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Identification of *cry1I*-Type Genes from *Bacillus thuringiensis* Strains and Characterization of a Novel *cry1I*-Type Gene

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A PCR-restriction fragment length polymorphism method for identification of *cry1I*-type genes from *Bacillus thuringiensis* was established by designing a pair of universal primers based on the conserved regions of the genes to amplify 1,548-bp *cry1I*-type gene fragments. Amplification products were digested with the *Bsp*119I and *Ban*I enzymes, and four kinds of known *cry1I*-type genes were successfully identified. The results showed that *cry1I*-type genes appeared in 95 of 115 *B. thuringiensis* isolates and 7 of 13 standard strains. A novel *cry1I*-type gene was found in one standard strain and six isolates. The novel *cry1I* gene was cloned from *B. thuringiensis* isolate Btc007 and subcloned into vector pET-21b. Then it was overexpressed in *Escherichia coli* BL21(DE3). The expressed product was shown to be toxic to the diamondback moth (*Plutella xylostella*), Asian corn borer (*Ostrinia furnacalis*), and soybean pod borer (*Leguminivora glycinivorella*). However, it was not toxic to the cotton bollworm (*Helicoverpa armigera*), beet armyworm (*Spodoptera exigua*), or elm leaf beetle (*Pyrrhalta aenescens*) in bioassays. Subsequently, the Cry protein encoded by this novel *cry* gene was designated Cry1Ie1 by the *B. thuringiensis* δ-endotoxin nomenclature committee.

Crystal proteins from the gram-positive spore-forming bacterium *Bacillus thuringiensis* are toxic to a wide variety of insects that are economically important as pests. Many different genes encoding the *B. thuringiensis* endotoxin have been isolated and characterized. The genes have been classified as *cry1* to *cry40*, *cyt1*, and *cyt2* and are ranked according to their homology (10, 21; see also the *B. thuringiensis* toxin nomenclature website at http://www.biols.susx.ac.uk/home/Neil_Crickmore/Bt/). Cry1 proteins that are active against lepidopteran insects are produced as crystalline parasporal inclusions during sporulation. Generally, the crystals are composed of protoxins of approximately 130 kDa, but *cry1I*-type genes are usually silent genes capable of encoding a protein of about 81 kDa in *B. thuringiensis* strains (9, 13, 21, 24). We decided to screen *B. thuringiensis* isolates for *cry1I* genes with the aim of finding novel *cry1I* genes, which could encode insecticidal proteins toxic to insensitive or resistant insect pests.

This screening approach included the development of an analysis protocol based on PCR-restriction fragment length polymorphism (RFLP). PCR-based methods have been developed to detect different *cry* genes from *B. thuringiensis* strains (1–8, 11, 13, 15, 17, 23). More than 80 primer pairs have been designed to identify entire groups and individual *cry* genes (19). Several specific primers and probes for Southern blotting were designed to detect *cry1I*-type genes. A wide distribution of *cry1I*-type genes among many different *B. thuringiensis* strains has been reported (13, 22, 25). However, the list of *cry* genes is increasing, and novel PCR primers are needed in order to identify some of the recently described genes (5).

The present study establishes a PCR-RFLP method for identifying *cry1I*-type genes from *B. thuringiensis* isolates. Both known and new *cry1I* genes can be determined by using the new method with universal primers that were designed based on the conserved regions of *cry1I*-type genes. One novel *cry1I*-type gene from a *B. thuringiensis* isolate, Btc007, was found and characterized by this method.

MATERIALS AND METHODS

Bacterial strains and plasmid. Thirteen *B. thuringiensis* standard serotype strains imported from the Pasteur Institute were supplied by the Bacterial Stock Center of the Chinese Academy of Forestry Sciences. *B. thuringiensis* isolates were obtained from the *B. thuringiensis* R&D Center of the Hubei Academy of Agricultural Sciences, the Bacterial Stock Center of the Chinese Academy of Forestry Sciences, and the Department of Plant Protection of Northeast Agriculture University, Harbin, China. Vector pET-21b(+) and *Escherichia coli* BL21(DE3) were purchased from Novagen Co.

Chemical reagents and enzymes. Chemical reagents were purchased from Sigma, and *Taq* DNA polymerase was purchased from Promega. Restriction enzymes were obtained from New England Biolabs, Inc., and MBI Fermentas.

Preparation of template and PCR-RFLP. *B. thuringiensis* strains were incubated overnight at 30°C and 220 rpm in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.0). A 1-ml volume of culture was collected after centrifugation, and the pellet was resuspended in 100 μl of purified water, boiled 5 min, and spun at 14,000 rpm for 5 min. The supernatant was collected as a template for PCR amplification. Based on the conserved regions of *cry1I* genes (*cry1Ia*, *cry1Ib*, *cry1Ic*, and *cry1Id*) (9, 10, 22, 25), a pair of universal primers (S5uni–S3uni) was designed (Table 1). The 50-μl PCR mixture was composed of 1 μl of templates with reaction buffer, 250 μM each deoxynucleoside triphosphate, 0.2 μM each primer, and 0.5 U of *Taq* DNA polymerase. PCR was carried out for 32 cycles (at 94°C for 1 min, 52°C for 1 min, and 72°C for 3 min). PCR products were digested with both *Bsp*119I and *Ban*I enzymes. The restriction fragments were separated in 1.5% agarose gels. Table 1 shows the predicted fragment sizes of PCR products for four kinds of known *cry1I* genes and the novel *cry1I*-type gene found in this study.

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TABLE 1. PCR-RFLP system of *cry1I* genes

Gene or primer	Accession no.	Sequence[b]	Positions	Coding region (nt)[c]	Product size (bp)	Fragment size (nt) (bp) with *Bsp*119I and *Ban*I
Primers						
S5uni		GCTGTCTACCATGATTCGCTTG				
S3uni		CAGTGCAGTAACCTTCTCTTGC				
Genes						
cry1Ia1	X62821	GCTGTCTACCATGATTCGCTTG	760–781	355–2514	1,584	141, 444, 571, 47, 381
		CAGTGCAGTAACCTTCTCTTGC	2343–2322			
cry1Ib1	U07642	GCcGTCTACCATGAaTCGCTTG	642–663	237–2396	1,584	1,156, 47, 381
		CAGTGCAGTAACCTTCTCTTGC	2225–2204			
cry1Ic1	AF056933	GCTGTCTACCATGAaTCGCTTG	406–427	1–2160	1,584	522, 634, 47, 381
		CAGTGCAGTAACCTTCTCTTGC	1989–1968			
cry1Id1	AF047579	GCTGTCTACCATGAaTCtCTTG	907–928	502–2661	1,584	141, 444, 999
		CAtTGCAGTAACtcTaTCTTGt	2490–2469			
cry1Ie1[a]	AF211190	GCTGTCTACCATGAaTCGCTTG	715–736	310–2469	1,584	585, 571, 47, 381
		CAGTGCAGTAACCTTCTCTTGC	2298–2277			

[a] The novel gene found in this study
[b] Lowercase letters indicate nucleotides that differ from those in the primer sequences.
[c] nt, nucleotides.

Preparation of DNA and Southern blotting. Plasmid DNA was prepared from a *B. thuringiensis* isolate Btc007 cell grown to an optical density at 600 nm of 1.0. Cells were pelleted by centrifugation and resuspended in protoplast buffer (20 mg of lysozyme/ml in 0.3 M sucrose, 25 mM Tris-Cl [pH 8.0], 25 mM EDTA). After incubation at 37°C for 1 h, protoplasts were lysed for 10 min by the addition of 9 volumes of a solution containing 10 mM Tris-Cl, 1 mM EDTA, 0.085 M NaOH, and 1% sodium dodecyl sulfate (SDS). One-half volume of 3 M potassium acetate (pH 4.8) was then added, and the cellular material was neutralized overnight at 4°C. After centrifugation, the plasmid DNA was precipitated from the supernatant with isopropanol and was purified by isopycnic centrifugation on cesium chloride-ethidium bromide gradients by the method described by Sambrook et al. (20). For Southern blot analysis, 2 μg of plasmid DNA was digested with the restriction enzymes *Hin*dIII, *Pst*I, and *Cla*I and electrophoresed on a 0.8% agarose gel. The digested DNA was denatured and transferred to a nylon membrane (Hybond N+; Amersham) by using 0.4 M NaOH. The product amplified from Btc007 with primers S5uni and S3uni was labeled with [^{32}P]dCTP by using a random-priming labeling kit (Promega) as a probe. Prehybridization and hybridization were carried out as described by Sambrook et al. (20).

Cloning of a novel insecticidal crystal protein gene. Southern blotting was performed against plasmid DNA purified from *B. thuringiensis* Btc007 digested with *Hin*dIII, *Pst*I, and *Cla*I. Among the positive signals obtained in the Southern blot analysis, a 4.8-kb *Cla*I fragment from *B. thuringiensis* Btc007 plasmid DNA was cloned into the pUCP19 *Cla*I site and transformed into *E. coli* DH5α according to the procedure of Sambrook et al. (20). To obtain subclones for analysis of the DNA sequence, a restriction enzyme map was constructed using various restriction enzymes such as *Cla*I, *Eco*RI, *Hin*dIII, *Xho*I, and *Kpn*I. Each of the DNA fragments of approximately 1 kb obtained by restriction with the *Eco*RI, *Hin*dIII, and *Cla*I enzymes existing on the cloned DNA was eluted to facilitate analysis of the DNA sequences and subcloned into pBlueScript II SK(+) with appropriate enzyme sites. Subcloned DNA fragments were sequenced with an automated DNA sequencer.

Expression of the *cry1Ie1* gene and protein analysis. To express the cloned insecticidal crystal protein gene, a pair of primers was designed to amplify the full-length gene. The sequence of the forward primer was 5′-CGCGGATCCG ATGAAACTA AAGAATCCAG-3′, with the restriction enzyme *Bam*HI at the 5′ end, and that of the reverse primer was 5′-ACGCGTCGACGGCAT GTTA CGCTCAATATGG-3′, with *Sal*I at the 5′ end. The PCR product was inserted into the pET-21b vector *Bam*HI and *Sal*I sites and transformed into *E. coli* BL21(DE3). *E. coli* BL21(DE3) harboring the cloned crystal protein genes was grown in LB medium overnight and was then inoculated at a 1% concentration into fresh LB medium containing the antibiotic ampicillin (100 μg/ml). The cultures were incubated at 37°C with shaking (250 rpm) until an optical density of 0.6 to 0.8 was reached, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.7 mM. The cultures were then maintained at 20°C for 12 h. Cry1Ie1 protein was purified and concentrated according to the method of Shin et al. (22). *B. thuringiensis* strains were incubated at 30°C and 220 rpm. Cultures for SDS-polyacrylamide gel electrophoresis (PAGE) analysis of total proteins were collected by centrifugation when the crystal was formed (about 30 h). The method of SDS-PAGE analysis described by Sambrook et al. (20) was used.

Insect bioassay. Insecticidal activity against first-instar larvae of the Asian corn borer (*Ostrinia furnacalis*), cotton bollworm (*Helicoverpa armigera*), and beet armyworm (*Spodoptera exigua*) was measured by incorporating a suspension containing twofold serial dilutions of purified inclusions into the artificial diet. Toxicity studies on larvae of the diamondback moth, *Plutella xylostella* (third-instar larvae), elm leaf beetle (*Pyrrhalta aenescens*) (second-instar larvae), and soybean pod borer (*Leguminivora glycinivorella*) (second-instar larvae) were conducted on fresh leaf disks by leaf dip bioassays (24). Disks cut from leaves of cabbages grown in the greenhouse were used for *P. xylostella*, disks cut from elm leaves were used for *P. aenescens*, and disks cut from green pods of soybean were used for *L. glycinivorella*. Ten larvae were each given an artificial diet or placed on a leaf disk, and their fates were monitored after 2 days for *P. xylostella* and after 7 days for the other insects. Bioassays were repeated at least twice, and 50% lethal concentrations (LC_{50}) were calculated by probit analysis (12).

Nucleotide sequence accession number. The nucleotide sequence data published in this paper were assigned GenBank accession number AF211190.

RESULTS

Identification of *cry1I* genes from *B. thuringiensis* strains. *cry1I* genes were detected in 13 standard strains and 115 isolates by use of this PCR-RFLP method. Seven standard strains contained *cry1I* genes (Table 2), and 95 isolates harbored *cry1I*

TABLE 2. *cry1I* gene types of *B. thuringiensis* strains

B. thuringiensis standard strain (serotype)	*cry1I* gene type[a]
B. thuringiensis subsp. *aizawai* HD-11 (H7)	*cry1Ia*
B. thuringiensis subsp. *alesti* HD-4 (H3a)	—
B. thuringiensis subsp. *canadensis* HD-224 (H5a5c)	—
B. thuringiensis subsp. *entomocidus* HD-9 (H6)	*cry1Ia*
B. thuringiensis subsp. *finitimus* HD-3 (H2)	—
B. thuringiensis subsp. *galleriae* HD-29 (H5a5b)	*cry1Ib*
B. thuringiensis subsp. *kurstaki* HD-73 (H3a3b)	—
B. thuringiensis subsp. *morrisoni* HD-12 (H8a8b)	*cry1Ib*
B. thuringiensis subsp. *pakistani* HD-395 (H13)	*cry1Ia*
B. thuringiensis subsp. *sotto* HD-770 (H4a4b)	—
B. thuringiensis isolate Btc007	*cry1Ie*
B. thuringiensis subsp. *thompsoni* HD-542 (H12)	*cry1Ia*
B. thuringiensis subsp. *thuringiensis* HD-2 (H1)	*cry1Ie*
B. thuringiensis subsp. *toumanoffi* HD-210 (H11a11b)	—

[a] —, no positive PCR signal.

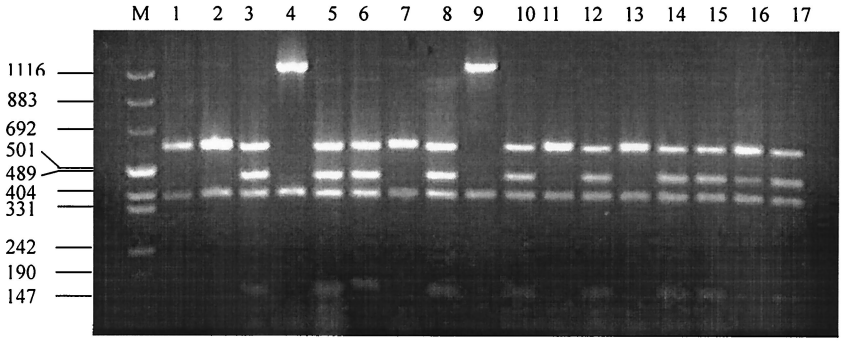

FIG. 1. PCR-RFLP patterns of *cry1I*-type genes from *B. thuringiensis* standard strains and isolates. Lanes: 1 and 10 to 17, *B. thuringiensis* isolates; 2, *B. thuringiensis* isolate Btc007; 3, *Bacillus thuringiensis* subsp. *aizawai* (H7); 4, *B. thuringiensis* subsp. *morrisoni* (H8a8b); 5, *Bacillus thuringiensis* subsp. *entomocidus* (H6); 6, *B. thuringiensis* subsp. *pakistani* (H13); 7, *B. thuringiensis* subsp. *thuringiensis* (H1); 8, *Bacillus thuringiensis* subsp. *thompsoni* (H13); 9, *B. thuringiensis* subsp. *galleriae* (H5a5b); M, Molecular mass marker (pUC mix).

genes based on a positive PCR signal. The *cry1I*-type genes of these strains were determined according to the patterns of fragments of products digested with *Ban*I and *Bsp*119I. Four kinds of *cry1I*-type RFLP patterns were found in the agarose gel (Fig. 1). Four standard strains and 71 isolates had a *cry1Ia*-type RFLP pattern. The gel revealed three main bands of 0.57, 0.44, and 0.38 kb, sizes which conformed to those of the predicted fragments of the *cry1Ia*-type genes (Fig. 1, lanes 3, 5, 6, 8, 10, 12, 14, 15, and 17). Two standard strains and 26 isolates showed a *cry1Ib*-type RFLP pattern, which had two main bands of 1.16 and 0.38 kb (Fig. 1, lanes 4 and 9). A novel *cry1I*-type pattern which had two main bands of 0.58 and 0.38 kb was found in *Bacillus thuringiensis* subsp. *thuringiensis* HD-2 and in six isolates, including *B. thuringiensis* Btc007 (Fig. 1, lanes 1, 2, 7, 11, and 13; Table 2). Four isolates had both *cry1Ia*-type and *cry1Ie*-type patterns (Fig. 1, lane 16). Eight isolates contained *cry1Ic* genes, and both *cry1Ia* and *cry1Ib* genes were found in five isolates. Three isolates contained both *cry1Ib* and *cry1Ic* genes (data not shown). No *cry1Id* genes were detected.

Cloning and sequence analysis of the *cry1Ie1* gene. The *Cla*I fragment from *B. thuringiensis* Btc007 was cloned. Subcloned DNA fragments of about 1 kb were sequenced with an automated DNA sequencer. The sequence analysis showed the presence of an open reading frame encoding a protein of 719 amino acid residues with a predicted molecular mass of 81 kDa. Homology analysis with four known holotype Cry1I proteins indicated that the sequence of Cry1Ie1 protein showed the maximum identity (94.9%) with the Cry1Ib1 protein sequence (Cry1Ie1 sequence identities with the other Cry1I proteins were 93.4% [Cry1Ia1], 91.6% [Cry1Ic1], and 87.7% [Cry1Id1]). Five common conserved regions of Cry protein (14, 21) existed in the Cry1Ie1 sequence; they were located between amino acid residues 186 and 202, 253 and 298, 491 and 526, 557and 567, and 634 and 643.

Expression of the *cry1Ie1* gene. A full-length *cry1Ie1* gene containing a *Bam*HI site at the 5′ end and a *Sal*I site at the 3′ end was amplified with a pair of primers. This fragment was inserted into *Bam*HI and *Sal*I sites of the pET-21b vector, generating pETB-1IE. After sequencing analysis proved the sequence of the fragment to be identical to that of *cry1Ie1*, the recombinant *E. coli* BL21(DE3) harboring vector pETB-1IE was induced to express the product of the *cry1Ie1* gene. The product expressed formed an inclusion in *E. coli*. SDS-PAGE analysis indicated that the expressed product had a molecular mass of 84.2 kDa (more than 81 kDa of Cry1I protein), because the product was fused with 14 amino acid residues at the N terminus containing a T7 tag and 15 amino acid residues at the C terminus containing a His tag (Fig. 2). More than 70% of the total protein expressed by recombinant *E. coli* BL21(DE3) harboring the *cry1Ie1* gene was the 84.2-kDa fusion protein (Fig. 2, lane 3). The purified Cry1Ie1 inclusion is shown in Fig. 2, lane 2. The *cry1Ie1* gene from isolate Btc007 was overexpressed in *E. coli* BL21(DE3) cells. No 81-kDa expressed product was apparent after the crystal was formed in the Btc007 cells containing *cry1Ie1* (Fig. 2, lane 1).

Insect bioassay. The purified fusion protein Cry1Ie1 was tested for insecticidal activities against five lepidopteran insects and one coleopteran insect. Cry1Ie1 was highly active against the Asian corn borer, with an LC_{50} of 2.22 μg/ml (95% confidence interval [95% CI], 1.77 to 2.75); the diamondback moth, with an LC_{50} of 0.20 μg/ml (95% CI, 0.13 to 0.27); and

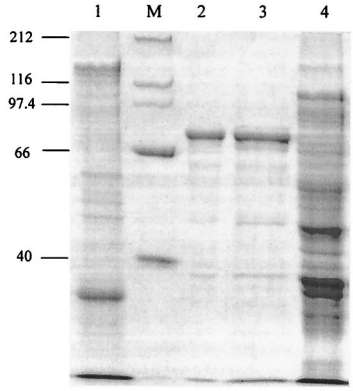

FIG. 2. Electrophoretic analysis of proteins from recombinant *E. coli* BL21(DE3) and *B. thuringiensis* isolate Btc007 on an SDS–10% polyacrylamide gel. Lanes: M, high-molecular-mass protein marker; 1, total proteins from *B. thuringiensis* isolate Btc007 after the formation of the crystal; 2, purified inclusions from recombinant *E. coli* BL21(DE3) harboring the *cry1Ie1* gene; 3, total proteins from recombinant *E. coli* BL21(DE3) harboring the *cry1Ie1* gene; 4, total proteins from *E. coli* BL21(DE3).

the soybean pod borer, with an LC_{50} of 9.02 μg/ml (95% CI, 3.50 to 23.24). Cry1Ie1 protein showed no toxicity against the cotton bollworm, the beet armyworm, or the elm leaf beetle (data not shown).

DISCUSSION

Several PCR-based methods for *cry* gene identification of *B. thuringiensis* strains have been developed (1–8, 11, 13, 15, 17, 23). Specific-primer PCR and multiplex PCR can directly detect known *cry* genes from *B. thuringiensis* (2, 4, 6–8). Exclusive PCR and PCR-RFLP can identify not only known *cry* genes but also novel *cry* genes (3, 5, 11, 15, 17, 23). We established a PCR-RFLP method to identify *cry1I* genes by using the conserved regions of four known *cry1I* genes as a basis for designing a pair of universal primers for *cry1I*-type genes which were different from the primers previously reported (18, 19, 22). According to restriction analysis of the sequences of predicted PCR fragments from four genes, two restriction enzymes, *Bsp*119I and *Ban*I, were used to produce the predicted RFLP patterns. Many *B. thuringiensis* strains were detected by this method.

We could not detect any *cry1I*-type gene in *Bacillus thuringiensis* subsp. *kurstaki* HD-73, *Bacillus thuringiensis* subsp. *alesti* HD-4, *Bacillus thuringiensis* subsp. *canadensis* HD-224, *Bacillus thuringiensis* subsp. *finitimus* HD-3, *Bacillus thuringiensis* subsp. *sotto* HD-770, or *Bacillus thuringiensis* subsp. *toumanoffi* HD-201 (Table 2), although *B. thuringiensis* subsp. *alesti* HD-4 and *B. thuringiensis* subsp. *toumanoffi* HD-201 showed weak signals in response to a *cry1I*-specific probe (22). Also, *B. thuringiensis* subsp. *alesti* HD-4 and *B. thuringiensis* subsp. *toumanoffi* HD-201 have been reported to produce no PCR products when PCR was performed with a *cry1I*-specific probe (13). *B. thuringiensis* subsp. *kurstaki* HD-73 was reported to contain a *cry1I* gene (25), but the present study showed it to harbor no *cry1I*-type gene. A *cry1Ib*-type gene was detected in *Bacillus thuringiensis* subsp. *morrisoni* HD-12, which showed weak signals by hybridization (22). *Bacillus thuringiensis* subsp. *galleriae* (HD-8) produced a PCR fragment, which was not cleaved by *Kpn*I (13). A *cry1Ia*-type gene was found in *Bacillus thuringiensis* subsp. *pakistani* HD-395, and a novel *cry1I*-type gene—*cry1Ie*—was found in *B. thuringiensis* subsp. *thuringiensis* HD-2. This is surprising, because neither strain showed any signal by hybridization (22).

Our studies did not detect the 81-kDa protein by SDS-PAGE analysis after the crystal was formed in Btc007 cells (Fig. 2, lane 1). Some investigators have reported that many *cry1I*-type genes are silent in *B. thuringiensis* strains because they are often located downstream of the *cry1* genes and a strong *cry1* transcriptional terminator is present in the interval sequence between the *cry1* and *cry1I* genes (13, 22, 25). However, Northern blot analysis showed that the mRNAs of *cry1I* genes in *B. thuringiensis* strains were detected at both the T2 and T5 stages of sporulation (18, 26). Cry1Ia is a secreted protein because of the presence of a putative signal peptide in the N-terminal domain I, so it was not accumulated and detected in the cell after the T5 stages of sporulation (16). Thus, we could not determine why no 81-kDa protein was expressed in Btc007 cells. The Northern blot analysis results and the interval sequence between the *cry1* and *cry1Ie1* genes in Btc007 should be further studied.

Although the first Cry1Ia1 protein reported by Tailor et al. had dual larvicidal activity against *Lepidoptera* and *Coleoptera* spp. (25), the Cry1I-type proteins found later had larvicidal activity only against lepidopteran insects (9, 13, 16, 22). Compared with Cry1Ia1, two Cry1Ia proteins (CGCryV and Cry732) that had single amino acid differences in domain I showed insecticidal activity against *Lepidoptera* and exhibited no activity against *Coleoptera* (9, 16). Cry1Ia3 (CryV1), Cry1Ib1 (CryV465), and Cry1Id1 proteins from recombinant *E. coli* were active only against lepidopteran insects (13, 22). Similarly, Cry1Ie1 showed no insecticidal activity against coleopteran insects. We found that the Cry1Ie1 protein was highly toxic to the soybean pod borer, though no insecticidal activity of any Cry protein against this pest had been reported.

Although many toxins have been found in *B. thuringiensis* strains, only a few of them have been used to effectively control some determined insect pests. Moreover, some insect pests have developed resistance against some *B. thuringiensis* toxins. In order to solve these problems, isolation of new strains and toxins is crucial. This study has provided a PCR-RFLP method for the rapid identification of *cry1I*-type genes that can be used not only to retrieve information on the presence of these genes in new isolates but also to discover novel *cry1I* genes. We also found a novel *cry1I*-type gene with new insecticidal properties.

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