Cloning and sequencing of the gene for a putatively nematode-toxic crystal protein, Cry21Ba1, from *Bacillus thuringiensis* serovar *roskildiensis*

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We have cloned and sequenced a gene encoding a novel crystal (Cry) protein, Cry21Ba1, from *Bacillus thuringiensis* serovar *roskildiensis*. The gene, *cry21Ba1*, was amplified by polymerase chain reaction using template (alkaline-cell lysate or plasmid DNA fraction of the *Bacillus*) and primers (pairs of oligomeric DNAs designed from the conserved amino-acid sequence blocks of Cry5-Cry12-Cry13-Cry14-Cry21-type Cry proteins), and was cloned followed by sequencing. The gene, *cry21Ba1*, should be functional, because it has putative promoter sequences upstream of the initiation codon and an inverted repeat downstream of the stop codon. The open reading frame (ORF) of *cry21Ba1* is a 3,858-bp DNA encoding Cry21Ba1 consisting of 1,286 amino acids. The nucleotide sequence of the *cry21Ba1* ORF is 75% identical with that of the *cry21Aa1* (the gene for a known nematode-toxic Cry protein, Cry21Aa1) ORF. The amino acid sequence of Cry21Ba1 is 67% identical with that of Cry21Aa1. High amino-acid-sequence similarity between these Cry proteins indicates that Cry21Ba1 is putatively nematode-toxic. Jpn. J. Nematol. 34 (2), 79-88 (2004).

Key words: *Bt*, Cry protein, *cry* gene, *cry21Ba1*, plant-pathogenic nematode.

INTRODUCTION

Crystal (Cry) proteins produced by *Bacillus thuringiensis* (*Bt*) are the most widely used biological insecticides, because they are insecticidal but practically nontoxic to vertebrates (Schnepf et al., 1998). At present, some *cry* genes are successfully used to introduce insect-resistance into plants (Schnepf et al., 1998). If Cry proteins potentially toxic to plant-pathogenic nematodes are found from *Bt* strains, the *cry* genes may be used to introduce nematode-resistance into plants (Atkinson et al., 2003), because many species of

agronomically important plant-pathogenic nematodes parasitize plant tissue (Sasser and Freckman, 1987).

However, at present, studies on the nematode-toxic Cry proteins are not so general, and only four Cry proteins (i.e., mutually amino-acid sequence-similar Cry5B, Cry14A, and Cry21A (Marroquin et al., 2000; Griffiths et al., 2001; Wei et al., 2003), and another Cry protein, Cry6A (Wei et al., 2003)) have been proved to be toxic to certain free-living and rat-intestinal nematodes. Furthermore, information on the cloning and sequencing of the nematode-toxic *cry* genes is scarce, perhaps because all these *cry* genes reported until now are patented materials, i.e., *cry5Aa1* (Sick et al., 1994), *cry5Ab1* and *cry12Aa1* (Narva et al., 1991), *cry5Ba1* (Payne and Michaels, 1995), *cry13Aa1* (Schnepf et al., 1992), *cry14Aa1* (Payne and Narva, 1994),

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cry21Aa1 (Payne et al., 1996), *cry21Aa2* (Feitelson, 1997), *cry6Aa1* (Narva et al., 1993) and *cry6Ba1* (Narva et al., 1991). In this situation, enrichment of such information may contribute to the advance of studies on the nematode-toxic Cry proteins, and this advance may lead to finding *Bt* strains carrying new *cry* genes potentially toxic to plant-pathogenic nematodes.

In this paper, we will present the information on the cloning and sequencing of a gene encoding a novel Cry protein, Cry21Ba1, from *Bacillus thuringiensis* serovar *roskildiensis*.

MATERIALS AND METHODS

Bacterial strains:

Sixty-nine *Bacillus thuringiensis* (*Bt*) strains of the IEBC (International Entomopathogenic *Bacillus* Centre at Institut Pasteur, Paris, France) Collection (Lecadet et al., 1999) were shake-cultured at 30°C for 24-48 h in Bacto Tryptose Phosphate Broth (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA).

Template preparation for PCR:

Alkaline-cell lysate or plasmid DNA fraction was prepared from *Bt* cells, and was used as the template for polymerase chain reaction (PCR) amplification. Alkaline-cell lysate was prepared by the method of O'Reilly et al. (1992) with some modifications, as follows. Five µl of *Bt* culture was added to 100 µl of ice-cold 0.1-N NaOH in a microtube and left on ice for 5 min. The cell lysate obtained was neutralized by adding 20 µl of 1-M ammonium acetate, and was immediately used as the template for PCR. Plasmid DNA fraction was prepared from the culture of *B. thuringiensis* serovar *roskildiensis* with QIAprep Spin Miniprep Kit (QIAGEN GmbH, Hilden, Germany) by the manufacturer's method with some modifications. Briefly the *Bt* cells were collected by low-speed centrifugation ($5,000 \times g$ for 2 to 3 min) from 4 ml of the culture, and were suspended in 225 µl of RNase-A-supplemented

Buffer P1 (QIAGEN). The cell suspension obtained was added with 25 µl of lysozyme solution (10 mg/ml in 10-mM Tris-HCl, pH 8.0), and was incubated at 37°C for 0.5-1.0 h to lyse the cells. The plasmid DNA fraction prepared from the lysate was stored at -20°C until used for PCR or the southern hybridization analysis.

PCR:

Primers (oligomeric DNAs showed in Table 1) for PCR were designed from the conserved amino-acid sequence blocks of two distinct Cry protein groups (i.e., Cry1 group and Cry5-Cry12-Cry13-Cry14-Cry21 group; Schnepf et al., 1998) with a primer-analysis software, OLIGO 4.0 Macintosh (Molecular Biology Insights Inc., Cascade, Colorado, USA). PCR was performed using *Ex Taq* polymerase (Takara Bio Inc., Otsu, Shiga, Japan), alkaline-cell lysate or plasmid-DNA fraction as template, and an appropriate pair of forward and reverse primers, with the stepdown program (Hecker and Roux, 1996) as follows: the denaturation time and temperature was 10 sec at 98°C, the annealing time and temperature was 30 sec at 72-36°C (decreased by 4°C intervals), the extension time and temperature was 2 min (for the amplification of < 1 kb) or 5 min (for the amplification of ≥ 1 kb) at 72°C, each cycle with the identical annealing temperature was repeated 3 times, and the total cycle number was 30.

Cloning and sequencing of PCR products:

PCR products were cloned using a cloning vector pCR-TOPO (Invitrogen Corporation, Carlsbad, California, USA). Subsequently, the PCR products were sequenced by the dye-terminator method (Lee et al., 1992) with a capillary-based sequencer ABI 310 (Applied Biosystems, Foster City, California, USA) or a polyacrylamide gel-based sequencer ABI 377 (Applied Biosystems) using template (pCR-TOPO vector containing the PCR product), primer (M13 Forward Primer, 5'-GTAAAACGACGGCCAG, or

Table 1. Primers used for the PCR-amplification of *cry21Ba1*.

Primer	Sequence*	Position	Direction of priming	Cry gene recognized
CRY21II	5'-ATGGCAGATCTAACTGAACATAAC	positions 397-420 of <i>cry21Ba1</i>	forward	<i>cry21Ba1</i>
CRY21F5D2	5'-WRBYRYAWCMWNKNHHCATAATGT ¹	positions 413-437 of <i>cry21Ba1</i> ⁴	forward	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21R5D1	5'-TAAATGGGTCAAAAATAGAAATC	positions 460-481 of <i>cry21Ba1</i>	reverse	<i>cry21Ba1</i>
CRY21R5D2	5'-GGGAAAATAAAGTCTACAAACATA	positions 678-701 of <i>cry21Ba1</i>	reverse	<i>cry21Ba1</i>
CRY21F1-1	5'-GGMGRACWWTMRWCTTWWYAKYAYATCA ²	positions 1099-1127 of <i>cry21Ba1</i> ⁵	forward	<i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21R1-1	5'-CCAWSTWYWSMRAATTWKWATATA ²	positions 1132-1155 of <i>cry21Ba1</i> ⁵	reverse	<i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY5FA3-1	5'-GARYVKWWTAAATGGWRMNMATGC ¹	positions 2083-2105 of <i>cry21Ba1</i> ⁴	forward	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY5F4-1	5'-TVNNRNTNMGHWHKCGWTAATGC ¹	positions 2169-2189 of <i>cry21Ba1</i> ⁴	forward	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY1R5	5'-RGAAYAAWTTTCAAWTYKRTCTAT ³	positions 2318-2340 of <i>cry1Aa1</i> ⁶	reverse	<i>cry1</i>
		(positions 2416-2438 of <i>cry21Ba1</i>)	(reverse)	
CRY5R5-1	5'-GRWAYAAAYTCWAKWCGRTCTA ¹	positions 2417-2438 of <i>cry21Ba1</i> ⁴	reverse	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21R8-1	5'-WCCHACATCRATRCRTARYTRAAGAAATG ¹	positions 3433-3462 of <i>cry21Ba1</i> ⁴	reverse	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21F8-2	5'-GGYATYGAAWTTBGGTCTTCGTATT ¹	positions 3487-3510 of <i>cry21Ba1</i> ⁴	forward	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21R8-3	5'-WARTGGACGDTCTTCWHKAATTTC ¹	positions 3547-3570 of <i>cry21Ba1</i> ⁴	reverse	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21F3D2	5'-GATTTTGAAGAAACAGAAATAC	positions 4024-4047 of <i>cry21Ba1</i>	forward	<i>cry21Ba1</i>
CRY21F3D3	5'-TTGAAGAAAGAAACAGAAATACA	positions 4028-4048 of <i>cry21Ba1</i>	forward	<i>cry21Ba1</i>
CRY21R3D5	5'-YTCRAARTARAANGTYTGYTCTTG ¹	positions 4147-4170 of <i>cry21Ba1</i> ⁴	reverse	<i>cry5</i> , <i>cry12</i> , <i>cry13</i> , <i>cry14</i> and <i>cry21</i>
CRY21S1	5'-TTGTGATTCGATTCCTTTGTTTATT	positions 4253-4276 of <i>cry21Ba1</i>	reverse	<i>cry21Ba1</i>

*Genetic code of degenerate DNA is as follows: B = C, G or T; H = A, C or T; K = G or T; M = A or C; N = A, C, G or T; R = A or G; S = C or G; V = A, C or G; W = A or T; Y = C or T. ¹Cry genes used for design of the degenerate primer are as follows: *cry5Aa1* (accession number L07025), *cry5Ab1* (L07026), *cry5Ac1* (I34543), *cry5Ba1* (U19725), *cry12Aa1* (L07027), *cry13Aa1* (L07023), *cry14Aa1* (U13955) and *cry21Aa1* (I32932). The cry genes can be cited from the DNA sequence database (DDBJ, <http://www.ddbj.nig.ac.jp>; NCBI, <http://www.ncbi.nlm.nih.gov>; or EMBL, <http://www.ebi.ac.uk>) at each accession number. ²Cry genes used for design of the degenerate primer are *cry13Aa1* and *cry21Aa1*. ³Cry genes used for design of the degenerate primer are as follows: *cry1Aa1* (M11250), *cry1Ab1* (M13898), *cry1Ac1* (M11068), *cry1Ba1* (X06711) and *cry1Ca1* (X07518). ⁴The corresponding positions of *cry5Aa1*, *cry5Ab1*, *cry5Ac1*, *cry5Ba1*, *cry12Aa1*, *cry13Aa1*, *cry14Aa1* and *cry21Aa1* can also be recognized. ⁵The corresponding positions of *cry13Aa1*, *cry14Aa1* and *cry21Aa1* can also be recognized. ⁶The corresponding positions of *cry1Aa1*, *cry1Ab1*, *cry1Ac1*, *cry1Ba1* and *cry1Ca1* can also be recognized.

M13 Reverse Primer, 5'-CAGGAAACAGCTAT-GAC; Invitrogen) and BigDye Terminator Cycle v2.0 Sequencing Ready Reaction Kit (Applied Biosystems). At least five clones of each PCR product were subjected to sequencing.

Southern hybridization analysis:

Plasmid DNA fraction of *B. thuringiensis* serovar *roskildiensis* was digested with each of thirteen restriction enzymes: *Apa*I, *Bgl*II, *Eco*RI, *Kpn*I, *Not*I, *Pst*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I, *Sph*I, *Xba*I and *Xho*I. The restriction-enzyme fragments were separated by electrophoresis with a 1.0% agarose gel, and then vacuum-transferred to a positive-charged nylon membrane Hybond-N+ (Amersham Biosciences Corp., Piscataway, New Jersey, USA) immersed in 20 × SSC (3-M NaCl, 0.3-M sodium citrate, pH 7.0). The transferred restriction-enzyme fragments were fixed to the membrane by UV irradiation. Two probes were amplified and used as follows: (1) a 2,364-bp DNA (positions 1099-3462 of *cry21Ba1*; Figs 1 and 2) was PCR-amplified with primers, CRY21F1-1 and CRY21R8-1, from the plasmid DNA fraction, and was used for the detection of the 5' flanking region of *cry21Ba1*; (2) a 684-bp DNA (positions 3487-4170 of *cry21Ba1*; Figs 1 and 2) was PCR-amplified with primers, CRY21F8-2 and CRY21R3D5, from the plasmid DNA fraction, and was used for the detection of the 3' flanking region of *cry21Ba1*. Labeling of the probes with horseradish peroxidase and subsequent detection of the DNA fragments which hybridized with either of the labeled probes were performed with ECL Direct Nucleic Acid Labeling and Detection System (Amersham Biosciences) according to the manufacturer's instructions. For the southern hybridization analysis of intact plasmid DNAs, the plasmid DNA fraction was electrophoresed with a 1.0% agarose gel, and subsequently, the separated plasmid DNAs were treated as described above. Sequencing of the flanking regions of *cry21Ba1*:

*Eco*RI (3.5 kb) and *Xba*I (5.5 kb) fragments were excised from the plasmid DNA fraction of *B. thuringiensis* serovar *roskildiensis*, and were purified by agarose-gel electrophoresis with a 0.9% low-melting point agarose (SeaPlaque GTG Agarose; Cambrex Corporation, East Rutherford, New Jersey, USA) gel. The purified *Eco*RI and *Xba*I fragments were blunted with DNA Blunting Kit (Takara Bio), and were cloned with a cloning vector pCR-Blunt (Invitrogen). The 5' flanking region was sequenced with template (the plasmid-DNA fraction or pCR-Blunt vector containing the 3.5-kb *Eco*RI fragment) and primer (CRY21R5D1, CRY21R5D2, M13 Forward Primer [Invitrogen] or M13 Reverse Primer [Invitrogen]). The 3' flanking region was sequenced with template (the plasmid-DNA fraction or pCR-Blunt vector containing the 5.5-kb *Xba*I fragment) and primer (CRY21F3D2 or CRY21F3D3). The sequencing was performed by the dye-terminator method as described above.

Sequence alignment:

Alignment of *cry21Ba1* with *cry21Aa1* for the nucleotide sequence or the deduced amino-acid sequence was performed with a gene-information analysis software, GENETYX-MAC 8.0 (GENETYX Corporation, Tokyo, Japan).

RESULTS

Cloning and sequencing of PCR products:

Initially, we tried to amplify a 300-400-bp DNA corresponding to the amino acid sequence between the alternate conserved amino-acid sequence block 3 and the conserved amino-acid sequence block 5 of Cry5-Cry12-Cry13-Cry14-Cry21-type Cry protein (Table 1 and Fig. 1). Consequently, a 356-bp DNA was PCR-amplified from *B. thuringiensis* serovar *roskildiensis* by the following two steps: (1) the first PCR was performed using template (the alkaline-cell lysate) and primers (CRY5FA3-1 and CRY5R5-1; Fig. 2);

Fig. 2. Sequencing strategy for *cry21Ba1* and its flanking regions. (A) The finally sequenced DNA (4,380 bp) is schematically shown. The coding region (positions 397-4257) of *cry21Ba1* is indicated by a rectangle. The flanking regions (1-396 and 4258-4380) are indicated by straight lines. Nucleotide sequence coding for each of the eight conserved blocks is indicated as [alt 1], [alt 2], [alt 3], [4], [5], [6], [7] or [8], respectively (see the legend to Fig.1). The nucleotide sequence is indicated by a black box. The length of the nucleotide sequence is drawn to scale. The initiation and stop codons are indicated by boxed letters. The sites for restriction enzymes, *EcoRI* and *XbaI*, are abbreviated as E and X, respectively. (B) The nucleotide sequence of the positions 413-4170 of *cry21Ba1* was clarified by a series of sequencings of PCR products from (1) to (5). PCR products, (5) and (6), were used as probes for the southern hybridization analysis. *EcoRI* fragment (3.5 kb; 7a) was used as template for the sequencings, i.e., (7b) and (7c), of the 5' flanking region. *XbaI* fragment (5.5 kb; 8a) was used as template for the sequencings, i.e., (8b) and (8c), of the 3' flanking region. Amplification of the *cry21Ba1* ORF is indicated as (9). An arrow indicates the direction and extent of the amplification reaction. The primer used is indicated on the 5' or 3' end of the PCR product. The regions each PCR product spans are indicated below the 5' and 3' ends of the PCR product.

sequenced a 3,758-bp DNA coding for the positions 413-4170 (the nucleotide sequence lying between two primers, CRY21F5D2 and CRY21R3D5) of the *cry21*-like gene (i.e., *cry21Ba1*). This 3,758-bp DNA is lacking in both 5' and 3' termini of the *cry21*-like gene.

Southern hybridization analysis:

Southern hybridization analysis was performed in order to identify plasmid DNA(s) carrying the *cry21*-like gene and restriction-enzyme fragments of the plasmid DNA(s) containing the coding region or the 5' or 3' flanking region of the gene. The *cry21*-like gene was detected on 11-kb plasmid DNA(s) (data not shown). The coding region of the gene was detected on several restriction-enzyme fragments of the plasmid DNA(s) including 5.5-kb *Xba*I fragment(s). The 5' and 3' flanking regions of the gene were detected on several restriction-enzyme fragments of the plasmid DNA(s) including 3.5-kb *Eco*RI fragment(s) and 5.5-kb *Bgl*III fragment(s), respectively.

The complete sequence of *cry21Ba1*:

On the basis of the result of the southern

hybridization analysis, the 5' and 3' flanking regions were sequenced as shown in Fig. 2, and finally, a 4,380-bp DNA containing the open reading frame (ORF) of the *cry21*-like gene was sequenced. The *cry21*-like-gene ORF is a 3,858-bp DNA encoding a Cry21-like protein consisting of 1,286 amino acids. The *cry21*-like gene now called *cry21Ba1* (Crickmore et al., 2004) is shown in Fig. 1.

PCR-amplification of *cry21Ba1* ORF:

The *cry21Ba1* ORF was successfully PCR-amplified with primers (CRY21II and CRY21SI) from template (the plasmid DNA fraction or pCR-Blunt vector containing the 5.5-kb *Xba*I fragment), and the nucleotide sequence of the ORF was confirmed by sequencing (Fig. 2). Therefore, it is possible to express *cry21Ba1* with recombinant-DNA expression systems such as the acrySTALLIFEROUS *B. thuringiensis* system (Sasaki et al., 1996) and the *E. coli* system (Wei et al., 2003).

Characterization of *cry21Ba1*:

The gene, *cry21Ba1*, has putative promoter sequences upstream of the initiation codon and

(A)

Cry21Ba1	MAULTELYPSVHMLAPLRLDSIPDFIDIDNALKGWEFAKTYKDPLESLKIAN	60	Cry21Ba1	EDTVISIRLPFGQITLILININDQLELRLTEVPIPTLPTNPTISIPKIDSPKDSK	704
Cry21Aa1	MTNPTLTPSVHMLAPLRLDSIPDFIDIDNALKGWEFAKTYKDPLESLKIAN	60	Cry21Aa1	ENITISIRLPFGQITLILININDQLELRLTEVPIPTLPTNPTISIPKIDSPKDSK	693
				[5]	
Cry21Ba1	ASQNTIDIALTKASISPIGLITPDADAVPPIDMFVDITFPLKPGESQNSQADFFEL	120	Cry21Ba1	VIMEASFOIPAIANTITLTVGSVDPADITFELYKGNMVSPIKPGPIRSHGNYVSC	764
Cry21Aa1	TSQNTIDIALTKASISPIGLITPDADAVPPIDMFVDITFPLKPGESQNSQADFFEL	120	Cry21Aa1	-----PTS-----	696
Cry21Ba1	ITISAKKEIVQKFRNPLNLLDLDGATTEIFPCNDVQIATCGQSGDILDERHPPC	180	Cry21Ba1	SGGILSYNNKPKVLDGFDQLRINISDFSDNSGCDTKNQYSAEIKINPLSATID	824
Cry21Aa1	ITISAKKEIVQKFRNPLNLLDLDGATTEIFPCNDVQIATCGQSGDILDERHPPC	180	Cry21Aa1	-----TNFVEPES-----	705
Cry21Ba1	FTTNHLSVSKESFNAKISITIVLTHFQKFI---DNRTPGNSDLYLLTPATTAAT	238	Cry21Ba1	ERTNQWQLPTSSQTEPANNITIDIRHLLDMKDALNNVEYKKALEKLVNANQV	884
Cry21Aa1	FTTNHLSVSKESFNAKISITIVLTHFQKFI---DNRTPGNSDLYLLTPATTAAT	240	Cry21Aa1	ERTNQWQLPTSSQTEPANNITIDIRHLLDMKDALNNVEYKKALEKLVNANQV	765
		[alt 1]		[6]	
Cry21Ba1	NLLAQCHIQFQKQSVNDEAFINQKADLRIGEGYTVSTVYFEXKPTU---SNK	296	Cry21Ba1	SKARNVAGZNPKEKHEVIGREATINNELFEGDHLLEPPITLPSYAYKGLDSSLLK	944
Cry21Aa1	NLLAQCHIQFQKQSVNDEAFINQKADLRIGEGYTVSTVYFEXKPTU---SNK	299	Cry21Aa1	SKARNVAGZNPKEKHEVIGREATINNELFEGDHLLEPPITLPSYAYKGLDSSLLK	825
		[alt 1] continued		[6] continued [7]	
Cry21Ba1	SNITNRYVYSMTNIDIRAKNLLNLYVSGSHPIQLQGLRLENNVGRPEANDDI	356	Cry21Ba1	SNITRYVSGSHPIAEPHLPVIVRYKGVKMDLVPRALPTSSDSPPWCKDPTQDPS	1004
Cry21Aa1	SNITNRYVYSMTNIDIRAKNLLNLYVSGSHPIQLQGLRLENNVGRPEANDDI	359	Cry21Aa1	SNITRYVSGSHPIAEPHLPVIVRYKGVKMDLVPRALPTSSDSPPWCKDPTQDPS	885
		[alt 2]		[7] continued	
Cry21Ba1	STVTPRSFMYSKGIDLVNSATQGIN---VPLRLSKVCFYTHDRGGRHAYTSEF	413	Cry21Ba1	CDGSQPSGIDFYSYTVSGSVSTANIGLPEGRITAKENSPAKINLEITEPELITDQIK	1064
Cry21Aa1	---TGNIDLVLPITNIGIPGESSDRHPTVYRIELQMGKSHQYVNSKSEVATYSDQ	415	Cry21Aa1	CDGSQPSGIDFYSYTVSGSVSTANIGLPEGRITAKENSPAKINLEITEPELITDQIK	945
				[8]	
Cry21Ba1	NITFNKNSMSA---KQDES---ATADSPLTARTIMNANSONSVYVYSSININDG	466	Cry21Ba1	KIQRBQKTRASDQDAVAATFOPTQLDINALYQNKNGSLRHPTVHLSSANPLP	1124
Cry21Aa1	KINYKNTTACVSNIPESQNKNGYVINSITIDINASSONSVLILNVMWAGGCK	475	Cry21Aa1	KIQRBQKTRASDQDAVAATFOPTQLDINALYQNKNGSLRHPTVHLSSANPLP	1005
Cry21Ba1	GGGSAFPPS-YQSSNPLINQKINVFVPGSSAHPIDPHITDPTDFLQVSSHIFYL	525	Cry21Ba1	LPKPHGMDRSHGVGVVTOCALDRGQOQRNGINGSPANKLIMVITGDAIC	1184
Cry21Aa1	VTCGFLSGVGNNAALNOKNIIY---SVQSNKPEGHADYTRDQVSSHIFYL	531	Cry21Aa1	LPKPHGMDRSHGVGVVTOCALDRGQOQRNGINGSPANKLIMVITGDAIC	1065
Cry21Ba1	TEQNKIDPTDPTQPSLLKGFPAKGGKSGIYSEPLNGDAKLTINDIEMOVIN	585	Cry21Ba1	TIYFDEVLFLAHQASVSTTEIMDFEERTYKLRKRGKGTVTVQHGEELEMTIN	1244
Cry21Aa1	VENYKIDPTDPTQPSLLKGFPAKGGKSGIYSEPLNGDAKLTINDIEMOVIN	591	Cry21Aa1	TIYFDEVLFLAHQASVSTTEIMDFEERTYKLRKRGKGTVTVQHGEELEMTIN	1125
		[alt 3]			
Cry21Ba1	LTQVYQIRFVATNNTTASVPHICNNQDIN-HSPDIPPRNNHVFQNGKRY	644	Cry21Ba1	TTSPFTQETVTFEGQTYDAPVSKNPTLVVSSVLELVEVR	1286
Cry21Aa1	QTVYVYRFTVATNNTTASVPHICNNQDIN-HSPDIPPRNNHVFQNGKRY	651	Cry21Aa1	TTSPFTQETVTFEGQTYDAPVSKNPTLVVSSVLELVEVR	1167
		[4]			

(B)

Fig. 3.(A) Amino-acid sequence alignment of Cry21Ba1 with Cry21Aa1. Identical amino acids are neutrally shaded, and structurally similar amino acids are palely shaded. The conserved blocks, [alt 1], [alt 2], [alt 3], [4], [5], [6], [7] and [8], are defined in the legend to Fig. 1. Each sequence block is underlined in the amino-acid sequence. The amino-acid sequences of Cry21Ba1 and Cry21Aa1 can be cited from the DNA sequence database (DDBJ, NCBI or EMBL) at the accession numbers AB088406 (or BAC06484) and I32932, respectively (for the domain name, see the footnote to Table 1). (B) Comparison of the structures of Cry21Ba1 and Cry21Aa1. Positions of the domains (I, II and III) and the C-terminal extension are each indicated by a double-headed arrow. The conserved blocks, [alt 1], [alt 2], [alt 3], [4], [5], [6], [7] and [8], are each indicated by a black box. The length of the amino acid sequence is drawn to scale.

an inverted repeat downstream of the stop codon (Fig. 1), indicating that *cry21Ba1* is a functional gene. The nucleotide sequence of the *cry21Ba1* ORF is 75% identical with that of the *cry21Aa1* ORF. The deduced amino acid sequence of Cry21Ba1 exhibits 67% identity with the amino acid sequence of Cry21Aa1 (Fig. 3A). High similarity in each sequence of the N-terminal region (the region from the N-terminus to the alternate block 2) and the C-terminal region (the region from the block 6 to the C-terminus) is observed between Cry21Ba1 and Cry21Aa1 (Fig. 3A). On the other hand, the sequences of the internal region (the region from just behind the alternate block 2 to just before the block 6) show relatively low similarity between these Cry proteins (Fig. 3A). Such differences in the amino acid sequences between Cry21Ba1 and Cry21Aa1 suggest that each of these Cry proteins has a distinct nematode-toxicity.

DISCUSSION

In the present study, we have cloned and

sequenced a novel *cry* gene, *cry21Ba1*, that shows close similarity to *cry21Aa1*. The gene, *cry21Ba1*, has been classified into the *cry5-cry12-cry13-cry14-cry21* group as a new *cry*-gene subtype (Crickmore et al., 2004).

According to Schnepf et al. (1998) and de Maagd et al. (2001), it is assumed that the protoxin of Cry21Ba1 consists of three structural domains (domains I, II and III) and a protoxin-specific C-terminal extension (Fig. 3B). The region from the N-terminus to the alternate block 2 corresponds to domain I. The region from the alternate block 2 to just before the alternate block 3 corresponds to domain II. The region from the alternate block 3 to the block 5 corresponds to domain III. The region from just behind the block 5 to the C-terminus corresponds to the C-terminal extension. In reference to the structure-function interaction, it has been suggested that domain I is involved in membrane insertion and pore formation, and domains II and III are both involved in receptor recognition and binding (Schnepf et al., 1998; de

Maagd et al., 2001). The C-terminal extension is not part of the active Cry toxin. The C-terminal extension of the protoxin of Cry21Ba1 may be accurately digested by protease(s) in the nematode intestine. Cry21Ba1 differs significantly from Cry21Aa1 in the amino acid sequence of the region comprised of domains II and III (Fig. 3A and B), indicating the differences between these Cry proteins in the properties of receptor recognition and binding. Therefore, if Cry21Ba1 is nematode-toxic, the toxicity of Cry21Ba1 should differ from that of Cry21Aa1 in the sensitivity spectrum of nematodes and the extent of toxicity.

Is it possible to find *Bt* strains carrying *cry* genes toxic to plant-pathogenic nematodes? In fact, recently, a new *Bt* strain, *B. thuringiensis* serovar *jordanica*, whose Cry proteins are toxic to root-knot nematodes, *Meloidogyne incognita* and *M. javanica*, has been reported (Khyami-Horani et al., 2003). In the near future, *Bt* strains carrying new *cry* genes potentially toxic to plant-pathogenic nematodes may be found.

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