Characteristics of Bacteroids in Indeterminate Nodules of the Leguminous Tree Leucaena glauca

HIRONOBU ISHIHARA\textsuperscript{1}, HIROKI KORIYAMA\textsuperscript{2}, ATSUSHI OSAWA\textsuperscript{2}, GRIGOR ZEHIROV\textsuperscript{1}, MASATOMI YAMAURA\textsuperscript{1}, KEN-ICHI KUCHO\textsuperscript{1}, MIKIKO ABE\textsuperscript{1}, SHIRO HIGASHI\textsuperscript{2}, EVA KONDOROSI\textsuperscript{1,3}, PETER MERGAERT\textsuperscript{3}, and TOSHIKI UCHIUMI*\textsuperscript{4}

\textsuperscript{1}Graduate School of Science and Engineering, Kagoshima University, 1–21–35 Korimoto, Kagoshima 890–0065 Japan; \textsuperscript{2}Faculty of Science, Kagoshima University, 1–21–35 Korimoto, Kagoshima 890–0065 Japan; \textsuperscript{3}Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France; and \textsuperscript{4}Institute for Plant Genomics, Human Biotechnology and Bioenergy, Bay Zoltan Foundation for Applied Research, 6726 Szeged, Hungary

(Received January 14, 2011—Accepted February 21, 2011—Published online March 25, 2011)

Rhizobia establish symbiosis with legumes. Bacteroids in indeterminate nodules of Inverted Repeat Lacking Clade (IRLC) legumes undergo terminal differentiation caused by Nodule-specific Cysteine-Rich peptides (NCRs). Microscopic observations of bacteroids and the detection of NCRs in indeterminate nodules of the non-IRLC legume Leucaena glauca were performed. A portion of the bacteroids showed moderate cell elongation, loss of membrane integrity, and multiple nucleoids. The symbiosome contained multiple bacteroids and NCR-like peptides were not detectable. These results indicate that bacteroid differentiation in L. glauca is different from that in IRLC legumes although both hosts form indeterminate nodules.

**Key words:** Leucaena glauca, Bradyrhizobium, bacteroid, nodule

Root nodules of leguminous plants are formed with soil bacteria belonging to the *Rhizobaceae* (rhizobia). Rhizobia are released into the cytoplasm of nodule cells from infection threads via an endocytosis-like process, resulting in organelle-like structures called symbiosomes containing one or more bacteria enclosed by a peribacteroid membrane of plant origin (5). In the symbiosome, rhizobia differentiate into an endosymbiotic form, bacteroids, and fix atmospheric nitrogen to produce ammonia.

The morphology of nodules can be categorized into two major types, determinate and indeterminate. Indeterminate nodules have a tip-localized persistent meristem, resulting in a continuously growing nodule with a cylindrical shape. The internal structure of an indeterminate nodule is divided into four zones; the meristem (zone I), the infection zone (zone II), the nitrogen-fixing zone (zone III), and the senescence zone (zone IV). Nodule cells in zone II, where rhizobia are released from infection threads, differentiate by endoreduplication-driven cell enlargement (16). Leguminous plants belonging to the Inverted Repeat Lacking Clade (IRLC) (17) form indeterminate nodules. Symbiosomes in nodule cells of IRLC legumes are occupied by single bacteroids. Bacteroids in nodules of IRLC legumes show remarkable characteristics such as enlargements of cell size, genome amplification, and loss of membrane integrity and reproductive capacity (9). This terminal bacteroid differentiation takes place in nodule zone II and is more advanced on the basal side (zone III and IV) than tip side (zone II) of the nodule (9).

*Lotus* (belonging to the robiniioid clade), *Glycine, Phaseolus* (belonging to the millettioid clade) (17), and other leguminous species form determinate nodules. Determinate nodules have no meristem and are spherical in shape. The symbiosomes are occupied by multiple bacteroids. No significant morphological differentiation is detectable in the bacteroids of determinate nodules which are reversibly differentiated as they maintain their capacity for cell division (9).

Leguminous woody plants and actinorhizal plants are key pioneer species in ecological succession and have a variety of uses (6, 7, 13, 18). *Leucaena glauca* is a leguminous tree belonging to the mimosoid clade which branches early on the phylogenetic tree of legumes (17). Thus, studying the symbiosis of *L. glauca* is worthwhile not only for molecular and physiological research but also for an evolutionary insight of legume-rhizobium symbiosis. However, bacteroid differentiation in *L. glauca* has not been characterized in detail. In this study, bacteroids of *L. glauca* nodules were compared with those of nodules of *Lotus japonicus* and Medicago sativa. Nodule-specific peptides were also analyzed by SDS polyacrylamide gel electrophoresis.

Seeds of *L. glauca* were soaked in sulfuric acid for 30 min and in 0.2% sodium hypochloride with 0.1% Tween 20 for 40 min. They were washed with sterilized water and germinated on 0.8% agar plates. Within a week, seedlings were transferred to expanded vermiculite with B & D medium (2) and inoculated with the microsymbiont *Bradyrhizobium* sp. OK-79A (MAFF210194). Acetylene reduction activity (ARA) as nitrogen-fixing activity of nodules was measured at 3, 4, 5 and 6 weeks after inoculation. Because ARA almost reached a plateau at 4 weeks after inoculation (data not shown), the same as in nodules of *M. sativa* and *L. japonicus*, the nodules were harvested as mature nodules at 28 days after inoculation and used for further investigation. The nodules were fixed with 2.5% glutaraldehyde, embedded in 5% water agar, and cut into 20-µm sections. Infected rhizobia in the nodule cells were stained with 0.01% toluidine blue in 5% perchloric acid and observed under a light microscope.

* Corresponding author. E-mail: uttan@sci.kagoshima-u.ac.jp; Tel: +81–99–285–8164; Fax: +81–99–285–8163.
blue and observed by light microscopy. Additionally, transmission electron microscopy was performed as described previously (4). The nodules of L. glauca were cylindrical (Fig. 1A) with a meristem at their tip (Fig. 1A and 1D, zone I), the same as the indeterminate nodules of M. sativa (Fig. 1B and 1E) but not the determinate nodules of L. japonicus (Fig. 1C). These observations clearly indicate the nodules of L. glauca to be indeterminate. However, the tissue organization was unlike that of the indeterminate nodules of IRLC legumes.

A zone structure was clearly evident in the M. sativa nodules (Fig. 1B and 1E) and the size of infected cells increased from zone I to zone III. In zone III, the major axis of infected cells was significantly (1.91 times) longer than that of uninfected cells (Fig. 1E). In the nodules of L. glauca, the zone structure was unclear (Fig. 1A asterisk) and the infected cells were almost the same size (0.93 times the length of the major axis) as uninfected cells (Fig. 1D). Moreover, electron microscopy of the central area of the nodules revealed that the symbiosomes were occupied by multiple bacteroids (Fig. 1F) similar to those in determinate nodules of L. japonicus (Fig. 1G). Together, this indicates that the morphology of the indeterminate nodules of L. glauca is different from that of IRLC legumes.

To characterize further the morphological differentiation of bacteroids, nodules of L. glauca were cut into two, a tip and a basal part. Each part was crushed in bacteroid extraction buffer (125 mM KCl, 50 mM Na-succinate, and 50 mM TES, pH 7.0) with 1% BSA. To remove the debris of plant cells, the suspension was centrifuged at 100 × g for 10 min. The supernatant was centrifuged at 3,000 × g for 10 min to precipitate the bacteroids. The precipitate was used as a bacteroid fraction and was observed with a differential interference contrast (DIC) microscope. The DIC images showed that bacteroids isolated from the basal part of the L. glauca nodules were elongated more extensively than those isolated from the tip. Only 20% of the bacteroids were longer than 3 µm in the tip, compared to 60% in the basal area (Fig. 2). This indicates that the microsymbiont of L. glauca changes morphologically in the nodule cells.

The morphological and cytological characteristics of the bacteroids of L. glauca were compared to those of the M. sativa and L. japonicus bacteroids. The bacteroids isolated from individual whole nodules were stained with 4',6-diamidino-2-phenylindole (DAPI) (50 µg mL−1) and propidium iodide (PI) (2 µg mL−1), and observed with the DIC and a fluorescence microscope. Although the bacteroids varied in size from 1 to 5 µm (Fig. 3), almost 90% were shorter than 4 µm in L. glauca, almost 75% were longer than 4 µm in M. sativa, and no bacteroid was longer than 4 µm in L. japonicus (Fig. 3 and Fig. 4A). Fluorescence images of the DAPI-stained bacteroids in L. glauca showed that some contained multiple fluorescent particles indicating polyploidy, but the intensity and frequency were low (11.4% in the bacteroids observed) (Fig. 3). Almost all of the bacteroids were polyploids in M. sativa and no polyploid bacteroid was observed in L. japonicus (Fig. 3). Fluorescence images of the PI-stained bacteroids showed that 19% had lost membrane integrity in the nodules of L. glauca, 8% in L. japonicus, and 46% in M. sativa (Fig. 4B). These results indicate that the morphological and cytological changes to bacteroids in nodules of L. glauca were milder than those in IRLC legumes.
Ishihara et al. 158

Legumes.

In Medicago truncatula (an IRLC legume), genes of the Nodule-specific Cysteine-Rich peptide family (NCRs) are strongly and specifically expressed in nodules (1, 8, 15). NCRs resemble antimicrobial peptides (1, 8) and were demonstrated to be the plant factors that induce terminal bacteroid differentiation (15). So far, NCR genes have been found only in IRLC legumes suggesting that terminal bacteroid differentiation induced by NCRs is unique to IRLC legumes.

To investigate the product ion of peptides similar to NCRs in L. glauca nodules, Tricine SDS-polyacrylamide gel electrophoresis and silver staining were performed. Soluble protein fractions were prepared from roots, nodules, bacteroids and cultured rhizobia as described previously (15), purified by ultrafilter Amicon Ultra-0.5 Ultracel-100 (Millipore, Carrigtwohill, Cork, Ireland), and analyzed by Tricine SDS-polyacrylamide gel electrophoresis (12, 15). Silver staining was performed as reported (14) with a minor change (the oxidation step with periodic acid was skipped).

In the proteins prepared from nodules and bacteroids of M. sativa, NCRs were detected at 5 kDa with strong intensity as in a previous report (15), whereas no bands were detected in the roots of M. sativa, in cultured Sinorhizobium meliloti, and in any tissues of L. glauca (Fig. 5). We can not rule out the presence of small amounts of NCR-like peptides. However, small peptides do not seem to act on bacteroid differentiation in the nodules of L. glauca, differ-

Fig. 3. Microscopic observation of rhizobia. The cultured bacteria and bacteroids were stained with DAPI/PI and observed with a DIC and fluorescence microscope. Culture, cultured bacteria; Bacteroid, bacteroids isolated from nodules; DIC, DIC micrographs; DAPI, fluorescent micrographs of DAPI-stained bacteria; PI, fluorescent micrographs of PI-stained bacteria. Arrowheads indicate multiple nucleoids in a bacteroid cell. Bars = 4 µm.

Fig. 4. Distribution of bacteroids in size (A) and the ratio of PI-positive bacteroids (B). All nodules were harvested at 28 days after inoculation. Data represent the average for three independent experiments with the standard error. One hundred bacteroids were observed in each experiment.

Fig. 5. Analysis of nodule-specific peptides. Soluble proteins were extracted from tissues or bacterial cells and 3 µg of protein was analyzed. The proteins in the gels were detected by silver staining. NCRs in lane Ms are indicated by asterisks. No stained band is detectable in lane Lg. Ms, M. sativa; Lg, L. glauca; Sm, S. meliloti; Br, Bradyrhizobium sp. OK-79A.
ent from *M. sativa*.

In summary, the differentiation of host nodule cells of *L. glauca* was not marked, in contrast with the substantial enlargement of the symbiotic cells in nodules of IRLC legumes (3, 16). In addition, the distribution of size and the ratio of polyploids and PI positive-bacteroids in indeterminate nodules of *L. glauca* were different from those in nodules of IRLC legumes, although bacteroids in *L. glauca* undergo morphological and cytological changes. In IRLC legumes, single bacteroids are present in symbiosomes, which may be caused by the bacterial cytokinesis-inhibiting function of NCRs (8, 15). In *L. glauca*, multiple bacteroids were observed per symbiosome the same as in *L. leucocephala* (11). This feature is identical to that in determinate nodules (15) and consistent with our finding that NCR-like peptides were not present in *L. glauca* nodules and bacteroids. Thus, the symbiotic features in *Leucaena* nodules are ‘intermediate’ between those of the indeterminate nodules of *M. sativa* and determinate nodules of *L. japonicus*. Legumes have developed several strategies to control and dominate the endosymbiotic rhizobia (10). In IRLC legumes, NCR peptides induce extreme bacteroid differentiation. In other legumes like *Lotus*, *Glycine* and *Phaseolus*, no significant morphological differentiation is detectable and the host-plant factors involved in bacteroid differentiation have yet to be identified. *Leucaena* bacteroids show a moderate but distinguishable differentiation phenotype and small peptides may not function properly. These features may be helpful to identifying new plant molecules that induce bacteroid differentiation.

**Acknowledgements**

*Bradyrhizobium* sp. OK-79A (MAFF210194) was provided by the National Institute of Agrobiological Sciences (NIAS) Genebank. Work in our laboratories is supported by the French Agence Nationale de la Recherche, grant ANR-09-BLAN-0396-01, by the Hungarian National Office for Research and Technology, grants OMFB-00441/2007 and OMFB-00128/2010, and by the Japan Society for the Promotion of Science.

**References**