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ORIGINAL ARTICLE

Characterisation and expression of a novel haplotype *cry2A*-type gene from *Bacillus thuringiensis* strain JF19-2

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Abstract The *Bacillus thuringiensis* isolate JF19-2, a native organism found in soil samples from the Sichuan Basin in western China, contains a bipyramidal crystal harbouring one insecticidal crystal protein (about 70 kDa). Interestingly, a novel *cry2A*-type gene, encoding a 635 amino acid protein with 92% homology to *cry2Ab1*, was found and cloned from this strain. According to the nomenclature principles adopted for *B. thuringiensis* insecticidal crystal proteins, this gene represents a novel haplotype *cry* gene and was designated as *cry2Ag1* by the *Bacillus thuringiensis* Pesticide Crystal Protein Nomenclature Committee. *Cry2Ag1* was expressed in *Escherichia coli* BL21(DE3)pLysS cells, and the results of insecticidal activity assays showed that Cry2Ag1 was toxic to both Dipteran (*Aedes aegypti*) and Lepidopteran (*Plutella xylostella* and *Helicoverpa armigera*) pests. These results strongly suggest that the cloning of the *cry2Ag1* gene is not only of interest to researchers studying insecticidal crystal genes, but may ultimately serve as a solution to the increasing resistance of pests to currently used insecticides.

Keywords *Bacillus thuringiensis* · Characterisation · *cry2Ag1* · Expression · Insecticidal activity

Introduction

Bacillus thuringiensis (Bt) is a member of a group of crystalliferous spore-forming Gram-positive bacteria of the family *Bacillaceae*. This bacterium is able to produce parasporal protein crystals (Cry and Cyt, encoded by the *cry* and *cyt* genes) that exhibit specific insecticidal activity against insects belonging to the orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, and Mallophaga (as well as some invertebrates) but are benign to the environment (Schnepf et al. 1998). Moreover, these insecticidal crystal protein genes can be genetically engineered into crops to provide constant protection (Romeis et al. 2006). Since the first cloning of the *cry1Aa* gene from *B. thuringiensis* subsp. *kurstaki* HD-1, 463 such protein genes have been isolated and cloned (to 17 September 2009; http://www.lifesci.sussex.ac.uk/home/NeilCrickmore/Bt). Notwithstanding the many different *cry* genes that have been cloned, and the many insecticidal toxins that have been successfully used for controlling pests (http://www.uniprot.org), there have been some problems with this approach, such as narrow insecticidal spectrum and insect resistance (McGaughey 1985; Schnepf et al. 1998). Hence the isolation of new Bt strains and toxins remains an efficient way to resolve these problems. Consequently, efforts to discover novel Bt strains and toxins active against particular groups of pests have been increasing.

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The *cry2A*-type genes encode mainly 60–75 kDa proteins that exhibit a wide spectrum of toxicity to insect pests (Diptera and Lepidoptera) and have been widely applied in transgenic plants (Schnepf et al. 1998). Cry2Aa has been reported to have high larvicidal activity against Dipteran (*Aedes aegypti*) and Lepidopteran (yellow stem borer and striped stem borer) pests, whereas Cry2Ab, Cry2Ac, Cry2Ad, Cry2Ae, and Cry2Af were toxic only to Lepidopteran pests (http://www.uniprot.org). Several researchers have reported that Cry2A-type proteins differ from the Cry1-type in both structure and pesticidal mechanism (Grochulski et al. 1995; Sims 1997; Karim and Dean 2000; Morse et al. 2001; Alcantara et al. 2004), making them a useful resource in the search for beneficial genes leading to insect-resistant transgenic plants (Chen et al. 2005).

The Sichuan Basin, one of the four biggest basins in China, is a unique area situated in western China with complicated geomorphological characteristics (i.e. mountains, pastures, gorges, virgin forests, highlands, hursts, glaciers, and plains) and unique biodiversity. These distinctive features and the associated diversity of insects provide the opportunity to isolate novel Bt strains and *cry* genes (Zhu et al. 2009). Recently, we isolated a novel Bt strain (JF19-2), native to the Sichuan Basin, which contained only one *cry2A*-type gene and exhibited a wide range of insecticidal activity (toxic to Dipteran and Lepidopteran pests; not shown). The present report describes the characterisation of this Bt isolate, including the cloning and sequence analysis of a novel haplotype crystal protein gene, *cry2Ag1*. In addition, the *cry2Ag1* gene was successfully expressed in *Escherichia coli* BL21 (DE3)pLysS cells. We found that the Cry2Ag1 protein is toxic to both Dipteran (*A. aegypti*) and Lepidopteran pests (*Plutella xylostella* and *Helicoverpa armigera*).

Materials and methods

Bacterial strains and plasmids

Bt isolate JF19-2 was added to Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0) and incubated at 30°C, 220 rpm. *E. coli* DH5α and BL21(DE3) pLysS (Tiangen, Beijing, China) strains were grown at 37°C in LB medium supplemented with the appropriate antibiotic: ampicillin and/or chloramphenicol. Plasmids pGEM-T (Tiangen) and pET22b (Novagen, Madison, WI) were used for cloning and expression as described by the manufacturer.

Scanning electron microscopy

JF19-2 was grown in 5 ml LB at 30°C, 220 rpm, for approximately 72 h, then collected by centrifugation at 10,000 rpm for 5 min. The pellet was washed with 1 M NaCl and then with distilled water. The spore-crystal mixture was placed on aluminium stubs and fixed in 1% OsO_4. The sample was then sputter-coated with gold in an IB-5 ion coater (Hitachi, Tokyo, Japan) for 5 min.

Isolation of crystal proteins

JF19-2 was grown in 20 ml LB at 30°C, 220 rpm, for approximately 72 h. Crystals, spores, and debris were collected by centrifugation at 10,000 g for 20 min, and the pellet was washed with 1 M NaCl containing 0.1% Triton X-100 and then with distilled water. The crystal was dissolved in 5 ml 50 mM Na_2CO_3 (pH 9.6) containing 0.1% 2-mercaptoethanol by incubating the preparation for 2 h at 25°C and then centrifuged at 10,000 rpm for 20 min, to remove the insoluble debris. Total proteins in the supernatant were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis as described by Sambrook et al. (2002).

Identification of the *cry2A*-type gene using PCR-RFLP

A loopful of JF19-2 cells was transferred to 0.1 ml double distilled water, frozen for 20 min at −70°C, and boiled for 10 min in water to lyse the cells. The cells were briefly spun (10,000 g at 4°C for 10 s), and 15 μl supernatant was collected for PCR amplification. Based on the conserved regions of each class of *cry2*-type genes, the primers II (+): 5′-TAAAGAAAGTGGGGAGTCTT-3′ and II (−): 5′-AACTCCATCGTTATTTGTAG-3′ were used for PCR-RFLP as previously described by Sauka et al. (2005). PCR products were purified with a Tiangen Midi Purification Kit as described by the manufacturer (Tiangen). The products were cloned into the vector pGEM-T and transformed into *E. coli* DH5α competent cells. The positive clones were selected by alpha-complementation, and the PCR products from positive clones were digested with the restriction endonuclease *Dde* I as described by Sauka et al. (2005). The Bt toxin nomenclature website and DNAStar were used to determine the expected restriction fragment sizes of the known *cry2A*-type genes (http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/). Positive clones were sequenced by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd (Shanghai, China).

Cloning and sequence analysis of the full-length *cry* gene

Based on the sequencing results, two specific primers: SP1: 5′-GAGACAGGAAGTTGGGCATT-3′ and SP2: 5′-AGAAATAAATGTTCGTGTTTGATT-3′, and one degenerate primer 5′-GGAGGNNNNNNNWWTG-3′ were designed to obtain the full-length *cry* gene using Tail-PCR with the following conditions: 5 min denaturation at 94°C; 15 cycles

of 2 cycles of 94°C for 30 s, 52°C for 50 s, and 72°C for 2 min and 1 cycle of 94°C for 30 s, 33°C for 50 s and 72°C for 2 min; extension at 72°C for 7 min. The amplification products were ligated into the cloning vector pGEM-T. Sequence homology was determined using the online services of BLAST at http://www.ncbi.nlm.nih.gov/BLAST. The ProDom service was used to analyse the domains of the Cry toxins (Servant et al. 2002). Neighbour-joining phylograms were constructed to analyse the phylogenesis of selected known Cry proteins using the CLUSTAL W program.

Expression of *cry2Ag1* in *Escherichia coli* and SDS-PAGE analysis

Using an E.Z.N.A.™ Gel Extraction Kit (Omega Bio-tek, Norcross, GA), a DNA product of approximately 2 kb purified from the plasmid containing the full-length sequence of *cry2Ag1* was digested with *Nco*I/*Xho*I, and then inserted into the multiple cloning site of the pET22b expression vector to generate the recombinant expression vector pET22-cry2Ag1. The inserted sequence and its reading frame were confirmed by *Nco*I/*Xho*I digestion and DNA sequence analysis. Following this, pET22-cry2Ag1 was transformed into *E. coli* BL21(DE3)pLysS cells. Transformants were cultured overnight in 100 ml LB with 100 μg ampicillin/ml at 37°C, subcultured into fresh medium (the volume ratio of 1:100) for 6 h, and then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4–6 h. Cells were harvested and resuspended in lysis buffer, sonicated and centrifuged. The pellets were washed in order with 10 ml 0.5 M NaCl and 2% Triton three times, 10 ml 0.5 M NaCl five times, and 10 ml double distilled water twice. After centrifugation at 10,000 rpm for 10 min, the pellet was diluted and analysed by SDS-PAGE.

Extraction of Cry2Ag1 proteins from *Escherichia coli* BL21(DE3)

The resulting supernatant was loaded, at a flow rate of 100 μl/min, onto a Sepharose CL-4B column precharged with Ni^{2+}-chelated His-Bind resin (Tiangen). The column was washed with about 20 ml wash buffer (50 mM Na_2HPO4/NaH_2PO_4, 300 mM NaCl, 8 M urea, 20 mM imidazole). Proteins were then eluted with about 5 ml elution buffer (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 8 M urea, 500 mM imidazole). Urea was removed from the purified protein fractions by dialysis for 16 h at 4°C against buffer 1 (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 6 M urea, 5 mM dithiothreitol) followed by dialysis against buffer 2 (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 3 M urea, 5 mM dithiothreitol) and buffer 3 (50 mM Na_2HPO_4/NaH_2PO_4, 300 mM NaCl, 5 mM dithiothreitol) in turn for 16 h at 4°C. The purified protein was stored at −20°C in buffer 3.

Insect toxicity assay

The activity of the Cry2Ag1 protein obtained from the recombinant *E. coli* cells was tested against the larvae of Dipteran (*A. aegypti*) and Lepidopteran (*P. xylostella* and *H. armigera*) pests, using non-induced BL21(DE3)pLysS cells as a negative control. Thirty early fourth-instar larvae of *A. aegypti* were placed into 100 ml dechlorinated water. Six concentrations (0.0625 to 2 μg/ml) of protein were added and the larvae were examined after 24 h. The insecticidal activity against first-instar larvae of *P. xylostella* and *H. armigera* was measured by incorporating a suspension containing two-fold serial dilutions of protein into their artificial diet (six concentrations: 3.125 to 100 μg/ml). Thirty larvae were used per treatment, and each treatment was replicated three times; mortality was monitored after 72 h. The mean 50% lethal concentration (LC_{50}) was estimated by probit analysis using statistical parameters.

Nucleotide sequence accession number

The *cry2Ag1* nucleotide sequence obtained in this study was deposited in the GenBank database under accession no. ACH91610.

Results and discussion

Characterisation of JF19-2 and identification of the *cry2*-type gene

The Bt JF19-2 isolate produced bipyramidal crystal inclusions observed under scanning electron microscopy (Fig. 1a). SDS-PAGE analysis of the crystal proteins clearly demonstrated that JF19-2 contains one major protein band with a molecular weight of about 70 kDa (Fig. 1b).

To detect the *cry2A*-type genes harboured in the JF19-2 genome, PCR analysis was performed with PCR-RFLP primer pairs. One amplification product (about 1.5 kb) was obtained using the primer pair II(+) and II(−). The PCR product was cloned into pGEM-T and transformed into *E. coli* DH5α competent cells. Ten positive clones were randomly selected, and then these PCR products were digested with *Dde* I as described by Sauka et al. (2005). The results showed that they had the same RFLP patterns, with three main bands about 1 kb, 0.35 kb and 0.15 kb (Fig. 2), which did not conform to the predicted fragments of other *cry2A*-type genes (Sauka et al. 2005). This result was a further indication that a novel *cry2A*-type gene could be found in JF19-2. Therefore, to determine the sequence of

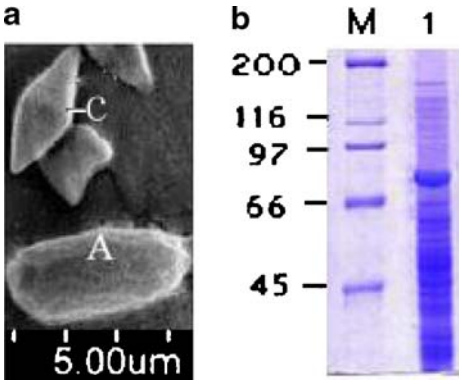

Fig. 1 **a** Scanning electron micrograph of the spore-crystal of *Bacillus thuringiensis* (Bt) JF19-2; *A* spore, *C* crystals. **b** SDS-PAGE analysis of the crystal protein; lanes: *M* protein marker, *1* Bt JF19-2

the novel *cry* gene, one amplification product was sequenced and the data analysed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and DNAStar software; the sequence had a maximum of 94% homology to *cry2Ab1*.

Cloning and sequence analysis of the novel *cry* gene

In order to obtain the full-length gene encoding this novel protein, the Tail-PCR upstream and downstream strategy was performed. We thus obtained an open reading frame of 1,905 bp encoding a 635-amino-acid protein with a predicted molecular mass of 70 kDa. Phylogenetic analysis of the selected known Cry proteins and the novel Cry protein showed that these Cry2A-type proteins might have evolved from a common ancestor (Fig. 3). Sequence alignment analysis revealed that the novel Cry protein corresponds to a putative Cry protein and has a maximum of 92% identity with Cry2Ab1. According to the nomenclature principles of insecticidal crystal proteins from Bt established by Crickmore et al. (1998), the novel *cry* gene was a haplotype *cry* gene designated as *cry2Ag1* by the Bt Pesticide Crystal Protein Nomenclature Committee.

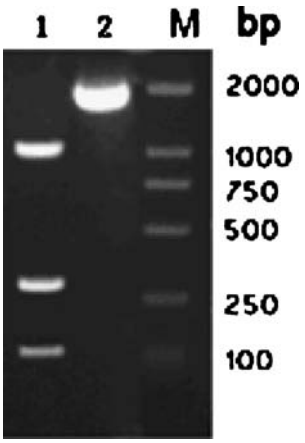

Fig. 2 PCR-RFLP analysis and PCR product of the novel *cry2*-type gene. Lanes: *1* RFLP patterns of the novel *cry2A*-type gene obtained from JF19-2, *2* PCR product of *cry2Ag1*, *M* D2000 ladder marker

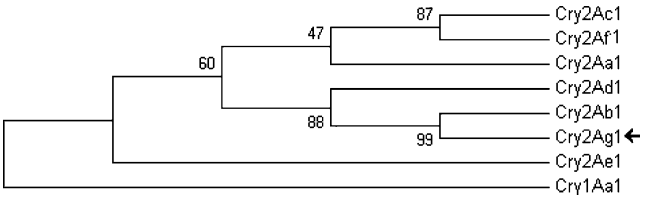

Fig. 3 Phylogenetic analysis of Cry proteins. Neighbor-joining phylograms were constructed after sequence alignment of the deduced amino acid sequences of selected known toxins and Cry2Ag1 protein by using the CLUSTAL W program. The tree was generated using Tree Explorer. Cry1Aa1, Cry2Aa1, Cry2Ab1, Cry2Ac1, Cry2Ad1, Cry2Ae1, and Cry2Af1 were obtained from the NCBI database and included as references. *Arrow* Position of the novel Cry sequence, *numbers at nodes* percentages of bootstrap resamplings based on 500 replicates

Analysis of the deduced amino acid sequence of the novel *cry* gene

A Clustal X comparison of the Cry2Ag1 protein with other known Cry protein sequences (http://www.biols.susx.ac.uk/Home/Neil-Crickmore/Bt/) helped to identify the characteristic Cry conserved blocks predicted by Schnepf et al. (1998). Although the novel Cry2Ag1 sequence had weak identity with other Cry-type proteins, a detailed analysis revealed that Cry2Ag1 also contained five conserved amino acid residue blocks (block 1 to block 5) that are present in almost all Cry proteins (not shown; Schnepf et al. 1998). Analysis of the Cry2Ag1 amino acid sequence using the ProDom service (Servant et al. 2002) demonstrated that the protein is composed of three distinct domains that have been previously reported for crystallised Cry proteins (i.e. Cry1Aa1, Cry2Aa1, Cry3Aa1, Cry3Bb1, and Cry4Ba1), but it does not contain the carboxyl-terminal half typical of the 130 kDa-type crystal proteins (Li et al. 1991;

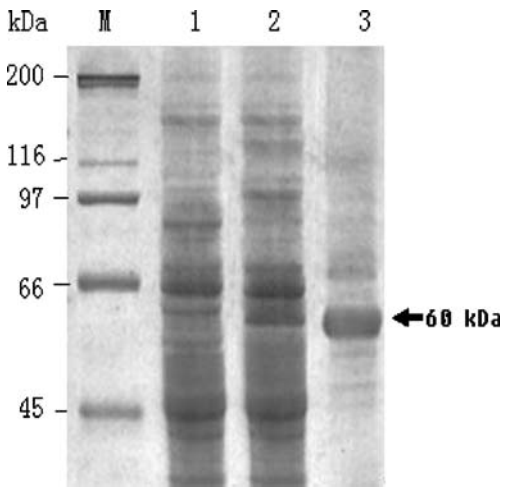

Fig. 4 SDS-PAGE analysis of the *cry2Ag1* gene expressed in *Escherichia coli* BL21(DE3):pLysS. Lanes: M Protein marker, *1 E. coli* BL21(DE3):pLysS (pET-22b), *2* lysate supernatant, *3* Cry2Ag1 protein in inclusion body

Grochulski et al. 1995; Galitsky et al. 2001; Boonserm et al. 2005). Most Cry proteins, such as the Cry1, Cry4, Cry5, Cry7, Cry8, Cry9, Cry12, and Cry14 protoxins, which have molecular weights around 120–140 kDa, consist of an N-terminal active area and a C-terminal inactive area. Their C-termini are truncated by the protease during hydrolysis, and a toxicity region of about 60 kDa then reforms (Schnepf et al. 1998). However, some other Cry proteins, such as Cry2, Cry3, Cry10, Cry11, Cry13, Cry16, Cry17, and Cry19, do not contain this typical C-terminus, but are deemed 'naturally truncated' proteins (Schnepf et al. 1998). The novel Cry2Ag1 protein only containing the N-terminal area may belong to this 'naturally truncated' group of proteins.

Expression of *cry2Ag1* in *Escherichia coli* and bioassays

According to the open reading frame of *cry2Ag1*, one pair of specific primers (cry2F: 5′-AAAACCATGGATAGTG TATTGAATACTG-3′ and cry2R: 5′-AAAACTCGAGT TAATAAAGTGGTGAAAT-3′), containing restriction sites for *Nco* I and *Xho* I, respectively, at their 5′ ends were designed and used to obtain a full-length *cry2Ag1* PCR product (Fig. 2). The amplified product was digested by *Nco* I+*Xho* I and inserted into the *Nco* I /*Xho* I site of the *E. coli* expression vector pET-22b to obtain the recombinant plasmid pET-cry2Ag1. The expression vector was then transformed into BL21(DE3)pLysS cells. SDS-PAGE analysis revealed that Cry2Ag1 was expressed as a 60-kDa protein in *E. coli* BL21(DE3):pLysS strain induced by IPTG (Fig. 4), while the molecular weight predicted from the sequence was 70 kDa. One possible explanation was that Cry2Ag1 contains only four cysteines (0.63% of total amino acids), thus the expressed protein may be digested easily by *E. coli* proteases, as shown by Lenin et al. (2001). The Cry2Ag1 protein was extracted from *E. coli* and assayed for insecticidal activity against *A. aegypti* (Diptera), *P. xylostella* (Lepidoptera), and *H. armigera* (Lepidoptera). The results showed clearly that Cry2Ag1 has a wide spectrum of insecticidal toxicity to Dipteran [*A. aegypti*: LC_{50}=2.541 μg/ml, 95% confidence, interval (CI) 1.707–3.432 μg/ml] and Lepidopteran (*H. armigera*: LC_{50}= 9.745 μg/ml, 95% CI, 7.171–13.738 μg/ml; *P. xylostella*: LC_{50}=23.478 μg/ml, 95% CI, 12.173–106.630 μg/ml) pests. This is similar to the activity of the CryIIA protein from Bt subsp. *kurstaki* (Sims 1997), which has been used successfully as a commercial product to control Dipteran and Lepidopteran pests in agriculture and medicine (Schnepf et al. 1998).

Several Bt toxins have been found, but only a few have been used for pest control. Unfortunately, some insects have developed resistance against several of the Bt toxins (McGaughey 1985). Cry1-type proteins have been widely applied in transgenic plants, but problems of narrow insecticidal spectrum and insect resistance have recently been observed due to lengthy use of high concentrations of a single Bt toxin (Romeis et al. 2006). In addition, the threat of secondary pests may result in the need for transgenic plants with high insecticidal activity and wide insecticidal spectrums. Cry2A is toxic to several of the main Lepidopteran pests such as yellow stem borer and striped stem borer (Karim and Dean 2000; Alcantara et al. 2004). Furthermore, biochemical studies showed that Cry2A did not share binding sites with Cry1A in brush border membrane vesicles from Lepidopteran pests (Karim and Dean 2000; Alcantara et al. 2004). Therefore, the isolation of novel Cry proteins with toxicity against a broader range of pests will be crucial for solving these problems. In this study, Cry2Ag1 exhibited high larvicidal activity against *A. aegypti* (Diptera), *H. armigera* (Lepidoptera), and *P. xylostella* (Lepidoptera), similarly to the Cry2Aa/Cry2Ab protein successfully used as commercial products to control Dipteran and Lepidopteran pests in agriculture and medicine (Liang and Dean 1994; Schnepf et al. 1998; Lenin et al. 2001). Thus, Cry2Ag1 appears to be an attractive alternative for controlling mosquitoes and crop pests, and for using in insect-resistant transgenic plants in the future. As such, it will be worthwhile to fully elucidate the insecticidal activity and insecticidal spectrum of this protein, and this work is now in progress.

In conclusion, although many Bt toxins have already been isolated, the cloning of additional novel *cry* genes continues to benefit the further development of Cry proteins as competitive biological insecticides. Our results strongly suggest that the *cry2Ag1* gene is not only a novel resource in the field of research into insecticidal crystal genes, but it may also serve as an alternative toxin to solve some of the potential problems associated with insect resistance.

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