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An Improved PCR-Restriction Fragment Length Polymorphism (RFLP) Method for the Identification of *cry1*-Type Genes

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The *cry1*-type genes of *Bacillus thuringiensis* represent the largest *cry* gene family, which contains 50 distinct holotypes. It is becoming more and more difficult to identify *cry1*-type genes using current methods because of the increasing number of *cry1*-type genes. In the present study, an improved PCR-restriction fragment length polymorphism (PCR-RFLP) method which can distinguish 41 holotypes of *cry1*-type genes was developed. This improved method was used to identify *cry1*-type genes in 20 *B. thuringiensis* strains that are toxic to lepidoptera. The results showed that the improved method can efficiently identify single and clustered *cry1*-type genes and can be used to evaluate *cry1*-type genes in novel strain collections of *B. thuringiensis*. Among the detected *cry1*-type genes, we identified four novel genes, *cry1Ai*, *cry1Bb*, *cry1Ja*, and *cry1La*. The bioassay results from the expressed products of the four novel *cry* genes showed that Cry1Ai2, Cry1Bb2, and Cry1Ja2 were highly toxic against *Plutella xylostella*, whereas Cry1La2 exhibited no activity. Moreover, Cry1Ai2 had good lethal activity against *Ostrinia furnacalis*, *Hyphantria cunea*, *Chilo suppressalis*, and *Bombyx mori* larvae and considerable weight loss activity against *Helicoverpa armigera*.

Bacillus thuringiensis, which is a Gram-positive bacterium, is known for its specific toxicity toward insect pests (1). This toxicity is largely attributed to the insecticidal crystal proteins encoded by the *cry* genes (2–4). The *cry1*-type genes of *B. thuringiensis* are highly toxic to lepidopteran pests, and some genes have been used to develop plants with resistance to insect pests (5–7). Because of their potential application and commercial value, much research has focused on the discovery of novel *cry1* genes; to date, approximately 258 *cry1* genes have been cloned and named, and 50 distinct holotypes have been classified (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Previous research has revealed that *cry1* genes are typically found in clusters; for example, *B. thuringiensis* strains HD12 and HD525 contain at least four different *cry1*-type genes (8).

PCR is a simple and convenient investigative method and has been widely used to identify the vast variety of *cry* genes by the use of different primers (9–12). PCR-restriction fragment length polymorphism (PCR-RFLP) is a modified PCR technique that is generally used for the identification of known and unknown *cry1*-type genes (except for *cry11*-type genes), and of parts of the *cry7*-type and *cry9*-type genes, according to the fragment lengths of digested PCR-amplified products described by Kuo and Chak (8). Some primers have been designed for the identification of *cry1*-type genes (13) and *cry8*-type genes (14) based on the PCR-RFLP method. However, it is becoming difficult to identify novel *cry1*-type genes using the PCR-RFLP method (8) because of the increase in the numbers of *cry1*-type genes.

To resolve this problem, an improved PCR-RFLP method was designed to directly identify the fourth class of *cry1*-type genes by dividing the *cry1*-type genes into four subgroups with relatively specific primers. In the present study, 22 *B. thuringiensis* strains were tested using the improved PCR-RFLP method; 2 of them, HD1 and HD29, were control strains, and others were isolated in our laboratory and are toxic to lepidoptera. The results showed that the improved PCR-RFLP method was effective and accurate at identifying *cry1*-type genes from *B. thuringiensis* strains.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Escherichia coli* JM110 cells [*dam dcm supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z*ΔM15)] were used for standard transformations.

B. thuringiensis subsp. *kurstaki* HD1 and *B. thuringiensis* subsp. *galleriae* HD29 were obtained from the Bacillus Genetic Stock Center (BGSC), Biological Sciences, Columbus, OH (http://www.bgsc.org/). Twenty native *B. thuringiensis* strains with high toxicity to lepidopteran pests were isolated by our laboratory. *E. coli* was incubated at 37°C in LB medium (1% NaCl, 1% tryptone, 0.5% yeast extract), and ampicillin (100 μg/ml) was added to the culture medium to select for antibiotic-resistant *E. coli*. The *B. thuringiensis* strains were grown at 30°C in peptone-beef extract (PB) medium (0.5% peptone, 0.3% beef extract; 15). The initial pH of all cultures was 7.2, and the cultures were incubated in a rotary shaker at 250 rpm.

DNA extraction. The *B. thuringiensis* strains were grown for 12 h on LB agar plates. Approximately 100 mg of cells (wet weight) was collected from the agar plates and washed with double-distilled water (ddH_2O). DNA was prepared from these cells using the methods described by Song et al. (13) and was used as the DNA template for PCR-RFLP.

Improved PCR-RFLP. After personal computer (PC) gene multialignment analysis, four sets of primers were designed to divide the *cry1*-type genes into four subgroups; the sequences of these four oligonucleotide primers as well as the alignment between primers and all published *cry1*-type genes are shown in Table S1 in the supplemental material. PCR was performed in 50 μl containing 10 ng template DNA, 0.4 mmol/liter deoxynucleotide triphosphate, 0.2 μM (each) primer, and 1.5 U *Taq* DNA

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polymerase (TaKaRa Corporation, China) in reaction buffer. The amplification was performed as follows: 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, which were followed by an additional extension at 72°C for 10 min. The PCR product was used for restriction endonuclease reactions, which were performed using a 20-μl mixture containing 10 μl PCR product and 1 U of the restriction endonuclease HinfI (according to analysis of restriction sites on the predicted DNA amplify fragment, HinfI could divide almost all *cry1*-type genes) in the appropriate reaction buffer. The PCR product was purified when nonspecific amplicons were present. The incubation temperature was set according to the manufacturer's instructions for the restriction endonuclease. The resulting restriction fragments were separated on 2% agarose gels. Table 1 shows the predicted fragment sizes of the PCR products for the known *cry1*-type genes.

Gene cloning and sequencing assay. For cloning the full-length *cry1*-type genes, primers were designed according to the reported genes (Table 2). PCR was performed using *Pfu* DNA polymerase (Tiangen, China) and a PTC-100 Peltier Thermal Cycler (MJ Research) as follows: 30 cycles of denaturation at 94°C for 60 s, annealing at 54°C for 50 s, and extension at 72°C for 4 min, which were followed by an additional extension at 72°C for 10 min. After amplification, the full-length PCR products were cloned into pSIMPLE-18 EcoRV/BAP vector (TaKaRa Corporation, China) for sequencing using an automated DNA sequencer (ABI-3730XL), and Vector NTI Suite 9 (Invitrogen; Carlsbad, CA) was used for sequence analysis.

Expression of *cry1*-type genes. To assay the toxicity of *cry1*-type genes, the genes were cloned and expressed in the *E. coli* Rosetta strain (DE3; Novagen). The primers listed in Table 2 were used for the amplification of full-length *cry1*-type genes. PCR was performed as described above. The products were cloned into the Ecl136II site of the pEB vector (16) and transformed into *E. coli* JM110 cells. Recombined plasmids containing in-frame *cry1*-type genes were transformed to the *E. coli* Rosseta strain (DE3). Expression was induced by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside) at a final concentration of 0.5 mmol/liter, and the cultures were incubated at 16°C at 220 rpm for 10 h. The cell pellet from a 10-ml culture of induced Rosetta (DE3) cells containing the recombinant expression vector was suspended in 1 ml of lysis buffer (20 mmol/liter Tris-HCl; pH 8.0) and sonicated (noise-isolating tamber; Ningbo Scientz Biotechnology Co., Ltd.) for 5 min (75% power; 3-s pulse on, 5-s pulse off). The lysate was centrifuged at 12,000 × *g* at 4°C for 20 min. The pellet (insoluble protein) was resuspended in 1 ml of lysis buffer (20 mmol/liter Tris-HCl; pH 8.0) and examined by SDS polyacrylamide gel electrophoresis (SDS-PAGE). ImageJ software (National Institutes of Health) was used to determine the intensity of the band against a bovine serum albumin (BSA) standard.

Insect bioassay. The insecticidal activities of the Cry1 proteins, which were tested against *Ostrinia furnacalis*, *Helicoverpa armigera*, *Hyphantria cunea*, and *Chilo suppressalis*, were assayed by exposing neonatal larvae to an artificial diet (17). Bioassays were performed in 24-well trays. Each well of the assay tray contained approximately 400 mg of diet. Each concentration was tested with 24 insects that were individually placed in each well, and the amount or total weight of the surviving insects was recorded after 4 days for *H. cunea* or 7 days for the others. Analysis of toxicity against *Plutella xylostella* second-instar larvae was conducted on fresh cabbage using the leaf-dip bioassay (18), and the number of surviving larvae was recorded after 2 days. Analysis of toxicity against *B. mori* neonate larvae was conducted on mulberry leaves also using the leaf-dip bioassay (18), and the number of surviving larvae was also recorded after 2 days. All bioassays were repeated at least three times using at least seven concentrations. The 50% lethal concentration (LC_{50}) and 50% weight loss concentration (WLC_{50}) values were calculated by Probit analysis (19).

Nucleotide sequence accession numbers. The sequences of the full-length *cry1Bb* and *cry1Ja* genes from WBT-2, the *cry1Ai* gene from SC6H8, and the *cry1La* gene from BtS6 have been deposited in the GenBank database, and the accession numbers are listed in Table 2.

TABLE 1 Predicted sizes of PCR products and the RFLP fragments of various *cry*-type genes

Primer pair and gene	Predicted size (bp) of PCR product(s)	
	Before digestion	After digestion with HinfI
CRY1P1 and CRY1PR		
cry1Aa	1,921	650, 561, 334, 330, 68
cry1Ab	1,924	724, 346, 334, 330, 212
cry1Ac	1,927	392, 346, 335, 334, 330, 212
cry1Ae	1,924	664, 494, 346, 230, 212
cry1Af	1,915	724, 346, 334, 321, 212
cry1Ag	1,921	576, 561, 334, 330, 74, 68
cry1Ai	1,924	561, 335, 334, 330, 318, 68
cry1La	1,894	762, 721, 240, 150, 43
CRY1P2 and CRY1PR		
cry1Ba	2,008	689, 322, 244, 168, 132, 118, 112, 72, 53, 48, 48, 24
cry1Bb	2,023	1,114, 221, 149, 144, 138, 137, 48, 48, 18, 15, 13
cry1Bc	2,023	1,114, 221, 149, 144, 138, 137, 48, 48, 18, 15, 13
cry1Bd	2,029	1,086, 658, 137, 48, 48, 46, 15, 13
cry1Be	2,017	1,003, 358, 215, 147, 108, 53, 48, 48, 26, 18, 15
cry1Bf	2,020	801, 358, 276, 215, 132, 53, 48, 48, 46, 26, 24, 15
cry1Bg	2,041	689, 358, 339, 215, 132, 112, 67, 53, 48, 26, 24
cry1Bh	2,023	822, 627, 176, 147, 108, 48, 48, 31, 18, 15, 5
cry1Ka	2,011	1,001, 505, 266, 106, 92, 48, 15
CRY1P3 and CRY1PR		
cry1Ad	1,921	718, 643, 561, 21
cry1Ah	1,924	643, 389, 346, 335, 212, 21
cry1Ca	1,948	702, 645, 517, 106
cry1Cb	1,909	521, 517, 302, 243, 181, 116, 51
cry1Da	1,876	860, 697, 276, 65
cry1Db	1,876	1,136, 585, 112, 65
cry1Dc	1,900	805, 712, 192, 127, 65, 21
cry1Ea	1,903	727, 709, 468, 21
cry1Eb	1,897	475, 296, 294, 287, 270, 128, 116, 53
cry1Fa	1,903	677, 476, 293, 140, 134, 114, 91
cry1Fb	1,900	489, 293, 283, 188, 153, 140, 114, 106, 91, 65
cry1Ga	1,879	552, 429, 403, 252, 131, 116, 18
cry1Gb	1,888	934, 350, 262, 258, 106
cry1Ha	1,897	825, 715, 192, 127, 39, 21
cry1Hb	1,888	828, 354, 225, 192, 130, 97, 48, 36
cry1Ja	1,885	516, 364, 349, 186, 168, 153, 106, 65
cry1Jb	1,888	702, 444, 322, 267, 73, 53, 49
cry1Jd	1,888	517, 448, 364, 274, 248, 59
CRY1P4 and CRY1PR		
cry1Ia	1,917	786, 315, 303, 145, 130, 111, 53, 48, 31, 17
cry1Ib	1,917	497, 315, 256, 211, 176, 147, 108, 92, 53, 48, 31, 5
cry1Ic	1,917	571, 497, 211, 181, 147, 108, 92, 53, 48, 31
cry1Id	1,917	624, 564, 303, 222, 147, 48, 31
cry1Ie	1,917	558, 315, 256, 228, 211, 130, 92, 53, 48, 31, 17
cry1Ma	1,908	1,270, 303, 130, 100, 95, 32

RESULTS

Evaluation of the improved PCR-RFLP method. Two strains, HD1 and HD29, containing several known *cry1*-type genes, were used to evaluate the improved method.

An RFLP pattern with 0.72-, 0.65-, 0.56-, 0.39-, 0.35-, 0.33-, and 0.21-kb fragments was obtained for strain HD1 using primer

TABLE 2 The four novel *cry1*-type genes cloned from the wild-type *B. thuringiensis* strain and the primers for amplification of the full-length genes

Gene type	Primers[a]	Source strain	Holotype gene (% identity)[b]	Toxin[c]	GenBank accession no.
cry1Ai	1AiF: ATGGATAACAATCCGAACATCAATG 1AiR: CTATTCTCCATAAGGAGTAATTCC	SC6H8	*cry1Ai1* (99)	Cry1Ai2	HQ439780
cry1Bb	1BbF: TTGACTTCAAATAGGAAAAATGAG 1BbR: CTATTCTTCCATGAGGAGTAGTTC	WBT-2	*cry1Bb1* (99)	Cry1Bb2	HQ439782
cry1Ja	1JaF: ATGGAGATAAATAATCAGAAGC 1JaR: TCTTATTCCTCCATGAGGAGTAATT	WBT-2	*cry1Ja1* (99)	Cry1Ja2	HM070030
cry1La	1 LaF: ATGGATAACAATCCGAAAATCCAG 1 LaR: TTATTCCTCCATAAGGAGTAATTC	BtS6	*cry1La1* (99)	Cry1La2	HM070031

[a] Oligonucleotide primers were designed according to the DNA sequence for full-length gene amplification.
[b] Data represent the identity score of the cloned gene compared with the relevant holotype gene.
[c] Toxins were named according to the *B. thuringiensis* Delta-Endotoxin Nomenclature Committee guidelines.

set one (CRY1P1 and CRY1PR) (Fig. 1, lane 1 in HD1), which suggested that the HD1 strain contained *cry1Aa*, *cry1Ab*, and *cry1Ac* genes. RFLP patterns obtained using primer set four (CRY1P4 and CRY1PR) (Fig. 1, lane 4 in HD1) conformed to the predicted fragments of *cry1Ia*. There were no products identified using primer set two or three. Thus, the HD1 strain was determined to contain four *cry1*-type genes, *cry1Aa*, *cry1Ab*, *cry1Ac*, and *cry1Ia*, which corresponded with known results (10, 20).

Strain HD29 produced a *cry1Ab* gene RFLP pattern (Fig. 1, lane 1 in HD29) using primer set one (CRY1P1 and CRY1PR). RFLP patterns obtained using primer sets two (CRY1P2 and CRY1PR) (Fig. 1, lane 2 in HD29) and four (CRY1P4 and CRY1PR) (Fig. 1, lane 4 in HD29) suggested that the HD29 strain contained *cry1Bd* and *cry1Ie* genes. Using primer set three (CRY1P3 and CRY1PR), HD29 produced a novel RFLP pattern (Fig. 1, lane 3 in HD29) that contained fragments of 1.14, 0.93, 0.59, 0.35, 0.26, and 0.11 kb, which corresponded to the predicted fragments of *cry1Db* and *cry1Gb* genes. Thus, the HD29 strain was determined to contain five *cry1*-type genes: *cry1Ab*, *cry1Bd*, *cry1Db*, *cry1Gb*, and *cry1Ie*.

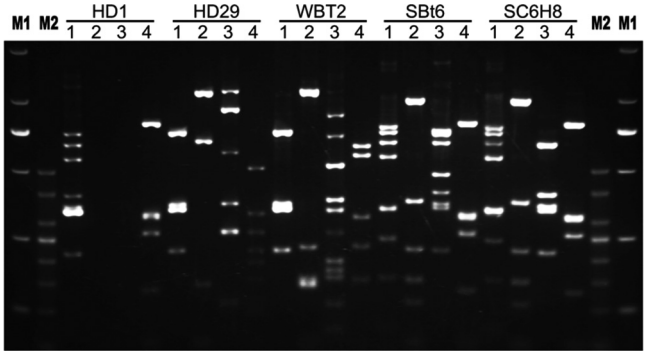

FIG 1 PCR-RFLP patterns of the distinct *cry*-type genes from *B. thuringiensis* strains HD1, HD29, SC6H8, WBT-2, and BtS6 generated using our improved method. The PCR-amplified fragments were digested with HinfI, and the RFLP patterns of the corresponding *cry1* genes were analyzed by 2% agarose gel electrophoresis. The numbers beneath the bars indicate RFLP patterns originating from the PCR products generated from different combinations of the primer pairs: 1, CRY1P1 and CRY1PR; 2, CRY1P2 and CRY1PR; 3, CRY1P3 and CRY1PR; 4, CRY1P4 and CRY1PR. M1, DNA molecular mass standard (bp): 2,000, 1,000, 750, 500, 250, and 100; M2, DNA molecular mass standard (bp): 500, 400, 300, 250, 200, 150, and 100.

Identification of *cry1*-type genes in 20 native *B. thuringiensis* strains using the improved method. Twenty native *B. thuringiensis* strains (listed in Table 3) isolated by our laboratory were identified using the improved PCR-RFLP method. Among these strains, 17 contained reported *cry1*-type genes, which are shown in Table 3 (RFLP patterns not shown), whereas the other 3 *B. thuringiensis* strains, WBT-2, BtS6, and SC6H8, were determined to have novel *cry1*-type genes (listed in Table 2 and Table 3).

The strain WBT-2 RFLP pattern (Fig. 1, lane 1 in WBT-2), which was obtained using primer set one (CRY1P1 and CRY1PR), was the same as that obtained with the HD29 strain, which suggested that the WBT-2 strain contained the *cry1Ab* gene. The RFLP pattern (Fig. 1, lane 2 in WBT-2) obtained using primer set two (CRY1P2 and CRY1PR) contained fragments of 1.11, 0.22, and 0.15 kb, which corresponded to the main specific fragments of *cry1Bb*. The RFLP pattern (Fig. 1, lane 3 in WBT-2) obtained using primer set three (CRY1P3 and CRY1PR) contained fragments of 0.86, 0.70, 0.52, 0.36, 0.35, 0.28, 0.19, 0.17, 0.15, and 0.10 kb,

TABLE 3 *cry1*-type gene identified in 20 native Chinese *B. thuringiensis* strains

Strain	*cry1*-type gene(s)
SC6H8	*cry1Aa*, *cry1Ah*, *cry1Ai*, *cry1Be*, *cry1Ia*, *cry1La*
WBT-2	*cry1Ab*, *cry1Bb*, *cry1Da*, *cry1Id*, *cry1Ja*
SC1-B2	*cry1Ia*
SC1-B3	*cry1Ac*, *cry1Ia*
SC1-G7	*cry1Ac*, *cry1Ia*, *cry1La*
PS9-A7	*cry1Ac*
PS9-A8	*cry1Aa*, *cry1La*, *cry1Ia*
PS9-C3	*cry1Ac*, *cry1Ia*
PS9-C12	*cry1Aa*, *cry1Ac*, *cry1Ia*
PS9-D1	*cry1Ia*
BtS6	*cry1Aa*, *cry1Ah*, *cry1Ai*, *cry1Be*, *cry1Ea*, *cry1Ia*, *cry1La*
PS9-E12	*cry1Aa*, *cry1La*
SC1-C3	*cry1Ac*, *cry1Ai*, *cry1Ia*
SC1-C9	*cry1Ac*
SC1-H3	*cry1Ac*, *cry1Da*, *cry1Ia*
FS18-1	*cry1Ac*, *cry1Ah*
PS2-E10	*cry1Ca*
mo3-B2	*cry1Ac*
mo3-B3	*cry1Ac*
mo3-D10	*cry1Fa*

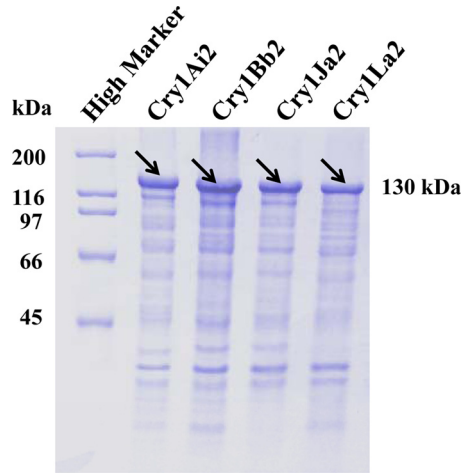

FIG 2 SDS-PAGE analysis of four new detected Cry1 toxins expressed in *E. coli* strain Rosetta (DE3).

which corresponded to the predicted fragments of *cry1Da* and *cry1Ja*. The RFLP pattern (Fig. 1, lane 4 in WBT-2) obtained using primer set four (CRY1P4 and CRY1PR) corresponded to the predicted fragments of the *cry1Id* gene. Thus, strain WBT-2 contains five *cry1*-type genes: *cry1Ab*, *cry1Bb*, *cry1Da*, *cry1Id*, and *cry1Ja* (Table 3).

The strain BtS6 RFLP pattern (Fig. 1, lane 1 in BtS6) obtained using primer set one (CRY1P1 and CRY1PR) contained fragments of 0.76, 0.72, 0.65, 0.57, 0.33, 0.31, 0.24, and 0.15 kb, which corresponded to the fragments predicted for the *cry1La*, *cry1Aa*, and *cry1Ai* genes. The RFLP pattern (Fig. 1, lane 2 in BtS6) observed using primer set two (CRY1P2 and CRY1PR) corresponded to the *cry1Be* gene. The RFLP pattern (Fig. 1, lane 3 in BtS6) obtained using primer set three (CRY1P3 and CRY1PR) contained fragments of 0.76, 0.72, 0.57, 0.33, 0.31, 0.24, and 0.15 kb, which corresponded to the predicted fragments of the *cry1Ah* and *cry1Ea* genes. The RFLP pattern (Fig. 1, lane 4 in BtS6) obtained using primer set four (CRY1P4 and CRY1PR) corresponded to the predicted fragments of the *cry1Ia* gene. Thus, BtS6 contains seven *cry1*-type genes: *cry1Aa*, *cry1Ah*, *cry1Ai*, *cry1Be*, *cry1Ea*, *cry1Ia*, and *cry1La* (Table 3).

The strain SC6H8 RFLP patterns (Fig. 1, lane 1 and 2 in SC6H8) obtained using primer sets one (CRY1P1 and CRY1PR) and two (CRY1P2 and CRY1PR) were similar to that obtained with BtS6 strain, which suggested that SC6H8 contains the *cry1La*, *cry1Aa*, *cry1Ai*, and *cry1Be* genes. The RFLP pattern (Fig. 1, lane 3 in SC6H8) obtained using primer set three (CRY1P3 and CRY1PR) corresponded to the predicted fragments of the *cry1Ah* gene. The RFLP pattern (Fig. 1, lane 4 in SC6H8) obtained using primer set four (CRY1P4 and CRY1PR) corresponded to the predicted fragments of the *cry1Ia* gene. Thus, SC6H8 contained six *cry1*-type genes: *cry1Aa*, *cry1Ah*, *cry1Ai*, *cry1Be*, *cry1Ia*, and *cry1La* (Table 3).

Cloning and sequencing assay of the *cry1*-type genes. The full-length *cry1Bb* and *cry1Ja* genes from WBT-2, *cry1Ai* gene from SC6H8, and *cry1La* gene from BtS6 were cloned into pSIMPLE-18 EcoRV/BAP vector and sequenced. *cry1Ai*, *cry1Bb*, *cry1Ja*, and *cry1La* are novel genes. Their sequences were submitted to the *B. thuringiensis* Delta-Endotoxin Nomenclature Committee, and logical names were assigned to their products as follows: Cry1Ai2, Cry1Bb2, Cry1Ja2, and Cry1La2, respectively.

Expression of *cry1* genes. The *cry1Ai*, *cry1Bb*, *cry1Ja*, and *cry1La* genes were successfully expressed in the *E. coli* Rosetta (DE3) strain. SDS-PAGE analysis showed that all four genes could be expressed and produced products that were approximately 130 kDa (Fig. 2).

Toxicity bioassay. The results of the toxicity bioassay against *P. xylostella* are shown in Table 4. As seen with the Cry1Ac toxin, Cry1Ai2, Cry1Bb2, and Cry1Ja2 exhibited strong toxicity against *P. xylostella* larvae, with LC_{50}s of 0.7 μg/ml, 0.32 μg/ml, and 1.01 μg/ml, respectively. However, neither weight loss nor mortality was detected with Cry1La2 even at concentrations up to 300 μg/ml.

In addition to *P. xylostella* larvae, the toxicity of Cry1Ai2 was evaluated against other lepidopteran insects (Table 5). The results indicated that Cry1Ai2 was highly active against *O. furnacalis* (50% lethal concentration [LC_{50}] = 5.09 μg/g of diet), *B. mori* larvae (LC_{50} = 1.01 μg/ml), *H. cunea* (LC_{50} = 11.14 μg/g), and *C. suppressalis* (LC_{50} = 9.69 μg/g) and showed considerable weight loss activity against *H. armigera* (WLC_{50} = 17.49 μg/g).

DISCUSSION

The potential commercial value of new biopesticides developed from *B. thuringiensis* strains or transgenic insect-resistant plants containing *cry* genes has made the isolation and identification of these strains routine in many laboratories (1, 21). The use of specific- or multiple-primer PCR can identify *cry* genes efficiently (22, 23); however, this method is not suitable for the identification of new *cry* genes because of the high specificity of the amplifications. The PCR-RFLP method can identify known and novel genes based on the distinct patterns produced by digestion of the PCR products amplified using conserved primers. However, the increasing numbers of *cry1*-type genes make it challenging to utilize PCR-RFLP identification. To solve this problem, an improved PCR-RFLP method was introduced by grouping the *cry1*-type genes into four smaller subfamilies with relatively specific primers. According to the computer-predicted RFLP patterns, the improved PCR-RFLP method is able to effectively and accurately identify 41 *cry1*-holotype genes. The characterization of two strains with known *cry1*-type genes indicated that the improved PCR-RFLP method is an effective technique to reveal *cry1*-type genes. The characterization of 20 native *B. thuringiensis* strains suggested that the improved PCR-RFLP method is a valuable tool

TABLE 4 Bioassay results for Cry1 toxins obtained against *P. xylostella*

Parameter	Value(s) or result for indicated toxin				
	Cry1Ac	Cry1Ai2	Cry1Bb2	Cry1Ja2	Cry1La2
LC_{50} (μg/ml)	4.92	0.70	0.32	1.01	NA[a]
95% confidence interval	3.80–7.00	0.20–1.43	0.25–0.43	0.72–1.38	

[a] NA, neither weight inhibition nor mortality was detected at the concentration of 300 μg/ml.

TABLE 5 Bioassay results for Cry1Ai2 toxin against certain lepidopteran insects

Parameter	Value(s) for insect				
	O. furnacalis	*B. mori*	*H. cunea*	*C. suppressalis*	*H. armigera*
LC_{50} (μg/g)	5.09	1.01	11.14	9.69	>500[a] (WLC_{50} = 17.49)
95% confidence interval	2.81–10.29	0.82–1.17	6.71–19.68	7.04–12.80	11.80–25.21

[a] The corrected mortality rate for Cry1Ai toxin at the concentration of 500 μg/g was still less than 50%.

to evaluate the resource of *cry1*-type genes in *B. thuringiensis* strains. Moreover, it is suitable for single *cry1*-type gene identification and complex *cry1*-type gene separation.

Using the improved method, we found that the results from strain HD1 were in agreement with previous reports, whereas those from strain HD29 were not. Previous reports on the combination of *cry*-type genes in *B. thuringiensis* subsp. *galleriae* were controversial because this subspecies has been reported to contain *cry1Da*, *cry9Aa*, and *cry7A* genes (24), *cry1Ab*, *cry1Ac*, *cry1Cb*, and *cry1Da* genes (25), or *cry1Aa*, *cry1Ca*, *cry1Cb*, and *cry1Fa* genes (8). The reason for these diverse results might be that we focused on the identification of *cry1*-type genes using different primers; what is more, it may because of evolution of strains caused by plasmid transfer and gene recombination. Anyway, the PCR-RFLP method is used for the identification of toxin genes.

To evaluate the four novel *cry1*-type genes (*cry1Ai2*, *cry1Bb2*, *cry1Ja2*, and *cry1La2*), bioassays against *P. xylostella* were performed. Three Cry1 proteins, Cry1Ai2, Cry1Bb2, and Cry1Ja2, were highly toxic against *P. xylostella* (Table 4). Because the Cry1A toxin is widely used for the control of lepidopteran insects, we evaluated Cry1Ai2 toxicity against five other lepidopteran insects, and the results show that it has highly lethal toxicity to *O. furnacalis*, *B. mori*, *H. cunea*, and *C. suppressalis* and weight loss activity against *H. armigera*. The result indicated that Cry1Ai2 was a worthy candidate for control of lepidopteran insects. Also, the Cry1Ai2 toxicity pattern showed its worth in insecticidal mechanism research. The Cry1Ai2 protein shares 91% amino acid identity with the Cry1Ac toxin; however, the 9% difference makes the two toxins different with respect to toxicity against *H. armigera* and *B. mori*. Cry1Ai has highly lethal toxicity against *B. mori* and low activity against *H. armigera*, while Cry1Ac has highly lethal toxicity against *H. armigera* (26) and low activity against *B. mori* (27). The further research of Cry1Ai and Cry1Ac may help in understanding the molecular basis of specificity and in the design of new biopesticides.

In conclusion, an improved PCR-RFLP method was established. This method can be used to identify single and clustered *cry1*-holotype genes and to evaluate the number of *cry1*-type genes in *B. thuringiensis* strains regardless of whether the *cry* genes are known or novel.

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