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Full Length Research Paper

Toxicity to *Eldana saccharina* of a recombinant *Gluconacetobacter diazotrophicus* strain carrying a truncated *Bacillus thuringiensis cry1Ac* gene

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The stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is a serious pest of sugarcane in Africa. Because *E. saccharina* larvae feed inside the stem of sugarcane, expression of insecticidal *Bacillus thuringiensis* Cry proteins in sugarcane endophytes, such as *Gluconacetobacter diazotrophicus*, would enable the Cry proteins to be produced in the feeding zones of burrowing larvae. To evaluate the potential of using a Cry-expressing *G. diazotrophicus* strain for the control of *E. saccharina,* a truncated *B. thuringiensis cry1Ac* gene was cloned into a *G. diazotrophicus* strain and the toxicity of the recombinant strain to *E. saccharina* larvae was evaluated. Bioassays showed that the recombinant *G. diazotrophicus* strain carrying the cloned *cry1Ac* gene caused significantly higher *E. saccharina* mortality and larval growth inhibition than the non-recombinant *G. diazotrophicus* strain. This study sets a foundation for *E. saccharina* control strategies that are based on the expression of truncated *cry1Ac* genes in *G. diazotrophicus.*

Key words: *Eldana saccharina, Bacillus thuringiensis,* Cry protein, toxicity, *Gluconacetobacter diazotrophicus,* recombinant strain.

INTRODUCTION

Bacillus thuringiensis (*Bt*) is an aerobic, spore-forming, Gram-positive bacterium that synthesizes one or more parasporal proteinaceous crystals during sporulation (Höfte and Whiteley, 1989; Schnepf et al., 1998). The *Bt* crystals may consist of either Cyt proteins or Cry proteins (Höfte and Whiteley, 1989; Schnepf et al., 1998). As a group, Cry proteins are toxic to certain species in the orders Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Hemiptera, and also to other invertebrates such as nematodes and mites (Schnepf et al., 1998; van Frankenhuyzen, 2009). Due to desirable characteristics, such as low toxicity to non-target organisms (Höfte and Whiteley, 1989), *Bt* insecticidal crystals have been used in several commercial products that are formulated for spray application using conventional spray equipment (Luttrell et al., 1999; Schnepf et al., 1998). Since *Bt* bioinsecticides do not have systemic activity in plants, they are not effective against insects that burrow into the host plant and feed in the stem.

The stalk borer *Eldana saccharina* Walker (Lepidoptera: Pyralidae) is the most destructive pest of sugarcane in southern Africa and causes severe sugarcane crop losses (Way, 2004). *E. saccharina* bores into the sugarcane stem and feeds internally for most of its life cycle. Primarily due to this feeding behaviour, *Bt* crystal-based formulations have not been effective against *E. saccharina* in sugarcane fields (D. Conlong, South African Sugarcane Research Institute, personal communication).

In an attempt to improve the efficacy of *Bt* against lepidopteran larvae that burrow into the sugarcane stem, considerable research effort has gone into alternative delivery strategies, such as the development of

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transgenic sugarcane or the use of plant-associated bacteria (PAB) as delivery vehicles for Cry proteins. Although transgenic sugarcane plants expressing Cry proteins have been constructed and tested against some sugarcane pests (Arencibia et al., 1997), a review of the literature revealed no published papers that evaluated Cry-expressing sugarcane for the control of *E. saccharina*. Both epiphytes (bacteria that live on the surface of plants) and endophytes (bacteria that inhabit, for at least one period of their life cycle, the interior of a plant) have been used as delivery vehicles for Cry proteins (Bora et al., 1994; Lampel et al., 1994; Salles et al., 2000). To target insects that burrow into a plant, endophytic bacteria are likely to be better vehicles for Cry proteins than epiphytic bacteria.

Gluconacetobacter diazotrophicus (*Acetobacteraceae*) is an obligate aerobic, Gram-negative, endophyte that has been isolated from the roots and stems of sugarcane (Gillis et al., 1989; Reis et al., 1994). Primarily due to its ability to fix atmospheric nitrogen, *G. diazotrophicus* has been the focus of intense study (Dong et al., 1997; Gillis et al., 1989; James et al., 1994; Lee et al., 2000; Reis et al., 1994; Saravanan et al., 2008; Sevilla and Kennedy, 2000). Salles et al. (2000) evaluated the potential of using *G. diazotrophicus* strain BR11281 as a vector to express a *Bt cry3A* gene for the control of coleopteran pests of sugarcane. Although the authors did not evaluate the insecticidal activity of the recombinant *G. diazotrophicus* strain, the study of Salles et al. (2000) suggested that *G. diazotrophicus* may have potential as a vector to express *Bt cry* genes for the control of sugarcane pests.

To our knowledge, no published studies have evaluated *G. diazotrophicus* as a vector to express lepidopteran-specific *cry* genes for the control of lepidopteran insects, such as *E. saccharina.* Because *G. diazotrophicus* inhabits the sugar solution in the intercellular-space apoplast of the stem cortex of sugarcane (Dong et al., 1997), it is a good candidate for the creation of a recombinant endophyte for the control of *E. saccharina* larvae. In this study, a novel *Bt cry1Ac* gene construct was cloned into a *G. diazotrophicus* strain and the toxicity of the recombinant strain to *E. saccharina* larvae was evaluated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bt isolate SB-4 (SB-4; *cry1Ac* gene GenBank accession number AY225453), which was previously isolated from *E. saccharina* larval cadavers and showed high insecticidal activity against *E. saccharina* larvae (G. Bouwer, unpublished study), was cultured at 30°C in Luria-Bertani (LB) medium. *E. coli* strains were cultured at 37°C in LB medium. *G. diazotrophicus* PAl 5 (hereafter referred to as PAl5) (ATCC 49037^T) was obtained from the American Type Culture Collection (ATCC) (Manassas, USA) and was cultured at 30°C in the medium described by James et al. (1994), hereafter referred to as JM medium.

General molecular techniques

General molecular techniques, such as ethanol precipitation of DNA and agarose gel electrophoresis (AGE), were performed as described by Sambrook et al. (1989). DNA was quantified with a DyNA Quant 200 Fluorometer (Amersham Biosciences, Piscataway, NJ) using Hoechst 33258 dye (Sigma, St. Louis, USA) and Calf Thymus DNA (Amersham Biosciences) as the standard. The DNA molecular weight marker used throughout the study was the 1 Kb plus DNA Ladder (Invitrogen, Carlsbad, USA). Plasmids were isolated from *E. coli* strains by using either a Plasmid Midi Kit (Qiagen) when large yields were required or by using a QIAprep Spin Miniprep Kit (Qiagen) for routine analysis or when small yields would suffice. Restriction enzymes were supplied by Roche Applied Biosciences (Indianapolis, USA). PCR products or linearized plasmids were prepared for cloning by electrophoretic separation in 0.7% (w/v) low melting temperature agarose gels (SeaPlaque GTG Agarose, Lonza Rockland, Rockland, USA), excision of the band of the desired size, and extraction of the DNA from the gel using a QIAquick Gel Extraction Kit (Qiagen, Valencia, USA). Commercial kits and equipment were used as per manufacturer instructions.

Cloning system

The Gateway Cloning System (Invitrogen) was used. The Gateway Cloning System is based on the site-specific recombination reactions of bacteriophage λ in *E. coli.* All Gateway reaction mixtures (BP or LR) were transformed into Library Efficiency DH5α competent cells (Invitrogen) and, after phenotypic expression in S.O.C. medium, transformed cells were identified on the basis of colony formation on LB plates containing the appropriate antibiotic. The plasmids from transformants were purified (as described above) and analysed by digestion with *Eco*RI and AGE.

PCR amplification of *cry1Ac* for cloning

An insecticidal crystal gene of SB-4 was previously characterized (G. Bouwer, unpublished study) and classified as a *cry1Ac* gene (GenBank accession number AY225453). Using conserved regions in the SB-4 *cry1Ac* gene, a primer set was designed to amplify the first (starting with "A" in the ATG start codon) 1908 bp of the *cry1Ac* gene. The forward primer (*cry1Ac*-*att*B1) had the following sequence:

5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAG AACCATGGATAACAATCCGAACATCAATG- 3'.

In addition to a sequence complementary to the 5' end of the *cry1Ac* gene, the forward primer also has a sequence that contains a ribosome recognition sequence (RRS) and an *att*B1 sequence which is required for cloning using the Gateway vectors. The reverse primer (*cry1Ac*-t-*att*B2) had the following sequence:

5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACCCTAG TTGGTTTGTAGACG- 3'.

In addition to a sequence that is complementary to the *cry1Ac* gene sequence (from position 1889 to 1908), this primer also contains a sequence that codes for a stop codon and an *att*B2 sequence. DNA analysis and primer design were done using DNAMAN version 4 (Lynnon BioSoft, Pointe-Claire, Canada).

The Expand High Fidelity PCR System (Roche Applied Science), a system that incorporates a proofreading polymerase, was used for PCR. PCR was performed on a PCR-Express Thermocycler (Thermo Hybaid/Thermo Scientific, Rockford, USA). The program for the primer set was 2 min at 94°C followed by 30 cycles of 15 s

at 94°C, 30 s at 55°C and 2 min at 72°C, and a final single extension cycle of 7 min at 72°C. Twenty nanogram of SB-4 plasmid DNA, purified using a Plasmid Midi Kit, was used as the template and all other reaction concentrations and volumes were as per the Expand High Fidelity PCR System manual. Amplification was confirmed by AGE, with PCR product sizes compared to the molecular weight markers.

Cloning of truncated *cry1Ac* into an expression vector

The basic outline of the protocol used to clone the truncated *cry1Ac* gene construct (including the RRS and stop codon; the construct is hereafter referred to as *cry1Ac*-t) into an expression vector is provided below. The PCR product (*cry1Ac*-t-*att*B) obtained with the primer set was cloned into pDONR201 (Invitrogen) using the PCR Cloning System and the Gateway BP Clonase enzyme mix. The BP reaction mixture was transformed into DH5α and transformants were selected on LB plates containing 50 µg/ml kanamycin (Km). The resultant construct was pENTR-cry1Ac-t. Cloning of *cry1Ac*-t-*att*B into pDONR201 was performed to flank *cry1Ac*-t with *att*L sites, thus enabling *cry1Ac*-t to be cloned into Gateway *att*R-containing expression vectors using the LR reaction. The *cry1Ac*-t construct was cloned from pENTR-cry1Ac-t into pDEST14 (T7 promoter expression vector; Invitrogen) using the Gateway *E. coli* Expression System and the Gateway LR Clonase enzyme mix. The LR reaction mixture was transformed into DH5α and transformants were selected on LB plates containing 100 µg/ml ampicillin (Ap). The resultant construct was pEXP14-cry1Ac-t. The cloning of *cry1Ac*-t into pDONR201 (creating pENTR-cry1Ac-t) and subsequent transfer into pDEST14 (creating pEXP14-cry1Ac-t) was confirmed by *Eco*RI digestion and analysis of the fragments by AGE.

pEXP14-cry1Ac-t, with *cry1Ac*-t located downstream of a T7 promoter, was transformed into *E. coli* BL21-SI competent cells (Invitrogen) and, after phenotypic expression in S.O.C. medium, transformed cells were identified on the basis of colony formation on LBON (LB without salt; Invitrogen) plates containing 100 µg/ml Ap. The BL21-SI strain carrying pEXP14-cry1Ac-t is hereafter referred to as BL21-SI(pEXP14-cry1Ac-t).

Analysis of protein expression in *E. coli*

BL21-SI(pEXP14-cry1Ac-t) cells were used for the *E. coli* protein expression studies. The protein expression studies were performed as per the protocol provided with the BL21-SI competent cells (Invitrogen), with LBON (containing 100 µg/ml Ap) as the culture medium. Proteins in cell pellets were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970), as modified by Davis et al. (1986). The molecular weights of the proteins were estimated by comparing their relative mobilities to those of calibration proteins (Roche Applied Science). Gels were fixed and stained with Coomassie Brilliant Blue R250 (Sigma).

Conversion of plasmid pKT230 into a Gateway destination vector

Plasmid pKT230 (Bagdasarian et al., 1981) was obtained from the Netherlands Culture Collection of Bacteria (NCCB) (Utrecht, The Netherlands). The rest of the vector conversion was performed using Gateway Cloning Technology and the Gateway Vector Conversion System (Invitrogen). The basic outline of the procedure is provided below. A Gateway *att*R cassette (a cassette with the *att*R sites used in Gateway destination vectors), was ligated with the linearized (*Sma*I digestion), dephosphorylated pKT230 using T4 DNA Ligase (Promega, Madison, USA) in ligase buffer. The ligation reaction was transformed into Library Efficiency DB3.1 competent cells (Invitrogen) and, after phenotypic expression in S.O.C. medium, transformed cells were identified on the basis of colony formation on LB plates containing 30 µg/ml chloramphenicol (Cm). The plasmids from transformed cells were analysed with *Eco*RI digestion. The resultant construct was named pDESTN230.

Cloning of truncated *cry1Ac* into pDESTN230

In accordance with Invitrogen's recommendations, pDESTN230 was linearized (*Not*I digestion) in preparation for cloning. The truncated *cry1Ac* gene was cloned from pENTR-cry1Ac-t into pDESTN230 using the Gateway LR Clonase enzyme mix. During the LR reaction, *cry1Ac*-t was transferred via site-specific recombination from pENTR-cry1Ac-t to pDESTN230 (creating pEXP230-cry1Ac-t). The LR reaction mixture was transformed into DH5α and transformants were selected on LB plates containing 40 µg/ml streptomycin (Sm). pEXP230-cry1Ac-t, with the truncated *cry1Ac* gene under control of the pKT230 Km^r promoter, was used to transform PAI5. Because there is a single *Eco*RI site in pDESTN230 and two *Eco*RI sites in *cry1Ac*-t, the cloning of *cry1Ac*-t was confirmed by the presence of three fragments (approximately 10.4, 2.8, and 0.7 kb) after *Eco*RI digestion.

Transformation of *G. diazotrophicus* PAI5 with pEXP230-cry1Ac-t

PAI5 was cultured in JM medium and made electrocompetent using the Procedure for High Efficiency Electro-transformation of *E. coli* - Preparation of Cells protocol provided in the Pulse Controller Operating Instructions and Applications Guide (Bio-Rad, Hercules, USA). Electroporation was performed with a Gene Pulser fitted with the Pulse Controller accessory (Bio-Rad). The Gene Pulser was set to 25 µF and 2.50 kV, and the Pulse Controller was set to 400 Ω. PAI5 (200 µl) was electro-transformed with 200 ng of pEXP230-cry1Ac-t. After phenotypic expression in JM medium for 3 h (140 rpm and 30°C), serial dilutions of the broth culture were prepared and transformed cells were identified on the basis of colony formation on JM plates containing 40 µg/ml Sm. Transformation was confirmed by AGE and PCR using the primer set described above, with purified plasmid as template. The PAI5 strain carrying pEXP230-cry1Ac-t is hereafter referred to as PAI5(pEXP230-cry1Ac-t). Proteins in PAI5 and PAI5(pEXP230-cry1Ac-t) cell pellets were analysed by SDS-PAGE as described above for the *E. coli* expression studies. The protein concentrations of samples were estimated by using a Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). Gels were stained by silver staining (Davis et al., 1986).

Toxicity bioassays

E. saccharina eggs were obtained from the South African Sugarcane Research Institute (Mt Edgecombe, South Africa). Insects were reared on a chickpea diet at 26 ± 2°C, 70 ± 4% relative humidity, and a 12:12 h (L:D) photoperiod. Neonate larvae were collected from egg hatching bags and used directly for neonate-based assays or were reared until they had reached the second instar. After adding an autoclaved agar suspension to the dry ingredients of the chickpea diet, the diet was allowed to cool to 56°C before lyophilized PAI5(pEXP230-cry1Ac-t) or PAI5 was mixed thoroughly with the medium. A multiple-concentration (0.5, 1.0, 2.0, 4.0, and 8.0 mg/ml) bioassay design was used. Bioassays were performed on both neonate and second instar larvae, with five replicates of 20 larvae per concentration. Mortality in each group was recorded 4 d after exposure and corrected for control (chickpea

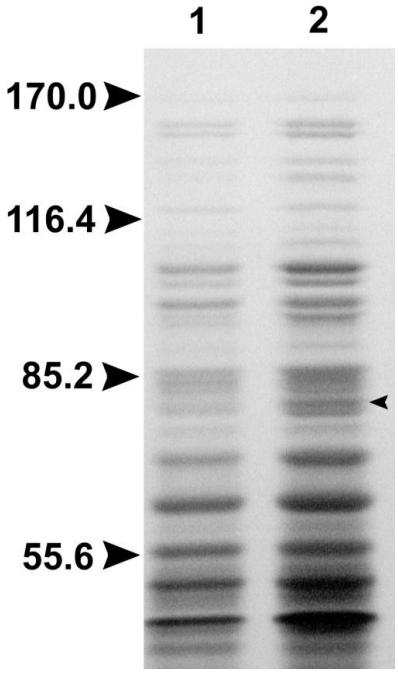

Figure 1. SDS-PAGE analysis of the expression of truncated *Bt* Cry1Ac protein in BL21-SI cells. An 8% (w/v) polyacrylamide gel and Coomassie Blue staining were used. Lane 1, protein profile of BL21-SI (control); lane 2, protein profile of BL21-SI(pEXP14-cry1Ac-t), with the arrow indicating the position of the truncated Cry1Ac protein (72 kDa). Arrow and number combinations alongside the gel show the positions and sizes (kDa) of the molecular weight markers. BL21-SI samples were prepared for SDS-PAGE by harvesting the cells from 1 ml broth culture, freezing the pellet overnight and re-suspending the cells in 100 µl ×1 loading buffer. For each BL21-SI sample, 20 µl was loaded per lane.

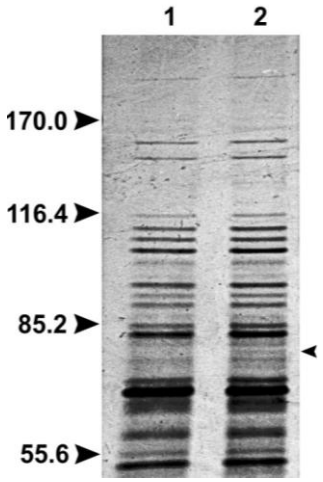

Figure 2. SDS-PAGE analysis of the expression of the truncated *Bt* Cry1Ac protein in *G. diazotrophicus* PAl5(pEXP230-cry1Ac-t). An 8% (w/v) polyacrylamide gel and silver staining were used. Lane 1, protein profile of *G. diazotrophicus* PAl5; lane 2, protein profile of *G. diazotrophicus* PAl5(pEXP230-cry1Ac-t), with the arrow indicating the position of the truncated Cry1Ac protein (72 kDa). Arrow and number combinations alongside the gel show the positions and sizes (kDa) of the molecular weight markers. Lanes were loaded with 10 µg total protein.

diet only) mortality using Abbott's formula (Abbott, 1925). Larvae that did not move when prodded were recorded as dead. Second instar larvae that survived treatment were individually weighed upon completion of the bioassays. ANOVA was use to analyse the bioassay data. When F values were significant, the Tukey HSD test was used to analyse differences in means (Statistica 7.1, Statsoft, Tulsa, USA). Percentage mortality was transformed by Anscombe's transformation prior to ANOVA (Anscombe, 1948; Zar, 1999).

RESULTS

Protein expression in *E. coli*

Analysis of the protein profiles of induced BL21-SI(pEXP14-cry1Ac-t) cells showed a novel (not present in BL21-SI cells) protein with an SDS-PAGE-estimated size of 72 kDa (Figure 1). Since *cry1Ac*-t codes for 636 amino acids with a theoretical molecular weight of 71.1 kDa (estimated with DNAMAN), the size of the novel 72 kDa protein is consistent with that expected from cloned *cry1Ac*-t. The SDS-PAGE therefore confirmed the functionality of both the *cry1Ac*-t construct and pEXP14-cry1Ac-t.

Expression of *cry1Ac* in *G. diazotrophicus* PAl5

Electroporation was used to transform PAl5 with pEXP230-cry1Ac-t. To confirm the presence of *cry1Ac*-t in transformed PAl5, plasmids were purified from transformed PAl5 and used as the template for PCR. The PCR resulted in a 2.0 kb product (data not shown), a product consistent with *cry1Ac*-t. The size of the extracted plasmid (13.9 kb) and the results of the PCR confirmed that PAl5 had been successfully transformed with pEXP230-cry1Ac-t. SDS-PAGE analysis of the recombinant strain PAl5(pEXP230-cry1Ac-t) confirmed the presence of a protein with a molecular weight of 72 kDa (Figure 2). This size is consistent with the

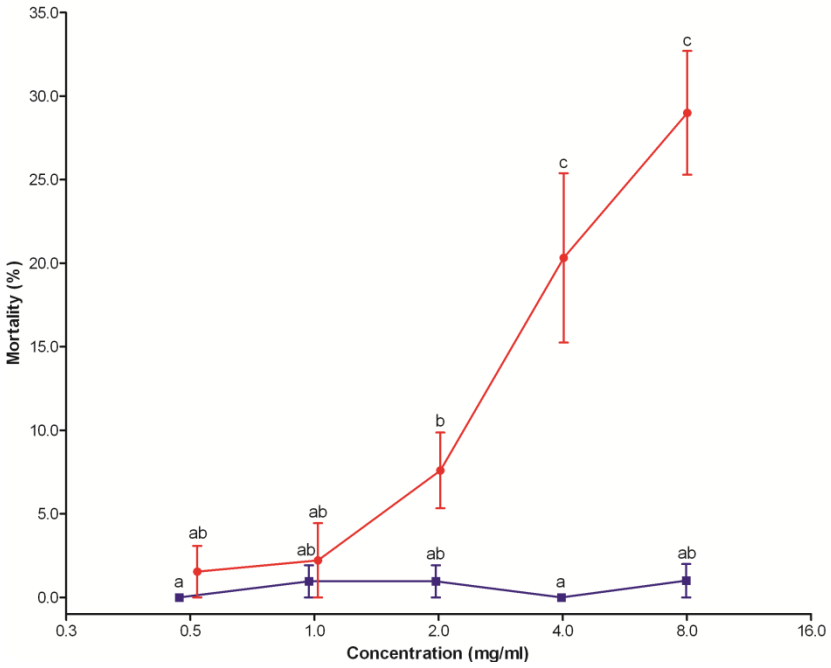

Figure 3. Insecticidal activity of *G. diazotrophicus* PAl5(pEXP230-cry1Ac-t) (●) and *G. diazotrophicus* PAl5 (■) against *E. saccharina* neonate larvae. Data points (mean ± standard error; 5 replicates of 20 larvae) with the same letters are not significantly different [P>0.05; Tukey HSD test for unequal sample size, performed after ANOVA showed there was a significant interaction ($F_{4,40}$=11.57, P<0.000001) between concentration and treatment, i.e. PAl5(pEXP230-cry1Ac-t) or PAl5]. Although statistical analyses were performed on transformed data [Anscombe's transformation for percentage (Anscombe, 1948; Zar, 1999)], for clarity the untransformed values are presented in the graph. X-axis is presented on a log base 2 scale.

polypeptide expressed from pEXP14-cry1Ac-t in BL21-SI cells (Figure 1) and with the theoretical molecular weight of the truncated Cry1Ac encoded by *cry1Ac*-t.

Toxicity bioassays

The toxicity of recombinant *G. diazotrophicus* PAl5(pEXP230-cry1Ac-t) to neonate larvae was compared to that of non-recombinant PAl5. At both 4.0 and 8.0 mg/ml lyophilized cells, corresponding to 9.1×10^5 and 1.8×10^6 cells/ml respectively, the mortality caused by PAl5(pEXP230-cry1Ac-t) was significantly higher (P<0.05) than that caused by PAl5 (Figure 3). For PAl5(pEXP230-cry1Ac-t), *E. saccharina* mortality at 4.0 or 8.0 mg/ml was significantly higher (P<0.05) than that recorded at the other concentrations evaluated (Figure 3). In contrast, no significant difference in neonate mortality was observed between different concentrations of PAl5 (Figure 3). Because PAl5(pEXP230-cry1Ac-t) did not kill second instar *E. saccharina* larvae, the effect of PAl5(pEXP230-cry1Ac-t) on 4-days-post-treatment weight was taken as a measure of toxicity. The interaction between treatment (PAl5(pEXP230-cry1Ac-t) or PAl5) and concentration had a significant effect (ANOVA, $F_{4.888}$ =34.2, P<0.00001) on larval weight. At every concentration evaluated, the weights of larvae reared on diet containing PAl5(pEXP230-cry1Ac-t) were significantly lower (P<0.05) than those of larvae reared on diet containing PAl5 (Table 1). In contrast to the results obtained for PAl5(pEXP230-cry1Ac-t), there was a general trend of higher larval weight at higher concentrations of PAl5 (Table 1).

DISCUSSION

With the aim of evaluating the potential of controlling *E. saccharina* larvae with a *G. diazotrophicus* strain expressing a *Bt* insecticidal Cry protein, a truncated *cry1Ac* gene was cloned into *G. diazotrophicus* PAl5. Since only the first 612 amino acids of the 1178 amino acid Cry1Ac protein are required to produce an active toxin (Adang et al., 1985), a truncated *cry1Ac* gene was used in this study. To determine if the truncated *cry1Ac* gene construct (*cry1Ac*-t) would be expressed in Gram-negative bacteria, the construct was tested in *E. coli* using Gateway expression vectors. The production in

Table 1. Concentration-larval weight responses of *E. saccharina* second instar larvae to *G. diazotrophicus* PAl5 and *G. diazotrophicus* PAl5(pEXP230-cry1Ac-t).

G. diazotrophicus concentration (mg/ml)	*E. saccharina* larval weight (mg)[a]	
	PAl5	PAl5(pEXP230-cry1Ac-t)
0.5	5.34 ± 0.23 a (n = 60)	2.17 ± 0.07 a (n = 94)
1.0	5.09 ± 0.18 a (n = 91)	1.99 ± 0.06 ab (n = 90)
2.0	5.30 ± 0.20 a (n = 95)	1.42 ± 0.05 bc (n = 91)
4.0	6.46 ± 0.22 b (n = 94)	1.16 ± 0.04 c (n = 93)
8.0	7.05 ± 0.22 b (n = 96)	1.19 ± 0.04 c (n = 94)

[a]Mean ± standard error; values in a column followed by the same letter are not significantly different (P>0.05; Tukey HSD test for unequal sample size). At each concentration, the mean weight of insects reared on a diet containing PAl5(pEXP230-cry1Ac-t) was significantly lower (P<0.05; Tukey HSD test for unequal sample size) than that of insects reared on a diet containing PAl5. Values in brackets are the sample sizes.

BL21-SI(pEXP14-cry1Ac-t) of a protein with the size expected (71 kDa) from *cry1Ac*-t confirmed the functionality of the *cry1Ac*-t construct. Since pENTR-cry1Ac-t was used to produce pEXP14-cry1Ac-t, and the functionality of *cry1Ac*-t was confirmed in pEXP14-cry1Ac-t, pENTR-cry1Ac-t was used to provide the truncated *cry1Ac* gene construct for cloning into pKT230. By cloning the *cry1Ac*-t construct contained in pENTR-cry1Ac-t into pDESTN230 (pKT230 converted into a Gateway-compatible vector) by λ-mediated site-specific cloning, a construct of known functionality was cloned in the desired orientation and reading frame into the vector pDESTN230. The resultant plasmid, pEXP230-cry1Ac-t, was used to transform PAl5.

PAl5(pEXP230-cry1Ac-t) was significantly more toxic to neonate larvae than PAl5, with 4.0 and 8.0 mg/ml lyophilized cells of PAl5(pEXP230-cry1Ac-t) causing 4-days-post-treatment average mortalities of 20.3 and 29.0% respectively. In comparison, a *cry1Ac*-expressing recombinant strain Nal1(pMT7) of the obligate endophytic bacterium *Herbaspirillum seropedicae* caused 48% average mortality 4-days-post-treatment when assayed at 3 mg/ml lyophilized cells against *E. saccharina* neonate larvae (Downing et al., 2000). However, in contrast to PAl5, non-recombinant *H. seropedicae* strain Nal1 caused considerable neonate mortality (12%), indicating that the control-corrected mortality for the recombinant strain Nal1(pMT7) was approximately 36% in the study of Downing et al. (2000).

Sublethal effects (feeding inhibition and growth inhibition) are considered more sensitive indicators of Cry protein toxicity than lethal effects (van Frankenhuyzen, 2009). Larvae that survived after treatment with PAl5(pEXP230-cry1Ac-t) showed significant stunting in their growth compared to larvae treated with PAl5. At each concentration evaluated, the weights of second instar larvae feeding on PAl5(pEXP230-cry1Ac-t) were statistically lower than those of second instar larvae feeding on PAl5. Examination of larvae and diet plugs confirmed that the failure to gain weight was due in part to cessation of feeding. Although they were too small to weigh successfully, neonate larvae that survived appeared to stop feeding and did not grow visibly when provided with diet containing more than 1.0 mg/ml PAl5(pEXP230-cry1Ac-t). In contrast, neonates that were fed with diets containing PAl5 continued to feed and developed rapidly. These findings are important because from a pest control perspective cessation of feeding is highly beneficial.

The expression of *cry1Ac-t* in PAl5 was relatively low, especially when compared to the expression obtained in *E. coli*, and may have been related to the vector pKT230 and the Km^r promoter used. However, previous studies have shown that pKT230 is suitable for the expression of promoterless heterologous genes in *G. diazotrophicus* and for the expression of *cry* genes in plant-associated Gram-negative bacteria (Sevilla and Kennedy, 2000; Skøt et al., 1990). Sevilla and Kennedy (2000) used pKT230 to express a promoterless 3.8 kb *uidA:cat* gene cassette in *E. coli* and PAl5. The *uidA:cat* gene cassette was cloned in the *Sma*I site of the Km^r gene. Expression was detected in *E. coli* and PAl5, however, expression in PAl5 was estimated to be about 50% less than that in *E. coli* (Sevilla and Kennedy, 2000). Their results confirmed the general suitability of expressing promoterless genes under control of the pKT230 Km^r promoter in *G. diazotrophicus*. However, the expression in PAl5 of a *gfp* gene under control of the pKT230 Km^r promoter was about 90% less than in *E. coli* (Sevilla and Kennedy, 2000). Poor *gfp* expression was also obtained with PAl5 carrying pTB93F (Sevilla and Kennedy, 2000), a *gfp*-carrying plasmid that was successfully used in *Rhizobium meliloti* (Gage et al., 1996). The results of Sevilla and Kennedy (2000) suggest that the characteristics of the heterologous gene may have a significant effect on expression levels in PAl5.

The average G+C content of PAl5 is 66% (Bertalan et al., 2009; Gillis et al., 1989), with the well-characterized *nifHDK* (nitrogenase) genes of PAl5 having an average G+C content of 61% (Lee et al., 2000). In contrast, the

G+C content of the truncated *cry1Ac* gene is 38%. Analysis of the genes that Sevilla and Kennedy (2000) used in their study shows that *uidA* (GenBank S69414) and *gfp* (GenBank AF108217) have G+C contents of 52% and 39% respectively. Since both the *gfp* and *cry1Ac*-t were expressed poorly in PAl5, there appears to be an association between low G+C content and poor expression in PAl5. In contrast, both *gfp* and *cry1Ac*-t were expressed well in *E. coli*, a host with a G+C content of approximately 51%. Large differences in G+C content suggest differences in codon usage and codon bias. Comparison of the codon usage in *cry1Ac*-t with that in the *uidA* and *nifD* genes of PAl5 shows significant differences. For example, the codon usage frequency for asparagine is: *uidA* (AAT 25% and AAC 75%), *nifD* (AAT 30% and AAC 70%), and the truncated *cry1Ac* (AAT 71% and AAC 29%). Several studies have shown that protein production in bacteria could be increased by replacing low-usage codons with high-usage codons (Mohsen and Vockley, 1995).

Studies have shown that *cry* expression may be improved by: (1) including a *cry* gene transcription terminator (which increases mRNA half-life by protecting the mRNAs from exonucleolytic degradation) (Wong and Chang, 1986), and (2) coexpression of Cry proteins with P20, an accessory protein from *Bacillus thuringiensis* subsp. *israelensis* which has been shown to stabilise some Cry proteins (Khasdan et al., 2007; Rang et al., 1996; Shao and Yu, 2004). In addition to testing whether expression of *cry1Ac-t* in PAl5 may be improved by replacing *cry1Ac* codons with *G. diazotrophicus*-preferred codons, further development of the strategy presented in this study should evaluate the effects of a *cry* gene transcription terminator and the *p20* gene on the insecticidal activity of recombinant PAl5 strains. Increased expression of a *cry* gene in an endophytic bacterium does not necessarily mean that the recombinant bacterium has better pest control potential. For example, high levels of constitutively expressed *cry1Ac* caused plasmid instability and no detectable protein expression in both *H. seropedicae* and *P. fluorescens* (Downing et al., 2000). An endophyte that is sensitive to high levels of Cry1Ac is unlikely to compete with non-recombinant endophytes *in planta*. A fine balance must be achieved between *cry1Ac* expression levels, toxicity to the host, and toxicity to the target pest.

In conclusion, recombinant *G. diazotrophicus* PAl5 carrying a truncated *cry1Ac* gene from *Bt* was toxic to *E. saccharina* larvae. In theory, inoculation of sugarcane stems with the recombinant *G. diazotrophicus* PAl5 strain would result in Cry1Ac protein being produced in the feeding zones of burrowing larvae and thus reduce damage caused by *E. saccharina*. Further studies, such as evaluation of the colonization of sugarcane by recombinant *G. diazotrophicus* strains, are required to determine the feasibility of controlling *E. saccharina* in the field with recombinant *G. diazotrophicus* strains expressing a truncated *cry1Ac* gene.

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