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The *Rj4* allele in soybean represses nodulation by chlorosis-inducing bradyrhizobia classified as DNA homology group II by antibiotic resistance profiles

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Summary. To determine the relationship between nodulation restriction by the *Rj4* allele of soybean, rhizobitoxine-induced chlorosis, and taxonomic grouping of bradyrhizobia, 119 bradyrhizobial isolates were tested in Leonard jar culture for nodulation response and chlorosis induction. In addition to strain USDA 61, the strain originally reported as defining the *Rj4* response, eight other isolates (i.e., USDA 62, 83, 94, 238, 252, 259, 260, and 340) were discovered to elicit the nodulation interdiction of the *Rj4* allele. Only 16% of all the bradyrhizobial strains tested induced chlorosis, but seven of the nine strains (78%) interdicted by the *Rj4* allele were chlorosis-inducing strains. Furthermore, in tests for antibiotic resistance profile, eight of the nine interdicted strains (89%) were classed in DNA homology group II. This evidence suggests that the *Rj4* allele has a positive value to the host plant in shielding it from nodulation by certain chlorosis-inducing bradyrhizobia of a DNA homology group with impaired efficiency of nitrogen fixation with soybean.

Key words: Genetics – Symbiosis – Nitrogen fixation – Coevolution

Introduction

Vest and Caldwell (1972) reported the identification in soybean [*Glycine max* (L.) Merr.] of a qualitatively inherited gene, designated *Rj4*, conditioning an ineffective nodulation response specifically with bradyrhizobial strain USDA 61 of the Beltsville Culture Collection. The *Rj4* allele, first identified in the cultivar "Hill", produced an ineffective nodulation response described as the presence of numerous small cortical proliferations or protuberances on the roots. Evidently, nodulation was initiated but aborted at an early stage, although occasionally a few nodules of normal appearance were produced. Ineffectively nodulated plants were reduced in growth and had the same appearance as N-deficient uninoculated control plants. The cultivar Hill nodulates normally when inoculated with other strains of bradyrhizobia such as USDA I-110 or USDA 142. Strain USDA 61 is capable of nodulating and fixing nitrogen with soybean cultivars homozygous for the recessive allele *rj4*.

Traditionally, the *Rj4* allele has been regarded as an aberration, supposedly resulting from a mutation that blocks the normal process of nodulation and nitrogen fixation. Devine (1987) reported that ineffective nodulation response with bradyrhizobial strain USDA 61 was the predominant response (63%) in *Glycine soja* (Sieb. and Zucc.), the wild progenitor species of the domesticated soybean. In a survey of over 800 domesticated soybean lines from 12 Asiatic nations, Devine and Breithaupt (1980b, 1981) found that the ineffective nodulation response with strain USDA 61 was the predominant response in lines from the Southeast Asian nations: Burma and Malaya (71.7%), Indonesia (64.8%), Thailand (66.9%), and Vietnam (97.4%). A lower frequency was found in lines from northern Asia. The fact that the *Rj4* response was the most common response occurring in the wild progenitor species of soybean and in lines from southeast Asia suggests the possibility that some selection pressure has been exerted in the ecosystem to maintain the gene frequency at such a level.

Some strains of bradyrhizobia that nodulate soybean, including strain USDA 61, also produce a chemical

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termed rhizobitoxine (Owens and Wright 1965; Devine and Breithaupt 1980a). Rhizobitoxine interferes with chlorophyll synthesis in young soybean leaves, producing a distinct foliar clorosis. Chlorosis-inducing strains of bradyrhizobia nodulate and fix nitrogen in symbiosis with cowpea in such a manner as to produce very vigorous growth, but do not produce chlorosis symptoms on cowpea (Devine 1988). Thus, these bradyrhizobia would appear to have a physiology that is more compatible with cowpea than soybean.

There is conclusive genetic evidence at the molecular DNA level that these chlorosis-inducing bradyrhizobia are significantly different from non-chlorosis-inducing bradyrhizobia. Hollis et al. (1981) used DNA:DNA homology to characterize a number of the bradyrhizobia that nodulate soybean. They found two major subgroups and suggested that the magnitude of the difference between these subgroups warranted separate taxonomic classification. Devine et al. (1988) have shown that none of the strains in group I produce chlorosis symptoms, but many of the strains in group II produce chlorosis. Apparently, then, the bradyrhizobia producing rhizobitoxine-induced chlorosis symptoms on soybean belong to a distinct DNA homology group. Devine et al. (1983) established that several chlorosis-inducing strains form at least rudimentary nodules on peanut. Therefore, a separate group of bradyrhizobia, more properly compatible with legumes other than soybean, have an extended ability to nodulate soyean and subsequently fix nitrogen, albeit imperfectly, with soybean. The most striking manifestation of the lack of adequate compatibility of these bradyrhizobia with soybean is the production of chlorosis. This foliar chlorosis was reported as having occurred on soybeans in U.S. production fields in the southeastern states of Alabama, Arkansas, Florida, Georgia, Mississippi, North and South Carolina, and Tennessee, and in California (Erdmann et al. 1957). Thus, in the southeastern Unites States a significant portion of the bradyrhizobial population potentially capable of nodulating soybean may be poorly adapted to efficient symbiosis with the soybean host.

To study the underlying biological relationships between rhizobitoxine production and taxonomic grouping of the microsymbiont and the soybean nodulation restrictive gene, *Rj4*, precisely defined genetic stocks were required. A 10-year program of backcrossing and self-pollination has produced a set of isogenic soybean lines differing in the *Rj4* versus *rj4* alleles (Devine and O'Neill 1986). These lines are calculated to be 99.95% identical in their nuclear DNA and completely identical in their cytoplasmic DNA. Since these isolines essentially differ only in the allele present at the *Rj4* locus, any difference between the lines in nodulation response with individual rhizobial strains can be attributed specifically to the *Rj4* allele.

With these lines as biological tools, it is possible to ascertain whether other strains of bradyrhizobia, in addition to USDA 61, specifically elicit the *Rj4* ineffective nodulation response. This study was undertaken to determine if other strains elicited the *Rj4* response and, if so, identify the characteristics of such strains.

Materials and methods

Eighty-seven bradyrhizobial strains recently isolated from Asia and 32 strains from the Beltsville Rhizobium Culture Collection (Keyser and Griffin 1987) were tested for their response to the soybean nodulation incompatibility gene *Rj4* and for their ability to produce a rhizobitoxine-induced foliar chlorosis on a rhizobitoxine-sensitive soybean line.

Bacterial strains

Strains were grown in A1E broth (Kuykendall 1979) amended with 0.1% gluconate (A1EG) at 30 °C for 6–8 day prior to inoculation of seeds.

Plant lines

Three soybean lines were used: BARC-2(*Rj4*), BARC-3(*rj4*), and N53-3494. BARC-2(*Rj4*) and BARC-3(*rj4*) are near-isogenic lines differing in the allele present at the *Rj4* locus, i.e. *Rj4* versus *rj4* (Devine and O'Neill 1986). These lines were used to determine the reaction of the bacterial strains specifically to the particular allele present at the *Rj4* locus. A strain that produced effective nodulation on the BARC-3(*rj4*) line established its capacity to nodulate plants with the host genetic background common to both isogenic lines. If the same strain produced an ineffective nodulation response with the isoline carrying the *Rj4* allele, i.e., BARC-2(*Rj4*), then that response can be ascribed to the interaction of that strain specifically with the *Rj4* allele. The soybean line N53-3494 has a propensity to exhibit rhizobitoxine-induced chlorosis and was used to test for the in vivo expression of this response (Johnson and Means 1960).

Plant tests

Seeds of each of the three lines were surface-sterilized by immersion in 50% (v/v) ethanol for 25 s, rinsed, and planted in sterile Leonard jar assemblies using vermiculite as the support medium (Leonard 1943). Individual Leonard jar assemblies were planted with six seed of a single plant line and inoculated with a stationary phase broth culture of a single bacterial strain. After planting, the surface of vermiculite was covered with 1/2 inch of sterilized perlite as a protection from air-borne contamination. All 119 bradyrhizobial strains were tested on each of the three plant lines in this manner. Strain USDA 61 was used as a reference control. USDA 61 is the type strain for the *Rj4* reaction and also produces rhizobitoxine-induced foliar chlorosis at approximately 4–5 weeks of growth. Scoring for nodulation response with BARC-2(*Rj4*) and BARC-3(*rj4*) was done after 28 days plant growth. Observations for chlorosis induction were made after 4, 5, and 6 weeks growth. Bradyrhizobial strains producing a differential nodulation response on BARC-2(*Rj4*) and BARC-3(*rj4*) or rhizobitoxine-induced chlorosis on N53-3494 were retested to confirm the observations. Chi-square analysis was used to test for the independence of occurrence in the bradyrhizobia of chlorosis induction and reaction to the *Rj4* allele.

Antibiotic resistance tests

These determinations were made as described previously (Kuykendall et al. 1988), except that erythromycin was used at a concentration of 500 μg/ml. Resistance to a particular concentration of antibiotic was defined as the ability of a strain to form colonies at that concentration on an A1EG solid agar medium containing that antibiotic.

Results

In addition to the strain originally reported as defining the *Rj4* response, strain USDA 61, eight other strains were discovered to elicit this response (Table 1 and 2). Strains interdicted by the *Rj4* allele were found among both Asian and U.S. isolates, as were chlorosis-inducing strains. Seven, or 78%, of the nine strains interdicted by the *Rj4* allele were chlorosis-inducing strains, although only 19 of the total 119 strains tested, or 16%, were chlorosis-inducing strains. Only 2% of the non-chlorosis-inducing strains were interdicted by the *Rj4* allele, although 36% of the chlorosis-inducing strains were interdicted.

If the occurrence of chlorosis induction was independent of the occurrence of reaction to the *Rj4* allele, then the frequency of chlorosis induction would be expected to be the same in *Rj4* reactive strains as in the total population. Using this frequency (16%) to calculate the expected values, the observed classification for chlorosis induction of the nine interdicted strains was tested by Chi-square analysis (Table 3). The substantial deviation from the expected that was obtained clearly indicates that chlorosis induction and interdiction by the *Rj4* allele are associated traits.

We used the antibiotic resistance profiles as described by Kuykendall et al. (1988) to distinguish the DNA homology group I and II strains. Bradyrhizobial strains 61, 83, 94, 238, 252, 260, and 340 possessed high level intrinsic resistances to each of the following antibiotics: erythromycin, chloramphenicol, tetracycline, streptomycin, and rifampicin. Strain 62 was sensitive to these antibiotics. Therefore, eight of the nine *Rj4*-interdicted strains discovered, or 89%, were evidently DNA homology group II strains based on their antibiotic resistance profile characteristics.

Table 1. Interaction with the *Rj4* allele in soybean of chlorosis-inducing and non-chlorosis-inducing bradyrhizobial strains isolated from Asia or other locations

Rj4 response	Chlorosis-inducing	Non-chlorosis-inducing	Total
Asiatic isolates			
Interdicted	4	1	5
Noninterdicted	2	80	82
Total	6	81	87
Other isolates			
Interdicted	3	1	4
Noninterdicted	10	18	28
Total	13	19	32
Total isolates			
Interdicted	7	2	9
Noninterdicted	12	98	110
Total	19	100	119

Table 2. The origin and rhizobitoxine-induced chlorosis reaction of bradyrhizobial strains eliciting the nodulation interdiction response of the *Rj4* allele

Strain	Origin	Chlorosis-induction
USDA 61	North Carolina, USA	+
USDA 62	North Carolina, USA	−
USDA 83	Maryland, USA	+
USDA 94	North Carolina, USA	+
USDA 238	Hokkaido, Japan	+
USDA 252	Yentai, China	+
USDA 259	Shanghai, China	+
USDA 260	Shanghai, China	+
USDA 340	Japan	−

Table 3. Test for the independence of *Rj4* interdiction and chlorosis induction by Chi-square analysis using the ratio of chlorosis-inducing versus non chlorosis-inducing strains of bradyrhizobia among the *Rj4* reactive strains

	Rj4 interdicted strains	$\chi 2^a$	*P*
Chlorosis-inducing strains	7	21.5	
Nonchlorosis-inducing strains	2	4.1	
Total	9	25.6	$<.001$

[a] Expected 119:19 ratio based on the frequency of chlorosis-inducing strains in the total population sampled

Discussion

The *Rj4* allele has been considered to be a strain-specific nodulation restriction gene arising from a random mutation. In this paper we have demonstrated a broad range of restriction, principally among strains that induce chlorosis, suggesting that the *Rj4* allele functions as a host range specificity gene. The frequency of the *Rj4* allele among *G. soja* lines lends credence to this view, i.e., that the *Rj4* allele represents the norm while the *rj4* allele represents a mutation from the norm.

Fuhrmann (1990) collected 360 bradyrhizobial isolates from nodules in soybean production fields at 18 farms in Delaware, USA. Fully one-third of these isolates had the colony morphology (designated LW) that was characteristic of the chlorosis-inducing bradyrhizobia and was positively correlated with DNA homology

group II. This indicates that a significant portion of the bradyrhizobial population nodulating the soybean crop in Delaware are DNA homology group II strains. In Fuhrmann's study, the symbiotic nitrogen fixation by the LW group was significantly inferior to the fixation by the other colony types. Thus, the symbiosis formed by the DNA homology group II strains with soybean is defective in the complex and central function of nitrogen fixation. It would appear, then, that the expression of rhizobitoxine-induced chlorosis is a particularly obvious manifestation of a more pervasive metabolic maladaptation of the chlorosis-inducing bradyrhizobia and the soybean macrosymbiont.

Devine et al. (1988) established that in vivo induction of chlorosis was frequently characteristic of bradyrhizobia in DNA homology group II, referred to by Hollis et al. (1981) as *Bradyrhizobium* species. In the present study, eight of the nine strains interdicted by the *Rj4* allele have been identified as members of this group by antibiotic resistance profiles. This group, in which chlorosis induction frequently occurs, also appears to carry genetic material that interacts with the *Rj4* allele resulting in the interdiction of normal nodulation.

In the research presented here, we have established that the *Rj4* allele principally interdicts nodulation by chlorosis-inducing bradyrhizobia, and thus would have a positive value to the plant in protecting it from nodulation by bradyrhizobial strains poorly adapted to symbiotic N fixation with this host germ plasm. Therefore, the traditional view of the *Rj4* allele as a mere genetic aberration is clearly unwarranted. Rather, the *Rj4* allele is an example of a host gene that functions to exclude from nodulation rhizobial strains lacking a suitable genetic profile for efficient symbiosis with a particular host population, as suggested by Devine (1985b).

The *Rj4* allele did not interdict nodulation by all chlorosis-inducing bradyrhizobia (only 36%), suggesting that the *Rj4* exclusion mechanism is not elicited specifically by the rhizobial gene(s) conditioning rhizobitoxine production in symbiosis per se, but by some other genetic component carried in high frequency in bradyrhizobial strains of DNA homology group II. This genetic component is apparently not exclusive to DNA homology group II, since one strain not in this group (USDA 62) was found to be interdicted by the *Rj4* allele.

Previous research has established that the ineffective nodulation response conditioned by the *Rj4* allele is more frequent than the effective nodulation response in the wild undomesticated soybean and in domesticated *G. max* lines from Southeast Asia (Devine 1987). This suggests that the bradyrhizobial populations in some areas of Asia exert a selection pressure on the soybean population to maintain the *Rj4* allele at such a high frequency. The recessive allele, *rj4*, that permits nodulation with chlorosis-inducing strains may be derived from an ancestral *Rj4* allele. This research provides evidence that genetic systems have evolved in nature that repress nodulation by less desirable bacteria. This evidence provides hope that breeders may construct similar systems for agronomic advantage (Devine and Breithaupt 1980b; Devine 1985a).

Assays of the lines in both the U.S. preliminary and uniform tests indicated that a progressive diminution in the frequency of the *Rj4* allele has occurred concomitant with breeding for agronomic performance in North America (Devine and Breithaupt 1981). If present trends continue, the *Rj4* allele may be lost from future cultivars. Soybean breeders, particularly those developing cultivars for the southeastern United States, should give serious consideration to conserving the *Rj4* allele in their breeding programs.

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