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Expression of insecticidal activity in Rhizobium containing the δ-endotoxin gene cloned from *Bacillus thuringiensis* subsp. *tenebrionis*

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Abstract

Bacillus thuringiensis subsp. *tenebrionis* produces a 65 kilodalton polypeptide toxin which is lethal to various coleopteran insect larvae. The gene encoding this toxin was cloned in *E. coli* in the broad host range vector pKT230 and subsequently transferred to *Rhizobium leguminosarum* by conjugation. Western blot analysis showed that the toxin gene was expressed in the free living state of *Rhizobium* producing two major polypeptides of 73 and 68 kilodalton in size. The level of expression of the toxin gene in *Rhizobium* varied from strain to strain. Cell extracts from toxin-producing rhizobia were toxic to larvae of *Gasterophysa viridula*. Bioassays also showed that the δ-endotoxin was toxic to larvae of the clover weevil *Sitona lepidus*. Furthermore, pea (*Pisum sativum*) and white clover (*Trifolium repens*) plants suffered less root and nodule damage by *Sitona* larvae when they were inoculated with *Rhizobium* strains containing the toxin gene. This suggests that such rhizobia could be useful in the biological control of this important legume pest.

Abbreviations: B.t.t. – *Bacillus thuringiensis* subsp. *tenebrionis*; *IPTG* – isopropyl-β-D-thiogalactoside

Introduction

Bacillus thuringiensis is a Gram positive bacterium which during sporulation produces a proteinaceous crystalline inclusion body with entomocidal activity (Bulla *et al.*, 1980). Different strains of *B. thuringiensis* produce crystal proteins which are active against different insect species. The majority of the known strains are toxic to either lepidopteran or dipteran insect larvae (Aronson *et al.*, 1986) and these strains have been used commercially as insectides (Klein, 1988). Toxin encoding genes have been cloned in *Escherichia coli* from *B. thuringiensis* strains with toxic activity against lepidopteran larvae (Held *et al.*, 1982; Höfte *et al.*, 1986; Klier *et al.*, 1982; Schnepf and Whiteley, 1981; Shibano *et al.*, 1985) and from strains with toxic activity against dipteran larvae (Angsuthanasombat *et al.*, 1987; Ward and Ellar, 1988; Ward *et al.*, 1984). The toxin genes were expressed in *E. coli* and the gene products retained their insecticidal activity. Furthermore, tomato and tobacco plants have been transformed with lepidopteran toxin genes cloned from *B. thuringiensis*. These transgenic plants expressed the

toxin genes and prevented damage from feeding by larvae of the tobacco hornworm (Fischhoff *et al.*, 1987; Vaeck *et al.*, 1987).

Recently, two new strains of *B. thuringiensis* have been isolated, namely *B.t.* subsp. *tenebrionis* (*B.t.t.*) (Krieg *et al.*, 1983) and *B.t.* subsp. *san diego* (Herrnstadt *et al.*, 1986). These strains produce a crystalline protein which is toxic towards coleopteran larvae. Bernhard (1986) showed that crystals from the *tenebrionis* strain contained polypeptides of 68 and 50 kilodalton. The *san diego* strain contained a 64 kDa polypeptide (Herrnstadt *et al.*, 1986). The toxin encoding gene from *B.t.t.* has been cloned and expressed in *E. coli* (Jahn *et al.*, 1987; McPherson *et al.*, 1988; Sekar *et al.*, 1987). The gene encodes a 73 kDa polypeptide (Höfte *et al.*, 1987; McPherson *et al.*, 1988; Sekar *et al.*, 1987), the nucleotide sequence of which is identical to the sequence obtained from the *san diego* gene (Herrnstadt *et al.*, 1987) as well as to a coleopteran toxin gene cloned from another *B. thuringiensis* isolate (Donovan *et al.*, 1988).

One of the main factors limiting the wider use of these genes in biological control of insect pests is the lack of suitable transformation systems for many of the most important crop species. In this respect, legumes may provide a unique method for sidestepping this problem. The clover, pea and bean weevil (*Sitona* spp.) is an important pest of forage and grain legumes such as white clover (*Trifolium repens*), pea (*Pisum sativum*) and broad bean (*Vicia faba*). Adult feeding on foliage is particularly damaging to establishing seedlings, but more importantly, the larvae invade and eat the root nodules inhabited by symbiotic nitrogen fixing bacteria of the genus *Rhizobium*. There is evidence to suggest that this can seriously affect the yield and nitrogen fixation capacity of these legume plants (McEwan *et al.*, 1979; Witty *et al.*, 1980). The aim of this work was to try to exploit the larval feeding habit by introducing the cloned *B.t.t.* toxin gene into *Rhizobium* and demonstrate that the recombinant bacteria have toxic activity against *Sitona*. Thus, expression of the toxin gene within root nodules or by free-living rhizobia on the root surface should provide a source of toxin which *Sitona* larvae would find it difficult to avoid.

Materials and methods

Strains and plasmids

These are listed in Table 1.

Enzymes and reagents

These were used according to the manufacturers' instructions. Restriction endonucleases, T4-DNA ligase, polynucleotide kinase, nick-translation kit, (γ-^{32}P)ATP and (α-^{32}P)dCTP were from Amersham International. Calf intestinal phosphatase was from Boehringer. Swine anti-rabbit immunoglobulins coupled to horse radish peroxidase were obtained from DAKO Ltd, UK.

Manipulation of DNA

Extraction of DNA

Cloning vectors and recombinant plasmids from *E. coli* were extracted and purified according to the method of Lev (1987) except that the alkaline lysis method of Birnboim and Doly (1979) was used to obtain the cleared lysate. Plasmid DNA from *B. thuringiensis* was isolated as described by Kronstad *et al.* (1983) from cells grown to the late exponential phase in PWYE (Herrnstadt *et al.*, 1986) at 30°C. Total DNA from *Rhizobium* was isolated from a 5 mL PA culture (Hirsch *et al.*, 1980) grown at 28°C to the late exponential phase (3 days). After centrifugation the cells were resuspended in 1.5 mL 50 m*M* Tris HCl pH 8.0 containing 20 m*M* EDTA; 0.5 mL 5% (w/v) Sarkosyl and 0.5 mL pronase (2.5 mg mL^{-1}) was added and the mixture was incubated at 37°C until lysis. The lysate was extracted with phenol, phenol/chloroform/isoamyl alcohol (25:24:1; v/v) and chloroform/isoamyl alcohol (24:1; v/v). The DNA was then precipitated with ethanol and redissolved in 10 m*M* Tris HCl pH 8.0 containing 1 m*M* EDTA. Visualisation of rhizobial plasmids on agarose gels was obtained by an *in situ* lysis method originally described by Eckhardt (1978) with modifications as described (Simon, 1984).

Cloning of the toxin gene

Plasmid DNA from *B. thuringiensis* was digested

Table 1. List of strains and plasmids

Strain	Genotype	Source or reference
B. thuringiensis subsp. *tenebrionis*		Krieg *et al.*, (1983)/ P. Jarrett[a]
E. coli		
JM83	*ara*$^-$, d(*lac-pro*AB), *rps*L(= *str*A), ϕ 80, *lac*ZdM15	Vieira and Messing (1982)
S17-1	MM294, *endo*I, *hsd*R, *pro*, (::RP4dTnl, tet::Mu, kan::Tn7)	Simon *et al.* (1983)
R. leguminosarum		
bv. *viciae*		
larf	rif^r	Skøt (1983)
1045rf	rif^r	Rothamsted
bv. *trifolii*		
RCR46sp	spc^r	Rothamsted
plasmids		
pKT230	Broad host range cloning vector	Bagdasarian *et al.* (1981)
pUC12	cloning vector	Vieira and Messing (1982)
pUC18	cloning vector	Yanisch-Perron *et al.* (1985)
pPBS3	5.9 kbp *Bam*H1 fragment from *B.t.t.* containing toxin gene cloned in pUC12	This work
pPBS7 and pPBS8	3.0 kbp *Hin*d111 toxin-gene fragment from *B.t.t.* cloned in pKT230 in opposite orientations	This work
pPBS10	3.0 kbp *Hin*d111 toxin-gene fragment from *B.t.t.* cloned in pUC18	This work

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with *Bam*Hl and fragments between 5.5 and 6.3 kbp were electroeluted after agarose gel electrophoresis and subsequently ligated to a *Bam*Hl linearised and dephosphorylated pUC12 vector (Maniatis *et al.*, 1982). Following transformation of *E. coli*, JM83 recombinants containing the toxin gene were detected by colony-hybridisation (Maniatis *et al.*, 1982). The probe was a 27 base oligonucleotide specific for the coding region of the *B.t.t.* toxin gene as published by Sekar *et al.* (1987) and had the following sequence: 5′-TAT AAA AGA CAA CTA AAA CTT ACG CAA-3′. The oligonucleotide was obtained from Peninsula Laboratories, Merseyside, U.K. It was end-labelled with (γ-^{32}P) using polynucleotide kinase as described by Maniatis *et al.* (1982). Unincorporated nucleotides were separated from the labelled oligonucleotide using a Whatman DE-52 cellulose column as described by Wallace and Miyada (1987).

Southern blot hybridisation

DNA fragments or plasmids were separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond N, Amersham). Hybridisation to nick-translated ^{32}P-labelled probes and subsequent washings were performed by using the Hybaid-I blot processing system as described by the manufacturer (Hybaid Ltd).

Subcloning of the toxin gene and transfer to Rhizobium

A 3.0 kbp toxin-gene fragment was subcloned in the *Hin*d111 site of the broad host range vector

pKT230 and transformed into *E. coli* S17-1. The recombinant plasmid was transferred conjugatively to *Rhizobium* using membrane filter crossings as described (Buchanan-Wollaston *et al.*, 1980). Transconjugants were obtained by selection for resistance to streptomycin (200 μg mL^{-1}).

Crystalline protein isolation

B. thuringiensis was grown in NYSM medium (Herrnstadt *et al.*, 1986) for two days at 30°C to induce sporulation. The resulting spore-crystal mixture was harvested by centrifugation (12,000 *g*, 10 min, 4°C) and the pellet washed four times with distilled H_2O followed by one 1 *M* NaCl wash and a final wash with distilled H_2O. The crystals were subsequently separated from the spores by two successive centrifugations in 55–88% (w/v) sucrose gradients in 50 m*M* Tris HCl, 10 m*M* KCl at 113,000 *g* using an MSE Europa 65 ultracentrifuge with a swinging bucket rotor MSA 30.25 (MSE Scientific Instruments, England). The crystals were solubilised in 100 m*M* $NaHCO_3$, pH 10.5.

Immunoblot analysis

Cell extracts from *E. coli* and *Rhizobium* were obtained by ultrasonic disintegration. *E. coli* cultures were grown overnight in LB (Maniatis *et al.*, 1982). *Rhizobium* cultures were obtained by suspending cells from 3–4 day old TY agar plates (Beringer, 1974). The polypeptides were separated by a SDS-polyacrylamide (10%) gel electrophoresis using a mini-Protean II Cell (Bio-Rad Laboratories Ltd, UK) and transferred to nitrocellulose by electroblotting. Immunodetection was obtained by using a primary rabbit antiserum raised against the solubilised crystal protein isolated from *B.t.t.* and swine antirabbit immunoglobulins coupled to horse radish peroxidase (Davies *et al.*, 1989).

Insect bioassays

Rearing of Sitona larvae

Sitona lepidus Gyll. adults were trapped from white clover plants in the field and identified according to Kevan (1959) on the basis of the shape of the head, elytra and scales on the elytra. Fifty adults were reared and fed with fresh clover leaves. Eggs were removed and surface sterilised by dipping them in 0.1% (w/v) sodium-hypochlorite for one min. followed by washing with sterile water. The eggs were incubated at 22°C on moist sterile filter paper and eggs hatched after 13 days. Only neonate larvae less than 24 h old were used in the experiments.

Assays with Sitona

Peas (*Pisum sativum* cv Dark Skinned Perfection) were grown in vermiculite in pots placed on top of an upside down saucer. The assembly was autoclaved. Pea seeds were surface sterilised in 10% (w/v) sodium-hypochlorite for 5 min and then thoroughly rinsed with sterile water. Immediately after planting, the pots were inoculated with a 3 day old suspension of the appropriate *Rhizobium* strain in YM broth (Vincent, 1970). The plants were grown in a containment greenhouse and watered with a half strength nitrogen-free nutrient solution (Ryle *et al.*, 1978). Three weeks after planting, the shoots were removed just above the seeds and the root systems were transferred to Petri plates (diameter: 9 cm) lined with moist filter paper and larvae of *Sitona* were added. The plates were then left in the dark at room temperature. Larval mortality was recorded after 48 h. Two root systems were used for each treatment, and 4 larvae were added to each.

White clover (*Trifolium repens* L. cv Menna) seeds were scarified and surface sterilised (as above). They were then planted in Petri plates (10 seedlings per plate) with a half strength nitrogen-free Jensens agar medium and inoculated with YM broth cultures of *Rhizobium* (Vincent, 1970). Two weeks following inoculation, *Sitona* larvae were added to the clover seedlings. The plates were left in the dark at room temperature. Larval mortality was recorded after 96 h.

Insect bioassay with purified toxin

Individual root nodules formed by wild type rhizobia from white clover plants grown in the greenhouse were transferred to small plastic vials. Each nodule was then painted with 20 μl of *B.t.t.* toxin solution (45 μg) or with 20 μl

buffer only. One larva was added to each nodule, and the vials were closed and left in the dark at room temperature. Larval mortality was recorded after 72 h.

Bioassay with Gasterophysa viridula *Deg.* (*Fam.* Chrysomelidae)
Eggs were collected from the leaves of dock plants (*Rumex* spp) and placed on moist filter paper in a Petri plate at 22°C and hatched in 3–4 days. Neonate larvae less than 24 h old were used in the experiment. Leaf discs of 1.5 cm in diameter cut from fresh dock leaves were painted on one side with 50 μl of protein extracts from recombinant *E. coli*, *Rhizobium* or δ-endotoxin solution and placed in Petri plates (diameter: 9 cm) lined with moist filter paper. Five larvae were released on each disc in 2–3 replications and larval mortality was recorded after 48 h. The results of all bioassays were subjected to Chi squared analysis.

Statistical analysis of the bioassay data

Numbers of living and dead insect larvae, or damaged and undamaged nodules and roots, were counted in control and toxin treatments. Chi squared values were computed from 2*2 contingency tables of the form:–

	Toxin	Control	Total
Alive	a	b	a + b
Dead	c	d	c + d
Total	a + c	b + d	N = a + b + c + d

$$\text{Where } X_c^2 = \frac{N(|ad - bc| - N/2)^2}{(a+b)(c+d)(a+c)(b+d)}$$

This formula includes a correction for continuity to prevent overestimation of significance where class frequencies are small (Snedecore and Cochran, 1967). In tests where comparable material was used and where the results showed similar trends, data was pooled for Chi squared analysis to avoid problems associated with expected class frequencies falling below 5. Where this occurs it will be indicated in the text. Finally, it should be noted that there are a priori reasons for expecting that where toxin treatments have an effect, then they will alter ratios in a predictable direction. Consequently, probabilities are based on single, rather than two-tail tests.

Results and discussion

Molecular cloning of the toxin gene

In order to establish the location of the toxin gene in our version of the *tenebrionis* strain a Southern blot of restriction enzyme digested *Bacillus* plasmid DNA was probed with a ^{32}P-labelled oligonucleotide specific to the coding region (see Materials and Methods) of the coleopteran toxin gene (Sekar *et al.*, 1987). The oligonucleotide hybridised to a 5.9 kbp *Bam*H1, a 3.0 kbp *Hin*d111 and a 7.3 kbp *Eco*R1 fragment. Furthermore, agarose gel electrophoresis of uncut *tenebrionis* plasmid DNA revealed the presence of at least 5 plasmid bands ranging in size from 10–200 kbp. One of these plasmids (ca 150 kbp) hybridised to the oligonucleotide probe (Fig. 1). The other hybridising smear probably represents sheared plasmids and chromosomal DNA. Colony-hybridisation of clones containing

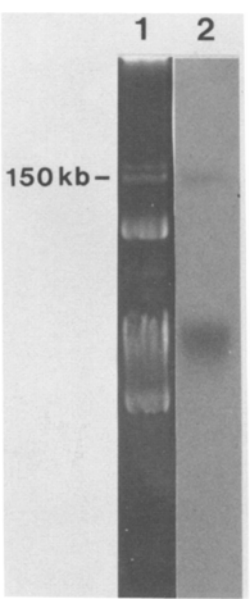

Fig. 1. Plasmid profile of *B. thuringiensis* subsp. *tenebrionis* (lane 1) and Southern blot hybridisation to a ^{32}P labelled 27 base toxin-gene specific oligonucleotide (lane 2). Plasmid size was estimated by comparison to plasmid profiles of rhizobial strains.

*Bam*H1 fragments cloned in the vector pUC12 resulted in the isolation of one colony, PBS3, hybridising with the oligonucleotide probe. The plasmid pPBS3 contained a 5.9 kbp *Bam*H1 fragment. These results, together with restriction mapping using *Hin*dIII, *Pst*I and *Eco*RI, indicate strongly that this fragment is identical to the 5.9 kbp *Bam*H1 fragment cloned by Sekar *et al.* (1987). The internal 3.0 kbp *Hin*dIII fragment was subcloned in pUC18 to give pPBS10 and in the broad host range vector pKT230 giving pPBS7 and pPBS8 (fragment inserted in opposite orientation). The plasmid pPBS8 was transferred by conjugation to three different *Rhizobium leguminosarum* strains. Putative transconjugants containing pPBS8 were obtained by selection for streptomycin resistance. *In situ* lysis agarose electrophoresis for visualising plasmids in *Rhizobium* revealed the presence of two extra bands with a slightly lower mobility than the two bands found in rhizobia containing pKT230. These two bands hybridised strongly to a probe consisting of the 5.9 kbp *Bam*H1 fragment containing the toxin gene sequence, whereas no hybridisation was found to the controls containing pKT230 (Fig. 2). The presence of two hybridising plasmid bands is probably due to supercoiled and nicked versions of pPBS8. The presence of the toxin gene was further confirmed by Southern blot hybridisation analysis of *Hin*dIII digests of total DNA isolated from the *Rhizobium* transconjugants. Those containing pPBS8 had a single band of 3.0 kbp in size hybridising to the toxin gene probe (Fig. 3).

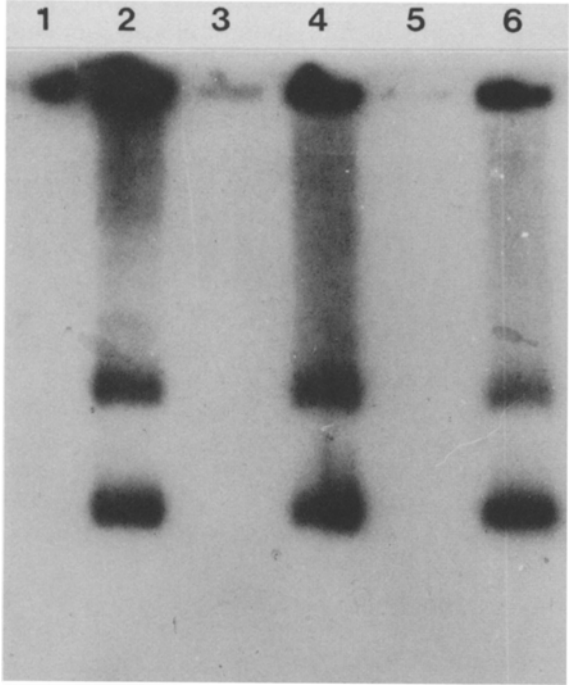

Fig. 2. Southern blot hybridisation of *Rhizobium* plasmid DNA to 5.9 kbp *Bam*H1 fragment containing the *B.t.t.* toxin-gene. Lanes 1: larf(pKT230); 2: larf(pPBS8); 3: 1045rf(pKT230); 4: 1045rf(pPBS8); 5: RCR46sp(pKT230); 6: RCR46sp(pPBS8).

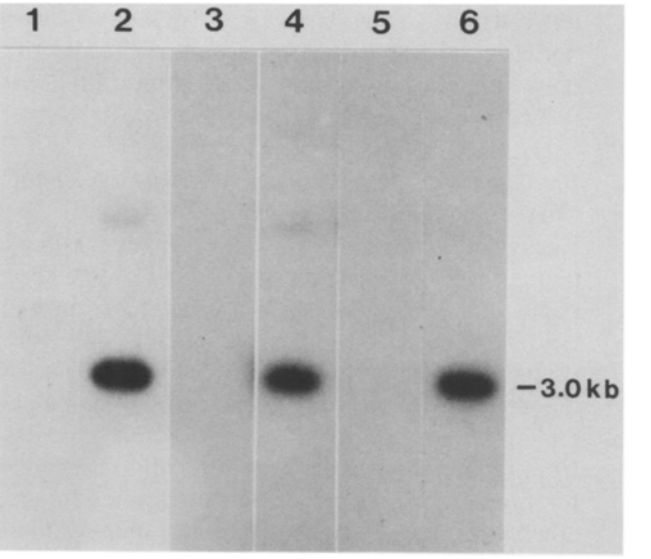

Fig. 3. Southern blot hybridisation of *Rhizobium* total DNA cut with *Hin*dIII to 3.0 kbp *Hin*dIII fragment containing the *B.t.t.* toxin-gene. Lanes 1: larf(pKT230); 2: larf(pPBS8); 3: 1045rf(pKT230); 4: 1045rf(pPBS8); 5: RCR46sp(pKT230); 6: RCR46sp(pPBS8).

Expression of the cloned toxin gene

Immunoblot analysis of *E. coli* and *Rhizobium* cell extracts showed that the toxin gene was expressed in these recombinant organisms. In pPBS3 the toxin gene was orientated in the same direction as the lacZ promotor on the vector (Sekar *et al.*, 1987), but the expression was low and addition of the inducer IPTG did not increase it (data not shown). In pPBS10, which has the 3.0 kbp *Hin*dIII toxin gene fragment inserted in the opposite orientation relative to the

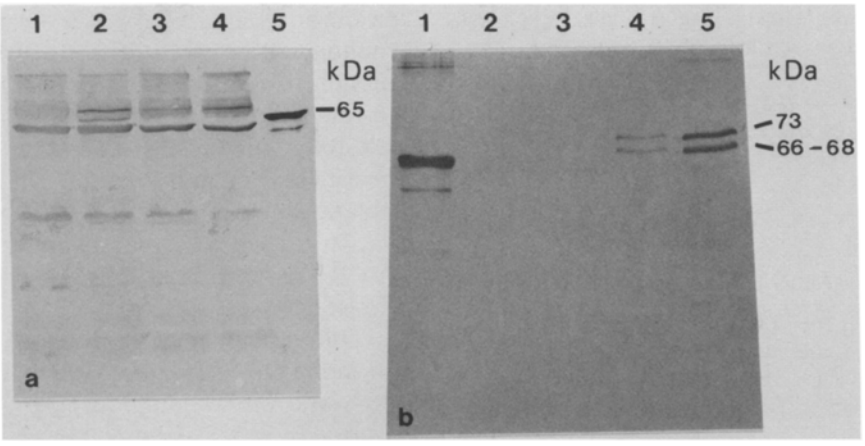

Fig. 4. Immunoblot analysis of toxin protein crystals from *B.t.t.* and of cell extracts from *E. coli* and *Rhizobium* strains. a: *E. coli* extracts. Lanes: 1, JM83(pUC18) (control); 2, JM83(pPBS10); 3, S17-1(pPBS7); 4, S17-1(pPBS8); 5, δ-endotoxin (0.6 μg protein). In lanes 1–3, 14 μg protein was applied; in lane 4, 6 μg. b: *Rhizobium* extracts. Lanes: 1, δ-endotoxin (1.0 μg protein); 2, larf(pRT230) (control); 3, larf(pPBS8); 4, RCR46Sp(pPBS8); 5, 1045rf(pPBS8). In lanes 2–5, 25 μg protein was applied.

lacZ promoter of the vector, a high level of expression occurred (Fig. 4a, lane 2). In pPBS8 the toxin gene was transcribed in the same direction as the interrupted kan^r gene of the vector. *E. coli* cells containing this plasmid produced more toxin than cells containing pPBS7, where the toxin gene was inserted in the opposite orientation (Fig. 4a, lanes 3 and 4). Two major antigenic polypeptides were detected. The larger one was 73 kDa. This is consistent with the molecular weight of the amino acid sequence predicted from the full length coding sequence of the *B.t.t.* toxin gene (McPherson *et al.*, 1988; Sekar *et al.*, 1987). The smaller polypeptide co-migrated with the 65 kDa protein isolated from *B.t.t.* crystals. In *R. leguminosarum* the expression of the toxin gene varied between the host strains (Fig. 4b). The largest of the two major antigenic polypeptides produced in *Rhizobium* co-migrated with the 73 kDa polypeptide detected in *E. coli*, but the smaller one was approximately 68 kDa. This is similar to the molecular weight of the amino acid sequence predicted from the coding sequence starting at the second possible translation initiation codon identified by McPherson *et al.* (1988), and in the same reading frame as the full length sequence. Whether both these translation initiation sites are being used in *Rhizobium* is not yet known.

Insect bioassays

Preliminary bioassays were made to test the effect of toxin crystals isolated from *B.t.t* on *Sitona lepidus* larvae. Solutions containing the δ-endotoxin were painted onto detached wild type root nodules from white clover as described in Methods. Control treatments were painted with buffer devoid of toxin. Five successive replicated experiments were carried out. In each one, more larvae died in the presence of toxin than in the control. In total, 101 larvae were tested. Of the 48 larvae in the control treatment, 4 died (8%). Of the 53 larvae exposed to the toxin 19 died (35%). Chi squared analysis of the pooled data showed the difference to be highly significant ($X^2 = 9.34$ $P < 0.001$).

Following this result, three experiments were carried out to evaluate the insecticidal activity of *Rhizobium* strains containing the toxin gene. In the first, pea and white clover roots from plants grown as described in Methods were placed on Petri plates and exposed to *Sitona* larvae. The results are shown in Table 2. Analysis of the combined data showed that the presence of rhizobia containing the toxin gene was associated with a significant increase in larval mortality ($X^2 = 7.14$ $P < 0.001$). The second experiment was carried out with individual white clover root

Table 2. Effect of pea and clover roots nodulated by toxin-gene containing rhizobia on mortality of *Sitona* larvae

Treatment	Larvae added	Dead larvae
Pea		
1045rf(pKT230) (control)	8	1[a]
1045rf(pPBS8) (toxin)	8	5
White clover		
RCR46sp(pKT230) (control)	8	2[b]
RCR46sp(pPBS8) (toxin)	8	6

[a] Mortality recorded after 48 h.
[b] Mortality recorded after 96 h.
X^2 for the pooled data = 7.14 $P < 0.001$.

nodules in vials, but this time the control consisted of nodules formed by RCR46sp(pKT230) and the treatment was nodules formed by RCR46sp(pPBS8). After 72 hours, each nodule was examined for penetration by *Sitona*. In the controls, 23 out of 30 nodules were penetrated (73%) compared with 13 out of 30 (43%) in the toxin gene treatment. Although there was no significant difference in larval mortality, the difference in the number of nodules damaged was highly significant ($X^2 = 5.625$ $P < 0.001$). In the third experiment, peas nodulated by either RCR1045rf(pKT320) or RCR1045rf(pPBS8) and exposed to *Sitona* larvae for five weeks were examined for root and nodule damage. The results are summarised in Table 3. The difference in the number of plants with nodule or main root damage was again significant (X^2 on the pooled data = 8.29 $P < 0.001$). Interestingly, we also observed that all 8 control root systems were infected to some extent with fungi, whereas no such infections were found in the toxin treatment. Whether this was coincidental or secondary infections as a consequence of *Sitona* attack remains to be seen. There was no significant difference in shoot height between control plants which were infected with *Sitona* and those that were not. The same was the case for the plants nodulated by RCR1045rf(pPBS8). *Rhizobium* nodule isolates were obtained from 50 nodules formed on the 8 root systems inoculated with RCR1045rf(pPBS8). Forty-three isolates were resistant to streptomycin, indicating only a relatively small loss of pPBS8 in the nodulation process. All 50 isolates from RCR1045rf(pKT230) plants were streptomycin resistant.

During the course of these studies early natural mortality of *Sitona* larvae made these difficult to work with. Similar problems have been reported by other workers (see Aeschlimann, 1986, and references therein). We therefore examined other coleopteran species to see if we could find a 'model' species which would overcome these difficulties. Eight species were tested. Large, vigorous populations of larvae were most easily raised from *Gasterophysa viridula*. Results of dock leaf bioassays with this species are shown in Table 4. They demonstrate that the larvae were sensitive to the δ-endotoxin from *B.t.t.* Furthermore, cell extracts from *E. coli* containing the toxin gene were toxic to the larvae, whereas no larval mortality was observed in any of the controls. Analysis of the pooled data from the *Rhizobium* treatments also shows that the presence of the toxin gene significantly increased larval mortality ($X^2 = 13.795$ $P < 0.001$).

To summarize, these results demonstrate that the δ-endotoxin gene from *B.t.t.* is toxic to larvae of *S. lepidus* as well as to *G. viridula*. *Rhizobium* strains containing the toxin gene were expressing it in the free living state, and cell extracts from such strains were isecticidal. Furthermore, pea and white clover roots inoculated with toxin producing rhizobia suffered less root and nodule damage when exposed to *Sitona* larvae than the corresponding control roots. These results suggest that such *Rhizobium*

Table 3. Effect of *Rhizobium* containing the *B.t.t.* δ-endotoxin gene on root and nodule damage on pea plants

Rhizobium	Plasmid	Plants	Plants with nodule damage	Plants with main root damage
1045rf	pKT230	8	8	6
1045rf	pPBS8	8	5	0

X^2 for the pooled data = 8.29 $P < 0.001$.
Four replicate pots, each containing two plants were used, and the results were pooled. Twenty *Sitona* larvae were added to each pot three days after planting and immediately after inoculation with *Rhizobium*.

Table 4. Effect of δ-endotoxin and cell extracts from toxin producing *E. coli* and *Rhizobium* on larvae of *Gasterophysa viridula*

Treatment	Dosage μg protein cm^{-2}	Larvae added	% Mortality
δ-endotoxin	0.48	15	100
δ-endotoxin	0.24	15	60
E. coli			
S17-1(pKT230) (control)	385[a]	15	0
S17-1(pPBS8) (toxin)	385	15	100
R.l. bv *viceae*			
1045rf(pKT230) (control)	128[a]	15	0
1045rf(pPBS8) (toxin)	128	15	60[b]
R.l. bv *trifolii*			
RCR46sp(pKT230) (control)	128[a]	10	0
RCR46sp(pPBS8) (toxin)	64	10	40[b]

[a] Total protein dosage.
[b] X^2 for the pooled data on the *Rhizobium* tests = 13.795 $P < 0.001$.

strains could be used in the biological control of *Sitona* larvae in forage and grain legumes. However, we realise that more work needs to be done, particularly on bioassays under conditions which resemble the field situation more closely. Another potential problem is that of competition between *Rhizobium* strains. The toxin gene construct would have to be present in a *Rhizobium* strain which could successfully compete for nodule sites with the indigenous *Rhizobium* population. Recent field studies have confirmed the possibility of selecting such strains (Mytton, 1988). This result suggests that solutions to the problems of competition will be available. Furthermore, although expression of the toxin gene has clearly been demonstrated in the free living state it is not yet clear to what extent it is expressed in the symbiotic state within root nodules. The insect bioassays with *Sitona* suggest nodule expression although the results could be explained by larvae consuming free living rhizobia colonising the rhizosphere and the root nodule surface. However, no antigenic polypeptides were detected by immunoblot analysis of extracts obtained from pea root nodules formed by 1045rf(pPBS8). Whilst constitutive expression in *Rhizobium* may have the advantage of conferring resistance to attack over the whole root system, it also poses the problem of making toxic rhizobia available to other soil organisms. We are therefore making constructs in which expression of the toxin gene is under the control of a nif-gene promoter. This should ensure that the toxin is produced within the plant and is restricted to root nodules which are most susceptible to *Sitona* attack.

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