Limiting an Insect Infestation of Nitrogen-Fixing Root Nodules of the Pigeon Pea (*Cajanus cajan*) by Engineering the Expression of an Entomocidal Gene in Its Root Nodules

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A region of DNA which determined the production of the insecticidal toxin of *Bacillus thuringiensis* subsp. *israelensis* was cloned into a derivative of a broad-host-range group IncQ plasmid vector of gram-negative bacteria. The plasmid which we constructed was transferred by conjugative mobilization into a *Bradyrhizobium* species that nodulates pigeon peas. In this species the construction was maintained stably in the absence of selection and expressed the gene that was installed. Experiments in a greenhouse with the strain which we constructed indicated that this organism provides protection against root nodule damage by the larvae of the insect *Rivellia angulata* (Diptera).

The seeds of legume crops that are cultivated in the tropics often constitute an important part of human diets. One such crop is the pigeon pea (*Cajanus cajan*), which contains approximately 20% of its dry weight as protein. In several tropical regions, particularly in the Indian peninsula, this plant is an important source of protein, and current economic trends and statistical estimates of the Food and Agricultural Organization suggest that it will become more important. The pigeon pea can grow well under nitrogen-limiting conditions provided that its roots are nodulated by appropriate soil bacteria that are defined loosely as *Bradyrhizobium* species. These bacteria fix nitrogen in the nodules, and the fixed nitrogen is made available to the plants. In certain regions, the beneficial effects of this symbiotic nodulation and nitrogen fixation are thwarted by insects belonging to the species *Rivellia angulata* (Diptera: Platystomatidae) and other related species (3, 8). The larvae of these insects prefer feeding on the root nodule contents without causing any apparent damage to the roots, and it has been observed that young larvae that are denied access to nodules tend to die within a few days (3). Gowda and Siddapaji (S. T. K. Gowda and C. Siddapaji, Abstr. 2nd All-India Symp. Soil Biol. Ecol., p. 101, 1979) have also reported that they obtained increased pigeon pea yields when the number of *Rivellia* larvae was reduced by the application of soil insecticides. These reports and the observation that the soil bacterium *Bacillus thuringiensis* subsp. *israelensis* produces an endotoxin that upon ingestion is toxic to larvae belonging to the order *Diptera* prompted us to construct a derivative of an appropriate *Bradyrhizobium* sp. that carried and inherited a hybrid plasmid that expressed the insecticidal endotoxin. The ability of the *Bradyrhizobium* sp. which we constructed to control a *Rivellia* infestation and to prevent a decrease in crop growth was tested in greenhouse experiments. The results of these tests are encouraging and are reported in this paper.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and relevant properties of the *Bradyrhizobium* isolate and the *Escherichia coli* K-12 strains which we used or constructed and the

plasmid vectors which we used are shown in Table 1. Strains carrying plasmids or plasmid vectors are indicated below with the plasmid in parentheses.

Media and culture conditions. *E. coli* strains were grown in LB medium (6), *Bradyrhizobium* sp. was grown in YEM medium (13), and *B. thuringiensis* was grown in antibiotic medium no. 3 (Penassay broth; Difco Laboratories, Detroit, Mich.). All media were adjusted to pH 7.0, and liquid cultures were grown with aeration (shaking) at 37°C (*E. coli*) or 30°C (all other organisms). When antibiotics were used as supplements, they were added to presterilized (autoclaved) media at the following concentrations: ampicillin, 40 µg/ml; carbenicillin, 200 µg/ml; chloramphenicol, 50 µg/ml; and kanamycin, 50 µg/ml.

Other chemicals. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., T4 DNA ligase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and materials for molecular hybridization were purchased from Amersham Corp., Arlington Heights, Ill. Molecular weight standards for proteins were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

Bacterial mating. Hybrid plasmid pBRK4013 (A. Hamel, M. S. thesis, Carleton University, Ottawa, Canada, 1988), which carries the broad-host-range conjugative transfer region of plasmid RK2, the narrow-host-range replicon of pBR322 (2), and a gene for carbenicillin resistance, was used as a helper to provide functions for the transfer of the plasmids which we constructed from *E. coli* to *Bradyrhizobium* sp. Both the plasmid which we constructed and the helper plasmid were in the same *E. coli* strain, and the matings between *E. coli* and *Bradyrhizobium* sp. were performed by using a ratio of donors to recipients of 1:10. A total of approximately 10⁹ viable cells of the mixture were collected by suction on the surfaces of filters (pore size, 0.45 µm; Millipore Corp., Bedford, Mass.); the filters were placed with their cell sides up onto the surfaces of YEM agar plates, and the preparations were incubated at 30°C for 12 to 16 h. After this, the filters were agitated (vortexed) in 1.0 ml of sterile distilled water and plated onto the surfaces of YEM agar plates containing chloramphenicol and kanamycin for the selection of transconjugant *Bradyrhizobium* colonies. Colonies that grew were further purified, and replicas of

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TABLE 1. Bacterial strains and plasmids

Bacterium or plasmid	Relevant phenotype or phenotype conferred ^a	Source ^b	Reference
<i>Bradyrhizobium</i> sp. strain IC3554	Nodulates pigeon pea, Cm ^r	J. V. D. K. Rao, ICRISAT	
<i>B. thuringiensis</i> subsp. <i>israelensis</i> ONR 608	Toxic for larvae of dipterans	BGSC	
<i>E. coli</i> HB101	<i>hds20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	H. W. Boyer	2
Plasmids			
pKT230	Rep(RSF1010;pACY177) Km ^r Sm ^r Mob	M. Bagdasarian	1
pBRK4013	Rep(pMB1) Tra(RK2) Ap ^r in vitro cointegrate of pBR322 and pRK2013	A. Hamel	— ^c
pSY368	Rep(pMB1) Tox Ap ^r ; 6-kilobase DNA fragment containing the entomocidal gene(s) cloned into pUC13	T. J. Pollock	12

^a Abbreviations: Ap^r, ampicillin resistance; *ara*, arabinose; *gal*, galactose; *hds*, host specificity; Km^r, kanamycin resistance; *lac*, lactose; *mtl*, mannitol; *rec*, recombination; Rep, replicon; *rps*, ribosomal protein small; Tox, entomocidal toxin; Tra, conjugative transfer; *xyl*, xylose; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Mob, mobilization; *pro*, proline.

^b ICRISAT, International Crop Research Institute for the Semi-Arid Tropics, Patancheru, India; BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus.

^c A. Hamel, M.S. thesis.

these organisms were tested for their genetic markers and for the presence of the delta-endotoxin region by differential colony hybridization with DNA probes made from pSY368 and pBR322 (the vector used in the construction of pSY368).

Endotoxin production in *Bradyrhizobium* sp. Total cellular proteins were isolated from *Bradyrhizobium* strains as described by Xu and Gross (14) and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12.0% polyacrylamide gels (4). Samples were electrophoresed overnight at 50 V, electroblotted onto nitrocellulose filters (pore size, 0.2 µm), and probed with a polyclonal antibody prepared against the delta-endotoxin isolated from the *B. thuringiensis* strain (5). Immunochemical staining was performed by using a type RPN23 kit purchased from Amersham Corp. and by following the procedures suggested by the manufacturer, except that the incubation time to block nonspecific binding was 15 h at 4°C.

Insect infestation tests. Insect infestation tests were conducted as described by Sithanathan et al. (10) by using four pigeon pea seedlings (cultivar ICPL80) per 8-cm-diameter pot and replicating each treatment (Table 2), which was arranged in a factorial randomized block design in five pots. Approximately 10¹¹ viable cells of a *Bradyrhizobium* strain were inoculated into each pot before the seeds were sown. At 22 days after sowing, the pots with the growing plants were enclosed in a muslin cage. Then 50 to 70 adult *Rivellia* flies collected in the field were released into the cage daily for 20 days, after which the potted plants were grown in a growth chamber with a 10-h day length at a day temperature of 28°C and a night temperature of 18°C. A similar set of plants was grown without insect infestation. The plants were supplied daily with a nitrogen-free nutrient solution (7) for 46 days and then harvested and examined. Individual root nodules that were more than 2 mm in diameter were examined for damage. The nitrogen contents of the leaves of the plants were determined as described previously (7). A limitation imposed by the known habits of the insects was that plants could not be examined conveniently for nodule damage at early stages; both the adult insects and the larvae (even more so) were susceptible to lethality upon trapping, and only a fraction of them survived.

RESULTS AND DISCUSSION

Construction of the plasmid containing the DNA region specifying delta-endotoxin production. Plasmids belonging to

the groups N, P, Q, and W are known to have broad host ranges among the gram-negative eubacteria (11). From preliminary studies (data not shown) with a plasmid vector belonging to each of these groups, we selected one plasmid, which was based on the group Q plasmid pKT230 (1), as the plasmid that would be the most suitable and convenient plasmid for cloning a DNA fragment carrying the endotoxin gene originating from pSY368 (Fig. 1). The vector which we used was a modified version of pKT230 called pMA530 (Fig. 1). In pMA530, there is a cluster of root nodulation genes (*nodABCIX*) which are under the control of a *nod* box, a regulatory region that controls transcription of this entire region in a *Rhizobium leguminosarum* strain (S.-W. Ma, Ph.D. thesis, Carleton University, Ottawa, Canada, 1988).

TABLE 2. Comparison of the ability of *Rivellia angulata* larvae to infest and damage root nodules of pigeon peas induced by *Bradyrhizobium* sp. strain IC3554 and strain IC3554(pSM4) carrying the entomocidal gene

Bacterial inoculant	Average no. of nodules per pot ^a	% of nodules damaged ^{b,c}	% of nitrogen in leaves at 40 days after plants were removed from the insect cage ^b
Plants exposed to caged insects ^d			
None	148	90 (72) ^e	1.2
Strain IC3554	234	86 (68)	1.4
Strain IC3554(pKT230)	189	79 (61)	1.2
Strain IC3554(pSM4)	207	46 (42)	2.9
Plants not exposed to caged insects: all three inoculants ^f	257	0	3.2

^a Each pot contained four plants.

^b Data are averages of values from 20 plants.

^c Only nodules that were 2 mm in diameter or larger were scored.

^d The least significant difference values for the plants exposed to caged insects were as follows ($P = 0.05$): average number of nodules per pot, 52; percentage of nodules damaged (after arcsine transformation), 12.9%; and percentage of nitrogen in leaves at 40 days after plants were removed from the insect cage, 0.54%.

^e The values in parentheses are the percentages obtained when the data were analyzed after arcsine transformation.

^f The three inoculants were also used separately with plants that were not exposed to caged insects. Since there was no significant difference among the data for the three inoculants, the average results are given.

FIG. 1. Construction of pSM4. pMA530 is a plasmid carrying a region containing a cluster of root nodulation genes (*nodABCIJX*) under the transcriptional control of a regulatory sequence called the *nod* box. (Ma, Ph.D. thesis). The source of the DNA fragment carrying the entomocidal toxin gene was pSY368 (12). This fragment, which was bounded by *EcoRI* (E) and *Sall* (Sa) sites, was linked to the vector region of pMA530 to yield pSM4.

In the construction of pSM4 (Fig. 1) most of this nodulation region was replaced by a DNA fragment from pSY368 that was known to specify the insecticidal toxin (12); pSM4 was transformed into the *E. coli* strain carrying conjugative helper plasmid pBRK4013. We verified that the resulting *E. coli* strain harbored pSM4 and pBRK4013 in a stable manner and then used this strain in a mating reaction with strain IC3554. Transconjugants were selected and screened to be *Cm*^r (strain IC3554 marker), *Km*^r (pSM4 marker), and *Cb*^s (absence of helper plasmid pBRK4013). Transconjugant colonies obtained in this manner were then used in colony hybridization reactions with radioactive pSY368 DNA and pBR322 DNA as probes. A replicate of a colony that hybridized to pSY368 DNA but not to pBR322 DNA was designated strain IC3554(pSM4).

Stability of pSM4 in strain IC3554. pSM4 was found to be as stable as pKT230 in strain IC3554. This was established

FIG. 2. Evidence for the production of a polypeptide (arrow) by strain IC3554(pSM4) (lane A) which is not detected in strain IC3554 (lane B) and which reacts with antibody produced against the insecticidal endotoxin of *B. thuringiensis* subsp. *israelensis*. The basis of the nonspecific reactions (bottom two bands) has not been investigated.

by growing and subculturing each strain in the liquid medium without antibiotics for approximately 50 generations, plating out dilutions of the culture for the development of single colonies on antibiotic-free media, and then replicating 200 colonies on media containing kanamycin. None had lost kanamycin resistance. The nodule-forming abilities of strain IC3554 and the strain IC3554 derivatives containing the plasmid vectors were examined in a presterilized sand-vermiculite medium (7). Under these conditions, strain IC3554(pSM4) formed an average of 12 nodules per plant, and strain IC3554 formed 20 nodules per plant (average of 20 plants). Visually, there was no difference in the appearance of the nodules. Thus, the engineered strain retained nodulating ability although its relative nodulating efficiency in unsterilized soil could be less than that of strain IC3554 or of other strains indigenous to the soil. We concluded that strain IC3554(pSM4) was a suitable candidate for further tests, but in order to overcome any possible deficiency in nodulating ability compared with indigenous bacteria in unsterilized soil, we decided to use large populations of this engineered bacterium in the subsequent experiments which were intended to determine whether plant protection could occur in natural but potted soil that had not been sterilized.

Detection of the endotoxin polypeptide in *Bradyrhizobium* sp. Figure 2 shows the polypeptides from strain IC3554 (pSM4) (lane A) and strain IC3554 (lane B) that reacted with the endotoxin-antibody probe. Nonspecific binding to *Bradyrhizobium* proteins was detected. However, the highest-molecular-weight polypeptide present in strain IC3554(pSM4) was absent in the control. This result was reproducible. We estimated that the molecular weight of this polypeptide was 45×10^3 , which is in reasonable agreement with expectations for the delta-endotoxin polypeptide of *B. thuringiensis* subsp. *israelensis*. These observations indicated that strain IC3554(pSM4) could produce the endotoxin polypeptide when it was grown ex planta. We assumed (but have not demonstrated) that this polypeptide is also produced in the root nodules. Nevertheless, the observation that the polypeptide was produced ex planta encouraged us to perform the plant infestation tests described below.

Ability of the *Bradyrhizobium* construction to provide protection against *Rivellia* infestation. The larvae of *Rivellia*

FIG. 3. Visual evidence of protection of pigeon peas against root nodule damage by *Rivellia angulata* larvae. The plant on the left was a control: the seeds of this plant were treated with *Bradyrhizobium* sp. strain IC3554. The seeds of the plant on the right were treated with strain IC3554(pSM4). Photographs were taken 40 days after the plants were removed from the insect cage.

species are difficult to collect and to rear successfully (Gowda and Siddapaji, Abstr. 2nd All-India Symp. Soil Biol. Ecol.; B. A. Foote, personal communication). We confirmed previous observations and could not obtain viable larvae in sufficient numbers to permit direct bioassays with the insecticidal toxin. Furthermore, the larvae are not easily transferred without loss of viability and reportedly feed only on root nodules. These limitations (which will need to be overcome to address mechanisms of insecticidal action) suggested assays for plant protection that were patterned after the pot assays of Sithanantham et al. (10). At 20 days after exposure to *Rivellia* sp., the pigeon pea plants growing in pots seeded with strain IC3554 began to show visible symptoms of nitrogen deficiency (yellowing), while the plants in pots inoculated with strain IC3554(pSM4) were healthy and green. After 40 days, only the plants inoculated with strain IC3554(pSM4) had green leaves (Fig. 3). Nodules were examined at this time. Only 46% of the nodules of plants growing in pots containing strain IC3554(pSM4) showed evidence of larval infestation while in the pots containing strain IC3554, 85% of the nodules showed evidence of larval infestation. These differences are statistically significant (Table 2). It should be noted that the experiments were conducted with unsterilized soil that was collected locally to simulate natural conditions. Under these circumstances, nodules were formed by other *Bradyrhizobium* strains indigenous to the soil, and these nodules were susceptible to the added *Rivellia* larvae (Table 2). Thus, in the soil that received strain IC3554(pSM4) not all nodules would have been initiated by this inoculant. It is remarkable that protection was evident despite this limitation, a limitation which is also likely to be present in natural situations.

Two aspects of this study which need to be investigated further and which will require refined methodology relate to

the control of biosynthesis of the polypeptide in the root nodules and the monitoring of early stages of infection. However, the observations which we report in this paper do suggest that the installation of engineered insecticidal genes into *Bradyrhizobium* species and the planned introduction of such constructed organisms into natural environments may have potential benefits.

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