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Photosynthetic *Bradyrhizobium* sp. Strain ORS285 Is Capable of Forming Nitrogen-Fixing Root Nodules on Soybeans (*Glycine max*)

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The ability of photosynthetic *Bradyrhizobium* strains ORS285 and ORS278 to nodulate soybeans was investigated. While the *nod* gene-deficient ORS278 strain induced bumps only on soybean roots, the *nod* gene-containing ORS285 strain formed nitrogen-fixing nodules. However, symbiotic efficiencies differed drastically depending on both the soybean genotype used and the culture conditions tested.

Aside from their ability to form nitrogen-fixing nodules on both the roots and stems of tropical aquatic legumes of the genus *Aeschynomene*, the photosynthetic bradyrhizobia exhibit several other remarkable features. First, their photosynthetic character, which is a rare property among rhizobia, contributes to symbiotic efficiency by providing energy to the bacteria that can be used for nitrogen fixation (1). Second, they are able to fix nitrogen in the free-living state (2). Third, they are able to develop a natural endophytic association with an African wild rice species *Oryza breviligulata* and this can be beneficial for plant growth and grain production (3). Lastly, these bacteria are able use a novel Nod factor (NF)-independent mechanism to interact symbiotically with a restricted number of *Aeschynomene* species (4).

Two specificity groups can be distinguished among the photosynthetic bradyrhizobia. Group I strains, including strain ORS285, contain the canonical *nodABC* genes found among other rhizobia and have a broad host range that extends to all stem-nodulated *Aeschynomene* species. In contrast, group II strains, including ORS278 and BTAi1, lack *nodABC* genes and are able to nodulate only a few species of *Aeschynomene*, including *A. sensitiva* and *A. indica* (4). Interestingly, *nod* gene-containing photosynthetic strains such as ORS285 are able to use both NF-dependent and NF-independent symbiotic processes, depending on the host plant nodulated (5).

The major NF synthesized by *Bradyrhizobium* sp. strain ORS285 is a pentameric lipochitooligosaccharide (LCO) with a 2-O-methylfucose at the reducing end. This is the same major NF synthesized by the nonphotosynthetic *Bradyrhizobium japonicum* USDA 110 strain that nodulates soybean (*Glycine max*) roots (6). It was recently shown that the *B. japonicum* strain USDA 110 is able to form N_2-fixing nodules on both the roots and stems of *A. afraspera*, an *Aeschynomene* species that requires NF to initiate symbiosis (6). Taken together, these data indicate that photosynthetic and nonphotosynthetic bradyrhizobia share some common symbiotic properties.

In this study, we examined the ability of photosynthetic *Bradyrhizobium* strains to establish a symbiotic relationship with soybean. Two photosynthetic *Bradyrhizobium* sp. strains were examined: the *nod* gene-lacking strain ORS278 and the *nod* gene-containing bacterium ORS285. Since several studies have shown that different soybean genotypes can differentially restrict nodulation by specific rhizobial strains, three cultivars were tested: Williams 82, Peking, and Lambert (7–10). Moreover, since host-controlled restriction of nodulation of soybean is temperature dependent, we examined the influence of plant incubation temperature on the symbiosis between the photosynthetic bradyrhizobia and soybeans (9).

The nodulation assays were performed in modified Leonard jar assemblies (11, 12) containing vermiculite and perlite (3:1). Each Leonard jar assembly was planted with two surface-sterilized seeds of *G. max* cultivar Williams, Peking, or Lambert in three hills, and the seedlings were thinned to one seedling of each genotype per hill 3 days after emergence. Seedlings in five replicate Leonard jars were inoculated with 1.0 ml (about 1×10^7 cells) of an AG-grown (12), stationary-phase culture of *Bradyrhizobium* strain ORS278, ORS285, or USDA 110. Noninoculated plants served as negative controls, and plants inoculated with *B. japonicum* strain USDA 110 served as positive nodulation controls. After inoculation, the seeds were covered with vermiculite and a 1-cm layer of sterilized paraffin-coated sand and watered with Keyser's nitrogen-free nutrient solution (13). Plants were grown in growth chambers under two different temperature conditions: (i) a high-temperature condition of 28°C for 16 h (light)/23°C for 8 h (dark) and (ii) a low-temperature condition of 20°C for 16 h (light)/15°C for 8 h (dark). The plants were harvested 35 days after inoculation. Nodule number and dry mass, as well as nitrogen fixation as measured by the acetylene reduction assay (ARA), were determined as previously described (14).

Results in Table 1 show that the *nod* gene-containing ORS285 strain formed bona fide nodules on the three *G. max* varieties tested under high-temperature (28°C) conditions that looked like nodules elicited by strain USDA 110 (Fig. 1A and B). However, the numbers of nodules, their sizes, and their nitrogen fixation efficiencies differed drastically depending on both the soybean genotype and the culture conditions tested (Table 1). The most effective symbiosis was obtained with soybean cv. Peking, where the

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TABLE 1 Nodulation responses of photosynthetic bradyrhizobia and *B. japonicum* USDA 110 on three soybean cultivars

Culture condition	Inoculant	Nodulation response on[a]: *G. max* cv. Williams			*G. max* cv. Peking			*G. max* cv. Lambert		
		No. of nodules	Nodule fresh mass (mg)	ARA[b]	No. of nodules	Nodule fresh mass (mg)	ARA	No. of nodules	Nodule fresh mass (mg)	ARA
28°C[c]	USDA 110	10 ± 2.3	83.2 ± 23.1	94 ± 0.02	3 ± 1	38.4 ± 2.4	48.0 ± 7.7	8 ± 1.4	11.5 ± 2.4	16.6 ± 2.9
	ORS285	24 ± 11	89.5 ± 24.1	4.1 ± 2.5	13 ± 4	52.5 ± 8.2	41.1 ± 12.5	28 ± 2.1	18.3 ± 5.6	0.06 ± 0.02
	ORS278	0	0	0	0	0	0	0	0	0
	NI[d]	0	0	0	0	0	0	0	0	0
20°C[e]	USDA 110	31 ± 11.4	39.3 ± 7.2	76.8 ± 7.8	26 ± 10	135.3 ± 13.7	123.6 ± 12	9 ± 2.1	44.3 ± 3.4	95.2 ± 15.6
	ORS285	0	0	0	37 ± 10	80 ± 11.1	75.3 ± 6.6	0	0	0
	ORS278	0	0	0	0	0	0	0	0	0
	NI	0	0	0	0	0	0	0	0	0

[a] Values are the means of the results of five replicates ± standard errors. Numbers of nodules and mass values (in mg) are per plant.
[b] ARA values are expressed in μmol C_2H_4/produced/h/plant.
[c] High temperature: 28°C for 16 h (light)/23°C for 8 h (dark).
[d] NI, not inoculated.
[e] Low temperature: 20°C for 16 h (light)/15°C for 8 h (dark).

ARA activity measured was comparable to that obtained with strain USDA 110.

We took advantage of the ability of the ORS285 strain to stably maintain a plasmid harboring the green fluorescent protein (GFP) gene to analyze colonization of cv. Peking nodules by the bacteria (5). Confocal microscopy revealed that the central tissue accumulated a brown pigment that autofluoresced in the red spectrum (excitation, 488 nm; emission, 600/660 nm) under high-temperature culture conditions (Fig. 1D and G). This is similar to the accumulation of polyphenol compounds that are generally associated with plant defense responses (15). In contrast, these plant defense-like reactions were never observed under low-temperature culture conditions—the central tissue was completely invaded by bacteria, indicating that the symbiotic interaction was compatible (Fig. 1H). Unexpectedly, although infection threads could be seen, extensive intercellular proliferation of the bacteria was also observed in a few nodules (Fig. 1I and J). This dual infection of soybean nodules, to our knowledge, has never been described before. This suggests that intercellular and intracellular infection processes in soybean can simultaneously occur in rare cases.

In contrast, the *nod* gene-lacking strain ORS278 failed to induce nodules on any of the tested *G. max* cultivars, regardless of the incubation temperature (Table 1). These data substantiate previous results indicating that Nod factors are absolutely required for the establishment of nitrogen-fixing root-nodule symbioses with soybean (16). However, under the two temperature conditions tested, small bumps were generally produced at the emergences of lateral roots on all three cultivars (Fig. 1C). Cytological analysis showed that these structures were devoid of bacteria (data not shown). They could have resulted from the bacterial synthesis of phytohormones, such as auxin and cytokinin, which have previously been shown to play essential roles in the control of cell proliferation during nodule formation (17–19).

Electron microscopic analyses of the nodules formed on cv. Peking by strains ORS285 and USDA 110, grown under low-temperature culture conditions, showed that USDA 110 bacteroids accumulated a large quantity of poly-β-hydroxybutyrate (PHB) granules, whereas it was rare to observe such granules in ORS285 bacteroids (Fig. 1K and L). Strain ORS285 does not lack the enzymatic activity necessary for the synthesis of PHB, since this storage material accumulates in this strain during symbiosis with *Aeschynomene* (5). The symbiosis-specific accumulation of PHB may be due to bacteroid metabolic differences that occur in different host backgrounds, or may reflect overall differences in carbon flow to nodules.

Taken together, these data demonstrate that the *nod* gene-containing photosynthetic *Bradyrhizobium* strains can establish an effective symbiotic relationship with *G. max*, but this occurs only with specific *G. max* genotypes and under specific temperature conditions. For example, strain ORS285 was able to nodulate the three soybean cultivars under high-temperature (28°C) conditions, but elevated nitrogen-fixing activity was observed only on soybean cv. Peking. Furthermore, only this cultivar formed nodules at 20°C. It has long been known that several host *Rj* genes control nodulation specificity in soybeans (10, 20), and some of these genes interact with bradyrhizobia in a temperature-dependent manner (9). The presence/absence or the dominance/recessiveness of one of these determinants could explain the symbiotic differences observed between the varieties tested. The pattern of flavonoids exuded by soybean roots is also a parameter to take into consideration. Indeed, it has previously been shown that the families of flavonoids that induce *nod* genes are greatly different between ORS285 and USDA 110 (6). It is possible that only *G. max* cv. Peking exudes the appropriate flavonoids at the two temperatures tested that can induce expression of the *nod* genes in ORS285. Temperature could also greatly impact the growth of the bacteria in the rhizosphere or modulate the pattern of glycoconjugates associated with the bacterial cell-wall, greatly influencing the response of the plant to invasion. Further studies are therefore necessary to completely understand what restricts the establishment of the symbiotic relationship between photosynthetic bradyrhizobia and soybeans.

Despite this lack of knowledge, however, we have already taken advantage of efficient nodulation of soybean cv. Peking by strain ORS285 to make progress in our understanding of a novel symbiotic interaction between this bacterium and soybeans. Indeed, a large amount of genomic information, as well as genetic tools, is

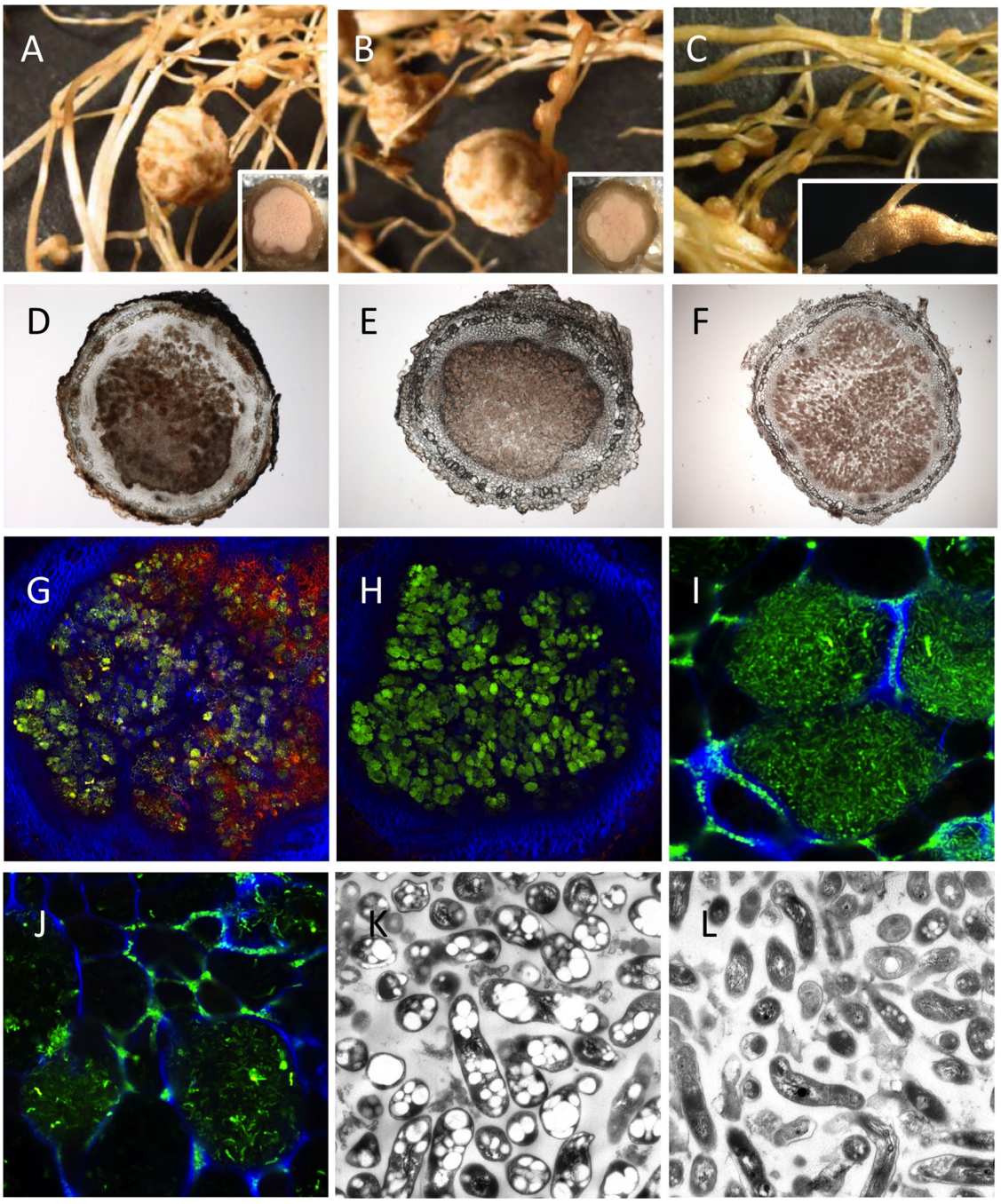

FIG 1 Symbiotic interaction between *Bradyrhizobium* strains USDA 110, ORS278, and ORS285 and *Glycine max.* (A to C) Response of *G. max* cv. Peking grown at low temperature (20°C) and inoculated with *B. japonicum* strain USDA 110 (A) and *Bradyrhizobium* sp. strains ORS285 (B) and ORS278 (C). Insets in panels A and B show a cross section of the nodules produced. The pink color in the central tissue is due to leghemoglobin and is an indicator of nitrogen-fixating efficacy. The inset in panel C shows, at higher magnification, the bumps elicited by strain ORS278. Panels D and E show cross sections of a nodule formed by ORS285 on *G. max* cv. Peking cultivated at high (D) and low (E) temperatures. Note in panel D the accumulation of brown pigment in the central tissue, similar to what is seen in plant defense reactions. Panel F shows a cross-sectional view of a nodule formed by USDA 110 on *G. max* cv. Peking cultivated at high temperature. Panels G through J are confocal microscopic images of the nodules formed by ORS285 on *G. max* cv. Peking cultivated at high temperature (G) and low temperature (H, I, and J). Panels I and J are higher magnifications of panel H and demonstrate that the nodule tissue is infected both intercellularly and intracellularly. Panels K and L show representative transmission electron micrographs of infected cells of mature nodules on *G. max* cv. Peking elicited by USDA 110 (K) and ORS285 (L) cultivated at low temperature. Note the accumulation of poly-ß-hydroxybutyrate granules (light areas) in USDA 110 bacteroids. Microscopic observations were performed on 30-to-40-μm-thick vibratome (Leica VT1000S) sections from fresh nodule samples using a fluorescence stereo microscope (Nikon AZ100) (D to F), a confocal laser scanning microscope (Carl Zeiss LSM 700) (G to J), or a transmission electron microscope (Jeol 100CX II) (K and L).

now available concerning the photosynthetic bradyrhizobia. In particular, a plasmid that originated from *Rhodopseudomonas palustris* is stably maintained in ORS285 (5, 21). Thanks to this plasmid, into which we introduced the GFP-encoding gene, it was possible to show in this study that intercellular invasion of soybean nodules could occur, although at a low frequency. To date, making "bright" GFP-expressing soybean bradyrhizobia has been difficult, and this likely limited past observations. Thus, one important conclusion from this study is that the photosynthetic *Bradyrhizobium* strains can be used to provide complementary data to address important functional questions concerning the soybean symbiosis. This will likely advance our understanding of this agronomically and biologically important symbiotic system.

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