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Visualizing Glutamine Accumulation in Root Systems Involved in the Legume–Rhizobia Symbiosis by Placement on Agar Embedded with Companion Biosensor Cells

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ABSTRACT

Microbial symbiotic nitrogen fixation (SNF) occurs inside root nodules, where fixed-N (NH_4^+) from rhizobia is first assimilated into the amino acid glutamine (Gln). Visualization of Gln dynamics in nodulated root systems of different plant species would require re-engineering transgenic Gln reporters specific for each rhizobia/host genotype. Here we demonstrate the use of companion biosensor cells called *GlnLux* (*Escherichia coli* auxotrophic for Gln and constitutively expressing lux) to image Gln accumulation in nodulated root systems across a diversity of legume/rhizobia species. Companion *GlnLux* cells are embedded into agar (*GlnLux* agar) upon which legume root systems are placed following freeze-thawing to cause Gln leakage. Photons released from nearby activated biosensor cells are captured using a photon capture camera. Using split root systems, we demonstrate that in diverse amide-exporting legumes (alfalfa, lentil, and green pea) and a ureide-exporting legume (soybean) that *GlnLux* agar imaging is sufficiently sensitive to detect Gln release from individual nodules and can differentiate root systems with active *nif*+ from inactive *nif*– nodules. The assay permits visualization of both source and sink dynamics of nodule Gln, specifically, Gln import into nodules from roots (for nodule growth and/or amino acid cycling), Gln assimilated from fixed nitrogen that accumulates inside nodules, and Gln export from nodules into roots from this assimilatory-N. *GlnLux* agar-based imaging is thus a new research tool to localize the accumulation and transfer of a critical amino acid required for rhizobia symbionts within legume phytobiomes. We discuss the ability of this technology to open new frontiers in basic research and its limitations.

Within the legume phytobiome, symbiotic interactions between legume plants and compatible rhizobia play a key role in adding nitrogen (N) inputs to agroecosystems and promoting sustainability. Legumes convert atmospheric nitrogen (N) gas into bioavailable forms of N (NH_4^+) through symbiotic associations with rhizobia bacteria inside root nodules (Udvardi and Poole 2013). Symbiotic nitrogen fixation (SNF) underlies the ability of legume crops to provide critical amino acids to humans especially in developing nations (Foyer et al. 2016), protein-rich feed for livestock (Graham and Vance 2003), and promotes deposition of organic fertilizer into soil (Thilakarathna et al. 2015, 2016a). Nodule nitrogen fixation is highly variable due to various biotic and abiotic factors. Among the biotic factors, legume species/cultivar (Carlsson and Huss-Danell 2003; Herridge et al. 2008; Thilakarathna et al. 2016b) and rhizobia strain genotype (Ji et al. 2017; Thilakarathna and Raizada 2017) have significant impacts on nodule SNF activity, in part due to compatibility issues. Soil nutrient availability (macro and micro nutrients), temperature, water availability, soil pH, and salinity/alkalinity are the major abiotic factors that directly influence nodule SNF activity (Giller 2001; Hungria and Vargas 2000; Schubert 1995; Thilakarathna and Raizada 2018; Zahran 1999).

Inside legume root nodules, bacteroid-fixed ammonium is assimilated into the amino acid glutamine (Gln) via glutamine synthetase (GS) (Betti et al. 2012; Lodwig and Poole 2003). GS_1 is the major isoform responsible for assimilating ammonium in nodules (Morey et al. 2002; Seabra and Carvalho 2015). In order to maintain

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Conflict of interest statement: We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. However, the *GlnLux* technology has a U.S. patent pending.

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bacteroid nitrogen fixation activity, assimilated amino acids inside the root nodules need to be exported from nodules to other plant tissues (Carter and Tegeder 2016; Chungopast et al. 2014; Day et al. 2001). Depending on the form of fixed nitrogen transported from nodules to shoots, legumes can be categorized as amide exporters (e.g., alfalfa, lentil, and pea) or ureide exporters (e.g., soybean, bean, and cowpea) (Unkovich et al. 2008). Inside the root nodules of amide-exporting legumes, fixed N (NH_4^+) from rhizobia is primarily exported as asparagine (major fraction) and Gln (minor fraction) (Prell and Poole 2006; Tegeder 2014) (Fig. 1A). In ureide-exporting legumes, fixed N from rhizobia is first assimilated into Gln, and then primarily converted into allantoin and allantoic acid for export (Carter and Tegeder 2016). Even among the ureide-exporting legumes (e.g., soybean), it has been shown that xylem Gln comes from nitrogen fixation and is a very useful indicator of SNF activity (Amarante and Sodek 2006). Furthermore, it was found that the Gln concentration in soybean xylem sap can represent up to 23% of the total amino acid fraction during nodule growth, which correlates with nodule formation (Amarante and Sodek 2006).

However, nodules are both a source and sink for Gln. As already noted, nodules export amino acids including Gln through xylem (e.g., legume lateral roots), but import them through root phloem from other tissues (Parsons et al. 1993). In terms of a sink, nodules demand amino acids during nodule development and differentiation of symbiotic rhizobia into bacteroids (Dunn 2015; Mulley et al. 2011; Peiter et al. 2004; Schubert 1986). Gln acts as both a carbon and N source for bacterial growth (Patriarca et al. 2002). Furthermore, nodule growth and its activity can be regulated by the amino acids supplied into the nodules by the phloem (Neo and Layzell 1997; Parsons et al. 1993). Nitrogen import into the nodules depends on plant growth and N status of the legume host, whereby adequate N availability in the plant promotes the channeling of N into nodules through phloem in order to shut down the energy expensive SNF process (Parsons et al. 1993). The major sources of N that are translocated as amino acids for developing nodules and roots are the N remobilized from senescing leaves and the N assimilated from photorespiration (Bernard and Habash 2009; Parsons et al. 1993). Legumes have more complex amino acid cycling, whereby a plant can channel amino acids such as glutamate (from Gln) to bacteroids to shut down ammonium assimilation, keeping symbiosis dominated by the host (Lodwig et al. 2003).

Researchers have used different analytical chemistry methods such as HPLC (Amarante and Sodek 2006), capillary electrophoresis (Gil-Quintana et al. 2012), and ^{15}N methodologies (Lodwig et al. 2003; Molero et al. 2014) to trace/quantify Gln in different tissues of legumes (e.g., nodules, roots, xylem, and phloem). However, these methodologies lack spatial detail, and are instead based on making extracts from ground tissues. There is interest in new imaging assays to help researchers reveal root-system level mechanisms involved in the plant–rhizobia symbiosis including environmental, genetic and physiological factors that affect nodule SNF activity, N assimilation, N import into the growing nodule, and export of fixed N from nodules.

Previous transgenic whole-cell biosensors have been engineered to sense different organic and inorganic compounds upon which they express visible reporters such as green fluorescent protein (GFP) and lux based on the concentration of the target compound (Goron and Raizada 2014; Renella and Giagnoni 2016; Somers

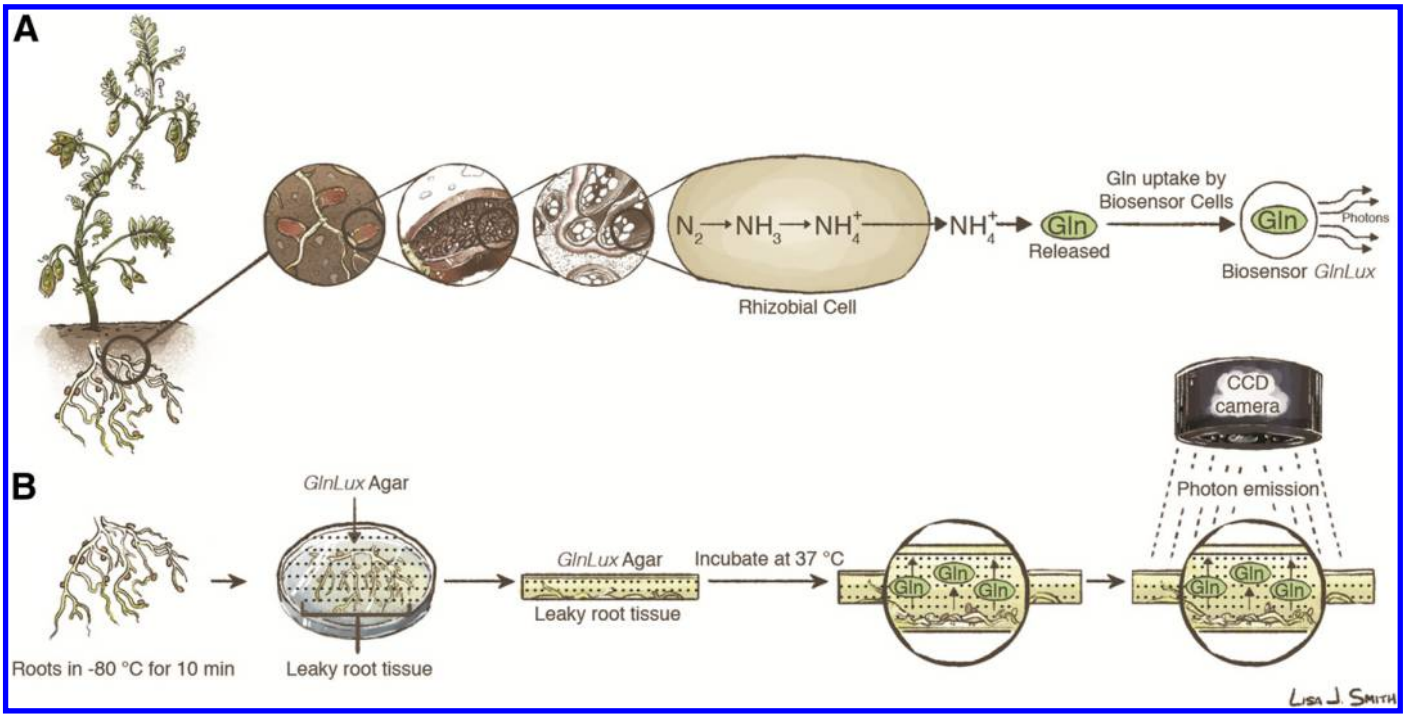

Fig. 1. Strategy to image glutamine (Gln) derived from symbiotic nitrogen fixation (SNF) in legume root systems using the *GlnLux* agar method. **A,** Principle underlying the imaging assay. Legume plants have root nodules that host rhizobia, which following differentiation into bacteroids, convert atmospheric nitrogen into NH_4^+, which is then assimilated into Gln. Gln is released from root systems into a medium containing companion *Escherichia coli* biosensor cells that are auxotrophic for Gln (strain *GlnLux*). The absorbed Gln stimulates the biosensor cells to become metabolically active and divide, proportionally activating a constitutive lux operon, resulting in photon emission. **B,** *GlnLux* agar assay to image SNF activity in nodules. Legume roots are frozen at –80°C and thawed at room temperature for 1 min to cause Gln leakage. Root systems are pressed down on agar pre-embedded with companion *GlnLux* reporter cells, referred to as *GlnLux* agar. Plates are inverted and imaged at the zero time point, then incubated for 1 to 6 h at 37°C to allow for the released plant Gln to activate adjacent biosensor cells. Plates are imaged hourly with 1,000 s exposure times using a luminescence imaging system (charged-coupled-device camera).

et al. 2004). Common transgenic whole-cell biosensors consist of promoter-reporter fusions or alternatively strains that are auxotrophic for the target compound (Goron and Raizada 2014).

Here we propose and demonstrate a method to visualize Gln dynamics in legume root systems using a microbial biosensor (Fig. 1). Specifically, we hypothesized that a whole cell biosensor that we previously developed for Gln called *GlnLux* (Tessaro et al. 2012) might be sufficiently sensitive to detect Gln leaking out of individual nodules (following freeze-thawing), by converting and amplifying this metabolic signal into photons for visualization using a charge coupled device (CCD) camera. *GlnLux* was created by transforming a mutant *Escherichia coli* strain, auxotrophic for Gln, with a constitutive lux reporter (Tessaro et al. 2012). We previously imaged freeze-thawed maize roots and shoots placed on agar embedded with *GlnLux* cells (*GlnLux* agar) and demonstrated that the underlying biosensor cells released photons proportional to N fertilization (Goron and Raizada 2016, 2017; Tessaro et al. 2012). We have not previously applied the *GlnLux* agar technology to image nodulated root systems.

Here we tested whether *GlnLux* agar based imaging can be used to visualize Gln source-sink dynamics in nodulated root systems, specifically whether it is (i) sufficiently sensitive to detect Gln released from individual root nodules of both amide- and ureide-exporting legumes; (ii) can distinguish root systems with *nif*+ nodules (active N-fixing, acting as Gln sources) from *nif*– nodules (non-N-fixing, acting solely as Gln sinks); and (iii) whether the technology can detect Gln import/export through associated lateral roots, all under low-N input, controlled conditions. Split root assays were conducted using three amide-exporting legume crops: alfalfa (*Medicago sativa* L.), an important forage legume in temperate regions (Graham and Vance 2003); lentil (*Lens culinaris* Medik.), a critically important legume in South Asia, the Middle East, and East Africa (Sharpe et al. 2013); and green pea (*Pisum sativum* L.), a high nitrogen fixing legume (Peoples et al. 2009); along with a ureide-exporter, soybean (*Glycine max* (L.) Merr), the most widely grown legume crop in the world (Herridge et al. 2008). Nodulated root systems, following freeze-thawing, were incubated with *GlnLux* agar, and then photons were captured using a CCD camera (Fig. 1B).

MATERIALS AND METHODS

Plant materials and *Rhizobium* strains. For the split root experiments, the crop varieties and respective rhizobia strains are noted in Table 1.

Split root experiments. *Plant growth conditions.* Split root experiments were conducted with lentil, alfalfa, green pea, and soybean. Surface sterilized seeds were pregerminated on sterilized, moistened filter paper in the dark at 28°C for 2 days as previously described (Thilakarathna et al. 2017). Seedlings were transferred to germination pouches (17.8 × 16.5 cm, Mega International, Minneapolis, MN) on light shelves containing 50 ml of double distilled water. Taproots were trimmed to induce lateral root growth. After 1 week of growth, the root systems of individual plants were divided into two and allowed to grow in two growth pouches, which were connected with paper clips. Plants were supplied with quarter strength N-free Hoagland's nutrient solution (pH = 6.8, adjusted using KOH) (HOP03-50LT, Caisson Labs, UT). Rhizobia treatments were applied 3 days after root growth in the split root systems. For the inoculated versus uninoculated treatments in alfalfa, lentil and green pea, one side of each root system was inoculated with wild-type N-fixing (*nif*+) rhizobia, while the other side remained uninoculated (negative control). For the second split root experiment to test active versus inactive nodules in amide exporters (alfalfa, lentil, and green pea) and one ureide exporter (soybean), one side of each root system was inoculated with a wild-type *nif*+ rhizobia strain, while the other side was inoculated with the corresponding *nif*– mutant strain.

Each rhizobia strain was grown in a specific liquid media in 50-ml plastic tubes for 3 days at 28°C as follows: *R. leguminosarum* biovar *viciae* (tryptone-yeast [TY] extract), *S. meliloti* (Luria-Bertani [LB]) and *B. japonicum* (modified arabinose-gluconate medium [MAG]). To prepare the inoculums, each rhizobia culture was centrifuged at 2,500 rpm for 10 min, the supernatant was decanted, and the pellet was resuspended in sterilized ddH_20, adjusted to a cell density of $OD_{595} = 0.1$. Plants were inoculated with 1 ml of each inoculum, pipetted directly onto the roots. Plants were grown under supplemental lighting (range: 180 to 200 µmol m^{-2} s^{-1} at the top of the growth pouch, EcoLux SP65, 40W, F40SP65ECO), at 23 ± 2°C, maintaining a photoperiod of 16 h/8 h light/dark cycles (Thilakarathna et al. 2017).

Root sampling. Root systems were collected from alfalfa, lentil, green pea, and soybean at 4 weeks following inoculation (or buffer treatment). Roots were immediately frozen in –80°C for 10 min before imaging using *GlnLux* agar.

Biosensor strain. The design and engineering of the *GlnLux* biosensor strain was previously reported (Tessaro et al. 2012). The Gln-auxotrophic *E. coli* strain (JW3841-1, KanR) was engineered by inserting a kanamycin cassette into *GlnA* [glnA732(del)::kan] (Baba et al. 2006) and subsequent transformation with ampicillin-resistant plasmid pT7-lux (Meighen and Szittner 1992). pT7-lux consists of a constitutive T7 promoter driving expression of the *luxCDABE* operon from *Vibrio fischeri* to create strain *GlnLux*. *GlnLux* luminesces when exogenous, free Gln is supplied.

***GlnLux* bacterial growth media.** *GlnLux* bacteria were grown in LB medium (consisting of yeast extract [DF0127179, Fisher

TABLE 1
Crops, crop varieties, and respective rhizobia strains used for the split root experiments

Crops	Varieties	Rhizobia strains	References
Alfalfa	OAC Minto (Canada)	*Sinorhizobium meliloti* Rm 1021 (*nif*+)	Kassaw et al. (2015)
		Sinorhizobium meliloti Rm 1312 (*nif*–)	Kassaw et al. (2015)
Lentil	Simal (Nepal)	*Rhizobium leguminosarum* biovar *viciae* 3841 (*nif*+)	Vanderlinde et al. (2011)
		Rhizobium leguminosarum biovar *viciae* 3940 (*nif*–)	Garcia-Fraile et al. (2015)
Green pea	Sparkle (USA/Canada)	*Rhizobium leguminosarum* biovar *viciae* 3841 (*nif*+)	Vanderlinde et al. (2011)
		Rhizobium leguminosarum biovar *viciae* 3940 (*nif*–)	Garcia-Fraile et al. (2015)
Soybean	Puja (Nepal)	*Bradyrhizobium japonicum* USDA 110 (*nif*+)	Kaneko et al. (2002)
		Bradyrhizobium japonicum USDA 510 [MC617] (*nif*–)	Bhagwat et al. (1991)

Scientific] at 5 g/liter, NaCl [BP358-212, Fisher Scientific] at 5 g/liter, and tryptone [BP1421-500, Fisher Scientific] at 10 g/liter, with or without Bacto-Agar [BD, DF0140010, Fisher Scientific] at 12 g/liter) at 37°C, pH 7.2. All liquid and solid plate media, including visualization agar, was supplemented with carbenicillin disodium salt (C346, PhytoTech) at 100 µg/ml and kanamycin monosulfate (K378, PhytoTech) at 50 µg/ml to select for the reporter plasmid and disrupted *glnA* chromosome, respectively.

Preparation of *GlnLux* agar and imaging using a photon capture camera. The procedure was modified from a previous protocol (Goron and Raizada 2016; Tessaro et al. 2012). *GlnLux* bacteria were cultured for 16 h in 200 ml of LB in a 500 ml flask, supplemented with 400 µl of 2.0 M glucose and 200 µl of each of kanamycin at 50 mg/ml, carbenicillin at 100 mg/ml, and 0.2 M Gln (37°C, 250 rpm). The culture was spun down (2,500 rpm, 25°C, 10 min, Sorvall Legend XTR centrifuge with Thermo Scientific FIBERLite F15-8 × 50cy rotor), and cells were washed three times in 0.01 M potassium phosphate buffer (pH 7.0) with centrifugation as above. Finally, the cells were resuspended in sterile modified M9 medium at OD_{595} = 0.5. Modified M9 medium consisted of 2 M

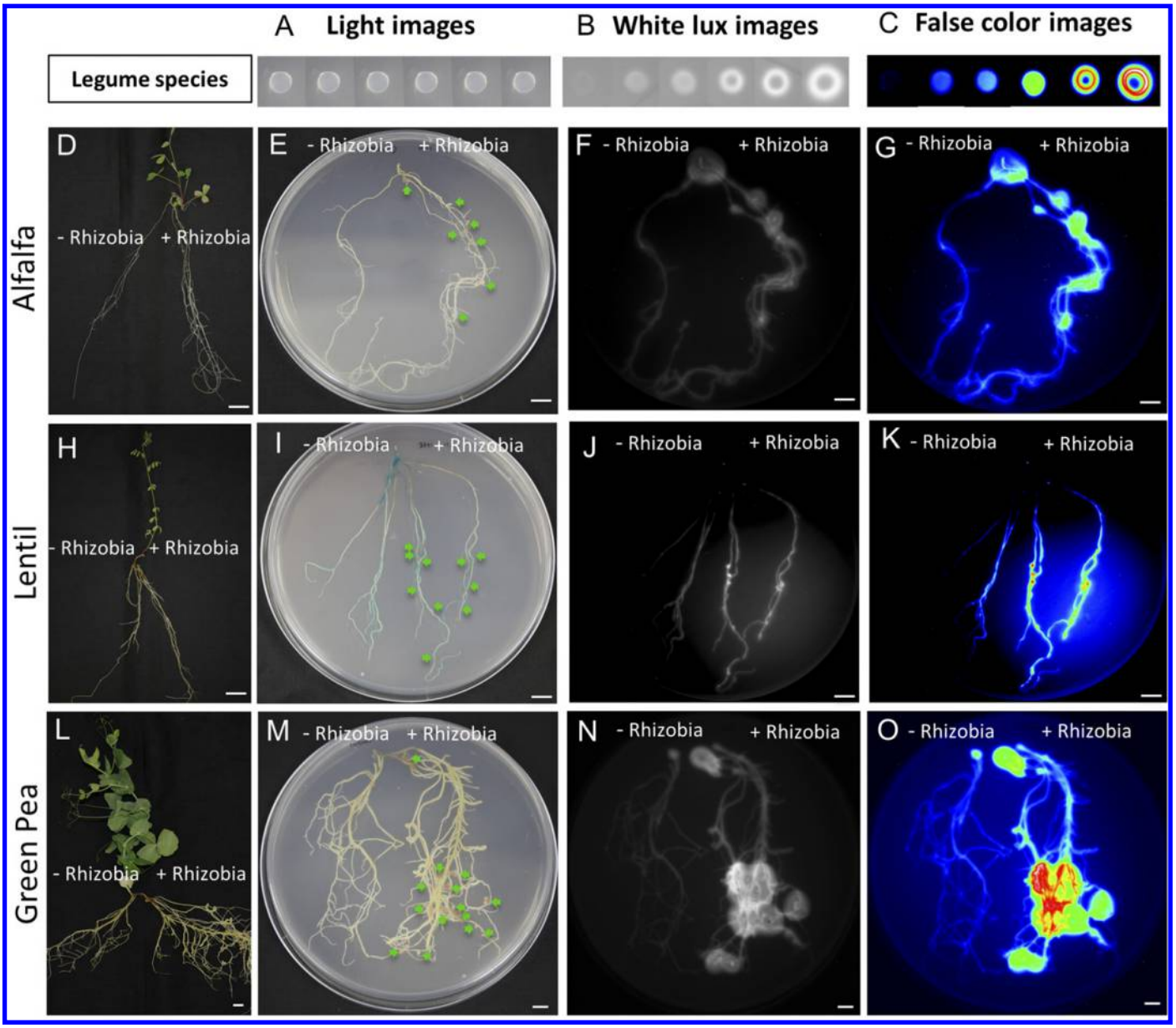

Fig. 2. Test to determine whether the *GlnLux* agar imaging assay is sufficiently sensitive to detect glutamine (Gln) from amide-exporting legume root systems including nodules. **A to C,** Luminescence imaging of Gln standards. Agar discs (agar at 20 g/liter) were prepared with a concentration gradient of Gln (from left to right: 0, 0.625, 1.25, 2.5, 5, and 10 mM) and placed on *GlnLux* agar. The opposite agar surface was then imaged using a photon capture charged-coupled-device (CCD) camera. **A,** The light image, **B,** white lux image, and **C,** false-colored lux images after 4 to 6 h of incubation and 1,000 s exposure. **D to O,** Split root systems inoculated with different *nif*+ (wild-type) rhizobia strains (right) or without inoculation (control, left) and then assayed using *GlnLux* agar. Split root systems of 5-week-old **D,** alfalfa, **H,** lentil, and **L,** pea plants were freeze-thawed to cause Gln leakage and then placed on *GlnLux* agar. The opposite agar surface was then imaged using a photon capture CCD camera. Shown are the corresponding: **E, I, and M,** light images, **F, J, and N,** white lux images, and **G, K, and O,** false-colored lux images after 4 to 6 h of incubation and 1,000 s exposure. Bright green-yellow-red color areas with halos represent the plant tissues with the highest Gln content, which correspond to active sites of nitrogen fixation. Green arrows (**E**, **I**, and **M**) indicate the location of nodules. Scale bars correspond to 1 cm. Supplementary Figures provide replicate images from separate trials.

D-(+)-glucose at 10 ml/liter, 1 M $CaCl_2$ at 100 µl/liter, 1 M $MgSO_4$ at 2 ml/liter, and 5× M9 Salts (DF048517, Fisher Scientific) at 200 ml/liter. To prepare *GlnLux* agar plates, the M9 medium was mixed with autoclaved molten Bacto-Agar (10 g/liter final concentration), cooled to 42°C, and mixed with 10% (vol/vol) of the OD_{595} = 0.5 *GlnLux* culture before pouring 75 ml into 150-mm diameter Petri plates. A ChemiProHT Luminescence Imaging System (ChemiPro, Roper Scientific) was used with Winview 32 software for lux imaging (Chinnusamy et al. 2002). The charged-coupled-device (CCD) chip camera was prechilled to −80°C in order to reduce dark noise (Christenson 2002).

Roots frozen at −80°C were thawed at room temperature for one min. Roots were placed in contact with *GlnLux* agar, and tissues were pressed down to improve contact. Inverted plates were imaged at the zero time point, then placed at 37°C for 2 to 6 h with hourly imaging using 1,000 s exposure times. For each legume, three individual split root systems were imaged using *GlnLux* agar plates to test (i) inoculated versus uninoculated treatment and (ii) inoculated with *nif*+ *versus nif*– rhizobia strains (n = 3). For the three amide-exporting legumes, *GlnLux* images from one plate (a split root system) are presented in the results section, whereas the *GlnLux* images from the second and third replicated plates are shown in the Supplementary figures. For soybean, *GlnLux* images comparing *nif*+ versus *nif*– rhizobia strains from three split root systems (n = 3) are presented.

Image quantification for lux. For lux quantification, CCD images were analyzed with the imaging software Winview 32 (Chinnusamy et al. 2002). All the visible nodules (*nif*– and *nif*+) within a plate were analyzed by selecting an area of 50-by-50-pixels around nodules. For the uninoculated roots, an area of 50-by-50-pixels was randomly selected on the root systems, where the number of selected areas was equal to the number of selected nodules (inoculated treatment) within a plate. Background subtraction for lux was performed by selecting a 50-by-50-pixel area lacking root organs. Data are expressed as total lux intensity per nodule and mean intensity per pixel (pxl).

Nodule contact surface area. The contact surface area of the nodules with the agar surface (individual nodules and nodules in clusters) was estimated by manual tracing from light images using ImageJ software (Version 1.47, Wayne Rasband, NIH, United States A).

Statistical analysis. For the statistical analysis, three completely independent root systems were used per treatment, grown at separate times and imaged at different times on independent *GlnLux* plates. For each trial, the n values (number of nodules/nodule clusters) are noted in each figure legend. In split root assays, total lux intensity per nodule and mean intensity per pxl of the *GlnLux* images were compared for the (i) inoculated versus uninoculated treatment and (ii) *nif*+ versus *nif*– rhizobia treatments, using t tests set at $P < 0.05$. Correlation analysis for total lux intensity (relative light units [RLU] pxl^{-1}) versus nodule contact area was performed using the Pearson correlation test. All statistical analyses were performed using GraphPad Prism Software (v7, GraphPad Software, United States).

RESULTS

Visualization of root nodules. The strategy to detect nodule-associated Gln visually consisted of placing root systems, leaking Gln from nodules and associated roots, onto agar pre-embedded with *GlnLux* cells (*GlnLux* agar) (Fig. 1B). Gln concentration standards incubated on *GlnLux* agar were used for assay optimization (Fig. 2A to C). To investigate whether nodules from amide-exporting legumes would release sufficient Gln onto the *GlnLux* agar to generate a detectable signal, root systems were split into two growth pouches, and one side inoculated with a *nif*+ rhizobia strain (rhizobia with wild-type nitrogenase), with the second side remaining uninoculated (negative control) (Fig. 2D, H, and I). Plants tested were alfalfa (Fig. 2D), lentil (Fig. 2H), and green pea (Fig. 2I). Intact root systems were freeze-thawed to cause Gln leakage, placed on *GlnLux* agar (Fig. 2E, I, and M), incubated for 2 to 6 h, and then imaged for 1,000 s.

GlnLux agar exposed to root systems with nodules emitted noticeably more photons than root systems with absence of nodules based on direct photon capture (Fig. 2F, J, and N) or false color conversion (Fig. 2G, K, and O, with blue-green being the lowest intensity, red-orange the highest, and a halo reflecting diffused Gln). *GlnLux* images of replicate root systems from independent trials showed similar results (Supplementary Figs. S1 and S2). Lux quantification of the images confirmed the visual observations, namely that root systems with nodules emitted significantly more photons compared with root systems lacking nodules based on total lux intensity per nodule (or corresponding root section for uninoculated roots) (Fig. 3A, C, and E) and mean intensity per pixel (Fig. 3B, D, and F). Furthermore, the highest intensity foci corresponded to the locations of the nodules (compare Fig. 2E, I, and M to Fig. 2F to G, J to K, and N to O). Photon emission was also detected associated with the lateral roots of the three legumes,

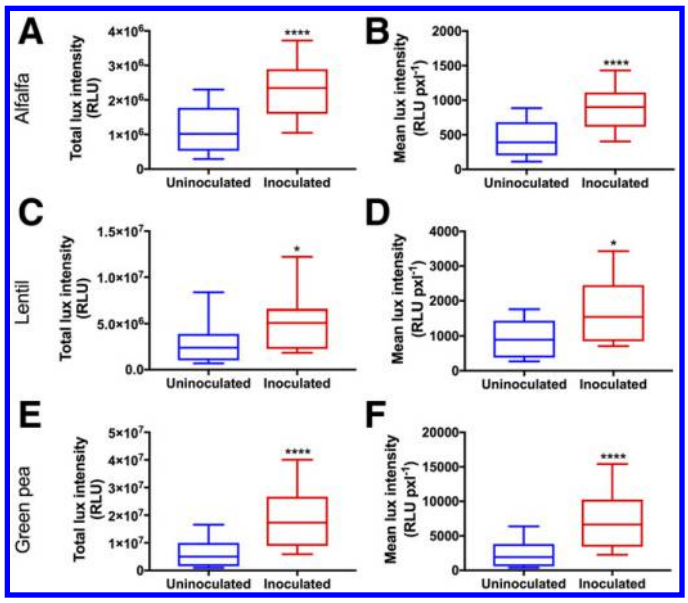

Fig. 3. Quantitative comparison of luminescence emitted from *GlnLux* agar-embedded cells exposed to inoculated versus uninoculated root systems of legume plants: **A and B,** alfalfa, **C and D,** lentil, and **E and F,** green pea. Box-plots showing **A, C, and E,** total lux intensity per nodule and **B, D, and F,** mean intensity per pixel in alfalfa, lentil, and pea, respectively. All the visible nodules (inoculated treatment) within a plate were quantified for lux emission by selecting an area of 50-by-50-pixels around each nodule. For the uninoculated roots, 50-by-50-pixel areas were randomly selected around the roots, where the number of selected areas was equal to the number of nodules selected (inoculated treatment) within the plate. Background subtraction for lux was performed by selecting a similar area of 50-by-50-pixels, where no roots were visible. A total of n = 15 to 19 individual nodules/nodule clusters (inoculated) or root segments (uninoculated) were quantified, each from three completely independent *GlnLux* agar plates from plants grown and imaged at separate times (Fig. 2). Asterisks indicate significant differences between uninoculated and inoculated roots for luminescence (t test, * = $P < 0.05$; and **** = $P < 0.0001$). RLU, relative light units.

especially close to the *nif*+ nodules (Fig. 2). We conclude that *GlnLux* agar is sufficiently sensitive to image Gln accumulation in root nodules in diverse amide-exporting legumes. Compared with alfalfa (Fig. 2F and G) and lentil nodules (Fig. 2J and K), pea nodules (Fig. 2N and O) resulted in the highest signal intensity. The lux quantification data also confirmed this latter trend (Fig. 3).

Visualization of root systems with *nif*+ versus *nif*– nodules in amide-exporting legumes. To investigate whether the *GlnLux* agar method could distinguish root systems inoculated with N-fixing versus nonfixing rhizobia, root systems of amide-exporting legumes were split into two growth pouches and either inoculated with a *nif*+ strain (active N-fixers) or corresponding nonfixing *nif*– mutant (with nodules present but inactive). Plants tested were again alfalfa (Fig. 4A), lentil (Fig. 4E), and green pea (Fig. 4I). *GlnLux* agar exposed to nodules inoculated with *nif*+ strains emitted noticeably more photons than *nif*– nodules based on direct photon capture (Fig. 4C, G, and K) or false color conversion (Fig. 4D, H, and L). *GlnLux* images of replicate root systems from independent trials showed similar results (Supplementary Figs. S3 and S4). Lux quantification of the images confirmed the visual observations, based on total lux intensity per nodule (Fig. 5A, C, and E) and mean intensity per pixel (Fig. 5B, D, and F). Similar to the earlier experiment, photon emission was also detected associated with the lateral roots of the three legumes, especially in root segments containing *nif*+ nodules (Fig. 4). We conclude that *GlnLux* agar imaging can distinguish root systems with potentially N-fixing nodules from those with non-fixing nodules in diverse amide-exporting legumes.

Visualization of *nif*+ versus *nif*– nodules in a ureide-exporting legume. To investigate whether the *GlnLux* agar method could similarly distinguish N-fixing versus nonfixing root systems of a ureide-exporting legume, in three independent trials, root systems of soybean were also split into two growth pouches and either inoculated with a *nif*+ strain or corresponding nonfixing *nif*– mutant (Fig. 6A, E, and I). Similar to amide-exporting legumes,

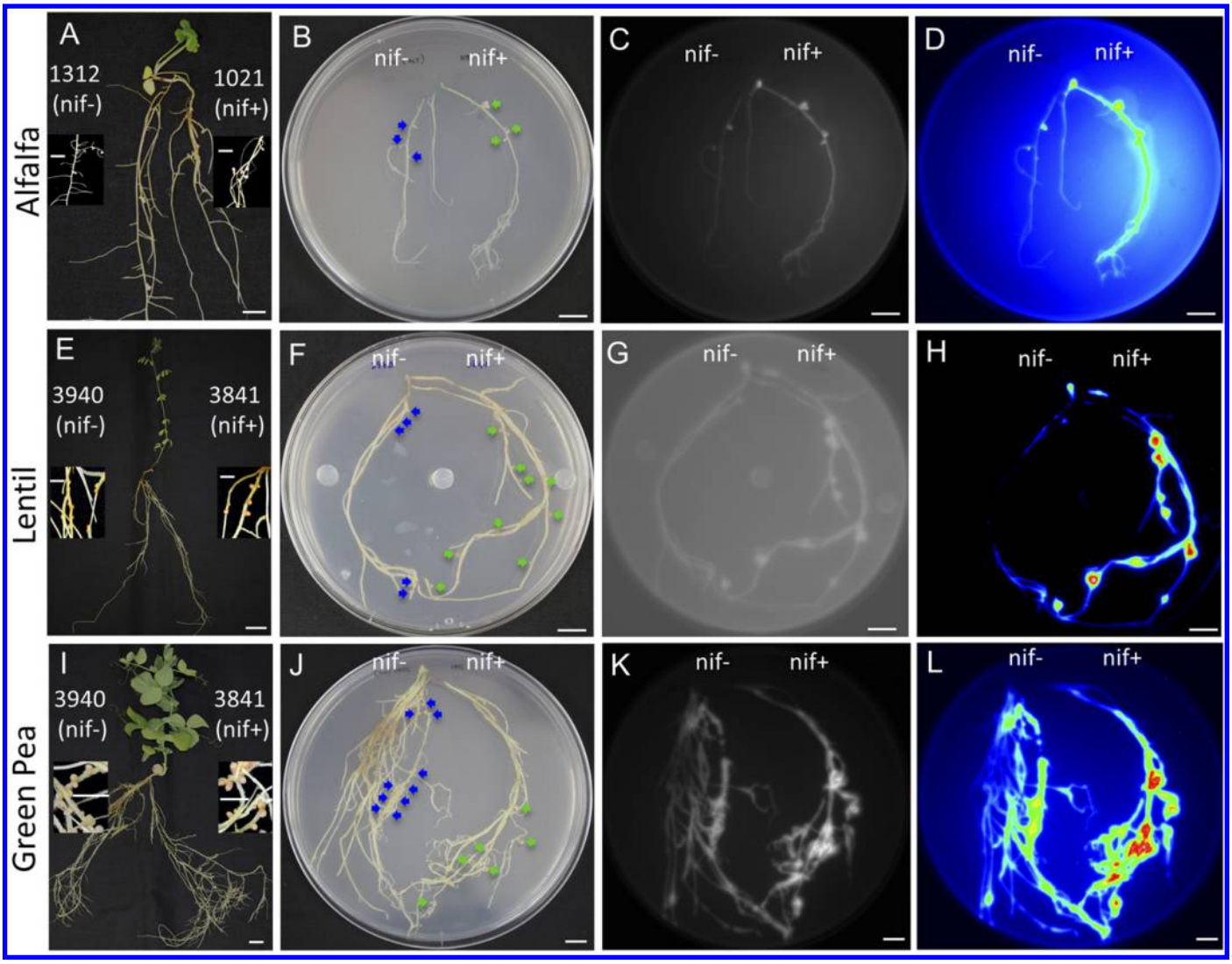

Fig. 4. Test to determine whether the *GlnLux* agar imaging assay can distinguish potentially active from inactive nodules of amide-exporting legume root systems. Potentially active nodules were those inoculated with *nif*+ wild-type rhizobia, while inactive nodules were those inoculated with mutant *nif*– rhizobia. Split root systems of 5-week-old **A,** alfalfa, **E,** lentil, and **I,** pea plants were freeze-thawed to cause Gln leakage and then placed on *GlnLux* agar. The opposite agar surface was then imaged using a photon capture charged-coupled-device camera. The inset images are magnifications to show the presence of nodules. Shown are the corresponding **B, F, and J,** light images, **C, G, and K,** white lux images, and **D, H, and L,** false-colored lux images after 4 to 6 h of incubation and 1,000 s exposure. Bright green-yellow-red color areas represent the tissues with the highest Gln content, which correspond to active sites of nitrogen fixation. Blue and green arrows (**B**, **F**, and **J**) indicate the location of nodules. Green and blue arrows indicate *nif*+ (active) and *nif*– (inactive) nodules, respectively. Scale bars correspond to 1 cm. Supplementary Figures provide replicate images from separate trials.

GlnLux agar exposed to nodules inoculated with *nif*+ strains emitted noticeably more photons than *nif*– nodules based on direct photon capture (Fig. 6C, G, and K) or false color conversion (Fig. 6D, H, and L). Lux quantification of the images confirmed the visual observations based on total lux intensity per nodule (Fig. 7A) and mean intensity per pixel (Fig. 7B). Similar to the amide-exporting legumes, photon emission was also detected from the lateral roots of soybean (Fig. 6), more prominent in roots with *nif*+ nodules but with considerable variation observed, from low (Fig. 6H) to high (Fig. 6L). We conclude that *GlnLux* agar imaging can distinguish root systems with potentially N-fixing nodules from those with nonfixing nodules in a ureide-exporting legume.

Correlation between nodule contact surface area and lux output. There was concern that the *nif*+ versus *nif*– imaging results may have been an artifact, since the *nif*– nodules were frequently smaller than *nif*+ nodules, and thus had lower contact surface area with *GlnLux* agar. To test for this possibility, the surface area of contact of individual nodules with *GlnLux* agar was measured and compared with the corresponding lux output of alfalfa, lentil, green pea and soybean nodules. In the three amide-exporting legumes, *nif*– contact surface areas had a smaller size distribution compared with *nif*+ nodules (Fig. 8A to C), whereas the distributions were more similar for soybean (Fig. 8D). However, when comparing nodule contact areas of a similar size (individual nodules or averaged for clusters), possible in alfalfa and lentil (both amide exporters) and especially in soybean (a ureide exporter), the highest lux-eliciting nodules tended to be *nif*+ not *nif*– though some exceptions were observed (Fig. 8A and B). However, some small *nif*+ nodules elicited similar or less *GlnLux* expression than corresponding-size *nif*– nodules (Fig. 8B and D). Significant positive correlations were found between nodule contact surface area and mean lux intensity for alfalfa *nif*+ nodules (Fig. 8A), lentil *nif*+ and *nif*– nodules (Fig. 8B), and pea *nif*– nodules (Fig. 8C). There was no significant correlation for nodule contact surface area and mean lux intensity in either *nif*– or *nif*+ nodules in soybean, where there was tremendous variation among *nif*+ and *nif*– nodules (Fig. 8D).

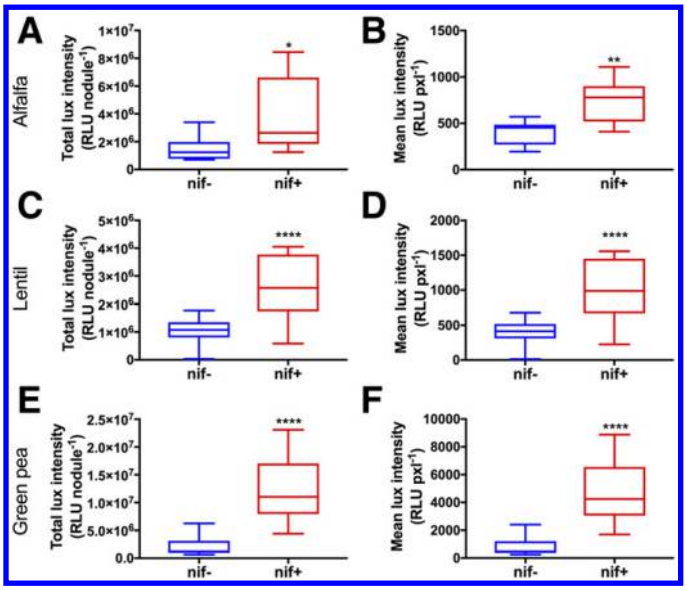

Fig. 5. Quantitative comparison of luminescence emitted from *GlnLux* agar-embedded cells exposed to amide-exporting legume root systems inoculated with *nif*– versus *nif*+ rhizobia strains: **A and B,** alfalfa, **C and D,** lentil, and **E and F,** green pea. Box-plots showing **A, C, and E,** total lux intensity per nodule and **B, D, F,** mean intensity per pixel in alfalfa, lentil, and pea. All the visible nodules (*nif*+ and *nif*–) within a plate were analyzed for lux quantification by selecting an area of 50-by-50-pixels around each nodule. Background subtraction for lux was performed by selecting a similar area of 50-by-50-pixels, where no roots were visible. *N* (number of nodules/nodule clusters) = 8 to 23 (*nif*–) and 10 to 20 (*nif*+), each from three completely independent *GlnLux* agar plates from plants grown and imaged at separate times (Fig. 4). Asterisks indicate significant differences between *nif*+ versus *nif*– roots for luminescence (*t* test, * = $P < 0.05$; ** = $P < 0.01$; and **** = $P < 0.0001$). RLU, relative light units.

DISCUSSION

SNF is mediated by the bacterial enzyme nitrogenase which is encoded and regulated by various *nif* and *fix* genes (Oldroyd et al. 2011; Raymond et al. 2004; Starker et al. 2006). Previously, transgenic rhizobia strains have been engineered in which promoters of these genes have been fused to visible reporters to create whole cell biosensors to understand factors that regulate nitrogenase activity and hence nitrogen fixation (Acosta-Jurado et al. 2016; Alfaro-Espinoza and Ullrich 2015; Barnett et al. 1998; Frederix et al. 2014; Gavrin et al. 2014; Horváth et al. 2015; Kim et al. 2015; Liu et al. 2014; Pini et al. 2017; Sánchez-Cañizares and Palacios 2013; Timmers et al. 2000; Vernie et al. 2008; Wang et al. 2017). Biosensors have also been used to understand additional components of plant–microbial interactions especially related to nodule formation (Brencic and Winans 2005). For example, quorum-sensing between different rhizobia strains has been extensively studied using biosensors that can detect *N*-acyl-homoserine lactones (Krysciak et al. 2011; Nievas et al. 2012; Pongsilp et al. 2005; Rodelas et al. 1999; Suárez-Moreno et al. 2008; Wielbo et al. 2010; Wisniewski-Dyé and Downie 2002). Initial rhizobia colonization through root hair infection threads has been visualized using GFP and DsRed-expressing *Sinorhizobium meliloti* mutant strains (Gage 2002; Wielbo et al. 2010) and nitric oxide biosensors (Del Giudice et al. 2011). In addition, biosensors have been used to study a number of interactions including how root exudates affect legume–rhizobia interactions in the rhizosphere (using *gfp* fused to a *melA* promoter that is induced by galactose and galactosides) (Bringhurst et al. 2001); the defense response to soil rhizobia and subsequent nodulation (using salicylic acid based biosensors) (Jiménez-Guerrero et al. 2015); bacteroids within nodules (using *gfp* expressing rhizobia) (Auriac and Timmers 2007); and stress induced nodule senescence (using nitric oxide biosensors) (Cam et al. 2012). Recently Pini et al. (2017) developed bacterial lux fusion bioreporters using *Rhizobium leguminosarum* bv. *viciae* specific for sugars, polyols, organic acids, and flavonoids as well as amino acids (phenylalanine, GABA).

Here, to detect Gln, rather than having to engineer Gln bioreporters in multiple rhizobia strains or construct different transgenic plants, we used an alternative approach wherein a companion *E. coli* biosensor strain, specific for Gln, was embedded into agar upon which nodulated root systems were placed and imaged for Gln release. The *GlnLux* agar method was sufficiently sensitive to detect Gln release from individual nodules, and the assay represents a new tool for legume–rhizobia symbiosis researchers. Furthermore, the new assay was validated for the visualization of SNF activity in three amide-exporting legumes and a ureide-exporting legume. The latter observation was surprising, since in ureide-exporting legumes, the majority of fixed N is transported as ureide compounds (allantoin and allantoic acid) (Carter and Tegeder 2016) not amino acids. However, in an earlier study of ureide-exporting legumes grown under different stress conditions (Amarante et al. 2006), it was found that the Gln concentration in the xylem follows the same trend as the ureide concentration (Amarante and Sodek 2006)

which, here, may explain why soybean root system Gln was a good indicator of SNF activity.

***GlnLux* agar imaging reveals Gln source-sink relationships in legume nodules.** We previously demonstrated that *GlnLux* cells, co-incubated with legume leaf punch extracts in 96-well plates, emitted photons that strongly correlated with the proportion of N derived from SNF (measured using the ^{15}N assay) in both amide and ureide-exporting legumes (Thilakarathna et al. 2017). This earlier study confirmed that *GlnLux* cells could accurately report assimilated Gln transported from nodules following SNF under minimal external N supply. However, we did not previously attempt legume tissue imaging, including root systems and individual nodules, using this technology.

Here, the *GlnLux* agar assay allowed imaging of source-sink relationships pertaining to nodule Gln in the legume–rhizobia symbiosis. Nodules elicited *GlnLux* cells to emit photons; and nodules could be interpreted as acting as a source or sink (or both) for Gln depending on the genotype of the rhizobia inoculant.

nif+ *nodules.* Among the *nif*+ nodule population in amide-exporting legumes (mainly alfalfa and lentil), the modest positive correlation observed between the nodule contact surface area and mean lux intensity could be an artifact of the number of *GlnLux* cells that could be induced, given their proximity to the Gln source; alternatively, the lux results could reflect real differences in total Gln accumulation in large versus small nodules. In the latter case, the Gln released from *nif*+ nodules could have predominantly originated from SNF activity within the nodule (thus, a Gln source) but possibly also Gln imported from other plant tissues (e.g., remobilization from leaves) to feed bacteroids (Lodwig et al. 2003; Patriarca et al. 2002) and nodule growth/metabolism (Parsons et al. 1993). It is likely that most of the observed Gln in many of the larger *nif*+ nodules was the result of SNF activity, since Gln was elevated

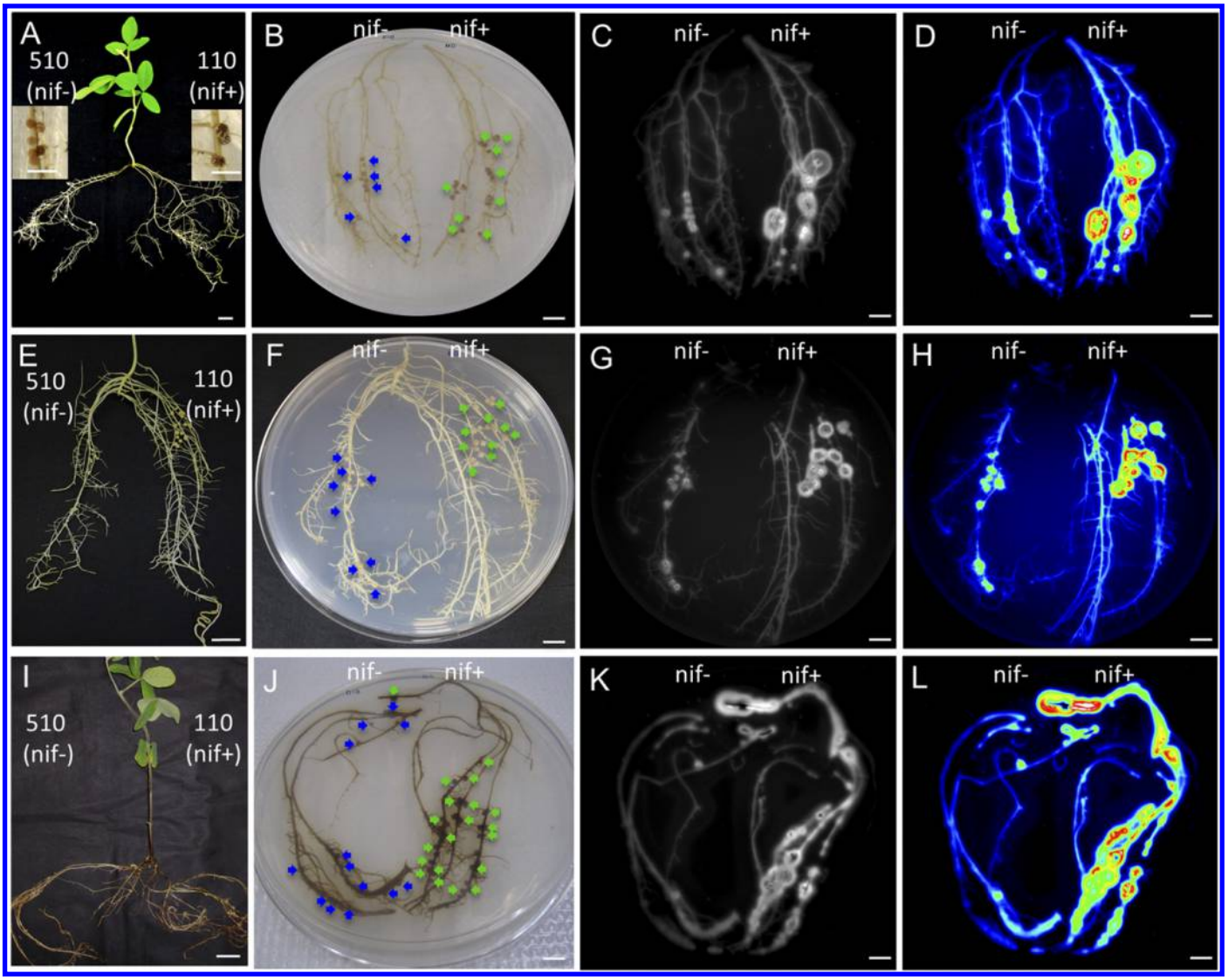

Fig. 6. Test to determine whether the *GlnLux* agar imaging assay can distinguish potentially active from inactive nodules from root systems of a ureide-exporting legume (soybean). Potentially active nodules were those inoculated with *nif*+ wild-type rhizobia, while inactive nodules were those inoculated with mutant *nif*– rhizobia. Shown are replicates from three separate trials: **A, E, and I,** split root systems of 5-week-old soybean plants were freeze-thawed to cause Gln leakage and then placed on *GlnLux* agar. The opposite agar surface was then imaged using a photon capture CCD camera. The inset images are magnifications to show the presence of nodules. Shown are the corresponding **B, F, and J,** light images, **C, G, and K,** white lux images, and **D, H, and L,** false-colored lux images after 4 to 6 h of incubation and 1,000 s exposure. Bright green-yellow-red color areas represent the tissues with the highest Gln content, which correspond to active sites of nitrogen fixation. Green and blue arrows indicate the location of *nif*+ (active) and *nif*– (inactive) nodules, respectively. Scale bars correspond to 1 cm.

in leaf veins of these plants compared with *nif*– plants or uninoculated plants (Supplementary Figs. S5 and S6). Furthermore, when comparing the contact areas of a similar size between *GlnLux* agar and *nif*+ versus *nif*– nodules (individual nodules or averaging of nodule clusters), most of the highest lux-eliciting nodules were *nif*+ in both amide- and ureide-exporting legumes (Fig. 8). These results suggest that the interpretation that *nif*+ nodules predominately release Gln originating from SNF activity is reasonable, and not an artifact of nodule size (since *nif*+ nodules tend to be larger than *nif*– nodules). However, subsets of *nif*+ nodules within each root system elicited similar photon emission intensities compared with *nif*– nodules, especially in soybean (Fig. 6D). One explanation is that the newly emerged *nif*+ nodules may have been developmentally immature and not yet have established active nitrogen fixation. Overall, the results suggest that *GlnLux* agar-based imaging cannot diagnose nodule SNF activity on an individual nodule basis but can on a population (root system) basis.

Consistent with the interpretation that *nif*+ nodules are exporting Gln derived from SNF activity, photon emission was also detected from the lateral roots (Figs. 2, 4, and 6), especially adjacent to *nif*+ nodules, possibly capturing the transfer of assimilated Gln from nodules to other plant tissues through root xylem (Molero et al. 2014; Prell and Poole 2006; Udvardi and Poole 2013). The *GlnLux* agar assay thus permits an integrated view of Gln accumulation in legume root systems from nodules, through roots, to the stem base.

nif– *nodules.* By contrast, as no nitrogen fertilizer was applied and since *nif*– nodules are not able to fix atmospheric nitrogen, the released Gln from *nif*– nodules had to be imported from other plant tissues to feed nodule development (e.g., seed or *nif*+ nodules since it was a split root-inoculated system), clearly uncovering nodule sink dynamics. Furthermore, nodule sink demand for amino acids increases during bacterial differentiation into bacteroids (Dunn 2015; Mulley et al. 2011). The plant also provides bacteroids with glutamate, derived from Gln, in part to shut down bacteroid ammonium assimilation in favor of ammonium export to their plant hosts (Lodwig et al. 2003) (this nodule sink demand for Gln donors may be greater in *nif*+ nodules). Interestingly, weak-moderate positive correlations were found between the nodule surface area and mean lux intensity in inactive *nif*– nodules in lentil and pea (Fig. 8), suggesting that *nif*– nodules can act as sinks for Gln based on their size. An interesting question is why such a correlation was not observed in *nif*– nodules of alfalfa and soybean.

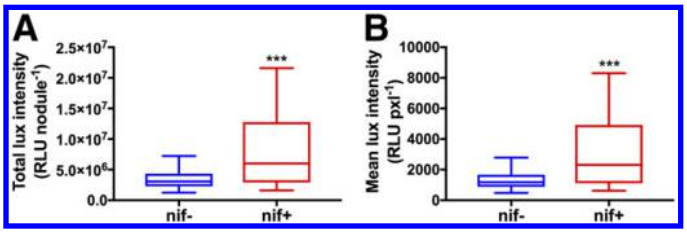

Fig. 7. Quantitative comparison of luminescence emitted from *GlnLux* agar-embedded cells exposed to root systems of a ureide-exporting legume (soybean) inoculated with *nif*– versus *nif*+ rhizobia strains. Boxplots showing **A,** total lux intensity per nodule and **B,** mean intensity per pixel. All the visible nodules (*nif*+ and *nif*–) within a plate were analyzed for lux quantification by selecting an area of 50-by-50-pixels around each nodule. Background subtraction for lux was performed by selecting a similar area of 50-by-50-pixels, where no roots were visible. *N* (number of nodules/nodule clusters) = 22 (*nif*–) and 30 (*nif*+), each from three completely independent *GlnLux* agar plates from plants grown and imaged at separate times (Fig. 6). Asterisks indicate significant differences between *nif*+ versus *nif*– roots for luminescence (*t* test, *** = $P < 0.001$). RLU, relative light units.

Variation in associated *GlnLux* photon emission between plant genotypes. In general, the photon emission was much higher in pea (Figs. 2O and 4L) and soybean (Figs. 6D and H and 4L) nodules compared with those of alfalfa (Figs. 2G and 4D) and lentil (Figs. 2K and 4H). Generally, pea (Figs. 2M and 4J) and soybean (Fig. 6B, F, and J) roots have larger nodules compared with alfalfa (Figs. 2E and 4B) and lentil (Figs. 2I and 4F), hence had more contact area with the *GlnLux* agar to activate more *GlnLux* cells.

A lack of correlation between the nodule surface area and mean lux intensity in soybean, a ureide exporter, could be due to the determinate nature of its nodules such that the age of each nodule cannot be inferred from its size: an immature nodule, a bacteroid-differentiating nodule, and an SNF-active nodule could all be of a similar size. This contrasts with the indeterminate nodules of the amide exporters (alfalfa, lentil, and pea) where their size and developmental age are more correlated (Kondorosi et al. 2005). Hence, a small alfalfa nodule, which was generally associated with low lux emission, may not have undergone bacteroid differentiation, in contrast to large nodules which may have been SNF active and hence are associated with high Gln release (Fig. 8).

Success and limitations. The success of this new imaging tool was enabled by several design features. First, the biosensor strain was auxotrophic for Gln and hence specific for exogenous Gln (Tessaro et al. 2012), in this case derived from nodulated root systems. The biosensor strain contained a lux reporter under the control of a constitutive promoter (Tessaro et al. 2012). These two design features allowed the biosensor to be highly sensitive to Gln released from individual nodules. Furthermore, as rhizobia and plant cells do not emit photons, any detected photons were derived from the biosensor or external sources (light leakage, camera heat) (Goron and Raizada 2014). The *luxCDABE* gene cassette encodes all the components required for luminescence and hence was independent of the host (Close et al. 2012).

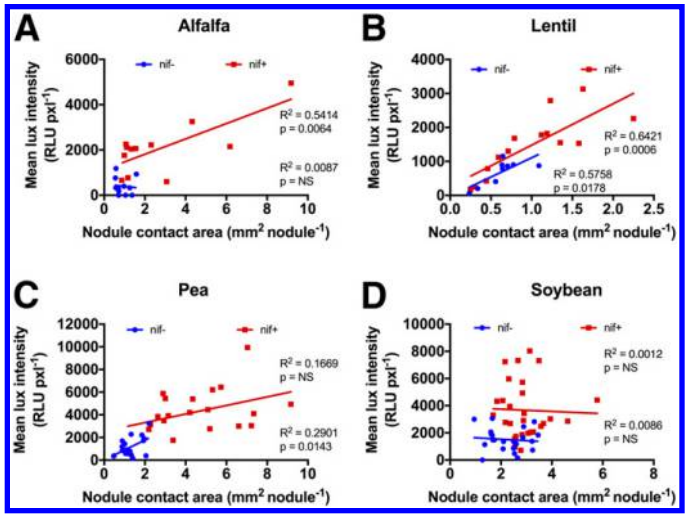

Fig. 8. Test for correlation between mean lux intensity emitted from *GlnLux* agar-embedded cells exposed to nodules versus their contact surface area with *GlnLux* agar. Shown are the correlation analysis between the nodule contact surface area (mm^2 nodule^{-1}) and mean lux intensity (relative light units [RLU] pixel^{-1}) for **A,** alfalfa, **B,** lentil, **C,** pea, and **D,** soybean nodules. All the visible nodules (*nif*– and *nif*+) within a plate were analyzed for lux quantification by selecting an area of 50-by-50-pixels around each nodule. Background subtraction for lux was performed by selecting a similar area of 50-by-50-pixels, where no roots were visible. When the nodules were clustered together, total contact surface area of nodules and total lux intensity around nodules were normalized by dividing them with the number of nodules within the cluster. *N* (number of nodules/nodule clusters) = 23 to 52. R^2, Pearson correlation coefficient. NS, no significant difference at $P < 0.05$.

A limitation of the *GlnLux* agar method is that it visualizes only Gln output, which is the immediate assimilatory product of SNF (Prell and Poole 2006; Tegeder 2014). However, Gln is the minor export form of fixed N in nodule-associated xylem with the major form being Asn in amide-exporters, and ureides in other legumes (Prell and Poole 2006; White et al. 2007). In the future, engineering of companion Asn/ureide biosensors would be useful, as would a companion biosensor for glutamate (Glu) which is imported into bacteroids as a dicarboxylic acid donor (Lodwig et al. 2003); *GlnLux* is specific for Gln, defective in glutamine synthetase and hence unresponsive to Glu (Tessaro et al. 2012). Another challenge in legumes is that environmental N from soil or fertilizer is also assimilated into Gln (Unkovich et al. 2008). Hence, the *GlnLux* assay may be most suited to controlled environments with minimal external N supply. An additional limitation is that some nodules and roots can have poor contact with *GlnLux* agar, which can create artifacts (e.g., SNF-active nodules that do not touch the agar would appear to be inactive). Given these observations, combined with the earlier points, it is critical to note that *GlnLux* agar imaging cannot diagnose SNF activity on an individual nodule basis, though it is informative on a root system scale.

Summary and future applications. The root system observations suggest that, in both amide and ureide exporters, the *GlnLux* agar assay potentially permits visualization of net Gln imported into nodules from roots (for nodule growth, bacteroid differentiation and as a source for glutamate-amino acid cycling), Gln assimilated from fixed nitrogen inside nodules, and Gln exported from this assimilatory N from nodules into roots. Especially noteworthy is that the *GlnLux* agar method is sufficiently sensitive to image nodule Gln from amide-exporting and ureide-exporting root nodules at a population level, able to differentiate root systems with active *nif*+ nodules from inactive *nif*– nodules. Thus, *GlnLux* agar based imaging is a new visualization tool to observe accumulation and transfer of a critical amino acid in legume root systems.

With respect to basic research, this new method has the potential to help researchers elucidate how plant and microbial genetic and environmental factors affect Gln accumulation in nodules and subsequent import and export from/to roots, respectively. Different abiotic stress conditions (e.g., drought, waterlogging, and heat stress) have been shown to alter Gln concentrations in the nodules and roots of legumes (Amarante et al. 2006; Gil-Quintana et al. 2012; Hungria and Kaschuk 2014), thus the *GlnLux* agar assay may create a new opportunity to visualize root systems for Gln under different stress conditions. The assay has the potential to help characterize plant or rhizobia mutant collections including in model legumes, for example to phenotype mutant nodules that accumulate but do not export fixed nitrogen (Gln). The new method has the potential to understand different mechanisms involved in the maintenance of symbiotic nitrogen fixation, as well as the dynamics of SNF during development from nodule establishment to senescence. Finally, at an earlier stage of development than the current study, the assay may be able to help characterize the dynamics of amino acid exudation from roots, involved in the stimulation of rhizobia in soil (Patriarca et al. 2002; Paynel et al. 2001; Thilakarathna et al. 2018). Now a single reporter strain can be used to visualize nodule/root/rhizosphere Gln across diverse amide- and ureide-exporting legume species, crop varieties, and rhizobia partners within legume phytobiomes, within the confines of the limitations noted.

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