

1    **Running head:** Spatiotemporal mapping of legume root exudation

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5    **\*Address correspondence for this author:**

6    Philip S. Poole

7    Department of Plant Sciences, University of Oxford

8    South Parks Road, OX1 3RB Oxford, United Kingdom

9    Telephone: +44 - (0)1865 - 275023

10   E-mail: philip.poole@plants.ac.ox.uk

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23 **Title:** Bacterial biosensors for in vivo spatiotemporal mapping of root  
24 secretion

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27 Francesco Pini<sup>a</sup>, Alison K. East<sup>a,b</sup>, Corinne Appia-Ayme<sup>b</sup>, Jakub Tomek<sup>c</sup>,  
28 Ramakrishnan Karunakaran<sup>b</sup>, Marcela Mendoza-Suárez<sup>a,b</sup>, Anne Edwards<sup>b</sup>, Jason J.  
29 Terpolilli<sup>b</sup>, Joshua Roworth<sup>a</sup>, J. Allan Downie<sup>b</sup> and Philip S. Poole<sup>a,b\*</sup>

30

31 <sup>a</sup>Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1  
32 3RB, UK

33 <sup>b</sup>Department of Molecular Microbiology, John Innes Centre, Norwich Research Park,  
34 Norwich NR4 7UH, UK

35 <sup>c</sup>Department of Physiology, Anatomy and Genetics, University of Oxford,  
36 Sherrington Road, Oxford OX1 3PT, UK

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42 **One sentence summary:**

43 Development of a suite of rhizobial *lux* reporters to map *in vivo* root exudation,  
44 spatially and temporally.

45 **Author Contributions**

46 Conceived and designed the experiments: FP, AKE, and PSP. Performed the  
47 experiments: FP, RK, MMS, CAA, AKE, JJT, JR, and AE. Analyzed the data: FP,  
48 AKE, and PSP. Software development: JT. Wrote the paper: FP, AKE, JAD, and PSP.

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65 Address correspondence to philip.poole@plants.ac.ox.uk

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68 in this article in accordance with policy described in the Instructions for Authors  
69 ([www.plantphysiol.org](http://www.plantphysiol.org)) is:

70 Philip S. Poole ([philip.poole@plants.ac.ox.uk](mailto:philip.poole@plants.ac.ox.uk))

71

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## 78    **Abstract**

79    Plants engineer the rhizosphere to their advantage by secreting various nutrients and  
80    secondary metabolites. Coupling transcriptomic and metabolomic analysis of the  
81    *Pisum sativum* rhizosphere, a suite of bioreporters has been developed in *Rhizobium*  
82    *leguminosarum* bv. *viciae* 3841, and these detect metabolites secreted by roots in  
83    space and time. Fourteen bacterial *lux*-fusion bioreporters, specific for sugars,  
84    polyols, amino acids, organic acids or flavonoids, have been validated *in vitro* and *in*  
85    *vivo*. Using different bacterial mutants (*nodC*, *nifH*), the process of colonization and  
86    symbiosis has been analyzed, revealing compounds important in the different steps of  
87    the rhizobial-legume association.

88    Dicarboxylates and sucrose are the main carbon sources within the nodules; in  
89    ineffective (*nifH*) nodules, particularly low levels of sucrose were observed  
90    suggesting that plant sanctions affect carbon supply to nodules. In contrast, high *myo*-  
91    inositol levels were observed prior to nodule formation and also in *nifH* senescent  
92    nodules. Amino-acid biosensors showed different patterns: a GABA biosensor was  
93    active only inside nodules, whereas the phenylalanine bioreporter showed a high  
94    signal also in the rhizosphere. The bioreporters were further validated in vetch,  
95    producing similar results. In addition, vetch exhibited a local increase of *nod*-gene  
96    inducing flavonoids at sites where nodules subsequently developed. These  
97    bioreporters will be particularly helpful to understand the dynamics of root exudation  
98    and the role of different molecules secreted into the rhizosphere.

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101    **Additional keywords:** Biosensor, Rhizosphere, Root secretion, Legume nodulation,  
102    *Pisum*, *Vicia*, Exudate

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## 105    **Introduction**

106    Due to root secretion, the narrow zone surrounding roots known as the rhizosphere, is  
107    a nutrient-rich region where plants encounter a diversity of microbes, fungi,  
108    invertebrates and the roots of other plants (Turner et al., 2013a). The constant  
109    improvement of sequencing techniques has rapidly increased acquisition of  
110    knowledge about rhizosphere communities (Turner et al., 2013b). It is now evident  
111    that there is a two-way dialogue, with plants actively shaping their rhizosphere  
112    community; this in turn, profoundly alters plant growth e.g. by improving plant  
113    nutrient uptake (Philippot et al., 2013). Secretion patterns from roots differ between  
114    plants (Biedrzycki and Bais, 2009) and despite much research on the role of chemical  
115    signals in mediating belowground interactions (Huang et al., 2014), many factors  
116    have not yet been identified (Badri et al., 2009). In addition, spatial and temporal  
117    variations in root secretions have never been clearly elucidated. Plant roots secrete  
118    large amounts of different compounds into the soil and about 20% of photosynthate is  
119    released through the roots (Kaiser et al., 2015). As a consequence of this, compared to  
120    bulk soil, the rhizosphere is a rich source of compounds sustaining bacterial growth.  
121    This results in the attraction to the rhizosphere of many different microorganisms,  
122    amongst them both pathogens and plant growth-promoting bacteria (Huang et al.,  
123    2014). Different techniques have been used to study this complex environment; e.g.  
124    proteomics, metabolomics and transcriptomics, (reviewed by: Sørensen et al., 2009;  
125    Oburger and Schmidt, 2016), but the results obtained with many of these different  
126    methodologies is that they give only a single ‘snapshot’ as it has not been possible to  
127    follow the same plant during the course of its development. Current 2D and 3D non-  
128    invasive imaging techniques to examine the physical architecture of the plant roots  
129    using radiation-based techniques of X-ray microfocus-computed tomography or  
130    synchrotron tomography have been described (Mooney et al., 2011). These techniques  
131    are powerful at revealing root architecture and development but do not yield  
132    information about chemical secretion by roots.

133        In response to plant secretions, bacteria modify expression of specific genes  
134    based on the molecules present in the rhizosphere (Ramachandran et al., 2011).  
135    Linking this with a method to monitor gene expression, we have used bacteria as  
136    biosensors to detect where and when specific molecules are secreted by plant roots.  
137    Bioluminescence is non-invasive and allows measurement of *in situ* differences in the

138 secretion of specific compounds in a semi-quantitative way. Lux biosensors have  
139 already been successfully used (Darwent et al., 2003), but lack of a simple system for  
140 image acquisition and complex experimental settings have so far limited routine use.  
141 Improvement of the technologies available and an increased knowledge of the  
142 bacterial transcriptomic response to roots, now gives us the chance to develop a suite  
143 of biosensors. These biosensors have been constructed using *Rhizobium*  
144 *leguminosarum* biovar *viciae*. Rhizobia are alpha-proteobacteria, ubiquitous within  
145 soil and able to establish nitrogen-fixing symbioses with specific legumes. Perception  
146 of environmental signals plays a pivotal role in association between plants and  
147 bacteria (Pini et al., 2011) and *R. leguminosarum* biovar *viciae* modifies its  
148 transcriptomic profile in different rhizospheres (Ramachandran et al., 2011).  
149 Moreover, the association of *R. leguminosarum* biovar *viciae* with peas has been  
150 studied in depth (Oldroyd et al., 2011; Terpolilli et al., 2012; Udvardi and Poole,  
151 2013), making *R. leguminosarum* biovar *viciae* one of the best candidates for  
152 biosensor development to investigate the rhizosphere. Pea and vetch plants have been  
153 used allowing us to monitor both the rhizosphere and the process of nodulation.  
154 Applications of this methodology will be multiple and are not restricted to  
155 leguminous plants; e.g. screening plant mutant libraries for those altered in secretion  
156 from roots or observing different exudations during seed germination.  
157



## 159 RESULTS

### 160 Solute Specificity of Biosensors

161 To spatially and temporally investigate secretion in the rhizosphere, we need  
162 biosensors able to detect compounds exuded into this environment. The idea that  
163 effective biosensors could be constructed using the control elements for expression of  
164 specific bacterial genes, often encoding components of solute transporters or enzymes  
165 with precise substrate recognition, has been previously described (Tecon and van der  
166 Meer, 2006; Yagi, 2007; Sørensen et al., 2009). Induction of expression of genes  
167 encoding transporters in the presence of their transported solute led to the  
168 identification of the substrates of many ATP-binding cassette (ABC) and tripartite  
169 ATP-independent periplasmic (TRAP) transporter systems of *Sinorhizobium meliloti*  
170 (Mauchline et al., 2006). Building on these data, Ramachandran *et al.* (Ramachandran  
171 et al., 2011) determined which genes of *R. leguminosarum* biovar *viciae* strain 3841  
172 (Rlv3841) are differentially expressed in the rhizospheres of pea, alfalfa and sugar  
173 beet, leading to identification of metabolites inducing expression of genes encoding  
174 both transport systems and metabolic enzymes. As many of these genes were induced  
175 in the rhizosphere in response to specific solutes, we sought to use their expression  
176 profiles to develop a suite of biosensors. These biosensors can be grouped by  
177 classification of inducer: i) sugars and polyols, ii) organic acids, iii) amino acids and  
178 iv) flavonoids (Table I). How each biosensor was selected is described below.

179 Eleven genes whose expression was induced in the rhizosphere during plant  
180 colonization by Rlv3841 of the pea rhizosphere (Ramachandran et al., 2011) were  
181 selected for biosensor development (Table I). These include seven solute-specific  
182 transport systems (which may transport plant-derived compounds, or those from any  
183 source e.g. fungi, other bacteria, within the rhizosphere), and four enzymes from  
184 metabolic pathways that are up-regulated during rhizosphere colonization (Table I).  
185 To increase the suite of biosensors, an additional two genes were included on the  
186 basis of microarray data that show differential expression in response to pea root  
187 exudate (Table I) (Ramachandran et al. 2011). The fourteenth biosensor is based on a  
188 salicylic acid-inducible export system for salicylic acid (Tett et al., 2014), which is  
189 known to be an important signaling molecule in plant defense. Whilst an inducer of  
190 gene expression was already known for many of these, the only previous  
191 characterization of pRL90085, RL4218, and pRL120556 was that their expression



192 was induced either in the rhizosphere or by pea-root exudate (Table II).

193

194 To develop the *lux*-based induction biosensors, the promoter regions upstream  
195 of the selected genes were used, often including the whole coding sequence of any  
196 upstream regulatory gene. Following PCR amplification, each region was cloned into  
197 the luminescence vector (pIJ11268, a plasmid stably inherited in rhizobia), in front of  
198 the bacterial *luxCDABE* operon (Frederix et al., 2014) (Supplemental Table S1). As  
199 these biosensors are plasmid-based it is possible to transfer them into different  
200 bacterial backgrounds, e.g. mutant strains of *R. leguminosarum* or other species of  
201 bacteria, although their expression in heterologous hosts may be limited by regulatory  
202 elements (Galardini et al., 2015).

203 Each of the fourteen *lux* reporters was tested on 26 different sugars and  
204 polyols and/or on a set of 18 selected compounds (organic acids, amino acids and  
205 plant metabolites) to establish the specificity of induction of *lux* expression from each  
206 of the bioreporters (Table I, Supplemental Table S2). Nine of the biosensors were  
207 induced by only a single compound tested: i.e. the polyols erythritol (pRL90085) and  
208 *myo*-inositol (RL4655; *intA*); the organic acids formate (RL4393; *fdsG*), malonate  
209 (RL0992; *matA*), tartrate (RL0996) and salicylic acid (RL1329; *sala* encoding an  
210 export system for salicylic acid induced by the presence of this molecule (Tett et al.,  
211 2014)); the amino acids phenylalanine (RL1860; *phhA*) and GABA (RL0102; *gabT*);  
212 and the flavonoid hesperetin (pRL100185; *nodABC*) (Table I). These solutes are  
213 considered to be specific inducers, i.e. the specific luminescence is  $\geq 10$ -fold the  
214 specific luminescence induced by the other solutes tested (apart from bioreporters for  
215 erythritol, formate, and GABA which have relatively high expression in the presence  
216 of a variety of chemically unrelated substrates (background), but show  $\geq 4$ -fold the  
217 specific luminescence of other solutes). However, this definition of specificity, in no  
218 way excludes the possibility that these bioreporters may also react with other  
219 compounds similar to the molecules which elicit the primary response, but not tested  
220 in this work, e.g. it is known that *nod* genes are induced by a variety of different  
221 flavonoids (Maj et al., 2010; Maxwell et al., 1989). Another two sugar biosensors  
222 were induced by two closely related compounds tested (Table I): i) by two  
223 aldopentoses, xylose or lyxose (RL2720; *rbsC*) or ii) induction by the di-saccharide  
224 sucrose was also achieved by the tri-saccharide raffinose (pRL120556), presumably  
225 because either the biosensor recognizes the sucrose 1-2  $\beta$  linkage in the di-/tri-

saccharide or raffinose is metabolized to sucrose. A further two bioreporters were induced by three closely related compounds; i) the C4-dicarboxylic organic acids succinate, malate and aspartate all cause induction of a single biosensor (RL3424; *dctA*) and ii) the polyol mannitol (C6) is the main inducer in biosensor (RL4218) although, a weaker induction is elicited also by its isomer sorbitol, and C5 adonitol (Table I) Sorbitol and adonitol are not considered specific inducers as their specific luminescence is <40% that of mannitol (Table I). One bioreporter was induced by six of the tested sugars and polyols, with the main inducer being the mono-saccharide fructose (RL0489; *frcB*). Induction was also seen with di-saccharides lactulose (4-O- $\beta$ -D-galactopyranosyl- $\beta$ -D-fructofuranose) and sucrose ( $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-fructofuranoside). These sugars all share a common structure containing a molecule of  $\beta$ -D-fructofuranose. Furthermore, this biosensor was induced by the polyols mannitol and sorbitol and the monosaccharide mannose. In this case, it is likely that induction occurs due to the catabolism of these compounds to fructose. The fourteen biosensors were used in the following experiments and are henceforth referred to by the compound that elicits the highest-fold change in *lux* expression (Table I). Provided rhizobial survival is not affected, these biosensors should be able to detect the presence of the compound/s for which they are specific, not only in the rhizosphere, whether they are derived from plants, from other bacteria or another source, but also in many other environments.

To discover the limits of detection for each biosensor, the sensitivity (the minimum concentration which causes induction) of the main inducer was determined using a range of different solute concentrations. The sensitivity ranges from the ability to detect levels of 1  $\mu$ M for mannitol and hesperetin to 10 mM for formate and malonate (Table I).

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## 252 **Data Extraction from Images using NightCROP**

Plants were analyzed using the NightOWL, a molecular imaging system linked to a sensitive charged-coupled device (CCD) camera, which allows measurement of light output from the Lux proteins. Images collected were first analyzed with the IndiGO software but although it was used to obtain data for nodules and vetch plants (output in cps / mm<sup>2</sup>), a limitation of this software is that it is unable to do an automatic segmentation of the picture, so NightCROP, a custom MATLAB

script, was developed. The script uses the SeNeCA algorithm (Tomek et al., 2013) to segment roots from the background in the light intensity channel, discarding those segments smaller than 1000 pixels. The information on the position of the roots is then used on the luminescence channel to specifically detect the fluorescence signal coming from the roots (Supplemental Fig. S1).

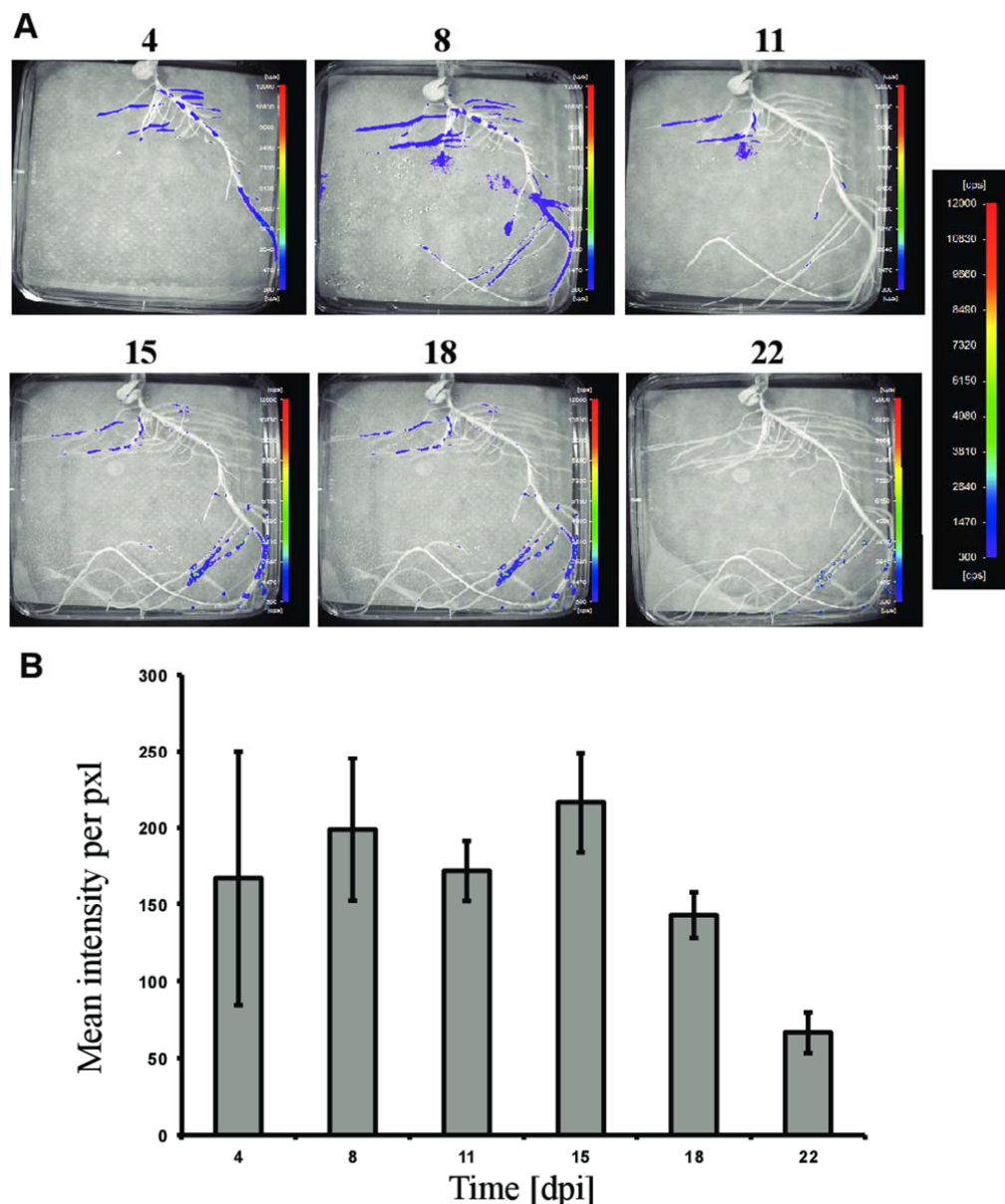
### ***In vivo* Mapping of Bacterial Colonization of Pea Roots**

Before using the suite of bioreporters to map the rhizosphere, we examined colonization by *R. leguminosarum* bv *viciae* of pea roots *in planta* using Lux mapping. Rlv3841 containing pIJ11282, expressing Lux constitutively under the control of the *nptII* promoter (Frederix et al., 2014), was used to inoculate pea seedling roots and the luminescent images revealed the location on the root of metabolically active Rlv3841. Lux expression relies on the bacteria having enough energy in the form of ATP to drive this energetically expensive production of light. Pea roots were imaged every 3-4 days until 22 dpi, with nodules becoming visible to the naked eye at 11-15 dpi.

At 4 and 8 dpi, luminescence from Rlv3841 colonization was mainly detected in the elongation zone of the lateral and primary roots (Fig. 1). At 11 dpi, overall luminescence was reduced probably due to energy depletion of the bacteria, (Fig. 1), although a signal was still detectable at a lower level (Supplemental Fig. S2). After 15 dpi, there was an increase of the signal concurrent with nodule development, and luminescence was then stable in nodules until 18 dpi, decreasing at 22 dpi, probably due to a general decline of plant health under these growth conditions. This constitutive Lux fusion is an energy sensor that is very effective at showing initial colonization and the total energy available to the bacteria, but must be used with caution in longer-term imaging, due to depletion of bacterial energy reserves resulting in loss of signal.

### ***In vivo* Mapping of Metabolites on Pea Roots and in Nodules**

The composition of pea root exudate was determined by metabolomic analysis of hydroponically-grown plants. Although plants grown in different conditions may differ in the composition of their root exudates, metabolomic analysis of these exudates is able to give important indications on compounds that it may be possible to retrieve from the rhizosphere. With the exception of formate, salicylic acid, and



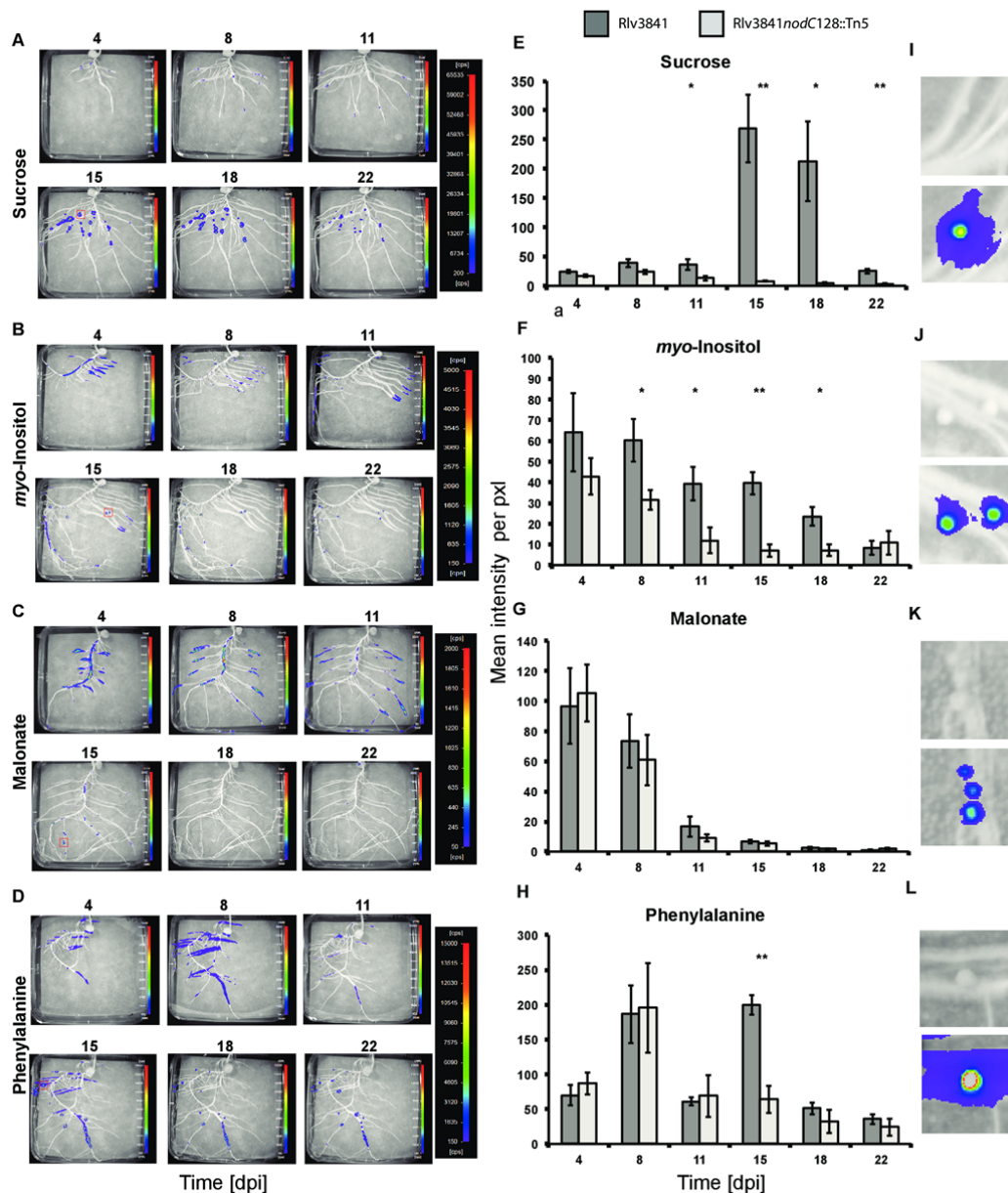
**Figure 1.** In vivo spatial and temporal mapping images of pea root colonization and nodulation with wild-type Rlv3841 luminescently-labelled with a constitutive neomycin phosphotransferase promoter controlling Lux expression in pIJ11282 (Frederix et al., 2014). A, Images were acquired at 4, 8, 11, 15, 18 and 22 dpi, with nodules visible to the naked eye between 11 and 15 dpi. B, Mean luminescence (pxl mm<sup>-2</sup>) with standard errors shown by bars.

hesperetin, the targets of our biosensors were among the 376 compounds detected in exudate from 23 d-old peas (Table II, Supplemental Table S3). A microarray experiment comparing Rlv3841 grown with and without addition of the same 23 d-old pea root exudates used for the metabolomic analysis was also performed. Results

from this are compared to a microarray where *R. leguminosarum* was inoculated into the pea rhizosphere and then harvested 1 dpi from 21 d-old plants (Ramachandran et al., 2011) (Table II). Of the fourteen genes used to develop biosensors, eleven (all except those detecting malonate, salicylic acid and GABA) showed increased (> 3-fold) expression with added pea root exudate and/or in pea rhizosphere-grown cells (Ramachandran et al., 2011) (Table II), however, relative increases in expression in these two experiments do not correlate particularly well. This discrepancy is likely to be due to differences in concentrations of solutes in the two experiments. Indeed, compounds collected from the root (exudate sample) and then diluted into liquid media are very unlikely to be present at the same concentrations as those in the experimental pea rhizosphere. In addition, the exact chemical composition of root exudates will also reflect the two different plant growth conditions. Despite these caveats, expression of approx. 80% of the genes selected to make biosensors are elevated by the conditions of the microarray experiments with added root exudate and/or the pea rhizosphere, which suggests that they will be useful in investigating the pea rhizosphere *in situ*.

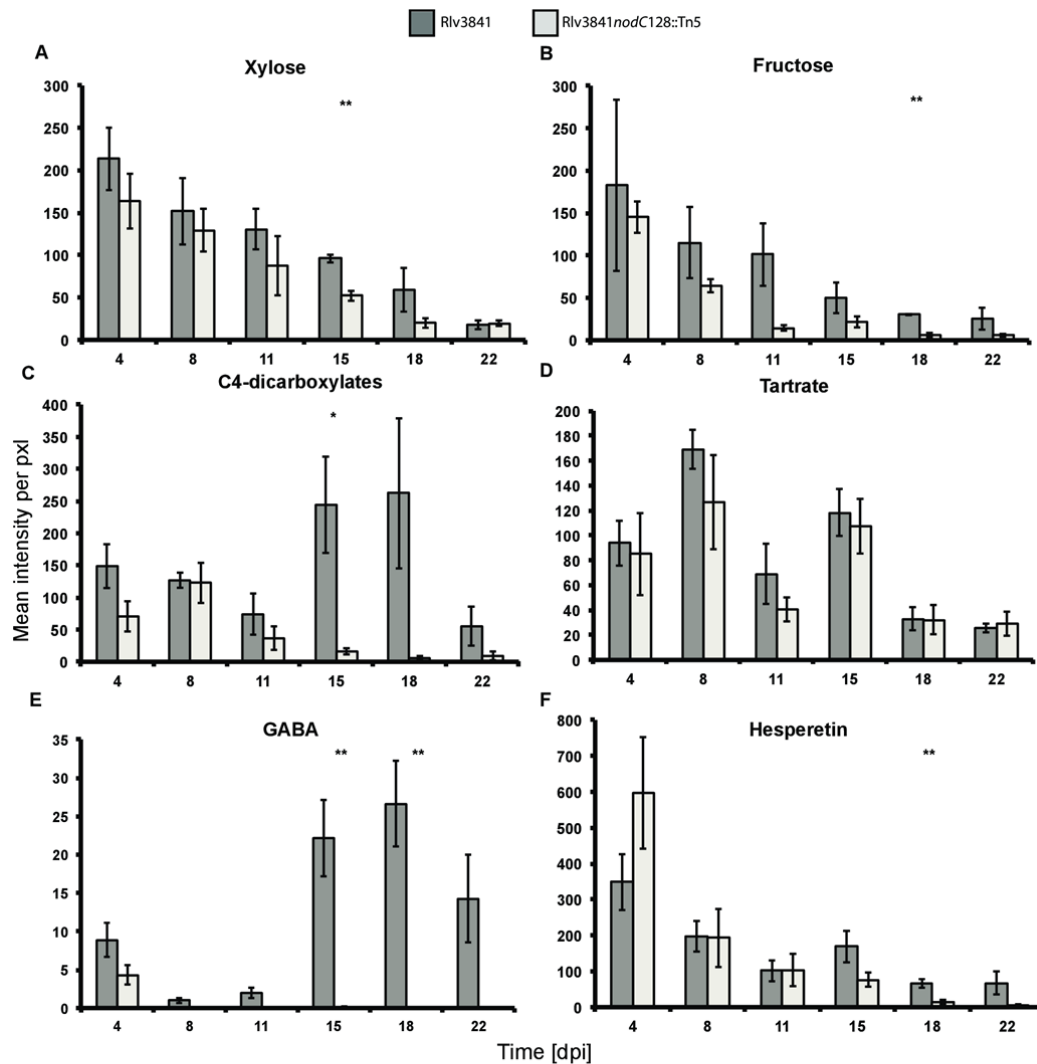
We analyzed *lux* expression on roots *in vivo* with each of the fourteen biosensors in Rlv3841. To ensure that expression of each biosensor is due to chemicals released from the plant roots, each bioreporter was spotted onto FP agar plates (supplemented with pyruvate and ammonium chloride) and incubated for 7 days (no growing plant present). The images (Supplemental Fig. S3) show no luminescence, thus indicating that the inducing compound is coming from the plant roots. To confirm that the biosensors respond to the same inducers on roots (*in vivo*) as they do *in vitro*, flooding experiments were undertaken. Pea roots at 4 dpi with a specific biosensor were flooded with a solution of a compound shown to induce Lux expression *in vitro* (Table I). Inoculation with the xylose, sucrose or GABA biosensor and flooding with xylose, sucrose or GABA, respectively, induced a Lux signal (Supplemental Fig. S4, A-C). Moreover, flooding the sucrose biosensor-inoculated roots with GABA, and the GABA biosensor-inoculated roots with sucrose showed no increase in Lux expression (Supplemental Fig. S4, D-E).

Images representative of those obtained on pea roots for the biosensors for sucrose (a sugar), *myo*-inositol (a polyol), malonate (an organic acid) and phenylalanine (an amino acid) are shown in Fig. 2, A-D. Luminescence images revealed that each of these four biosensors detected target metabolites during the 22



**Figure 2.** In vivo spatial and temporal mapping images of pea roots with biosensors detecting: sucrose (A) (scale: 200-65,535 cps), myo-inositol (B) (scale: 150-5,000 cps), malonate (C) (scale: 50-2,000 cps), and phenylalanine (D) (scale: 150-15,000 cps). Images were acquired at 4, 8, 11, 15, 18 and 22 dpi, with nodules visible to the naked eye between 11 and 15 dpi. These images are representative of those from biosensors in wild-type Rlv3841 background which nodulates peas. Comparison of mean luminescence intensity per pixel from pea roots inoculated with biosensors in wild-type Rlv3841 background (dark gray) and Rlv3841 nodC128::Tn5 background (light gray). Only wild-type Rlv3841 can form nodules. The biosensors detect: sucrose (E), myo-inositol (F), malonate (G) and phenylalanine (H). Standard errors are shown by bars, stars indicate significant differences between a biosensor in wild-type Rlv3841 and Rlv3841 nodC128::Tn5 backgrounds (t-test, \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ). Differences between each time point (ANOVA with post hoc Tukey test,  $p < 0.05$ ) are reported in Supplemental Tables S4 and S5. For representative images from Rlv3841 nodC128::Tn5 background see Supplemental Fig. S6. Close-up light-field photograph and luminescence of roots showing nodules at 15 dpi inoculated with biosensors detecting: sucrose (I), myo-inositol (J), malonate (K), and phenylalanine (L).

330 dpi period, and the results gave not only the location of the detected compound, but  
 331 also, by following the same plant over time, the changes that occur over the course of  
 332 an experiment. Bearing in mind that the bacterial cells containing the bioreporter need  
 333 to be metabolically active to generate a Lux signal, it is possible to get a false  
 334 negative result, i.e. the inducing compound is present at levels above the minimum  
 335 sensitivity, but the cells do not have the energy required to produce the signal. The

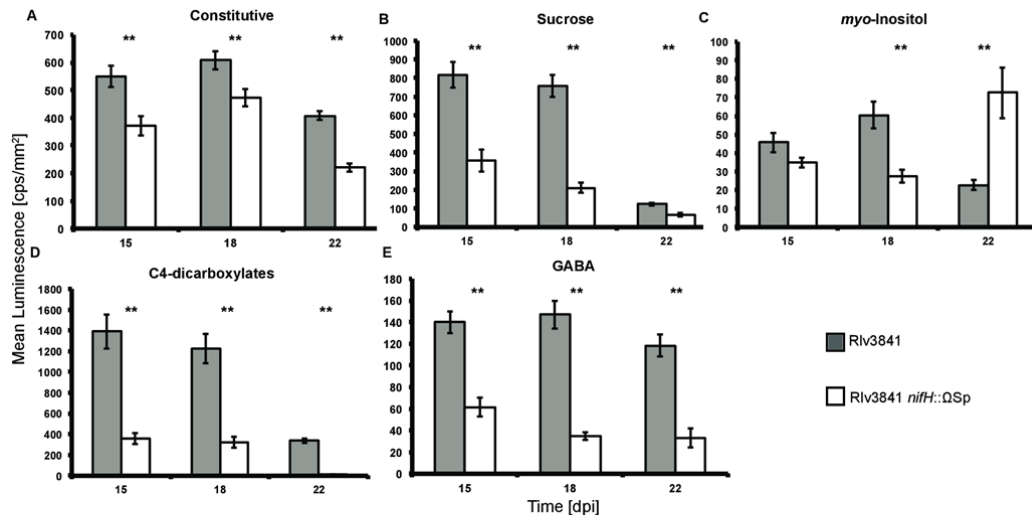


**Figure 3.** Comparison of mean luminescence intensity per pixel from pea roots inoculated with biosensors in wild-type Rlv3841 (dark gray) or the Rlv3841 nodC128::Tn5 mutant (light gray). Only wild-type Rlv3841 can form nodules. Biosensors detect: xylose (A), fructose (B), C4-dicarboxylates (C) tartrate (D), GABA (E) and hesperetin (F). Standard errors are shown by bars, stars indicate significant differences between a biosensor in wild-type Rlv3841 and Rlv3841 nodC128::Tn5 (t-test, \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ). Differences between each time point (ANOVA with post hoc Tukey test,  $p < 0.05$ ) are reported in Supplemental Tables S4 and S5. For representative images from Rlv3841 and Rlv3841 nodC128::Tn5 background see Supplemental Figs. S6 and S7, respectively.

336 signal from a constitutive Lux fusion fades over several days because it places a  
 337 substantial energy drain on cells. However, if Lux is detected, it indicates the  
 338 inducing compound is present. Analysis of the localization of luminescence in the  
 339 images (4 or more plants) and the different temporal patterns of metabolite detection  
 340 during the colonization and nodulation process (Fig. 2, E-H, Fig. 3) revealed that  
 341 there were similar detection profiles which could be grouped to aid analysis, although  
 342 the scale and maximum values observed are different for each bio-reporter.

343 The biosensor for the polyol, *myo*-inositol (able to detect  $\geq 100 \mu\text{M}$  *myo*-inositol,  
 344 Table I), was induced in the rhizosphere, mostly on the primary root and near the tips  
 345 of lateral roots (Fig. 2, B and F), with a steady decrease in expression over time.





**Figure 4.** Comparison of mean luminescence (cps mm<sup>-2</sup>) from pea nodules of different ages with biosensors in wild-type Rlv3841 (dark gray) or Rlv3841 *nifH::ΩSp* mutant (white) background. Nodules formed by Rlv3841 fix nitrogen whereas those formed by these mutant strains are unable to do so. Biosensors are specific for: constitutively active (A), sucrose (B), myo-inositol (C), C4-dicarboxylates (D) and GABA (E). Standard errors are shown by bars and stars indicate significant differences between a biosensor in wild-type Rlv3841 and the Rlv3841 *nifH::ΩSp* mutant (t-test, \* = p < 0.05; \*\* = p < 0.01). Differences between each time point (ANOVA with post hoc Tukey test, p < 0.05) are reported in Supplemental Tables S6 and S7.

346 Expression of the *myo*-inositol reporter that was seen 15 dpi was mostly in nodules. A  
 347 similar pattern was seen for the reporters detecting xylose (able to detect  $\geq 1$  mM  
 348 xylose), fructose (able to detect  $\geq 10$   $\mu$ M fructose) and the flavonoid, hesperetin (able  
 349 to detect  $\geq 1$   $\mu$ M hesperetin) (Fig. 3, A-B and F, Supplemental Fig. S5, A-B and F).  
 350 Biosensors for these compounds were highly expressed in the rhizosphere at 4-8 dpi,  
 351 usually localized at and above lateral root tips and then, despite a general decrease in  
 352 luminescence over the whole root, the compounds were then detected almost  
 353 exclusively in nodules 15-22 dpi (Supplemental Fig. S5, A-B and F). Expression of  
 354 the organic acid C4-dicarboxylate biosensor indicated that succinate/malate/aspartate  
 355 (able to detect  $\geq 100$   $\mu$ M/  $\geq 10$   $\mu$ M/  $\geq 10$  mM of succinate/malate/aspartate,  
 356 respectively) are present in the rhizosphere and then found specifically localized to  
 357 nodules at 15-18 dpi, with levels dropping by 22 dpi (Fig 4D, Supplemental Fig.  
 358 S5C).

359 A second expression profile, although similar to that described above, is that of  
 360 biosensors that gave a strong signal on roots but were barely detectable within  
 361 nodules. For example, the biosensor for malonate (able to detect  $\geq 10$  mM malonate)  
 362 (Fig. 2, C and G) was detected only in the rhizosphere (4-8 dpi), both on primary and  
 363 lateral roots, with the highest levels appearing just before the root tips (Fig. 2C). The  
 364 expression of this reporter fell over the time course (Fig. 2G) and was barely  
 365 detectable in early nodules (11-15 dpi) and undetectable in older nodules (18-22 dpi)  
 366 (Fig. 2, C and G).



367 A third profile, typified by the phenylalanine biosensor (able to detect  $\geq 10 \mu\text{M}$   
368 phenylalanine), showed two peaks of total luminescence (Fig. 2H) similar to that seen  
369 with the constitutive promoter (Fig. 1B), one at 8 dpi with the signal localized to the  
370 root elongation zone of lateral roots, and a second peak both in the rhizosphere and in  
371 nodules (15 dpi) (Fig. 2, D and H). Although the total signal from the phenylalanine  
372 reporter detection fell over time, at 18-22 dpi the luminescence was confined to  
373 nodules. By following the pattern of luminescence of a number of individual nodules,  
374 we conclude that the phenylalanine concentration peaks in nodules and then falls as  
375 the nodule senesces. As new nodules are being initiated constantly over the time  
376 course analyzed, there were numerous bright spots, which got brighter as the nodule  
377 developed and then faded as nodules got older (Fig. 2D). The tartrate sensor (able to  
378 detect levels  $\geq 100 \mu\text{M}$  tartrate), was expressed in a temporal pattern similar to that of  
379 phenylalanine with a similar dip in total levels of detection as nodules form 11 dpi  
380 (Fig. 3D), although, in contrast to the phenylalanine sensor, the tartrate reporter was  
381 expressed largely on the primary root (4-15 dpi) (Supplemental Fig. S5D); there may  
382 be low levels of expression of the tartrate reporter in mature nodules (22 dpi)  
383 (Supplemental Fig. S5D).

384 A fourth profile was high expression of the reporter in nodules once they are  
385 formed, with very weak or no luminescence in the rhizosphere on pea roots in general  
386 prior to that. The biosensor for sucrose typifies this group (Fig. 2, A and E). The total  
387 levels of expression of the sucrose reporter (able to detect levels  $\geq 100 \mu\text{M}$  sucrose),  
388 did not peak until the nodules were more mature (15-18 dpi) and then fell as the  
389 nodules senesced (22 dpi) (Fig. 2E). In the same way, expression of the GABA  
390 reporter (able to detect levels  $\geq 500 \mu\text{M}$  GABA), was hardly detected in the  
391 rhizosphere, but was induced specifically in nodules, with total levels peaking at 15-  
392 18 dpi (Fig. 3E, Supplemental Fig. S5E).

393 The fifth profile was seen with a group of biosensors that gave results too low to  
394 properly evaluate, because luminescence was routinely detected below a mean  
395 intensity *per pixel* of approx. 30 (Supplemental Fig. S6). The polyol reporters for  
396 erythritol (able to detect levels  $\geq 1 \text{ mM}$  erythritol), and mannitol (able to detect levels  
397  $\geq 1 \mu\text{M}$  mannitol), were barely detected in either the rhizosphere or in nodules of pea  
398 plants (Supplemental Fig. S6, A-B). The reporter for formate (able to detect levels  $\geq$   
399 10 mM formate), was expressed at very low levels and expression of the salicylic acid

reporter (able to detect levels  $\geq 1$  mM salicylate), was not detectable (Supplemental Fig. S6, C-D). This last result is not unexpected, because formate and salicylic acid were not found in the metabolomic analysis of pea root exudate (Supplemental Table S3).

Based on the spatial localization on pea roots of the reporters, we can draw conclusions about metabolites found in the rhizosphere prior to nodule formation (Table III). Xylose ( $\geq 1$  mM), fructose ( $\geq 10$   $\mu$ M), *myo*-inositol ( $\geq 100$   $\mu$ M), phenylalanine ( $\geq 10$   $\mu$ M) and hesperetin ( $\geq 1$   $\mu$ M) are largely exuded by the elongation zone of primary and lateral roots (Fig. 2, B and D, Supplemental Fig. S5, A-B and F). Malonate ( $\geq 10$  mM) was detected on both the uppermost portion of the primary root and the elongation zone of primary and lateral roots (Fig. 2C). Tartrate ( $\geq 100$   $\mu$ M) exudation was exclusively localized to the uppermost portion of the primary root, with little or no tartrate being detectable on the lateral roots (Supplemental Fig. S5D). Although present in the pea rhizosphere, the localization of C4-dicarboxylates was not clear, because detection in different regions varied over time with no clear pattern being observed (Supplemental Fig. S5C).

Between 11 and 15 dpi nodules became visible on pea roots. The reporters indicate that xylose ( $\geq 1$  mM), fructose ( $\geq 10$   $\mu$ M), sucrose ( $\geq 100$   $\mu$ M), *myo*-inositol ( $\geq 100$   $\mu$ M), C4-dicarboxylates ( $\geq 10$   $\mu$ M -10 mM, see Table I), phenylalanine ( $\geq 10$   $\mu$ M), GABA ( $\geq 500$   $\mu$ M) and hesperetin ( $\geq 10$   $\mu$ M) were present in nodules at 15 dpi (Table III, Fig. 2 A, B, D-F, and H, Fig. 4 B-E, and Supplemental Fig. S7). Malonate ( $\geq 10$  mM), and tartrate ( $\geq 100$   $\mu$ M), were detectable in nodules at 15 dpi, but at very low levels of bio-reporter expression (towards the lower limit of detection) (Table III, Fig. 2 C and G, and Supplemental Fig. S5D). For both phenylalanine and hesperetin, levels were highest in nodules at 15 dpi (dropping to 18 dpi, and dropping further to 22 dpi) (Table III, Supplemental Fig. S5F, and Supplemental Fig. S7, C-D). In most of the biosensors, the signal from nodules was strongly reduced by 22 dpi probably due to the general plant growth conditions, the only exception being the fructose reporter where the signal increased until 22 dpi (Table III and Supplemental Fig. S7B).

### Effect of Nodulation on Rhizosphere Metabolites

Since wild-type Rlv3841 induces nodule formation on pea roots, the effects of

433 nodulation on pea root secretion were investigated by comparing the induction of the  
434 biosensors in wild-type with their induction in a mutant unable to induce nodulation  
435 (a derivative of Rlv3841 carrying *nodC128::Tn5* (Downie et al., 1985)). Rhizobia  
436 produce Nod factors enabling recognition by legumes, and *nodC* mutants are unable  
437 to enter the plant or induce nodule formation (Udvardi and Poole, 2013). By  
438 comparing results obtained with wild-type and mutant backgrounds it is possible to  
439 separate the processes and metabolite changes of root colonization and nodule  
440 formation (Fig. 2 and 4, Supplemental Fig. S5, S8 and S9). Notably, in the pre-nodule  
441 formation stage, 4-8 dpi, no significant differences occurred between any biosensor in  
442 the two different backgrounds, other than with the *myo*-inositol reporter 8 dpi (Fig.  
443 2F). This suggests that the presence of Nod factor in itself does not alter either the  
444 amount or the localization of root secretions prior to nodule formation for the sugars  
445 xylose, fructose, and sucrose, the organic acids malonate, C4-dicarboxylates, and  
446 tartrate or the amino acids phenylalanine, and GABA.

447         With the *nodC* mutant that is unable to form nodules, there was a significant  
448 decrease in detection of *myo*-inositol at 8 dpi relative to wild-type (Fig. 2F). In a wild-  
449 type strain at this time point, nodule initiation has begun, although nodules are not yet  
450 visible to the naked eye. The conclusion that *myo*-inositol (at concentrations  $\geq 100$   
451  $\mu\text{M}$ ) is present in developing and very young nodules can be drawn from the relative  
452 decrease in *lux* expression of *myo*-inositol biosensor on roots inoculated with the  
453 *nodC* mutant. There was also a significant decrease in expression of the *myo*-inositol  
454 reporter in roots inoculated with the *nodC* mutant at each time point from 8 to 18 dpi,  
455 suggesting the *myo*-inositol detected in the roots inoculated with wild-type is due to  
456 nodule formation (Fig. 2F). Indeed, expression of the *myo*-inositol reporter can be  
457 seen clearly localized to nodules (Fig. 2B). *Lux* output from the sucrose reporter  
458 (detecting concentrations  $\geq 100 \mu\text{M}$  sucrose), was reduced significantly at 11-22 dpi  
459 in roots inoculated with the *nodC* mutant compared with wild-type (Fig. 2E); the  
460 expression of this reporter in roots inoculated with Rlv3841 was clearly seen localized  
461 to nodules at 15-22 dpi and this is consistent with sucrose from the shoot being  
462 supplied to nodules to support nitrogen fixation by rhizobial bacteroids (Fig. 2A).  
463 Expression of the phenylalanine reporter (detecting concentrations of  $\geq 10 \mu\text{M}$   
464 phenylalanine), was reduced significantly only at 15 dpi in roots inoculated with the  
465 *nodC* mutant compared to wild-type (Fig. 2H), suggesting phenylalanine is abundant

466 in nodules of this age. For fructose, C4-dicarboxylates, GABA, and hesperetin  
467 bioreporters, levels are significantly lower in the *nodC* mutant background at 18 dpi,  
468 15 dpi, 15 and 18 dpi, and 18 dpi, respectively (Fig. 3, B-C, and E-F), indicating the  
469 presence of these metabolites (fructose  $\geq 10$   $\mu$ M, C4-dicarboxylates  $\geq 10$   $\mu$ M –  
470 10mM, GABA  $\geq 500$   $\mu$ M, hesperetin  $\geq 1$   $\mu$ M) in pea nodules.

471

#### 472 **Effect of Symbiotic Nitrogen Fixation on Nodule Metabolites**

473 Biosensors detecting sucrose (sugar), *myo*-inositol (polyol), C4-dicarboxylates  
474 (organic acid) and GABA (amino acid) were used to examine levels of metabolites  
475 within effective and ineffective nodules by transferring each to a *nifH* mutant  
476 background (Karunakaran et al., 2009). The *nifH* mutant induces normal nodule  
477 formation, but is totally defective for nitrogen fixation. Since *nifH* encodes one of the  
478 components of nitrogenase, the enzyme complex that carries out nitrogen fixation,  
479 interruption of this gene by mutation means no functional nitrogenase is produced by  
480 the bacteria.

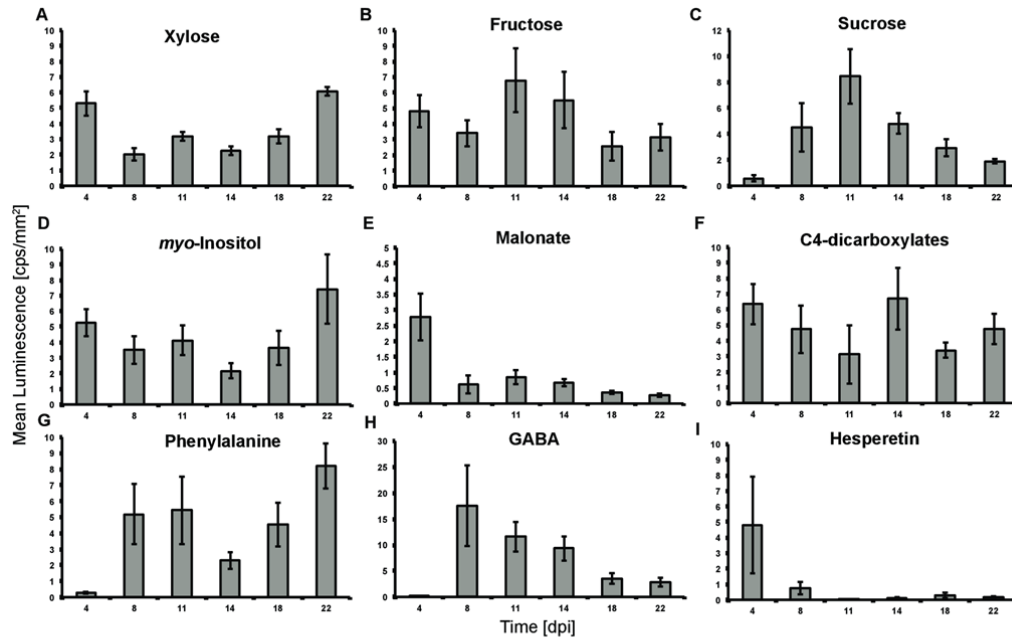
481 Levels of Lux expression from a constitutive promoter were approx. 30% lower  
482 in the Rlv3841 *nifH* mutant background relative to wild-type (Fig. 4A), presumably  
483 because ineffective nodules do not contain as many, and/or as metabolically active,  
484 bacteroids. Levels of detection of sucrose, *myo*-inositol, C4-dicarboxylates and  
485 GABA in effective (wild-type) and ineffective (*nifH* mutant-background) nodules  
486 were compared 15, 18, and 22 dpi (Fig. 4, B-E). Once corrected for overall lower  
487 activity in the *nifH*-mutant background (Supplemental Fig. S10), lower levels of  
488 sucrose (approx. 65% at 15 dpi, approx. 35% at 18 dpi), C4-dicarboxylates (approx.  
489 35% at 15-18 dpi, > 10% at 22 dpi), and GABA (approx. 65% at 15 dpi, approx. 30%  
490 at 18 dpi and approx. 50% at 22 dpi) were detected in ineffective nodules. The  
491 reduction of sucrose, C4-dicarboxylates and GABA in ineffective nodules suggests  
492 that plants sanction nodules that cannot fix nitrogen by decreasing their carbon supply  
493 (Fig. 4, B, and D-E). In contrast, the level of *myo*-inositol detected was higher in  
494 ineffective senescing nodules at 22 dpi (approx. 600% at 22 dpi) (Fig. 4C,  
495 Supplemental Fig. S10).

496 Malonate catabolism is not required for nitrogen fixation (Karunakaran et al.,  
497 2013). Transcriptomic data did not show a significant change in the expression of  
498 *matABC* genes in the rhizosphere of pea plants, but a significant difference was found

in the alfalfa rhizosphere (Ramachandran et al., 2011). The malonate biosensor indicates that malonate (at concentrations  $\geq 10$  mM) is present in the pea rhizosphere, although over time its level decreases to being barely detectable in nodules 15 dpi (Fig. 2, C and G). With evidence that  $\geq 10$  mM malonate is present in the rhizosphere of peas, colonization of roots by *R. leguminosarum* mutants defective for malonate metabolism was explored to see if an inability to metabolize malonate affects this process. Root attachment assays and nodule competition (as a measure of effective root colonization) were assayed using *matC* and *matA* mutants that are impaired in malonate transport and catabolism, respectively (Karunakaran et al., 2013). Although both mutants, defective in either malonate transport or catabolism, are less efficient at root attachment than the wild-type, there were no significant differences in pea root colonization compared with wild-type (Supplemental Fig. S11). We conclude from this that malonate uptake and its subsequent bacterial catabolism play a part in the initial attachment of *R. leguminosarum* to pea roots. However, although attachment might be the first step of bacterial colonization, in overall colonization assays, ability to either take-up or metabolize malonate has no effect.

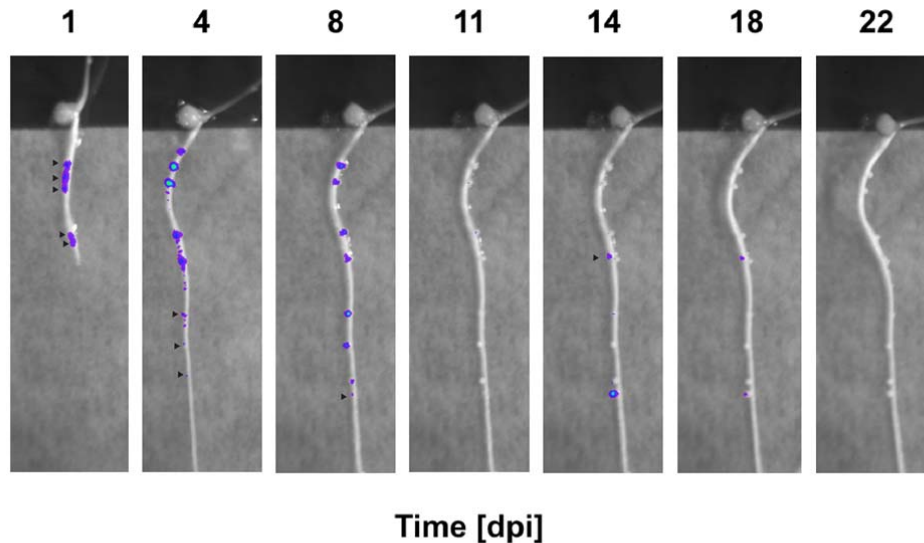
#### ***In vivo* Mapping of Metabolites on Roots and within Nodules of *V. hirsuta***

To investigate a different legume root and its rhizosphere, similar analyses were performed with *V. hirsuta*, on which Rlv3841 is also able to form nodules. Images were acquired at similar time points as for pea (up to 22 dpi), but with vetch plants the nodules appear to the naked eye earlier, at about 8 dpi (Fig. 5 and Table IV). With the exception of the polyols, erythritol and mannitol, and the organic acids, formate, tartrate and salicylic acid, all other metabolite were detected in the vetch rhizosphere over the time course (Table IV). For the compounds we failed to detect, we can't exclude the possibility that they may be present, but at levels too low for their detection, i.e. levels of erythritol at  $\leq 1$  mM, mannitol at  $\leq 1$   $\mu$ M, formate at  $\leq 10$  mM, tartrate at  $\leq 100$   $\mu$ M and salicylic acid at  $\leq 1$  mM. This differs from pea only in the case of the tartrate, which was detected in the pea rhizosphere but not in that of vetch. Metabolites xylose ( $\geq 1$  mM), fructose ( $\geq 10$   $\mu$ M), *myo*-inositol ( $\geq 100$   $\mu$ M), malonate ( $\geq 10$  mM), C4-dicarboxylates ( $\geq 10$   $\mu$ M -10 mM, Table I), and hesperetin ( $\geq 1$   $\mu$ M) were detected in the vetch rhizosphere at 4 dpi (Fig. 5, A-B, D-F, and I). These were also all detected on pea roots (Table III), but a difference between these two legume rhizospheres is that phenylalanine was barely detected in the vetch



**Figure 5.** Comparison of mean luminescence (cps mm<sup>-2</sup>) from vetch roots inoculated with biosensors in wild-type Rlv3841 background. Biosensors detect: xylose (A), fructose (B), sucrose (C), myo-inositol (D), malonate (E), C4-dicarboxylates (F), phenylalanine (G), GABA (H) and hesperetin (I). Standard errors are shown by bars. Differences between each time point (ANOVA with post hoc Tukey test,  $p < 0.05$ ) are reported in Supplemental Table S8. Nodules are visible to the naked eye from 8 dpi.

rhizosphere at 4 dpi (Fig. 5G) (the limit of detection is  $\geq 10 \mu\text{M}$  phenylalanine). In vetch, as in pea, sucrose (able to detect  $\geq 100 \mu\text{M}$ ), and GABA (able to detect  $\geq 500 \mu\text{M}$ ) were hardly detected on the roots prior to nodule formation but were detected within nodules: for sucrose, the maximum level was observed at 11 dpi, while that of GABA peaked at 8 dpi (Fig. 5, C and H). This pattern is similar overall to that seen on pea roots for both sucrose (Fig. 2E) and GABA (Fig. 3E), taking into account that pea nodules form later ( $>11$  dpi). Malonate ( $\geq 10 \text{ mM}$ ) was detected in the vetch rhizosphere prior to nodule formation, but once nodules were formed ( $> 8$  dpi), it was detected only at a very low level (8-22 dpi) (Fig. 5E), again, a similar pattern to that of pea (Fig. 2G). Hesperetin ( $\geq 1 \mu\text{M}$ ) was detected in vetch, with levels peaking at 4 dpi, and then falling once nodule development was initiated (8 dpi onwards) (Fig. 5I). Images from 1-22 dpi for detection of hesperetin on vetch roots (Fig. 6) show that, even at 1-4 dpi, prior to any sign of nodule formation, there are foci where hesperetin is detected. At later time points, nodules developed at these exact locations illustrating the power of this technique in pinpointing the spatial and temporal secretion of this flavonoid (Fig. 6).

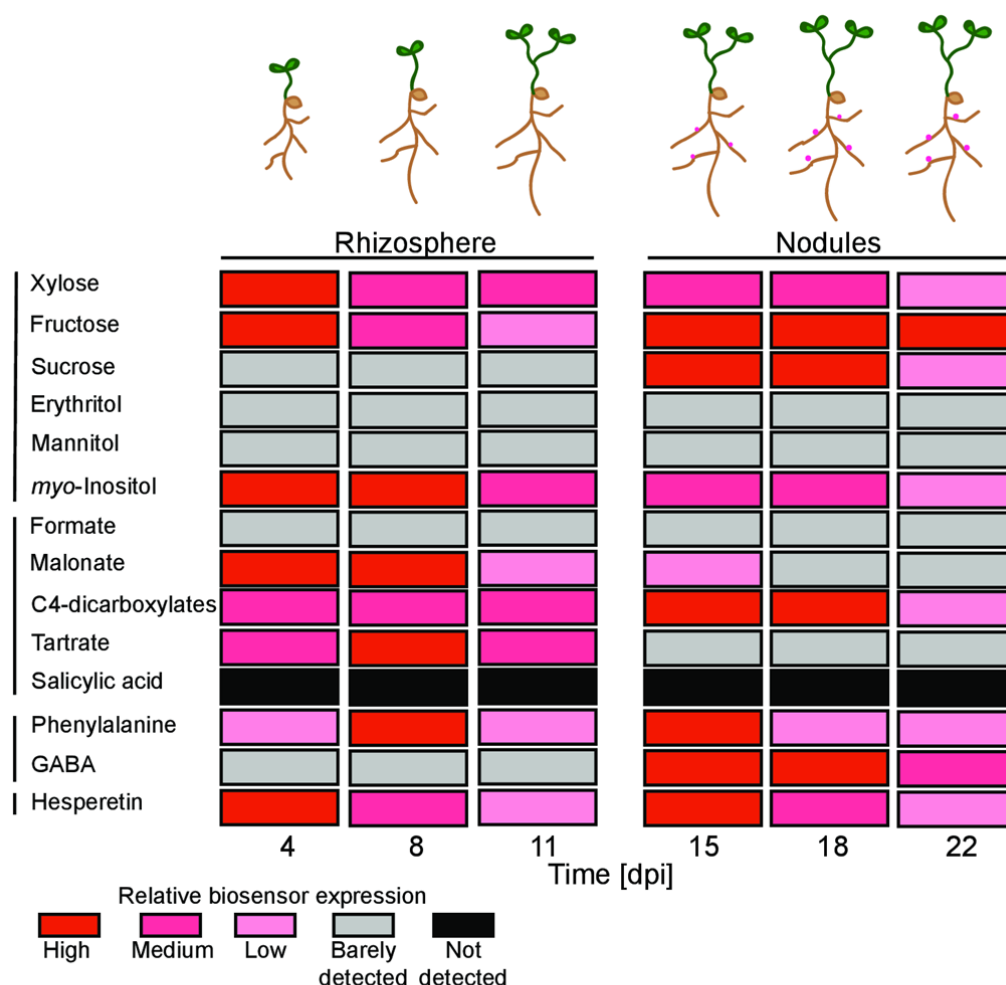


**Figure 6.** Time-course of hesperetin detection on a vetch seedling root from 1 to 22 dpi. Arrows indicate spots where luminescence is concentrated and a nodule forms later.

## DISCUSSION

Owing to the hidden nature of the rhizosphere and its complexity, a major problem encountered in its study is the intrinsic difficulty in sampling (Bais et al., 2006). Most of the techniques used require either sacrifice of the specimen, or at least require its manipulation, making it impossible to non-invasively follow the same sample over time. The bacterial *lux* gene cassette has been widely used in several different applications, including the visualization of gene expression, as a tool for cellular population monitoring and as bioreporter target through activation under specific, predetermined conditions (Close et al., 2012). In this work, we have constructed a suite of Lux biosensors able to detect a variety of key sugars, polyols, organic acids, amino acids and flavonoids that are commonly found in root exudates. The presence of these compounds was confirmed using a metabolomics approach, allowing us to identify 376 compounds present in pea root exudate. Lux-based reporter plasmids have been transferred into Rlv3841, an alpha-proteobacterium, which is generally associated with leguminous plants and is ubiquitous in soil (Udvardi and Poole, 2013). Pea and vetch plants, both hosts of *R. leguminosarum*, on which it forms nitrogen-fixing nodules, have been used to test the efficiency of this system as a proof-of-concept. However, the ability of rhizobia to colonize non-legume plants (Chabot et al., 1996) should allow the use of these bioreporters in other systems.

A constitutive promoter was used to examine rhizobial colonization of plant



**Figure 7.** Summary of metabolite detection on pea roots; in the rhizosphere ( $\leq 11$  dpi) and within nitrogen-fixing nodules ( $\geq 15$  dpi). Lines on the left-hand side group similar chemicals into; sugars and polyols, organic acids, amino acids, and flavonoids. Color shows level detected in rhizosphere and nodules: high, red; medium, dark pink; low, pale pink; barely detected, gray; not detected, black.

571 roots. Bacteria colonize the whole of the root system but the strongest Lux signals are  
 572 visible from the elongation zone of the primary and lateral roots (Fig. 1, Supplemental  
 573 Fig. S2). Heavy colonization at the root elongation zone is to be expected, as this is an  
 574 area of actively growing root where many metabolites are secreted and exuded. Low  
 575 Lux signal from the root cap is probably due to the reduced colonization of this area,  
 576 which generally secretes antimicrobial phytochemicals (Baetz and Martinoia, 2014).  
 577 The level of Lux signal detected in the rhizosphere was constant until 11 dpi and then  
 578 reduced, possibly due to a general decrease in root exudation caused by plant growth  
 579 conditions and/or to the physiological status of the bacterial population. Constitutive  
 580 *lux* expression drains energy reserves in bacterial cells and reduces the light output  
 581 over time. In a wild-type bacterial background, after 11 dpi the Lux signal was mainly  
 582 localized in the nitrogen-fixing nodules formed on the legume roots which is densely



583 populated with metabolically active bacteria. It is important to consider where  
584 bacteria are located. Although the whole root is colonized by rhizobia (Figure S2),  
585 these are unevenly distributed i.e. more bacteria or more metabolically active bacteria  
586 are in the root elongation zone. The overall levels of metabolites detected in the  
587 rhizosphere before 11 dpi and in nodules (after 11 dpi) for pea roots are summarized  
588 in Fig. 7.

589       On pea and vetch roots, xylose ( $\geq 1$  mM), fructose ( $\geq 10$   $\mu$ M), *myo*-inositol ( $\geq$   
590 100  $\mu$ M), phenylalanine ( $\geq 10$   $\mu$ M) (not detected in the vetch rhizosphere), and  
591 hesperetin ( $\geq 1$   $\mu$ M) were detected largely at the elongation zone (just behind the root  
592 tip) of lateral roots, while malonate ( $\geq 10$  mM) (at least initially) and tartrate ( $\geq 100$   
593  $\mu$ M) (not detected on vetch roots) were mainly localized on the primary root (Fig. 2C  
594 and Supplemental Fig. S5D). In Rlv3841, malonate transport and metabolism has  
595 been shown to have no role in nitrogen fixation in peas (Karunakaran et al., 2013), but  
596 does seem to be involved in attachment of Rlv3841 to pea roots as mutants in  
597 malonate uptake and metabolism show a reduced attachment phenotype, although no  
598 change in overall colonization of pea roots is observed. Malonate at  $\geq 10$  mM is  
599 localized to the primary root at 4 dpi during initial colonization (although levels of  $\leq$   
600 10 mM malonate may well be present on other parts of the root but are below the  
601 levels of detection by this method). Given the role of malonate in attachment, it is  
602 possible that bacteria attach to the primary root before colonizing the lateral roots,  
603 where most of the nodules subsequently appear. C4-dicarboxylates  
604 (succinate/malate/aspartate with detection levels  $\geq 100$   $\mu$ M/  $\geq 10$   $\mu$ M/  $\geq 10$  mM,  
605 respectively) were detected in the rhizosphere of both pea and vetch, but showed no  
606 clear spatial pattern on pea roots (Supplemental Fig. 3C).

607       In pea and vetch nodules, xylose ( $\geq 1$  mM), fructose ( $\geq 10$   $\mu$ M), sucrose ( $\geq$   
608 100  $\mu$ M), *myo*-inositol ( $\geq 100$   $\mu$ M), C4-dicarboxylates (succinate/malate/aspartate, ( $\geq$   
609 10  $\mu$ M – 10 mM), phenylalanine ( $\geq 10$   $\mu$ M), and GABA ( $\geq 500$   $\mu$ M) were present  
610 (Table III, Table IV, Fig. 7). Although nodules formed on pea and vetch have not  
611 been analyzed by metabolomic studies, MALDI mass spectrometric analysis of  
612 *Medicago truncatula* nodules formed by *S. meliloti* (Ye et al., 2013) revealed many of  
613 these same compounds present: sucrose, C4-dicarboxylates (succinate/malate/  
614 aspartate), and GABA, but also salicylic acid was detected, which, if present in pea or  
615 vetch nodules, is below the limits of detection ( $\leq 1$  mM) of Lux-based salicylic acid

616 detection. In pea, hesperetin ( $\geq 1 \mu\text{M}$ ) was detected in mature nodules (Fig. 3F), while  
617 in vetch it was detected only before nodules could be seen with the naked eye or in  
618 very young nodules, with none detected in mature nodules (i.e. levels  $\leq 1 \mu\text{M}$ ) (Fig.  
619 6). Sucrose is supplied from the shoot to nodules, where it is converted to C4-  
620 dicarboxylates and supplied to bacteroids as their primary energy source for nitrogen  
621 fixation (Poole and Allaway, 2000). In the ineffective nodules of a *nifH*-mutant  
622 background, the levels of sucrose and C4-dicarboxylates detected were lower,  
623 suggesting that the plant sanctions supply of carbon to nodules unable to provide them  
624 with nitrogen. While the *nifH*-mutant showed lower levels of the constitutive  
625 promoter, presumably because of reduced bacteroid numbers and metabolic activity,  
626 (Kiers et al., 2003; Berrabah et al., 2015), the levels of sucrose, C4-dicarboxylates and  
627 GABA were still substantially reduced when the decrease in activity of the  
628 constitutive promoter was accounted for. Furthermore, levels of some metabolites,  
629 such as *myo*-inositol, increased dramatically in the *nifH* mutant possibly due to  
630 environmental/osmotic stress.

631 Use of these tools has allowed us to draw a spatial and temporal map of key  
632 compounds present in the legume rhizosphere and to monitor the relative supply of  
633 specific metabolites inside nodules (e.g. sucrose, C4-dicarboxylates, GABA). We  
634 have demonstrated that with this system it is possible to follow the same plant for  
635 days, gathering data non-invasively and it is relatively easy to set up. Moreover, it  
636 will be possible in the future to expand the set of reporters to include many different  
637 compounds. We believe that this is an excellent tool, which can be adapted to  
638 investigate the role of specific root exudates in many different plant growth  
639 conditions (e.g. stress; both abiotic and biotic). As *R. leguminosarum* spp. colonize  
640 root systems of non-leguminous plants (Schloter et al., 1997), it will be possible to  
641 monitor root exudates of other plant species using this series of biosensors. By  
642 combining this methodology with plant mutant collections, screening specific  
643 exudate-related phenotypes in genome-wide association studies could be performed.  
644 Finally, by co-inoculating biosensors with other bacteria and/or fungi, it will be  
645 possible to observe the effects of other microorganisms on the plant secretome.

## 647 MATERIALS AND METHODS

648

## 649 **Bacterial Strains and Growth Conditions**

650 The bacterial strains and plasmids used in this study are listed in Supplemental  
651 Table S1. *Escherichia coli* strains were grown in liquid or solid Luria–Bertani (LB)  
652 medium (Sambrook et al., 1989) at 37°C supplemented with appropriate antibiotics:  
653 tetracycline (10 µg ml<sup>-1</sup>) and kanamycin (20 µg ml<sup>-1</sup>). Rlv3841 carrying  
654 *nodC128::Tn5* was isolated by transduction using phage RL38 propagated on strain  
655 6015 carrying *nodC128::Tn5* on pRL1JI (Downie et al., 1985). Rlv3841 strains were  
656 grown in tryptone yeast (TY) agar or broth (Beringer, 1974) or universal minimal  
657 salts (UMS) at 28°C. UMS is derived from AMS (Poole et al., 1994) with the changes  
658 being: EDTA-Na<sub>2</sub> (1 µM), CoCl<sub>2</sub>·6H<sub>2</sub>O (4.2 µM), FeSO<sub>4</sub>·7H<sub>2</sub>O (0.04 mM) and  
659 CaCl<sub>2</sub>·2H<sub>2</sub>O (0.51 mM); UMS was supplemented with 30 mM pyruvate and 10 mM  
660 ammonium chloride as the carbon and nitrogen sources, unless otherwise stated  
661 (Supplemental Table S2). UMA is UMS with the addition of 16 g l<sup>-1</sup> agar. Antibiotics  
662 were added when necessary at the following concentrations: streptomycin (500 µg  
663 ml<sup>-1</sup>), tetracycline (2 µg ml<sup>-1</sup> in UMS, 5 µg ml<sup>-1</sup> in TY), spectinomycin (100 µg ml<sup>-1</sup>)  
664 and neomycin (20 µg ml<sup>-1</sup>).

665

## 666 **Strain Construction and General Techniques**

667 The promoter region (often including the complete upstream regulator) of  
668 each of the candidate genes was amplified using primers listed in Supplemental Table  
669 S9 with Phusion High-Fidelity DNA Polymerase (ThermoFischer, Waltham, MA,  
670 USA) according to manufacturer's instructions. Fragments were purified and double  
671 digested with *KpnI* or *XhoI* (at the 5'-end) and *XhoI* or *BamHI* (at the 3'-end)  
672 (ThermoFischer). Restriction fragments were cloned into pIJ11268 (Frederix et al.,  
673 2014) digested with the same enzymes. Plasmids (Supplemental Table S1) were  
674 transferred into wild-type (Rlv3841), Rlv3841 *nodC128::Tn5*, Rlv3841 *nifH::ΩSp*  
675 (Karunakaran et al., 2009), Rlv3841 *matA::pK19* and Rlv3841 *matC::pK19*  
676 (Karunakaran et al., 2013) backgrounds by tri-parental mating according to Figurski  
677 and Helinski (Figurski and Helinski, 1979). All plasmids are available from addgene  
678 (<https://www.addgene.org>).

679

## 680 **Determining Solute Specificity**

Each biosensor was grown for 3 d on an UMA slope with antibiotics, re-suspended in UMS with no added carbon or nitrogen and washed three times. Each was then diluted to an OD<sub>600</sub> of 0.01 in a final volume of 5 ml UMS with a sugar (10 mM) as sole carbon source, or with pyruvate (30 mM) as carbon source in presence of a specific compound (Supplemental Table S2) for 17 h. Luminescence (in relative luminescence units (RLU)) and OD<sub>600</sub> were measured using a GloMax®-Multi+ Detection System (Promega, Fitchburg, WI, USA). For each compound, the fold-induction is defined as the ratio of RLU/OD<sub>600</sub>, when grown in the presence of that compound (Supplemental Table S2 gives concentrations of each solute), with that obtained in control conditions (UMS with pyruvate and ammonia). The solute(s) that give the highest fold-induction and specific luminescence (Table I) are described as inducer(s). Biosensor induction by a solute is described as specific if the specific luminescence is  $\geq 10$ -fold that observed from the other solutes tested. The biosensors for erythritol, formate, and GABA have a relatively high expression with a variety of non-related solutes (background) and they are described as being specific for these solutes, respectively, with specific luminescence  $\geq 4$ -fold values obtained with other solutes. More than one compound is considered inducing when the fold-induction is  $> 40\%$  of the maximum fold-induction for a biosensor (Table I). For each biosensor grown on each solute, three independent cultures were measured. Supplemental Table S10 shows the expression of each gene used in biosensor construction in microarray experiments performed under seventy-three different conditions.

702

### 703 **Plant Growth Conditions**

Seeds of *Pisum sativum* cv Avola and *Vicia hirsuta* were surface sterilized and germinated on distilled water agar plates. Plates with pea seedlings were put into black bags and incubated for 6 d at room temperature. Vetch seedlings were incubated overnight at 4°C and for 3 d at room temperature. Seedlings were transferred to 10-cm square Petri dishes containing FP agar (Somasegaran and Hoben, 1994) covered with sterile filter paper (one seedling per plate for peas, six per plate for vetch). Each biosensor was analyzed with at least 4 plates for pea (corresponding to 4 plants) and 1 plate for vetch (corresponding to 6 plants). Biosensors were grown on UMA slopes for 3 d at 27°C, washed 3 times in UMS without any additions and inoculated directly on the seedling root. Each seedling was inoculated with  $5 \times 10^7$  or  $2 \times 10^7$  colony forming units (cfu), for vetch and pea plants, respectively. Plates were covered with

715 aluminum foil to prevent exposure of roots to light and placed in a growth chambers  
716 at 23°C with a 16 h/8 h day/night for 22 d. In flooding experiments, peas were grown  
717 for 4 dpi with a biosensor before the plate was flooded with 10 ml of 10 mM solution  
718 of substrate and poured off. Plates were imaged before, and 5 min, 3 hr, and 21 hr  
719 post-flooding. Background luminescence in experiments with plants was evaluated by  
720 spotting  $2 \times 10^7$  cfu ml<sup>-1</sup> bacteria (the same amount used for pea root inoculation) onto  
721 FP plates supplemented with pyruvate and ammonium chloride. Plates were imaged  
722 after 7 d.

723

## 724 **Image Acquisition**

725       Plates were photographed using a NightOWL camera (Berthold Technologies,  
726 Bad Wildbad, Germany) 4, 8, 11, 15, 18, and 22 d post inoculation (dpi). CCD images  
727 of light output were exposed for 120 s. Each CCD image consisted of an array 1024  
728 by 1024 pixels, and after acquisition pictures were post-processed for cosmic  
729 suppression and background correction. Images were analyzed with the imaging  
730 software IndiGO (Berthold Technologies) and with the custom software, NightCROP.  
731 NightCROP first segments an image using the SeNeCA algorithm (Tomek et al.,  
732 2013) into roots and background using a bright-field image, discarding objects  
733 smaller than 1000 pixels. All subsequent analysis uses the respective fluorescence  
734 image. The script subtracts background intensity: background in the image is defined  
735 as a set of pixels given by logical inverse of the mask containing roots, subsequently  
736 morphologically eroded with a disk structural element of radius 9 to filter out signal  
737 from the edges of roots. Then, for each pixel belonging to a root, mean intensity of  
738 background pixels in a 200-by-200-pixel square is subtracted. The output from  
739 NightCROP for a given image is expressed as mean intensity of pixels labeled as  
740 roots after the background subtraction. Exact values of parameters (minimum root  
741 size, erosion radius and background size) may be freely selected, based on the  
742 resolution and nature of data (Fig. S1). Data are expressed as the ratio of  
743 luminescence/surface; cps mm<sup>-2</sup> (counts per second mm<sup>-2</sup>; IndiGO) or intensity pxl<sup>-1</sup>  
744 (NightCROP).

745

## 746 **Software Development and Use**

747       NightCROP was developed as a script for MATLAB environment (MATLAB  
748 version 8.5.0 r2015a, (MathWorks Inc., Natick, MA, USA)). Before using the script

an image processing toolbox should be installed in MATLAB. The script is available upon request to the corresponding author and works on any operating system which supports MATLAB.

### **Extraction of Exudate from Roots**

Glass jars (0.5 l) were prepared by filling one third of the jar with glass beads (6 mm diameter; Atlas Ball & Bearing Co. Ltd., Walsall, UK) and adding water (reverse osmosis highest quality) to cover all except the top layer of beads (approx. 150 ml). Jars were covered with a metal lid containing a foam bung and sterilized by autoclaving. Six sets of twenty sterilized and germinated peas with 1 cm roots were transferred into six sterilized glass jars, with each jar (of twenty plants) representing one biological replicate. The jars were wrapped with black plastic up to the level of the beads and the peas grown for 21 d at 20°C for 16 h light /18°C for 8 h dark. The liquid was then decanted into sterile glass bottles and sampled for sterility by plating 100 µl aliquots on TY plates. Samples were filtered through a 0.2-µm nitrocellulose filter. Samples for metabolite profiling were freeze-dried and re-suspended in 800 µl of sterile water prior to downstream analysis.

### **Metabolite Profiling Analysis**

The metabolomic profile of six biological replicates of pea root exudates was analyzed using non-biased, global metabolome profiling technology based on GC/MS and UHLC/MS/MS<sup>2</sup> platforms (Lawton et al., 2008; Evans et al., 2009; Terpolilli et al., 2016) developed by Metabolon ([www.metabolon.com](http://www.metabolon.com)). Samples from the six biological replicates were extracted using the automated MicroLab STAR® system (Hamilton, [www.hamiltoncompany.com](http://www.hamiltoncompany.com)). Recovery standards (Evans et al., 2009) were added prior to the first step in the extraction process for QC purposes. The protein fraction was removed using methanol extraction, which allows maximum recovery of small molecules. The resulting extract was divided into two fractions: one for analysis by LC and one for analysis by GC. Organic solvent was removed by placing samples on a TurboVap® (Zymark). Each sample was frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS.

The LC/MS portion of the platform was based on a Waters ACQUITY UHPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an

electrospray ionization source and linear ion-trap mass analyzer. The sample extract was split into two aliquots, dried and then reconstituted in acidic or basic LC-compatible solvents, each of which contained 11 or more injection standards at fixed concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient-eluted using water and methanol both containing 0.1% (v/v) formic acid, while the basic extracts, which also used water/methanol, contained 6.5 mM  $\text{NH}_4\text{HCO}_3$ . The MS analysis alternated between MS and data-dependent MS<sup>2</sup> scans using dynamic exclusion.

Samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 h prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide. The GC column was 5% phenyl and the temperature ramp was 40°C to 300°C, over a 16-min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole gas chromatograph mass spectrometer using electron impact ionization. For metabolite profiling, identification of known chemical entities was based on comparison to metabolomic library entries of purified standards as previously described (Evans et al., 2009; Yobi et al., 2012).

### **RNA Isolation and Microarray Analysis**

Rlv3841 was grown overnight in 10 ml of UMS supplemented with pyruvate/ammonia. Cultures were split in two and concentrated root exudates (5 mg/ml) were added to one culture. After 3 h of induction RNA was extracted from three biological replicates, amplified and hybridized as previously described (Karunakaran et al., 2009). Microarray data were deposited in the ArrayExpress database ([www.ebi.ac.uk/arrayexpress](http://www.ebi.ac.uk/arrayexpress)) under accession number E-MTAB-4790.

### **Root Attachment Assay**

Surface-sterilized pea seeds were germinated on distilled water agar plates for 5 d. Bacteria were grown on UMA slopes for 3 d, re-suspended and washed twice in phosphate-buffered saline and then 10 ml of a suspension ( $\text{OD}_{600} = 0.1$ ) was added to 50 ml glass tubes containing 7-10 pea roots. Tubes were incubated on a rocking platform for 1 h at room temperature at 50 rpm. Roots were washed by dipping six

817 times in PBS and then individual roots were transferred into a 1.5 ml tube, weighed  
818 and their luminescence measured using a GloMax®-Multi Jr Single-Tube Multimode  
819 Reader (Promega). Luminescence was scored as relative light units. Data are the  
820 average of individual roots from at least 3 different experiments; relative light units  
821 (RLU) were normalized for the weight of the roots and the average luminescence of  
822 roots incubated with bacteria not expressing the *lux* cassette (LMB542) was  
823 subtracted. Luminescence of the pure culture after 1 h was used to calculate the  
824 relative luminescence of a single bacterial cell. Differences between the three groups  
825 were calculated by one-way ANOVA with post hoc Tukey test.

826

### 827 **Root Competition Assay**

828 Pea plants were grown on FP plates as described above (Plant Growth  
829 Conditions), with the addition of a wet filter paper to cover the roots. Strains were co-  
830 inoculated in 1:1 ratio ( $1 \times 10^7$  cfu of each strain), using combinations of Rlv3841  
831 with either Rlv3841 [pIJ11282] or Rlv3841 *matC*::pK19 [pIJ11282] and Rlv3841  
832 *matC*::pK19, with either Rlv3841[pIJ11282] or Rlv3841 *matC*::pK19 [pIJ11282]. At  
833 7 dpi, plants were imaged using a NightOWL camera and processed using the  
834 NightCROP script.

835

### 836 **Biosensor Sensitivity Assay**

837 Bacterial biosensors from UMA slopes (with appropriate antibiotics) grown  
838 for 3 d at 28°C, were washed three times before re-suspension in 3 ml UMS. Bacteria  
839 were added to 50 ml of molten (cooled to 42°C) UMA, to give a final concentration of  
840  $1 \times 10^8$  cfu ml<sup>-1</sup>. The agar containing bacteria was then poured into a 12-cm square  
841 Petri dish and allowed to set. 25 µl droplets (n=5) of 10-fold dilutions of solute,  
842 concentrations ranging from 10 mM to 1 µM (including a distilled water control)  
843 were spotted onto the agar and incubated at 28°C. Sensitivity of each biosensor was  
844 defined as the lowest concentration of solute which gave a signal when imaged using  
845 a NightOWL camera at 4 h post-spotting.

846

## 847 **ACKNOWLEDGEMENTS**

848 We would like to acknowledge the technical assistance of Elisabeth Nowak in  
849 this work.





## Figure Legends

**Figure 1.** *In vivo* spatial and temporal mapping images of pea root colonization and nodulation with wild-type Rlv3841 luminescently-labelled with a constitutive neomycin phosphotransferase promoter controlling Lux expression in pIJ11282 (Frederix et al., 2014). A, Images were acquired at 4, 8, 11, 15, 18 and 22 dpi, with nodules visible to the naked eye at between 11 and 15 dpi (scale: 300-12,000 cps). B, Mean luminescence (pxl mm<sup>-2</sup>) with standard errors shown by bars.

**Figure 2.** *In vivo* spatial and temporal mapping images of pea roots with biosensors detecting: sucrose (A) (scale: 200-65,535 cps), *myo*-inositol (B) (scale: 150-5,000 cps), malonate (C) (scale: 50-2,000 cps), and phenylalanine (D) (scale: 150-15,000 cps). Images were acquired at 4, 8, 11, 15, 18 and 22 dpi, with nodules visible to the naked eye between 11 and 15 dpi. These images are representative of those from biosensors in wild-type Rlv3841 background which nodulates peas.

Comparison of mean luminescence intensity *per* pixel from pea roots inoculated with biosensors in wild-type Rlv3841 background (dark gray) and Rlv3841 *nodC128::Tn5* background (light gray). Only wild-type Rlv3841 can form nodules. The biosensors detect: sucrose (E), *myo*-inositol (F), malonate (G) and phenylalanine (H). Standard errors are shown by bars, stars indicate significant differences between a biosensor in wild-type Rlv3841 and Rlv3841 *nodC128::Tn5* backgrounds (t-test, \* = *p* < 0.05; \*\* = *p* < 0.01). Differences between each time point (ANOVA with post hoc Tukey test, *p* < 0.05) are reported in Supplemental Tables S4 and S5. For representative images from Rlv3841 *nodC128::Tn5* background see Supplemental Fig. S8.

Close-up light-field photograph and luminescence of roots showing nodules at 15 dpi inoculated with biosensors detecting: sucrose (I), *myo*-inositol (J), malonate (K), and phenylalanine (L).

**Figure 3.** Comparison of mean luminescence intensity *per* pixel from pea roots inoculated with biosensors in wild-type Rlv3841 (dark gray) or the Rlv3841 *nodC128::Tn5* mutant (light gray). Only wild-type Rlv3841 can form nodules. Biosensors detect: xylose (A), fructose (B), C4-dicarboxylates (C) tartrate (D), GABA (E) and hesperetin (F). Standard errors are shown by bars, stars indicate significant differences between a biosensor in wild-type Rlv3841 and Rlv3841 *nodC128::Tn5* (t-

test, \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ). Differences between each time point (ANOVA with post hoc Tukey test,  $p < 0.05$ ) are reported in Supplemental Tables S4 and S5. For representative images from Rlv3841 and Rlv3841 *nodC128::Tn5* background see Supplemental Figs. S5 and S9, respectively.

**Figure 4.** Comparison of mean luminescence (cps mm<sup>-2</sup>) from pea nodules of different ages with biosensors in wild-type Rlv3841 (dark gray) or Rlv3841 *nifH::ΩSp* mutant (white) background. Nodules formed by Rlv3841 fix nitrogen whereas those formed by these mutant strains are unable to do so. Biosensors are specific for: constitutively active (A), sucrose (B), *myo*-inositol (C), C4-dicarboxylates (D) and GABA (E). Standard errors are shown by bars and stars indicate significant differences between a biosensor in wild-type Rlv3841 and the Rlv3841 *nifH::ΩSp* mutant (t-test, \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ). Differences between each time point (ANOVA with post hoc Tukey test,  $p < 0.05$ ) are reported in Supplemental Tables S6 and S7.

**Figure 5.** Comparison of mean luminescence (cps mm<sup>-2</sup>) from vetch roots inoculated with biosensors in wild-type Rlv3841 background. Biosensors detect: xylose (A), fructose (B), sucrose (C), *myo*-inositol (D), malonate (E), C4-dicarboxylates (F), phenylalanine (G), GABA (H) and hesperetin (I). Standard errors are shown by bars. Differences between each time point (ANOVA with post hoc Tukey test,  $p < 0.05$ ) are reported in Supplemental Table S8. Nodules are visible to the naked eye from 8 dpi.

**Figure 6.** Time-course of hesperetin detection on a vetch seedling root from 1 to 22 dpi. Arrows indicate spots where luminescence is concentrated and a nodule forms later.

**Figure 7.** Summary of metabolite detection on pea roots; in the rhizosphere ( $\leq 11$  dpi) and within nitrogen-fixing nodules ( $\geq 15$  dpi). Lines on the left-hand side group similar chemicals into; sugars and polyols, organic acids, amino acids, and flavonoids. Color shows level detected in rhizosphere and nodules: high, red; medium, dark pink; low, pale pink; barely detected, gray; not detected, black.

## 918 **Supplemental Data**

919 The following supplemental data are available

920 **Supplemental Figure S1.** Workflow of the NightCROP image processing script, the  
921 picture is a pea inoculated with a constitutively expressed bioreporter at 15 dpi.

922 **Supplemental Figure S2.** *In vivo* spatial and temporal mapping images of pea root  
923 colonization and nodulation with wild-type Rlv3841.

924 **Supplemental Figure S3.** Luminescence is not detected in the absence of plant roots.

925 **Supplemental Figure S4.** *In vivo* spatial and temporal mapping images of pea roots  
926 inoculated with the xylose biosensor.

927 **Supplemental Figure S5.** *In vivo* spatial and temporal mapping images of pea roots  
928 with biosensors detecting: sugars, xylose and fructose.

929 **Supplemental Figure S6.** Comparison of pea roots inoculated with biosensors in  
930 wild-type Rlv3841 and Rlv3841 *nodC128::Tn5* mutant background.

931 **Supplemental Figure S7.** Comparison of mean luminescence (cps mm<sup>-2</sup>) of pea  
932 nodules from 15 to 22 dpi, with biosensors in wild-type Rlv3841 background.

933 **Supplemental Figure S8.** *In vivo* spatial and temporal mapping images of pea roots  
934 with biosensors detecting: sucrose, *myo*-inositol, malonate and phenylalanine.

935 **Supplemental Figure S9.** *In vivo* spatial and temporal mapping images of pea roots  
936 with biosensors detecting: sugars, xylose and fructose, organic acids, C4-  
937 dicarboxylates and tartrate, amino acid, GABA and flavonoid, hesperetin.

938 **Supplemental Figure S10.** Comparison of mean luminescence (cps mm<sup>-2</sup>) from pea  
939 roots inoculated with biosensors in wild-type Rlv3841 background (dark gray) and  
940 Rlv3841 *nifH::ΩSp* mutant background (white).

941 **Supplemental Figure S11.** Investigation of the role of malonate during root  
942 attachment.

943 **Supplemental Table S1.** Strains and plasmids used in this work. St (streptomycin),  
944 Neo (neomycin), Tc (tetracycline) and Sp (spectinomycin).

945 **Supplemental Table S2.** Conditions used to test specificity the bioreporter library.

946 **Supplemental Table S3.** Metabolomic data from root exudates of *P. sativum*.

947 **Supplemental Table S4.** ANOVAs with post hoc Tukey test (p <0.05) on total  
948 luminescence for bioreporters in Rlv3841 inoculated onto pea plants (Fig. 2 and 3).

949

950 **Supplemental Table S5.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on total  
 951 luminescence for bioreporters in Rlv3841 *nodC128::Tn5* inoculated onto pea plants  
 952 (Fig. 2 and 3).

953 **Supplemental Table S6.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on nodules  
 954 luminescence for bioreporters in Rlv3841 inoculated onto pea plants (Fig. 4).

955 **Supplemental Table S7.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on nodules  
 956 luminescence for bioreporters in Rlv3841 *nifH::ΩSp* inoculated onto pea plants (Fig.  
 957 4).

958 **Supplemental Table S8.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on total  
 959 luminescence for bioreporters in Rlv3841 inoculated onto vetch plants (Fig. 5).

960 **Supplemental Table S9.** Primers used in this work.

961 **Supplemental Table S10.** Relative expression in microarray experiments under  
 962 seventy-three different conditions of the *R. leguminosarum* genes whose promoters  
 963 were used for construction of biosensors.

964

965 **Supplemental Figure S1.** Workflow of the NightCROP image processing script, the  
966 picture is a pea inoculated with a constitutively expressed bioreporter at 15 dpi. A,  
967 NightOWL output photographic image of the pea root system. B, NightOWL output  
968 luminescent image of same area captured in A. C, NightOWL superimposed image of  
969 pictures A and B with false colors. D, NightCROP segmentation of picture A, the  
970 program detects difference in light intensity and draws a segmentation mask over the  
971 root system. E, Segmentation mask is applied on picture B and luminescence intensity  
972 is specifically extracted from the segmented area (pxl/mm<sup>2</sup>). F, Mean background  
973 luminescence is calculated around each segmented pixel and, subtracting this from the  
974 intensity value of the pixel.

975

976 **Supplemental Figure S2.** *In vivo* spatial and temporal mapping images of pea root  
977 colonization and nodulation with wild-type Rlv3841 luminescently-labelled with a  
978 constitutive neomycin phosphotransferase promoter controlling Lux expression in  
979 pIJ11282 (Frederix et al., 2014). Images were acquired at 4, 8, 11, 15, 18 and 22 dpi,  
980 with nodules visible to the naked eye at between 11 and 15 dpi (scale: 50-12,000 cps).  
981

982 **Supplemental Figure S3.** Luminescence is not detected in the absence of plant roots.  
983 Luminescence of biosensors on FP media supplemented with pyruvate and  
984 ammonium chloride after 7 days' growth (scale: 50-1,000 cps).

985

986 **Supplemental Figure S4.** *In vivo* spatial and temporal mapping images of pea roots  
987 inoculated with the xylose biosensor, flooded with xylose at 4 dpi (A); the sucrose  
988 biosensor, flooded with sucrose at 4 dpi (B); the GABA biosensor, flooded with  
989 GABA at 4 dpi (C); the sucrose biosensor, flooded with GABA at 4 dpi (D) and the  
990 GABA biosensor, flooded with sucrose at 4 dpi (E) (scale: 500-5,000 cps). For each,  
991 images were taken before, and 5 min, 3 hr, and 21 hr post-flooding.

992

993 **Supplemental Figure S5.** *In vivo* spatial and temporal mapping images of pea roots  
994 with biosensors detecting: sugars, xylose (A) (scale: 400-12,000 cps) and fructose (B)  
995 (scale: 300-12,000 cps), organic acids, C4-dicarboxylates (C) (scale: 400-65,535 cps)  
996 and tartrate (D) (scale: 200-15,000 cps), amino acid, GABA (E) (scale: 500-10,000  
997 cps) and flavonoid, hesperetin (F) (scale: 250-13,000 cps). Images were acquired at 4,

998 8, 11, 15, 18 and 22 dpi with nodules visible to the naked eye at between 11 and 15  
999 dpi. These images are representative of those from biosensors in wild-type Rlv3841  
1000 background.

1001

1002 **Supplemental Figure S6.** Comparison of pea roots inoculated with biosensors in  
1003 wild-type Rlv3841 and Rlv3841 *nodC128::Tn5* mutant background. Only wild-type  
1004 Rlv3841 strains are able to form nodules. Biosensors detect: polyols, erythritol (A, E  
1005 and I) (scale: 100-1,000) and mannitol (B, F and J) (scale: 50-700), the organic acids,  
1006 formate (C, G and K) (scale: 100-2,000) and salicylic acid (D, H and L) (scale: 100-  
1007 2,000). A-D, Images representative of biosensors in Rlv3841 background. E-H,  
1008 Comparison of mean luminescence ( $\text{pxl mm}^{-2}$ ) from pea roots inoculated with  
1009 biosensors in wild-type Rlv3841 background (dark gray) and Rlv3841 *nodC128::Tn5*  
1010 background (light gray). Standard errors are shown by bars. I-L, Images  
1011 representative of biosensors in Rlv3841 *nodC128::Tn5* mutant background.

1012

1013 **Supplemental Figure S7.** Comparison of mean luminescence ( $\text{cps mm}^{-2}$ ) of pea  
1014 nodules from 15 to 22 dpi, with biosensors in wild-type Rlv3841 background.  
1015 Biosensors are specific for: xylose (A), fructose (B), phenylalanine (C) and hesperetin  
1016 (D). Standard errors are shown by bars.

1017

1018 **Supplemental Figure S8.** *In vivo* spatial and temporal mapping images of pea roots  
1019 with biosensors detecting: sucrose (A) (scale: 200-65,535 cps), *myo*-inositol (B)  
1020 (scale: 50-2,000 cps), malonate (C) (scale: 50-2,000 cps) and phenylalanine (D)  
1021 (scale: 150-15,000 cps). Images were acquired at 4, 8, 11, 15, 18 and 22 dpi. These  
1022 images are representative of those from biosensors in Rlv3841 *nodC128::Tn5*  
1023 background and are unable to nodulate peas.

1024

1025 **Supplemental Figure S9.** *In vivo* spatial and temporal mapping images of pea roots  
1026 with biosensors detecting: sugars, xylose (A) (scale: 400-12,000 cps) and fructose (B)  
1027 (scale: 300-12,000 cps), organic acids, C4-dicarboxylates (C) (scale: 400-65,535 cps)  
1028 and tartrate (D) (scale: 200-15,000), amino acid, GABA (E) (scale: 500-10,000 cps)  
1029 and flavonoid, hesperetin (F) (scale: 250-13,000 cps). Images were acquired at 4, 8,  
1030 11, 15, 18 and 22 dpi. These images are representative of those from biosensors in  
1031 Rlv3841 *nodC128::Tn5* background and are unable to nodulate peas.

1032  
1033 **Supplemental Figure S10.** Comparison of mean luminescence (cps mm<sup>-2</sup>) from pea  
1034 roots inoculated with biosensors in wild-type Rlv3841 background (dark gray) and  
1035 Rlv3841 *nifH*::ΩSp mutant background (white). Values for *nifH*::ΩSp mutant are  
1036 adjusted for the lower level of constitutive Lux expression in this background (Fig. 4)  
1037 to show equivalent expression in a wild-type background. Only wild-type Rlv3841  
1038 strains are able to form nitrogen-fixing nodules. Biosensors detect: sucrose (A), *myo*-  
1039 inositol (B), C4-dicarboxylates (C), and GABA (D). Standard errors are shown by  
1040 bars and stars indicate significant differences between a biosensor in wild-type  
1041 Rlv3841 and Rlv3841 *nifH*::ΩSp mutant background (t-test, \* = *p* <0.05; \*\* = *p*  
1042 <0.01).

1043  
1044 **Supplemental Figure S11.** Investigation of the role of malonate during root  
1045 attachment. A, Whole root attachment assay showing the number of bacteria attached  
1046 after 1 h incubation for wild-type Rlv3841[pIJ11282], Rlv3841 *matC*::pK19  
1047 [pIJ11282] and Rlv3841 *matA*::pK19[pIJ11282], mutated in malonate transport and  
1048 catabolism respectively. Plasmid pIJ11282 has constitutively expressed *lux* genes  
1049 (Frederix et al., 2014). Standard errors are shown by bars. Different letters are used to  
1050 indicate significant difference (ANOVA with post hoc HSD Tukey test, *p* < 0.05)  
1051 between groups. B, Competition test on pea plants at 7 dpi. Histograms represent  
1052 luminescence/area of constitutive reporter pIJ11282. Strains Rlv3841[pIJ11282] or  
1053 Rlv3841 *matC*::pK19 [pIJ11282] were inoculated in ratio 1:1 with Rlv3841 or  
1054 Rlv3841 *matC*::pK19. Standard errors are shown by bars.

1055  
1056 **Supplemental Table S1.** Strains and plasmids used in this work. St (streptomycin),  
1057 Neo (neomycin), Tc (tetracycline) and Sp (spectinomycin).

1058  
1059 **Supplemental Table S2.** Conditions used to test specificity the bioreporter library.

1060  
1061 **Supplemental Table S3.** Metabolomic data from root exudates of *P. sativum*.

1062  
1063 **Supplemental Table S4.** ANOVAs with post hoc Tukey test (*p* <0.05) on total  
1064 luminescence for bioreporters in Rlv3841 inoculated onto pea plants (Fig. 2 and 3).

1065



1066 **Supplemental Table S5.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on total  
1067 luminescence for bioreporters in Rlv3841 *nodC128::Tn5* inoculated onto pea plants  
1068 (Fig. 2 and 3).  
1069  
1070 **Supplemental Table S6.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on nodules  
1071 luminescence for bioreporters in Rlv3841 inoculated onto pea plants (Fig. 4).  
1072  
1073 **Supplemental Table S7.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on nodules  
1074 luminescence for bioreporters in Rlv3841 *nifH::ΩSp* inoculated onto pea plants (Fig.  
1075 4).  
1076  
1077 **Supplemental Table S8.** ANOVAs with post hoc Tukey test ( $p < 0.05$ ) on total  
1078 luminescence for bioreporters in Rlv3841 inoculated onto vetch plants (Fig. 5).  
1079  
1080 **Supplemental Table S9.** Primers used in this work.  
1081  
1082 **Supplemental Table S10.** Relative expression in microarray experiments under  
1083 seventy-three different conditions of the *R. leguminosarum* genes whose promoters  
1084 were used for construction of biosensors.

1085  
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**Table I.** Characterization of biosensors from *R. leguminosarum*

| Biosensor                 | Inducer(s)            | Fold-induction                                    | Sensitivity (mM) | Specific luminescence (RLU/OD <sub>600</sub> ) | Gene                   | Name        | Function of protein encoded by gene                  |
|---------------------------|-----------------------|---------------------------------------------------|------------------|------------------------------------------------|------------------------|-------------|------------------------------------------------------|
| <b>Sugars and polyols</b> |                       |                                                   |                  |                                                |                        |             |                                                      |
| <b>Xylose</b>             | Xylose                | 7.20 (±0.34)                                      | 1                | 1.10 x 10 <sup>7</sup>                         | RL2720 <sup>b</sup>    | <i>rbsC</i> | Transport, permease of ABC, CUT2                     |
|                           | Lyxose                | 6.99 (±0.37)                                      |                  | 1.12 x 10 <sup>7</sup>                         |                        |             |                                                      |
| <b>Fructose</b>           | Fructose              | 16.13 (±1.07)                                     | 0.01             | 2.84 x 10 <sup>7</sup>                         | RL0489 <sup>c</sup>    | <i>frcB</i> | Transport, SBP of ABC, CUT2                          |
|                           | Lactulose             | 11.10 (±0.87)                                     |                  | 1.97 x 10 <sup>7</sup>                         |                        |             |                                                      |
|                           | Mannitol              | 8.94 (±0.26)                                      |                  | 1.51 x 10 <sup>7</sup>                         |                        |             |                                                      |
|                           | Mannose               | 8.41 (±0.46)                                      |                  | 1.47 x 10 <sup>7</sup>                         |                        |             |                                                      |
|                           | Sorbitol              | 8.94 (±0.58)                                      |                  | 1.61 x 10 <sup>7</sup>                         |                        |             |                                                      |
|                           | Sucrose               | 7.00 (±0.21)                                      |                  | 1.18 x 10 <sup>7</sup>                         |                        |             |                                                      |
| <b>Sucrose</b>            | Sucrose               | 36.35 (±1.77)                                     | 0.1              | 2.94 x 10 <sup>7</sup>                         | pRL120556 <sup>c</sup> |             | Transport, SBP of ABC, CUT1                          |
|                           | Raffinose             | 34.19 (±4.43)                                     |                  | 2.93 x 10 <sup>7</sup>                         |                        |             |                                                      |
| <b>Erythritol</b>         | Erythritol            | 4.72 (±0.50)                                      | 1                | 2.47 x 10 <sup>5</sup>                         | pRL90085 <sup>b</sup>  |             | Transport, SBP of ABC, CUT2                          |
| <b>Mannitol</b>           | Mannitol              | 1.71 x 10 <sup>6</sup> (±1.50 x 10 <sup>6</sup> ) | 0.001            | 5.51 x 10 <sup>12</sup>                        | RL4218 <sup>b</sup>    |             | Transport, solute binding protein (SBP) of ABC, CUT1 |
|                           | Sorbitol <sup>a</sup> | 28.52 (±3.53)                                     |                  | 3.45 x 10 <sup>7</sup>                         |                        |             |                                                      |
|                           | Adonitol <sup>a</sup> | 13.01 (±2.39)                                     |                  | 1.45 x 10 <sup>7</sup>                         |                        |             |                                                      |
| <b>myo-Inositol</b>       | myo-Inositol          | 216.84 (±13.80)                                   | 0.1              | 4.00 x 10 <sup>7</sup>                         | RL4655 <sup>b,c</sup>  | <i>intA</i> | Transport, SBP of ABC, CUT2                          |
| <b>Organic acids</b>      |                       |                                                   |                  |                                                |                        |             |                                                      |
| <b>Formate</b>            | Formate               | 27.20 (±2.13)                                     | 10               | 2.02 x 10 <sup>6</sup>                         | RL4393 <sup>b</sup>    | <i>fdsG</i> | Metabolism, formate dehydrogenase                    |
| <b>Malonate</b>           | Malonate              | 540.48 (±155.87)                                  | 10               | 5.69 x 10 <sup>6</sup>                         | RL0990 <sup>b</sup>    | <i>matA</i> | Metabolism                                           |
| <b>C4-dicarboxylates</b>  | Succinate             | 87.83 (±3.46)                                     | 0.1              | 2.28 x 10 <sup>7</sup>                         | RL3424 <sup>b</sup>    | <i>dctA</i> | Transport, dicarboxylate transporter                 |
|                           | Malate                | 57.19 (±9.18)                                     | 0.01             | 2.86 x 10 <sup>7</sup>                         |                        |             |                                                      |

|                       |                |                   |       |                        |                          |               |                                              |
|-----------------------|----------------|-------------------|-------|------------------------|--------------------------|---------------|----------------------------------------------|
| <b>Tartrate</b>       | Aspartate      | 33.18 (±3.02)     | 10    | 1.28 x 10 <sup>7</sup> | RL0996 <sup>b</sup>      |               | Transport, permease of MFS uptake system     |
|                       | Tartrate       | 188.68 (±10.51)   | 0.1   | 2.32 x 10 <sup>7</sup> |                          |               |                                              |
| <b>Salicylic acid</b> | Salicylic acid | 1024.87 (±161.97) | 1     | 1.78 x 10 <sup>7</sup> | RL1329                   | <i>sala</i>   | Transport, MFS efflux system                 |
| <b>Amino acids</b>    |                |                   |       |                        |                          |               |                                              |
| <b>Phenylalanine</b>  | Phenylalanine  | 52.10 (±8.43)     | 0.01  | 4.08 x 10 <sup>7</sup> | RL1860 <sup>b</sup>      | <i>phhA</i>   | Metabolism, phenylalanine-4-hydroxylase      |
| <b>GABA</b>           | GABA           | 3.56 (±1.28)      | 0.5   | 5.34 x 10 <sup>4</sup> | RL0102 <sup>d</sup>      | <i>gabT</i>   | Metabolism, 4-aminobutyrate aminotransferase |
| <b>Flavonoids</b>     |                |                   |       |                        |                          |               |                                              |
| <b>Hesperetin</b>     | Hesperetin     | 256.44 (±68.68)   | 0.001 | 1.63 x 10 <sup>7</sup> | pRL100185 <sup>b,c</sup> | <i>nodABC</i> | Metabolism, Nod factor synthesis             |

Abbreviations used: RLU (relative luminescence units), ABC (ATP binding cassette transporter); CUT1, CUT2 (carbohydrate uptake transporter-1 and -2); SBP (substrate binding protein); MFS (major facilitator superfamily). Inducer(s) are solute(s) that give the highest fold-induction and specific luminescence. Biosensor induction by a solute is described as specific if the specific luminescence is ≥10-fold that observed from the other solutes tested. The biosensors for erythritol, formate, and GABA have a relatively high expression with a variety of non-related solutes (background) and they are described as being specific for these solutes, respectively, with specific luminescence ≥4-fold values obtained with other solutes. More than one compound is considered inducing when the fold-induction is > 40% of the maximum fold-induction for a biosensor. Fold-induction is the ratio of RLU/OD<sub>600</sub> when grown in the presence of solute (Supplemental Table S2 gives solute concentration), to RLU/OD<sub>600</sub> when grown in absence of solute. Sensitivity is the minimum concentration of substrate spotted onto bacteria growing in agar to give a luminescent signal visible with a NightOWL camera after 4 h incubation. <sup>a</sup> Fold-induction is <40% that of the best inducer and therefore not considered to be specific induction. <sup>b</sup> indicates genes induced >3-fold, p ≤ 0.05 in the pea rhizosphere (Ramachandran et al. 2011). <sup>c</sup> indicates genes induced >3-fold, p ≤ 0.05 by pea exudate (Ramachandran et al. 2011). <sup>d</sup> indicates genes induced >3-fold, p ≤ 0.05 in the alfalfa rhizosphere (Ramachandran et al. 2011).

1100 **Table II.** Results from microarrays comparing the effect of pea root exudate and the  
 1101 pea rhizosphere on expression of genes used to develop biosensors  
 1102

| Biosensor                         | Gene      | Fold-expression<br>with added 23 d-old<br>pea root exudate <sup>a,b</sup> | Fold-expression in<br>21 d-old pea<br>rhizosphere <sup>b,c</sup> |
|-----------------------------------|-----------|---------------------------------------------------------------------------|------------------------------------------------------------------|
| <b>Sugars and polyols</b>         |           |                                                                           |                                                                  |
| <b>Xylose</b>                     | RL2720    | 1.21 <sup>†</sup>                                                         | 4.00                                                             |
| <b>Fructose</b>                   | RL0489    | 18.52                                                                     | 1.65                                                             |
| <b>Sucrose</b>                    | pRL120556 | 3.31                                                                      | 1.03 <sup>†</sup>                                                |
| <b>Erythritol</b>                 | pRL90085  | 1.34 <sup>†</sup>                                                         | 5.74                                                             |
| <b>Mannitol</b>                   | RL4218    | 4.30 <sup>†</sup>                                                         | 3.18                                                             |
| <b>myo-Inositol</b>               | RL4655    | 26.94                                                                     | 2.71                                                             |
| <b>Organic acids</b>              |           |                                                                           |                                                                  |
| <b>Formate<sup>d</sup></b>        | RL4393    | 0.88 <sup>†</sup>                                                         | 7.52                                                             |
| <b>Malonate</b>                   | RL0992    | 1.48 <sup>†</sup>                                                         | 1.31 <sup>†</sup>                                                |
| <b>C4-dicarboxylates</b>          | RL3424    | 0.67 <sup>†</sup>                                                         | 23.00                                                            |
| <b>Tartrate</b>                   | RL0996    | 3.34 <sup>†</sup>                                                         | 5.70                                                             |
| <b>Salicylic acid<sup>d</sup></b> | RL1329    | 1.18 <sup>†</sup>                                                         | 2.27                                                             |
| <b>Amino acids</b>                |           |                                                                           |                                                                  |
| <b>Phenylalanine</b>              | RL1860    | 0.86 <sup>†</sup>                                                         | 46.28                                                            |
| <b>GABA</b>                       | RL0102    | 2.90                                                                      | 1.14 <sup>†</sup>                                                |
| <b>Flavonoids</b>                 |           |                                                                           |                                                                  |
| <b>Hesperetin<sup>d</sup></b>     | pRL100185 | 49.15                                                                     | 2.09                                                             |

1103  
 1104 <sup>a</sup> Microarrays performed in triplicate on free-living Rlv3841 cells grown with and  
 1105 without the addition of pea root exudate (part of the sample used for metabolomic  
 1106 analysis). <sup>b</sup>  $p \leq 0.05$ , unless marked <sup>†</sup> when  $p > 0.05$ ; fold-expression  $>1$  are an  
 1107 increase in expression under the condition tested. <sup>c</sup> Microarrays performed in  
 1108 triplicate on Rlv3841 extracted from rhizosphere compared with free-living cells  
 1109 grown in glucose/ammonia (Ramachandran et al., 2011). <sup>d</sup> Compound not found in  
 1110 metabolomic analysis of pea root exudate.  
 1111

**Table III.** Summary of compounds detected by biosensors in the pea rhizosphere and within nodules of Rlv3841

| Compound                      | Rhizosphere<br>4-11 dpi | Location on roots                                                          | Nodules<br>11-22 dpi | Nodule age at which<br>detection peaks<br>(dpi) |
|-------------------------------|-------------------------|----------------------------------------------------------------------------|----------------------|-------------------------------------------------|
| <b>Sugars and polyols</b>     |                         |                                                                            |                      |                                                 |
| Xylose                        | Yes                     | Primary and lateral root tips                                              | Yes                  | 15-18                                           |
| Fructose                      | Yes                     | Primary and lateral root tips                                              | Yes                  | 15-22                                           |
| Sucrose                       | No                      |                                                                            | Yes                  | 15-18                                           |
| Erythritol <sup>a</sup>       | No                      |                                                                            | No                   |                                                 |
| Mannitol <sup>a</sup>         | No                      |                                                                            | No                   |                                                 |
| myo-Inositol                  | Yes                     | Primary root and lateral root tips                                         | Yes                  | 15-18                                           |
| <b>Organic acids</b>          |                         |                                                                            |                      |                                                 |
| Formate <sup>a,b</sup>        | No                      |                                                                            | No                   |                                                 |
| Malonate                      | Yes                     | Uppermost portion of primary root and lateral roots, just before root tips | Low                  | 15                                              |
| C4-dicarboxylates             | Yes                     | No clear pattern                                                           | Yes                  | 15-18                                           |
| Tartrate                      | Yes                     | Uppermost portion of primary root only                                     | Low                  | 15                                              |
| Salicylic acid <sup>a,b</sup> | No                      |                                                                            | No                   |                                                 |
| <b>Amino acids</b>            |                         |                                                                            |                      |                                                 |
| Phenylalanine                 | Yes                     | Root elongation zone of lateral roots                                      | Yes                  | 15                                              |
| GABA                          | No                      |                                                                            | Yes                  | 15-18                                           |
| <b>Flavonoids</b>             |                         |                                                                            |                      |                                                 |
| Hesperetin <sup>b</sup>       | Yes                     | Lateral roots                                                              | Yes                  | 15                                              |

<sup>a</sup> Luminescence from biosensor detecting this compound was very low throughout the time course examining the pea rhizosphere. <sup>b</sup> Compound not listed as present in metabolomics analysis of pea exudate. dpi, days post-inoculation.

**Table IV.** Summary of compounds detected by biosensors in the vetch rhizosphere and within nodules of Rlv3841

| Compound                    | Rhizosphere<br>4 dpi | Nodules<br>8-22 dpi | Nodule age at which<br>detection peaks<br>(dpi) <sup>a</sup> |
|-----------------------------|----------------------|---------------------|--------------------------------------------------------------|
| <b>Sugars and polyols</b>   |                      |                     |                                                              |
| Xylose                      | Yes                  | Yes                 | 22                                                           |
| Fructose                    | Yes                  | Yes                 | (8) 11 (14-22)                                               |
| Sucrose                     | No                   | Yes                 | (8) 11 (14-22)                                               |
| Erythritol <sup>b</sup>     | No                   | No                  |                                                              |
| Mannitol <sup>b</sup>       | No                   | No                  |                                                              |
| myo-Inositol                | Yes                  | Yes                 | 22                                                           |
| <b>Organic acids</b>        |                      |                     |                                                              |
| Formate <sup>b</sup>        | No                   | No                  |                                                              |
| Malonate                    | Yes                  | No                  |                                                              |
| C4-dicarboxylates           | Yes                  | Yes                 | (8-11) 14 (18-22)                                            |
| Tartrate <sup>b</sup>       | No                   | No                  |                                                              |
| Salicylic acid <sup>b</sup> | No                   | No                  |                                                              |
| <b>Amino acids</b>          |                      |                     |                                                              |
| Phenylalanine               | No                   | Yes                 | (18) 22                                                      |
| GABA                        | No                   | Yes                 | 8 (11-14)                                                    |
| <b>Flavonoids</b>           |                      |                     |                                                              |
| Hesperetin                  | Yes                  | No                  |                                                              |

<sup>a</sup> Brackets indicate when there is less than the maximum detected. <sup>b</sup> Luminescence from biosensor detecting this compound was very low throughout the time course examining the vetch rhizosphere. dpi, days post-inoculation.

## Parsed Citations

**Badri DV, Weir TL, van der Lelie D, Vivanco JM (2009) Rhizosphere chemical dialogues: plant-microbe interactions. Curr Opin Biotechnol 20: 642-650**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Baetz U, Martinoia E (2014) Root exudates: the hidden part of plant defense. Trends Plant Sci 19: 90-98**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57: 233-266**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Beringer JE (1974) R factor transfer in Rhizobium leguminosarum. J Gen Microbiol 84: 188-198**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Berrabah F, Ratet P, Gourion B (2015) Multiple steps control immunity during the intracellular accommodation of rhizobia. J Exp Bot 66: 1977-1985**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Biedrzycki ML, Bais HP (2009) Root secretions: from genes and molecules to microbial associations. J Exp Bot 60: 1533-1534**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Chabot R, Antoun H, Kloepper JW, Beauchamp CJ (1996) Root colonization of maize and lettuce by bioluminescent Rhizobium leguminosarum biovar phaseoli. Appl Environ Microbiol 62: 2767-2772**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Close D, Xu T, Smartt A, Rogers A, Crossley R, Price S, Ripp S, Sayler G (2012) The evolution of the bacterial luciferase gene cassette (lux) as a real-time bioreporter. Sensors (Basel) 12: 732-752**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Darwent MJ, Paterson E, McDonald AJ, Tomos AD (2003) Biosensor reporting of root exudation from Hordeum vulgare in relation to shoot nitrate concentration. J Exp Bot 54: 325-334**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Downie JA, Knight CD, Johnston AWB, Rossen L (1985) Identification of genes and gene products involved in the nodulation of peas by Rhizobium leguminosarum. Molecular and General Genetics 198: 255-262**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E (2009) Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. Anal Chem 81: 6656-6667**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Figurski DH, Helinski DR (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A 76: 1648-1652**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Frederix M, Edwards A, Swiderska A, Stanger A, Karunakaran R, Williams A, Abbruscato P, Sanchez-Contreras M, Poole PS, Downie JA (2014) Mutation of praR in Rhizobium leguminosarum enhances root biofilms, improving nodulation competitiveness by increased expression of attachment proteins. Mol Microbiol 93: 464-478**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Galardini M, Brilli M, Spini G, Rossi M, Roncaglia B, Bani A, Chianciani S, Merotto M, Engelen K, Bassi G, Pini F, Biondi EG,**

**Bazzicalupo M, Mengoni A (2015) Evolution of intra-specific regulatory networks in a multipartite bacterial genome. PLoS Comput Biol 11: e1004478**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Huang X-F, Chaparro JM, Reardon KF, Zhang R, Shen Q, Vivanco JM (2014) Rhizosphere interactions: root exudates, microbes, and microbial communities. Botany 92: 267-275**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Kaiser C, Kilburn MR, Clode PL, Fuchslueger L, Koranda M, Cliff JB, Solaiman ZM, Murphy DV (2015) Exploring the transfer of recent plant photosynthates to soil microbes: mycorrhizal pathway vs direct root exudation. New Phytol 205: 1537-1551**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Karunakaran R, East AK, Poole PS (2013) Malonate catabolism does not drive N(2) fixation in legume nodules. Applied and Environmental Microbiology 79: 4496-4498**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Karunakaran R, Ramachandran VK, Seaman JC, East AK, Mouhsine B, Mauchline TH, Prell J, Skeffington A, Poole PS (2009) Transcriptomic analysis of Rhizobium leguminosarum biovar viciae in symbiosis with host plants Pisum sativum and Vicia cracca. J Bacteriol 191: 4002-4014**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Kiers ET, Rousseau RA, West SA, Denison RF (2003) Host sanctions and the legume-rhizobium mutualism. Nature 425: 78-81**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Lawton KA, Berger A, Mitchell M, Milgram KE, Evans AM, Guo L, Hanson RW, Kalhan SC, Ryals JA, Milburn MV (2008) Analysis of the adult human plasma metabolome. Pharmacogenomics 9: 383-397**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Maj D, Wielbo J, Marek-Kozaczuk M, Skorupska A (2010) Response to flavonoids as a factor influencing competitiveness and symbiotic activity of Rhizobium leguminosarum. Microbiol Res 165: 50-60**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Mauchline TH, Fowler JE, East AK, Sartor AL, Zaheer R, Hosie AH, Poole PS, Finan TM (2006) Mapping the Sinorhizobium meliloti 1021 solute-binding protein-dependent transportome. Proc Natl Acad Sci U S A 103: 17933-17938**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Maxwell CA, Hartwig UA, Joseph CM, Phillips DA (1989) A Chalcone and Two Related Flavonoids Released from Alfalfa Roots Induce nod Genes of Rhizobium meliloti. Plant Physiol 91: 842-847**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Mooney SJ, Pridmore TP, Helliwell J, Bennett MJ (2011) Developing X-ray Computed Tomography to non-invasively image 3-D root systems architecture in soil. Plant and Soil 352: 1-22**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Oburger E, Schmidt H (2016) New Methods To Unravel Rhizosphere Processes. Trends Plant Sci 21: 243-255**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45: 119-144**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH (2013) Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev Microbiol 11: 789-799**

Pubmed: [Author and Title](#)



CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Pini F, Galardini M, Bazzicalupo M, Mengoni A (2011) Plant-bacteria association and symbiosis: are there common genomic traits in alphaproteobacteria? Genes (Basel) 2: 1017-1032**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Poole P, Allaway D (2000) Carbon and nitrogen metabolism in Rhizobium. Adv Microb Physiol 43: 117-163**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Poole PS, Schofield NA, Reid CJ, Drew EM, Walshaw DL (1994) Identification of chromosomal genes located downstream of dctD that affect the requirement for calcium and the lipopolysaccharide layer of Rhizobium leguminosarum. Microbiology 140 ( Pt 10): 2797-2809**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Ramachandran VK, East AK, Karunakaran R, Downie JA, Poole PS (2011) Adaptation of Rhizobium leguminosarum to pea, alfalfa and sugar beet rhizospheres investigated by comparative transcriptomics. Genome Biol 12: R106**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning : a laboratory manual, Ed 2nd. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Schlöter M, Wiehe W, Assmus B, Steindl H, Becke H, Hoflich G, Hartmann A (1997) Root colonization of different plants by plant-growth-promoting Rhizobium leguminosarum bv. trifolii R39 studied with monospecific polyclonal antisera. Appl Environ Microbiol 63: 2038-2046**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Somasegaran P, Hoben HJ (1994) Handbook for Rhizobia: Methods in Legume-Rhizobium Technology. Springer-Verlag, New York**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Sørensen J, Haubjerg Nicolaisen M, Ron E, Simonet P (2009) Molecular tools in rhizosphere microbiology—from single-cell to whole-community analysis. Plant and Soil 321: 483-512**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Tecon R, van der Meer JR (2006) Information from single-cell bacterial biosensors: what is it good for? Curr Opin Biotechnol 17: 4-10**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Terpolilli JJ, Hood GA, Poole PS (2012) What determines the efficiency of N<sub>2</sub>-fixing Rhizobium-legume symbioses? Adv Microb Physiol 60: 325-389**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Terpolilli JJ, Masakapalli SK, Karunakaran R, Webb IU, Green R, Watmough NJ, Kruger NJ, Ratcliffe RG, Poole PS (2016) Lipogenesis and Redox Balance in Nitrogen-Fixing Pea Bacteroids. J Bacteriol 198: 2864-2875**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Tett AJ, Karunakaran R, Poole PS (2014) Characterisation of SalRAB a salicylic acid inducible positively regulated efflux system of Rhizobium leguminosarum bv viciae 3841. PLoS One 9: e103647**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Tomek J, Novak O, Syka J (2013) Two-Photon Processor and SeNeCA: a freely available software package to process data from two-photon calcium imaging at speeds down to several milliseconds per frame. J Neurophysiol 110: 243-256**

Pubmed: [Author and Title](#)  
CrossRef: [Author and Title](#)  
Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Turner TR, James EK, Poole PS (2013a) The plant microbiome. *Genome Biol* 14: 209**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Turner TR, Ramakrishnan K, Walshaw J, Heavens D, Alston M, Swarbreck D, Osbourn A, Grant A, Poole PS (2013b) Comparative metatranscriptomics reveals kingdom level changes in the rhizosphere microbiome of plants. *ISME J* 7: 2248-2258**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Udvardi M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. *Annu Rev Plant Biol* 64: 781-805**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Yagi K (2007) Applications of whole-cell bacterial sensors in biotechnology and environmental science. *Appl Microbiol Biotechnol* 73: 1251-1258**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Ye H, Gempferline E, Venkateshwaran M, Chen R, Delaux P-M, Howes-Podill M, Ane J-M, Li L (2013) MALDI mass spectrometry-assisted molecular imaging of metabolites during nitrogen fixation in the *Medicago truncatula*-*Sinorhizobium meliloti* symbiosis. *The Plant Journal* 75: 130-145**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

**Yobi A, Wone BWM, Xu W, Alexander DC, Guo L, Ryals JA, Oliver M, Cushman JC (2012) Comparative metabolic profiling between desiccation-sensitive and desiccation-tolerant species of *Selaginella* reveals insights into the resurrection trait. *The Plant Journal* 72: 983-99**

Pubmed: [Author and Title](#)

CrossRef: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)