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Responses of Rj_1 and rj_1 Soybean Isolines to Inoculation with *Bradyrhizobium japonicum*[1]

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ABSTRACT

We evaluated the symbiotic phenotypes of nodulation-restrictive and normal soybean isolines by inoculating Clark (genotypically Rj_1Rj_1) and mutant Clark-rj_1 (genotypically rj_1rj_1) seedlings in plastic growth pouches. Nodules first appeared on Clark seedlings inoculated with *Bradyrhizobium japonicum* USDA 94 after 6 days. The mean number of nodules per plant was 13.9 ± 0.8 after 24 days. In contrast, Clark-rj_1 seedlings first nodulated at 12 days, and the mean number of nodules per plant was only 1.7 ± 0.3 at 24 days. Segments from infectible zones of primary roots, *i.e.* near the position occupied by the root tip at the time of inoculation, were sectioned serially. Clark roots contained cortical cell divisions and a few infection threads in question mark-shaped root hairs by 2 days after inoculation. Typical nodules developed soon thereafter. Analogous serially sectioned segments from Clark-rj_1 roots lacked these responses. This prompted us to section nodules and adjacent tissues from other parts of Clark and Clark-rj_1 roots. Clark roots contained cortical cell divisions, many associated with infected root hairs. Cortical cell divisions occasionally were present in Clark-rj_1, and a few infection threads were visible in surface cells. The presence of infection threads within Clark-rj_1 nodules was confirmed by transmission electron microscopy. Thus, although *B. japonicum* USDA 94 fails to elicit the wild-type spectrum of responses in the infectible zones of primary roots, it can infect Clark-rj_1 via infection threads.

Symbiotic interaction between legume roots and rhizobia culminates in the formation of nitrogen-fixing nodules. These complex structures are the products of two interacting genetic systems—that of the plant and that of the microbe. Molecular analysis of bacterial genes required for nodulation is well under way, and the symbiotic phenotypes of several genetically defined *Rhizobium* mutants have been examined in some detail. These include failure to nodulate, delayed or attenuated nodulation, abnormal nodule development, and altered host range (for example, 1, 9, 16, 30).

Although not well understood, symbiotically altered plant mutants occur naturally and can be induced by chemical treatments (13, 19, 20). One of the most widely recognized such mutants is the so-called nonnodulating soybean first described by Williams and Lynch (31). Such plants carry a spontaneous mutation at the allele designated rj_1. Homozygous recessive individuals do not nodulate in the field. Under experimental conditions, however, they form varying, but usually small numbers of apparently normal nodules in combination with certain *Bradyrhizobium* strains (5, 7, 8, 10, 17, 21). The relatively inefficient, strain-specific nodulation of rj_1rj_1 genotypes can be explained in two ways. One is that the rj_1rj_1 genotype reduces the frequency of nodulation and somehow renders it strain-specific, but does not influence the normal nodule developmental sequence. Alternatively, rj_1rj_1 may inactivate the normal nodulation pathway and thereby unmask a second nodulation mechanism that is similar to that described in peanut, a legume that also is nodulated by bradyrhizobia (6).

Normal nodule development in soybean is reasonably well understood and involves bacterially induced divisions of cortical cells, root hair curling, infection thread biogenesis and elongation, release of bacteria into dividing cortical cells, and continued proliferation of plant and bacterial cells to form the functional nodule (3, 4, 18, 26, 27). We systematically compared inoculated roots of rj_1rj_1 soybean with those of normal Rj_1 soybean. Our objective was to discover how the rj_1rj_1 genotype influences the root's response to rhizobia, especially with respect to nodule initiation.

MATERIALS AND METHODS

Bradyrhizobium japonicum USDA 94 and USDA 110 were from H. H. Keyser, USDA-ARS, Beltsville, MD. The bacteria were stored in glycerol at −70°C and maintained as stock cultures on YEM[2] slants (28) at 4°C. Seeds of Clark soybean (*Glycine max* [L.] Merr.) and its isoline, L63-1889, were from R. L. Bernard, USDA-ARS, University of Illinois, Urbana, and D. A. Phillips, Department of Agronomy and Range Science, University of California, Davis. Clark is genotypically Rj_1Rj_1 and nodulates normally with USDA 94 and USDA 110. The isoline, here designated Clark-rj_1, is genotypically rj_1rj_1. It forms a few nodules with USDA 94 and none with USDA 110 (21).

Seeds were surface-disinfested by consecutive 5 min immersions in 50% ethanol and 1.05% sodium hypochlorite followed by copious rinses in deionized water. Disinfested seeds were transferred aseptically to water agar and germinated in the dark for 2 to 3 d. Bacterial inoculum was produced in liquid gluconate-mannitol medium as described previously (21). Bacteria were harvested by centrifugation at 7000*g* and adjusted turbidimetrically to 5×10^8 to 1×10^9 cells/ml (except where noted) in Jensen's nitrogen-free solution (28). Seedling roots were dipped into the bacterial suspensions and the seedlings immediately transferred to autoclaved plastic growth pouches (Northrup King, Minneapolis, MN) containing Jensen's solution. The position of the primary RT was marked on the surface of the pouch, and the plants were incubated as described previously (23). For time

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[2] Abbreviations: YEM, yeast extract-mannitol; RT, root tip; TEM, transmission electron microscopy.

course experiments, seedling roots were examined on alternate days between 6 and 24 d after inoculation. The position of each new nodule was marked on the surface of the pouch with waterproof ink.

Three groups of tissue samples were examined by light microscopy. The first consisted of segments of primary roots extending from 1 cm above to 2 cm below the RT mark. These segments were harvested 4 d after inoculation. After staining with toluidine blue, each segment was bisected longitudinally with a scalpel. The sections then were examined for the presence of curled root hairs and infection threads (23). Tissues to be used for serial sectioning were harvested 2, 4, 10, and 14 d after inoculation. Segments of the primary root extending from 1 mm above to 1 cm below the RT mark were excised and fixed overnight in 100 mM sodium cacodylate buffer (pH 7.0) containing 2% glutaraldehyde. Nodulated segments from other parts of roots inoculated with USDA 94 were fixed similarly. After rinses in the buffer, the tissues were dehydrated in an ascending ethanol series, transferred to chloroform, and infiltrated with Paraplast (24). Serial, 15-μm longitudinal sections were affixed to slides and stained with safranin-fast green (24). Sections were examined at magnifications ranging from ×40 to 1000 with a Leitz Dialux 20 microscope. Freehand sections of nodules also were examined by light microscopy. The distal tips of individual nodules were excised with a sharp scalpel, stained with toluidine blue, and examined immediately by interference-contrast microscopy.

Nodules from plants inoculated with USDA 94 were examined by TEM. Nodules from Clark seedlings (harvested 5–15 d after inoculation) and from Clark-rj_1 plants (harvested 9–32 d after inoculation) were submerged and quartered in 100 mM sodium cacodylate buffer (pH 6.8) containing 3% glutaraldehyde. Tissues were fixed for 4 h at 4°C and postfixed in pH 6.8 buffer containing 1% OsO_4. The samples were dehydrated in a graded ethanol series, infiltrated, and embedded in Spurr's resin. Ultrathin sections (600–800 Å) were cut with an LKB microtome and stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 100B microscope operating at 100 kV.

RESULTS

Time Course of Nodule Appearance. The time-course of nodulation of Clark and Clark-rj_1 soybean in growth pouches is given in Figure 1. Nodules on Clark first were visible 6 d after inoculation. Nodule number per plant increased rapidly until 10 d after inoculation, when a plateau was reached. The mean number of nodules per Clark plant at 24 d after inoculation was 13.9 ± 0.8 (SE). Primary root nodules as a percentage of total nodules declined with time, from 100% (6 d) to 60 to 65% (12–24 d). Clark-rj_1 seedlings formed only a few nodules under these conditions (Fig. 1). The first nodules appeared 12 d after inoculation, but the mean total number of nodules per plant was only 1.7 ± 0.3 at 24 d. Although the first few Clark-rj_1 nodules were on primary roots, lateral roots contained more than 75% of the nodules by 20 d after inoculation.

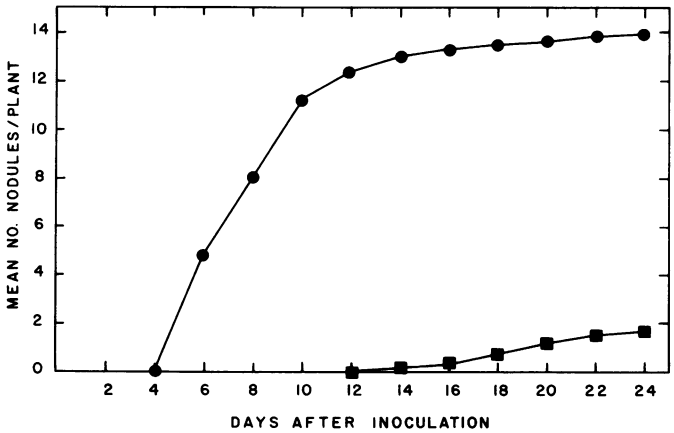

FIG. 1. Time course of nodule appearance on Clark (●) and Clark-rj_1 (■) soybean roots inoculated with USDA 94. Each point represents the mean value from a total of 42 plants in two replicate experiments.

Responses of Primary Roots to Inoculation. Tissues from the vicinity of the RT mark initially were surveyed 4 d after inoculation by the rapid procedure of Pueppke (23). Short root hairs, curled into the shapes of question marks, were apparent on each of 18 Clark seedlings inoculated with USDA 94 and on each of 18 seedlings inoculated with USDA 110. Infection threads were visible within one or more such root hairs on the majority of these seedlings. In contrast, 27 Clark-rj_1 seedlings that had been inoculated with USDA 94 lacked both question mark-shaped root hairs and infection threads.

Serial sectioning of tissues embedded in paraffin was used to quantitatively and systematically analyze the responses of roots to inoculation. The data from 11 Clark seedlings inoculated with USDA 94 are summarized in Table I. Two hundred forty-two infected root hairs were resolved, including some in tissues harvested only 2 d after inoculation. All infection threads were associated with centers of cortical cell divisions, which were classified according to the scheme described by Calvert *et al.* (4). Sixty to 70% of the infection threads were in division centers classified as stage II, *i.e.* the outer cortical cells had divided only six to eight times. Another 5 to 10% were in stage III division centers, in which inner cortical cells had begun to divide (Table I). These percentages remained relatively constant at different times after inoculation, indicating that most infections in Clark are arrested at an early developmental stage. Analogous root segments from a total of six uninoculated Clark seedlings (two each at 2, 4, and 10 d after inoculation) were devoid of cortical cell divisions, infection threads, and nodules.

A total of 24 Clark-rj_1 seedlings were serially sectioned. Seedlings inoculated with USDA 94 were harvested 2 d (four plants), 4 d (eight plants), 10 d (three plants), and 14 d (three plants) after inoculation. Pairs of uninoculated controls were harvested at each time. All 24 root segments were devoid of centers of cortical cell division, infection threads, and nodules. There were no obvious anatomical differences between these roots and those of Clark.

Nodule Development. Failure to detect any responses of Clark-rj_1 tissues to inoculation in the vicinity of the RT mark prompted us to examine root segments containing nodules of various sizes, irrespective of their positions on roots. Clark nodules of similar sizes were harvested as controls. Infection threads initially were sought by making freehand sections of fresh tissues. Distal tips of small nodules (<2 mm in diameter) were excised, stained, and examined immediately by interference-contrast microscopy. This method permits rapid identification of root hairs if they are positioned distally on the nodule. Curled root hairs with infection threads were present in sections from 13 of 20 Clark nodules (65%). Similar sections of 27 Clark-rj_1 nodules were examined, but none contained curled root hairs with identifiable infection threads. This negative result could be explained in three ways: (a) infection threads are absent; (b) infection threads are present but not in curled root hairs; (c) infection threads are present in curled root hairs, but the sites of infection always are lateral or near the bases of the nodules.

We distinguished among these possibilities by serially sectioning about two dozen paraffin-embedded Clark-rj_1 root segments containing nodules that ranged in size from <1 to about 3 mm in diameter. Similarly nodulated Clark root segments were harvested as controls. We realize that the quality of preservation of such tissues is inferior to that of materials embedded in plastic,

Table I. *Infected Centers of Cortical Cell Division in Clark Soybean Inoculated with B. japonicum USDA 94*

Time after Inoculation	No. Plants Examined	Percentage of Infection Threads in Division Centers of Stage[a]						Total No. Threads
		I	II	III	IV	≥ V	NA	
d								
2	4	13	68	13	6	0	0	16
4	5	2	60	16	9	7	6	143
10	2	4	61	16	6	7	6	83

[a] Centers of division are classified according to the scheme of Calvert *et al.* (4). Stage I, 4 to 8 daughter cells in hypodermis; stage II, 6 to 8 divisions of outer cortical cells; stage III, inner cortical cells have begun to divide; stage IV, nodule meristem visible. Later stages describe erumpent nodules of progressively greater degrees of development. NA, not assignable.

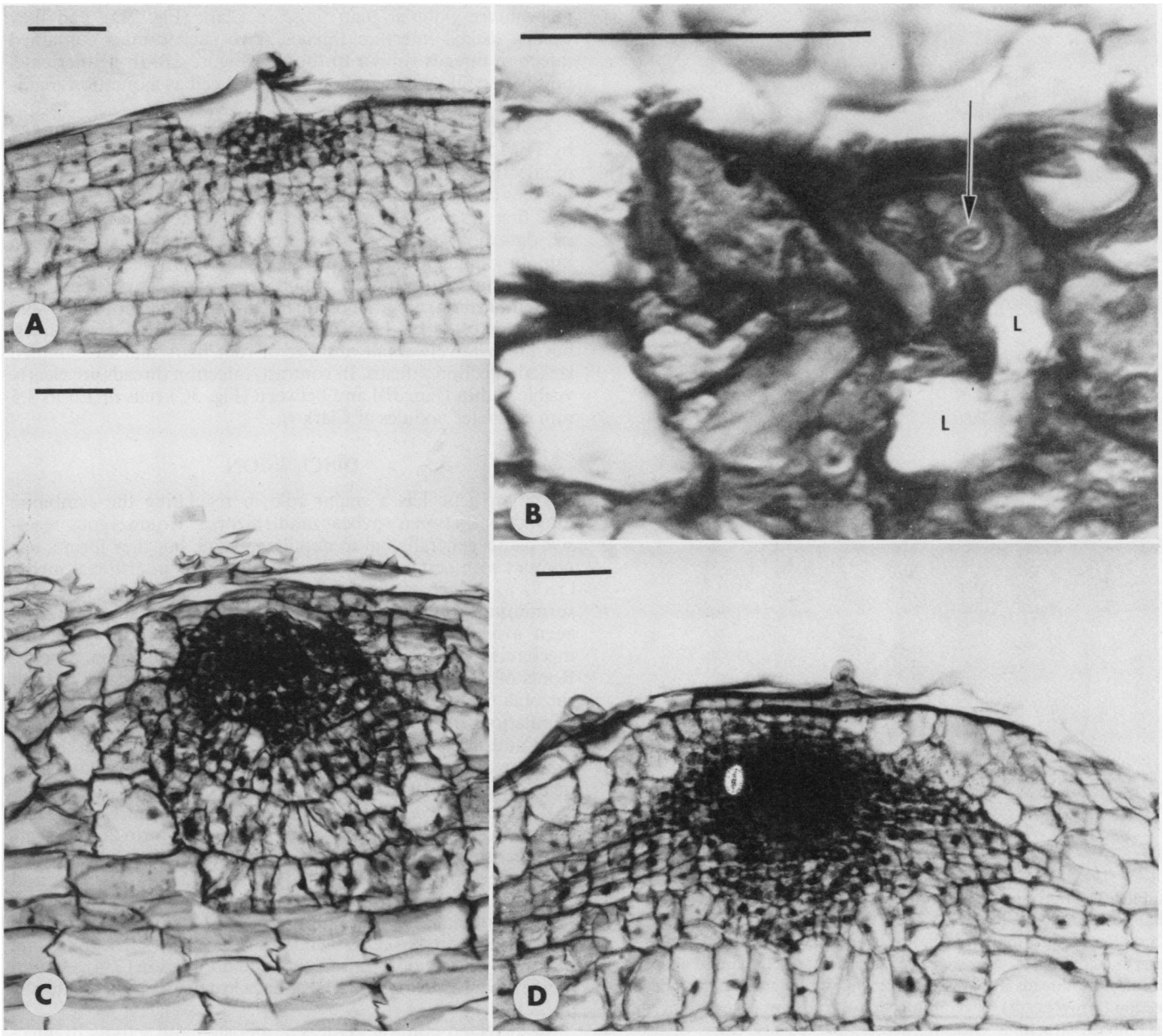

FIG. 2. Responses of Clark and Clark-rj_1 roots to inoculation with *B. japonicum* USDA 94. Tissues were harvested 22 to 25 d after inoculation. A, Center of cortical cell division in Clark. A question mark-shaped root hair containing stalked infection threads is centered above the zone of dividing cells, which has not yet organized into the nodule meristem. B, Coiled infection threads (arrows) within a surface cell of the distal tip of a Clark-rj_1 nodule. A pair of locules (L) is adjacent to the infected cell. C, A globular nodule meristem embedded in the outer cortex of Clark-rj_1 lateral root. D, A shield-shaped nodule meristem just distending the root surface of Clark. Scale bars = 20 μm.

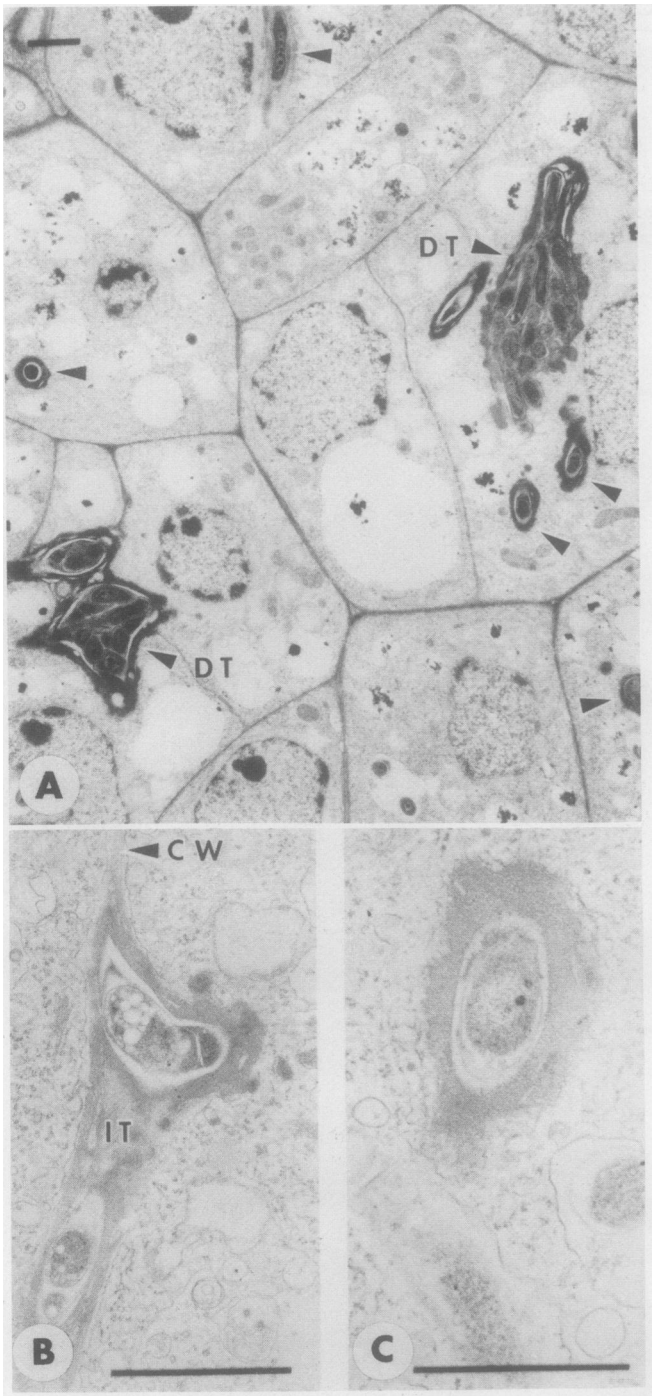

FIG. 3. Transmission electron microscopy of nodules (1.5–3.5 mm in diameter) produced by USDA 94. Clark nodules were harvested 13 to 15 d after inoculation, and Clark-rj_1 nodules were harvested 17 to 19 d after inoculation. A, Meristematic cells in Clark contain an abundance of infection threads in both cross-section and longitudinal or tangential section (arrow heads). Several infection threads are distended or appear as bulbous structures encasing densely packed aggregates of bacteria (DT). B, Tangential section of an intercellular infection thread (IT) in Clark-rj_1. CW = well wall. C, Cross-section of an intracellular infection thread in Clark-rj_1. Scale bars = 2 μm (A and C) and 1 μm (B).

but chose paraffin because it facilitated detailed, systematic analysis of many samples.

As expected, nodule development in Clark was morphologically similar to that reported previously in other soybean cultivars (3, 4, 18; data not shown). Young centers of Cell division were abundant and often associated with prominantly infected root hairs (Fig. 2A). Although most Clark-rj_1 nodules lacked curled root hairs that could be identified as sites of infection, threads were observed in a few small nodules. Figure 2B shows a group of coiled infection threads in a cell on the distal surface of a 0.5 mm diameter Clark-rj_1 nodule. Because the plane of sectioning was perpendicular to the nodule surface, we could not identify the infected cell as a deformed root hair or a simple epidermal cell. A few preemergent centers of cortical cell division were found adjacent to Clark-rj_1 nodules. An example of the youngest such division centers is shown in Figure 2C. Such centers appear to be more globular than those in Clark (Fig. 2D), and they usually lacked infection threads. Two such centers contained infection threads similar to those in Figure 2B. In neither case, however, could we identify the infected cell as a question mark-shaped root hair.

Nodules produced by USDA 94 on Clark-rj_1 were examined by TEM to provide high resolution confirmation of the presence of infection threads. Reference sections of newly emergent Clark nodules contained curled root hairs with infection threads of the type first described ultrastructurally by Turgeon and Bauer (26, 27; data not shown). More mature nodules of Clark (1.5–3.5 mm in diameter) appear to be typical examples of soybean nodules. Infection threads, which sometimes were distended by packed bacteria, are abundant (Fig. 3A). Curled root hairs were readily detected by TEM on the surfaces of Clark-rj_1 nodules, but the relatively small number of root hairs that we sampled lacked infection threads. In contrast, infection threads are clearly visible within (Fig. 3B) and between (Fig. 3C) cells in 1.5 to 3.5 mm diameter nodules of Clark-rj_1.

DISCUSSION

The rj_1 gene has a major role in regulating the symbiotic interaction between soybean and rhizobia. Homozygous recessive plants generally fail to nodulate in soil, but they form a few nodules with certain *Bradyrhizobium* strains in artificial media (5, 7, 31). Such strains are termed overcomers because they surmount the effects of the rj_1 gene. Although rj_1 soybeans have been available since the early 1950s, little is known of the mechanism by which the gene alters the nodulation process. Roots of rj_1 plants are not defective in their capacities to bind rhizobia (21), and Rj_1 and rj_1 root systems support equivalent populations of rhizobia (5). Efforts to correlate the rj_1 genotype with unusual physiological characteristics of the plant or the presence of substances that inhibit rhizobia generally have been inconclusive (5, 10–12, 14, 15, 25).

We sought to define the symbiotic consequences of rj_1 by comparing the responses of wild-type Clark soybean and its near-isogenic isoline, Clark-rj_1, to *B. japonicum* USDA 94, an overcomer. Seedlings were incubated in plastic growth pouches and infectible zones, *i.e.* root segments that either lacked root hairs or were populated by immature root hairs at the time of inoculation, were analyzed. In wild-type soybean, such zones are maximally susceptible to infection and nodulation (2, 4, 23), and their responses to rhizobia have been catalogued systematically (4, 22).

We detected multiple centers of meristematic activity in infectible zones of Clark roots by 2 d after inoculation. Curled root hairs were associated with many of the division centers, and a few such hairs already were infected. Typical nitrogen-fixing soybean nodules (3, 18, 29) appeared soon thereafter. Although our observations of inoculated Clark seedlings generally agree

with those of Calvert *et al.* (4), who examined the interaction of *B. japonicum* I-110ARS with Williams soybean, an unusually large number of infections in Clark ceased development very early, at stage II.

Evaluation of an analogous series of 24 Clark-rj_1 root segments inoculated with USDA 94 failed to reveal any cellular responses, even though such segments can nodulate infrequently after long periods of incubation (21). We found no evidence for attempted or unsuccessful infection of young primary roots, *i.e.* aborted infection threads or abnormal centers of cell division. Thus, one obvious characteristic of the rj_1rj_1 genotype is blockage of the onset of wild-type cellular responses in the infectible zone of primary roots.

The observed lack of responsiveness of infectible zones of Clark-rj_1 to inoculation raises intriguing questions about the mechanism by which overcomers occasionally form nodules on this cultivar. We serially sectioned a series of nodule containing root segments of Clark-rj_1 to determine if infection threads are present. Although infection threads were observed, the infected cells were not obviously curled root hairs, and threads were very difficult to detect by light microscopy. This suggests that the rj_1rj_1 genotype also influences infection thread development. The presence of threads in inner nodule cells, however, was confirmed by TEM. This observation rules out the hypothesis that the rj_1rj_1 condition abolishes infection via threads, leading to obligate nodulation via crack entry, as in peanut (6).

We conclude that rj_1rj_1 genotypes display complicated symbiotic abnormalities. The overall effect is regulatory: nodule number is greatly reduced, and nodulation is delayed and shifted to lateral roots. Others have shown that rj_1 renders the nodulation process strain-specific and sensitive to the presence of soil (5, 6). We have provided evidence here that overcoming strains fail to elicit cellular responses to inoculation at the expected times and places. The overcoming strain, nevertheless, forms a few nodules, at least some of which contain infection threads.

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