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# $Rj_1$ and $rj_1$ Soybean Isolines: Adsorption of Rhizobia to Roots and Distribution of Primary Root Nodules

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**Abstract**

We studied the temperature-sensitivity of nodule distribution and the adsorption of rhizobia to roots of the soybean cultivar Clark and its nodulation-restrictive isoline, Clark-$rj_1$. Five tested *Rhizobium japonicum* strains nodulated Clark in plastic growth pouches. At 22°C or 27°C, most primary root nodules were clustered near the position occupied by the root tip at the time of inoculation. At 32°C, the nodules were positioned much lower on the root, the extent of clustering was reduced, and the total number of nodules per plant was decreased. Clark-$rj_1$ was nodulated by strains 61, 84, 94, and 119, but not by 110. The temperature-sensitivity of nodulation of Clark-$rj_1$ was similar to that of Clark, but secondary roots were preferentially nodulated. Although a few nodules formed near the position occupied by the root tip at the time of inoculation, the total number of nodules per Clark-$rj_1$ plant was much less than that of the normal isoline. Adsorption of strain 94 to both isolines and of strain 110 to Clark was roughly linear during a 120 min incubation period at 27°C. Adsorption in the nonnodulating combination of strain 110 and Clark-$rj_1$ was more rapid, and the total number of bound bacteria was greater. Reduction of the temperature from 27°C to 22°C, a treatment that enhances nodulation of Clark-$rj_1$ by strain 94, strongly reduced the adsorption of strain 94.

Keywords: *Glycine max*, nodule profiles, *Rhizobium*

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## 1. Introduction

During August of 1947, the leaves of a group of soybeans in an Illinois breeding plot unexpectedly turned yellow (Williams and Lynch, 1954). These plants yielded poorly, and additional studies in 1948 revealed that the roots of sibling plants were not nodulated. The nonnodulating character, which evidently arose by spontaneous mutation, is conditioned by an allele originally named *no* for nonnodulating. The allele subsequently was redesignated $rj_1$ to conform to modern terminology for soybean genetics (Caldwell, 1966).

In early experiments, $rj_1rj_1$ plants failed to nodulate in the presence of rhizobia (Williams and Lynch, 1954; Caldwell, 1966). Now it is clear that although most strains of *Rhizobium japonicum* do not nodulate these plants, several strains produce a variable number of nodules on plants grown under suitable conditions (Clark, 1957; Devine and Weber, 1977; Devine, 1984; Lafavre and Eaglesham, 1984). These strains are termed "overcoming" because they surmount the effects of the $rj_1$ gene. Devine and Breithaupt (1980) reported that temperature influences nodulation of the soybean cultivar Clark and its isoline Clark-$rj_1$ by two overcoming strains, USDA 61 and 76. Nodulation of Clark-$rj_1$ by strain 61 is roughly equivalent at 21°C and 27°C, but strain 76 appears to have a distinct optimum at 21°C.

Although several processes that regulate nodulation of wild-type soybean have been identified, little is known of the factors that restrict nodulation of $rj_1$ soybean in a strain-specific manner. Most nodules on the primary root of wild-type soybean are clustered near the position that corresponded to the location of the root tip at the time of inoculation (Bhuvaneswari et al., 1980; Bhuvaneswari et al., 1983). Because this area lacks mature root hairs at the time of inoculation, Bhuvaneswari et al. (1980) hypothesized that infection is restricted to immature root hairs. Pueppke (1983) and Calvert et al. (1984) subsequently confirmed that the formation of infection threads is the developmentally restricted event. The binding of rhizobia to host roots also is thought to facilitate nodule initiation (Dazzo, 1980; Pueppke, 1984a). *R. japonicum* cells can be visualized on the surface of elongated root hairs of soybean and wild soybean (Stacey et al., 1980), and some (but not all) nonnodulating mutants appear to lose their capacities to bind to soybean roots (Law et al., 1982; Stacey et al., 1982).

Here we describe a series of experiments designed to measure the effect of the nodulation-restrictive plant gene, $rj_1$, on factors thought to regulate nodule initiation. Our objectives were (a) to compare nodule distribution on $rj_1$ and $Rj_1$ soybean and its sensitivity to temperature and (b) to determine if $rj_1$ functions to restrict the adsorption of nonnodulating strains to soybean roots.

## 2. Materials and Methods

*Bacteria*

*Rhizobium japonicum* 61, 84, 94, and 119 are overcoming strains that nodulate $rj_1$ soybean; control strain 110 does not. All strains nodulate $Rj_1$ soybean and were obtained from the U.S. Department of Agriculture (Beltsville, MD). The bacteria were stored at 4°C on yeast extract-mannitol agar slants (Vincent, 1970). Inoculum was produced from log-phase cultures grown in liquid gluconate-mannitol medium (Bhuvaneswari et al., 1977) at 28°C on a rotary shaker operating at 120 rpm.

*Plants*

Seeds of *Glycine max* (L.) Merr. cultivar Clark-L1 ($Rj_1Rj_1$) and the nodulation-restrictive isoline of Clark-L1, L63-1889 ($rj_1rj_1$), were obtained from R.L. Bernard (U.S. Department of Agriculture, Univ. of Illinois-Urbana) and from D.A. Phillips (Dept. of Agronomy and Range Science, University of California-Davis). The isolines are designated Clark and Clark-$rj_1$ according to the nomenclature of Devine and Breithaupt (1980). Seeds were disinfested by soaking in 50% ethanol for 2 min with agitation, rinsing in deionized water, and then shaking in 0.5% aqueous sodium hypochlorite for 2 min. Seeds were washed for 20 min in running deionized water and were germinated aseptically in the dark on water agar for 4 to 5 days at 22°C, 27°C, or 32°C, depending on experimental requirements.

*Nodule distribution*

Bacterial cultures were centrifuged at 7500× g for 10 min, and the bacteria were resuspended in sterile nitrogen-free Jensen's solution (Vincent, 1970). Cell concentration was adjusted turbidimetrically to $5 \times 10^8$ cells/ml. Seedlings with roots approximately 4 cm long were inoculated by immersing the roots in the bacterial suspension. After 10 min, pairs of seedlings were transferred to autoclaved plastic growth pouches (Northrup King; Minneapolis, MN) containing 15 ml of Jensen's solution. The surface of each pouch was marked at the location of the primary root tip of each plant (Bhuvaneswari et al., 1980). This mark was designated the root tip mark (RTM).

Plants were grown for 30 days at a constant temperature of 22°C, 27°C, or 32°C with a 10 hr light/dark cycle in a Conviron growth chamber with 900 $E/m^2/sec$ (400 to 700 nm) irradiance at canopy height. Each temperature experiment was repeated 3 times with 6 plants of Clark and 10 plants of Clark-$rj_1$ tested for each *R. japonicum* strain in each experiment. Control

plants sham-inoculated with sterile Jensen's solution were included in each experiment. Roots were moistened as necessary with deionized water.

The distance (to the nearest mm) from the RTM to each nodule on the primary root was measured on each plant at harvest. The number of nodules on secondary roots also was determined. Data were subjected to analysis of variance using the general linear models procedures of the Statistical Analysis System (SAS Institute; Cary, NC).

*Bacterial adsorption*

Adsorption of rhizobia to soybean roots was measured as described previously (Pueppke, 1984b). Briefly, seedlings were germinated and transferred to autoclaved plastic growth pouches. After 1 day, pairs of plants were removed and suspended so that their roots were submersed in suspensions containing approximately $10^4$ bacteria/ml of Jensen's solution. After prescribed time intervals, individual seedlings were removed, their roots washed vigorously in a stream of Jensen's solution, and the distal 2 cm of the primary root severed. Pairs of segments were homogenized and aliquots of the homogenate were plated to determine colony-forming units (cfu). Data were collected as the mean number of cfu formed on 5 replicate plates spread with homogenates from each set of root segments. All data were normalized by dividing treatment means by a ratio representing the turbidimetrically estimated inoculum ($10^4$ cells/ml) divided by the cfu/ml of inoculum (Pueppke, 1984b). Normalized data are expressed as the number of bacteria adsorbed per plant.

Most binding assays were completed at an ambient air temperature of ca. 27°C. The Clark-$rj_1$ × strain 94 combination also was tested at 22°C. In these experiments, the test tubes containing bacterial inoculum were placed in a water bath and equilibrated at 22°C for 30 min before seedling roots were immersed in the suspension.

Two kinds of control experiments were conducted. In the first, known numbers of bacteria were ground with root tissue to determine the effect of grinding and of plant tissue constituents on bacterial cfu; neither significantly influenced the recovery of bacteria. In the second, seedlings that had been incubated for 2 hr with bacteria were washed as described above and then directly placed into prepared growth pouches. These seedlings were examined after 30 days for the presence of nodules.

## 3. Results

*Nodulation and nodule distribution*

The mean number of nodules per plant on Clark varied between 2 and 8 at 32°C and increased to between 15 and 19 at 22°C (Fig. 1). The mean number of nodules on Clark-$rj_1$ inoculated with overcoming strains was less than one per plant at 32°C and increased to 0.7 to 2.2 nodules per plant at 22°C. No nodules were formed on Clark-$rj_1$ inoculated with control strain 110 or on plants sham-inoculated with Jensen's solution, regardless of temperature. Analysis of variance was conducted with the number of nodules per plant as the dependent variable. In a preliminary test, nodule numbers on Clark and Clark-$rj_1$ were demonstrated to be significantly different. The 2 genotypes thus were treated separately in all subsequent analyses to avoid heterogeneity of variance. Temperature significantly ($p < 0.01$) influenced nodule number for each plant genotype, but $F$-values for strain and replication were not significant.

The effect of temperature on position of nodules on Clark-$rj_1$ is dramatically emphasized by expression of the data as the percentage of inoculated plants developing at least 1 nodule (Table 1). All of the strains preferentially nodulate secondary roots. Nodulation of primary roots is more strongly attenuated by temperature. Nodulation by strain 119 is most sensitive to temperature and is abolished even at 27°C. At each temperature a total of 120 plants was inoculated with 1 of the 4 overcoming strains; in aggregate, 4% of the plants were nodulated at 32°C, 23% at 27°C, and 44% at 22°C.

Table 1. Percentage of Clark-$rj_1$ soybean plants nodulated by *Rhizobium japonicum* at three temperatures

| Strain | Percentage of plants with one or more nodules* | | | | | |
|---|---|---|---|---|---|---|
| | 22°C | | 27°C | | 32°C | |
| | Primary | Secondary | Primary | Secondary | Primary | Secondary |
| 61 | 23 | 73 | 10 | 50 | 3 | 3 |
| 84 | 3 | 30 | 7 | 30 | 0 | 10 |
| 94 | 13 | 27 | 0 | 10 | 0 | 0 |
| 119 | 3 | 27 | 0 | 0 | 0 | 0 |

* Each experiment was repeated three times with 10 plants per treatment

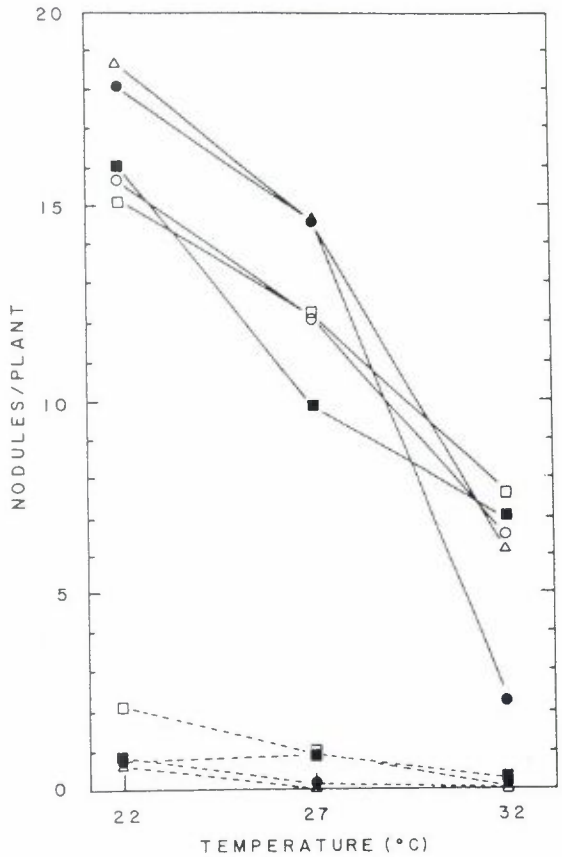


Figure 1. Influence of temperature on the nodulation of Clark and Clark-$rj_1$ soybean by *R. japonicum*. Plants were dip-inoculated in bacterial suspensions ($5 \times 10^8$ cells/ml), placed in plastic growth pouches, and grown at constant temperature for 30 days. For each strain at each temperature, the experiment was replicated 3 times with 6 plants per treatment for Clark and 10 plants per treatment for Clark-$rj_1$. □ = strain 61, ■ = strain 84, ● = strain 94, ○ = strain 110, and Δ = strain 119. (——) = Clark, (– – –) = Clark-$rj_1$. Sham-inoculated plants and Clark-$rj_1$ plants inoculated with strain 110 did not nodulate.

The positions of every primary root nodule in 3 representative strain interactions with Clark are mapped in Fig. 2. At 22°C and 27°C, all three strains produce the expected peak of nodules near the position occupied by the root tip at the time of inoculation, i.e., the RTM. Strain 94, however, was the only strain that formed a significant number of nodules well above the RTM. The 32°C temperature flattened each of the nodule peaks and displaced the

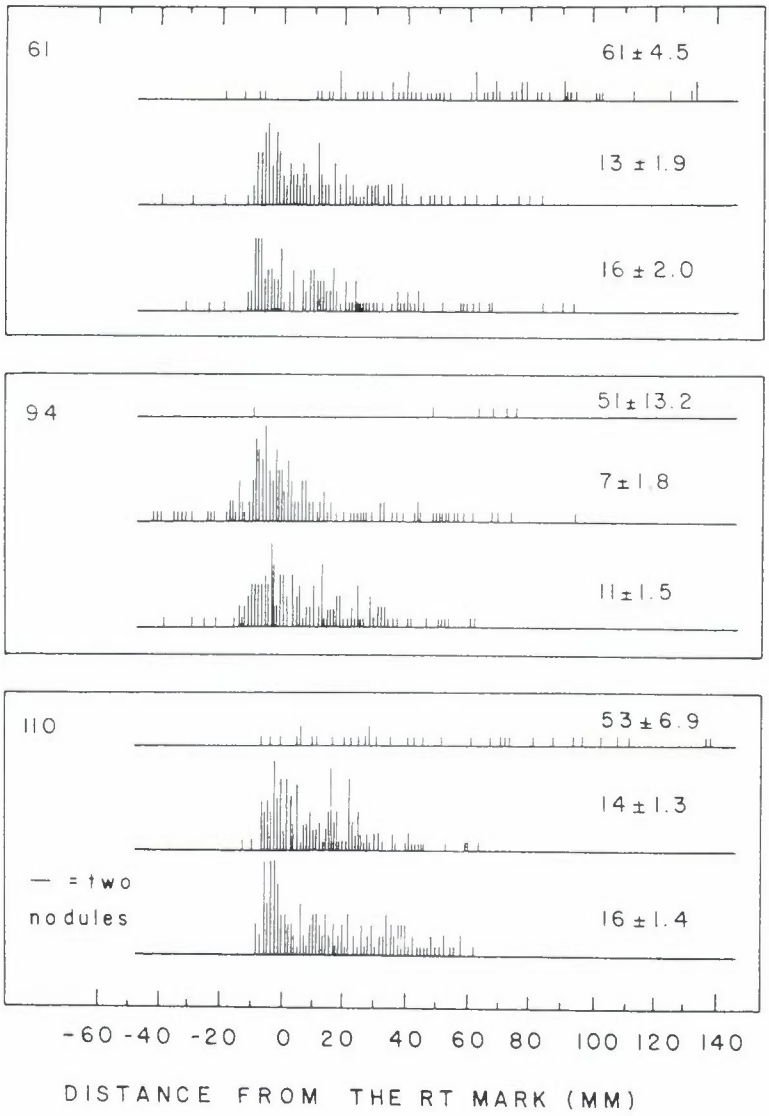


Figure 2. Effect of temperature on the distribution of nodules on the primary roots of Clark soybean. Plants were dip-inoculated with *R. japonicum* and grown at constant temperature for 30 days. The distance of each primary root nodule from the RTM (designated 0 on the x-axis) then was measured. Nodules above the mark are assigned negative values. The frequency diagrams are for plants from 3 repeat experiments at each temperature with 6 plants per treatment. The upper, middle, and lower diagrams in each panel represent 32°C, 27°C, and 22°C temperatures, respectively. The value above each diagram is the mean nodule distance (± SE) below the RTM.

populations of nodules downward (Fig. 2). This downward displacement is clearly evident when the data are summarized as the mean distance of all primary root nodules from the RTM (Fig. 2). In every case, the mean distance at 32°C is 37 mm or more below that at lower temperatures. Profiles of strains 84 and 119 on Clark are similar to those of strains 61 and 110 and are not shown.

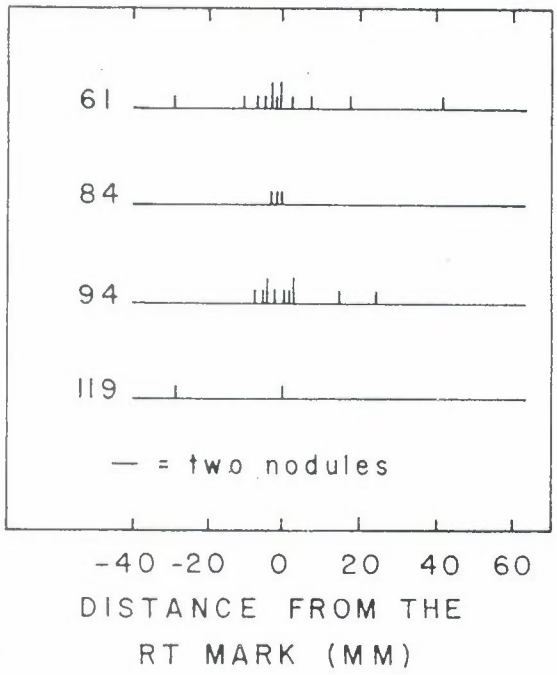


Figure 3. Distribution of nodules on the primary roots of Clark-$rj_1$ soybean at 22°C. Plants were dip-inoculated with *R. japonicum* and grown for 30 days. The distance of each primary root nodule from the RTM (designated 0 on the x-axis) then was measured. Nodules above the RTM are assigned negative values. The frequency diagrams are for plants from 3 repeat experiments with 10 plants per treatment. No nodules formed on plants that were sham-inoculated or inoculated with strain 110.

Figure 3 contains maps of Clark-$rj_1$ inoculated with four strains. Data from the 27°C and 32°C experiments were omitted because most plants lacked primary root nodules. Although nodulation at 22°C was sparse, all strains formed nodules near the RTM. The nodules produced by strains 61 and 94 appear to be clustered around this mark.

*Bacterial adsorption*

Both bacterial strains bind to Clark soybean roots in roughly similar numbers with a near linear increase over time (Fig. 4). Nodules formed on 100% of the seedlings that were rinsed after the 2 hr incubation period and then returned to growth pouches. The capacity of Clark-$rj_1$ to adsorb overcoming strain 94 is similar to that of Clark, even though only 10% of the Clark-$rj_1$ plants nodulated when returned to growth pouches. Significantly *greater* numbers of bacteria, however, bind in the nonnodulating combination of Clark-$rj_1$ × strain 110. The mean number of strain 110 cells bound per Clark-$rj_1$ plant is nearly 100 at 30 min, more than twice that in other combinations.

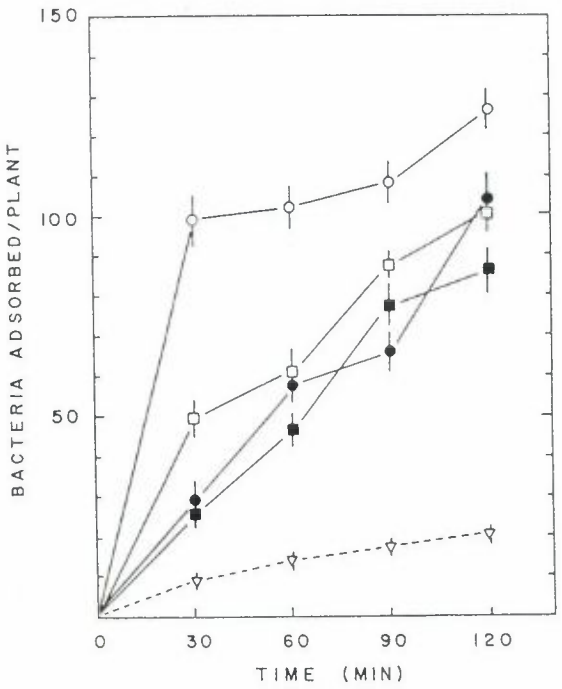


Figure 4. Adsorption of *Rhizobium japonicum* to soybean roots. Each point represents the mean from 5 experiments with 4 pairs of plants tested in each experiment. Bars indicate standard errors. Seedling roots were incubated in bacterial suspensions ($10^4$ cells/ml) for the times indicated and rinsed vigorously. The terminal 2 cm of each primary root was excised, homogenized, and aliquots plated for determination of cfu. □ = Clark × strain 110, 27°C; ■ = Clark × strain 94, 27°C; ○ = Clark-$rj_1$ × strain 110, 27°C; ● = Clark-$rj_1$ × strain 94, 27°C; ▽ = Clark-$rj_1$ × strain 94, 22°C.

Although reduction of temperature from 27°C to 22°C markedly enhances nodulation of Clark-$rj_1$ by strain 94 (Table 1), it has the opposite effect on bacterial adsorption (Fig. 4). The lower temperature reduces the number of bacteria bound after 30 min by about 65% and after 2 hr by about 80%. At 22°C, this combination has a greater number of nodules, a greater percentage of the plants with nodules, and a greater number of nodules on the primary root.

## 4. Discussion

Although temperature is known to influence the number of nodules per soybean plant under various conditions (Weber and Miller, 1972; Manevar and Wollum, 1982; Devine and Breithaupt, 1984), ours is the first study to examine the effect of temperature on the distribution of nodules, a phenomenon that appears to reflect precise regulation of successful infections (Bhuvaneswari et al., 1980; Pueppke, 1983; Calvert et al., 1984; Heron and Pueppke, 1984). The pattern of nodulation on primary roots of Clark soybean at 22°C and 27°C is similar to that reported by others for compatible interactions and follows that predicted by the model of transient susceptibility to nodulation (Bhuvaneswari et al., 1980). This model suggests that nodules result from infections that are developmentally constrained to areas of the primary root that lack root hairs or contain immature root hairs at the time of inoculation. Strain 94, like *R. fredii* strain 191 and *Rhizobium* sp. strain 3G4b16, can produce an appreciable number of nodules well above this region (Heron and Pueppke, 1984). Although the presence of such nodules indicates that this region may retain immature root hairs for an unusually long time period, it is difficult to understand how these hairs could be infected by only certain strains of rhizobia.

High temperature reduces nodule number on Clark and obliterates the peaks observed at 22°C and 27°C. The resulting nodule pattern is analogous to an anomalous pattern on Vicoja soybean inoculated with *R. fredii* strain 191 (Heron and Pueppke, 1984). Delayed nodulation mutant HS111 produces a similar scattered and downwardly displaced pattern on Essex soybean (Halverson and Stacey, 1984). The striking similarity of these diverse combinations indicates that strain, environment, and strain X cultivar interactions all exert a major influence on regulation of successful infections.

Nodule number varies as an inverse function of temperature in all strain X isoline combinations, suggesting that the $rj_1rj_1$ genotype does not alter the inherent thermal sensitivity of nodulation of Clark. In comparison to Clark, however, Clark-$rj_1$ preferentially forms nodules on secondary roots. Nodulation of the primary roots of this isoline consequently is sparse, and

interpretation of the nodule profiles is difficult. The majority of the primary root nodules on $rj_1$, nevertheless, are positioned near the RTM. Thus, although the final number of nodules is attenuated in the $rj_1$ isoline, nodules are initiated in the usual places (Bhuvaneswari et al., 1980). It remains to be seen whether nodule initiation events are similar at the cellular level in the 2 isolines.

The kinetics of adsorption of the two tested *Rhizobium* strains to Clark roots are roughly similar to those of four different strains to Hardee soybean roots (Pueppke, 1984b). The $rj_1$ gene appears to markedly influence *Rhizobium* adsorption, but it does so in an unexpected manner. Binding of nonnodulating strain 110 is significantly elevated, but that of overcoming strain 94 is unaffected by the gene. Thus, although adsorption of strain 94 to Clark-$rj_1$ is temperature-sensitive, the temperature-sensitivity of nodulation is precisely opposite that of adsorption. Similar numbers of rhizobia are recovered from the roots of $Rj_1$ and $rj_1$ plants in the greenhouse and field (Clark, 1957). Moreover, Clark-$rj_1$ actually maintains substantially higher populations of rhizobia in the rhizosphere than does Clark for 45 of the first 60 days of growth (Elkan, 1962). These observations, in conjunction with our results, suggest that the limiting step in nodulation of the $rj_1$ isoline occurs postadsorption.

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