Biology of *Rhizobium trifolii* Bearing the Hairy Root Plasmid

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We have transferred the hairy root plasmid (pRi) from *Agrobacterium rhizogenes* into *Rhizobium trifolii*. The plasmid fully expressed its root-inducing activity in the *R. trifolii* (pRi) transconjugant. Generally, the *R. trifolii* (pRi) transconjugant was unable to nodulate clover. However, occasionally a spontaneous mutant(s) of the *R. trifolii* (pRi) transconjugant arose which was capable of nodulating clover and of inducing root proliferation in a number of plant species. The mutant(s) induced significantly more nodules than the wild-type bacterium, but significantly less N₂ was fixed by these nodules.

INTRODUCTION

Recently, we have introduced the Ri plasmid of *Agrobacterium rhizogenes* into *Rhizobium meliloti* (Strobel *et al.*, 1985). The Ri plasmid is responsible for the induction of the hairy root syndrome in dicotyledonous plants (Moore *et al.*, 1979; White & Nester, 1980*a, b*). In order to accomplish the direct transfer of the Ri plasmid into *R. meliloti*, a mutant derivative of *A. rhizogenes* strain TR105 was prepared by Tn5 transposon mutagenesis. This derivative carries a Tn5 insertion in its Ri plasmid which permits a much higher efficiency of transfer than that obtained with the wild-type Ri plasmid (B. Lam, unpublished). The *R. meliloti* transconjugants prepared by this procedure were unable to cause root proliferation in any of the dicotyledonous plants that were tested. However, the transconjugants were capable of nodulating alfalfa seedlings to a much greater extent than wild-type *R. meliloti*. The increased nodulation also resulted in significantly more N₂ being fixed than by either the wild-type or antibiotic-resistant mutants of *R. meliloti*. Furthermore, there were no ultrastructural differences between the nodules produced by the wild-type *R. meliloti* and those produced by the transconjugants.

In this paper we report the construction and characteristics of Ri plasmid transconjugants of *Rhizobium trifolii*. The transconjugants differed in several ways from those of *R. meliloti*.

METHODS

Bacterial strains and culture conditions. The strains of *Agrobacterium* spp. and *R. trifolii* used are listed in Table 1. All bacteria were grown on yeast extract/mannitol (YM) medium and the antibiotic concentrations used were as previously reported (Strobel *et al.*, 1985). All bacteria used to inoculate plants were grown from single colonies.

Bacterial mating. Matings between *A. rhizogenes* and *R. trifolii* were done on sterile Millipore filters placed on nutrient agar medium. The *A. rhizogenes* donor (BL311), derived from TR105, contains only the Ri plasmid into which there is a Tn5 insertion which permits a higher efficiency of transfer (B. Lam, unpublished; Strobel *et al.*, 1985) and allows growth on a medium containing kanamycin. The *R. trifolii* recipient (LH3) is a spontaneous streptomycin- and rifampicin-resistant derivative of the wild-type *R. trifolii* strain 2067. Back mating of *R. trifolii* (pRi) transconjugants with *Agrobacterium radiobacter* NT1 was done as described above; however, selection of the *A. radiobacter* transconjugants, i.e. LH16, was done on an agrobacterium selective medium containing 500 µg kanamycin ml⁻¹ (Strobel & Nachmias, 1985).

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Table 1. *Bacterial strains*

Strain	Relevant characteristics*	Source
<i>A. rhizogenes</i>		
TR105	Wild-type (producing roots)	L. Moore, Oregon State Univ.
BL311	TR105(pRiTR105-2::Tn5) Kan ^r , transfers pRi at high frequency (donor parent)	Our collection
<i>A. radiobacter</i>		
NT1	Derived from <i>A. tumefaciens</i> C58 Lacks the Ti plasmid	L. Moore, Oregon State Univ.
LH16	NT1(pRiTR105-2::Tn5) Prepared by conjugation of NT1 and <i>R. trifolii</i> LH11	This paper
<i>R. trifolii</i>		
2067	Wild-type	USDA, Beltsville, Md.
LH3	Spontaneous, two step, Rif ^r Str ^r derivative of strain 2067 (recipient parent)	This paper
LH11	} Rif ^r Str ^r Kan ^r Contain pRiTR105-2::Tn5 (pRi transconjugants)	This paper
LH12		
LH11-Ro		
	Spontaneous mutant of LH11 Produces numerous small nodules on clover	This paper

* Str, streptomycin; Kan, kanamycin; Rif, rifampicin.

Identification of bacteria. To ensure that the recipients of the Ri plasmid were really transconjugants, we compared the banding patterns of total soluble proteins separated by isoelectric focusing of the *R. trifolii* and *A. radiobacter*-transconjugants to the proteins of the donor and recipient bacteria. Cultures (200 ml) at late exponential phase were centrifuged at 10000 *g* for 15 min at 4 °C. The supernatant liquid was discarded and the pellet resuspended in 50 ml 50 mM-potassium phosphate buffer pH 7.5 made 0.14 M with respect to sodium chloride. The bacterial cells were broken by sonication (4 × 45 s, 200 W) and the pellet was discarded. The homogenate was centrifuged at 20000 *g* for 30 min at 4 °C. The supernatant solution was concentrated to a final protein concentration of 15–20 mg ml⁻¹ using an Amicon B-15 macrosolute concentration device, and stored in 25 µl portions at -20 °C. Protein was determined by the Lowry method using bovine serum albumin as a standard. Isoelectric focusing was done using 1% IEF-agarose and Pharmalyte 3–10 (both from Pharmacia), in accordance with the supplier's guidelines.

DNA procedures. The Ri plasmid was isolated from *A. rhizogenes* strain TR105 according to the procedures of Currier & Nester (1976). The *Bgl*II (New England Biolabs) restricted pattern of the authentic Ri plasmid was compared to that of the putative Ri plasmid in the *A. radiobacter* transconjugant (LH16), using a modification of the mini-plasmid preparation procedure of Silhavy *et al.* (1984). Electrophoresis of digested DNA was done according to David *et al.* (1980). Other DNA procedures, used on parent and transconjugant bacteria, were done as described by Strobel *et al.* (1985).

Electron microscopy. The techniques and procedures used for transmission electron microscopy and scanning electron microscopy of nodules and root segments were those described by Strobel *et al.* (1985). At least six nodules produced on clover roots by each bacterial strain were examined.

Plant growth and analyses. Seeds of clover (*Trifolium pratense* L. cv. Redland) were surface disinfected and rinsed in sterile distilled H₂O according to the procedures previously used for alfalfa (Strobel *et al.*, 1985). After the seeds had germinated they were inoculated with bacteria and planted in sterilized medium grade white sand in containers, which were placed in racks in a growth chamber. The racks and the chamber had been thoroughly scrubbed with Chlorox followed by 70% (v/v) ethanol solutions. The emerging plants were watered on alternate days with a sterilized Hoagland solution minus nitrogen and grown with 12 h darkness at 22 °C and 12 h light at 27 °C with a light intensity of 170 µE m⁻² s⁻¹. The numbers of nodules of all sizes on each plant were counted after 30 d. Between 12 and 20 plants were used in each treatment category and the experiments were replicated at least three times. Kjeldahl analysis was done on groups of five to seven whole dried plants. Root-initiating ability of various bacteria was tested on root discs of various plants prepared and inoculated according to Moore *et al.* (1979).

RESULTS AND DISCUSSION

Ri plasmid transfer

We used *A. rhizogenes* strain BL311 as a donor of the Ri plasmid to *R. trifolii* LH3. Strain BL311 carries an Ri plasmid mutant with elevated transferability due to a Tn5 insertion. There was no detectable transfer of the Ri plasmid from *A. rhizogenes* TR105 to *R. trifolii* LH3.

Fig. 1. Carrot root discs inoculated with (a) *R. trifolii* 2067 (same response given with *R. trifolii* LH3, parent bacterium), (b) *R. trifolii* (pRi) transconjugant (LH11), (c) *A. radiobacter* NT1 (parent) and (d) *A. radiobacter* (pRi) transconjugant (LH16). The transconjugants were prepared and inoculated onto the discs as described in Methods.

Furthermore, under the conditions of these mating experiments, no spontaneous antibiotic-resistant mutants arose.

When strain BL311 was mated with *R. trifolii* LH3, transconjugants were obtained at a frequency of 10^{-4} to 10^{-5} per parental bacterium. Banding patterns obtained after isoelectric focusing of soluble proteins and gel separation of *Eco*RI digests of total DNA confirmed that the transconjugants possessed the genetic background of *R. trifolii* (data not shown). Likewise, when the *R. trifolii* transconjugant LH11 was mated with *A. radiobacter* NT1 (the frequency of plasmid transfer was 10^{-3} per parental bacterium), the transconjugant (LH16) possessed the same gross protein and *Eco*RI restriction banding patterns as *A. radiobacter* NT1. Furthermore, a plasmid was isolated from the *A. radiobacter* transconjugant LH16 which possessed the same gross *Bgl*II restriction pattern as that given with the authentic Ri plasmid. The results showed that successful transfer of the Ri plasmid had occurred from *A. rhizogenes* BL311 to *R. trifolii* LH3, and from *R. trifolii* LH11 to *A. radiobacter* NT1, resulting in *A. radiobacter* LH16. Southern blotting techniques, using labelled Ri plasmid as a probe, also confirmed that all of the Ri plasmid was successfully transferred to each of these recipient bacteria (data not shown). Therefore, *A. rhizogenes* BL311 can serve as an effective transfer vector of the Ri plasmid to species of *Rhizobium* other than *R. meliloti*. The efficiency of transfer, however, is about an order of magnitude lower in the case of *R. trifolii* than *R. meliloti*.

Root induction

Both the wild-type *A. rhizogenes* strain TR105 and the plasmid-transfer mutant *A. rhizogenes* BL311 caused root proliferation in carrot, rutabaga, turnip, sweep potato and parsnip. However, in contrast to the *R. meliloti* (pRi) transconjugant (Strobel *et al.*, 1985), the *R. trifolii* (pRi) transconjugant also caused root proliferation in all of the fleshy dicotyledonous plants that were tested (for example, see Fig. 1). Thus, the rooting characteristics of the Ri plasmid are expressed in *R. trifolii*, but not in *R. meliloti*, a totally unexpected result. As expected, the *A. radiobacter* NT1(pRi) transconjugant (LH16), prepared by mating *A. trifolii* LH11 with *A. radiobacter* NT1, expressed the rooting characteristic of the Ri plasmid on all of the plants listed above (for example, see Fig. 1).

Table 2. *Growth chamber experiments with clover*

The conditions were 12 h darkness at 22 °C and 12 h light at 27 °C with a light intensity of 170 $\mu\text{E m}^{-2} \text{s}^{-1}$. The plants were watered on alternate days with sterile Hoagland's solution minus a nitrogen source. With the exception of LH12 and 'no cells' the data are means of three independent experiments, with at least 12–20 plants per treatment. Means followed by the same letters are not significantly different at $P \leq 0.05$ as judged by an LSD multiple comparisons procedure. This analysis was preceded by an analysis of variance which reached a significance at the $P < 0.01$ level for nodulation and $P < 0.05$ for total N per plant. The SEM was 0.81 for nodulation and 0.01 for total N per plant.

Organism	No. of nodules per plant	Total N per plant (mg)
<i>R. trifolii</i> wild-type (2067)	14.6 ^a	1.12 ^d
<i>R. trifolii</i> parent (LH3)	14.5 ^a	1.02 ^d
Mixed inoculation:		
<i>R. trifolii</i> (2067) plus		
<i>R. trifolii</i> transconjugant (LH11)	17.7 ^b	0.91 ^d
<i>R. trifolii</i> transconjugant (LH11-Ro)	21.5 ^c	0.39 ^e
<i>R. trifolii</i> (pRi) transconjugant (LH11)	0	0.075*
<i>R. trifolii</i> (pRi) transconjugant (LH12)	0	0.08*
<i>A. rhizogenes</i> TR105 (pRiTR105-2::Tn5) (BL311)	0	0.085*
<i>A. radiobacter</i> NT1 (pRiTR105-2::Tn5) (LH16)	0	0.068*
No cells	0	0.05*

* These results were not included in the statistical analysis because the treatments produced no nodules and did not result in biological N_2 fixation.

Nodulation and nitrogen fixation

R. trifolii (pRi) transconjugants LH11 or LH12 did not nodulate clover roots, whereas wild-type *R. trifolii* 2067 or *R. trifolii* LH3 (Rif^r Str^r) did (Table 2). However, in a preliminary test, four plants of 20 developed numerous small nodules on their roots after having been inoculated with *R. trifolii* (pRi) transconjugant LH11. The organism in these small nodules was isolated on YM medium containing rifampicin, streptomycin and kanamycin and was designated LH11-Ro. It apparently arose by spontaneous mutation and consistently produced significantly more nodules per plant than any of the other rhizobia tested; however, significantly less N_2 was fixed by these plants than by plants inoculated with *R. trifolii* strains 2067, LH16, or 2067 and LH11 (Table 2). There seemed to be no question that strain LH11-Ro was an *R. trifolii* (pRi) transconjugant since it was consistently isolated (in 80–100% of cases) from the small nodules on medium containing three antibiotics with few, if any, contaminants. No bacterial growth was observed on this medium when nodules from plants inoculated with strain 2067 were placed on it. Furthermore, LH11-Ro caused root formation in all of the dicotyledonous plants tested, in a manner and rate comparable to LH11. When LH11-Ro was mated with *A. radiobacter* NT1, using the agrobacterium selective medium containing 500 μg kanamycin ml^{-1} , transconjugants were obtained that caused rooting on the dicotyledonous plant species previously mentioned. This suggested that if a mutation had occurred in LH11 to yield LH11-Ro, this had not affected the rooting activity of the Ri plasmid since it was successfully transferred back to *A. radiobacter* without any noticeable effect on its biological activity. Additional work is needed to characterize LH11-Ro.

It should also be mentioned that there was some difficulty in keeping the 'no cell' treatments free of nodulating bacteria during the plant tests, and quite often plants in this group became nodulated even though the strictly aseptic conditions were kept. On some occasions minor cross contamination between the treatments (i.e. 1–5%) was observed, and this was taken into consideration.

Fig. 2. Electron micrographs of bacteroids in representative nodules from clover plants treated with (a) *R. trifolii* 2067 (wild-type), (b) *R. trifolii* LH3 (parent bacterium), (c) mutant *R. trifolii* (pRi) transconjugant (LH11-Ro) and (d) both *R. trifolii* 2067 and *R. trifolii* (pRi) transconjugant (LH11). Note that the electron transparent granules (probably poly β -hydroxybutyrate) visible in (a), (b) and (d) are absent in (c). Bar, 0.5 μ m.

These results on nodulation with *R. trifolii* (pRi) transconjugants are quite different from those observed with *R. meliloti* (Strobel *et al.*, 1985). The *R. meliloti* (pRi) transconjugants consistently produced more nodules than wild-type *R. meliloti*, and this apparently resulted in more N₂ fixed. However, when transconjugant strain LH11 was mix-inoculated with wild-type *R. trifolii*, nodulation was greater than that with wild-type alone (Table 2). The transconjugant strain could be isolated from about 50% of these surface disinfected nodules. Although this observation is interesting, we have no good explanation for the effect.

As expected, no nodules were produced on any of the plants treated with the *Agrobacterium* strains, or on untreated plants when free of contaminating bacteria. Furthermore, no N₂ was fixed in any of these cases (Table 2).

As is true for the *R. meliloti* transconjugants (Strobel *et al.*, 1985), it seems highly unlikely that we selected, by the single colony technique, a strain of *R. trifolii* that is not capable of nodulating clover, since both LH11 and LH12 behaved in an identical manner. Furthermore, putative pRi transconjugants of *R. trifolii* 2063, made in the same way as described in this report, also failed to nodulate clover; this is in extreme contrast to the wild-type bacterium (G. A. Strobel, unpublished).

Morphological and cytological effects

No differences were observed by scanning electron microscopy between the nodules produced by mutant *R. trifolii* (pRi) transconjugant LH11-Ro and the nodules produced by either the wild-type *R. trifolii* 2067 or the *R. trifolii* parent (LH3), except that the nodules produced by LH11-Ro were generally about half the size of those produced by 2067 or LH3. Examination of photographs made by transmission electron microscopy showed that there were gross structural differences between the bacteroids in the nodules produced by the mutant transconjugant LH11-Ro and the other *R. trifolii* strains used as inoculants, i.e. parent strain LH3, wild-type strain 2067 and the mixed inoculant, 2067 plus transconjugant LH11. Numerous electron transparent polyhydroxybutyrate storage deposits, which are generally a characteristic feature of nodules produced by rhizobia (Newcomb, 1981; Jordan & Allen, 1974), were observed in the bacteroids of the rhizobia that caused nodulation, except for the bacteroids of LH11-Ro, where they were absent (Fig. 2). A general decrease in N₂ fixation by LH11-Ro seemed to manifest itself in the small size of the nodules that the bacterium produced, and in the small capacity for storage of an energy source by the bacteroids. However, co-inoculation of LH11 with the wild-type bacterium resulted in significantly more nodules than with the wild-type alone, and the nodules again appeared to have normal ultrastructural characteristics (Table 2, Fig. 2).

General observations

Both *R. meliloti* and *R. trifolii* are considered to be fast-growing species of rhizobia. However, when representative strains of each of these organisms carry and maintain the Ri plasmid they behave differently to each other. When carrying the Ri plasmid, *R. meliloti* enhances nodulation and fixes more N₂, but does not express the root-inducing effects of the plasmid, while *R. trifolii* does not nodulate its host, but is capable of causing rooting on the fleshy roots of several dicotyledonous plants. However, spontaneous mutants of the *R. trifolii* (pRi) transconjugant may arise which cause numerous small nodules to form on clover, but N₂ fixation by these nodules is significantly decreased when compared to the wild-type. Thus, it appears that the expression of the Ri plasmid in *Rhizobium* spp. is under the control of either the bacterial genome, or its endogenous plasmids, or both. To date, the Ri plasmid has not been transferred to any of the slow-growing rhizobia. We have shown that many biological consequences can result from the Ri plasmid transfer to rhizobium, from increased nodulation and N₂ fixation to no nodulation whatever, and it seems that there is no method by which we can predict the outcome of such a transfer.

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