Nematicidal Activity of Cry1Ea11 from *Bacillus thuringiensis* BRC-XQ12 Against the Pine Wood Nematode (*Bursaphelenchus xylophilus*)

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Accepted for publication 8 September 2017.

ABSTRACT

The nematicidal activity of 92 *Bacillus thuringiensis* strains against the pine wood nematode *Bursaphelenchus xylophilus*, one of the world's top 10 plant-parasitic nematodes, was determined. The insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* BRC-XQ12 were the most toxic to *Bursaphelenchus xylophilus*, with a lethal concentration 50 (LC₅₀) of 32.13 µg/ml. Because the ICPs expressed by *Bacillus thuringiensis* BRC-XQ12 were closest to Cry1Ea6 and *B. thuringiensis* BRC-XQ12 contained four kinds of *cryI* subgenes (*cryIAa*, *cryICb*, *cryIEa*, and *cryIIa*), Cry1Ea was most likely to be the key active component against the nematode. The 3,516-bp *cryIEa11* gene from BRC-XQ12, as designated by the *B. thuringiensis* δ-endotoxin nomenclature committee, was expressed in *Escherichia coli*. Purified Cry1Ea11 showed an LC₅₀ of 32.53 and 23.23 µg/ml at 24 and 48 h,

with corresponding virulence equations of $Y = 32.15X + 1.38$ ($R^2 = 0.9951$) and $Y = 34.29X + 3.16$ ($R^2 = 0.9792$), respectively. In order to detect the pathway of *B. thuringiensis* Cry1Ea11 into *Bursaphelenchus xylophilus*, the nematode was fed with NHS-rhodamine-labeled GST-Cry1Ea11. The results of confocal laser-scanning microscopy showed that the 159-kDa GST-Cry1Ea11 could be detected in the stylet and the esophageal lumen of the pine wood nematode, indicating that GST-Cry1Ea11 could enter into the nematode through the stylet. As far as we know, no Cry1 proteins have been shown to have activity against plant-parasitic nematodes before. These results demonstrate that Cry1Ea11 is a promising nematicidal protein for controlling pine wilt disease rendered by *B. xylophilus*, further dramatically broadening the spectrum of *Bacillus thuringiensis* ICPs.

Plant-parasitic nematodes are important crop pathogens, causing yearly losses of approximately \$10 billion in the United States and \$78 to 125 billion worldwide (Batish et al. 2008; Chitwood 2003). Thus far, more than 4,100 species of plant-parasitic nematodes have been described (Jones et al. 2013). Of these nematodes, *Bursaphelenchus xylophilus* (Steiner & Buhner) Nickle (pine wood nematode [PWN]) was one of the world's top 10 plant-parasitic nematodes and a major invasive organism causing pine wilt disease (PWD), leading to significant losses of 2×10^6 m³ of timber under some environmental conditions (Jones et al. 2013; Zhao et al. 2014).

B. xylophilus is unique in that it can live on living trees and the fungi colonizing dead or dying trees in its life cycle (Kikuchi et al. 2011). It has propagative and reproduction forms in its life cycle (Aikawa et al. 2003). With pine wilting or death, the second-instar larvae will mature to third-instar larvae, passing the winters with their vector beetle *Monochamus alternatus* (Japanese pine sawyer) in the xylem (Akbulut and Stamps 2012). Then, the fourth-instar larvae move with *M. alternatus* to healthy pine (Jung et al. 2010; Zhao et al. 2014). Because PWD can cause rapid death in a variety of pine species and *B. xylophilus* has plant and fungal feeding styles, prevention of PWD is extremely difficult (Jones et al. 2013). Thus, many countries pay a high level of attention to its control (Yang et al. 2014).

Because the pathogenesis of PWD has not been fully clarified, the control of the plant-parasitic nematode is largely dependent on chemical nematicides such as fumigants; however, most of them have been banned by the European Commission (D'Addabbo et al. 2014).

Additionally, extensive use of chemical pesticides has caused the insurmountable “3 R” problem. Therefore, given the current shortcomings of *B. xylophilus* control, it is necessary to develop new strategies such as biological control for the sustainable control of *B. xylophilus* (Chu et al. 2015; Kim et al. 2008; Seo et al. 2010).

Bacillus thuringiensis is a well-known biopesticide and contains a variety of biologically active substances such as insecticidal crystal proteins (ICPs) (Bravo et al. 2011; Dai et al. 2016; Deng et al. 2014; Sanahuja et al. 2011). *B. thuringiensis* ICPs are active against members of the orders Hymenoptera, Lepidoptera, Coleoptera, and Diptera; plus protozoa, nematodes, platyhelminths, schistosomiasis, malaria parasites, mites, and others (Bravo et al. 2011; Kotze et al. 2005; Sanahuja et al. 2011; Wei et al. 2003). However, the nature of most of the components active against nematodes remains unknown (Wang et al. 2014; Yu et al. 2015). This study attempts to screen *B. thuringiensis* strains active against the PWN *Bursaphelenchus xylophilus* and to further identify the active component using mass spectrometry (MS), polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), heterologous expression, and bioassays. Herein, we first showed that Cry1 was active against the plant-parasitic nematode *B. xylophilus*. The nematode was further fed with NHS-rhodamine-labeled glutathione-S-transferase (GST)-Cry1Ea11 and observed with confocal laser-scanning microscopy (CLSM) to make sure the nematode could take up such a large protein and to detect the pathway of *Bacillus thuringiensis* Cry1Ea11 into *Bursaphelenchus xylophilus*. This work may lay a foundation for the effective control of *B. xylophilus*-like plant-parasitic nematodes.

MATERIALS AND METHODS

Bacterial strains and nematode. *Escherichia coli* JM109 and BL21 (DE3) and expression plasmid pGEX-KG were stocked

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*The e-Xtra logo stands for “electronic extra” and indicates that six supplementary figures and three supplementary tables are published online.

in the State Key Laboratory of Ecological Pest Control for Fujian and Taiwan Crops, China. *B. xylophilus* was reared by the same lab. Because *B. xylophilus* is classified as an internal and external important quarantine object, it is impossible for foreign counterparts to share this experimental material. *Bacillus thuringiensis* strains used in this study are listed in the Supplementary Table S1.

Media and reagents. Luria-Bertani (LB), potato dextrose agar (PDA), and peptone-medium (PM) (0.2% yeast extract, 1% glucose, 1% tryptone, 0.1% KH_2PO_4 , 0.1% K_2HPO_4 , 0.002% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002% $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.002% $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$ [pH 7.2]) were used in this study. Taqase, PCR product recovery kits, T4 DNA ligase, restriction enzymes, and pMD18-T vector were from TaKaRa Co., Ltd.. Vent DNA polymerase was from New England Biolabs. Proteinase K was from Boehringer Mannheim Co, Ltd.. Lysozyme was from Shanghai Bioasia Co, Ltd.. Other reagents were analytical pure products purchased in China.

Culturing and isolation of *Bursaphelenchus xylophilus*. *Bursaphelenchus xylophilus* was isolated from susceptible *Pinus thunbergii* using the Baermann funnel method (Beane and Hobbs 1983). Briefly, *Botrytis cinerea* was inoculated on a PDA plate at 25°C. When the whole plate was covered with the mycelia of *B. cinerea*, the nematodes were inoculated onto the plates. After 7 days, the medium containing the nematodes was put in a Baermann funnel and rinsed with sterile Milli-Q water to wash out the nematodes. The nematodes were collected via centrifugation at 1,000 rpm, disinfected with 0.1% streptomycin sulfate, and concentrated to 10 μl of suspension.

Screening of *Bacillus thuringiensis* strains active against *Bursaphelenchus xylophilus*. *Bacillus thuringiensis* was incubated in 50 ml of LB at 200 rpm and 30°C for 3 days. Then, the fermentation broth containing ICPs was diluted to 1 (optical density at 600 nm) with sterile Milli-Q water. The activity was determined by a dipping method. Briefly, 100 μl of the above fermentation broth was transferred into each well of a 96-well plate. LB broth was used as a negative control. Then, 100 nematodes suspended in 100 μl of sterile Milli-Q water were added to each well. They were mixed well and cultured at 25°C under dark and wet conditions. After 48 h, the mortality was determined with a microscope. If the nematodes had C/J-like shapes or did not move after repeated stimulation with 50 μl of water, they were recorded as dead. If they were wavy, spiral curled, or “S” shaped, they were recorded as alive. The mortality and corrected mortality of *Bursaphelenchus xylophilus* were calculated. The mortality of the nematodes suspended in 100 μl of sterile Milli-Q water was used as the control group mortality. Corrected mortality (%) = (treatment group mortality – control group mortality) / (100 – control group mortality) $\times$ 100. Each treatment was repeated three times. The fermentation broths of the *Bacillus thuringiensis* strains with >80% corrected mortality were rescreened by diluting 30 times. The bioassay was performed as above.

Preparation of *B. thuringiensis* ICPs. Overnight culture (1%) of the *B. thuringiensis* strains was transferred to PM media and cultivated at 37°C with shaking at 220 rpm until ICPs formed. Then, the fermentation broth was centrifuged at 10,000 rpm and 4°C for 10 min. To wash the pellets, 1 M NaCl solution and sterile Milli-Q water were applied three times. Solution I (50 mM $\text{Na}_2\text{CO}_3/\text{HCl}$ and 10 mM dithiothreitol [DTT], pH 9.5) was used to dissolve the precipitate. After centrifugation, 1:3 diluted acetic acid was gradually added to the supernatants until the pH reached 4.6 to 4.8, and the precipitate was left overnight. After centrifugation, sterile Milli-Q water was used to wash the precipitate three times. After centrifugation, a 0.01 M NaOH solution was used to dissolve the precipitate. After centrifugation, the supernatants were loaded into dialysis bags with 0.01 M phosphate-buffered saline (pH 7.4), stirring overnight at 4°C. After centrifugation, the concentration of the supernatants was confirmed by the Bradford method (Bradford 1976).

Bioassay of *B. thuringiensis* ICPs on *Bursaphelenchus xylophilus*. Nematodes ($n = 100$) suspended in 100 μl of sterile Milli-Q water were added to each well of a 96-well plate. The above ICPs were

diluted in a twofold ratio. The dilutions (100 μl) were added to the above wells, with 0.01 M NaOH as a negative control. The percentage of live nematodes was calculated under a microscope every 12 h, as described above.

Effect of *Bacillus thuringiensis* ICPs on the proliferation of *Bursaphelenchus xylophilus*. *Alternaria* Nees was inoculated in PDA media at 25°C for 5 days. In 1 ml of sterile Milli-Q water, 5,000 nematodes were mixed with 19 and 43.5 μg of ICPs from BRC-ZY3 and BRC-XQ12, respectively. Sterile Milli-Q water was used as a negative control. After 12, 24, 36, and 48 h, every 200 nematodes isolated by the Baermann funnel method were put onto a PDA plate and incubated at 22 to 27°C for 7 days. The percentages of live nematodes were calculated as described above. The formula for the inhibition rate of proliferation was (live number of the control $\times$ live number of the treatment) / live number of the control $\times$ 100. Each treatment was repeated three times.

Detection of *Bacillus thuringiensis* ICPs using sodium dodecyl sulfate polyacrylamide gel electrophoresis, liquid chromatography with MS, and spectral library query. *Bacillus thuringiensis* ICPs were separated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Each target band in the SDS-PAGE gel and its corresponding position was recorded, and the bands were then cut out of the gel and individually placed in Eppendorf tubes. The bands were washed with 50 μl of double-distilled H_2O for 10 min twice. Then, the bands were repeatedly treated with 50 μl of 50 mM NH_4HCO_3 /acetonitrile solution (1:1) at 37°C for 20 min until the blue dye faded. The gel bands were dehydrated with 50 μl of acetonitrile until completely white. After vacuuming for 10 min, 20 μl of 10 mM DTT was supplemented to the gel band and treated at 56°C for 1 h. The gel bands were then cooled to room temperature and dried. Next, 20 μl of 55 mM iodoacetamide (IAM) was added, and the tubes were placed in a darkroom for 45 min. Then, the gel bands were dehydrated with 25 mM NH_4HCO_3 (2 $\times$ 10 min), 25 mM NH_4HCO_3 /50% acetonitrile solution (2 $\times$ 10 min), and acetonitrile (10 min) until completely white. After vacuuming for 10 min, the gel was digested with 2 to 3 μl of trypsin at 20 $\mu\text{g}/\text{ml}$ and placed on ice for 30 min. Then, 25 mM NH_4HCO_3 was added to the sample to a total volume of 10 to 15 μl and incubated at 37°C overnight; 0.2% formic acid was added to stop the reaction.

Liquid chromatography (LC) with pulse Q collision-induced dissociation (PQD) tandem MS (LC-PQD-MS/MS) was performed at the Agricultural Ecology Institute, Fujian Agriculture and Forestry University, China. Briefly, high-performance liquid chromatography (HPLC) was first run with a Thermo Scientific Surveyor System (Thermo Fisher Scientific Inc.). The HPLC conditions were as follows: BioBasic C18 Column (100 $\times$ 0.18 mm, particle size = 5 μm); 10 μl sample; mobile phase A, 0.1% formic acid in water, and phase B, 0.1% formic acid in acetonitrile; gradient condition = 5 to 35% B for 20 min and 35 to 95% B for 2 min; and flow rate of 2.5 $\mu\text{l}/\text{min}$. Then, the MS/MS was analyzed by an LTQ-XL linear ion trap mass spectrometer (Thermo Fisher Scientific Inc.). The MS conditions were as follows: mass spectrometer LTQ-XL, spray voltage = 3.5 kV, capillary temperature = 275°C, sheath gas flow rate = 15 arbitrary unit (arb), parent ion scan range = 400 to 2,000 m/z, and isolation width = 2 Da. The secondary MS conditions were: AGC Target 1e4, 1 microscan, and collision energy = 35% collision induced dissociation (CID). Proteome Discoverer 1.2 Software (Thermo Fisher Scientific) was used to retrieve the relative quantitative analysis and database match. The *Bacillus thuringiensis*.fasta protein library was downloaded from <https://www.ncbi.nlm.nih.gov/>.

Detection of cry genes from *B. thuringiensis* BRC-XQ12 by PCR-RFLP. Total DNA was extracted from *B. thuringiensis* BRC-XQ12, and its *cryI-cryII* gene profile was identified by PCR-RFLP (Huang and Guan 2017; Huang et al. 2014; Kuo and Chak 1996; Song et al. 1998, 2003).

Cloning, sequence analysis, and localization of *cryIEa* from *B. thuringiensis* BRC-XQ12. A pair of primers, cry1EF (5'-cgGGATCCATGGAGATAGTGAATAATCAGAATC-3') and cry1ER

(5'-acgcGTCGACTTATTCCTCCATAAGAAGTAATTC-3'), designed using the sequence of *cryIEa6* (GenInfo Identifier (gi) number 17979618), was used to amplify the *cryIEa* gene from *B. thuringiensis* BRC-XQ12 with high-fidelity vent DNA polymerase. The A tail was added to the PCR products by traditional PCR methods. Then, the PCR products were cloned into the pMD18-T vector to acquire the recombinant plasmid pMD-*cryIEa* and were sequenced. The gene was submitted to the *B. thuringiensis* toxin nomenclature committee (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/) and designated *cryIEa11*.

DNAMAN software was used to analyze the basic information of *cryIEa11*. BLAST was used to search for sequence homology. ExPaSy (<https://au.expasy.org>) was used to predict the protein physicochemical properties, including amino acid composition, isoelectric point (pI), instability coefficient, and fat index.

The plasmid DNA and total DNA of BRC-XQ12 were extracted as described previously (Wu et al. 2004). Because the strain did not contain the plasmids, the localization of *cryIEa11* was performed with the following modifications: its plasmid DNA was used as the template for PCR as the negative control and the localization of the *cryIEa11* gene was directly detected by PCR using the chromosomal DNA of BRC-XQ12 recovered from its total DNA by a 0.7% agarose gel as the template.

Expression of CryIEa from *B. thuringiensis* BRC-XQ12 in *E. coli*. The pMD-*cryIEa* harboring the *cryIEa11* gene and the expression plasmid pGEX-KG were double digested with *Bam*HI and *Sal*I. Then, they were ligated to obtain an *E. coli* containing the expression plasmid pGEX-*cryIEa11*. This strain, *E. coli* BL21 (DE3)-pGEX-*cryIEa11*, was registered with the China General Microbiological Culture Collection Center as number 7661. The *E. coli* strains containing pGEX-*cryIEa11* and pGEX-KG were cultured in LB and induced with 1 mM isopropyl-thio-galactopyranoside (IPTG) at 16°C for 30 h to express GST-CryIEa11 and GST, respectively. The proteins were further purified with GST*bind resin.

Activity of GST-CryIEa11 against *Bursaphelenchus xylophilus*. A *Bursaphelenchus xylophilus* suspension was prepared and the assay was performed as described above. After 24 and 48 h, the mortality, corrected mortality, and lethal concentration 50 (LC₅₀) were calculated. Purified GST and the elution buffer of GST-CryIEa were used as controls. The experiments were repeated three times.

Detection of the pathway of *Bacillus thuringiensis* CryIEa11 into *Bursaphelenchus xylophilus* with a CLSM. The experiments were performed as described previously, with some modifications (Yu et al. 2015; Zhang et al. 2012). Briefly, purified GST-CryIEa11 protein at 1 mg/ml was labeled with 3.5 µl of NHS-Rhodamine (10 mg/ml) according to the manual procedure of the product (Thermo Scientific 46406), and was dialyzed with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 8.0). *B. xylophilus* was cultured and isolated as described above. Resorcinol (1 mg/ml) and nystatin (100 µg/ml) were added to the nematode to induce stylet pulsing frequency and to avoid the contaminations of microorganisms, respectively. Then, 100 µl of labeled GST-CryIEa11 was mixed with 100 nematodes suspended in 100 µl of sterile Milli-Q water in

the wells of a 96-well plate. At 1 and 24 h postinjection (hpi), *B. xylophilus* was observed using a CLSM (Leica TCS SP8X DLS) at dual excitation laser wavelengths of 488 and 568 nm with two detectors (HyD1 at 500 to 550 nm and PMT 2 at 654 to 691 nm) to determine the pathway of CryIEa11 into the nematode.

RESULTS

Screening of *Bacillus thuringiensis* strains active against *Bursaphelenchus xylophilus*. Of the 92 *Bacillus thuringiensis* strains tested, the corrected mortality of the fermentation broth of 8, 13, 14, and 57 strains against *Bursaphelenchus xylophilus* was >80, 51 to 80, 31 to 50, and 10 to 30%, respectively. *Bacillus thuringiensis* strains BRC-CWS1, 140, BRC-SFR30, BRC-Bt2, BRC-XQ12, BRC-ZYR1, BRC-ZLL7, and BRC-ZY3 showed >80% corrected mortality of *Bursaphelenchus xylophilus*. The nematodes were active without treatment with the *Bacillus thuringiensis* strains (Fig. 1A). After treatment with the *B. thuringiensis* strains for 12 h, the nematodes began to show infection symptoms, including bradykinesia and body stiffness, swelling, and thickening, and they eventually died (Fig. 1B). BRC-XQ12 could even partially degrade part of the intestinal guts of the nematodes (Fig. 1C).

The above eight *B. thuringiensis* strains were selected for rescreening experiments. Using 30-fold diluted fermentation broth, the activities of the eight *B. thuringiensis* strains against the nematodes were in the order of BRC-ZY3 > 140 > BRC-ZLL7 > BRC-XQ12 > BRC-ZYR1 > BRC-Bt2 > BRC-CWS1 > BRC-SFR30, with corrected mortalities of 72.73, 63.33, 57.14, 56.31, 54.54, 52.94, 50.00, and 37.50%, respectively.

Effects of *B. thuringiensis* ICPs on the mortality and reproduction of *Bursaphelenchus xylophilus*. It is convenient and fast to use *Bacillus thuringiensis* fermentation broth for nematocidal activity screening. According to previous articles, ICPs may be the major ingredients of *B. thuringiensis* fermentation broth active against nematodes. Thus, the ICPs were extracted from *B. thuringiensis* strains BRC-ZY3, 140, BRC-ZLL7, and BRC-XQ12 for bioassays. The results showed that the ICPs of the four strains killed the nematodes with varied virulence and different toxic speeds. The LC₅₀ of *B. thuringiensis* 140 and BRC-XQ12 displayed a time-dependent decrease. The LC₅₀ after 24-h treatments of *B. thuringiensis* BRC-ZLL7 and BRC-ZY3 slightly increased compared with that of the 12-h treatments, which may be related to the recovery of suspended animation of the nematodes. The results showed that BRC-XQ12 had the best effect, with an LC₅₀ of 32.13 µg/ml after 48 h of treatment (Supplementary Fig. S1), indicating that its ICPs were responsible for the nematocidal activity.

The ICPs of BRC-XQ12 (87 µg/ml) inhibited the reproduction of *Bursaphelenchus xylophilus*. After treatment for 12, 24, and 36 h, the inhibition rates of reproduction were 43.200, 44.286, and 47.709%, respectively. BRC-XQ12 inhibited *B. xylophilus* reproduction up to 72.699% after treatment for 48 h.

Detection of the ICPs profile of BRC-XQ12 using SDS-PAGE, LCPQD-MS/MS, and PCR-RFLP. To further investigate what kinds of ICPs have nematocidal activity, the ICPs profile of BRC-XQ12

Fig. 1. Morphological changes of *Bursaphelenchus xylophilus* treated with *Bacillus thuringiensis* strains. **A**, *Bursaphelenchus xylophilus* without *Bacillus thuringiensis* treatment. **B**, *Bursaphelenchus xylophilus* treated with *Bacillus thuringiensis* strains for 12 h. **C**, Degradation of *Bursaphelenchus xylophilus* by the fermentation broth of *Bacillus thuringiensis* strain BRC-XQ12. Scale bars: 400 µm (A and B) and 200 µm (C).

was first determined by SDS-PAGE and LC-PQD-MS/MS. The SDS-PAGE results showed that the ICPs of BRC-XQ12 mainly ranged from 50 to 140 kDa, and the approximately 130-kDa proteins were abundantly expressed (Fig. 2). Based on the mass and secondary spectra, the bands matched peptide sequences including RSENITPTLGINVVQGVGFIQPNNAEVLRY, IAEELPIRGGELYDKIELILADATFEEDYDLER, and NRGYDEAYESNSSVHASVYEEK, which corresponded to CryIEa6 (gi number 17,979,619), a 133.2-kDa protein with an pI value of 5.11 (Tables 1 and 2).

Three *cry* gene types of BRC-XQ12 (the *cryI*, *cryII*, and *cry2* genes) were detected by PCR-RFLP using 10 pairs of universal primers (Fig. 3A). After treatment of the PCR products with the corresponding restriction endonucleases (Fig. 3B), the RFLP pattern showed products with sizes of approximately 1.10, 0.95, 0.75, 0.65, 0.50, 0.22, and 0.15 kb in lane 1; approximately 1.10, 0.72, 0.49, 0.30, and 0.25 kb in lane 2; approximately 0.57, 0.44, and 0.38 kb in lane 3; and approximately 0.79, 0.30, and 0.14 kb in lane 4. These were fit for the predicted RFLP sizes of *cryIAa*, *cryICb*, *cryIEa*, *cryIIa*, and *cry2Ab*, suggesting that BRC-XQ12 contained these five kinds of *cry* genes (Fig. 3).

According to the above SDS-PAGE, LC-PQD-MS/MS, and PCR-RFLP results, CryIEa is most likely responsible for the majority of the toxicity of BRC-XQ12 to *B. xylophilus* nematodes.

Fig. 2. Detection of insecticidal crystal proteins (ICPs) from *Bacillus thuringiensis* BRC-XQ12 using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Lane M, TaKaRa Unstained Protein Molecular Weight Markers, and lane 1, ICPs from BRC-XQ12.

Cloning, sequence analysis, and localization of *cryIEa11* from BRC-XQ12. A correct recombinant JM109-pMD-*cryIEa* containing the corresponding 3.5-kb *cryIEa* gene from BRC-XQ12 was obtained (Supplementary Fig. S2). After sequencing, the *cryIEa* gene with a GenBank accession number JQ652456 was designated *cryIEa11* by the *Bacillus thuringiensis* Toxin Nomenclature Committee. The *cryIEa11* gene is 3,516 bp and encodes 1,171 amino acids, showing the highest similarity (99%) with CryIEa6 (GenBank accession number AF202531.1). This is consistent with the results of LC-PQD-MS/MS.

The physicochemical properties of CryIEa11 were further predicted. The relative molecular weight of the CryIEa11 protein is 133.267 kDa, with a theoretical pI of 5.01, indicating that it is a weakly acidic protein. Of the 20 amino acids, the highest proportion of CryIEa11 is Leu (8.8%), while Met is present at the lowest percentage (0.7%) (Supplementary Fig. S3). It contains 165 acidic amino acids, 139 basic amino acids, 349 hydrophilic amino acids, and 518 hydrophobic amino acids (Supplementary Table S2). It has 165 negatively charged residues and 115 positively charged residues. The prediction of physicochemical properties provides a reference for subsequent protein expression and purification.

Because BRC-XQ12 did not contain the plasmids (Supplementary Fig. S4), its plasmid DNA was used as the template for *cryIEa11* PCR as the negative control. The localization of the *cryIEa11* gene was directly mapped to the chromosome of BRC-XQ12 by PCR using the chromosomal DNA of BRC-XQ12 recovered from its total DNA by a 0.7% agarose gel as the template.

Expression and purification of CryIEa11 and its bioassay using *Bursaphelenchus xylophilus*. A recombinant *E. coli* BL21 (DE3)-pGEX-*cryIEa11* containing *cryIEa11* from pMD-*cryIEa* was obtained (Supplementary Fig. S5). After IPTG induction, an approximately 160.0-kDa protein, equal to the size of the fusion protein GST-CryIEa11 (133 kDa plus 26 kDa), was expressed (Fig. 4A). The fusion protein GST-CryIEa11 was further purified by GST*bind resin (Fig. 4B). GST protein, the 26-kDa negative control of the bioassay, was purified from *E. coli* BL21 (DE3)-pGEX-KG using the same technique (Fig. 4C). Using GST protein as a negative control, the LC₅₀ values of CryIEa11 after treatment for 24 and 48 h were 32.53 and 23.23 µg/ml with virulence equations $Y = 32.15X + 1.38$ ($R^2 = 0.9951$) and $Y = 34.29X + 3.16$ ($R^2 = 0.9792$), respectively (Supplementary Table S3; Supplementary Fig. S6).

NHS-rhodamine labeled 159 kDa GST-CryIEa11 entered into the PWN via the stylet in vitro. GST-CryIEa11 was labeled with NHS-rhodamine in order to determine whether it could be ingested by the PWN and to detect the pathways that it entered into the nematode. The nematode was observed by a laser confocal microscope. The results showed that the red fluorescence of the rhodamine was not seen in the nematode treated with NHS-rhodamine

TABLE 1. Liquid chromatography with pulse collision energy-induced dissociation tandem mass spectrometry analysis of the insecticidal crystal proteins from *Bacillus thuringiensis* strain BRC-XQ12^a

Accession number	Cov	PSM	Pep	AA	MW (kDa)	pI	Score	Description (<i>B. thuringiensis</i>)
gi17979619	57.39	188	47	1,171	133.2	5.11	1,428.08	Cry032
gi37728716	46.74	151	38	1,181	133.3	5.10	1,225.92	Insecticidal crystal protein
gi33339672	47.72	160	39	1,182	133.9	5.29	1,211.28	δ-Endotoxin CryIA
gi23344760	55.06	145	39	1,177	133.1	5.14	1,132.98	Insecticidal crystal protein CryIAc
gi37781497	40.83	124	34	1,183	133.6	5.07	1,085.56	CryIAa
gi142875	21.68	73	17	1,181	133.7	5.08	722.38	δ-Endotoxin
gi1247711	17.81	63	16	1,179	133.3	5.15	632.11	PS81RR1
gi259156864	23.55	49	17	1,227	138.9	5.22	427.89	CryIBe2
gi3360519	25.26	45	17	1,227	139.0	5.20	406.69	CryIBe1 δ-endotoxin
gi228911585	17.96	57	15	1,164	131.9	5.43	374.11	Pesticidal crystal protein CryIAe
gi45387401	20.85	34	15	1,170	133.2	5.40	369.66	Crystal protein
gi296087	10.58	44	9	1,172	132.9	5.21	314.00	Crystal protein
gi126566926	11.73	30	10	1,228	139.6	5.17	247.79	Insecticidal protein CryIBa5

^a Abbreviations: Cov = coverage, PSM = peptide-spectrum match, Pep = number of peptides, AA = number of amino acids, MW = molecular weight, and pI = calculated isoelectric point.

labeled GST-CryIEa11 at 1 hpi (Fig. 5A). At 24 hpi, the red fluorescence of rhodamine was detected in the stylet and esophageal lumen of the nematode (Fig. 5B). This showed that 159-kDa GST-CryIEa11 can enter into the PWN via the stylet in vitro.

DISCUSSION

Although there were early reports of PWD symptoms in 1905 in Nagasaki, Japan, it was not until 1971 that *B. xylophilus* was identified as the new species responsible for pine wilt (Kikuchi et al. 2009; Maehara 2008; Mallez et al. 2013). By 2009, PWD had spread from North America to Asia and Europe (Portugal) (Jones et al. 2013; Kikuchi et al. 2009). PWD has strong pathogenicity in that it spreads rapidly and causes host death quickly. It became an international danger to pine forest-like ecosystems, resulting in huge economic losses (Jung et al. 2010; Kusunoki 1987).

Bacillus thuringiensis ICPs can effectively control a variety of animal and plant-parasitic nematodes (Holterman et al. 2006; Hui et al. 2012; Iatsenko et al. 2014a; Li et al. 2008; Wang et al. 2014; Wu et al. 2015; Yu et al. 2015). In the 1960s, it was discovered that *B. thuringiensis* showed specific activity against nematodes (Ciordia and Blzzell 1961). In 1972, *B. thuringiensis* was found to play a role in the control of plant-parasitic nematodes: it not only inhibits egg hatching but also has nematicidal activity against the larvae (Bottjer et al. 1985). With further studies, people continued to find a variety of *B. thuringiensis* that killed nematodes, such as *Caenorhabditis elegans*, *Pristionchus pacificus*, *Meloidogyne incognita*, *Panagrellus redivivus*, *Rotylenchulus reniformis*, *Aphelelchus avena*, *Heterodera glycines* and *Pratylenchus penetrans*

(Griffitts et al. 2005; Iatsenko et al. 2014a,b; Kho et al. 2011; Rae et al. 2010; Wang et al. 2014; Wei et al. 2003; Yu et al. 2015; Zuckerman et al. 1993). *B. thuringiensis* can also effectively reduce the rate of root-knot caused by nematodes (Zuckerman et al. 1993). Extensive studies have been carried out to clone the related antinematode genes. The main *B. thuringiensis* biologically active substances against nematodes are 4.4- to 153.5-kDa ICPs, whose corresponding genes belong to *cry5B*, *cry6A*, *cry14A*, *cry21A*, and *cry55A* (Guo et al. 2008; Wei et al. 2003). Recent studies showed that *B. thuringiensis* ICPs such as Cry5Ba3 can also kill *Bursaphelenchus xylophilus* (de Oliverira et al. 2004; Wang et al. 2012).

The current methods for characterization of *Bacillus thuringiensis* ICPs include bioassay, spore counting, HPLC methods, immunoassay, electrophoresis, MS, and genome mining. In this study, the main active component of BRC-XQ12, determined by combining bioassays, SDS-PAGE, and LC-PQD-MS/MS with PCR-RFLP, was identified as CryIEa11. The ICPs diversity detected by SDS-PAGE (Fig. 2) and the simplicity reported by LC-PQD-MS/MS (Table 1) were contradictory, in that *B. thuringiensis* ICPs are activated in insect alkaline guts by cutting off parts of their N-terminal and C-terminal amino acids into smaller proteins, which can be activated in vitro by NaOH or in vivo by alkali proteases. Thus, the small bands detected by SDS-PAGE were likely to be the activated smaller fragments from the 130-kDa proteins. MS technology has been recognized as an important means of identification of peptides and proteins in proteomic analysis. In 2009, the peptides matching Cry19Ba, Cry40 ORF2, Cry27Aa, and Cry20Aa from a *B. thuringiensis* strain containing 75- to 30-kDa ICPs were detected by nano-LC-electrospray ionization (ESI)-ion trap

TABLE 2. Insecticidal crystal proteins (ICPs) that highly matched the ICPs profile of *Bacillus thuringiensis* strain BRC-XQ12 detected by liquid chromatography with pulse collision energy-induced dissociation tandem mass spectrometry^a

CI	Sequence coverage	Accession	Prob	Xcorr	Δ Score	m/z (Da)	MH+ (Da)	Δ M (ppm)	RT (min)	Ions
High	RSENITPTLGINV VQGVGFIQPNNAEVLRY	17,979,619	115.93	7.81	0.41	1,100.88745	3,300.6478	573.65	59.68	46/116
High	IAEELPIRGGELYID KIELILADATFEEEDYDLER	17,979,619	300.00	7.10	0.55	1,322.95386	3,966.84702	206.66	62.46	40/132
High	IAEELPIRGGELYIDKIE LILADATFEEEDYDLER	17,979,619	105.78	6.94	0.53	1,323.13477	3,967.38974	343.43	62.36	42/132
High	IAEELPIRGGELYI DKIELILADATFEEEDYDLER	17,979,619	108.53	6.86	0.55	1,323.18616	3,967.54392	382.28	60.36	41/132
High	NRGYDEAYESSNVHASVYEEK	17,979,619	41.79	6.62	0.53	845.11078	2,533.31778	311.41	48.93	43/84
High	SRGTVDLSLNLPIDGENSELVGYSHR	17,979,619	121.09	6.41	0.54	905.90692	2,715.70621	137.29	54.98	42/96

^a Abbreviations: CI = confidence identity, Accession = GenBank protein gi number, Prob = probability, Xcorr = cross-correlation, MH+ = [M + H]⁺, delta M = delta M, RT = reaction time, and Ions = ions matched.

Fig. 3. Detection of *cry* genes from *Bacillus thuringiensis* strain BRC-XQ12 using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). **A**, PCR products of the insecticidal crystal protein genes amplified from BRC-XQ12. Lane M1, GeneRuler 100-bp DNA Ladder Plus; lane M2, GeneRuler 250-bp DNA Ladder Plus; lane M3, TaKaRa 250-bp DNA ladder (Dye Plus); lanes 1 to 10, PCR products amplified with K5un2 (5'-AGGACCAGGATTACAGGAGG-3')/K3un2 (5'-GCTGTGACACGAAGGATATAGCCAC-3') (for *cry1/cry7/cry9*), K5un3 (5'-CAATGCGTACCTTACAATTGTTAAGTAAT-3')/K3un3 (5'-CCTCCTGTAAATCC TGGTCTT-3') (for *cry1/cry7/cry9*), S5un1 (5'-GCTGTCTACCATGATTGCTTG-3')/S3un1 (5'-CAGTGCAGTAACCTTCTCTTG-3') (for *cry11*), S5un2 (5'-GGAAGAACTACTATTGTGATGC-3')/S3un2 (5'-AATAGTTTGAATTACCGCAGC-3') (for *cry2*), S5un3 (5'-ATGAATCCRAACAATCGAAG-3')/S3un3 (5'-GGTCCATCTGTTGTTTCTG-3') (for *cry3*), S5un4 (5'-GTGTCAAGAGAACCAACAGTATG-3')/S3un4 (5'-ACTAAGTCTCTCTGTATGACCAG-3') (for *cry4/cry10*), cry5F (5'-AGTGATYATGAKATTGAAGAAG-3')/cry5R (5'-TTRITRTTCATACTTGTATYGC-3') (for *cry5*), cry6F (5'-TATWATYTCWAARSAAGAATGGG-3')/cry6R (5'-TKTAAMGAYGTTKYTCKAAGAIT-3') (for *cry6*), cry8F (5'-ATGAGTCCAAATAATCAAAATG-3')/cry8R (5'-ATAACAWTCGTTTTTCATTTGG-3') (for *cry8*), and cry11F (5'-GCATTAATAGCAGTAGCTCC-3')/cry11R (5'-GTATCTTCTAARAATGATCTATC-3') (for *cry11*), respectively. **B**, RFLP patterns of *cry* genes from BRC-XQ12. Lanes 1 to 4, PCR-amplified fragments of K5un2/K3un2, K5un3/K3un3, S5un1/S3un1, and S5un2/S3un2 digested with *PstI/XbaI*, *EcoRI/PstI*, *Bsp119I/BanI*, and *HincII/MspI*, respectively; lane M1, GeneRuler 100-bp DNA Ladder Plus; and lane M2, GeneRuler 250-bp DNA Ladder Plus.

(IT)-time of flight (TOF)-MS (Roh et al. 2009). In 2011, a simple method combining LC-ESI-MS with antifungal tests was applied to identify the compound produced by *Bacillus subtilis* CCM1 1051 that was against filamentous fungi as a lipopeptide (Caldeira et al. 2011). In 2012, at least 72 differentially expressed proteins related to metabolic changes during ICPs synthesis in *B. thuringiensis* YBT-1520 were detected by comparative proteomic analysis based on matrix-assisted laser desorption-ionization time-of-flight MS (Gong et al. 2012). By using PQD in this study, information from low-quality ion fragments can be provided, while the high selectivity of MS/MS spectra is helpful for retrieving a better match in the database and library, thus improving

the reliability of the proof of structure. Therefore, MS technology is an important method for investigating the synthesis, properties, and other aspects of *B. thuringiensis* ICPs.

As a general description of strain BRC-XQ12, it is useful to perform plasmid profiling to check whether this strain carries plasmids and, if so, how many. This is important because the vast majority of *cry* genes are located on *B. thuringiensis* plasmids (Ye et al. 2012). In this study, however, the *cryIEa11* gene was localized on the chromosome of BRC-XQ12.

To the best of our knowledge, Cry1 have never been shown to have activity against plant-parasitic nematodes. Cry1Ea-like proteins were active against lepidoptera larvae of *Spodoptera exigua* (Visser et al. 1990), *S. frugiperda* (Rang et al. 2004), *Manduca sexta* (Barboza-Corona et al. 1998; Carmona et al. 2011), *Pectinophora gossypiella* (Tabashnik et al. 2000), and *Anticarsia gemmatilis* (Fiuza et al. 2013). This study found that Cry1Ea11 was toxic to *Bursaphelenchus xylophilus*, with an LC₅₀ value of 23.23 µg/ml, further dramatically broadening the spectrum of *Bacillus thuringiensis* ICPs.

Plant-parasitic nematodes use the stylet to feed, affecting the size and shape of the proteins ingested by them. To our knowledge, the size exclusion limit of the *Bursaphelenchus xylophilus* stylet remains unknown. However, the limit of other nematodes has been reported in the literatures. The nematode *H. schachtii* can only feed 28-kDa proteins. The potato nematode *Globodera rostochiensis* can ingest 32-kDa proteins. The transgenic expression of 54-kDa Cry6A and truncated 79-kDa Cry5B in tomato roots significantly affected the reproductive ability of *Meloidogyne incognita*, indicating that it could feed at least 79-kDa proteins in vivo (Li et al. 2007, 2008). Further research showed that 45- to 140-kDa proteins can be taken by the root-knot nematode in vitro through the stylet (Zhang et al. 2012). Our study indicated that *B. xylophilus* could feed 159-kDa GST-Cry1Ea11 protein via stylet in vitro.

Because plant-parasitic nematodes such as *B. xylophilus* live inside the plant body and rely on nutrients in plants, they are difficult to control, resulting in tremendous economic losses in many countries. Growing transgenic nematode-resistant plants is currently a recognized and feasible approach to control such nematodes. The *cryIEa11* gene obtained in this study will certainly provide a good foundation for breeding plants with antinematode genes. And it would be of considerable interest in the future researches to test whether *Bacillus thuringiensis* strain BRC-XQ12 and Cry1Ea11 are active against other nematodes to further broaden their application.

ACKNOWLEDGMENTS

This project was supported by the National Key R&D Program of China (numbers 2017YFD0200400 and 2011AA10A203), the Project of Fujian-Taiwan Joint Center for Ecological Control of Crop Pests (number Minjiaoke [2013] 51), the National Natural Science Foundation of China (numbers 31672084 and 31201574), the Leading Talents of Fujian Province College (number k8012012a), and the Study-Abroad Scholarship of Fujian Province to T. P. Huang. We thank the teachers and the students of the Biopesticide Research Center for their help; senior editor M. Wubben and the journal referees for their constructive comments; colleagues X. Cheng, F. Zhang, Z. Yu, X. Fan, and S. Ma for their help in the identification of the pathway of Cry1Ea11 entering into *Bursaphelenchus xylophilus*; and W. Rao for the data processing of LC₅₀ values.

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Fig. 4. Expression and purification of glutathione-S-transferase (GST)-Cry1Ea11 and GST. **A**, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) detection of the GST-Cry1Ea11 fusion protein expressed in *Escherichia coli* BL21 (DE3). Lanes 1 to 3, *E. coli* BL21 (DE3), *E. coli* BL21 (DE3)-pGEX-KG, and *E. coli* BL21 (DE3)-pGEX-*cryIEa11* induced by isopropyl-thiogalactopyranoside (IPTG), respectively; lane M, TaKaRa unstained protein molecular-weight markers. **B**, SDS-PAGE analysis of GST-Cry1Ea11 purified with GST*bind resin. Lane M, TaKaRa unstained protein molecular-weight markers; lane 1, supernatant of *E. coli* BL21 (DE3)-pGEX-*cryIEa11* induced with IPTG; and lane 2, fusion protein GST-Cry1Ea11 after GST-affinity column purification. **C**, SDS-PAGE analysis of GST purified by GST*bind resin. Lane M, TaKaRa unstained protein molecular-weight markers; and lane 1, GST protein expressed by *E. coli* BL21 (DE3)-pGEX-KG after GST-affinity column purification.

Fig. 5. Identification of the pathway of glutathione-S-transferase (GST)-Cry1Ea11 entering into *Bursaphelenchus xylophilus*. After *B. xylophilus* was incubated in NHS-Rhodamine-labeled GST-Cry1Ea11 for **A**, 1 h and **B**, 24 h, the pathway of the nematode feeding the 159-kDa GST-Cry1Ea11 was observed using confocal laser-scanning microscopy and imaged using a merged image. Abbreviations: s = stylet and el = esophageal lumen. Scale bars of the images were 10 µm (A) and 5 µm (B).

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