0273-2289/1559-0291 (Online)/06/128/0185/$30.00

Cloning, Characterization, and Expression of a New *cry2Ab* Gene From *Bacillus thuringiensis* Strain 14-1

Devendra Jain, V. Udayasuriyan,* P. Indra Arulselvi, Sona S. Dev, and P. Sangeetha

Department of Plant Molecular Biology and Biotechnology, Centre for Plant Molecular Biology, Tamil Nadu Agricultural University, Coimbatore-641 003, Tamil Nadu, India, E-mail: udayvar@yahoo.com

Received July 9, 2005; Revised August 29, 2005; Accepted September 9, 2005

Abstract

Bacillus thuringiensis is the major source for transfer of genes to impart insect resistance in transgenic plants. Cry2A proteins of *B. thuringiensis* are promising candidates for management of resistance development in insects owing to their difference from the currently used Cry1A proteins, in structure and insecticidal mechanism. The *cry2Ab* gene was found to lack a functional promoter and, hence, is cryptic in nature. The *cry2Ab7* gene was cloned from a new indigenous *B. thuringiensis* strain, 14-1. Nucleotide sequencing of the *cry2Ab* gene cloned from *B. thuringiensis* strain 14-1 revealed an open reading frame of 1902 bp. The deduced amino acid sequence of Cry2Ab of *B. thuringiensis* strain 14-1 showed a variation in three amino acid residues in comparison to the holotype sequence, Cry2Ab1. Expression of the newly cloned *cry2Ab* gene was studied in an acrystalliferous strain of *B. thuringiensis* (4Q7) by fusing the *cry2Ab* gene downstream of *cry2Aa* promoter and *orf1* + *orf2* sequences. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a spore–crystal mixture obtained from transformants of *B. thuringiensis* strain 4Q7 showed production of Cry2Ab protein of about 65 kDa. Alkali solubilized Cry2Ab7 protein showed toxicity against *Helicoverpa armigera* neonates.

Index Entries: *Bacillus thuringiensis*; δ-endotoxin; Cry2Ab7; *cry2Aa* promoter; cloning; *Helicoverpa armigera*.

Introduction

Bacillus thuringiensis is a well-known Gram-positive, spore-forming soil bacterium that forms insecticidal crystal proteins during the sporulation

*Author to whom all correspondence and reprint requests should be addressed.

phase of its growth cycle. These insecticidal crystal proteins are termed δ-endotoxin because of their intracellular location and have been used for many years as successful biological insecticides *(1)*. Schnepf and Whiteley *(2)* reported the cloning of the first crystal protein gene (*cry*) of *B. thuringiensis*. Since then more than 270 *cry* genes have been cloned and characterized, and their classification is based on amino acid sequence similarity of their proteins *(3)*. Significant sequence similarity exists among the *cry* genes of *B. thuringiensis*; however, individual gene products vary in their levels and spectra of toxicity to different insects *(4)*. The search for new *cry* genes is an ongoing effort worldwide. In the natural isolates of *B. thuringiensis*, the *cry2Aa* and *cry2Ac* genes are expressed as the third *orf* in the operon model, whereas *cry2Ab* gene is cryptic in nature *(5,6)*. Crickmore et al. *(7)* achieved the expression of *cry2Ab* gene in acrystalliferous *B. thuringiensis* using the promoter *orf1* and *orf2* of *cry2Aa*. The *orf2* of *cry2Aa* was necessary for crystal formation of Cry2Aa and Cry2Ab proteins in recombinant *B. thuringiensis* strains.

Transgenic crops that produce a Cry1A toxin can control some key lepidopteran pests *(8)*. Continuous exposure to a single kind of *B. thuringiensis* toxin can lead to the development of insect resistance. Routine replacement of *cry* genes or pyramiding of *cry* genes could be useful for effective control of insect pests by *B. thuringiensis*–transgenic plants. Owing to the difference in structure *(9)* and insecticidal mechanism *(10)*, *cry2A* genes are promising candidates for the management of insects in crop plants. The Cry1Ac-resistant *Helicoverpa armigera* (cotton bollworm) was not cross resistant to Cry2A proteins. The second version of *B. thuringiensis*–cotton, Bollgard® II, which expresses Cry1Ac and Cry2Ab, is expected to have positive implications for Cry1Ac-resistance management in cotton bollworm *(11)*. Bollgard II has already been released in the United States and is expected soon for commercial use in India. The toxicity of Cry2Ab protein against Indian populations of *H. armigera* has not been studied in detail. Generating baseline susceptibility data prior to commercialization of any new version of the *B. thuringiensis*–transgenic crop is essential for effective utilization of *B. thuringiensis* transgenics and to monitor resistance development in insects. Cloning and expression of a *cry* gene in *Escherichia coli* or an acrystalliferous strain of *B. thuringiensis* will provide basic material to study toxicity of the individual Cry protein against target pests. The present study describes the cloning, characterization, and expression of *cry2Ab7* gene from a new isolate of *B. thuringiensis*, 14-1.

Materials and Methods

Bacterial Strains, Plasmids, and Oligonucleotide Primers

The *B. thuringiensis* strains 14-1 and 22-4 used are from the collection of indigenous *B. thuringiensis* isolates *(12)* maintained by V. Udayasuriyan. The *B. thuringiensis* strain 4Q7 and the *B. thuringiensis*–*E. coli* shuttle vector pHT3101 *(13)* were obtained from Drs. D. R. Zeigler and D. H. Dean, Ohio State University, Columbus, respectively. Table 1 provides the oligonucleotide primers used.

Table 1
Primers Used for Amplification of *cry2Aa* Promoter, *orf1* + *orf2*, and *cry2Ab* Gene

Serial no.	Primer name	Sequence (5' → 3')[a]	Amplicon	Size (bp)
1	2Aaorf1F	G**GAATTC**AGAAATATGATGTTGATTCTTAGAG	*cry2Aa P*, orf1* + *orf2*	1800
2	2Aaorf2R	GC**TCTAGA**TTTATATTAAAACTAAACTCTTTTG		
3	2AabF	GC**TCTAGA**TATTTAAGGAGGAATTTTATATGAA	*cry2Ab orf* and terminator	2100
4	2DbR	AA**CTGCAG**CCATTTCTCCAATCTCAATCCAT		

[a]Primer sequences containing restriction endonuclease recognization sites for *Eco*RI (GAATTC), *Xba*I (TCTAGA), and *Pst*I (CTGCAG) are in boldface. P*, promoter.

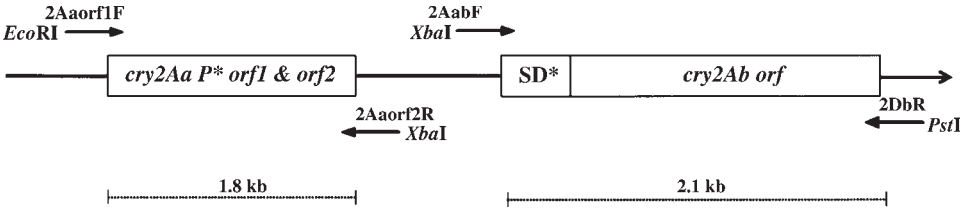

Fig. 1. Schematic diagram for construction of *cry2Ab* operon. The short arrows indicate the position and direction of the primers. The tail end and top of the arrows indicate the 5' restriction site and name of the primers, respectively. The dotted lines indicate the size of the amplicon. P*, promoter; SD*, Shine-Dalgarno sequence.

Amplification of B. thuringiensis *DNA by Polymerase Chain Reaction*

Genomic DNA isolated from *B. thuringiensis* strains 22-4 and 14-1 was used as template for polymerase chain reaction (PCR) amplification. The primers 2Aaorf1F and 2Aaorf2R correspond to the DNA sequence located 260 nucleotides (nt) upstream of the start codon of *cry2Aa orf1* and 90 nt downstream of the stop codon of *cry2Aa orf2*, respectively. These primers were used to amplify *cry2Aa* promoter, *orf1* + *orf2* (1.8 kb). Amplification of *cry2Ab orf* along with terminator sequences (2.1 kb) was done using 2AabF and 2DbR primers (Fig. 1). PCR for a DNA fragment >1.0 kb in size was carried out with a high-fidelity XT-PCR system (Bangalore Genei Pvt., Peenya, Bangalore, India) in a 40-µL reaction volume. Each 40-µL reaction mixture contained 100 ng of genomic DNA of *B. thuringiensis* strain, 50–100 ng of forward and reverse primers, each dNTP at a final concentration of 200–300 µ*M*, and 2.5 U of XT-Taq polymerase in 1X XT-Taq buffer (with 15 µ*M* $MgCl_2$). Amplification was accomplished with a thermalcycler (Eppendorf Mastercycler personal) using step-cycle program. First, the PCR was carried out for 10 cycles under the following conditions: 94°C for 2 min, 94°C for 40 s, 60°C for 40 s, and 72°C for 2 min. Second, the PCR was subjected to 20 cycles with a 20-s increment/cycle for extension. The PCR was performed for screening of *cry2Ab* gene (DNA fragments <1000 bp) with screening primers *(14)* and *Taq* Polymerase for 30 cycles as follows: 94°C for 1 min, 65°C for 45 s, and 72°C for 45 s. For both the step-cycle and normal PCR programs, the final extension was performed for 7 min at 72°C.

Recombinant DNA Procedures

Restriction digestion and ligation reactions were carried out according to the manufacturer's instructions. Agarose gel electrophoresis, preparation of *E. coli*-competent cells, and their transformation were done according to the standard procedure *(15)*. Transformation of the acrystalliferous strain of *B. thuringiensis* (4Q7) was performed as described previously by Lenin et al. *(16)*. DNA sequences were obtained using an automated sequencing service of Bangalore Genei. Sequences were compared with the databases using BLAST.

Preparation and Analysis of Cloned Crystal Protein

B. thuringiensis transformants were grown in T3 medium *(17)* containing 20 µg/mL of erythromycin at 30°C until cell lysis (48–72 h). The spore–crystal mixture was centrifuged and washed twice in 0.5 *M* NaCl and TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0, with 1 m*M* phenylmethylsulfonyl fluoride [PMSF]). Finally, the spore–crystal mixture was resuspended in a small volume of sterile ddH_2O with 1 m*M* PMSF and used for 8.0% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) *(18)*. The spore–crystal mixture of recombinant *B. thuringiensis* clones was solubilized in 50 m*M* NaOH for 30 min and centrifuged at 10,000*g*. The supernatants were analyzed by SDS-PAGE to detect solubilized proteins. Bioassays were carried out with the alkali-solubilized Cry2Ab protein to test its toxicity against neonates of *H. armigera*, as described earlier *(19)*.

Results and Discussion

Cloning of cry2Ab *From* B. thuringiensis *Strain 14-1 and Construction of* cry2Ab *Operon*

From the genomic DNA of *B. thuringiensis* strain 14-1, the *cry2Ab orf* along with terminator sequences (2.1 kb) was amplified with *Xba*I and *Pst*I sites at 5' and 3'ends, respectively, and cloned into the same sites of pUC18 (2.7 kb). In the present study, *cry2Aa* promoter along with *orf1* and *orf2* was used to express *cry2Ab* gene in the acrystalliferous *B. thuringiensis* strain 4Q7. For this purpose, the *cry2Aa* promoter, *orf1* + *orf2* (1.8 kb), of *B. thuringiensis* strain 22-4 was amplified with *Eco*RI and *Xba*I sites at 5' and 3' ends, respectively, and cloned into recombinant pUC18 (4.8 kb) at the same sites. Nucleotide sequence data obtained from the 5' and 3' regions of the constructed *cry2Ab* operon as well as fusion sites confirmed amplification and cloning of DNA from the expected regions (data not shown). Construction of *cry2Ab* operon was confirmed by restriction digestion of recombinant plasmid with *Eco*RI and *Pst*I that release the constructed *cry2Ab* operon.

Construction of the *cry2Ab* operon was achieved by introducing an *Xba*I site between the 3' end of *cry2Aa orf2* and the 5' region of *cry2Ab* gene. The *Xba*I site is introduced at 20 bp upstream to the start codon (ATG) as described by Crickmore et al. *(7)*. For this purpose, primers were designed that cause variation of three bases in the upstream sequence of *cry2Ab* gene and resulted in an *Xba*I restriction site (Table 2). The spacing between the Shine Dalgarno (S-D) sequence and the initiation codon is important for its optimum translation. Fusion of *cry2Aa* promoter, *orf1* + *orf2*, with *cry2Ab* gene did not result in either the addition of extra nucleotide sequences or displacement of S-D sequences from the start codon of *cry2Ab*. In the present study, the *cry2Ab* operon was constructed by amplification using PCR, whereas Crickmore et al. *(7)* constructed the *cry2Ab* operon by site-directed mutagenesis. The primers described in the present study could be used for cloning and characterization of cryptic *cry2Ab* from several new isolates of *B. thuringiensis* in a rapid manner.

Table 2
Comparison of Nucleotide Sequence at Fusion Site
of *cry2Aa* Promoter, *orf1 and 2*, and *cry2Ab orf* + Terminator

Location	Sequence (5' → 3')
Wild-type *cry2Aa* sequence	AAACTAGATATTTAA**GGAGG**AATTTTATG
Wild-type *cry2Ab* sequence	TTACTTAATATTTAA**GGAGG**AATTTTATG
DNA sequence of constructed *cry2Ab* operon	AA***TCTAGA***TATTTAA**GGAGG**AATTTTATG

[a]Shine Dalgarno sequences are in boldface, ATG codons are underlined, and the fusion site is boldface italic.

Analysis of cry2Ab *Sequence of* B. thuringiensis *Strain 14-1*

The newly cloned *cry2Ab* gene was sequenced by primer walking. The sequence revealed the presence of an open reading frame of 1902 nt (DQ119823). This sequence encodes a protein of 633-amino acids (Fig. 2). The computer-based homology search program of the National Center for Biotechnology Information revealed that it is a novel *cry2Ab* gene. It has 3, 3, 3, 1, 3, and 6 amino acid variation with Cry2Ab1, Cry2Ab2, Cry2Ab3, Cry2Ab4, Cry2Ab5, and Cry2Ab6, respectively (Table 3). The amino acid threonine at position 474 is unique in Cry2Ab of *B. thuringiensis* strain 14-1 in comparison with all other Cry2Abs reported so far *(20)*. As per the recent nomenclature, the *cry* genes whose products are different in amino acid sequence but are more than 95% identical to each other are given separate quaternary ranks by Arabic number at the end (e.g., *cry1Aa1, cry1Aa2).* Hence, the *B. thuringiensis* toxin nomenclature committee has assigned a new name, Cry2Ab7 *(20)*, to the Cry2Ab of *B. thuringiensis* strain 14-1.

Expression of cry2Ab *Gene of* B. thuringiensis *Strain 14-1 in Acrystalliferous* B. thuringiensis *Strain 4Q7*

The whole *cry2Ab* operon was ligated to pHT3101 (6.7 kb) at the *Eco*RI and *Pst*I sites and used to transform the *E. coli*. Recombinant clones were selected based on the restriction digestion with *Eco*R1 and *Pst*I that releases the 3.9-kb *cry2Ab* operon and 6.7-kb pHT3101 vector. The recombinant pHT3101 containing the *cry2Ab* whole operon was named pHT14-2B. The plasmid construct pHT14-2B was electroporated to the acrystalliferous *B. thuringiensis* strain 4Q7. Transformed *B. thuringiensis* colonies were selected on Luria-Bertani agar containing erythromycin. To check the crystal protein production of 4Q7 transformants, spore-crystal mixtures prepared from the recombinant 4Q7 *B. thuringiensis* strains were subjected to SDS-PAGE (8%) analysis. Protein profiles of recombinant 4Q7 strains harboring the constructed operon were distinguished in SDS-PAGE by the presence of a prominent band of approx 65 kDa, whereas there was no prominent band of approx 65 kDa in the case of the control, 4Q7 (Fig. 3).

Fig. 2. Nucleotide sequence and deduced amino acid sequence of *cry2Ab* gene of *B. thuringiensis* strain 14-1. The underlined ATG and TAA correspond to the start and stop codons, respectively. Boldface letters indicate the three amino acid differences between Cry2Ab1 (holotype) and Cry2Ab of *B. thuringiensis* strain 14-1.

Table 3
Comparison of Deduced Amino Acid Sequence
of Newly Cloned *cry2Ab* Gene With Already Reported Sequences

	Position of amino acid[a]						
Sequences	110	119	414	446	474	497	514
Cry2Ab1	L	G	N	E	I	S	E
Cry2Ab2	L	G	N	E	I	S	E
Cry2Ab3	L	G	N	E	I	S	E
Cry2Ab4	V	G	I	E	I	S	E
Cry2Ab5	L	E	N	E	I	S	E
Cry2Ab6	L	G	N	G	I	L	G
Cry2Ab (14-1)	V	G	I	E	T	S	E

[a]L, leucine; V, valine; G, glycine; E, glutamic acid; I, isoleucine; T, threonine; S, serine; N, asparagine.

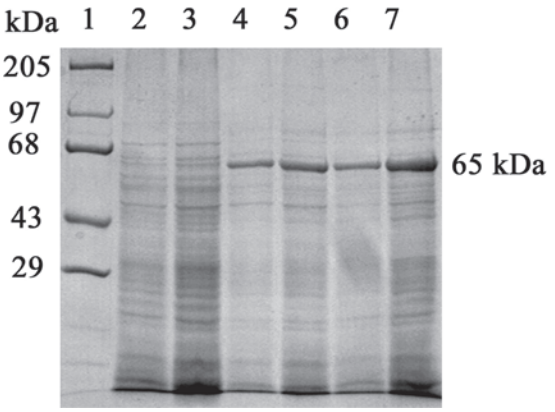

Fig. 3. SDS-PAGE of spore-crystal mixture isolated from transformants of *B. thuringiensis* strain 4Q7 harboring *cry2Ab* operon. Lane 1, protein molecular weight marker; lanes 2 and 3, 4Q7; lanes 4–7, transformants of 4Q7 (pHT14-2B).

A prominent band of approx 65 kDa was also visualized in SDS-PAGE of the supernatant obtained after alkali solubilization of Cry2Ab inclusions (data not shown), whereas in our previous study the Cry2Ab protein expressed in *E. coli* by T7 promoter was not soluble in alkaline pH. Solubilization of Cry proteins in alkaline pH is a prerequisite for exhibiting toxicity against lepidopteron insects such as cotton bollworm *(19)*.

Toxicity Analysis

Bioassay with the alkali-solubilized Cry2Ab protein isolated from the transformants of 4Q7 (pHT14-2B) showed 100% mortality after 3 d in *H. armigera* neonates fed an artificial diet coated with Cry2Ab protein at

400 ng/mL, whereas all the larvae on a control diet were alive until the 7th d. This result indicated that expression of *cry2Ab* gene by *cry2Aa* promoter, *orf1 + orf2*, in recombinant *B. thuringiensis* strain produced active Cry2Ab protein. The Cry2Ab protein produced from the newly cloned *cry2Ab* gene shows toxicity against *H. armigera*. Variation of a single amino acid can significantly influence the level of toxicity in Cry proteins *(21,22)*. Therefore, the *cry2Ab* genes from new isolates of *B. thuringiensis* could encode more toxic Cry2Ab proteins owing to variation in their sequences. Further studies are needed to compare the toxicity of the newly cloned Cry2Ab7 with that of other Cry proteins. Expression of cloned *cry2Ab* gene in an acrystalliferous *B. thuringiensis* strain is preferable because expression of *cry2Ab* gene in recombinant *E. coli* resulted in nontoxic protein in our previous study *(19)*. Moreover, autolysis of *B. thuringiensis* cells after sporulation is an advantage to isolate the expressed protein without sonication, which is essential in the case of recombinant *E. coli*. The recombinant *B. thuringiensis* strain developed in the present study could be used to study baseline susceptibility of Indian populations of *H. armigera* and other insect pests to Cry2Ab.

Conclusion

The *cry2Ab* gene was cloned from an indigenous isolate of *B. thuringiensis*, 14-1. The deduced amino acid sequence of Cry2Ab of *B. thuringiensis* strain 14-1 is different from all the already known six sequences of Cry2Ab. Hence, the Cry2Ab of *B. thuringiensis* strain 14-1 is designated Cry2Ab7. Because the *cry2Ab* gene is cryptic in nature, it requires a functional promoter for its expression. A *cry2Ab* operon was constructed by fusing the *cry2Ab7 orf* along with its terminator sequences downstream of the *cry2Aa* promoter, *orf1 + orf2*, of another *B. thuringiensis* strain. The protein profile of recombinant 4Q7 strain harboring the constructed *cry2Ab* operon was distinguished in SDS-PAGE by the presence of a prominent band of about approx 65 kDa. Alkali-solubilized Cry2Ab7 protein showed toxicity against *H. armigera*, which is a serious polyphagous pest of, among others, cotton, chickpea, pigeon pea, tomato, and sunflower. The newly cloned *cry2Ab* gene could be a valuable tool for transgenic technology to impart insect resistance in crop plants and to minimize the use of hazardous chemical pesticides in agriculture.

Acknowledgments

We gratefully acknowledge D. R. Sudha, senior research fellow, and Dr. M. Bharathi, professor of entomology, for their help in conducting the insect bioassays. This research was supported by grants from the Department of Biotechnology, Government of India, New Delhi; and the Rockefeller Foundation, New York, USA.

References

1. Schnepf, E., Crickmore, N., Van Rie, J., Lerecurs, D., Baum, J., Feitelson, J., Zeigler, J. D. R., and Dean, D. H. (1998), *Microbiol. Mol. Biol. Rev.* **62,** 775–806.
2. Schnepf, H. E. and Whiteley, H. R. (1981), *Proc. Natl. Acad. Sci. USA* **78,** 2893–2897.
3. Crickmore, N., Zeigler, D. R., Feitelson, J., et al. (1998), *Microbiol. Mol. Biol. Rev.* **62,** 807–813.
4. Hofte, H. and Whiteley, H. R. (1989), *Microbiol. Rev.* **53,** 242–255.
5. Winder, W. R. and Whiteley, H. R. (1989), *J. Bacteriol.* **171,** 965–974.
6. Wu, D., Cao, X. Y., Bai, Y. Y., and Aronson, A. I. (1991), *FEMS Microbiol. Lett.* **81,** 31–36.
7. Crickmore, N., Wheeler, V. C., and Ellar, D. J. (1994), *Mol. Gen. Genet.* **242,** 365–368.
8. Barton, K. A., Whiteley, H. R., and Yang, N. S. (1987), *Plant Physiol.* **85,** 1103–1111.
9. Morse, R. J., Yamamoto, T., and Stroud, R. M. (2001), *Structure* **9,** 409–417.
10. English, L., Robbins, H. L., Von Tersch, M. A., et al. (1994), *Insect Biochem. Mol. Biol.* **24,** 1025–1035.
11. Perlak, F. J., Oppenhuizen, M., Gustafson, M., et al. (2001), *Plant J.* **27,** 489–501.
12. Udayasuriyan, V., Chitra, V., Ananda, C. S., and Sadasivam, S. (1996), *TNAU News Lett.* **26,** 4.
13. Lereclus, D., Arantes, O., Chaufaux, J., and Lecadet, M. M. (1989), *FEMS Microbiol. Lett*. **60,** 211–218.
14. Ben-Dov, E., Zaritsky, A., Dahan, E., et al. (1997), *Appl. Environ. Microbiol.* **63,** 4883–4890.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, New York.
16. Lenin, K., Asia Mariam, M., and Udayasuriyan, V. (2001), *World J. Microbiol. Biotechnol.* **17,** 273–278.
17. Martin, P. A. W. and Travers, R. S. (1989), *Appl. Environ. Microbiol.* **55,** 2437–2442.
18. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
19. Kumar, S., Udayasuriyan, V., Sangeetha, P., and Bharathi, M. (2004), *Curr. Sci.* **86,** 566–570.
20. www.lifesci.susx.ac.uk/Neil_Crickmore/Bt.
21. Udayasuriyan, V., Nakamura, A., Mori, A., Masaki, H., and Uozumi, T. (1994), *Biosci. Biotechnol. Biochem.* **58,** 830–835.
22. Rajamohan, F. O., Alzate, J. A., Cotrill, A., Curtiss, A., and Dean, D. H. (1996), *Proc. Natl. Acad. Sci. USA* **93,** 14,338–14,343.