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Short Communication

Lotus japonicus Nodulates and Fixes Nitrogen with the Broad Host Range *Rhizobium* sp. NGR234

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***Lotus japonicus* possesses major advantages as a model legume for the study of plant-microbe interactions. The relative absence of genetic information on its normal microbial partner (i.e., *Mesorhizobium loti*) could limit its utility in research. Here we show for the first time that the broad host range *Rhizobium* strain NGR234 nodulates and fixes nitrogen in symbiosis with *Lotus japonicus* ecotypes "Gifu" and "Funakura". We demonstrate that bacterial mutants deficient in nodulation or nitrogen fixation possess the expected phenotype with *L. japonicus*. Nodulation of *L. japonicus* was sensitive to nitrate. Vermiculite was an efficient synthetic growth substrate, allowing axenic growth in Magenta jars. The genetic analysis of the *Lotus japonicus-Mesorhizobium* interaction should be accelerated through the use of this well-defined microsymbiont.**

Key words: DAF — ERIC — Genetics — Legume — Nitrogen fixation — Nodulation — Symbiosis.

It is clear that genetic analysis of symbiotic nitrogen fixation is essential to achieve a clearer understanding of structure-function relationships. The genetics of bacteria have revealed many integrated processes, by which plant development and function are channeled (Long 1996). Likewise, plant genetics, through induced or natural mutants, have revealed many mechanisms, such as the systemic control of nodulation through the shoot (Carroll et al. 1985, Gresshoff 1993) or the involvement of ethylene in infection control (Penmetsa and Cook 1997). Molecular biology helped through the description of novel proteins (called nodulins) which function in the nodule environment (Legocki and Verma 1979).

The significance of coupling genetics to plant physiology and development analysis is well illustrated in *Arabidopsis thaliana*. Unfortunately, this crucifer is unable to nodulate and fix nitrogen (Kolchinsky et al. 1994). As a member of the brassica family, it also lacks mycorrhizal associations (Wegel et al. 1998). These reasons, and the concomitant problems with existing crop legumes in terms of large genome sizes (e.g., 1×10^9 bp for soybean), the high degree of repeated DNA sequences, and difficulty of high efficiency transformation, have led to the acceptance of model legumes such as *Lotus japonicus* (Jiang and Gresshoff 1997, Handberg and Stougaard 1992) and *Medicago truncatula* (Pentmetsa and Cook 1997). The latter is particularly attractive because of the extensive bacterial genetics available in *Sinorhizobium meliloti*. In contrast, *L. japonicus* was deemed less attractive, because of the limited genetics of *M. loti*.

L. japonicus has genetic, biological and experimental advantages (Handberg and Stougaard 1992, Jiang and Gresshoff 1997). It is a true diploid (n=6) with a small genome (about 400 Mb per haploid genome), has a short generation time, large self-fertile flowers, large number of small seeds per pod, is easy to cross sexually, and is easily transformed by *Agrobacterium tumefaciens* and *A. rhizogenes* facilitating insertional mutagenesis and gene tagging (Thykjær et al. 1995, Stiller et al. 1997, Schauser et al. 1998, Oger et al. 1996). A skeletal molecular map containing arbitrary and microsatellite markers allowing the mapping of symbiotic mutations is available (Jiang et al. 1999). For example the hypernodulation and altered root mutant, *har-1* (Szczyglowski et al. 1998) was mapped to linkage group 2 close to two DNA amplification finger printing (DAF) markers (Jiang et al. 1999).

The fast-growing *Rhizobium* NGR234 strain nodulates more than 110 genera of legumes, as well as the non-legume Parasponia (Pueppke and Broughton 1999). Its 536-kb symbiotic plasmid pNGR234a (Perret et al. 1994, Freiberg et al. 1997) including most nodulation and nitrogen fixation genes, as well as novel type III secretion system genes (Viprey et al. 1998), has been entirely sequenced. The chemical structures of NGR234 synthesized lipo-oligosaccharide nod-factors are known, and sufficient amounts for physiological experiments are available (Price et al. 1992, Jabbouri et al. 1998). Large numbers of symbiotic mutants, as well as those altered in related metabolism (such as surface polysaccharide biosynthesis) have been isolated and characterized.

Here we demonstrate that this broad host range

Abbreviations: ARA, acethylene reduction assay; DAF, DNA amplification finger printing; ERIC, Enterobacterial repetitive intergenic consensus.

Rhizobium strain nodulates *L. japonicus* effectively and efficiently and fixes nitrogen, as measured by acetylene reduction, to the same extent as *M. loti* (Lopez Lara et al. 1995, Sullivan and Ronson 1998), thereby removing a limitation to further genetic and physiological analysis of plant-microbe interactions in this symbiosis.

Bacterial strains—Rhizobium sp. NGR234 (Fix^+), NGR(*ΔfixF* (Nod^+, Fix^-); Jabbouri et al. 1996), NGR(*ΔnodABC* (Nod^-, Relić et al. 1994) and *Mesorhizobium loti* NZP2235 (a gift from Dr. Frans de Bruijn, Michigan State University, East Lansing, MI, U.S.A.) were grown at 28°C in YMB medium (Handberg et al. 1994) with antibiotics at 100 mg $liter^{-1}$ rifampicin for NGR234; at 100 mg $liter^{-1}$ each rifampicin and streptomycin for NGR(*ΔfixF*) (Lewin et al. 1990) and NGR(*ΔnodABC*), and no antibiotics for NZP2235.

Plants—Lotus japonicus ecotypes B-129-S9 "Gifu" and B-581 "Funakura" seeds were originally obtained from the University of Aarhus, Denmark (Dr. Jens Stougaard), and then propagated in our greenhouses. Both ecotypes were the ones used as the parents for our mapping population and derived recombinant inbred lines (RILs) (Jiang and Gresshoff 1997, Jiang et al. 1999).

Seed sterilization—Seeds of *L. japonicus* were scarified by gentle treatment with sand paper, then sterilized for 15 min in 3% H_2O_2 in 70% ethanol followed by five rinses with sterile water. The sterilized seeds were germinated on a wet filter paper pile in Petri-dishes in the dark, at 24°C for 1 d.

Plant media—Germinated seedlings were transferred to plastic cups and growth pouches containing different commercially available media with sterile B&D solution (1/4 strength; Broughton and Dilworth 1971) plus 2 mM KNO_3. Three days after transfer, 2 ml inoculant per plant was added (1×10^9 cells ml^{-1}). All plants were grown in the greenhouse with an 18/6 h day/night cycle and 24°C/18°C day/night temperature regime for 4 weeks. 1/4B&D solution was added on alternative days to compensate for depleted plant nutrients and liquid. Ten plants were grown for each strain and ecotype. A preliminary experiment with "Gifu" and "Funakura" in vermiculite using different rates of potassium nitrate (0, 0.5, 1, 2, 4, 6, 8 and 10 mM) showed that addition of 2 mM nitrate with B&D was required for *L. japonicus* as basal dose, which did not hamper nodulation (Fig. 1) and growth (data not shown). The effect on nitrogen fixation was not measured.

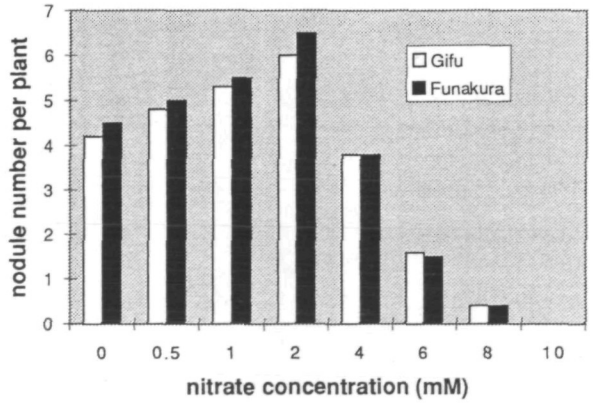

Fig. 1 Nodule numbers of *L. japonicus* as affected by different concentrations of nitrate (KNO_3, mM) in vermiculite after 4 weeks of seed germination.

After selecting the best medium for nodulation of *L. japonicus*, Magenta jars with 1/4B&D wetted vermiculite in the upper chamber and liquid 1/4B&D medium in the lower reservoir were used routinely for nodulation tests (Lewin et al. 1990).

Acetylene reduction assay—Plant roots with intact nodules were severed at the hypocotyl node and individually placed in 30 ml test tubes, sealed with a serum stopper, and 10 percent of the air was replaced with acetylene. Nodulated roots were incubated at room temperature and 1 ml subsamples were analyzed for ethylene production at 5 and 25 min after incubation by using a flame ionization gas chromatograph. After assaying, the nodules and roots were collected separately, weighed and used for further study.

Isolation and characterization of bacteria from nodules—The nodules were immerged in 90% ethanol for one min, then transferred to solution of hydrogen peroxide (5%), soaked for 5 min and washed three times with sterile saline solution (0.9%). Afterwards the nodules were crushed with a glass rod and diluted with saline solution. The suspension was streaked onto Yeast-mannitol agar plates containing antibiotics. Individual colonies were picked and tested for renewed nodulation ability on Magenta jar-grown "Gifu" and "Funakura" plants.

DNA isolation—DNA was extracted for 10 d old colonies the DNA of the isolates was extracted for DAF (Bassam et al. 1992). Bacterial genomic DNA was lysed by DNAzol (Chomczynski 1997) and the genomic DNA was precipitated from the lysate with 100% ethanol. Following a 95% ethanol wash, DNA was solubilized in water.

DAF and Enterobacterial Repetitive Intergenic Consensus (ERIC) analysis—DAF was used to evaluate the molecular genotype of parental and nodule isolates (Caetano-Anollés and Gresshoff 1994, Caetano-Anollés et al. 1991). The reaction mixture in a total volume of 10 ml contained 4 ng template DNA (2 ng μl^{-1}), 3 mM oligonucleotide primer (National Biosciences; Plymouth, MN, U.S.A.), 2 units Stoffel fragment polymerase (Perkin-Elmer, Emeryville, CA, U.S.A.), 0.2 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 10 mM KCl and 4 mM $MgSO_4$. The total reaction mixture was overlaid with a drop of heavy mineral oil and amplified in a thermocycler (Ericomp Inc., San Diego, CA, U.S.A.) for 35 cycles (using two-step

Table 1a Nodule number and total biomass production (dry matter) per plant in vermiculite medium

Strain	Gifu		Funakura	
	Nodule number	Dry matter (mg)	Nodule number	Dry matter (mg)
Control	0	18.8±0.6	0	34.8±1.0
NZP2235	5.2±0.4	15.6±0.7	5.8±0.4	31.4±1.4
NGR234	4.6±0.2	12.6±0.7	2.0±0.3	21.0±1.4
NGR*ΔfixF*	3.2±0.2	17.4±1.1	1.6±0.2	28.2±2.1
NGR*ΔnodABC*[a]	0	16.6±1.1	0	32.4±1.4

[a] Nod⁻ plants grew well because of limiting nitrate in the medium.

cycles of 1 s at 96°C and 1 s at 30°C, then one final cycle at 72°C for 5 min; heating and cooling rates were 23°C min^{-1} and 14°C min^{-1}, respectively). Amplification products were separated on 5% polyacrylamide gels (7 M urea), backed by Gel-Bond film (FMC, Rockland, ME, U.S.A.; for stability and later handling) by electrophoresis and silver stained (Bassam et al. 1991). Enterobacterial Repetitive Intergenic Consensus (ERIC) analysis (Versalovic et al. 1994) was performed on all parental material and nodule re-isolates to confirm the identity.

Growth medium selection and nodulation—M. loti NZP2235 and *Rhizobium* sp. NGR234 were used for nodulation of *L. japonicus* ecotypes "Gifu" and "Funakura" growing in different substrates. Significantly higher nodule numbers (about 6 per plant after 4 weeks of growth) and good plant growth (about 19 to 38 mg per plant dry weight) of both ecotypes were obtained in vermiculite medium. "Funakura" repeatedly grew faster than "Gifu", giving a plant mass nearly twice that of "Gifu" after 4 weeks of growth.

Plants could be grown and nodulated on sealed agar plates. However, roots exposed to light developed green nodules with low nitrogenase activity and delayed appearance (data not shown). We conclude that light exposure to roots is detrimental to *L. japonicus* nodulation.

Nodule numbers—In two chambered Magenta jars (being modified from Leonard jars), nodule number of both "Gifu" and "Funakura" did not differ when inoculated with NZP2235. In contrast, inoculation with two NGR234 strains [NGR234 (wild type) and NGR*Δfix* (fix⁻)], resulted in significantly lower nodule numbers in "Funakura" compared to "Gifu". There was a trend to decrease total biomass production when the plants were inoculated with either NZP2235 or NGR234 (Table 1a). Uninoculated plants, and those inoculated with NZP2235 showed no significant difference in root length, but showed a decrease when inoculated with NGR234 (Table 1b). This effect was independent of the nitrogen fixation capability of nodules.

Nitrate equally inhibited nodulation of "Gifu" and "Funakura" (Fig. 1). Plants inoculated with strain NZP2235 were grown in Magenta jars filled with vermiculite. Low levels of nitrate up to 2 mM increased nodule number per plant, presumably because of increased plant size. The nitrate inhibition curve was similar to that seen in soybean (Carroll et al. 1985). High nitrate (10 mM) completely eliminated nodulation and affected plant growth.

Nitrogen fixation and nodule occupancy—Specific ace-

Table 1b Shoot length (SL), root length (RL), and nodule fresh weight (NFW) per plant

Strain	Gifu				Funakura			
	SL (cm)	RL (cm)	NFW (mg)	Specific NFW (μg)	SL (cm)	RL (cm)	NFW (mg)	Specific NFW (μg)
Control	12.4±0.6	6.4±0.3	0	0	12.7±0.5	8.7±0.6	0	0
NZP2235	11.0±0.5	5.9±0.5	1.04±0.01	200	13.7±0.6	8.4±0.5	1.60±0.04	276
NGR234	11.3±0.6	4.8±0.4	1.63±0.01	354	11.5±0.8	6.2±0.4	0.83±0.01	415
NGR*ΔfixF*	12.8±0.7	5.0±0.4	0.97±0.02	303	12.6±0.6	5.8±0.3	0.19±0.00	369
NGR*ΔnodABC*	11.3±0.5	5.2±0.3	0	0	11.6±0.4	8.0±0.4	0	0

The nutrient solution (1/4 strength B&D medium with 2 mM nitrate) was changed every 2 d.
Mean±SE of 8–10 plants are shown.

Table 2 ARA of *L. japonicus*

ARA	Strain	Gifu	Funakura
Specific acetylene reducing activity[a]	NZP2235	34.5±0.7	7.1±0.1
	NGR	46.4±0.6	17.1±0.2
	NGR*ΔfixF*	0	0
Total acetylene reducing activity[b]	NZP2235	6.9±0.1	1.8±0.1
	NGR	11.6±0.3	5.7±0.1
	NGR*ΔfixF*	0	0

The data are means±SE of 8–10 plants.
[a] nmol C_2H_4 produced g^{-1} (FW) nodule h^{-1}.
[b] nmol C_2H_4 produced $plant^{-1}$ h^{-1}.

tylene reduction activity (ARA) and total ARA of "Gifu" was 2 to 4 times greater than that of "Funakura" in both NGR234 and NZP2235 inoculated plants (Table 2). There was no apparent explanation for this, but it is noteworthy in relation to the differential growth rate of "Gifu" always being slower than "Funakura" for the first 30–40 d after germination.

Genetic confirmation of NGR234 nodulation of Lotus japonicus—We tested Koch's postulates by colony re-isolation, testing, and re-infection. Results confirmed that strain NGR234 was the causative organism for *L. japonicus* nodules. Colonies derived from surface-sterilized nodules, grown on YMB plates, showed the same microbiological (growth rate, color, colony morphology, and antibiotic resistance profile) characteristics as those of the inoculant strains. DAF and ERIC analysis, using arbitrary primer technology or repetitive DNA sequences respectively, of the nodule isolates showed the same band pattern as the inoculant strains (Fig. 2).

The ability of strain NGR234 to nodulate and fix nitrogen in *L. japonicus* was demonstrated. Vermiculite provided the best and most easily attainable substrate for nodule and plant growth. This was a convenient alternative to the more optimal "pillow" system used by Szczyglowski

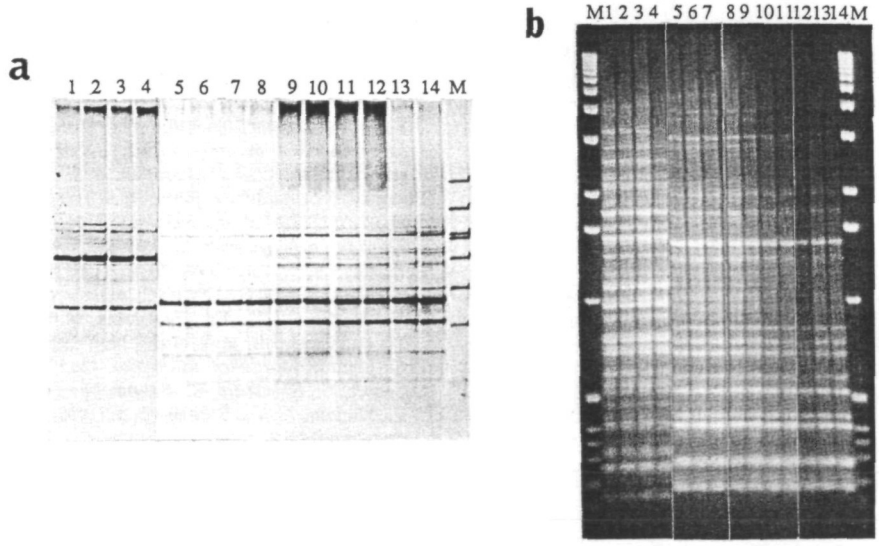

Fig. 2 DNA amplification (DAF) profile and ERIC analysis of *Lotus japonicus* nodule isolates and inoculant strains. A. DNA amplification profile of NZP2235, NGR234, NGR*ΔfixF* and NGR*ΔnodABC* using primer HpA41 (5'GCGAAAGCCCA3'). Lane 1, NZP2235; Lanes 2 to 4, Isolates from nodules inoculated with NZP2235; Lane 5, NGR234; Lanes 6 to 8, Isolates from nodules inoculated with NGR234; Lane 9, NGR*ΔfixF*; Lanes 10 to 12, Isolates from nodules inoculated with NGR*ΔfixF*; Lane 13 to 14, NGR*ΔnodABC*; Lane 15, Molecular weight marker. B. Box-PCR of NZP2235, NGR234, NGR*ΔfixF* and NGR*ΔnodABC*. Lane 1, NZP2235; Lanes 2 to 4, Isolates from nodules inoculated with NZP2235; Lane 5, NGR234; Lanes 6 to 8, Isolates from nodules inoculated with NGR234; Lane 9, NGR*ΔfixF*; Lanes 10 to 12, Isolates from nodules inoculated with NGR*ΔfixF*; Lane 13 to 14, NGR*ΔnodABC*; Lane M, Molecular weight marker.

et al. (1998).

We confirmed interesting differences of seedling growth rates (Jiang and Gresshoff 1997) between "Gifu" and "Funakura" and noted differences in nodule number per plant as well as specific and total nitrogen fixation rates. Specific ARA was similar to those measured for *M. truncatula* (Penmetsa and Cook 1997) and *Trifolium repens* (Carroll and Gresshoff 1983). All measurements, however, cannot be taken as absolute activities, as plants were not incubated in an open-flow system and may have been affected by trauma- or acetylene-induced oxygen barrier effects.

For optimal growth and nodulation of *L. japonicus* in the presence of *Rhizobium*, we suggest to use 2 mM nitrate as basal nitrogen supplement. "Gifu" developed more nodules with NGR234 than "Funakura".

The demonstration that strain NGR234 nodulates and fixes nitrogen with this model legume opens the entire genetic, chemical and microbiological aspects of this strain for further experimentation. Coupled with genetic approaches in the plant itself, we foresee a fertile period of analysis.

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