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USE OF ENDOPHYTIC DIAZOTROPHIC BACTERIA AS A VECTOR TO EXPRESS THE *cry3A* GENE FROM *BACILLUS THURINGIENSIS*

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ABSTRACT

The goal of this study was to evaluate the potential of endophytic diazotrophic bacteria as a vector to express a *cry* gene from *Bacillus thuringiensis*, envisaging the control of pests that attack sugarcane plants. The endophytic nitrogen-fixing bacteria *Gluconacetobacter diazotrophicus* strain BR11281 and *Herbaspirillum seropedicae* strain BR11335 were used as models. The *cry3A* gene was transferred by conjugation using a suicide plasmid and the recombinant strains were selected by their ability to fix nitrogen in semi-solid N-free medium. The presence of the *cry* gene was detected by Southern-blot using an internal fragment of 1.0 kb as a probe. The production of δ-endotoxin by the recombinant *H. seropedicae* strain was detected by dot blot while for *G. diazotrophicus* the Western-blot technique was used. In both cases, a specific antibody raised against the *B. thuringiensis* toxin was applied. The δ-endotoxin production showed by the *G. diazotrophicus* recombinant strain was dependent on the nitrogen fixing conditions since the *cry3A* gene was fused to a *nif* promoter. In the case of *H. seropedicae* the δ-endotoxin expression was not affected by the promoter (*rhi*) used. These results suggest that endophytic diazotrophic bacteria can be used as vectors to express entomopathogenic genes envisaging control of sugarcane pests.

Key words: *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, endophytic bacteria, diazotrophic, *cry* gene

INTRODUCTION

The development of recombinant DNA technology has allowed production of many biotechnological products envisaging the biological control of insects, and most of them are related to *Bacillus thuringiensis*. This bacterium is responsible for 98% of the world biopesticide market and produces crystals with entomopathogenic activity, composed by proteins (δ-endotoxins) coded by cry genes. Höfte and Whiteley (13) classified different δ-endotoxins according to their amino acids sequence and their target insect. Recently, a new nomenclature based only on the identity of the amino acids of these toxins was proposed (7).

Most of these biotechnological products were obtained through the transfer of a *cry* gene to other organisms such as plant (1), cyanobacteria (16), bacteria (21) and viruses (15). These strategies have the potential to improve the *B. thuringiensis* efficacy and persistence, eliminating certain undesirable characteristics of the crystals such as its fast degradation when exposed to sunlight, instability in water and the inability to control insects that feed on internal plant tissue (11).

The insertion of the *B. thuringiensis* genes into plant chromosomes allows the control of insects that have the habit of feeding internally within plant (1). However to enable the *cry* gene expression in plants, it is necessary to increase its percentage of G and C bases (synthetic gene) to values very close to those found in plants (9). Adang *et al.* (1) evaluated the δ-endotoxin production in transgenic carrot and corn protoplasts containing the native *cry3A* gene (64% A+T) or

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the synthetic version (55% A+T) and demonstrated that only the latter was transcribed in a stable manner.

The introduction of *cry* genes into plant-associated bacteria appears to be more worthwhile since its chromosomal integration is much simpler. Moreover, the similarity of G+C content between these organisms and *B. thuringiensis* makes unnecessary changes of the value in the inserted gene. The main restriction to release recombinant bacteria into the environment is the dispersal of a foreign gene to another organism and among the components of risk assessment are persistence and genetic stability. In this way, recombinant endophytic bacteria are good candidates for δ-endotoxin production since their low survival in soil decrease the possibility of *cry* gene transfer to other soil microorganisms. This strategy proved to be an efficient methodology to inoculate corn plants with *Clavibacter xyli* subsp. *cynodontis* containing the *cry1Ac* gene (14) to control the European corn borer (*Ostrinia nubilalis*).

This report describes the introduction and expression of the *cry3A* gene *from B. thuringiensis* into endophytic diazotrophic bacteria, envisaging the control of coleopteran pests in sugarcane. Among the main endophytic bacteria which colonise roots and aerial tissues of sugar cane are *Gluconacetobacter diazotrophicus* (6) and *Herbaspirillum seropedicae* (3). The former has restricted occurrence, being associated mainly with sugar-rich plants, whereas *H. seropedicae* has a wider host range and neither survive well in soil (2). The expression of the *cry3A* gene in endophytic diazotrophic bacteria opens new perspectives of a biotechnological technique to control internally plant-tissue feeding insects where chemical and other biological control agents are not effective.

MATERIALS AND METHODS

1. Strains, plasmids and growth conditions

Bacterial strains and plasmids are listed in Table 1. *Escherichia coli* strains were grown in LB medium (18), *Gluconacetobacter diazotrophicus* strains were culture either in Dygs medium (8), Potato agar (8), LGI-P semi-solid medium (8) or modified LGI medium (5 $g.l^{-1}$ glucose; 5 mM sodium glutamate; 4 $g.l^{-1}$ K_2HPO_4; 6 $g.l^{-1}$ KH_2PO_4; 0.2 $g.l^{-1}$ $MgSO_4.7H_2O$; 0.02 $g.l^{-1}$ $CaCl_2.2H_2O$; 0,002 $g.l^{-1}$ $NaMoO_4.2H_2O$; 0.01 $g.l^{-1}$ $FeCl_3.6H_2O$; pH 6.0). *Herbaspirillum seropedicae* strains were grown in JNFB medium (8), Potato agar (8) or modified JNFB medium (JNFB medium containing 5 mM sodium glutamate and without yeast extract and bromotymol blue).

2. Bacterial mating

The introduction of the *cry3A* gene into the recipient strains BR11281 and BR11335 was achieved by conjugation using as donors *E. coli* strains containing the plasmids pPBS70 and pPBS80 respectively. The plasmid pRK2013 was used as a helper. The mating was performed onto the surface of filter (0.45 μm.) using a ratio of recipient to donor to helper of 10:1:1.

Table 1: Strains and plasmids used in this work.

Bacterial strain	Relevant characters	References
E. coli		
JM105	Plasmid mobiliser strain	18
HB101	Plasmid mobiliser strain	18
G. diazotrophicus		
BR11281	ATCC 4937; Nal^r	6
JS5701	BR11281::Tn*5*:*nifHp-cry3A*; Km^r	This work
H. seropedicae		
BR11335	Nal^r	3
PG5801	BR11335::Tn*5*:*rhiAp-cry3A*; Km^r	This work
Plasmids		
pRK2013	Helper plasmid	10
pPBS22	pKK233-2 containing *trc-cry3A*; Km^r	Skøt, L. pers. com.
pPBS24	pUC18 containing *cry3A* gene; Amp^r	Skøt, L. pers. com.
pPBS70	pSUP1021 containing *nifHp-cry3A* fusion with Tn5, Km^r	20
pPBS80	pSUP1021 containing *rhiAp-cry3A* fusion with Tn5; Km^r	20

Amp^r:100 μg ml^{-1}; Km^r: 50 μg ml^{-1} ; Nal^r: 10 μg ml^{-1}.

The filters were placed on Dygs (*G. diazotrophicus*) or JNFB (*H. seropedicae*) medium and incubated overnight at 30°C. The mixture of cells was diluted up to 10^{-3} and plated on Dygs or JNFB medium containing nalidixic acid and kanamycin for selection of the recombinants. The colonies obtained were again transferred to the specific medium containing the antibiotics and also plated on Potato agar medium containing 10% sucrose (*G. diazotrophicus*) and Potato agar (*H. seropedicae*). The recombinants were then inoculated into semi-solid medium and the nitrogenase activity was determined by the acetylene reduction assay (20) to confirm their ability to fix nitrogen.

3. Extraction of DNA

Recombinant plasmids from *E. coli* were obtained by the alkaline lysis method (4). Genomic DNA from recombinant and type strains of both diazotrophics was extracted by the CTAB (cetylmethylammonium bromide) method. The cells were grown in 100 ml of specific medium, centrifuged and re-suspended in 2 ml 10 mM Tris HCl pH 8.0 containing 1 mM EDTA. Then were added 0.5 ml 10% SDS and 10 µl pronase (100 $mg.ml^{-1}$) and the mixture was incubated at 37°C for 1 hour. After the incubation, 1.8 ml 5 M NaCl and 1.5 ml CTAB/NaCl (0.7 M NaCl containing 10% CTAB) were added and the lysate was incubated for 20 min at 65°C. The lysate was extracted with chloroform/isoamyl alcohol (24:1, v/v), phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v). The DNA was then precipitated with isopropanol and redissolved in 10 mM Tris HCl pH 8.0 containing 1 mM EDTA.

4. Southern blot hybridisation

A 1,060 bp internal fragment of the *cry3A* gene, generated by PCR using the primers col2A/col2B (5), was used as a probe. The purified plasmid pPBS24 containing the whole *cry3A* gene was added to the PCR mixture (0.2 mM dNTP, 1.5 U of *Taq* DNA-polimerase [GIBCO BRL], 0.1 to 0.5 µM of each primer and 10x buffer). The amplification was performed in a thermal cycler (PTC-100, MJ Research, Inc.) by using a program consisting of the following steps: 1 cycle 94°C for 1 min, 50°C for 1 min and extension at 72°C for 1 min. This cycle was repeated 30 times. The PCR product of the correct size was labelled with digoxigenin according to the manufacturer recommendation (DIG DNA Labelling Kit, Boehringer Mannheim Biochemical).

Genomic DNA and plasmids were digested with *Pst*I (Promega) and *Hind*III (Pharmacia Biotech) restriction enzymes, separated by electrophoresis in a 0.8% agarose gel and transferred by capillarity to a positively charged nylon membrane (Boehringer Mannheim Biochemical). Hybridisation was performed overnight at 65°C and subsequent washings and detection were accomplished according to the manufacturer recommendation (DIG DNA Labelling Kit, Boehringer Mannheim Biochemical).

5. δ-endotoxin production

Recombinant bacteria grown in flasks containing 900 ml of modified LGI or JNFB medium during a period of 4 days were collected, ressuspended in an extraction buffer (50 mM Tris HCl pH 8.0 containing 25% sucrose, 1 mM PMSF and 60 $mg.ml^{-1}$ lisozyme) and disrupted by 10 cycles of freezing/thawing. The pellet was washed with 5 M NaCl, incubated in an extraction buffer for 30 min at 4°C and washed with 5 M NaCl. The δ-endotoxin was solubilized in 5 mM Na_2CO_3 buffer pH 10.5 containing 1 mM DTT for 4 hours at 37°C, and quantified using the Bradford method (22).

6. Immunoblot analysis

Total cellular proteins isolated from *G. diazotrophicus*, *H. seropedicae* and *E. coli* strains were separated by SDS-polyacrilamide (10%) gel electrophoresis, electroblotted onto nitrocellulose filters and probed with primary antibody raised against the solubilised crystal protein isolated from *Bacillus thuringiensis* subsp. *tenebrionis* and secondary antibody coupled to Alkaline Phosphatase (Sigma). The δ-endotoxin was visualised by using NBT (nitro-blue-tetrazolium-chloride) e BCIP (5-bromo-4-cloro-3-indoylil phosphate, toluidine salt), according the manufacturer instructions (Bio-Rad).

RESULTS AND DISCUSSION

The *cry3A* gene from *B. thuringiensis* was transferred to *G. diazotrophicus* strain BR11281 and *H. seropedicae* strain

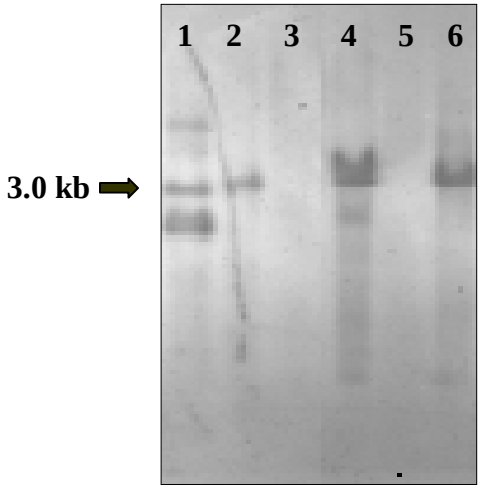

Figure 1: Detection of the *cry3A* gene in recombinant strains by Southern blot, using an internal fragment as a probe. The DNA was digested with *Eco*RI and *Hind*III restriction endonucleases. Lane 1, pPBS24; lane 2, pPBS70; lane3, BR11281; lane 4, JS5701; lane 5, BR11335; lane 6, PG5801.

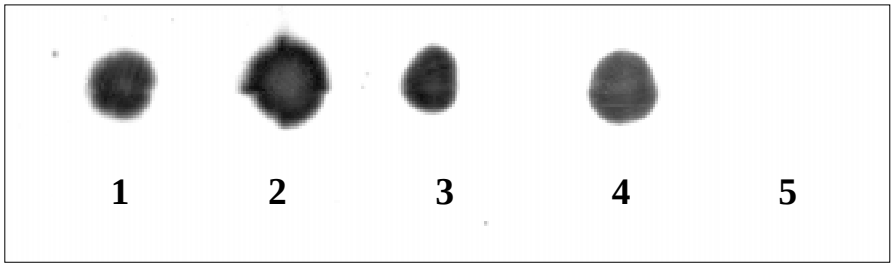

Figure 2: Dot blot analysis showing the presence of the *cry3A* gene in the recombinant *G. diazotrophicus* strain isolated from micropropagated sugar cane plants. The genomic DNA was directly applied on positively charged nylon membranes and hybridised with the *cry3A* probe. Lane 1, pPBS70; lane 2, pPBS22; lane 3, JS5701; lane 4, JS5701 isolated from sugar cane tissue; lane 5, BR11281.

BR11335 by conjugation, using the suicide plasmids pPBS70 and pPBS80 as vectors. In both constructions, the *cry3A* gene was hooked in the transposon Tn5. A transposable element instead of a plasmid was chosen as a vector to minimise the likelihood of losing the foreign gene. Skøt *et al.* (21) observed that transposons rather than plasmids improved the stability of the *cry3A* gene in the host genome, therefore decreasing its transference to other organisms.

To avoid selection of recombinant bacteria without the ability to fix nitrogen, all kanamycin resistant colonies obtained were checked. In the case of *H. seropedicae*, all km^r colonies retained their ability to fix nitrogen in semi-solid JNFB medium (data not shown). In contrast, this ability was lost in many of the *G. diazotrophicus* km^r colonies as well as the chocolate colour of the colonies when grown on Potato-P medium (data not shown). Two recombinant strains, one from *G. diazotrophicus* JS5701 containing the Tn5::*nifHp-cry3A* and one from *H. seropedicae* PG5801 containing the Tn5::*rhiAp-cry3A*, were used in this work. The presence of *cry3A* gene in JS5701 and PG5801 recombinant strains was confirmed by positive hybridisation signals at 3.0-kb size with the total DNA from both recombinant bacteria (Fig. 1). No signals were observed with total DNA from the original strain. The *Hind*III/*Pst*I fragment length (3 kb) is consistent with the *cry3A* putative sequence described by Sekar *et al*. (19).

The stability of the *cry3A* gene in the recombinant strain JS5701 was confirmed after reisolation of these inoculated strains from micropropagated sugarcane tissues maintained for 10 days in modified MS medium (17). All isolated colonies from *G diazotrophicus* were kanamycin resistant, indicating that the recombinants retained the inserted gene. This result was further confirmed by a Dot blot hybridisation of the genomic DNA from the reisolated strains with the *cry3A* probe (Fig. 2).

The δ-endotoxin production by the *H. seropedicae* recombinant strain was confirmed by Dot blot of total cellular proteins with the antibody raised against the Cry3A toxin (Fig. 3). In this recombinant strain, the production of the δ-endotoxin is regulated by a *rhi* promoter, which seems to be activated when the bacteria are colonising the plant rhizosphere.

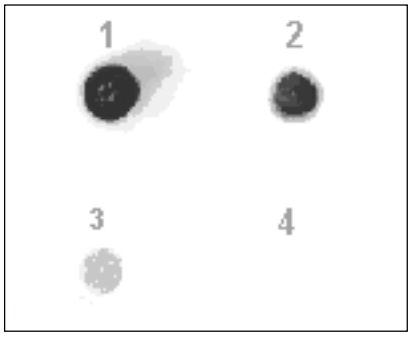

Figure 3: Dot blot analysis showing the δ-endotoxin production by the *H. seropedicae* recombinant strain. The Cry3A was detected using a specific antibody raised against the toxin. Lane 1, HB101(pPBS22); lane 2, HB101(pPBS22); lane 3, PG5801; lane 4, BR11335. The amount of total cellular proteins were not determined.

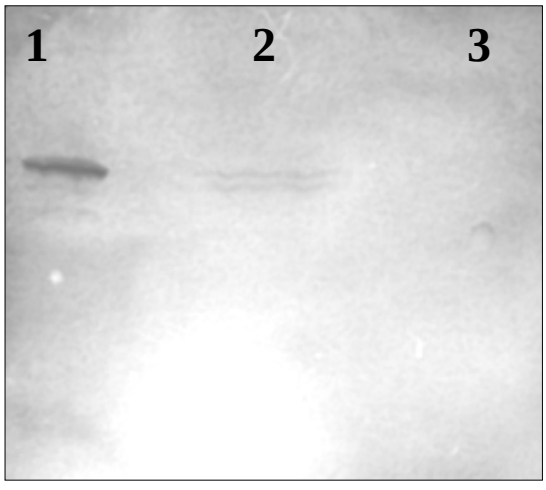

Figure 4: Western blot analysis of the total cellular proteins from G. *diazotrophicus* showing the presence of a 65 kDa band, which gave positive signal against the antibody specific to δ-endotoxin. Lane 1, JM105(pPBS22); lane 2, JS5701; lane 3, BR11281. The amount of total cellular proteins applied in each lane was 25, 25 and 20mg of protein, respectively.

Although the *cry3A* gene expression could be detected when the *H. seropedicae* recombinant strain was growing in culture medium, the δ-endotoxin production was higher when root exudates were added (data not shown). These results are in accordance with those obtained by Skøt *et al.* (21) who showed that even in the absence of root exudates, the *Rhizobium leguminosarum* recombinant strain, containing the *cry3A* gene under the control of the same promoter, was able to produce low amounts of δ-endotoxin. A possible explanation for the activation of the promoter *rhi* when the recombinant strain was grown in culture medium is the presence of growth factors or co-factors with functions related to those substances released by the root exudates.

The *cry3A* gene expression was observed for the *G. diazotrophicus* recombinant strain. A Western blot analysis of the total cellular proteins from *G. diazotrophicus* showed the presence of a 65 kDa band which gave positive signal against the antibody specific to δ-endotoxin (Fig. 4). Because the *cry3A* gene is fused to a *nif* promoter in *G. diazotrophicus*, the δ-endotoxin production was evaluated under nitrogen fixing conditions. The results showed that the nitrogenase activity of both the wild-type and recombinant strain increased during the first two days, reaching its maximum activity at the 3rd day and then decreased to a value similar to that found at the beginning of the experiment (Fig. 5a). The δ-endotoxin production of the recombinant strain increased daily and reached its maximum amount at the 4th day (Fig. 5b). Considering that the *cry3A* expression in *G. diazotrophicus* recombinant strain was regulated by the *nif* promoter it would be expected no δ-endotoxin production when the bacteria were

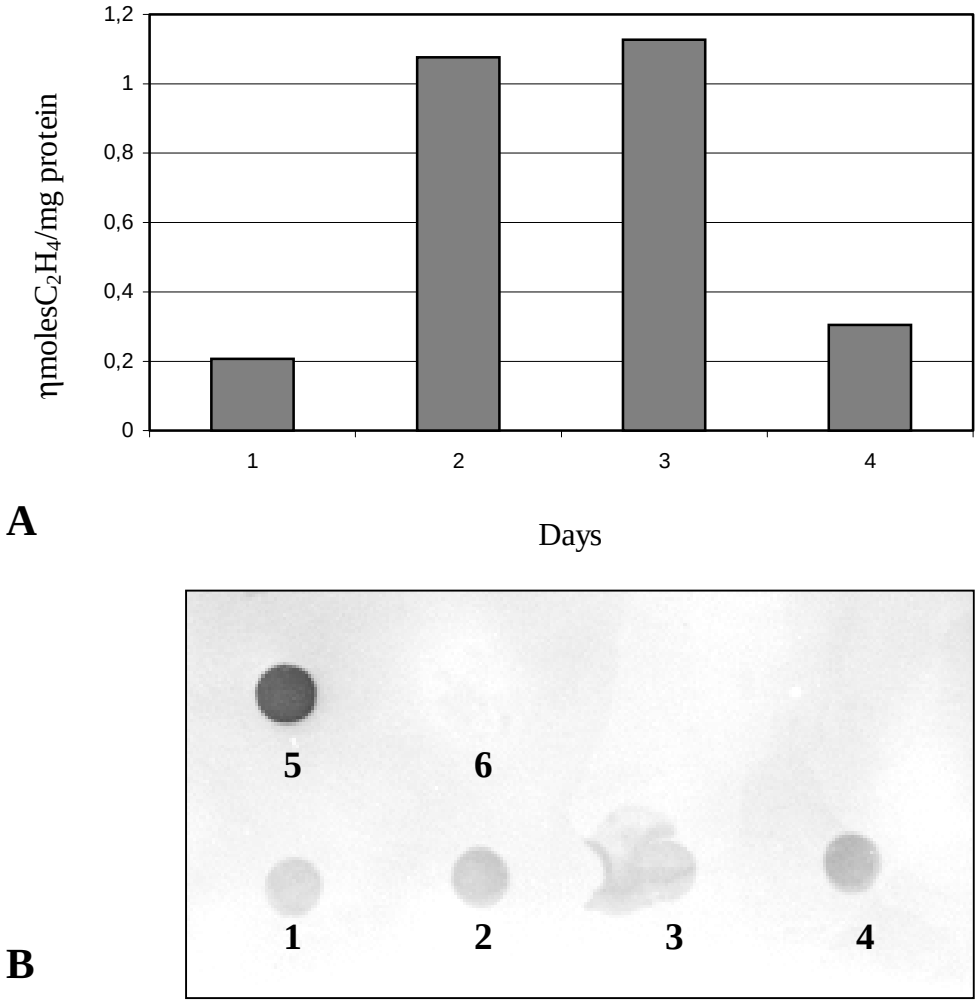

Figure 5: Evaluation of δ-endotoxin production in the *G. diazotrophicus* recombinant strain under nitrogen fixing conditions. A) Nitrogenase activity detected by acetylene reduction assay. B) Dot blot analysis showing the different amount of δ-endotoxin produced by the recombinant strain during a 4-day period. 1, 2, 3 and 4) δ-endotoxin production by JS5701 at the 1st, 2nd, 3rd and 4th day of growth, respectively; 5) JM105(pPBS22); 6) BR11281. The amount of total cellular proteins applied was 20mg, except for sample 5 (10mg of total cellular protein).

grown in medium containing an inorganic nitrogen source. The difference between the peaks of nitrogenase activity and the δ-endotoxin production during the experiment could be explained by the high stability of *cry3A* RNA messenger. In *B. thuringiensis* the half-life of the δ-endotoxin mRNA has an average of 10 minutes (12) and some *cis*-elements that act as mRNA stabilisers, located at both 3' and 5' end, are responsible for the high stability of δ-endotoxin transcript.

According to Wong and Chang (24), the *cis*-element that acts at the 3' extremity of the *cry* mRNA is composed of inverted repeated sequences. The transcription of which leads to the formation of a stem-and-loop structure that protects the mRNA from exonucleolytic degradation, increasing the half-lives of their transcripts and therefore enhancing gene expression (24). Udayasuriyan *et al*. (23) evaluated the *cry1Aa* expression in *E. coli* recombinants and observed that the absence of the structure was responsible for low δ-endotoxin production, probably due to the lower stability of the mRNA. The stem-and-loop structure was also detected at the 3' extremity of *cry3A* gene (19) and this region is present in the *cry3A* fragment used in the construction of pPBS70, which may explain the stability of *cry3A* mRNA in the *G. diazotrophicus* recombinant strain.

Our results confirmed the introduction of the *cry3A* gene and its expression in *G. diazotrophicus* and *H. seropedicae*, two diazotrophic bacteria known to colonise endophytically sugarcane. Although the promoters used in the plasmids pPBS70 and pPBS80 were not from *G. diazotrophicus* and *H. seropedicae* species, they were able to direct the *cry3A* transcription. In plasmids pPBS70 and pPBS80, the *cry3A* gene is regulated by the promoter of the operon *nifHDK* of *Rhizobium leguminosarum* biovar *trifolii* and by the promoter of the *rhiABC* operon in *Rhizobium leguminosarum* biovar *viciae*, respectively (21). The use of promoters dependent on regulatory gene-products may avoid the problem of insect resistance caused by the constant production of the δ-endotoxin, because they are activated only under specific conditions. Skøt *et al.* (21) introduced and expressed the *cry3A* gene in *Rhizobium leguminosarum* biovar *trifolii* strains under the control of the *nifHDK* operon. The recombinant bacteria were used to control *Sitona* spp., a coleopteran larvae that feeds on nitrogen fixing root nodules of several legume plants. The expression of the δ-endotoxin was dependent on the age of the nodules. Mature nodules expressed the gene while young nodules were unable to produce the toxin. The use of its own *nif* promoters would enhance the δ-endotoxin production of *G. diazotrophicus* and *H. seropedicae* species, therefore increasing the efficiency of the recombinant strains. The ability of these bacteria to colonise endophytically the plant tissues associated with their poor survival in soil are characteristics that make these recombinants good candidates to be used as vectors for the control of coleopteran and lepidopteran pests in sugarcane.

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RESUMO

Uso de bactérias diazotróficas endofíticas como vetores para expressar *cry*3A de *Bacillus thuringiensis*

Este estudo teve como objetivo avaliar o potencial de uso de bactérias diazotróficas endofíticas como vetores para a expressão de genes *cry* de *Bacillus thuringiensis*. As bactérias diazotróficas endofíticas *Gluconacetobacter diazotrophicus* estirpe BR11281 e *Herbaspirillum seropedicae* estirpe BR11335 foram usadas como modelo. O gene *cry3A* foi transferido através de um plasmídeo suicída por conjugação e os recombinantes foram selecionados pela sua capacidade de fixar nitrogênio em meio semi-sólido sem N. A presença do gene *cry3A* no genoma dos transconjugantes foi detectada através da técnica de "Southern blot" utilizando como sonda um fragmento de 1,0 kb, interno ao gene *cry3A*. A produção de δ-endotoxina pelos transconjugantes foi detectada por "Dot blot" em de *H. seropedicae* e por "Western blot" em *G. diazotrophicus*, usando-se o anticorpo específico para a toxina de *B. thuringiensis*. A avaliação da produção da δ-endotoxina mostrou que a expressão do gene *cry3A* em *G. diazotrophicus* é dependente do processo de fixação de nitrogênio por estar fusionado a um promotor *nif* nesta bactéria. No caso de *H. seropedicae* a expressão não foi influenciada pelo promotor de rizosfera (*rhi*) usado. Os resultados obtidos sugerem que estas bactérias diazotróficas endofíticas podem ser usadas como vetores para expressar genes com atividade entomopatogênica, visando o controle de pragas de cana-de-açúcar ou outras plantas de interesse econômico.

Palavras-chave: *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, bactérias endofíticas, diazotróficas, gene *cry*.

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