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NOTES

Expression of Symbiotic Genes of *Rhizobium japonicum* USDA 191 in Other Rhizobia†

EDWARD R. APPELBAUM,[1]* THOMAS J. McLOUGHLIN,[1] MICHAEL O'CONNELL,[2]‡ AND NICOLE CHARTRAIN[1]

Agrigenetics Advanced Research Division, Madison, Wisconsin 53716,[1] and Lehrstuhl für Genetik, Universität Bielefeld, 4800 Bielefeld 1, Federal Republic of Germany[2]

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A 200-megadalton plasmid was mobilized from *Rhizobium japonicum* USDA 191 to other *Rhizobium* strains either that cannot nodulate soybeans or that form Fix⁻ nodules on certain cultivars. The symbiotic properties of the transconjugants indicate that both soybean specificity for nodulation and cultivar specificity for nitrogen fixation are plasmid encoded.

Soil bacteria of the genus *Rhizobium* are classified by host range for ability to form nodules on the roots of leguminous plants. For example, *Rhizobium meliloti* forms nitrogen-fixing nodules on alfalfa (e.g., *Medicago* sp.), *R. leguminosarum* does so on peas (e.g., *Pisum* sp.), and *R. japonicum* does so on soybeans (e.g., *Glycine* sp.). The mechanism of host specificity is not well understood at the molecular level. Genes controlling host specificity of nodulation reside on symbiotic plasmids in fast-growing rhizobia such as *R. meliloti*, *R. trifolii*, and *R. leguminosarum* (3, 4, 7, 12, 13, 15). Host specificity genes have not been identified or associated with plasmids in slow-growing rhizobia such as *R. japonicum*.

A novel group of fast-growing, soybean-nodulating rhizobia from China was recently described (14). Most of these strains form nitrogen-fixing (Fix⁺) nodules on the genetically unimproved soybean cultivar Peking and Fix⁻ nodules on commerical soybean cultivars (14). One exceptional strain, USDA 191, forms Fix⁺ nodules on some commerical soybean cultivars such as Williams and Clark as well as on Peking and forms weakly Fix⁺ nodules on other commerical cultivars such as Calland (9, 25; T. McLoughlin, unpublished data). Analysis of symbiotic mutants (1) and hybridization studies with heterologous probes (1, 18) have shown that genes required for nodulation (*nod* genes) and for nitrogen fixation (*nif* genes) are located on a 200-megadalton (MDa) plasmid (pSym191) in strain USDA 191. In this study, we mobilized pSym191 to several other *Rhizobium* strains to determine whether genes controlling host specificity for nodulation and cultivar specificity for nitrogen fixation are located on this plasmid.

In preliminary experiments with a Tn*5*-marked Sym plasmid, we were unable to detect self-transfer of the plasmid from USDA 191 to other strains. We therefore used the Tn*5-mob* system described by Simon (22) to mobilize the plasmid, using RP4-4 as a helper plasmid. The transconjugants were confirmed as being derivatives of their respective recipient parents by examining their colony morphologies, antibiotic resistance markers, plasmid profiles, genomic restriction fragments, and reactions with strain-specific antisera (data not shown).

pSym191::Tn*5-mob* was transferred from USDA 191 to *R. meliloti* Rm2011 (21) and from Rm2011(pSym191::Tn*5-mob*) to a Nod⁻ deletion mutant of *R. leguminosarum* (strain 6015) (11, 13). Analysis of the plasmid content of the transconjugants by an in-gel cell lysis and electrophoresis technique (22) showed that each transconjugant had acquired a new 200-MDa plasmid which comigrated with the Sym plasmid of USDA 191 and a 40-MDa band corresponding to the RP4-4 helper plasmid (data not shown). The indigenous plasmids of strains Rm2011 and 6015 were unaltered. One such *R. meliloti* transconjugant and one *R. leguminosarum* transconjugant were then inoculated onto soybean cultivars Peking, Williams, and Calland, and the plants (at least five replicates) were grown in vermiculite in Leonard jars (24) with a nitrogen-free nutrient solution (6). After 4 weeks, the plants inoculated with these transconjugants were well nodulated (about 20 to 40 nodules per plant in all cases) (Table 1). Plants inoculated with the Rm2011 and 6015 parents had no nodules. Thus, pSym191 broadens the host range of *R. meliloti* and *R. leguminosarum* and therefore contains genes controlling host specificity for nodulation. This is the first report of nodulation of soybeans by *R. meliloti* or *R. leguminosarum* derivatives.

The nodules formed by the *R. meliloti* and *R. leguminosarum* transconjugants were markedly different in morphology from those induced by USDA 191 on these cultivars, and the plants were defective in nitrogen fixation (Table 1). The Fix⁻ phenotypes could be caused by loss or inactivation of pSym191 symbiotic (*sym*) genes in the new hosts. This was tested by transferring pSym191::Tn*5-mob* from these transconjugants to a pSym191 -cured derivative of USDA 191. These transconjugants were Fix⁺ on soybean cultivar Williams, indicating that pSym191 was functionally intact in both the *R. meliloti* and the *R. leguminosarum* hosts. The Fix⁻ phenotypes of the *R. meliloti* and *R. leguminosarum* transconjugants could be caused either by improper expression of pSym191 *sym* genes or by the

* Corresponding author.
† Agrigenetics Advanced Research Division manuscript no. 36.
‡ Present address: National Institute for Higher Education, Glasnevin, Dublin 9, Ireland.

TABLE 1. Results of plant tests

Strain[a]	Reference	Symbiotic properties[b] on cultivars:		
		Peking	Williams	Calland
USDA 191 *str-1*	This work	Fix^+	Fix^+	Fix^+
Rm2011	21	Nod^-	Nod^-	Nod^-
Rm2011(pSym191)	This work	Fix^-	Fix^-	Fix^-
6015	11, 13	Nod^-	Nod^-	Nod^-
6015(pSym191)	This work	Fix^-	Fix^-	Fix^-
ANU240	20	Fix^-	Fix^-	Fix^-
ANU265	20	Nod^-	Nod^-	Nod^-
ANU265(pSym191)	This work	Fix^+	Fix^+	Fix^+
USDA 257 *spc-1*	This work	Fix^+	Fix^-	Fix^-
USDA 257 *spc-1*(pSym191) type A	This work	Fix^+	Fix^-	Fix^-
USDA 257 *spc-1*(pSym191) type B	This work	Fix^+	Fix^+	Fix^+

[a] pSym191 in this table refers to the 200-MDa plasmid of USDA 191 containing the Tn*5-mob* transposon in a region that is not essential for symbiosis. USDA 191 *str-1* and USDA 257 *spc-1* are spontaneous streptomycin- and spectinomycin-resistant mutants of USDA 191 and USDA 257, respectively.

[b] Fix^+ plants were green and vigorous after 4 weeks of growth in nitrogen-free medium, were positive in acetylene reduction assays (2) for nitrogenase, and had nodules with wild-type morphology (large, spherical, striated, with internal pink color). Fix^- plants were yellow, were negative in acetylene reduction assays, and had nodules with aberrant morphologies (small, irregular, not striated, and lacking internal pink color). Viable bacteria could be recovered from Fix^+ but not from Fix^- nodules (except in the case of ANU240 Fix^- nodules). Fix^+ cultivar Calland plants showed paler green color and lower acetylene reduction activities than Fix^+ cultivar Peking and Williams plants. Nod^- plants had no nodules.

absence of *sym* genes which are located on the chromosome or on other plasmids in USDA 191.

pSym191::Tn*5-mob* was also transferred to ANU265. ANU265 is a Nod^-, pSym-cured derivative of ANU240 (20), which is a streptomycin-resistant derivative of the broad-host-range, fast-growing strain NGR234 (23). Gel electrophoresis showed that each transconjugant contained new plasmid bands corresponding to pSym191::Tn*5-mob* and to the RP4-4 helper plasmid. Two such transconjugants were tested on plants and found to be indistinguishable in their symbiotic properties from USDA 191 (Table 1). In contrast, ANU265 formed no nodules on any of the cultivars tested. ANU240 formed Fix^- nodules on the roots of all three cultivars. We conclude that pSym191 is expressed normally in the ANU265 genetic background and that pSym191 differs from the Sym plasmid of ANU240 in the ability to confer late nodule functions such as nitrogen fixation on soybeans. Transconjugants isolated from surface-sterilized nodules retained their antibiotic resistance markers and symbiotic properties when used in a second cycle of growth and plant inoculation, indicating that the transferred plasmid is stable during nodule development.

USDA 257 is a fast-growing *R. japonicum* strain (10) that forms Fix^+ nodules on soybean cultivar Peking and Fix^- nodules on commercial cultivars. Transfer of pSym191::Tn*5-mob* to strain USDA 257 *spc-1* resulted in two classes of transconjugants. Two of three transconjugants examined (type B in Table 1) had symbiotic properties identical to those of USDA 191. One transconjugant (type A) had properties identical to those of the USDA 257 parent. The existence of the type B transconjugants provides the first evidence that plasmid genes control cultivar specificity for nodule development and nitrogen fixation.

The USDA 257 *spc-1*(pSym191::Tn*5-mob*, RP4-4) transconjugant strains did not contain a 200-MDa plasmid. They did contain a plasmid of about 260 MDa that comigrated with the unique plasmid seen in USDA 257 in agarose gels (data not shown). The observation that the incoming pSym191::Tn*5-mob* was not maintained as a 200-MDa replicon raised the possibility that a recombination event had occurred, resulting in a hybrid plasmid containing both pSym191 and pSym257 sequences. (The unique plasmid in USDA 257 hybridizes to heterologous *nif* and *nod* probes [1] and is referred to here as pSym257.) Transfer of the 260-MDa plasmid from a type B transconjugant to a plasmid-cured USDA 191 derivative (selecting for Kan^r) resulted in a strain which had the same symbiotic phenotype as USDA 191 on all three cultivars and was shown by gel electrophoresis to contain a 260-MDa plasmid (data not shown). Thus, the genetic determinants of the ability to fix nitrogen on soybean cultivar Williams are located on this 260-MDa plasmid. The kanamycin resistance and *mob* genes of pSym191::Tn*5-mob* are also apparently present on this plasmid.

Blot hybridization experiments provided further evidence for the presence of both pSym191 and pSym257 sequences in a single recombinant plasmid in one type A and in one type B transconjugant. *Eco*RI-digested genomic DNA from transconjugant and parent strains was hybridized with ^{32}P-labeled pRmSL26 by standard techniques (17). pRmSL26 is a cosmid clone containing a 20-kilobase (kb) insert of *R. meliloti* DNA that includes several *nod* genes (16). This probe is capable of hybridizing to the Sym plasmids of USDA 191 and USDA 257 (see below and reference 1). Since the pattern of hybridizable fragments differs between these two strains, it is possible to use this probe to determine whether pSym191 or pSym257 sequences are present in the 260-MDa plasmid in the transconjugant strains. Three hybridizable *Eco*RI fragments (9.6, 7.3, and 4.5 kb) were present in strain USDA 191 *str-1* (Fig. 1, lane a) but were missing from USDA 257 *spc-1* (lane b) and from a pSym191-cured strain (data not shown). Thus, these fragments are found on pSym191 and not on pSym257. Analysis of two transconjugants (lanes c and d) showed that the 4.5-kb fragment is present in both strains but that the 7.3-kb fragment is only present in the Fix^+ transconjugant. Transfer of the 260-MDa plasmid from the Fix^+ transconjugant to a pSym191-cured derivative of USDA 191 resulted in the appearance of these fragments in the transconjugant (lane e).

These results show that different, overlapping segments of pSym191 are present on the 260-MDa plasmid in the Fix^+ and Fix^- transconjugants. Some other pRmSL26-hybridizable pSym191 sequences were not present on the 260-MDa recombinant plasmid (see the 9.6-kb fragment in Fig. 1 for example), indicating that these sequences are not responsible for the difference in cultivar specificity between USDA 191 and USDA 257. The presence of pSym257 sequences on this plasmid was confirmed by observing that other *Eco*RI fragments (8.7 and 2.3 kb in Fig. 1) were present in USDA 257 *spc-1* and in strains containing the 260-MDa plasmid but not in USDA 191 *str-1* (Fig. 1, lanes a through e). Some of the hybridizable fragments did not come from the Sym plasmids (e.g., the 11.5-kb fragment in USDA 191 and the 4.2- and 2.0-kb fragments in USDA 257) (data not shown). The observation that selection for pSym191 in USDA 257 resulted in recombination with pSym257 suggests that pSym191 is unable to replicate or segregate properly in USDA 257, possibly owing to incompatibility between these two plasmids. Recombination between Sym plasmids from *R. leguminosarum* and *R. trifolii* has also been attributed to incompatibility between the plasmids (5, 7). pSym191 is apparently compatible with the resident plasmids and megaplasmids of the other recipient strains.

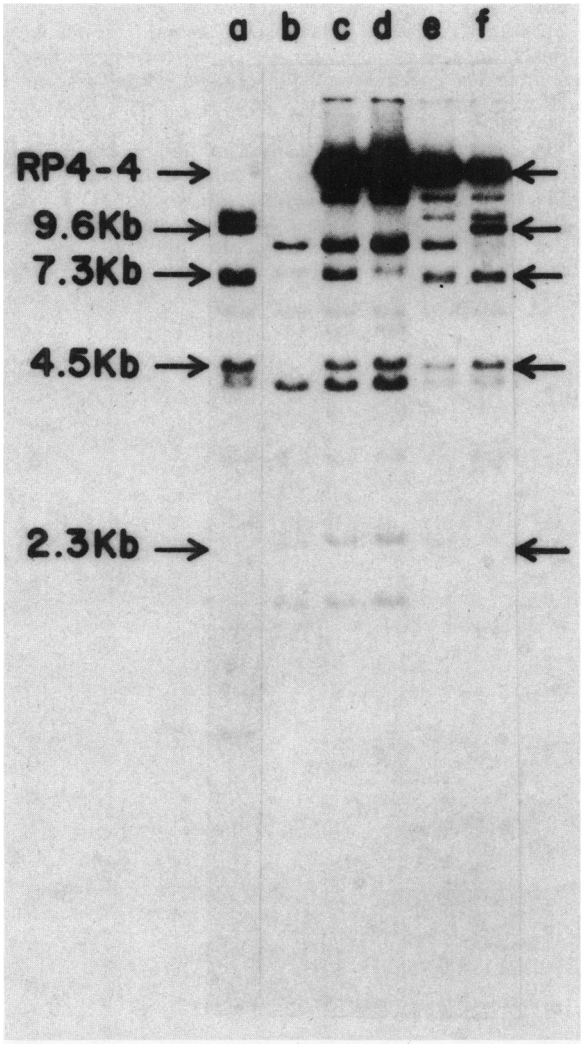

FIG. 1. Blot hybridization analysis of transconjugants. *Eco*RI restriction fragments of genomic DNA from each strain were hybridized with pRmSL26 probe. Lanes: a, USDA 191 *str-1*; b, USDA 257 *spc-1*; c, USDA 257 *spc-1*(pSym191) type B; d, USDA 257 *spc-1*(pSym191) type A; e, pSym-cured USDA 191 *str-1* containing pSym from USDA 257 *spc-1*(pSym191) type B; f, pSym-cured USDA 191 *str-1* containing pSym191 transferred from strain Rm2011(pSym191). The 7.3-kb fragment is the upper band in a doublet that is not fully resolved in lanes a, e, and f. The two largest fragments are present in all strains containing RP4-4 and Tn*5-mob* and presumably correspond to RP4-4 and Tn*5-mob* sequences that are homologous to the pLAFR1 vector sequences in the probe.

The ability of pSym191 to confer a Fix^+ phenotype in strains USDA 191, USDA 257, and ANU265, all of which were originally discovered in China or Papua New Guinea in nodules of soybeans or tropical legumes, may reflect a closer evolutionary relationship among these strains than between these strains and *R. leguminosarum* and *R. meliloti*, which are symbionts of temperate legumes. It has recently been reported that strain 6015 containing the Sym plasmid of ANU240 forms white epidermal outgrowths on the tropical legume siratro (19) that are similar to the nodules formed by 6015(pSym191) on soybeans in this study and that *Agrobacterium tumefaciens* containing pSym191 forms Fix^- nodules on soybeans (8).

The phenotypes of the transconjugants described here may be useful to design procedures for identifying clones of Sym plasmid genes that control soybean specificity of nodulation or cultivar specificity of nodule development and nitrogen fixation.

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