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Soybean Ureases, but Not That of *Bradyrhizobium japonicum*, Are Involved in the Process of Soybean Root Nodulation

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Supporting Information

ABSTRACT: Ureases are abundant in plants, bacteria, and in the soil, but their role in signaling between soybean and soil microorganisms has not been investigated. The bacterium *Bradyrhizobium japonicum* forms nitrogen-fixing nodules on soybean roots. Here, we evaluated the role(s) of ureases in the process of soybean nodulation. Chemotaxis assays demonstrated that soybean and jack bean ureases were more chemotactic toward bacterial cells than the corresponding plant lectins. The *eu1-a,eu4* soybean, deficient in urease isoforms, formed fewer but larger nodules than the wild-type, regardless of the bacterial urease phenotype. Leghemoglobin production in wild-type plants was higher and peaked earlier than in urease-deficient plants. Inhibition of urease activity in wild-type plants did not result in the alterations seen in mutated plants. We conclude that soybean urease(s) play(s) a role in the soybean−*B. japonicum* symbiosis, which is independent of its ureolytic activity. Bacterial urease does not play a role in nodulation.

KEYWORDS: *B. japonicum, soybean urease, leghemoglobin, nitrogen fixation*

INTRODUCTION

Urea is one of the most utilized nitrogen fertilizers in the world. Although plants can capture nitrogen directly from urea, through transporters,[1] most of this compound is rapidly degraded by the urease activity of soil microorganisms. The high urease activity in the soil leads to nitrogen loss upon NH_3 volatilization, leading to the use of urease inhibitors to reduce urea hydrolysis and improve nitrogen uptake by plants.[2,3] Besides the economic losses, inefficient use of urea, and nitrogen in general, leads to water and atmospheric pollution with nitrate, ammonia, and NO_x compounds.[3] Biological nitrogen fixation (BNF) is an inexpensive and efficient way to supply plants with nitrogen without the use of urea or other nitrogenous fertilizers. This process consists of the reduction of atmospheric nitrogen (N_2) to two molecules of ammonia (NH_3), catalyzed exclusively by prokaryotic microorganisms that have the nitrogenase enzyme complex. Symbiosis established between legumes and nitrogen-fixing bacteria leads to formation of root nodules containing bacteria. This phenomenon has been extensively investigated for soybean plants associated with *Bradyrhizobium* spp. The association starts with a chemotactic response of *B. japonicum* toward root exudates, containing a number of organic compounds, mucilage, and proteins.[4] Isoflavonoids in the root exudates, mainly genistein and daidzein, activate *nod* genes in *B. japonicum* and production of Nod factors, which are specifically recognized by kinase-like receptors of root epidermal cells.[5] In response to Nod factors, root cells divide and form infection threads that lead bacteria to the interior of the root where they are enveloped by membranes of the host, forming symbiosomes, in which they differentiate into nitrogen-fixing bacteroids.[6] Cell division and differentiation in the root cortex leads to formation of the root nodule, containing both infected and uninfected cells.

Ureases (urea amidohydrolase; EC 3.5.1.5) are nickel-dependent enzymes that catalyze urea hydrolysis into ammonia and carbon dioxide and are widespread among plants, fungi, and bacteria, in which they allow use of urea as a nitrogen source.[1] Evidence of toxicity of plant ureases, independent of urea hydrolysis, toward some insects and fungi has accumulated.[7−9] These previously overlooked properties of plant and microbial ureases, such as entomotoxic and fungitoxic activities, have widened the proposed biological roles of ureases.

Soybean produces an active seed urease, synthesized in the developing embryo,[10,11] designated embryo-specific urease (eSBU) and encoded by the *Eu1* gene (Glyma05g27840).[11−13] Loss of eSBU has no apparent physiological effect,[14] and eSBU is rapidly degraded upon germination and seedling development.[10] A possible nonmetabolic role of urease(s) in seed and embryo

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Table 1. Primers Used in This Work

name	sequence*
ureABC-F	GTTCGGACGCACTGCGATGG
ureABC-R	CGTAATGATTTCGACCTTCG
ureG-F	TGTGGTCGCCGCGATGCACG
ureG-R	CATGGACGTCGATCGCACGT
PS1	GTGTAGGCTGGAGCTGCTTC
PS2	CATATGAATATCCTCCTTAG
Delta ureABC-F	GCTGTGGTTGAATTGACGTGTTGAACGGAAGAGATTTCGCGTGTAGGCTGGAGCTGCTTC
Delta ureABC-R	AAACCGTCCCCGGACAGGCTCCGGGGACCGGGGCGCTATTCATATGAATATCCTCCTTAG
Delta ureG-F	GGGTCCTGGCTTTCGCCAGGACGACGATAGGAAAGAATCAGTGTAGGCTGGAGCTGCTTC
Delta ureG-R	GTTAACGGCGGAAAACCCCTGTGCCAAGTCGTCGCGTTCGCATATGAATATCCTCCTTAG

*Underlined sequences refer to PS1 and PS2.

defense was postulated.[15] Consistent with a role in defense, eSBU and jack bean seed urease display insecticidal effects, in part due to the action of a subpeptide released by cathepsin-type proteases in the gut of susceptible insects.[7,8]

Soybean expresses a second urease, the tissue-ubiquitous urease (uSBU), essential for assimilation of urea, either endogenously generated or environmentally available. uSBU is encoded by *Eu4* (Glyma11g37250), which is expressed in all tissues examined, including callus and the developing embryo.[11,16] Loss of uSBU activity leads to accumulation of urea in seeds and in leaves to the extent that leaf tips become necrotic,[14] a trait also observed in soybean plants deprived of nickel,[17] a component of the urease active site. Endogenous urea is produced by arginase action, for example, during mobilization of seed protein reserves.[18] Consistent with a general role of ureases in defense, uSBU-suppressed soybean plants showed increased susceptibility to fungal infection.[19]

Few, if any, studies have addressed the involvement of urease in the symbiosis of nitrogen-fixing bacteria and their host plants. In light of the now recognized multifunctionality of ureases,[7,20] the main objective of this study was to test the hypothesis that both plant and bacterial enzymes are involved in communication between the soybean plant and its symbiont *B. japonicum*.

MATERIALS AND METHODS

Plant Material. Seeds of *Glycine max* (L.) Merr cv. Williams 82 were commercially available (Missouri Seed Foundation). Soybean double mutant *eu1-a,eu4* (in the Williams 82 background) has a lesion in the *eu1* gene and is null for synthesis of eSBU protein[12,21] and transcript.[11] The *eu-1* allele was orignally termed *eu1-sun* (seed urease-null[11]) and is here termed *eu1-a*, consistent with designation of alleles of other soybean urease loci.[13] The second lesion is a missense alteration in the *eu4* gene, resulting in inactive ubiquitous urease.[16,22] Two homozygous double mutant strains were employed—both contained the same *eu1-a* null allele[11] but differed in the *eu4* allele (*aj3* vs *aj6*), each containing a unique single base missense mutation inactivating the ubiquitous urease.[16]

Bacterial Strains and Culture Conditions. *B. japonicum* strain USDA110 was routinely maintained on HM salts medium[23] supplemented with 0.5% yeast extract at 28 °C with shaking (200 rpm). When necessary, antibiotics were supplied at 150 $\mu g \cdot mL^{-1}$ (kanamycin) and 30 $\mu g \cdot mL^{-1}$ (chloramphenicol). *Escherichia coli* strains were cultured on Luria−Bertani medium at 37 °C.

Construction of B. japonicum Urease Mutants. Primers used in this work are described in Table 1. To generate mutants, the genes of interest were amplified with primers ureABC-F and ureABC-R or ureG-F and ureG-R. Polymerase chain reaction (PCR) products contained the full sequence of the genes of interest in addition to about 400 bp 5′ and 3′ flanking sequence. The PCR products were inserted into the suicide vector pKnockout,[24] generating PKO Ω−URE. A kanamycin cassette from pHP45 Ω−Km[25] was amplified with primers PS1 and PS2, and then, this product was used as the template for another PCR with primers Delta ureABC-F/R and Delta ureG-F/R, generating pKO Ω−URE−Km. These primers added to the kanamycin gene 40 base pairs identical to regions flanking the genes of interest. This construct was transferred from *E. coli* DH5α to *B. japonicum* USDA110 by triparental conjugation with the helper plasmid pRK2073.[26] Deletion mutant recombinants were selected by kanamycin resistance and confirmed as described in the Results section.

Canavalia ensiformis Urease (JBU). Urease type C-III from jack bean (Sigma−Aldrich) was used in all experiments. The protein (hexameric form, M_r = 540 kDa) was solubilized in 20 mM sodium phosphate buffer, pH 7.0.

Soybean Embryo-Specific Urease. eSBU was purified as described[27] from soybean flour (Sigma−Aldrich) and dialyzed against 20 mM phosphate buffer, pH 7.0 prior to use.

Soybean Ubiquitous Urease. Cells of *E. coli* BL21-RIL:pGEX-4T-2 containing the full length cDNA encoding the uSBU[28] were grown in 2xYT medium (16 $g \cdot L^{-1}$ tryptone, 10 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ sodium chloride, pH 7.0) containing ampicillin (150 $\mu g \cdot mL^{-1}$) and chloramphenicol (40 $\mu g \cdot mL^{-1}$) and maintained at 37 °C with shaking (200 rpm). When the culture reached 0.7 OD_{600}, the expression of uSBU fused to glutathione S-transferase at its N terminus (GST-uSBU) was induced by adding 0.55 mM IPTG, keeping the culture at 24 °C with shaking (200 rpm) for 15 h. Bacteria were collected by centrifugation, suspended in phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), and subjected to lysis by ultrasound. After centrifugation at 60 000*g* for 25 min, the supernatant was passed through a 0.45 μm membrane, and the filtrate was applied onto a Glutathione Sepharose 4B (GE Healthcare) column. Retained GST-uSBU was eluted with 10 mM reduced glutathione in 50 mM Tris−HCl, pH 8.0. The GST protein (not fused to uSBU) was used as a control and was obtained from *E. coli* BL21:pGEX-4T-2 grown in 2xYT medium supplemented with 150 $\mu g \cdot mL^{-1}$ ampicillin and maintained at 37 °C with shaking (200 rpm). When the culture reached 0.7 OD_{600}, expression of GST was induced by adding 1 mM IPTG, and the culture was maintained at 37 °C for 3 h with shaking (200 rpm). Purification of GST was performed by affinity chromatography as described above.

Urease Activity. The phenol-nitroprusside colorimetric determination of ammonia[29] was used to determine urease activity in soybean tissues and nodules. One unit of urease activity was defined as the amount of enzyme that produces 1 μmol of ammonia per minute at 37 °C. Alternatively, urease activity in whole cells or tissue samples was semiquantitatively evaluated in a weakly buffered urea solution containing a pH indicator. Samples were incubated in 1 mL of 100 mM urea, 1 mM EDTA, 10 mM KPO_4, pH 7.0, and 5 $mg \cdot L^{-1}$ cresol red and kept at room temperature. Urease-positive samples turned the solution sequentially from yellow to pink to vermillion[30] in a time frame dependent on urease level.

Urease Inactivation. JBU and eSBU were inactivated by treatment with *p*-hydroxy-mercurybenzoate (Sigma−Aldrich), as previously described.[27] Excess inhibitor was removed by extensive dialysis against

20 mM phosphate buffer, pH 7.0. In one set of experiments, urease-inhibited soybean plants were obtained by adding 100 μM phenylphosphorodiamidate (PPD) (Sigma–Aldrich) to the plant nutrient solution.[31] PPD is an analogue of urea that binds to the urease active site.[32]

Chemotaxis Assays. *B. japonicum* chemotaxis was assayed as described[33] with minor modifications, using a 48-well Boyden chamber (Neuroprobe Microchemotaxis System, Gaithersburg, MD) with 2 μm PVP-free polycarbonate filters. Chemotactic stimulation was tested in 50 μL containing 9 μM of one of the following: GST-uSBU, eSBU, JBU, soybean genistein (Sigma–Aldrich), lectins (Sigma–Aldrich) from *Phaseolus vulgaris*, *G. max*, and *C. ensiformis*, or bovine serum albumin (BSA). Individual putative attractants, diluted with 20 mM phosphate buffer, pH 7.0, were added to the top wells of the Boyden chamber. *B. japonicum* cells suspended in saline solution (10^6 cells·mL^{-1}) were added (28 μL) to the bottom chamber wells and incubated 60 min at room temperature, after which bacterial cells in the top wells were collected and plated counting of colony-forming units (CFUs).

Plant Growth and Inoculation. Soybean seeds were surface sterilized and germinated as described.[34] After 48 h, seedlings were inoculated with isogenic *B. japonicum* USDA110 urease-positive or urease-negative mutants for 30 min and then planted in autoclaved Leonard jars[35] containing a 1:1:1 mixture of vermiculite/perlite/sand supplied with a nitrogen-free plant nutrient solution.[36] During growth, sterile nutrient solution was added to the lower reservoir as needed. Plants were maintained in growth chambers at 28 °C and 16 h light. Nodules were harvested from the roots 16–42 d after planting and kept frozen at −80 °C until use.

Leghemoglobin Content. Nodules were transferred to 2 mL tubes containing a 5 mm steel ball. PBS was added, and nodules were macerated and homogenized in a TissueLyser system (Qiagen) with pulses of 3 min at a frequency of 30 Hz. Samples were subjected to two cycles of centrifugation, first at 3000*g* for 10 min and the second at 18 000*g* for 20 min. Leghemoglobin content (mg·mg^{-1} protein) in the second supernatant was estimated by absorbance at 410 nm and an extinction coefficient[37] of 33.9 × 10^3 L·mol^{-1}·cm^{-1}.

Protein Determination. The protein content of samples was determined by their absorbance at 280 nm or alternatively by the Coomassie dye method[38] using BSA as the standard.

Electron Microscopy. Nodule samples were fixed in 25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 h at room temperature and then kept at 4 °C for 16 h. Samples were then washed with three changes of 0.1 M phosphate buffer and postfixed in 2.0% osmium tetroxide for 1 h. Samples were again washed with buffer as above before being dehydrated through 30%, 50%, 70%, 95%, and 100% acetone. The tissues were then embedded stepwise in Epon resin. For polymerization, the material was kept at 60 °C for 72 h. The resulting blocks of embedded biofilm were sectioned in an ultramicrotome (Leica Ultracut UCT) with a diamond knife at a thickness of 800 nm and stained with aqueous toluidine blue and 1% basic fuchsin. Ultrathin sections (100 nm thick) were prepared using the same ultramicrotome and post contrasted with uranyl acetate and lead citrate. Samples were visualized under a transmission electron microscope (Philips model EM208S, Center of Electron Microscopy and Microanalysis, Universidade Luterana do Brasil, Canoas, RS).

Statistical Analysis. Data were evaluated using "one-way" ANOVA followed by the *t* test of Bonferroni or Tukey. A *p* value less than 0.05 was considered statistically significant. All analyzes were performed using GraphPad Prism software (version 5.0 for Windows).

RESULTS

Construction of *B. japonicum* Mutants. To examine the role of ureases in the interaction between soybean and *B. japonicum*, two mutants of *B. japonicum* were constructed. The mutant strain Δ*ureABC* is deleted in the three urease structural genes thus lacking urease protein. The mutant strain Δ*ureG* has a deletion of the gene encoding the accessory protein UreG, which participates in the assembly of the protein metallocenter, thus producing an enzymatically inactive apoenzyme, with no Ni^{2+} in its active site.[39] The approach used for construction of the mutants consists of inserting the gene that confers resistance to kanamycin via homologous recombination in regions flanking the target genes into the genome of *B. japonicum*. Mutants were confirmed by PCR (Figure 1) employing genomic primers

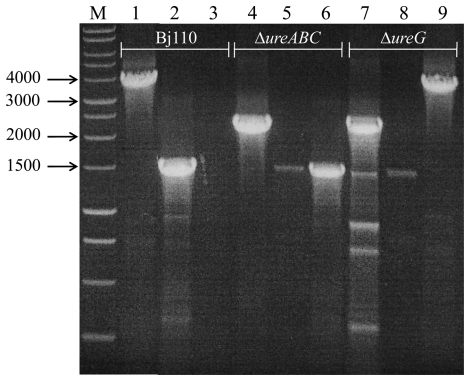

Figure 1. Validation of mutants by PCR. Genomic DNA from wild-type *B. japonicum* USDA110 (Bj110) and deletion mutants (Δ*ureABC* and Δ*ureG*) was subjected to PCR with specific primers. (1) Intact ureABC, (2) intact *ureG*, (3) Can^r, (4) interruption of *ureABC* by Can^r, (5) Can^r, (6) intact *ureG*, (7) interruption of *ureG* by Can^r, (8) Can^r, (9) intact *ureABC*.

flanking the putative insert and by the absence of ureolytic activity. In the PCR amplifications, the sizes of *ureABC* and *ureG* fragments in Bj110 are 3814 and 1491 bp, respectively. Homologous recombination in the regions flanking the genes of interest promotes the insertion of the kanamycin resistance gene (Can^r). Thus, in the genomic PCR amplifications, Δ*ureABC* generates a fragment of 2212 bp, while no change is seen in *ureG*. In turn, from the DNA of mutant Δ*ureG*, a fragment of 2287 bp is amplified without change in *ureABC*.

To verify the ureolytic activity, 1 mL of stationary-phase bacterial cells were collected by centrifugation and suspended in a solution containing weakly buffered 100 mM urea and pH indicator cresol red (Materials and Methods). Alkalinization of the medium by urea hydrolysis induced a color change of the pH indicator after 2 h at room temperature by wild-type bacterium Bj110. Mutants Δ*ureG* and Δ*ureABC* showed no ureolytic activity, since after incubation the medium kept the original yellow color for at least 16 h (Figure S1, Supporting Information). Lack of urease was further confirmed by the lack of growth of the mutant bacteria on minimal medium containing urea as the sole nitrogen source (data not shown).

Chemotaxis toward Plant Ureases. Plant ureases were found to be strongly chemotactic to *B. japonicum*. JBU, eSBU, and recombinant GST-uSBU induced chemotaxis of *B. japonicum* in the 0.1–9 μM concentration range. Figure 2 shows that increasing concentrations of JBU and GST-uSBU were accompanied by an increase in the CFUs of *B. japonicum* recovered in the upper compartment of the Boyden chamber. While recombinant GST-uSBU is fused to GST, recombinant GST alone induced a much weaker chemotaxis. The GST response peaked at 0.5 μM, whereas chemotaxis increased steadily up to 9 μM GST-uSBU.

It was shown previously that GST-uSBU is not active enzymatically,[28] most likely due to lack of incorporation of Ni into the urease active site by the *E. coli* host, lacking urease accessory proteins. The contribution of the ureolytic activity to chemotaxis was evaluated by testing enzymatically active plant

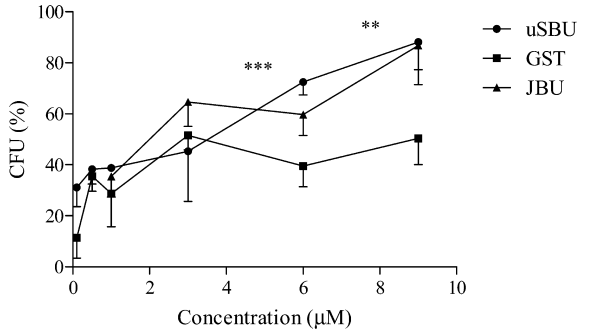

Figure 2. Response curve of *B. japonicum* to various concentrations of soybean ubiquitous urease (uSBU), jack bean urease (JBU), and glutathione-S-transferase (GST). The experiment was performed in a Boyden chamber containing 10^6 cells·mL^{-1} of Bj110 in the lower wells and different concentrations of uSBU or GST (control) in the upper wells. After 1 h at room temperature, the cells that migrated to the upper wells of the chamber were collected and plated for CFU counts. The points represent the mean ± SD of triplicates. Points marked with ** ($p < 0.01$) or *** ($p < 0.001$) were significantly different compared to control at the same dose by the Bonferroni test.

ureases eSBU and JBU before and after treatment with *p*-hydroxymercuribenzoate (*p*-HMB), an irreversible inhibitor of urease catalytic activity.[27] Ureases (3 μM) were added to the upper compartment of the Boyden chamber, and no significant differences were observed in their chemotactic effect, regardless of their ureolytic activities (Figure 3). Chemotaxis assays with

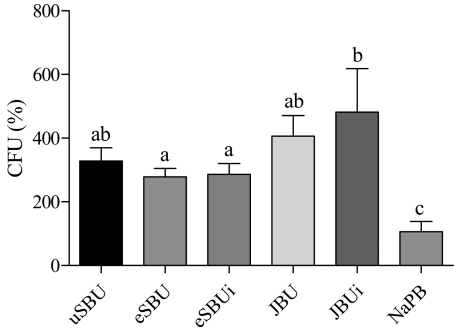

Figure 3. Effect of ureolytic activity in the chemotaxis of *B. japonicum* to plant ureases. Ureases were incubated at 4 °C for 16 h with 50 mM *p*-hydroxymercuribenzoate (*p*-HMB). Excess inhibitor was removed by dialysis prior to testing. All proteins were tested at a concentration of 3 μM. After 1 h at room temperature, the cells that migrated to the upper compartment of the chamber were collected and plated for CFU counts. (eSBU) soybean embryo-specific urease, (eSBUi) inactivated eSBU, (JBU) *C. ensiformis* urease, (JBUi) inactivated JBU, (uSBU) soybean recombinant ubiquitous urease. Bars represent the mean ± SD of triplicates.

mutants of *B. japonicum* indicated that the absence of the urease protein (Δ*ureABC*) or of its ureolytic activity (Δ*ureG*) in the bacteria did not alter their attraction to uSBU, as illustrated in Figure 4.

Finally, we compared the response of *B. japonicum* cells to the chemotactic effect of nonurease proteins (BSA, GST, and the lectins PHA-L from *P. vulgaris*, soybean agglutinin SBA, and jack bean concanavalin A) and the soybean flavonoid genistein. It can be observed in Figure 5 that soybean or jack bean ureases were the most potent chemotactic stimuli compared to the other tested compounds, followed by plant lectins. GST and BSA also

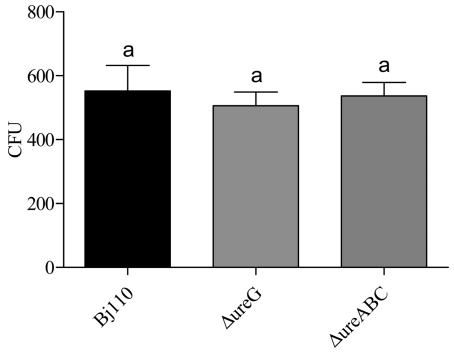

Figure 4. Chemotactic response of wild-type and urease deficient *B. japonicum* to uSBU. The experiment was performed in a Boyden chamber containing 10^6 cells·mL^{-1} of bacteria in the lower wells and 9 μM uSBU in the upper wells. After 1 h at room temperature, the cells that migrated to the upper wells of the Boyden chamber were recovered and plated for CFU counts. Bars represent the mean ± SD of six replicates.

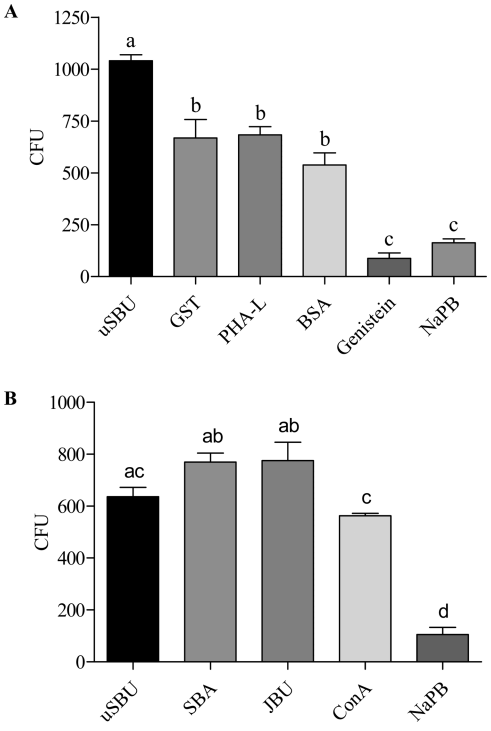

Figure 5. (A) Chemotactic response of *B. japonicum* USDA110 induced by 9 μM of the following: ubiquitous recombinant urease soybean (uSBU), the GST fusion protein, a lectin from *P. vulgaris* (PHA-L), bovine serum albumin (BSA), and genistein from soybean. (B) Chemotactic response of *B. japonicum* USDA110 induced by 9 μM ubiquitous recombinant urease soybean (uSBU), soybean lectin (SBA), *C. ensiformis* urease (JBU), and *C. ensiformis* lectin (ConA). Sodium phosphate buffer (NaPB, 20 mM) was used as a control. After 1 h at room temperature, the cells that migrated to the upper compartment of the Boyden chamber were recovered and plated for CFU counts. Columns with the same letter do not differ by the Tukey test ($p > 0.05$).

induced migration of *B. japonicum* but were significantly less chemotactic than ureases. Genistein was a very poor attractant.

Assays of Nodule Properties. To evaluate the role of plant and bacterial ureases in nodulation and nitrogen fixation, wild-type and urease-negative soybean were inoculated with wild-type and urease-negative *B. japonicum* mutants in all possible

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combinations. The number and fresh weight of nodules were determined 21 d after inoculation.

Wild-type plants showed a higher number of nodules when compared to mutant plants, regardless of the inoculated bacterial strain (Figure 6A). However, there was a marked increase in the size of nodules in mutant plants *eu1-a,eu4*, again regardless of the inoculated strain. No differences were seen in the total fresh mass of nodules per plant (Figure 6B).

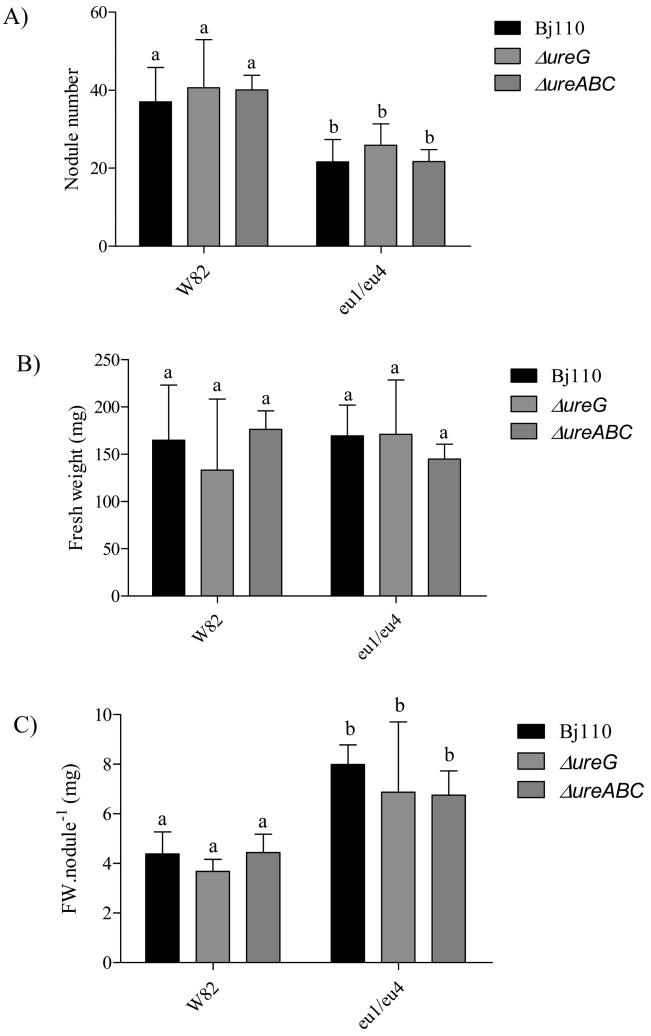

Figure 6. Wild-type soybean plants (W82) and a mutant deficient in embryo-specific and ubiquitous ureases (eu1/eu4) were inoculated with *B. japonicum* wild-type (Bj110), with a mutant strain that produces an apourease (Δ*ureG*), or a mutant strain that does not produce urease (Δ*ureABC*). Nodule number and weight were assessed 21 d after inoculation. (A) Number of nodules per plant. (B) Total fresh weight of nodules per plant. (C) Ratio between the total fresh weight of nodules and the number of nodules. Bars represent the mean of five Leonard pots, each containing 2−3 plants. Bars with the same letter do not differ by the Tukey test ($p > 0.05$).

To evaluate the importance of urease enzyme activity during soybean nodulation by *B. japonicum*, we used the urease inhibitor PPD, an analogue of urea that binds to the active site of the enzyme.[32] Wild-type soybean seedlings were inoculated with wild-type *B. japonicum* as described in Materials and Methods, and 100 μM PPD was added to the nutrient solution in the Leonard jars. Inhibition was maintained throughout the experimental period. Plants were analyzed 21 d after inoculation by counting and weighing the nodules formed. At the end of the experiment, urease activity was measured in extracts of leaves and nodules, confirming the inhibition of the enzyme by PPD (data not shown). Still, no differences were observed either in the number or fresh weight of nodules of PPD-treated urease-inhibited plants (data not shown).

Nodule Analysis by Optical and Transmission Electron Microscopies. Nodules of wild-type and *eu1-a,eu4* plants collected 35 d after inoculation with the *B. japonicum* wild-type strain were analyzed by optical and transmission electron microscopies. No alterations in architecture and histological organization of the nodules in the urease-deficient plants were observed (Figure S2, Supporting Information).

Leghemoglobin (LegHb) Content. The amount of LegHb in the supernatant of nodule macerates was estimated at times from 16 to 42 d after inoculation. As can be observed in Figure 7 (and Table S1, Supporting Information), the accumulation of LegHb from wild-type and mutant plants followed the same trend, regardless of the inoculant bacterial strain. Wild-type plants had lower nodule LegHb content at day 16, and it rose rapidly, peaking at 21 d after inoculation. On the other hand, nodules from mutant plants had a higher level of LegHb at 16 d after inoculation compared to wild-type but were slower to reach the maximal LegHb content, observed only at 35 d after inoculation (Figure 7). The altered pattern of accumulation of LegHb in *eu1-a,eu4* plants suggests that lesions in the structural genes of both soybean ureases, resulting in the absence of eSBU and in an inactivated uSBU, somehow compromised the symbiosis between the plant and the bacterium, delaying the LegHb peak from 21 d in the wild plant to about 35 d after inoculation.

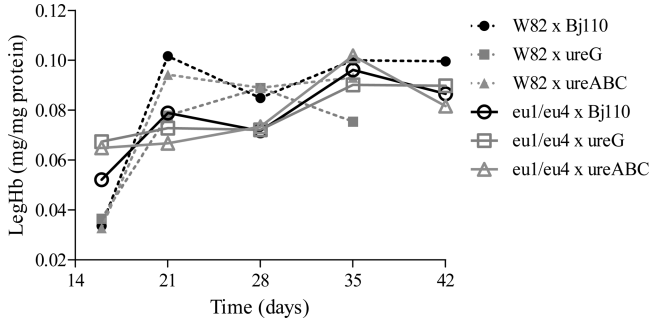

Figure 7. Leghemoglobin (LegHb) content of nodules versus time postinoculation. Wild-type (W82) and mutant (eu1/eu4) soybean plants were inoculated with wild-type *B. japonicum* (Bj110), with a mutant strain that produces an apourease (Δ*ureG*) or with a mutant strain that does not produce urease (Δ*ureABC*). LegHb content was measured by absorbance at 410 nm in extracts of nodules collected from 16 up to 42 d after inoculation. Each point is the average of three to six replicates.

DISCUSSION

Ureases have highly conserved structures and very similar catalytic mechanisms, reflective of their conservation throughout evolution.[40] In microorganisms, urease activity is involved in the assimilation of urea nitrogen, in pathogenicity in animals and humans, and in the metabolism of ruminants.[7] On the other hand, the physiological role(s) of this enzyme in plants is(are) still a matter of debate.[1,7] Addressing this question with in vivo studies using urease-deficient organisms may help to advance our understanding of the biological properties of this enzyme.

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Here, we investigated the hypothesis that ureases from soybean and/or *B. japonicum* could be involved in the signaling between plant and bacterium—a concept originally suggested by the common platelet aggregation properties of plant ureases and the urease of the soil bacterium, *Bacillus pasteurii*.[41] Previous results from our group with canatoxin,[42] an isoform of jack bean urease, and *Helicobacter pylori* urease[33] have shown that these proteins are chemotactic to neutrophils and other proinflammatory cells. Considering these data, we evaluated a possible chemotactic activity of SBU toward *B. japonicum*. Our results showed that *B. japonicum* cells are attracted to SBU isoforms and to JBU and that this response is independent of urease enzymatic activity or the urease status of the bacteria. Thus, plant ureases exert chemotaxis in bacteria, as well as in eukaryotic cells such as neutrophils and monocytes.

High molecular-weight components in root exudates, which include lectins,[43] can affect symbiosis between plants and nodule bacteria. Plant lectins are known to act as chemoattractants and to promote adhesion of rhizobia to root hairs, enhancing the symbiotic properties of root nodule bacteria. Not all steps of lectin−rhizobia interaction are species-specific.[44] Accordingly, here, *B. japonicum* migrated toward different legume lectins. As reported previously,[45] genistein behaved as a weak attractant to *B. japonicum*.

Chemotaxis induced by ureases is also not species-specific, as jack bean urease also attracted *B. japonicum*, and in the same micromolar concentration range observed for lectins. It is not known if ureases are present in root exudates. However, it is plausible to think of a gradient of urease originating from death/shedding of root (or root cap) cells, with concentrations decreasing with distance from roots. Chemotaxis of *B. japonicum* toward soybean urease may be indirectly implicated in the nodulation process, directing the bacteria toward the roots. On the other hand, GST and BSA stimulated chemotaxis of *B. japonicum* similarly to soybean lectin. Previously, Sytnikov et al.[46] reported that incubating *B. japonicum* with the nonplant protein human serum albumin (at 0.4 μM), resulted in soybean plants with increased nodule nitrogen-fixing activity.

The Boyden chamber allowed easy assay of migration after 1 h of simultaneous exposure to multiple attractants, within the same time frame as the Pfeffer's capillary tube method.[47] Both methods are much faster than the swarm plate assay,[48] for which migration is measured after 48 h. Only the bacteria that crossed the 0.2 μm filter and were recovered in the liquid medium in the opposite chamber were plated for CFU analysis.

To the best of our knowledge, there are very few published studies that evaluated the role of ureases in the symbiosis between plants and microorganisms. Toffanin and co-workers[49] used a *Rhizobium leguminosarum* strain mutated in the *ureE* gene to show that urease activity is not important for biological nitrogen fixation in pea. This mutation prevents the proper formation of the urease active site, since in the absence of accessory protein UreE, there is no incorporation of Ni^{2+} into the apoenzyme.[39] However, this mutation does not affect the synthesis of apoenzyme(s), which could be contributing to the process of nodulation independently of ureolytic activity.

In our study, we used urease-deficient mutants of soybean as well as *B. japonicum*. In agreement with the study by Toffanin and co-workers,[49] who concluded that the urease of *R. leguminosarum* was not implicated in nodulation, our data showed that neither *B. japonicum* apo-urease (in Δ*ureG*) nor active *B. japonicum* urease affected nodulation in soybean.

In contrast, we showed that in *eu1-a,eu4* soybean plants, nodulation by *B. japonicum* is compromised, yielding higher mass nodules in a reduced number with no gross alteration in morphology. This double mutant does not synthetize eSBU (*eu1* null allele[11]) and produces an enzymatically inactive uSBU.[16] Two missense point mutations *eu4* alleles have been described (G468E in the *aj3*/*aj3* allele and R201C in the *aj6*/*aj6* allele, now termed *eu4-a* and *eu4-b*, respectively[16]). Both changes are radical as all bacterial, fungal, and plant urease sequences showing significant alignments with uSBU have the wild-type Arg201 and Gly468 residues at these positions. Modeling of either mutated uSBU showed that the affected amino acids occur at different points of the surface of the protein likely contributing contacts at the interfaces of the urease trimer. Thus, both amino acids are probably important for maintaining urease structure and/or function.[16] It has been shown that leaves of *eu4*(*aj3*) plants produce 40% the wild-type urease antigen,[22] consistent with instability of the mutant uSBU in *eu4*(*aj3*).

It can be argued that mutagenesis to derive the *eu4* mutation resulted in an altered genetic background, influencing nodule size and number. Arguing against a "background" effect on nodule size/number, we employed two different *eu1-a,eu4* double mutants, each with a different *eu4* mutant allele and hence each derived from a separate mutagenic event.

Torisky and Polacco reported that the eSBU is abundant in roots of 4 d old soybean plantlets, both in the wild-type Williams 82 or in the *eu4*(*aj3*) mutant plant, with specific activities at the root/shoot junction region of about 1500 and 3000 units per milligram of protein, respectively.[10] In roots of 4 d old *eu1-a*/*eu1-a* plants, which produce only uSBU, the specific urease activity reached 20 units per milligram of protein. The eSBU found in the young radicles is derived from the cotyledons, and its levels in the roots decline as the plant grows, but 5% of the 4 d value still remains after the third week of development.[10] In our work, *B. japonicum* was inoculated in soybean seedlings 48 h after germination. Thus, the deleterious effects we observed in the double mutant *eu1-a,eu4* plants could be due to the absence of the eSBU in the initial phase of root development, or reflect the presence of a mutated uSBU, or be a sum of both traits. We did not measure the content of urease protein in the roots of the double mutants *eu1-a,eu4* plants.

We assessed the ability of nodules to fix nitrogen by quantifying LegHb. The presence of this protein in the nodules is connected to nitrogenase activity, which is extremely sensitive to O_2; a function of LegHb is to maintain a low O_2 environment inside the nodules.[50] Hence, the LegHb content is an indirect measure of nitrogenase activity and hence total biological nitrogen fixation at a given developmental stage.

The time course of LegHb accumulation was altered in nodules of soybean *eu1-a,eu4* plants. Mutation in both plant urease genes delayed the LegHb peak from 21 d in the wild plant to 35 d in mutant plants, independent of the bacterial strain, implicating a role of the plant ureases in the soybean−*B. japonicum* symbiosis.

In previous studies[31], we observed no changes in total protein content or ureides in inoculated soybean plants, treated or not treated with PPD. Accordingly, we reported here that the nodulation process was also not affected in inoculated wild-type soybean plants grown in the presence of PPD. These results indicate that any possible role of urease on nodulation would be independent of ureolytic activity, either from the plant or from the bacterial enzymes.

Taken together, our results show that a deficiency (quantitative and/or qualitative) of soybean ureases alters the physiology of the nodules with consequent changes in nitrogen fixation. In addition, altered urease levels/structure may potentially interfere with chemotactic signals triggered by soybean plants in the rhizosphere. None of these processes apparently involves the bacterial urease.

The data shown here represent a new approach in studies of ureases. The multifunctionality of this protein and the lack of consensus about its physiological function(s) make it important to develop studies that go beyond considering urease as solely a ureolytic enzyme, expanding information to understand the conservation of this macromolecule during evolution and its presence in the most diverse organisms.

■ ASSOCIATED CONTENT

Supporting Information

Figures showing the ureolytic activity of wild-type and urease mutants of *B. japonicum* and transmission electron micrographs of nitrogen-fixating nodules from soybean plants; table showing LegHb content of soybean nodules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.M.-S., M.P.B., and S.B.P. produced soybean ureases and collected data on chemotaxis, nodulation, and leghemoglobin; W.F. constructed *B. japonicum* mutants; K.N.S., D.W.E., and G.S. helped to design nodulation and leghemoglobin experiments; J.P.C. developed the mutant soybean lines; J.C.P. and C.R.C. conceived and supervised all phases of the work.

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Notes

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■ ABBREVIATIONS USED

BNF, biological nitrogen fixation; eSBU, embryo-specific urease; uSBU, soybean ubiquitous urease; JBU, jack bean urease; PCR, polymerase chain reaction; GST, gluthatione-S-transferase; PBS, phosphate-buffered saline; PPD, phenylphosphoro-diamidate; CFUs, colony-forming units; *p*-HMB, *p*-hydroxymercuribenzoate; SBA, soybean agglutinin; BSA, bovine serum albumin; LegHb, leghemoglobin

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