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# cDNA Cloning and Molecular Characterization of a Cysteine-rich Gene from *Campoletis chlorideae* Polydnavirus

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**Polydnavirus (PDV) of *Campoletis chlorideae* (CcIV) is very important for the successful development of the parasitoid progenies. Previous study revealed that the persistence and expression of CcIV in parasitized *Helicoverpa armigera* larvae continued for 5 days, and the 1.0 kb gene (CcIV 1.0) was most abundantly expressed. In this report, a cDNA library was constructed using the SMART™ cDNA Synthesis Method, and the CcIV 1.0 was cloned and identified by PCR, Southern blot hybridization and 5′ end amplification, this gene is 936 bp long and encodes 207 amino acids with a signal peptide and a cysteine motif. Sequence comparison shows CcIV 1.0 has high identity with VHv1.4, VHv 1.1 genes (86%, 88%) and WHv 1.6, WHv 1.0 genes (89%, 87%) of *Campoletis sonorensis* PDV, which might suggest that they have arisen from a common ancestral gene; the homology between CcIV and other PDV genes is not significant.**

*Keywords*: Polydnavirus; Gene; Cysteine rich motif; *Campoletis chlorideae*; *Helicoverpa armigera*; CcIV

## INTRODUCTION

Endoparasitoids lay eggs into lepidopterous larvae, where the eggs have to overcome the defence system of the hosts that normally triggers encapsulation by the circulating haemocytes (Salt, 1968; Lackie, 1988). Polydnaviruses (PDVs) are the most important strategies that parasitoids adopted for this purpose. PDVs have been found in some endoparasitoids in Braconidae and Ichneumonidae families, and are called bracovirus (BV) and ichnovirus (IV), respectively (Stoltz and Whitfield, 1992; Stoltz, 1993). PDVs are characterized by double-stranded, segmented, circular DNA molecules with heterogeneous sizes (Stoltz *et al.*, 1984), for example, *Campoletis sonorensis* IV (CsIV), which has been described to the greatest extent at the molecular level (Cui and Webb, 1996), consists of 28 or more segments ranging 6–21 kb, and letter designations A–W were used to identify the superhelical molecules (Krell *et al.*, 1982; Blissard *et al.*, 1986). The life cycle of PDVs is unique. They replicate only in the ovaries of symbiotic female wasps, and then act as pathogens in parasitoid hosts when they are injected into the hosts of the wasps along with eggs during oviposition.

PDV genes express in host haemocytes and other tissues to modify host physiological properties such as immune and endocrine systems so as to ensure the successful development of endoparasitic wasp (Lavine and Beckage, 1995; Strand and Pech, 1995a). Quantities of host physiological changes after parasitizition are directly related to the expression of PDVs. In many systems, PDVs change host haemocyte structure and affect host defence reaction against foreign substance. By this means PDVs prevent parasitoid eggs from being encapsulated (Davies *et al.*, 1987; Lavine and Beckage, 1995; Strand and Pech, 1995a; Asgari *et al.*, 1996). *Microplitis demolitor* BV (MdBV) enters *Pseudoplusia includens* granulocytes and induces apoptosis (Strand and Pech, 1995b). In *Heliothis virescens* larvae parasitized by *C. sonorensis*, viral

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genes VHv1.1 and VHv1.4 produced proteins disrupt host haemocyte cytoskeletons so as to reduce encapsulation rate of parasite eggs (Li and Webb, 1994; Cui *et al.*, 1997).

However, the identification of PDV genes progressed slowly since the large size of the viral genome and the complexity of viral gene expression in the host (Asgari *et al.*, 1996). Although approx 50 PDV species among the estimated 30,000 PDV species of parasitoids including ichneumonid and braconid endoparasitic wasps have been described before (Stoltz, 1993), the properties of the PDVs in much less species were known, including ichneumonid *C. sonorensis* (Blissard *et al.*, 1989; Dib-Hajj *et al.*, 1993; Cui and Webb, 1996; 1997), and braconids *Chelonus inanitus* (Albrecht *et al.*, 1994), *Cotesia congregata* (Harwood *et al.*, 1994, Savary *et al.*, 1997), *Cotesia kariyai* (Yamanaka *et al.*, 1995), *Cardiochiles nigriceps* (Varricchio *et al.*, 1999), *Cotesia rubecula* (Asgari *et al.*, 1996), *Microplitis demolitor* (Strand *et al.*, 1997), *Glyptapanteles indiensis* (McKelvey *et al.*, 1996); *Hyposoter didymator* (Volkoff *et al.*, 1999), *Tranosema rostrale* (Beliveau *et al.*, 2000), etc.

*Campoletis chlorideae* Uchida is an important early larval endoparasitoid of cotton bollworm *Helicoverpa armigera* in China. Recently we characterized *C. chlorideae* PDV (CcIV) and found that CcIV was composed of at least twenty different DNA segments ranging in size from 3 to 26 kb (Yin *et al.*, 2003). CcIV genes expressed abundantly during the first two days post parasitization (p.p.), and their expression peak was consistent with the parasitoid eggs free of encapsulation (Yin *et al.*, 2003). A gene larger than 900 bp (termed CcIV 1.0) was the most abundantly expressed, but its properties were unknown. To improve our understanding of the molecular interaction between CcIV and host insects and to determine the specific virus gene products involved in host immune reaction, we constructed a CcIV cDNA library from infected host haemocytes and cloned CcIV 1.0, which provides an experimental basis for further studying of the mechanism which PDVs overcome host immune system.

## MATERIAL AND METHODS

### Insect Culture

*H. armigera* and *Pseudaletia separata* larvae were reared on artificial diets at 26 ± 1°C and a 15 h light–9 h dark photoperiod. Since *C. chlorideae* was easy to be reared when *P. separata* larvae were used as host insects, *P. separata* was used to maintain the colony of *C. chlorideae* in laboratory. For *C. chlorideae* oviposition, the host larvae at late 2nd or early 3rd instar were stung by mated female wasps for one or two times. The parasitized host larvae were kept under the same condition. Adult wasps were fed with a 10% honey–water solution.

### Virus Purification and Viral DNA Extraction

PDVs were extracted from female wasp ovaries by the methods described by Yin *et al.* (2003). Viral DNA was extracted from CcIV using a method described by Harwood *et al.* (1994). Female wasps DNA isolation was the same as viral PDV extraction.

### Isolation of RNAs from *H. armigera* Haemocytes

About 20 *H. armigera* late 3rd instar larvae were parasitized. One day after parasitization, haemocytes were collected by bleeding the larvae from a cut proleg and total RNAs were isolated using Trizol RNA extracting kit (Gibco-BRL) in accordance with the manufacturer's protocols.

### Construction of cDNA Library

The cDNA library was constructed using SMART™ (switching mechanism at the 5′ end of the RNA transcript) technology according to the manufacturer's protocols (CLONTECH laboratories). Briefly, first-strand cDNAs were synthesized from 1 μg of total RNA isolated from parasitized *H. armigera* haemocytes using Powerscript Reverse Transcriptase, then the first-strand cDNAs were amplified according to LD (Long-distance) PCR protocol (CLONTECH laboratories) with Advantage 2 Polymerase, PCR was carried out on a DNA Thermal Cycler (Perkin Elmer 9600) using the following conditions: 95°C, 20 s and 20 cycles at 95°C for 5 s and 68°C for 6 min. The double strand cDNAs from LD PCR were digested by proteinase K to degenerate the thermostable DNA polymerases, then the cDNAs were extracted with phenol-chloroform. The products were digested with *Sfi*I enzyme and were then size fractioned by the column CHROMA SPIN-400 to remove the small fragments (<400 bp), the wanted fractions were extracted and cloned into *Sfi*I digested λ TriplEx2 vector, and packaged using Packagene extracts (Promega). The original library was amplified and titered according to the manufacture's protocol (CLONTECH laboratories).

The titer of the library was $2 \times 10^6$ pfu/ml, after amplifications it reached $9 \times 10^9$ pfu/ml. The percentage of the recombinant clones was 100% in more than 600 plaques tested.

### PCR Insert Screening

After plating, the well-isolated plaques were randomly picked with a Pasteur pipette and suspended in 100 μl λ dilution buffer (100 mM NaCl; 10 mM

$MgSO_4$; 35 mM Tris–HCl), the diluted phages were placed at 4°C overnight, then 3 μl of which was denatured at 95°C for 10 min and were used as templates, λ TriplEx2 5′ and 3′ LD-Insert Screening Amplimers(CLONTECH laboratories) were the primers, PCR reaction was initiated at 95°C, 3 min, 35 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 3.5 min, followed by the final extension at 72°C for 10 min. 5 μl of each PCR product was analyzed on 1.1% agarose gels to assess the insert lengths and recombinant rate.

### Southern Blot Hybridization

CcIV DNA digested with *Eco*RI and *Hind*III restriction enzymes was labeled with digoxigenin (DIG) using a DIG High Prime DNA Labeling and Detection Kit (Roche Molecular Biochemicals), the DIG labeled CcIV DNA was used as hybridization probe, 0.5 μg female wasps DNA which was digested with *Eco*RI and *Hin*dIII was used as positive control.

PCR products whose inserts were about 1 kb in size were transferred to nylon membrane through capillary. Hybridization was done at 39°C in DIG Easy Hyb Buffer (Roche Molecular Biochemicals) for 13 h following the instruction manual of Roche Molecular Biochemicals for DIG High Prime DNA Labeling and Detection Kit II. Filter washes and exposure were the same as Yin *et al.* (2003) described.

### Cloning of the CcIV 1.0

Diluted phages whose PCR products hybridized with the probe were converted to p TriplEx2 and plated in accordance with the manufacture's protocol (CLONTECH laboratories), then the well-isolated colonies were sequenced by ABI377 automated DNA sequencer (Applied Biosystems). The sequences were compared with the GenBank database using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

### Amplification of 5′ End of cDNA

To clone the 5′ end of the 1.0 kb cDNA, 0.5 μg total RNA from 20 parasitized *H. armigera* larvae at 24 h p.p was reverse transcribed using cDNA synthesis kit (ThermoScript™ RT–PCR system, Gibco-BRL) according to the manufacturer's protocol. 1 μl of the reverse transcription mixture was used for PCR. One degenerate primer was designed based on the N-terminal sequence of CsIV cysteine rich motif (cys-motif) (C--C--CC--C--C) gene, corresponding to the residues MKFLWFALV of VHv 1.4, it was (5′-ATG AAR TTY CTN TGG TTY GCN CTN GT-3′), the other one was CcIV 1.0 sequence-specific, it was (5′-ACG TCC AAA TCG CAC CGA TGT GCG A-3′). The reaction was carried out at 95°C, 3 min, 35 cycles at 94°C for 1 min, 45–57°C for 1 min, 72°C for 1 min, followed by the final extension at 72°C for 10 min. PCR products were gel purified using Wizard PCR Preps (Promega). Following overnight ligation into pGEM®-T Easy Vector, resulting plasmids were used to transform *E. coli* DH5α. Ten white colonies (recombinants) were picked for PCR under above-mentioned conditions. Appropriate colonies were sequenced.

## RESULTS

### Molecular Cloning of CcIV Gene

To identify viral genes among the cDNA clones, the library was screened by PCR, then the PCR products with lengths about 1 kb were transferred and hybridized to the DIG labeled, *Eco* RI and *Hin*dIII digested CcIV DNA probe (Fig. 1). Only the positive clones were converted and then sequenced (Fig. 2). By this means, 5 cDNA clones were isolated from more than 100 Southern blot screened cDNA clones and all corresponded to the same gene, named CcIV 1.0. The longest clone was 880 bp.

Comparing the nucleotide sequence of CcIV 1.0 with the gene family of CsIV using BLAST program in GenBank database, we found CcIV 1.0 has high similarity to CsIV cys-motif genes on segments V and W (VHv and WHv), the N-terminal sequences are

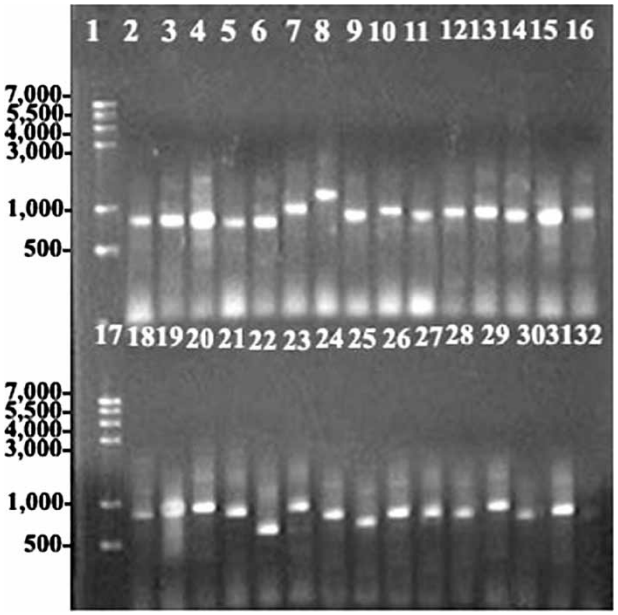


FIGURE 1 Gel electrophoretic profile of PCR insert which was about 1 kb in size. Lanes 2–16, 18–31 show the inserts about 1 kb using λ TriplEx2 5′ and 3′ LD-Insert Screening Amplimers, separated in a 1.1% agarose gel, stained with ethidium bromide, and visualized with UV light. (lane 1,17: MK; lanes 2–16, 18–31: PCR products; lane32: 0.5 μg female wasp DNA digested with *Eco*RI and *Hin*dIII.)

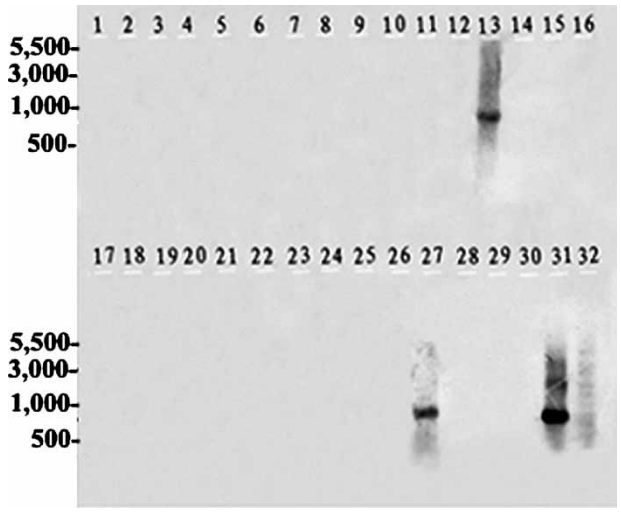


FIGURE 2 Southern blot analysis of PCR inserts. PCR insert which was about 1 kb in size was separated in a 1.1% agarose gel, the gel was blotted onto a nylon membrane, and the membrane was hybridized with DIG-labeled CcIV DNA which was digested with *Eco*RI and *Hin*dIII. (Lanes 1,17: MK; lanes 2–16,18–31: PCR products; lane 32: 0.5 μg *Eco* RI and *Hin*dIII digested female wasp DNA.)

much highly homologous between CsIV cys-motif genes, VHv 1.4 and VHv 1.1, so we used the highly conserved N-terminal sequence of VHv 1.4 to design the primer for CcIV 1.0, by PCR we cloned the 5′ end of CcIV 1.0, so we got the full lengh of CcIV 1.0.

CcIV 1.0 is 936 bp long, it contains a complete putative open reading frame (ORF) that includes 621 nucleotides, followed by a 3′ UTR where a consensus poly (A) signal sequence (AATAAA) and a 30-base poly (A) tail are present (Fig. 3; DDBJ accession number AB100268). The complete coding sequence of the CcIV 1.0 was determined at both 5′ and 3′ ends.

Calculated by GenBank, the overall identities of CcIV 1.0 to VHv 1.4 and VHv 1.1 (GenBank accession numbers: U41656 and U41655) are 86% and 88%, respectively, much higher than the identity between VHv1.4 and VHv1.1 (their cDNAs have 62% identity) (Cui and Webb, 1996), the identities of CcIV 1.0 to WHv 1.6 and WHv 1.0 (GenBank accession numbers: L08244 and L08243) are 89% and 87%, respectively (Dib-Hajj *et al.*, 1993). However, little homology was found with other PDV genes.

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1                                                                    T
2          ATGAAGTTTTTGTGGTTTGCTCTTGTCGCAGTGGTTTTAGTGGCGGCGCATCCTACGGC
1           M  K  F  L  W  F  A  L  V  A  V  V  L  V  A  A  H  P  T  A
62         CGGGAAATCAAGTGATAGGCCAGAGGCCGACGACGGGAAAATTTCCACCCCATTCGAGCC
21          G  K  S  S  D  R  P  E  A  D  D  G  K  I  S  T  P  F  E  P
122        AGGGTGCATCCGCAATTACCAACCTTGCATTGAGTCCACCAAGCCCTGCTGCCGACTTGA
41          G  C  I  G  N  Y  Q  P  C  I  E  S  T  K  P  C  C  R  L  E
182        AGATCGCACATCGGTGCGATTTGGACGTGAAGAGTACATCTGTCAACGATTCCTCGGCGG
61          D  R  T  S  V  R  F  G  R  E  E  Y  I  C  Q  R  F  L  G  G
242        ACTGTGTGCCCCATTAGAAGTCATTTCCAACCTTACACTGTATATAGAATTGAGTCGACG
81          L  C  A  P  L  E  V  I  S  N  L  T  L  Y  I  E  L  S  R  R
302        ATTGAACGAAACTAATTTGGCGGAACTCTCCAATCTGTATTCGCTAGCTGTAATGCCCAC
101         L  N  E  T  N  L  A  E  L  S  N  L  Y  S  L  A  V  M  P  T
362        GCCGGGAATGAAGCCAGAACCACCCAAGATATTAAACGCGGGAAACGTCGAGGAAGCCCT
121         P  G  M  K  P  E  P  P  K  I  L  N  A  G  N  V  E  E  A  L
422        GAAACAGAGTACGGGTGAGATGGAAATGAGTGCCGATACCGAACGTGAGCCTGACAAGTC
141         K  Q  S  T  G  E  M  E  M  S  A  D  T  E  R  E  P  D  K  S
482        AGCATCCAGAATAGAAGACGAGTTAAAACCAGACAAGGATTCGCCTACGAACAACAACGA
161         A  S  R  I  E  D  E  L  K  P  D  K  D  S  P  T  N  N  N  E
542        ATTGATTGATGCACGTAGAGGAGACCAAGGATACCAGCTCGAACCCGGAATGACAGACGG
181         L  I  D  A  R  R  G  D  Q  G  Y  Q  L  E  P  G  M  T  D  G
602        GCACGAATCAAATGGCAAATGGTGAGCAACGATTGCCATTTTCTTCGTTTTTCAACTGAA
201         H  E  S  N  G  K  W  ※
662        CTAGAGGAGGTTTTTGTTTTATACCTTTACTCTTAAATATTTTATGTGTTGCCTTAAGCA
722        TTTTTTAAAATTTTGTCTTTTCTTATATCAATAATTTTAGGTGTTGCCTTAAGCATTTTT
782        TTTAATTTTCTTTTTCCTTATATCAATAATTTTAGGTCGTATTTCGTTAAAATACTATTT
842        AGATTATTAAGAAGGAATAATGTAATGTTTCAAGATTTTTTTCAATAAAGAGTAATAATA
902        ATAACGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
                  **********************************
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FIGURE 3 Nucleotide and predicted amino acid sequences of the CcIV 1.0. CcIV 1.0 is 936 bp long and encodes a protein of 207 amino acids (in boldface type), it contains a consensus poly (A) signal sequence (AATAAA) and a 30-base poly (A) tail. 16 N-terminal amino acids encoding a signal peptide are double underlined, 41 amino acids encoding cys-motif are underlined. Asterisks indicate the 30-base poly (A) tail.

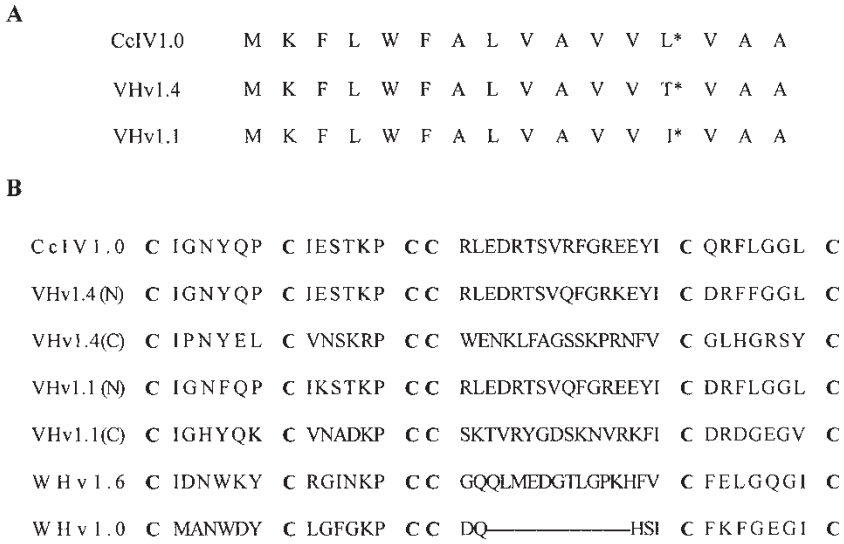


FIGURE 4 Alignments of predicted N-terminal signal peptides and cysteine-rich motifs. A: Alignment of predicted N-terminal signal peptide of CcIV 1.0, VHv1.4, and VHv 1.1. Asterisks indicate the only one different amino acid among them. B: Alignment of predicted cys-motifs of CcIV 1.0, VHv1.4, VHv 1.1 WHv1.6 and WHv 1.0. All the motifs have 41 amino acids except that the WHv 1.0 motif has only 30 amino acids. The cysteine residues are in boldface type. Gaps (–) are used to optimize alignment of the cysteine residues. N, N-terminal; C, C-terminal; VHv for *Campoletis sonorensis* IV segment V; WHv for *C. sonorensis* IV segment W.



CcIV 1.0 encodes a predicted protein with 207 amino acids, its N-terminal sequence is very hydrophobic and encodes a signal peptide according to the principle of Von Heijne (1986) (Fig. 3). The signal peptide of CcIV1.0 has high similarity to those of VHv1.4 and VHv1.1, in fact there is only one amino acid difference among them (Fig. 4A). The identity of CcIV 1.0 predicted protein to those of VHv1.4 and VHv 1.1 is 61% and 60%, respectively, and its identity to those of WHv 1.6 and WHv 1.0 is 37%. CcIV1.0 also encodes 1 cys-motif (Fig. 3, amino acids 42–82), with 41 amino acids, similar to the motifs of VHv1.4, VHv1.1 and WHv1.6 (Fig. 4B), only that VHv 1.4 and VHv1.1 both have 2 motifs, one is at N-terminal, the other is at C-terminal, while CcIV 1.0, WHv1.6 and WHv 1.0 have only1 cys-motif, besides, the motif of WHv 1.0 has only 30 amino acids (Fig. 4B). The cys-motif of CcIV 1.0 is 26–90% identical to those of CsIV gene family. The position of CcIV 1.0 cys-motif is near the N-terminal, and its structure is much more similar to the N-terminal cys-motifs of the CsIV genes ( $>$ 90% identical). The DNA structure of CcIV1.0 is quite different from that of BV, and the cys-motif of CcIV 1.0 is quite different from those of MdBV (C--C----C---C-C------C) both in structure and amino acids composition (GenBank accession numbers: U 76033,U 76034 for MdBV 1.0 and MdBV 1.5, respectively) (Strand *et al.*, 1997).

## DISCUSSION

Since the time Rotheram first isolated the PDV particle in 1965, the structure and function of PDV have always been the hot issue in the field of insect-parasitoid interactions and attracted a great deal of consideration. Upon the many described PDV species, most of the virus gene structure and sequence were unknown yet, maybe partially because of the complexity of the virus genome.

*C. chlorideae* is a key agent for suppressing population of the cotton bollworm (*H. armigera*) in cotton cultivated area of Huanghe River Valley, China. In our previous study, we have found that the ovaries of the female wasps contained abundant CcIVs, and the morphology and genome of CcIV were similar to those of CsIV reported previously (Krell *et al.*, 1982; Stoltz, 1993); also we demonstrated that CcIV existed in parasitized host haemocytes from 30 min to 6 d after parasitization (Yin *et al.*, 2003). Hybridization of cDNAs from parasitized host haemocytes with DIG-labeled CcIV DNA probe suggested that only part of CcIV genes were expressed in the parasitized host, furthermore, the overall peak CcIV gene expression was the first two days p.p. and continued for 5 days (Yin *et al.*, 2003). In present study, we further investigated the study to clone the most abundantly expressed CcIV gene through constructing the cDNA library by SMART™ method with 1 μg starting RNA. By PCR, Southern blotting and 5′ end amplification, we characterized the CcIV 1.0 expressed in parasitized hosts. Comparing the sequence of CcIV 1.0 with those of CsIV cys-motif gene family, VHv1.4 and VHv1.1 in GenBank, together with the position and composition of CcIV 1.0 cys-motif and N-terminal signal peptide, we know the ORF is complete, although there is only 1 nucleotide before the ATG codon.

VHv 1.1 protein had been confirmed that it was secreted (Li and Webb, 1994), WHv 1.6 and

WHv 1.0 proteins were also efficiently secreted from recombinant baculovirus-infected cells (Blissard *et al.*, 1989). Cui *et al.* proved that VHv1.4 appeared predominantly in the plasma fraction of haemolymph from parasitized hosts, which suggested this protein was secreted (Cui *et al.*, 1997). Just as VHv1.1 and VHv1.4, CcIV 1.0 also has a very similar signal peptide, which indicating this protein is destined for insertion into the membrane or secretion (Cui and Webb, 1996), but whether CcIV 1.0 protein is a secreted protein is not clear at present.

Comparison of CcIV 1.0 and CsIV cys-motif gene family revealed high similarities. In fact, CcIV 1.0 has greater identity to VHv 1.4 and VHv 1.1 than VHv 1.4 and VHv 1.1 themselves do. Existence of similar N-terminal signal peptides, cys-motif genes and highly homologous sequences in two *Campoleti*s species suggests that these genes might have arisen from a common ancestral gene. The comparison between CcIV 1.0 and other PDV genes especially those of BV showed no significant similarity. Despite all the difference, some IV and BV gene predicted proteins all contain cysteine-rich domains though the structure of the cys-motifs is different. Cysteine-rich proteins or peptides were also isolated from ω-conotoxins (Olivera *et al.*, 1990; 1991), the *ctl* gene product of *Autographa californica* nucleopolyhedrovirus (Eldridge *et al.*, 1992) and animal venoms of carnivorous snails, scorpions, spiders, snakes (Cui and Webb, 1996).

Cysteine residues provide conformational stability for the toxin proteins or peptides. The combination of hypervariable regions and a constant backbone of disulphide bridges produce high-affinity ligands with various specificities (Olivera *et al.*, 1990). The cysteine-rich gene VHv 1.1 protein has been proved to target host plasmatocytes and granulocytes (Li and Webb, 1994), while VHv 1.4 protein was found to bind to host haemocytes, most notably the granulocytes (Cui *et al.*, 1997). Specific binding of the proteins to haemocytes implies an important function in the suppression of host cellular immune response. Since VHv1.1 and VHv 1.4 have 2 cys-motifs, while CcIV 1.0 contains only 1 N-terminal cys-motif and lacks the C-terminal motif, so their functions might be different. However, we suppose that the cysteine residues might form a structural backbone for CcIV 1.0 protein to act as ligand for surface proteins of targeted cells, especially haemocytes, but this remains to be determined.

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