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Transference of a crystal protein gene from *Bacillus thuringiensis* and its expression in *Bradyrhizobium* sp. cells

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Summary

The plasmid pHT409 that harbours the *cryIA(a)* gene for the production of a δ-endotoxin (crystal protein) from *Bacillus thuringiensis* was transferred into *Bradyrhizobium* sp. A conjugal transfer system aiming to introduce the plasmid into the *Bradyrhizobium* sp. host from colonies of an *Escherichia coli* donor strain (DH5α::pHT409) has been developed. As a result exconjugants were obtained in which the transferred plasmid has been detected by both microbiological and electrophoresis techniques. The *cryIA(a)* gene when inside the new host had a low expression level which was detected by immunoblotting.

Introduction

Recombinant DNA technology has provided a whole new set of genetic recombination possibilities and has become a useful tool for the genetic improvement of some species. Much of this work is done to improve food quality with reduction of the usage of chemical pesticides.

In this context the *Bacillus thuringiensis* Berliner δ-endotoxin (crystal protein) gene manipulations fit quite well since the toxin is an attractive alternative to the use of the chemical insecticides which are highly toxic to humans and domestic animals and lead to the development of resistance in insect pests.

Bacillus thuringiensis spore-crystal preparations have been used for more then three decades as biological insecticides. However, widespread utilization is limited by high production costs and crystal protein instability when exposed to environmental conditions. These limitations resulted in the search for alternatives based on the development of genetic manipulation techniques aiming to overcome these difficulties by transferring to plants as well as to endophytic bacterial cells some of the *B. thuringiensis* crystal protein genes.

Bradyrhizobium cells are unable to perform genetic recombination through the transformation process because of the lack of membrane pores (Hattermann & Stacey 1990), so conjugation is the most commonly used method to transfer DNA molecules into these kinds of cells (Guerinot & Chelm 1986; So *et al.* 1987).

For comparison purposes it is interesting to point out that Nambiar *et al.* (1990) have cloned a DNA fragment containing the *B. thuringiensis* subsp. *israelensis* δ-endotoxin coding genes into a plasmid vector with a wide host range. This plasmid has been transferred by conjugation from an *E. coli* donor strain (HB101) into a *Bradyrhizobium* sp. strain IC3554 that nodulates pigeon pea (*Cajanus cajan*). The resultant exconjugants were able to produce the crystal proteins that reacted with the anti-serum produced against δ-endotoxin and in greenhouse trials they were able to provide nodule protection against the attack by larva of the coleopteran *Rivellia angulata*.

The objective of the present work was to introduce the plasmid pHT409 (Figure 1), in which Arantes *et al.* (1990) have cloned a DNA fragment containing the *cryIA(a)* gene, by conjugation assays into *Bradyrhizobium* sp. and to verify its expression by immunoenzymatic analysis.

Materials and Methods

Bacterial strains and plasmids

The bacterial and plasmid strains are listed on Table 1.

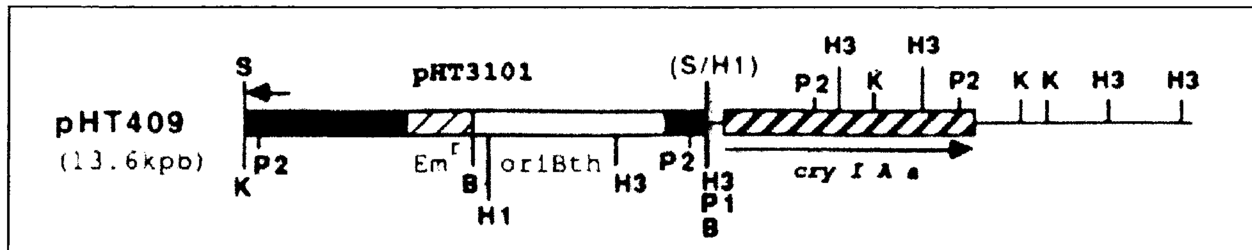

Figure 1. Map of the restriction enzymes sites on the plasmid pHT409 which harbours the gene *cryIA(a)*. The arrow indicates the transcription orientation. B = BamHI, K = KpnI; S = SmaI; P1 = PstI; P2 = PvuII; H1 = HpaI; H3 = HindIII (Lereclus *et al.* 1989).

Table 1. Strains of bacteria and plasmids.

Strains/plasmids	Relevant characteristics	Source
Escherichia coli		
DH5α	hsdR 17 recA1 Dam^+ Dcm^+	This lab
HB-101	hsdS20 recA13 lac Y1 Gal K2 Dam^+ Dcm^+	This lab
Bradyrhizobium sp. SEMIA 6033	Recommended for the use with *Arachis hypogea*	Niftal MIRCEN
Bacillus thuringiensis		
var. *kurstaki*-HD1	*Cry B⁻*	BGSC/USA
var. *kurstaki*-HD1	*Cry B⁻*::pHT408	BGSC/USA
var. *kurstaki*-HD1	*Cry B⁻*::pHT409	BGSC/USA
var. *kurstaki*-HD1	Cry^+	BGSC/USA
Plasmid pHT 409	Fusion of a 7-Kb fragment containing the *cryIA(a)* gene into the plasmid pHT3101	Lereclus *et al.* (1989) Arantes *et al.* (1990)

BGSC/USA – Bacillus Genetic Stock Center, United States of America.
MIRCEN – Microbiological Research Center, Brazil.
Niftal – Nitrogen Fixation in Tropical Agricultural Legumes Project, United States of America.

Plasmid extraction procedures

Plasmid isolation from *E. coli* cells was done as described in Sambrook *et al.* (1989). For plasmid isolation from *Bradyrhizobium* the culture cells were obtained by centrifugation (15,000 ×*g*; 30 min), the pellet was washed with 0.9% NaCl saline, and later with solution G (50 mM Tris, 1 mM EDTA and 10% glycerol, pH 8.0). The pelleted cells were submitted to lysis in the presence of 15% sucrose at 25 °C for 30–45 min and after these steps the Sambrook *et al.* (1989) plasmid isolation procedure was followed up to completion.

Crystal extraction

Bacillus thuringiensis strains were grown on a rotary shaker at 200 rev/min and 30 °C in 50 ml Erlenmeyer flasks containing C2 broth (Donovan *et al.* 1988) specific for sporulation, up to the sporulation phase. The spore-crystal mixture was harvested by centrifugation (6000 ×*g*; 10 min, at 4 °C) and the cellular material washed in NaCl 0.5 M. The pellet was then washed twice and suspended in ice-cold deionized water. The suspension was then taken to be sonicated in a Branson Sonifier, model 250 (5 min, 65 W).

The sonicated material was then submitted to ultracentrifugation on a sucrose gradient (67–79%) at 42,500 ×*g* for 10 min. The crystal band was harvested and suspended in two volumes of deionized water and washed three times, by centrifugation (6000 ×*g*; 10 min, 4 °C) with deionized water. The final suspension of purified crystals was lyophilized and kept at −20 °C.

Crystal protein polyacrylamide gel electrophoresis

The purified crystal protein samples obtained from the *B. thuringiensis* strains were diluted in sample buffer (Tris–HCl, 50 mM pH, 6.8 with 20% glycerol, 5% β-2 mercaptoethanol, 4% SDS, and 0.01% bromophenol blue). After boiling for 5 min, 50 μl of each sample was subjected to electrophoresis at 25 mA on 13% polyacrylamide slab gel containing 0.1% SDS.

Production of hyperimmune sera

For antisera production rabbits were injected subcutaneously with 0.5 ml of a suspension of purified crystals in water (4.0 mg/ml), every week during a period of 8 weeks. The animals were bled by puncture of the ear marginal vein and serum samples checked by immunodot reactions. When the reaction titre was 1:1000 (v/v) or higher the animals were bled by cardiac puncture and the sera obtained by fractionation and stored at −20 °C.

Immunoblotting procedure

Cells of different bacteria were sonicated as previously described and were then centrifuged (15,600 ×*g*, 10 min). The supernatants (soluble extract) were submitted to polyacrylamide gel electrophoresis as described above.

The electrophoresed samples were then transferred to nitrocellulose paper using the Bio-Rad blotting system loaded with the Tris–glycine buffer pH 6.8 (0.2 M Tris, 0.05 M glycine and 1.6% methanol). The transfer was carried out under constant voltage (80 V) at 4 °C for 4 h (Harlow & Lane 1988).

After the transfer the nitrocellulose membranes were incubated with the blocking reagent made of TBS (Tris–NaCl 0.05 M, pH 7.5) containing 0.05% Tween 20 and 5% nonfat dried milk. After this the anti-crystal protein serum diluted 1:200 (v:v), was added and the sheets were incubated at room temperature with slow agitation for 1 h. The membranes were then washed with TBS, and rabbit antigammaglobulin conjugated to alkaline phosphatase (Bio-Rad) diluted 1:3000 (v:v) with TBS was used as the second antibody. The incubation was carried out for 1 h with low agitation and at room temperature, then the membranes were washed with TBS and developed with nitro blue tetrazolium chloride (NBT) at 33% and 5-bromo-4-chloro-3indolyl phosphate (BCIP) at 16.5% were dissolved in Tris–NaCl 0.1 M buffer pH 9.5 containing 1.25 mM magnesium chloride.

Conjugation

The conjugation assays between the *E. coli* strain DH5α::pHT409 (donor) and the *Bradyrhizobium* sp. SEMIA 6033 (recipient) were performed according to Beringer *et al.* (1978). The *E. coli* strain was grown in Luria broth at 37 °C and the *Bradyrhizobium* was grown in yeast mannitol broth (YMB) at 30 °C, both on an rotary shaker regulated for 150 rev/min, overnight. After growth, the cells were mixed in a ratio of 1.0 from the donor (*E. coli*): 1.8 from the recipient (*Bradyrhizobium* sp.). After the conjugation the mixture of cells was submitted to centrifugation at 13,560 ×*g* for 3 min and washed with 1.0 ml of sterile deionized water containing 5% of Luria broth. The pelleted cells were resuspended in 200 μl of the same solution and 100 μl were plated, in replicates, on yeast mannitol agar (YMA) plates containing 110 μg/ml of ampicillin and 20 μg/ml of tetracyclin. Controls were plated under the same conditions but without prior cellular contact. All the plates were incubated at 30 °C for 48 h. The conjugation frequency was determined by the ratio between the number of exconjugant colonies and the total count on YMA plates.

Results and Discussion

The electrotransformed *E. coli* DH5α::pHT409 obtained was used in the conjugation assays with *Bradyrhizobium* sp. SEMIA 6033 with the purpose of evaluating the presence of cells with the proper plasmid resistance maker (Ap^r) and whether any of the exconjugants could express the *cryIA(a)* gene. Figure 2 shows colonies of the *Bradyrhizobium* sp. exconjugant strain, grown under antibiotic selective pressure.

Colonies of the exconjugant strain were isolated and grown under the proper antibiotic selective pressure on YML broth and nucleic acid was extracted and analysed by agarose gel electrophoresis. The plasmids profiles can be seen on Figure 3.

Figure 2. Growth of colonies on YMA plates containing 125 μg/ml ampicillin and 40 μg/ml of tetracyclin. (**a**) *E. coli* DH5α::pHT409, (**b**) *Bradyrhizobium* sp. SEMIA 6033::pHT409 (exconjugant) and (**c**) *Bradyrhizobium* sp. SEMIA 6033.

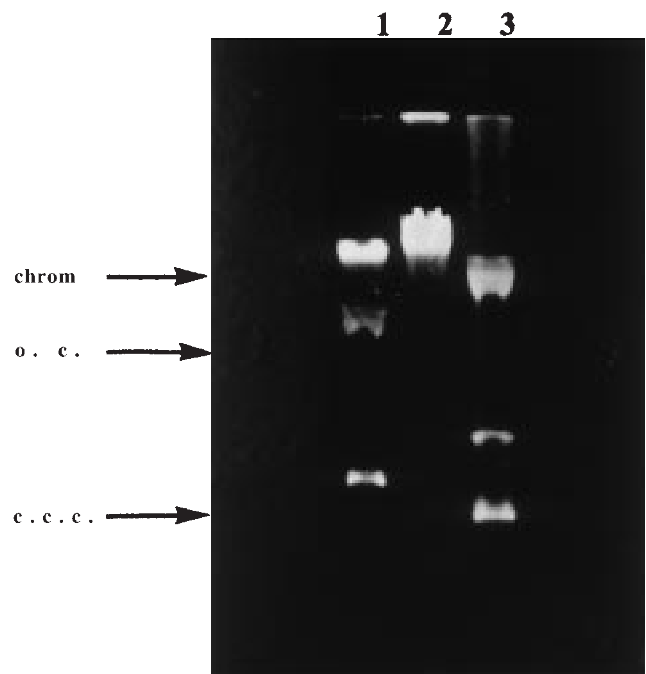

Figure 3. Electrophorogram made with DNA extracted from: (**1**) *Bradyrhizobium* sp. SEMIA 6033::pHT409, (**2**) *Bradyrhizobium* sp. SEMIA 6033 (recipient) and (**3**) *E. coli* DH5α::pHT409 (donor).

The frequency of conjugation was calculated as 4.9×10^{-5} and the bifunctionality of the pHT3101, meaning its ability to replicate in different hosts, was detected since such plasmid was able to replicate within the *Bradyrhizobium* sp. recipient strain (Figure 3, lane 3). Similar results were also described by Goze & Ehrlich (1980) and Schäfer *et al.* (1990), that succeeded in the replication of Gram-positive type of plasmids into Gram-negative cells. According to Batz quoted by Mazodier & Davies (1991), some DNA molecules might escape digestion by restriction enzymes because when they enter the recipient cells they are found in a transient special unifilamentous condition, which allows them to be less sensitive to the majority of restriction enzymes.

The self-transfer ability detected for the pHT409 plasmid has not been previously described, but it seems to be quite a general feature of some of the *B. thuringiensis* plasmids (Arantes *et al.* 1990).

The immunoblotting assays were developed in order to verify *cryIA(a)* gene expression in the exconjugants after the insertion of the pHT409 plasmid, Figure 4. The presence of proteins with molecular weight lower than 92 kDa in the crystal protein producer strains (*B. thuringiensis* var. *kurstaki* – HD1 *Cry*$^+$, *Bradyrhizobium* sp., SEMIA 6033::pHT409, *B. thuringiensis* var. *kurstaki* HD1 *CryB*$^-$::pHT408) might correspond to degradation products of the 130–140 kDa protein and to secondary initial or late translations such as observed by Sanchis *et al.* (1988).

The low molecular weight bands that were detected for the *E. coli* strains and for the strain *B. thuringiensis* var. *kurstaki cryB*$^-$ (Figure 4, lanes 1–5) can be explained since a polyclonal antibody serum was used in the assays. The antibody has been used as whole serum and because of that it contains the complete repertoire of circulating antibodies of the immunized animals at the time of serum collection. If the inoculated antigen is similar to those of bacterial or fungal origin that possibly might have infected the experimental animals, such antigens will be also detected during the immunoenzymatic assay (Harlow & Lane 1988).

These results have shown that the exconjugant strain of *Bradyrhizobium* sp. SEMIA 66033::pHT409 has expressed a low level of the 112 kDA polypeptide, that is related to the *B. thuringiensis* δ-endotoxin coded by the *cryIA(a)* gene inserted into the plasmid pHT409, Figure 4, lane 7 (arrow).

The exconjugant cells were also evaluated for the segregational stability of the pHT409 plasmid within the cells, Figure 5. The assays were carried out because of the importance of this characteristic for recombinant organisms in environmental use, and they show that after a period of 72 h the exconjugant cells still harboured the pHT409 plasmid, even when cultivated without selection pressure (lane 3).

We conclude that the bifunctional plasmid pHT 409 was able to transfer itself into *Bradyrhizobium* sp. SEMIA 6033 cells during the conjugation process (Figure 3, lane 1). Within its new host this plasmid was not restricted and showed a low level of expression of the 112 kDa polypeptide that reacted with the anti-crystal protein antibody (Figure 4, lane 7).

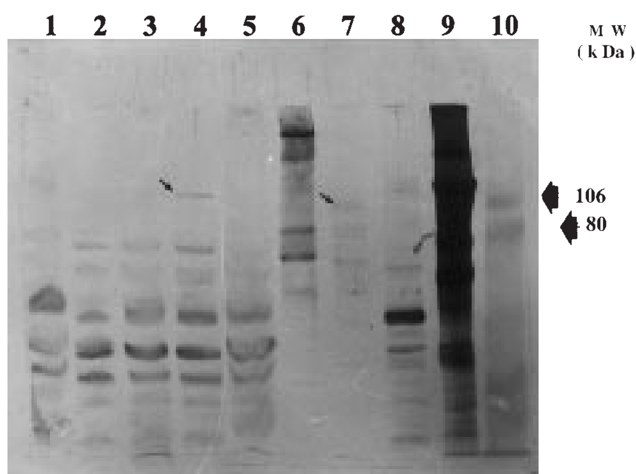

Figure 4. Immunoblotting from the soluble extract of the strains *E. coli.* DH5α (lane 1), *E. coli* DH5α::pHT409 (lane 2), *E. coli* HB101 (lane 3), *E. coli* HB101::pHT409 (lane 4), *B. thuringiensis* var. *kurstaki*-HD I *cryB*$^-$ (lane 5), *B. thuringiensis* var. *kurstaki*-HD I *Cry*$^+$ (lane 6), *Bradyrhizobium* sp. SEMIA 6033 (lane 7) *Bradyrhizobium* sp. SEMIA 6033::pHT409 (lane 8), *B. thuringiensis* var. *kurstaki*-HD I cry$^-$B::pHT408 (lane 9) and molecular weights (lane 10). Arrows indicate immunodetected crystal protein band.

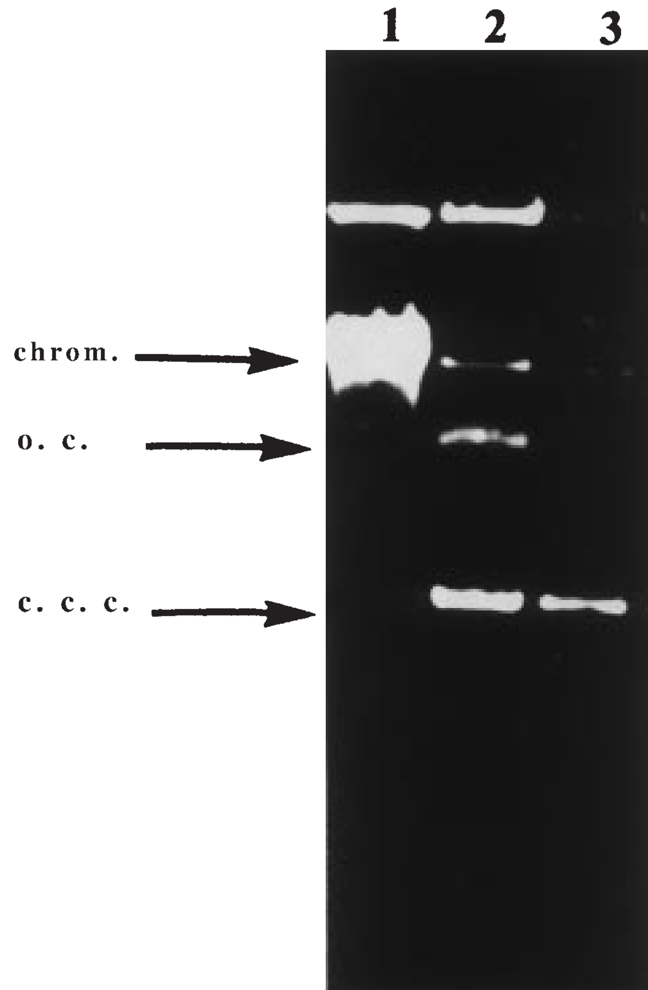

Figure 5. Stability of the plasmid pHT409 after 72 h without antibiotic selective pressure. (**1**) *Bradyrhizobium* sp. SEMIA 6033 genomic DNA, (**2**) *Bradyrhizobium* sp. SEMIA 6033::pHT409 after 72 h of cultivation with ampicillin, (**3**) *Bradyrhizobium* sp. SEMIA 6033::pHT409 after 72 h of cultivation without ampicillin.

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