

Strategy for amplification and sequencing of insecticidal *cryIA* genes from *Bacillus thuringiensis*

Diego H. Sauka · Ariel F. Amadio ·
Rubén O. Zandomeni · Graciela B. Benintende

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Abstract A feasible and fully described strategy, with a detailed list of primers, for amplifying, cloning and sequencing known and potentially novel *cryIA* genes harboured by a *Bacillus thuringiensis* strain was successfully established. Based on the analysis of conserved regions of the *cryIA* genes, the 1AF and 1UR oligonucleotide primers were designed to amplify the whole open reading frame of these genes. The PCR products obtained revealed the successful amplification of *cryIA* genes from 13 *B. thuringiensis* strains. These bacteria were previously known to harbour at least one *cryIA* gene. An Argentinean *B. thuringiensis* isolate INTA Mo1-12 was randomly chosen for cloning and sequencing of *cryIA* genes by using a primer set developed in this study. Both nucleotide and amino acid sequences similarity analysis revealed that *cryIAa* and *cryIAc* from *B. thuringiensis* INTA Mo1-12 are new natural variants, showing several differences with the other known *cryIA* subclasses. These genes were named by the *B. thuringiensis* Pesticidal

Crystal Protein Nomenclature Committee as *cryIAa15* and *cryIAc21* respectively.

Keywords *Bacillus thuringiensis* · *cryIAa15* · *cryIA* genes · *cryIAc21* · Hot start PCR · sequencing

Introduction

Bacillus thuringiensis (Bt) is an entomopathogenic, Gram-positive bacterium that is well known to produce, during the sporulation process, insecticidal crystalline inclusions composed of protoxins currently known as Cry proteins (Schnepf et al., 1998). These must be processed by insect midgut proteases to become activated toxins (Schnepf et al. 1998). Cry proteins are classified in 51 groups due to their amino acid sequence similarity (Crickmore et al. 1998; http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). Cry1A toxins are very important because of their high toxicity to lepidopteran pests and widespread distribution among Bt strains (Bravo et al. 1998; Li et al. 1995; Uribe et al. 2003). In addition, variation in toxicity and specificity due to minor amino acid substitutions exist among different Cry1A toxins (Tounsi et al. 1999). To date sequences of 62 *cryIA* genes, classified into *cryIAa* to *cryIAi* types, have been published (Crickmore et al. 1998; <http://www.lifesci.sus>

D. H. Sauka (✉) · A. F. Amadio ·
R. O. Zandomeni · G. B. Benintende
Area Bioinsumos Microbianos, Instituto de
Microbiología y Zoología Agrícola (IMYZA),
Instituto Nacional de Tecnología Agropecuaria
(INTA), De los Reseros y Las Cabañas s/nro. C.C. 25,
C.P. 1712 Castelar, Buenos Aires, Argentina
e-mail: dsauka@cni.inta.gov.ar

sex.ac.uk/home/Neil_Crickmore/Bt/). The *cryIAa*, *cryIAb* and *cryIAc* genes are the most frequently found in Bt strains and native isolates (Ben-Dov et al. 1997; Bravo et al. 1998; Juárez-Pérez et al. 1997; Uribe et al. 2003). Some Bt strains bear a single *cryIA* gene [e.g., strain HD-73, with only one *cryIAc* gene (González et al. 1981)] whereas others have been found to present complex gene profiles including several *cryIA* subclasses (Ben-Dov et al. 1997; Bravo et al. 1998; Juárez-Pérez et al. 1997; Uribe et al. 2003).

Bt strains belonging to the serovar *kurstaki* were developed and used as efficient control agents for lepidopteran pests. The commercial Bt product called Dipel® (Abbott) is a formulated product based on Bt serovar *kurstaki* HD-1. This strain, as well as others of the same serovar, has also been used as a source of *cryIA* genes for the construction of genetically modified plants or bacteria to protect against lepidopteran attack (Bora et al. 1994; Lampel et al. 1994; Vaeck et al. 1987). Polymerase chain reaction (PCR) and gene sequencing are an integral part in agronomical research and biomolecular product development. Both are essential for the finding of novel *cry* genes in Bt strains that could be used for the construction of genetically modified organisms (GMOs). To our knowledge, many studies have focused primarily on the use of *cry* genes of Bt strains with the final objective of protecting against lepidopteran pests but none detail a complete molecular strategy with a full description of all primers used for sequencing (Alberghini et al. 2005; Huang et al. 2004; Masson et al. 1998; Tounsi et al. 1999). Therefore, the amplification, subsequent cloning of the full-length *cry* genes from Bt and sequencing are important because they might provide genes encoding active toxins against insect pests that are capable of being introduced in GMOs (Alberghini et al. 2005; Huang et al. 2004; Masson et al. 1998; Tounsi et al. 1999).

The main objective of this study was to establish a complete strategy designed for the amplification, cloning and sequence determination of known and potentially novel *cryIA* genes harboured in a Bt strain and to prove its robustness. Using this method, we analyzed two novel *cryIA* genes from an Argentinean Bt isolate INTA Mo1-12.

Materials and methods

Bt strains and native isolates

Eight Bt reference strains known to harbour *cryIA* genes (Bt serovar *thuringiensis* HD-2, Bt serovar *kurstaki* HD-1, Bt serovar *kurstaki* HD-73, Bt serovar *kenyae* HD-5, Bt serovar *aizawai* HD-137, Bt serovar *aizawai* HD-133, Bt serovar *tolworthi* HD-125 and Bt serovar *wuhanensis* HD-525), and two known not to harbour *cryIA* genes (Bt serovar *kyushuensis* HD-541 and Bt serovar *israelensis* HD-567) (Cerón et al. 1994; Kuo and Chak 1996), were kindly provided by the United States Department of Agriculture (USDA) Agricultural Research Service (Peoria, USA). Five native Bt isolates collected from stored product dust from different agroecological regions of Argentina were obtained from the Instituto de Microbiología y Zoología Agrícola-Instituto Nacional de Tecnología Agropecuaria (IMYZA-INTA) Bacterial Collection. All these isolates harbour at least one *cryIA* gene (Table 1).

PCR primers

Two novel specific primers for the amplification of *cryIA* genes were designed based on the analysis of conserved regions by multiple alignments of all *cryI* DNA sequences in the Bt toxin nomenclature website using ClustalW (Thompson et al. 1994; <http://www.ebi.ac.uk/clustalw/>) and Oligoanalyzer 3.0 (<http://scitools.idtdna.com/scitools/Applications/OligoAnalyzer/>). Primers used for the amplification of the whole open reading frame of *cryIAa*, *cryIAb*, *cryIAc*, *cryIAe*, *cryIAf*, *cryIAG* and *cryIAi* were as follow: 1AF (forward;

Table 1 Native Bt isolates from the IMYZA-INTA Bacterial Collection used in this study

Native Bt isolates	<i>cryIA</i> content
INTA Mo29–1	<i>cryIAa</i> , <i>cryIAb</i> and <i>cryIAc</i>
INTA Mo14–3	<i>cryIAa</i> , <i>cryIAb</i> and <i>cryIAc</i>
INTA Mo1–12	<i>cryIAa</i> and <i>cryIAc</i>
INTA Mo27–1	<i>cryIAa</i> and <i>cryIAc</i>
INTA Mo23–2	<i>cryIAa</i> and <i>cryIAc</i>

The *cryIA* content of these isolates was identified by PCR with primer previously described (Cerón et al. 1994; Juárez-Pérez et al. 1997)

5'-ATGGATAACAATCCGAACATC-3') and 1UR (reverse; 5'-YTATTCYTCCATRAGRAS-TAR-3'). The degenerate bases were designated as follows: Y, C/T; R, A/G; S, G/C. The *cryIAd* and *cryIAh* genes were excluded because of several sequences dissimilarities at the extreme 5' end. The forward primer is class specific and was designed to begin at the ATG initiation codon. The reverse primer is degenerate and was designed from sequences at the 3' end of all *cryI* genes. It might also be used to amplify any *cryI* gene in combination with other class specific primer. Oligonucleotides were synthesized in a DNA synthesizer as specified by the manufacturer (alpha DNA).

Amplification of *cryIA* genes

All Bt reference strains and native isolates were grown on nutrient agar plates for 16 h. A loopful of cells was transferred to 100 µl of H₂O and boiled for 10 min to make Bt DNA accessible for PCR amplification. The lysate was centrifuged briefly (5 s at 15,000 g; Eppendorf model 5415R centrifuge), and 10 µl of supernatant was used as the DNA template in the reactions. Manual "hot start" PCR was performed with a final volume of 50 µl containing of 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 200 µM each deoxynucleoside triphosphate (dATP, dTTP, dGTP, and dCTP), 1 µM each primer and 2 mM MgCl₂. The PCR amplification consisted of an initial denaturation step of 2 min at 94°C, followed by 29 cycles of 5 s at 95°C, 20 s at 47°C, 4 min at 68°C and a final elongation step of 5 min at 68°C in a thermocycler (Eppendorf Mastercycler gradient). Five U of *Taq* DNA polymerase (Invitrogen) were added after the first denaturation step. Finally, the PCR product was analyzed by 1.0% agarose gel electrophoresis stained with ethidium bromide.

Cloning of *cryIA* genes from Bt INTA Mo1-12

The PCR product of Bt INTA Mo1-12 was purified from the agarose gel matrix using Wizard SV Gel and PCR Clean-Up System (Promega), cloned in pGEM-T Easy vector (Promega) and then transformed into competent *Escherichia coli*

XL-1 strain following the manufacturer protocols. Thirty white colonies were selected on X-gal IPTG containing selective LB agar plates. Verifying whether the clones contained inserts was accomplished by PCR of recombinant plasmid DNA using vector primer SP6 and T7. Afterwards, the *cryIA* subclasses of each clone were identified by multiplex PCR with the primers described previously (Cerón et al. 1994).

Sequencing primers and nucleotide sequencing of *cryIA* genes from Bt INTA Mo1-12

Five clones harbouring the *cryIAa* and five harbouring *cryIAc* genes were sequenced in both directions by primer walking using vector (SP6 and T7) and specific primers detailed in Table 2 by using DYEnamic ET Terminator chemistry (Amersham) in an ABI 373 DNA Automated Sequencer.

Sequence comparisons

The *cryIA* nucleotide sequences from Bt INTA Mo1-12 and their translations into amino acid sequences (with the ExpASY translate tool; <http://www.expasy.org/tools/dna.html>) were aligned separately with ClustalW (Thompson et al. 1994). The deposited sequences in GenBank of all *cryIA* subclasses were accessed through the Bt toxin nomenclature website (http://www.life.sussex.ac.uk/home/Neil_Crickmore/Bt/) and used as references.

Nucleotide sequence accession number

The DNA nucleotide sequences of *cryIAa* and *cryIAc* from Bt INTA Mo1-12 have been deposited in the GenBank databases (<http://www.ncbi.nlm.nih.gov>) under the accession numbers DQ062690 and DQ062689 respectively.

Results and discussion

The oligonucleotide primers, 1AF and 1UR, were successfully tested for the amplification of PCR

products of the expected size (about 3500 bp) using Bt reference strains and native isolates. Bt serovar *thuringiensis* HD-2, Bt serovar *kurstaki* HD-1, Bt serovar *kurstaki* HD-73, Bt serovar *kenyae* HD-5, Bt serovar *aizawai* HD-137, Bt serovar *aizawai* HD-133, Bt serovar *tolworthi* HD-125, Bt serovar *wuhanensis* HD-525, and the Bt native isolates INTA Mo29-1, INTA Mo14-3, INTA Mo1-12, INTA Mo27-1 and INTA Mo23-2, used as positive controls, yielded PCR products of the expected size. Bt serovar *kyushuensis* HD-541 and Bt serovar *israelensis* HD-567, used as negative controls, failed to produce any amplification. Fig. 1 shows the result of electrophoresis analysis of these amplifications.

In order to test the designed strategy, the native Bt isolate INTA Mo1-12 was randomly chosen for

cloning and sequencing of *cryIA* genes by using the vector and specific primers detailed in Table 2. To our knowledge, there are no previously published studies concerning *cry* genes with a full description of all the primers needed for sequencing. For these analyses, the less than full-length sequences resulting from cloned PCR fragments were trimmed to remove the common, conserved primer sequences, because the sequence hybridizing with the primer might not represent the amplified gene and amplification of certain combinations of degenerated bases in the reverse primer might have resulted in non-native sequence. The trimmed *cryIAa* and *cryIAc* sequences are approximately 99% of the full length.

The sequence of *cryIAa* from Bt INTA Mo1-12 was 3489 nucleotides long, encoding 1163

Table 2 Primers for sequencing of *cryIA* genes

<i>cryIAa</i> primers ^a	Oligonucleotide sequence ^b	Position ^c	Tm ^d	Gene/s recognized	Reference
1F, reg 5'	GCT GGT ACA ATA CGG GAT TAG A	625–647	54,0	<i>CryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIAf</i> , <i>cryIag</i> and <i>cryIAi</i>	This work
1R, reg 3'	TGG GAA ACT GTT CGA ATT GGA T	764–786	54,7	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIAf</i> , <i>cryIag</i> and <i>cryIAi</i>	This work
CJ1, reg 5'	TTA TAC TTG GTT CAG GCC C	1101– 1120	51,7	<i>cryIAa</i> , <i>cryIag</i> and <i>cryIAi</i>	Cerón et al. 1994
2F, reg 5'	TCA ACA ACT ATC TGT TCT TGA CGG	1130– 1154	54,9	<i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> and <i>cryIAf</i>	This work
CJ2, reg 3'	TTG GAG CTC TCA AGG TGT AA	1332– 1351	53,3	<i>cryIAa</i> , <i>cryIag</i> and <i>cryIAi</i>	Cerón et al. 1994
CJ5, reg 3'	CTC TTA TTA TAC TTA CAC TAC	1328– 1348	42,2	<i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> and <i>cryIAf</i>	Cerón et al. 1994
3Fc, reg 5'	GCT ACG TCA TTA GAT AAT CTA C	1657– 1679	47,9	<i>cryIAc</i> and <i>cryIAi</i>	This work
3Fab, reg 5'	GGA GTA ATT TAC AGT CCG GAA GC	1669– 1692	55,4	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAe</i> , <i>cryIAf</i> and <i>cryIag</i>	This work
2R, reg 3'	GTA AAC AGC KCA TTC ACC GC	1863– 1883	55,9	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIAf</i> , <i>cryIag</i> and <i>cryIAi</i>	This work
4F, reg 5'	ATT GTC CGA GAA AGT CAA ACA TGC	1995– 2019	56,1	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIAf</i> , <i>cryIag</i> and <i>cryIAi</i>	This work
3R, reg 3'	CAT CSC CTC CTT GGA TGG TAA T	2113– 2135	56,7	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIAf</i> , <i>cryIag</i> and <i>cryIAi</i>	This work
5F, reg 5'	AGA ATC TGT AGA TGC TTT AT	2712– 2732	45,7	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIAf</i> , <i>cryIag</i> and <i>cryIAi</i>	This work
4R, reg 3'	AAT CAC AGA CAG CTC AGG CAG	2826– 2847	57,2	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> and <i>cryIag</i>	This work
6F, reg 5'	GAA GCA GAA GTG TCA CAA GAA G	3030– 3052	53,8	<i>cryIAa</i> , <i>cryIAb</i> , <i>cryIAc</i> , <i>cryIAe</i> , <i>cryIag</i> and <i>cryIAi</i>	This work

^a reg, refers to the coding strand

^b Abbreviation for wobble positions: K, G/T; S, G/C

^c The A of the first ATG is numbered 1

^d Tm, melting temperature of the primer

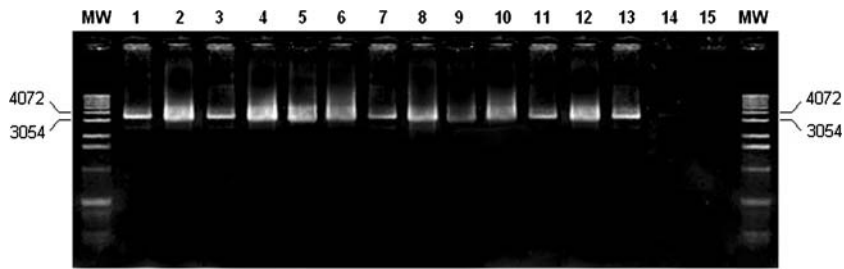


Fig. 1 Agarose gel electrophoresis analysis on 1.0% agarose gel of DNA sequences amplified by manual “hot start” polymerase chain reaction (PCR) assay by using primers 1AF and 1UR targeted *cryIA* genes. Lanes: 1, Bt INTA Mo1–12; 2, Bt INTA Mo27–1; 3, Bt INTA Mo23–2; 4, Bt INTA Mo14–3; 5, INTA Bt Mo29–1; 6, Bt serovar *thuringiensis* HD-2; 7, Bt serovar *kurstaki* HD-1; 8, Bt

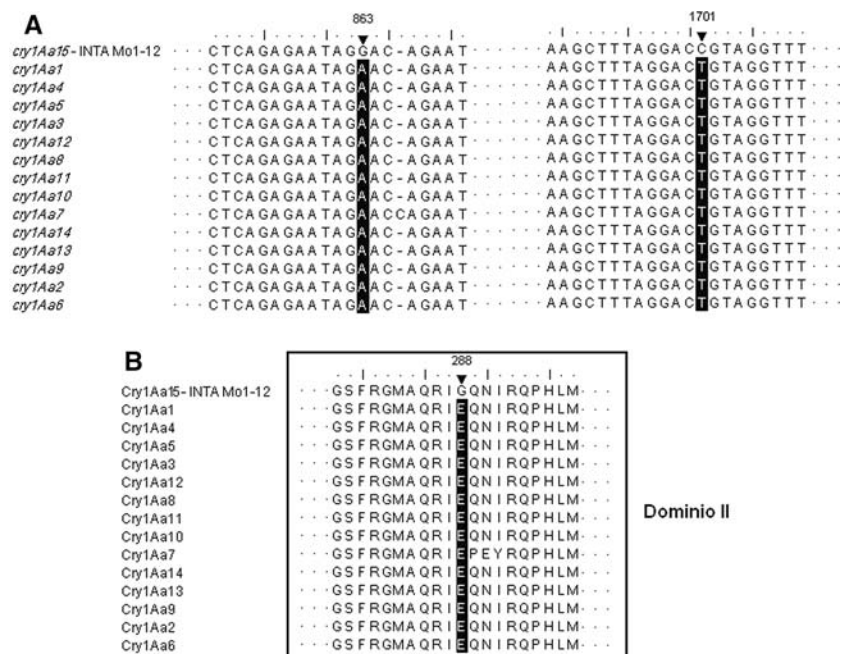
serovar *kurstaki* HD-73; 9, Bt serovar *kenyae* HD-5; 10, Bt serovar *aizawai* HD-137; 11, Bt serovar *aizawai* HD-133; 12, Bt serovar *tolworthi* HD-125; 13, Bt serovar *wuhanensis* HD-525; 14, Bt serovar *israelensis* HD-567; 15, Bt serovar *kyushuensis* HD-541. MW, molecular weight marker with sizes indicated on left and right (bp)

amino acid residues showing 98.0–99.9% identity with those of the known *cryIAa* genes. The *cryIAa* gene from INTA Mo1–12 could be grouped together with *cryIAa1*, *cryIAa3*, *cryIAa5*, *cryIAa7*, *cryIAa8*, *cryIAa10* and *cryIAa12* genes that also encode 1163 amino acids, whilst another group, containing *cryIAa9*, *cryIAa13* and *cryIAa14* genes, encode 1167 amino acids. Incomplete sequences of genes *cryIAa2* and *cryIAa6* are available but were not

used. The search for sequence similarity with the previously known *cryIAa* genes using ClustalW revealed that it is a new *cryIAa* gene (Crickmore et al. 1998; http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/). There were substitutions at position 863 of A for G (transversion), resulting in the substitution of Glu⁽²⁸⁸⁾ for Gly and at 1701 of T for C (silent mutation) (Fig. 2).

The crystal structures of several Cry toxins have been solved (Derbyshire et al. 2001;

Fig. 2 Alignment of *cryIAa15* gene from INTA Mo1–12 (A) and their deduced amino acid sequence (B) in the regions where differences were found. The vertical downward arrows and the black boxes indicate the nucleotides and amino acid showing variation. The Glu⁽²⁸⁸⁾ observed in Cry1Ac from Bt INTA Mo1–12 was embedded in domain II. Nucleotides and amino acids are numbered from those of the corresponding position in all known Cry1Aa sequences



Grochulski et al. 1995; Li et al. 1991). The seven α -helices that form the N-terminal domain I have been implicated in the pore formation. Domain II consists of three antiparallel β -sheets with exposed loop regions, whereas domain III is a β -sandwich. Domains II and III are important in receptor recognition. Based on the structure assignment of Cry1Aa (Grochulski et al. 1995), domain I of Cry1Aa from INTA Mo1-12 consists of 221 residues (Tyr⁽³³⁾–Arg⁽²⁵³⁾), domain II of 197 residues (Arg⁽²⁶⁵⁾–Phe⁽⁴⁶¹⁾) and domain III of 147 residues (Asn⁽⁴⁶³⁾–Thr⁽⁶⁰⁹⁾). The substitution of Glu⁽²⁸⁸⁾ for Gly observed in Cry1Ac from INTA Mo1-12 was embedded in β -1 (third sheet). This observed change may affect the salt bridge formed at the interface between domain I and II (Arg⁽²³³⁾–Glu⁽²⁸⁸⁾) in the other Cry1Aa proteins and therefore its stability might be affected at this point.

On the other hand, the sequence of *cryIac* from Bt INTA Mo1-12 was 3492 nucleotide long, encoding 1164 amino acid residues showing 99.5–99.9% identity with those of the known *cryIac* genes. For this analysis, the incomplete sequence of *cryIac3* was excluded, although the 1833 nucleotides available through the Bt toxin nomenclature Web site showed 100% identity. Additionally, incomplete published sequences of the genes *cryIac6* and *cryIac13*, and two truncated *cryIac* genes (*cryIac12* and *cryIac17*) exist but were not used. A deletion of three nucleotides (AAT) at positions 1303-1305 was found to be present in *cryIac* of Bt INTA Mo1-12. This characteristic was also observed in

cryIac2, *cryIac3*, *cryIac6*, *cryIac12*, *cryIac15* and *cryIac17*, but not in *cryIac1*, *cryIac4*, *cryIac5*, *cryIac7*, *cryIac11* and *cryIac16*. When *cryIac* of INTA Mo1-12 was compared with the full available 3492 nucleotides of three *cryIac* genes (*cryIac2*, *cryIac14* and *cryIac15*), these analysis finally revealed that it is a new *cryIac* gene. Variations in *cryIac2*, *cryIac14* and *cryIac15* genes and in their deduced amino acid sequences make *cryIac* from INTA Mo1-12 different and unique (Fig. 3). Sequence analysis of the regions within these *cryIac* deduced amino acid sequences suggested that the new gene might have arisen by rearrangement among them (Fig. 3).

The Cry1 protoxins are activated through the proteolytic removal of an N-terminal peptide of 25–30 amino acids and approximately half of the remaining protein from the C terminus (Bravo et al. 2002; Tojo and Aizawa 1983). The changes observed beyond domain III (Asn⁽⁴⁶³⁾–Thr⁽⁶⁰⁹⁾) (Fig. 3) comprise a region that is cleaved in the activated toxin and therefore a role in target insect specificity and level of toxicity is not expected. Excluding this region and considering the substitutions at positions Phe⁽¹⁴⁸⁾ and His⁽²⁰⁶⁾ in Cry1Ac15 and Cry1Ac2 respectively that are embedded in domain I (Tyr⁽³³⁾–Arg⁽²⁵³⁾) (Fig. 3), the activated Cry1Ac from INTA Mo1-12 was identical to Cry1Ac14.

Due to multiple differences in their nucleotide and deduced amino acid sequences, the *cryIac* and *cryIac* genes from Bt INTA Mo1-12 reported in this paper are natural variants of

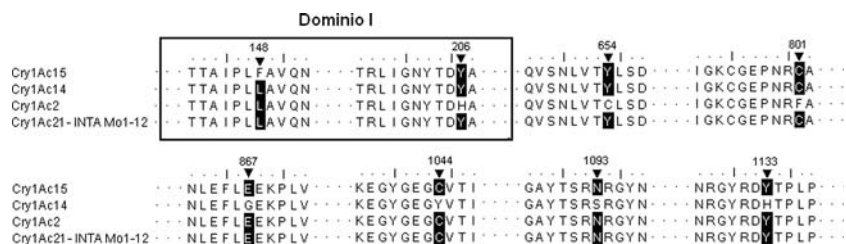


Fig. 3 Alignment of the deduced amino acid sequence encoded by *cryIac21* gene from Bt INTA Mo1-12 with those encoded by other *cryIac* genes. Only the regions containing differences are presented. The vertical downward arrows and the black boxes indicate the amino acid showing variation. Variations in Cry1Ac15, Cry1Ac14 and

Cry1Ac2 make Cry1Ac from INTA Mo1-12 different and unique. The box indicates that this region was embedded in domain I. Nucleotides and amino acids are numbered from those of the corresponding position in all known Cry1Ac sequences

these gene subclasses and they were named by the Bt Pesticidal Crystal Protein Nomenclature Committee as *cryIAa15* and *cryIac21* respectively (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/).

In summary, we have established a feasible strategy, with a fully detailed list of primers, for amplifying, cloning and sequencing known and potentially novel *cryIA* genes harboured in a Bt strain. Following this strategy, novel *cryIAa* and *cryIac* genes from Bt INTA Mo1-12 were identified.

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