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...
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 K₂HPO₄; 6 g.l⁻¹ KH₂PO₄; 0.2 g.l⁻¹ MgSO₄.7H₂O; 0.02 g.l⁻¹ CaCl₂.2H₂O; 0.002 g.l⁻¹ NaMoO₄.2H₂O; 0.01 g.l⁻¹ FeCl₃.6H₂O; 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.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Relevant characters</th>
<th>References</th>
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<tbody>
<tr>
<td>E. coli</td>
<td>Plasmid mobiliser strain</td>
<td>18</td>
</tr>
<tr>
<td>JM105</td>
<td>Plasmid mobiliser strain</td>
<td>18</td>
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<tr>
<td>HB101</td>
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<td></td>
</tr>
<tr>
<td>G. diazotrophicus</td>
<td>ATCC 4937; Na⁺</td>
<td>6</td>
</tr>
<tr>
<td>BR11281</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JS5701</td>
<td>BR11281::Tn5::Hp-cry3A; Km⁻</td>
<td>This work</td>
</tr>
<tr>
<td>H. seropedicae</td>
<td>Na⁺</td>
<td>3</td>
</tr>
<tr>
<td>BR11335</td>
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<td></td>
</tr>
<tr>
<td>PG5801</td>
<td>BR11335::Tn5::rhiAp-cry3A; Km⁻</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
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<tr>
<td>pRK2013</td>
<td>Helper plasmid</td>
<td>10</td>
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<tr>
<td>pPBS24</td>
<td>pUC18 containing cry3A gene; Amp'</td>
<td>Skøt, L. pers. com.</td>
</tr>
<tr>
<td>pPBS70</td>
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</tr>
<tr>
<td>pPBS80</td>
<td>pSUP1021 containing rhiAp-cry3A fusion with Tn5; Km⁻</td>
<td>20</td>
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</tbody>
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Amp':100 µg ml⁻¹; Km⁻: 50 µg ml⁻¹; Na⁺: 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 diazotrophs was extracted by the CTAB (cetyltrimethylammonium bromide) method. The cells were grown in 100 ml of specific medium, centrifuged and resuspended in 2 ml 10 mM Tris HCl pH 8.0 containing 1 mM EDTA. Then were added 0.5 ml 10% SDS and 10 \mu l 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 \mu M 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 PstI (Promega) and HindIII (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. \delta-endotoxin production

Recombinant bacteria grown in flasks containing 900 ml of modified LGI or JNFB medium during a period of 4 days were collected, resuspended in an extraction buffer (50 mM Tris HCl pH 8.0 containing 25% sucrose, 1 mM PMSF and 60 mg.ml^{-1} lisoyme) 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 \delta-endotoxin was solubilized in 5 mM NaCO3 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, electrobotted onto nitrocelulose 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 \delta-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.

3.0 kb

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 EcoRI and HindIII restriction endonucleases. Lane 1, pPBS24; lane 2, pPBS70; lane3, BR11281; lane 4, JS5701; lane 5, BR11335; lane 6, PG5801.
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⁻ 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⁻ 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 HindIII/PstI 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.

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**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.

**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 20 mg 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
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. Skott 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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Cry3A gene from *B. thuringiensis*


