tables**

11 **Table S1.** RNA-seq data validation using *q*RT-PCR.

12 **Table S2.** 36 and 35 *Sinorhizobium fredii* HH103 ORFs are regulated by genistein through *nod* (NB) and *tts* (TB) boxes respectively.

13 **Table S3.** 30 *Sinorhizobium fredii* HH103 ORFs are regulated by genistein but are not dependent on *nod* or *tts* boxes.

14 **- Supplementary Data 1.** General features of the total sequenced and mapped reads.

15 **- References cited in Supplementary Data 4.**

16

17 **Table S1. RNA-seq data validation using *q*RT-PCR (*q*PCR).** The expression of 19 individual genes was measured in the wild-type strain and  
 18 in its *nodDI* and *ttsI* mutant derivatives, both in the presence or absence of genistein. For *q*RT-PCR, fold-change values were calculated using  
 19 the  $\Delta\Delta C_t$  method and normalized to the reference 16S rRNA gene.

Gene name	Locus Tag / Promoter Sequences	RNA-seq			<i>q</i> PCR		
		Wild-type	NodDI <sup>-</sup>	TtsI <sup>-</sup>	Wild-type	NodDI <sup>-</sup>	TtsI <sup>-</sup>
-	psfHH103d_373 / NB1	19.3	1.58	32.44	15.45	2.30	100.19
<i>nodA</i>	psfHH103d_126 / NB8	17.65	-1.59	14.68	30.31	1.49	20.42
-	psfHH103d_161 / NB10	21.58	-1.26	22.37	15.85	2.34	33.05
-	psfHH103d_448 / NB13	27.06	-1.15	28.73	171.06	2.48	144.34
-	psfHH103d_229 / NB14	37.99	-1.21	23.89	39.26	1.59	89.26
<i>y4wE</i>	psfHH103d_257 / NB15	24.69	-1.3	31.5	63.79	1.37	118.87
-	psfHH103d_208 / NB17	17.43	-1.31	39.14	25.93	1.81	86.62
<i>syrM</i>	psfHH103d_367 / NB19	5.81	-1.72	9.29	6.51	1.71	8.47
<i>nopI</i>	SFHH103_04163 / TB2	3.13	-1.22	1.82	24.82	2.39	2.06
<i>gunA</i>	psfHH103d_292 / TB5	5.21	-1.17	2.11	5.49	2.06	1.64
<i>nopL</i>	psfHH103d_327 / TB9	4.56	-2.85	2.31	43.01	1.9	1.84
<i>nopC</i>	psfHH103d_3504 / TB12	6.29	-2.52	1.63	22.52	2.14	2.33
-	psfHH103d_255 / -	3.39	-1.14	3	3.65	1.2	9.51
-	psfHH103d_275 / -	3.45	-1.45	1.42	1.51	-1.2	-1.88
-	psfHH103d_306 / -	20.53	1.21	23.27	103.01	1.3	165.04
<i>flgJ</i>	SFHH103_00346 / -	7.57	-1.3	2.07	5.65	-1.51	-2.38
-	SFHH103_02192 / -	6.9	-1.63	11.64	68.04	-1.23	19.2
-	SFHH103_05321 / -	0.18	0.24	0.17	0.14	0.15	0.1
<i>fbpA</i>	SFHH103_00387	1.03	0.29	0.11	1.07	0.03	0.03

21 **Table S2.** 36 and 35 *Sinorhizobium fredii* HH103 ORFs are regulated by genistein through *nod* (NB) and *tts* (TB) boxes respectively<sup>a,b</sup>.

NB/TB <sup>c</sup>	Locus-tag (gene name)	Functions (putative functions)	Fold change <sup>d</sup>
NB1	psfHH103d_373 to 370	Hypothetical proteins (Carotenoid synthesis)	19.3 to 4.1
NB2	psfHH103d_381 to 379 ( <i>nodZnoeLnolK</i> )	Fucosylation of LCOs	3.7 to 2.6
NB8	psfHH103d_126 to 132 ( <i>nodABCINolO'noeI</i> )	Synthesis of LCOs backbone / LCO export / Methylation of LCOs	17.7 to 2.4
NB9	psfHH103d_118, 116	Hypothetical proteins	10.3, 4.5
NB10	psfHH103d_161	Hypothetical proteins (Polysaccharide inhibition)	21.6
NB13	psfHH103d_448, 447	Hypothetical proteins (Gene expression regulation?)	27.1, 5.3
NB14	psfHH103d_229 to 224 ( <i>fixABCX</i> )	Hypothetical proteins/ Electron transfer to nitrogenase	38 to 6.3
NB15	psfHH103d_257 ( <i>y4wE</i> )	Indole Acetic Acid (IAA) biosynthesis	24.7
NB17	psfHH103d_208	Hypothetical proteins (ABC-type transport)	17.4
NB18	psfHH103d_323 ( <i>ttsI</i> ) to 326	Transcriptional activator of the T3SS / Components of the T3SS apparatus / Conserved putative lipoprotein/(Transposase)	10.1 to 1.5
NB19	psfHH103d_367 ( <i>syrM</i> ) to 369	Transcriptional regulator of symbiotic genes / Hypothetical protein (Transcriptional regulator) / Hypothetical protein (Reduction of ethylene levels)	5.8 to 2.7
TB1	SFHH103_04201 ( <i>nopM2</i> )	E3 ubiquitination	2.1
TB2	SFHH103_04163 ( <i>nopI</i> ) to 04167	Putative effector/Hypothetical proteins	3.7 to 3.1
TB3	psfHH103d_90 ( <i>nopM</i> )	E3 ubiquitination	2.2
TB4	psfHH103d_287( <i>nopD</i> )	(C48 peptidase activity)	7.7
TB5	psfHH103d_292 ( <i>gunA</i> ), 293	Cellulase activity /Hypothetical protein	5.2, 6.5
TB8	SFHH103_335 ( <i>nopX</i> ) to 329	Translocation of T3SS effectors/Hypothetical proteins	10.3 to 3.3
TB9	psfHH103d_327 ( <i>nopL</i> )	(Host MAPK signalling interference)	4.6

TB10	psfHH103d_337 to 348 ( <i>nopBrhcInolUrhcLNQRSTU</i> )	Components of the T3SS apparatus	25.3 to 4.2
TB11	psfHH103d_349 ( <i>nopP</i> )	(Host MAPK signalling interference)	5.5
TB12	psfHH103d_3504, 3506, 351 to 353 ( <i>nopCAy4yPrhcVy4yS</i> )	Effector protein/Component of the T3SS apparatus/Component of the T3SS apparatus	7.2 to 4.8
TB13	psfHH103d_363( <i>nopT</i> )	C46 peptidase activity	4.5

22 <sup>a</sup> Genes driven by NB are dependent on NodD1. Genes driven by TB are dependent on both NodD1 and TtsI.

23 <sup>b</sup> With the exception of genes linked to TB1 and TB2, all these genes are located on the pSym (pl. d, pSfHH103d).

24 <sup>c</sup> According to Vinardell et al. [20].

25 <sup>d</sup> Fold induction with respect to non-induced cultures. In operons, the first and second values correspond to the first and last gene of the transcriptional unit respectively.

26

27 **Table S3.** 30 *Sinorhizobium fredii* HH103 ORFs are regulated by genistein but are not dependent on *nod* or *tts* boxes.

<p><b>17 genes depending on NodD1 (12/5)<sup>a</sup></b></p>	<p><b>Induced:</b> SFHH103_00841 (<i>ligE</i>), SFHH103_02192, SFHH103_06013, psfHH103d_255, psfHH103d_306 to 311, psfHH103d_322 to 319 (including <i>nodD2</i>)</p> <p><b>Repressed:</b> SFHH103_00450, SFHH103_00451, SFHH103_00799, SFHH103_01317, SFHH103_02323 (<i>hmuU</i>)</p>
<p><b>7 genes depending on NodD1 and TtsI (7/0)</b></p>	<p><b>Induced:</b> SFHH103_00346 (<i>flgJ</i>) to 00348, SFHH103_00844, SFHH103_01920, psfHH103d_275 and psfHH103d_274</p>
<p><b>6 genes no depending on NodD1 and/or TtsI (3/3)</b></p>	<p><b>Induced:</b> SFHH103_03274<sup>b</sup>, SFHH103_03749<sup>c</sup>, SFHH103_03875</p> <p><b>Repressed:</b> SFHH103_05321 to 05319</p>

28 <sup>a</sup> The number of genes induced/repressed is shown between brackets.

29 <sup>b</sup> The induction of this genes was 3.0, 2.4, and 2.7 in the wt, *nodD1* and *ttsI* backgrounds respectively.

30 <sup>c</sup> This gene only shows partial induction in the *ttsI* mutant background

31

**Supplementary Data 1. General features of the total sequenced and mapped reads.**

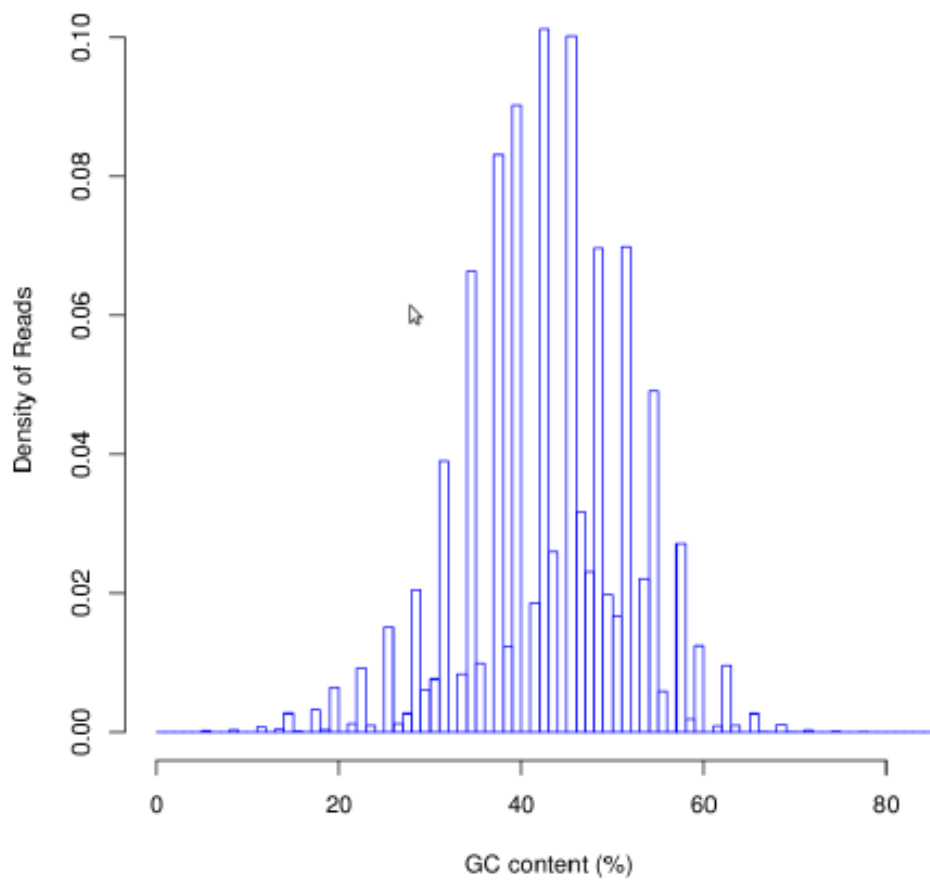
**Total Number of Reads:** Reads were mapped and pair using a Bayesian inference using Cufflinks v2.11 software. Worst quality reads were removed by means of Picard Tools.

<b>Sample condition</b>	<b>#Mapped Reads</b>	<b>High Quality Mapped Reads</b>	<b>Properly Pair</b>
Wild-type_1	43,514,666	41,856,055	40,447,758
Wild-type_2	47,976,366	46,001,800	43,889,282
Wild-type_GEN_1	41,545,412	39,919,415	38,189,900
Wild-type_GEN_2	44,649,058	43,054,550	41,288,654
NodD1 <sup>-</sup> _1	42,853,472	40,660,931	38,861,148
NodD1 <sup>-</sup> _2	39,211,312	37,460,139	35,672,278
NodD1 <sup>-</sup> _GEN_1	43,075,850	41,447,019	39,962,702
NodD1 <sup>-</sup> _GEN_2	44,443,662	42,776,374	40,794,302
TtsI <sup>-</sup> _1	46,494,272	44,688,429	43,212,764
TtsI <sup>-</sup> _2	46,671,594	38,746,887	35,760,244
TtsI <sup>-</sup> _GEN_1	41,472,444	39,520,075	37,767,084
TtsI <sup>-</sup> _GEN_2	45,623,306	13,074,049	12,332,926

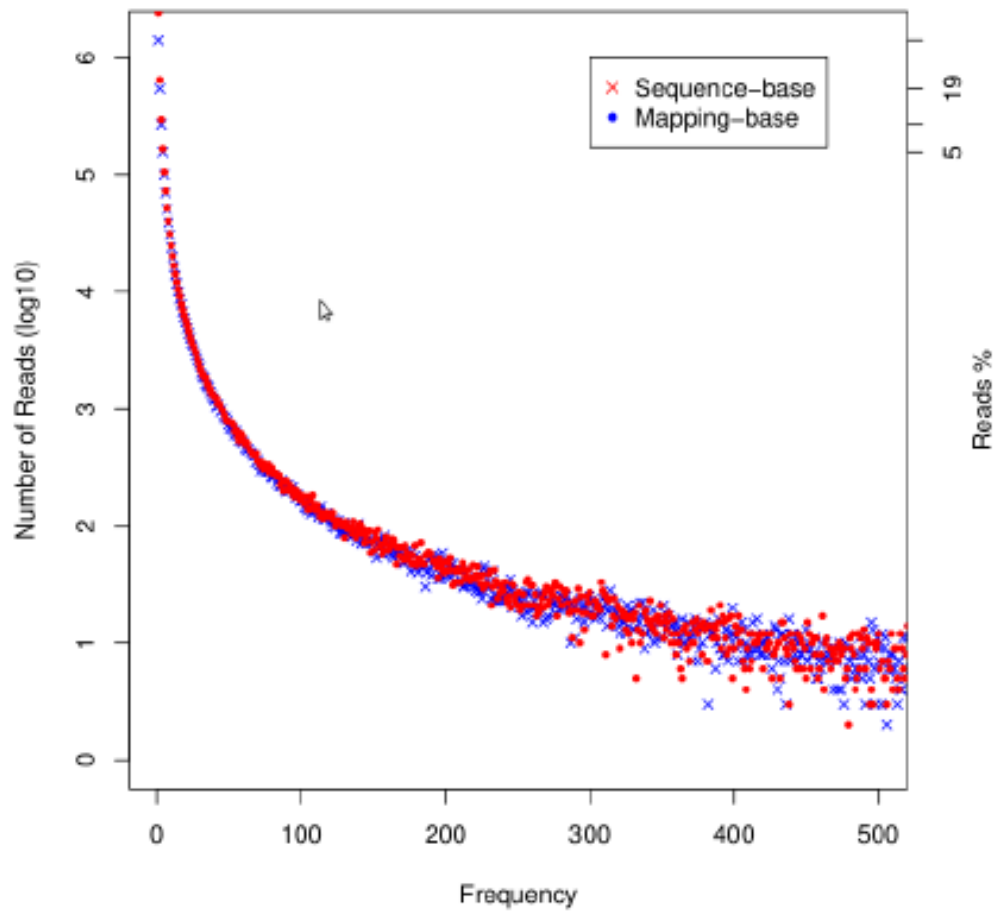
Two biological and independent experiments were carried out for each condition.

**Quality Read Controls:** Three different controls were performed to ensure the quality of reads: GC content, duplicate distribution and distribution respect genetic coordinates.

- *GC content:* distribution of GC content on mapped reads. A normal distribution around 45-55% is expected.

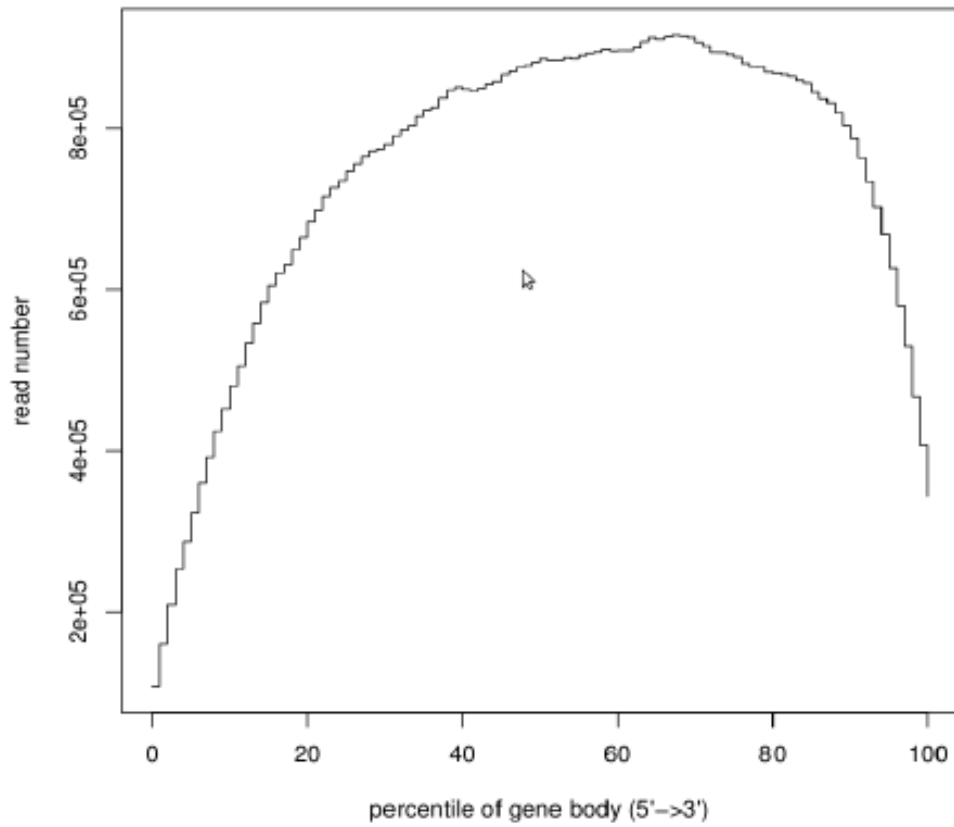


- *Duplicate distribution*: common distribution of duplicates in a RNA-Seq experiment shows a small number of reads with high levels of duplicates and a high number of reads with low levels of duplicates. All samples presented optimal values of duplicate distributions.



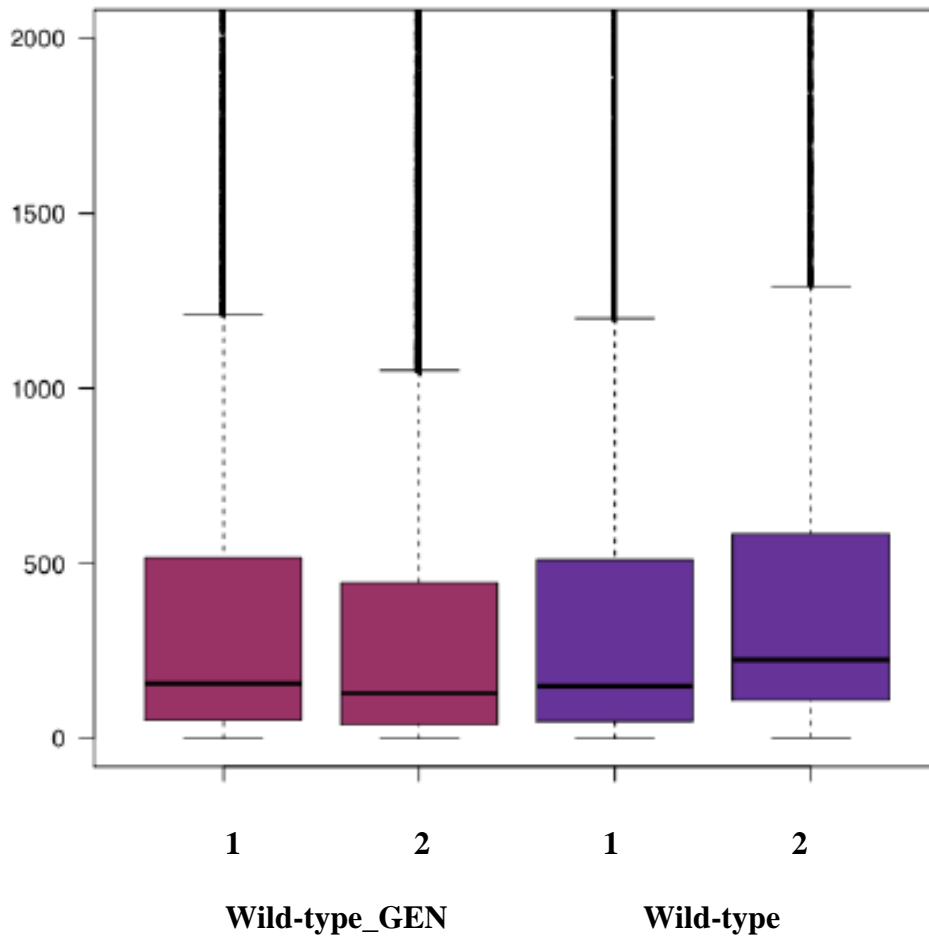


- *Distribution respect genetic coordinates*: centralization around 50 percentile of gene body is expected in high quality samples. Read concentration in both ends could be indicative of RNA degradation.

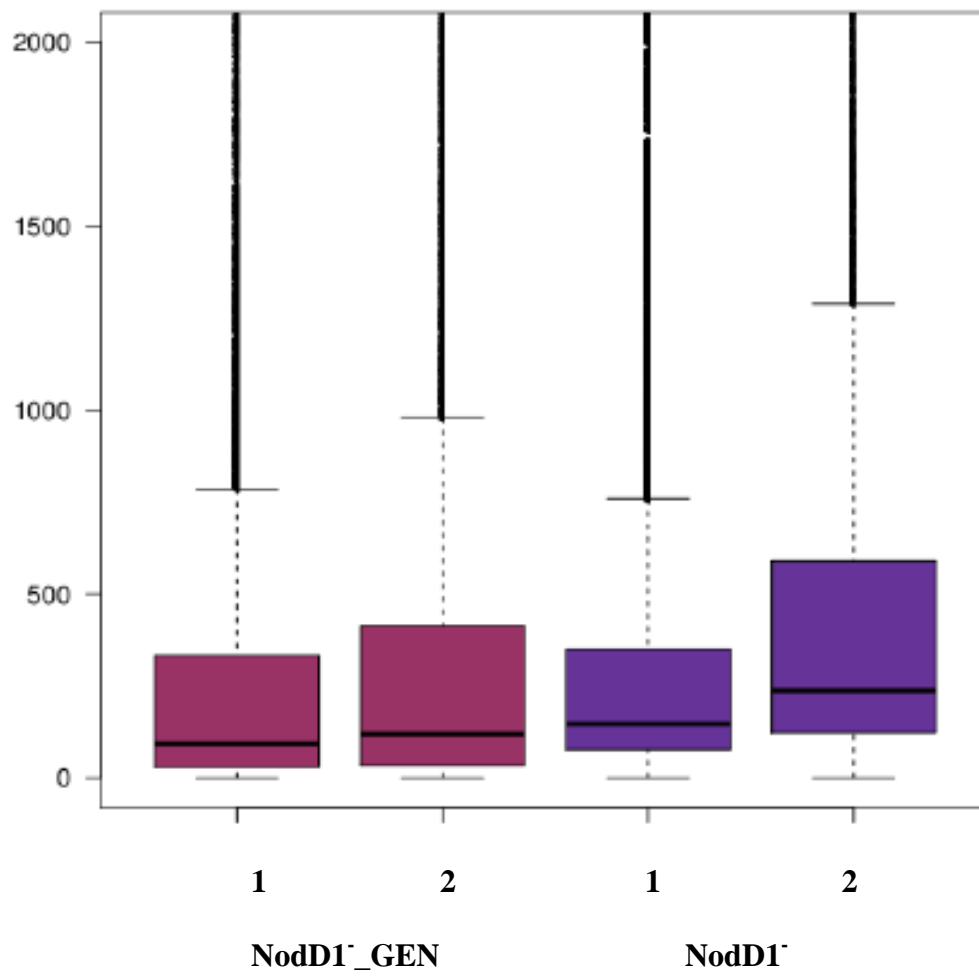


**Normalization:** normalization is needed to avoid statistical deviations due to differences in library sizes.

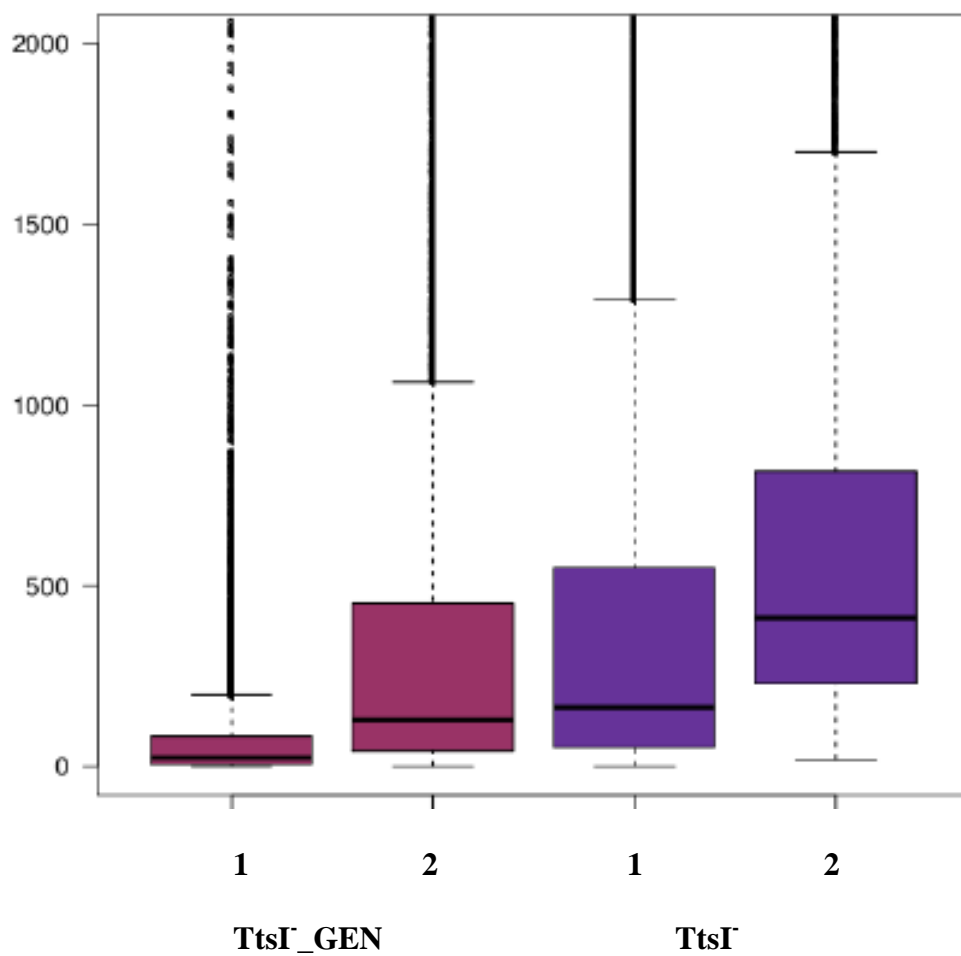
Number of reads per gene **before** normalization.



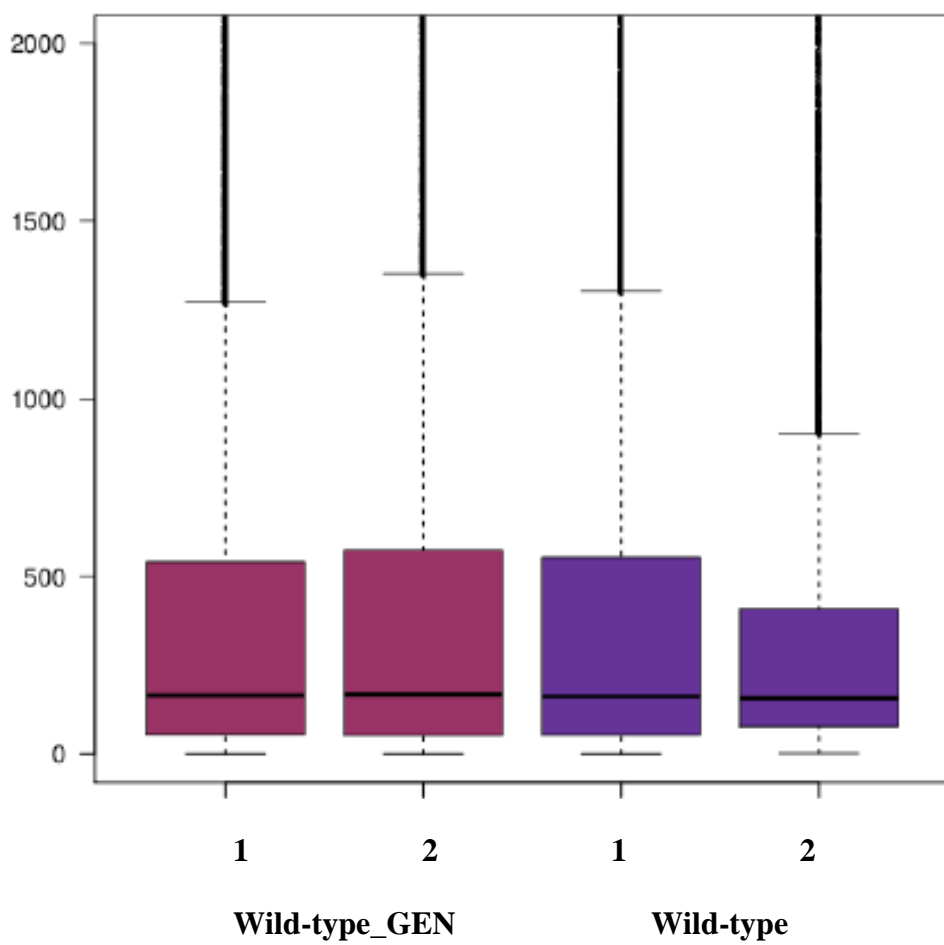
Number of reads per gene **before** normalization.



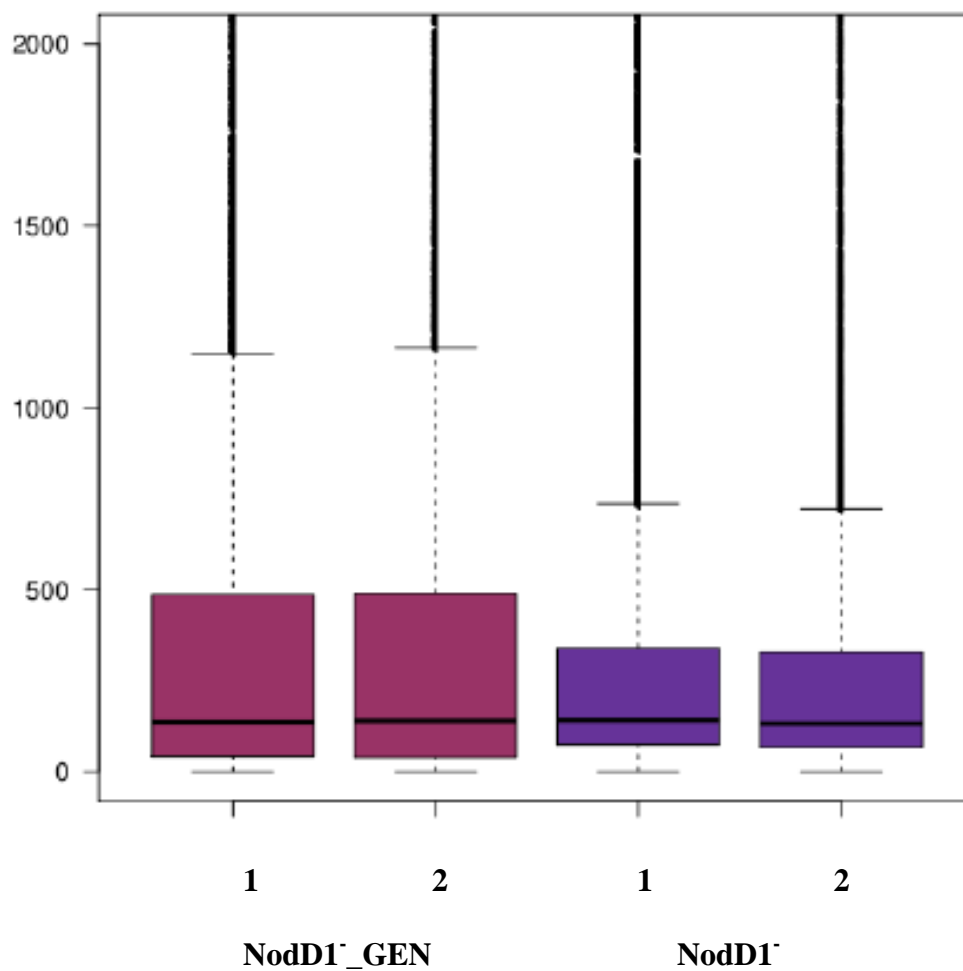
Number of reads per gene **before** normalization.



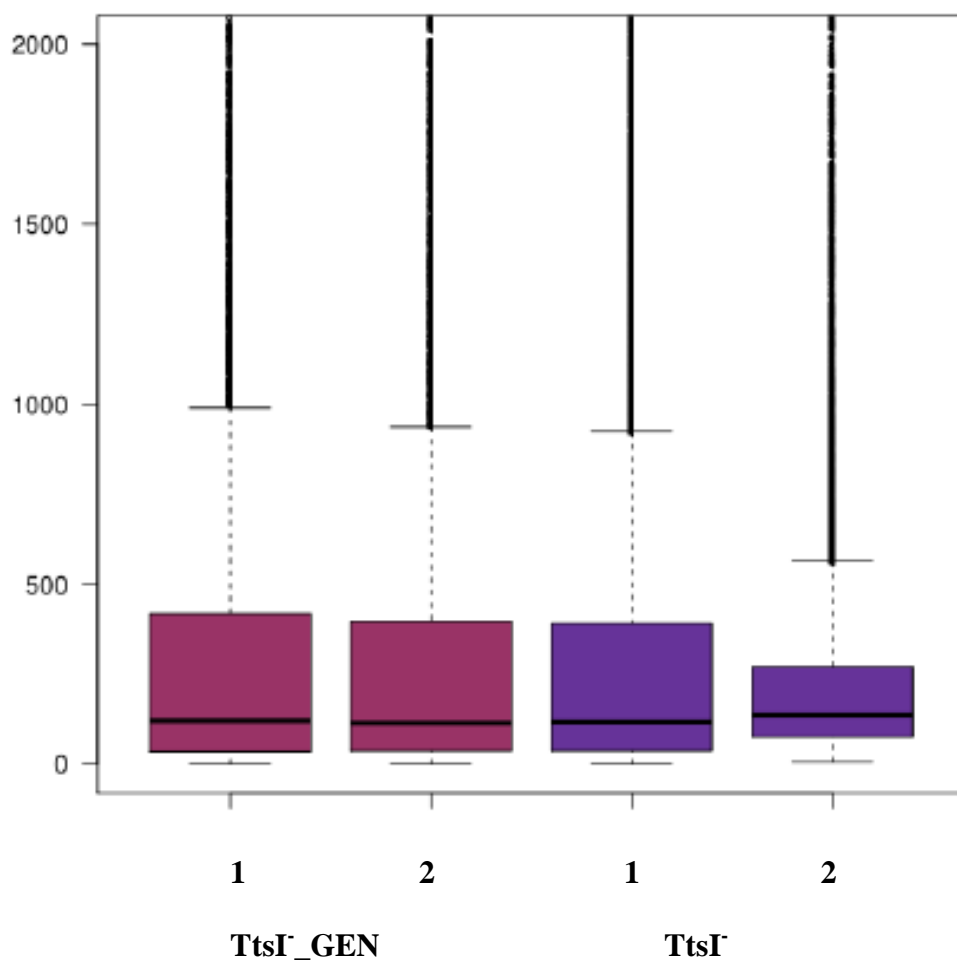
Number of reads per gene **after** normalization.



Number of reads per gene **after** normalization.



Number of reads per gene **after** normalization.



1 **References cited in Supplementary Data 4**

- 2 Ausmees, N. et al. Characterization of NopP, a type III secreted effector of *Rhizobium*  
3 sp. strain NGR234. *J. Bacteriol.* **186**, 4774-4780 (2004).
- 4 Bartsev, A. V., Boukli, N. M., Deakin, W. J., Staehelin, C. & Broughton, W. J.  
5 Purification and phosphorylation of the effector protein NopL from *Rhizobium* sp.  
6 NGR234. *FEBS Lett.* **554**, 271-274 (2003).
- 7 Bartsev, A. V. et al. NopL, an effector protein of *Rhizobium* sp. NGR234, thwarts  
8 activation of plant defense reactions. *Plant Physiol.* **134**, 871-879 (2004).
- 9 Bellato, C., Krishnan, H. B., Cubo, T., Temprano, F. & Pueppke, S. G. The soybean  
10 cultivar specificity gene *nolX* is present, expressed in a *nodD*-dependent manner, and of  
11 symbiotic significance in cultivar-nonspecific strains of *Rhizobium* (*Sinorhizobium*)  
12 *fredii*. *Microbiology.* **143**, 1381-1388 (1997).
- 13 Caldelari Baumberger, I., Fraefel, N., Göttfert, M. & Hennecke, H. New NodW- or  
14 NifA-regulated *Bradyrhizobium japonicum* genes. *Mol. Plant Microbe Interact.* **16**, 342-  
15 351 (2003).
- 16 Dai, W. J., Zeng, Y., Xie, Z. P. & Staehelin, C. Symbiosis-promoting and deleterious  
17 effects of NopT, a novel type 3 effector of *Rhizobium* sp. strain NGR234. *J. Bacteriol.*  
18 **190**, 5101-110 (2008).
- 19 de Lyra, Mdo C. et al. Inactivation of the *Sinorhizobium fredii* HH103 *rhcJ* gene  
20 abolishes nodulation outer proteins (Nops) secretion and decreases the symbiotic  
21 capacity with soybean. *Int. Microbiol.* **9**, 125-133 (2006).



22 Deakin, W. J., Marie, C., Saad, M. M., Krishnan, H. B. & Broughton, W. J. NopA is  
23 associated with cell surface appendages produced by the type III secretion system of  
24 *Rhizobium* sp. strain NGR234. Mol. Plant Microbe Interact. **18**, 499-507 (2005).

25 Earl, C. D., Ronson, C. W. & Ausubel, F. M. Genetic and structural analysis of the  
26 *Rhizobium meliloti* *fixA*, *fixB*, *fixC*, and *fixX* genes. J. Bacteriol. **169**, 1127-1136 (1987).

27 Hubber, A., Vergunst, A. C., Sullivan, J. T., Hooykaas, P. J. & Ronson, C. W.  
28 Symbiotic phenotypes and translocated effector proteins of the *Mesorhizobium loti*  
29 strain R7A VirB/D4 type IV secretion system. Mol Microbiol. **54**, 561-574 (2004).

30 Jabbouri, S, et al. *nolO* and *noeI* (HsnIII) of *Rhizobium* sp. NGR234 are involved in 3-  
31 O-carbamoylation and 2-O-methylation of Nod factors. J. Biol. Chem. **273**, 12047-  
32 12055 (1988).

33 Jiang, G. & Krishnan, H. B. *Sinorhizobium fredii* USDA257, a cultivar-specific soybean  
34 symbiont, carries two copies of *y4yA* and *y4yB*, two open reading frames that are  
35 located in a region that encodes the type III protein secretion system. Mol. Plant  
36 Microbe Interact. **13**, 1010-1014 (2000).

37 Jiménez-Guerrero, I., Pérez-Montaña, F., Medina, C., Ollero, F. J. & López-Baena, F. J.  
38 NopC is a *Rhizobium*-specific type 3 secretion system effector secreted by  
39 *Sinorhizobium (Ensifer) fredii* HH103. PLoS One **10**, e0142866 (2015).

40 Kambara, K. et al. Rhizobia utilize pathogen-like effector proteins during symbiosis.  
41 Mol. Microbiol. **71**, 92-106 (2009).

42 Kim, W. S. & Krishnan, H. B. A *nopA* deletion mutant of *Sinorhizobium fredii*  
43 USDA257, a soybean symbiont, is impaired in nodulation. Curr. Microbiol. **68**, 239-246  
44 (2014).

45 Kondorosi, E. et al. Involvement of the *syrM* and *nodD3* genes of *Rhizobium meliloti* in  
46 *nod* gene activation and in optimal nodulation of the plant host. *Mol Microbiol.* **5**, 3035-  
47 3048 (1991).

48 Kovács, L. G., Balatti, P. A., Krishnan, H. B. & Pueppke, S. G. Transcriptional  
49 organization and expression of *noIXWBTUV*, a locus that regulates cultivar-specific  
50 nodulation of soybean by *Rhizobium fredii* USDA257. *Mol Microbiol.* **17**, 923-933  
51 (1995).

52 Krishnan, H. B. & Pueppke, S. G. Flavonoid inducers of nodulation genes stimulate  
53 *Rhizobium fredii* USDA257 to export proteins into the environment. *Mol. Plant Microbe*  
54 *Interact.* **6**, 107-113 (1993).

55 Krishnan, H. B. et al. Extracellular proteins involved in soybean cultivar-specific  
56 nodulation are associated with pilus-like surface appendages and exported by a type III  
57 protein secretion system in *Sinorhizobium fredii* USDA257. *Mol. Plant Microbe*  
58 *Interact.* 2003 **16**, 617-625 (2003).

59 Kulkarni, G. et al. Specific hopanoid classes differentially affect free-living and  
60 symbiotic states of *Bradyrhizobium diazoefficiens*. *MBio.* **6**, 5 (2015).

61 Lamrabet, Y. et al. Mutation in GDP-fucose synthesis genes of *Sinorhizobium fredii*  
62 alters Nod factors and significantly decreases competitiveness to nodulate soybeans.  
63 *Mol. Plant Microbe Interact.* **12**, 207-217 (1999).

64 López-Baena, F. J. et al. Regulation and symbiotic significance of nodulation outer  
65 proteins secretion in *Sinorhizobium fredii* HH103. *Microbiology* **154**, 1825-1836  
66 (2008).

67 López-Baena, F. J. et al. The absence of Nops secretion in *Sinorhizobium fredii* HH103  
68 increases GmPR1 expression in Williams soybean. *Mol. Plant Microbe Interact.* **22**,  
69 1445-1454 (2009).

70 Lorio, J. C., Kim, W. S. & Krishnan, H. B. NopB, a soybean cultivar-specificity protein  
71 from *Sinorhizobium fredii* USDA257, is a type III secreted protein. *Mol. Plant Microbe*  
72 *Interact.* **17**, 1259-1268 (2004).

73 Lorio, J. C., Chronis, D. & Krishnan, H. B. Y4xP, an open reading frame located in a  
74 type III protein secretion system locus of *Sinorhizobium fredii* USDA257 and  
75 USDA191, encodes cysteine synthase. *Mol. Plant Microbe Interact.* **19**, 635-643 (2006).

76 Madinabeitia, N. et al. *Sinorhizobium fredii* HH103 has a truncated *nolO* gene due to a -  
77 1 frameshift mutation that is conserved among other geographically distant *S. fredii*  
78 strains. *Mol. Plant-Microbe Interact.* **15**, 150-159 (2002).

79 Marie, C. et al. Characterization of Nops, nodulation outer proteins, secreted via the  
80 type III secretion system of NGR234. *Mol. Plant Microbe Interact.* **16**, 743-751 (2003).

81 Meinhardt, L. W., Krishnan, H. B., Balatti, P. A. & Pueppke, S. G. Molecular cloning  
82 and characterization of a sym plasmid locus that regulates cultivar-specific nodulation  
83 of soybean by *Rhizobium fredii* USDA257. *Mol Microbiol.* **9**, 17-29 (1993).

84 Mercante, V. et al. The absence of protein Y4yS affects negatively the abundance of  
85 T3SS *Mesorhizobium loti* secretin, RhcC2, in bacterial membranes. *Front. Plant Sci.* **6**,  
86 12 (2015).

87 Miller, S.H. , Elliot, R. M., Sullivan, J. T. & Ronson, C. W. Host-specific regulation of  
88 symbiotic nitrogen fixation in *Rhizobium leguminosarum* biovar *trifolii*. *Microbiology.*  
89 **153**, 3184-3195 (2007).

90 Okazaki, S., Kaneko, T., Sato, S. & Saeki, K. Hijacking of leguminous nodulation  
91 signaling by the rhizobial type III secretion system. Proc. Natl. Acad. Sci. USA. **110**,  
92 17131-17136 (2013).

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

95 Silipo, A. et al. Covalently linked hopanoid-lipid A improves outer-membrane  
96 resistance of a *Bradyrhizobium* symbiont of legumes. Nat. Commun. **5**, 5106 (2014).

97 Skorpil, P. et al. NopP, a phosphorylated effector of *Rhizobium* sp. strain NGR234, is a  
98 major determinant of nodulation of the tropical legumes *Flemingia congesta* and  
99 *Tephrosia vogelii*. Mol. Microbiol. **57**, 1304-1317 (2005).

100 Staehelin, C. & Krishnan, H. B. Nodulation outer proteins: double-edged swords of  
101 symbiotic rhizobia. Biochem. J. **470**, 263-274 (2015).

102 Theunis, M., Kobayashi, H., Broughton, W. J. & Prinsen, E. Flavonoids, NodD1,  
103 NodD2, and *nod*-box NB15 modulate expression of the *y4wEFG* locus that is required  
104 for Indole-3-Acetic Acid Synthesis in *Rhizobium* sp. strain NGR234. Mol. Plant  
105 Microbe Interact. **17**, 1153-1161 (2004).

106 Tittabutr, P., Awaya, J. D., Li, Q. X. & Borthakur, D. The cloned 1-aminocyclopropane-  
107 1-carboxylate (ACC) deaminase gene from *Sinorhizobium* sp. strain BL3 in *Rhizobium*  
108 sp. strain TAL1145 promotes nodulation and growth of *Leucaena leucocephala*. Syst.  
109 Appl. Microbiol. **31**, 141-150 (2008).

110 Tsurumaru, H. et al. A Putative Type III Secretion System Effector Encoded by the  
111 MA20\_12780 Gene in *Bradyrhizobium japonicum* Is-34 Causes Incompatibility with  
112 Rj4 Genotype Soybeans. Appl. Environ. Microbiol. **81**, 5812-5819 (2015).

113 van Rhijn, P. & Vanderleyden, J. The *Rhizobium*-plant symbiosis. Microbiol. Rev. **59**,  
114 124-142 (1995).

115 Viprey, V., Del Greco, A., Golinowski, W., Broughton, W. J. & Perret, X. Symbiotic  
116 implications of type III protein secretion machinery in *Rhizobium*. Mol. Microbiol. **28**,  
117 1381-1389 (1998).

118 Xin, D. W. et al. Functional analysis of NopM, a novel E3 ubiquitin ligase (NEL)  
119 domain effector of *Rhizobium* sp. strain NGR234. PLoS Pathog. **8**, e1002707 (2012).

120 Zhang, L., Chen, X. J., Lu, H. B., Xie, Z. P. & Staehelin, C. Functional analysis of the  
121 type 3 effector nodulation outer protein L (NopL) from *Rhizobium* sp. NGR234:  
122 symbiotic effects, phosphorylation, and interference with mitogen-activated protein  
123 kinase signaling. J. Biol. Chem. 2011 **286**, 32178-32187 (2011).