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Construction of a Transposon-mediated Baculovirus Vector Hanpvid and a New Cell Line for Expressing Barnase

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In this study we developed the transposon-mediated shuttle vector 'Hanpvid', which composed of HaNPV (*Heliothis armigera* nuclear polyhedrosis virus) genomic DNA and a transposon cassette from Bacmid of Bac-to-Bac system. Hanpvid replicates in *E. coli* in the same way as Bacmid and retains infective function in cotton bollworm cells (Hz-AM1). Using Hanpvid we constructed a recombinant virus, which could infect Hz-AM1 cells and generate recombinant HaNPV (rHa-Bar) containing the *barnase* gene, a ribonuclease gene from *Bacillus amyloliquefaciens*. Since the expression vector carrying *barnase* gene cannot replicate in the absence of *barstar*, a specific inhibitor of *barnase*, we constructed a new cotton bollworm cell line (AM1-NB) using the marker rescue method. In AM1-NB *barstar* was integrated into the cellular chromosome to sustain the replication of rHa-Bar. To screen out recombinant HaNPV for potential use as biopesticide, Hz-AM1 and AM1-NB cell lines were infected with rHa-Bar, respectively. The results obtained indicate that Viral progenies in AM1-NB were 23 and 160 times greater than those in Hz-AM1 48 h and 72 h after infection, respectively. With additional insertion of the *polyhedron* gene from AcNPV (*Autographa californica* nuclear polyhedrosis virus) into the Hanpvid genome, rHa-Bar regained the polyhedron phenotype and its pest-killing rate greatly improved. Toxic analysis showed that the lethal dosages (LD_{50}) and the lethal time(s) (LT_{50}) of rHa-Bar were reduced by 20% and 30%, respectively, compared to wt-HaNPV in the third instar larvae of cotton bollworm. This study shows that in AM1-NB barnase can be effectively produced and used as pest-killing agent for the biological control of cotton pests.

Keywords: *barnase* gene, *barstar* gene, *Heliothis armigera* nuclear polyhedrosis virus, Shuttle vector Hanpvid, Transgene cell line

Introduction

Today, baculoviruses are being successfully used to control a wide range of insect pests. Baculoviruses infect only arthropods and individual virus strains infect only one or a limited number of species, which makes these viruses attractive as biological control agents (Chakraborty *et al.*, 1996). *Heliothis armigera*, also known as the "cotton bollworm", is a major pest of many crops worldwide. For the past twenty years, the wild type *Heliothis armigera* nuclear polyhedrosis virus (wt-HaNPV) has been utilized as a biological insecticide in China and has extensively improved the severe environmental situation that resulted from chemical insecticide use (Cuyno *et al.*, 2001; Lu *et al.*, 2002). However, in terms of its commercial value, wt-HaNPV has failed to compete with common chemical insecticides due to its low pest-killing speed. In recent years, a number of strategies have been investigated to solve this inefficiency by employing various genetic manipulation techniques based on the concept of improving the virus's ability to express target genes that are deleterious to the insect.

In 1983, *Autographa californica* nuclear polyhedrosis virus (AcNPV)/Sf-21 was first developed as an expression system to highly express IFN-β driven by the polyhedrin gene (*polh*) promoter (Smith *et al.*, 1983). A large variety of foreign genes have been expressed by different baculovirus expression vectors, which primarily utilize an effective promoter of the very late gene *polh* or p10 (Patel *et al.*, 1992; Possee *et al.*, 1997) The past decade has witnessed the development of several strategies for generating recombinant baculoviruses. The most common strategy involves constructing a vector carrying foreign genes downstream of a viral promoter, which

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is flanked by baculovirus DNA derived from a non-essential locus. Recombinant viruses are obtained by homological recombination after introducing this vector into insect cells with wild-type viral genomic DNA (Possee *et al.*, 1997). However, using this strategy, only 0.1-1% of the resulting progeny are selected as recombinant viruses. In addition, several rounds of recombinant plaque purification are required. Accordingly, many attempts have been made to increase the proportion of recombinant progeny, such as, the "linearization" technique (Kitts *et al.*, 1990; 1993) and "*in vitro* enzymatically mediated recombination" (Peakman *et al.*, 1992). However, it still takes an excessive time to perform multi-rounds of plaque identification and separation of recombinant viral stock from the wild type virus.

A transposon-mediated strategy (Bac-to-Bac system) that generates the recombinant virus in *E. coli* has also been described (Luckow *et al.*, 1993). It is based on the site-specific transposition of an expression cassette into AcNPV genomic DNA to then allowing it to propagate in *E. coli* as a shuttle vector (bacmid). Recombinant bacmids generated in *E. coli* are purified and used to transfect insect cells to express target genes at high levels. The Bac-to-Bac system markedly reduces the time required to identify and purify a recombinant virus, since recombinant virus DNA harbored in selected bacterial colonies is not contaminated by parental or non-recombinant viruses without multiple rounds of plaque identification. However, due to host species preferences, bacmid in the Bac-to-Bac system cannot replicate in silkworm cells (BmN) or in cotton bollworm cells (Hz-AM1), which considerably limits its application range.

In previous work, *via* gene homological recombination, we constructed a recombinant baculovirus containing AcNPV genomic DNA that can infect silkworms (Zhu *et al.*, 1998a). We developed a transposon-mediated vector, Hanpvid, similar to bacmid in the Bac-to-Bac system that replicates in *E. coli* as a large plasmid (Hanpvid), but which retains infectivity toward *Heliothis armigera* cells. Hanpvid is composed of HaNPV genomic DNA and a cassette that includes several distinct genetic elements (*i.e. mini-attTn7*) inserted into the frame within the *lacZ* region, including a selective kanamycin resistance gene and an element conserving rep/par function derived from the F factor.

Using the Hanpvid system, it is easy to screen out recombinant HaNPV with potential for the development of a *Heliothis armigera* controlling pesticide in a pool of diversiform toxic genes.

Barnase, a major extra-cellular ribonuclease, and *barstar*, a specific inhibitor of *Barnase*, are both isolated from *Bacillus amyloliquefaciens* (Hartley and Rogerson, 1972; Hartley *et al*., 1972; 1989). In the absence of *barstar* expression, the expression of *barnsase* is lethal to cells (Buckle *et al.*, 1994; Raghunathan *et al.*, 1994). Inhibition by *barstar* involves the formation of a stable and nonequivalent one-to-one complex with *barnase* (Hartley *et al.*, 1973). Without Barstar protein co-expression, *barnase* gene (*bar*) can only be cloned in an inactive form, such as in *E. coli*, either with the insertion derived from the transposon Tn917 (Paddon *et al.*, 1986) or with point mutations, which allow the expressions of inactive *bar* mutants (Paddon *et al.*, 1987). However, in the same vector, the assembly of *bar* with the *barstar* gene (*bst*) will counteract the function of Barnase. Therefore, it is important to find a system in which the vector containing only *bar* can be stably replicated. In order to normally propagate recombinant HaNPV containing bar (rHa-Bar) but not *bst*, a transgenic cotton bollworm cell line AM1-NB with *bst* integrated into the chromosome was constructed. In AM1-NB, rHa-Bar could be regularly propagated on a large scale. We predict that Barnase expression in insect larvae could increase the speed of pest-killing and provide a more useful biological control agent than wt-HaNPV.

Materials and Methods

Plasmids, insect, cells, and reagents The plasmid pTS and pFaGP with HaNPV *polh* (polyhedrin) gene and *gfp* (green fluorescence protein) gene inserted into pFastBac1 were provided by Dr. Zhu (Zhu *et al.*, 1998b). Prof Zhang, Wuhan Institute of Virology, Chinese Academy of Science, provided wt-HaNPV. Transposon vector, pFastBacHTb, carrying six histidines was purchased from GIBCO/BRL Co., and Prof. Hartley (NIH, Bethesda, USA) (Hartley *et al.*, 1972b) donated plasmid pMT416 (Paddon *et al.*, 1987). The plasmid pBlue*polh*, containing a subcloned polyhedrin gene of AcNPV inserted into pBluescript (sk+), and pBlueIE1, containing a promoter of AcNPV IE1 gene, were from Dr. Liu, Wuhan University, P R China. Plasmid pIE1Neo, containing the neomycin resistance gene driven by IE1 promoter, was from Prof. Javis (Texas A & M University, USA) (Javis *et al.*, 1990). Dr. Du, Wuhan University, P. R. China, provided the cotton bollworm cell line, Hz- AM1. Graces medium, lipofectin reagent, fetal bovine serum (FBS), geneticin (G418), and T4 DNA ligase were purchased from GIBCO/BRL Co. (Gaithersburg, USA) Proteinase K was purchased from Promega Co. (Madison, USA). The DIG DNA labeling detection reagent kit was purchased from Boehringer MannheinCo. (Mannheim, Germany). Yeast tRNA was purchased from Yuanpin Co.(Beijing, P.R. China,) and the PCR reagent kit, restriction endonucleases, and antibiotics were purchased from Huamei Co (P R China).

Plasmid and virus constructs Plasmid pTS contains a transposition/shuttle cassette (*kan/lacZ/attTn7/miniF*) and is flanked by the *polh* sequence, which was derived from AcNPV bacmid purified from *E. coli* DH10Bac by *Bam*HI partial digestion. The restricted fragment of bacmid was ligated by T4 DNA ligase and then transformed into *E. coli* DH5α. On the plate containing *Kan/IPTG/X-gal* (Kan: 50 μg/ml; IPTG: 100 μg/ml; X-gal: 200 μg/ml), blue bacterial colonies were selected as positive colonies harboring pTS.

The pTS DNA was introduced into Hz-AM1 cells along with wt-HaNPV genomic DNA. Since relatively high homology exists between the *polh* of AcNPV and that of HaNPV, the transposition/shuttle cassette in pTS can be site-specific and integrated into the

polh site in the HaNPV genome by homological recombination to produce a Hanpvid shuttle plasmid containing both the transposition/shuttle cassette and the entire genome of HaNPV. Hanpvid shuttle plasmid, which is the total DNA extracted from the transfected Hz-AM1 cells, was transformed into *E. coli* DH5α, and Hanpvid was selected on plates containing X-gal/IPTG/kanamycin with no wt-HaNPV contamination, since wt-HaNPV cannot replicate in *E. coli*. Another plasmid pHelper purified from *E. coli* DH10Bac1, which has an in *trans* function necessary for the transposon, was transformed into *E. coli* DH5α harboring Hanpvid, with positive colonies selected by kanamycin and tetracycline resistance. The transformed *E. coli* harboring both Hanpvid and pHelper was designated *E. coli* DH5Ha.

0.5 kb *bar* in the plasmid pMT416 (Paddon *et al.*, 1986) was amplified by PCR. Transposon vector, pFastBacHTb, comprised of ampicillin, gentamycin resistance gene, promoter of the *polh* gene (P*polh*), multiple cloning sites (MCS), and 3' and 5' ends flanked by the left and right arms of transposon Tn7, was digested by *BamHI-XhoI* and ligated to 1.3kb *polh* from plasmid pBlue*polh* digested by *XhoI-PstI* together with the *bar* PCR product digested by *BamHI-PstI*. After being transformed into *E. coli* TG1, recombinant plasmid pFb-Bar carrying *bar* and *polh* genes was selected on a plate containing Ap^r/Gm^r and verified by restriction enzyme digestion.

Recombinant pFb-Bar or pFaGP was then transformed into *E. coli* DH5Ha competent cells. The *bar* or *gfp* gene flanked by the left and right arms of Tn7 was transposed to the Tn7 att site in target Hanpvid with assistance in *trans* by the pHelper. Positive colonies harboring the recombinant Hanpvid named pHa-Bar or pHa-FaGP were white colored versus a comparative background of blue colonies on plates containing kanamycin, tetracycline, gentamycin, X-gal and IPTG. Recombinant HaNPV rHa-Bar or rHa-FaGP was obtained after pHa-Bar or pHa-FaGP was extracted from positive colonies and then transfected into Hz-AM1 cells.

Recombinant plasmid pIE-*bst*, which carries *bst* of 0.45 kb from pMT416 and the immediate early gene promoter (Pie1) of AcNPV from pBlueIEI, was constructed by subcloning both gene fragments into the *EcoRI-HindIII* sites of pBluescript. The 1.3 kb fragment containing Pie1 and the neomycin resistance gene from plasmid pIE1Neo, were then subcloned into pIE-*bst* digested with *SmaI* to produce another construct, pIE1-NB, carrying *bst* and *neo*, both driven by IE1 promoter.

Amplification of the *barnase* gene by PCR According to the sequence of the plasmid pMT416 (Paddon *et al.*, 1986), two primers were designed for PCR to amplify *bar*. The sequences of these two primers were ***a*** 5'-TCACAGGATCCGCACAGG-3ºC and ***b*** 5'-TTCAGCTGGACGATGTC-3ºC. The PCR procedure was as follows: denaturing for 5 min at 95ºC; 30 amplification cycles (1 min at 95ºC; 2 min at 55ºC; 3 min at 72ºC). Products were purified in low melting point agarose.

Dot-blotting of DNA and RNA DNA and RNA were denatured with alkaline or glyoxal, respectively. Samples were dotted on a nylon membrane and fixed by baking for 2 h at 80ºC. Hybridization was carried out using the procedure described by Boehringer Mannheim Co. (Mannheim, Germany) with prepared dig-labeled *bst* or *bar* probes.

Cell line transformation Cotton bollworm cells Hz-AM1 were washed twice with serum free Grace's medium without antibiotics and then covered with lipofectin-DNA complex (10 μg pIE1-NB DNA and 10 μl lipofectin). After 6 h at 28ºC, the transfection mixture was removed and replaced with 4 ml fresh Grace's medium supplemented with 10% fetal bovine serum (10% FBS) and 1.2 mg/ml G418. After more than three generations, both *bst* and *neo* were integrated into the chromosome of Hz-AM1 cell line. The transformed cell line was designated AM1-NB.

Plaque analysis The concentration of a virus is expressed as $TCID_{50}$, and the multiplicity of infection (MOI) represents the quantity of virus available for infection. The following formula can be used to convert $TCID_{50}$ to MOI: MOI = PFU/ml × cell infected volume/number of cells; $TCID_{50}$/ml × 0.69 = PFU/ml.

In our work, 10 MOI of HaNPV was added to Hz-AM1 cells. After incubation for various times (1, 6, 12, 24, 48 and 72 h), 1 ml of the old medium containing budded virus (BV) was poured onto AM1-NB and Hz-AM1, respectively, for the plaque assay. After incubation for 1 h at 27ºC, the medium was removed and replaced with 2 ml fresh medium containing low melting point agarose. After incubation for 6 days at 27ºC, the cells were stained with neutral red and counted.

Toxic assays *Heliothis armigera* eggs were treated in 5% HCH_2OH for 30 min, dried and treated with 0.1% NaOH for 3 min, and then washed and dried. Third instar larvae incubated for 5-6 days at 28ºC were selected and fed with 3 g of artificial diet in every small bottles.

rHa-Bar and wt-HaNPV were purified and the virus suspension of concentration 10^7 PIB/ml ((PIB: polyhedrin inclusion bodies; the concentration was determined by counting inclusion bodies under a microscope) was obtained with sterilized water. A virus suspension of purified recombinant and wild type virus were added to the diet, respectively, with the following gradient of viral concentrations: 2×10^5, 2×10^4, 2×10^3, 2×10^2, 2×10.

RNase activity of barnase Sensitivity test circle papers were soaked in 100 μl of supernatant solution of Hz-AM1 or AM1-NB cell cultures, respectively, infected by variant HaNPV. Then the papers were picked out and placed on agarose plates (containing 0.5% yeast tRNA as a substrate of Barnase) overnight. After the papers had been treated with 1 M H_2SO_4 for 3 min black circles were observed on the papers.

Results

Construction of Hanpvid and its application for the cloning of the *gfp* gene Please refer to *Materials and Methods* for Hanpvid construction details. To identify Hanpvid, pFaGP was transformed carrying *gfp* and *polh* into *E. coli* DH5Ha containing Hanpvid and pHelper. *bar* or *gfp* gene flanked by the left and right arms of Tn7 was transposed to the Tn7 att site in target Hanpvid with assistance from *trans* by pHelper, to produce recombinant Hanpvid pHa-FaGP.

Recombinant virus rHa-FaGP virions were harvested 6

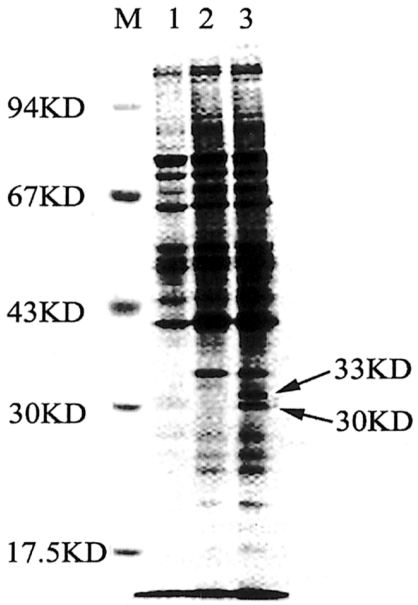

Fig. 1. The rHa-FaGP virions were harvested 6 days after transfecting recombinant Hanpvid pHa-FaGP into Hz-AM1 cells. Viral stocks were then used to infect Hz-AM1 cells for SDS-PAGE analysis. M: protein marker; 1: mock-infected cells; 2:cells infected with Hanpvid; 3:cells infected with rHa-FaGP.

Fig. 2. Expression of green fluorescent protein in *Heliothis armigera* larvae. *Heliothis armigera* larvae were orally infected with rHa-FaGP containing foreign *polh*. 72 h later, *Heliothis armigera* larvae with green fluorescence could be seen by the naked eye in bright light b_2: Normal *Heliothis armigera* larvae; b_1: *Heliothis armigera* larvae infected with recombinant virus rHa-FaGP.

days after recombinant Hanpvid pHa-FaGP had been transfected into Hz-AM1 cells. Viral stocks were then used to infect Hz-AM1 cells for protein expression. SDS-PAGE analysis suggested that there were two distinct bands representing polyhedrin and GFP with molecular weights of 33 and 30 kDa, respectively. Both proteins were highly expressed 48 h post infection (Fig. 1).

The third instar *Heliothis armigera* larvae reared at 28 were orally infected with rHa-FaGP, which contained a foreign *polh*. 72 h later green fluorescing *Heliothis armigera* larvae could be seen with the naked eye under bright light (Fig. 2).

Application of Hanpvid to the cloning of the *bar* gene To screen out potential recombinant Hanpvid expressing a toxic protein, *bar* and *polh* were inserted into Hanpvid to generate rHa-Bar using rHa-FaGP-construction methodology.

Using a 0.5 kb *bar* probe, dot blotting was carried out to identify the construction of rHa-Bar, containing HaNPV genome*/Kan*^{r}*/Gm*^{r} resistance genes*/miniF ori/LacZ/bar/polh*. Results show that there were hybridization signals in the DNA of pMT416, pGM-Bar, pFbBar, pHa-Bar, DNA, and RNA of transfected cells, but no signals in the DNA of Hanpvid, pBlue*polh*, pFastBacHTb, pGEM-3Zf, DNA, and the RNA of normal-cells (Fig. 3). By constructing recombinant Hanpvids carrying *gfp* or *bar* genes, we have shown that this novel transposon-mediated shuttle vector, Hanpvid, derived from the Bac-to-Bac system, offers a feasible and straightforward means of recombinant baculovirus construction.

Identification of transformation cell line AM1-NB integrated with *bst* To propagate large-scale recombinant virus (rHa-Bar) containing *bar*, we constructed a transgenic cell line AM1-NB by integrating a foreign *bst* gene into the chromosome of Hz-AM1 cells. The identification of AM1-NB by dot blotting with *bst* as a probe showed hybridization signals to the DNA and RNA of transformed AM1NB, and the DNAs of pMT416, pIE-*bst*, and pIE1-NB, but no hybridization signals to the DNA or RNA of normal cotton bollworm cells Hz-AM1, or to the DNA of pBluescript, pBlueIE1, or pIE-neo (Fig. 4). These results suggest that *bst* was successfully integrated into the chromosome of the cotton bollworm cells Hz-AM1. We designated this novel transgenic cell line AM1-NB.

Expression of *barnase* in both AM1-NB and Hz-AM1 Cells Both AM1-NB and Hz-AM1 cells were infected with recombinant HaNPV rHa-Bar and wt-HaNPV, respectively. The infected cells were harvested 72 h later for SDS-PAGE (Fig. 5). A *barstar* polypeptide band with a molecular weight of 12 kDa was observed in the lane representing AM1-NB cells infected by rHa-Bar but not in Hz-AM1 cells infected by wt-HaNPV. Polyhedrin protein, with a molecular weight of 33 kDa, was expressed both in Hz-AM1 cells infected with wt-HaNPV and AM1-NB cells infected with rHa-Bar, and result from the insertion of an extra copy of *polh* into the rHa-Bar genome. *Barnase* polypeptide expressed by rHa-Bar in AM1-

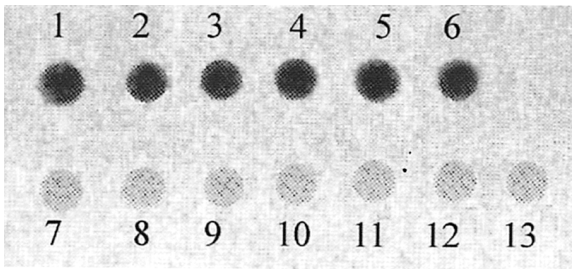

Fig. 3. With 0.5 kb *bar* probe, dotblotting was carried out to identify the construction of rHa-Bar. 1: pMT416 DNA; 2: pGM-Bar DNA; 3: pFb-Bar DNA; 4: pHa-Bar DNA; 5: transfected cellular DNA; 6: transfected cellular RNA; 7: Hanpvid DNA; 8: pBluepolh DNA; 9: pFastBacHTb DNA; 10,11: pGEM 3Zf DNA; 12: normal cellular DNA; 13: normal cellular RNA.

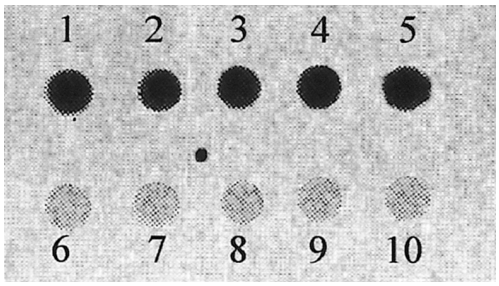

Fig. 4. A transgenic cell line AM1-NB was constructed by integrating a foreign *bst* gene into the chromosome of Hz-AM1 cells, and then identify AM1-NB by dotblotting with *bst* as a probe. 1: pMT416; 2: pIE-bst; 3: pIE1-NB; 4: transformed-cellular DNA; 5: transformed-cellular RNA; 6: pBluescript; 7: pBlueIE1; 8: pIE-neo; 9: normal cellular DNA; 10: normal cellular DNA/RNA.

NB cells was present at the same position because both polyhedron and *barnase* have similar molecular weights. Abnormally low rHa-Bar expression was observed in Hz-AM1 cells, and rare bands on SDS-PAGE were due to the expression of *bar* and to the degradation of intracellular RNA.

Black circles could be seen around the test papers due to tRNA degradation by Barnase in the culture media of AM1-NB (Fig. 6.1) and Hz-AM1 cells (Fig. 6.2) infected with rHa-Bar. However, the black circles were larger and more significant in AM1-NB cells than in Hz-AM1 cells, suggesting a higher expression of Barnase in AM1-NB cells than in Hz-AM1 cells. Conversely, no black circles around sensitivity test papers containing culture medium of both AM1-NB cells (Fig. 6.3) and Hz-AM1 cells (Fig. 6.4) infected with wt-HaNPV were observed due to a lack of *bar* gene expression.

Replication of rHa-Bar in AM1-NB Normal cotton bollworm cells Hz-AM1 were infected with rHa-Bar at a multiplicity of infection of 10 (MOI 10). 24 h post-infection CPE was apparent, but by 48h post-infection all cells had died (Fig. 7C), which suggests that Barnase expressed by rHa-Bar

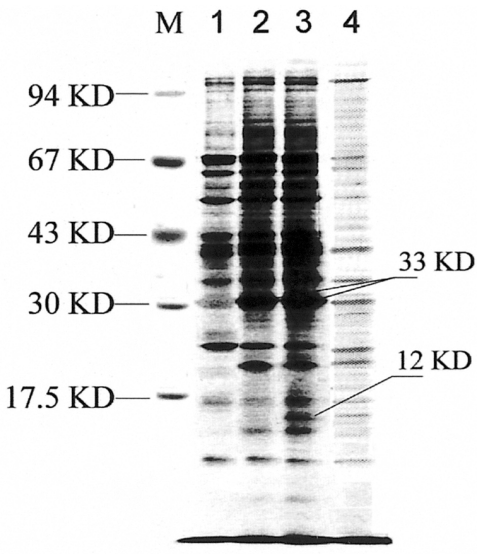

Fig. 5. AM1-NB and Hz-AM1 cells were infected with rHa-Bar and wt-HaNPV , respectively. Infected cells were harvested 72 h later for SDS-PAGE analysis. M: Marker. 1: Hz-AM1 cells without infection; 2: Hz-AM1 cells infected with wt-HaNPV; 3: AM1-NB cells infected with rHa-Bar; 4: Hz -AM1 cells infected with Ha-Bar.

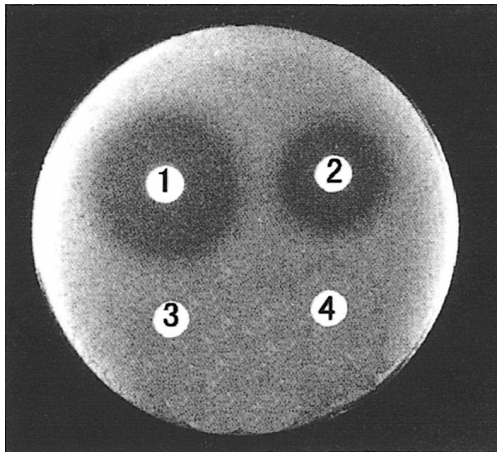

Fig. 6. Activity of Barnase Expressed by rHa-Bar in cotton bollworm cells. 1: supernatant of AM1-NB infected with rHa-Bar; 2: supernatant of Hz-AM1 infected with rHa-Bar; 3: supernatant of Hz-AM1 infected with wt-HaNPV; 4: supernatant of uninfected Hz-AM1.

was directly cytotoxic to host cells. On the other hand, at 24 h post-infection in AM1-NB with rHa-Bar typical cytopathic symptoms were observed, which indicated that the replication of the recombinant virus was regular. Nuclei became swollen and cells rounded, and this was accompanied by obvious polyhedrosis 48 h post-infection (Fig. 7B). In addition, wt-HaNPV was inoculated in both AM1-NB and Hz-AM1 cells. Plaque assay results at different times after infection suggested that the replication of wt-HaNPV in both cotton boll worm cells were continuous (Fig. 8). This further

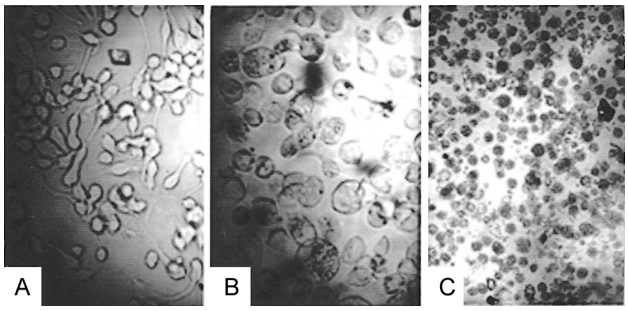

Fig. 7. AM1-NB and Hz-AM1 cells infected with rHa-Bar. A: Hz-AM1 cells without infection; B: AM1-NB cells infected with rHa-Bar, 48 h; C: Hz-AM1 cells infected with rHa-Bar, 48 h.

indicates that viral-proliferation was normal in AM1-NB cells, and that yields of rHa-Bar in AM1-NB were similar with wt-HaNPV in Hz-AM1 as a result of the integration of *bst* into the cellular chromosomes. Whereas the normal cotton bollworm cell line Hz-AM1 and transformed cell line AM1-NB were infected with rHa-Bar, 48 h and 72 h post-infection, viral progenies in AM1-NB were 23 and 160 times greater than in Hz-AM1, respectively.

Pest-killing activity with rHa-Bar

Heliothis armigera larvae were infected with different HaNPVs and larval death statistics were performed 3 days after infection, whilst ruling out deaths with no symptoms of virus infection. Six days after infection experimental mortality rates were calculated. And the adjusted lethal rate was determined after adjusting versus appropriate controls (Table 1). The lethal dose (LD_{50}) and lethal time (LT_{50}) of both recombinant-HaNPV and wt-HaNPV were calculated. The results obtained show that the LD_{50} of wt-HaNPV was 2186 PIB/g, and that of rHa-Bar was 1749 PIB/g. These results represent a reduction of 20% compared to wt-HaNPV. The LT_{50} of wt-HaNPV was 7.2 days, and that of rHa-Bar was 5.1 days, the latter of which represents a reduction of 30% versus wt-HaNPV. These results suggest that rHa-Bar has potential use as a viral pesticide.

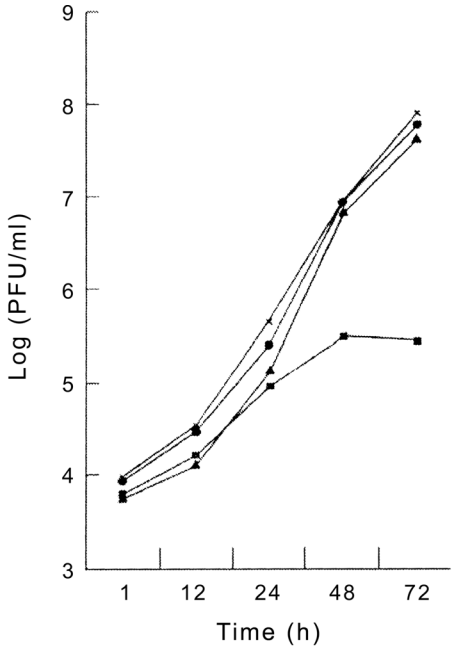

Fig. 8. Normal cotton bollworm cell line Hz-AM1 and transformed cell line AM1-NB were infected with rHa-Bar and wt-HaNPV, respectively. ■ : Hz-AM1 infected with rHa-Bar; ●: AM1-NB infected with wt-HaNPV; ▲ : AM1-NB infected with rHa-Bar; ×: Hz-AM1 infected with wt-HaNPV.

Discussion

The AcNPV Bac-to-Bac system (Luckow *et al*., 1993) provides a rapid and efficient method of generating recombinant AcNPV. In our previous work, an alternative Bac-to-Bac system, Bonpvid, was constructed by using hybrid p143 gene (helicase gene) to expand the host range of AcNPV to BmN cell line and silkworms (Zhu *et al.*, 1998a). Bonpvid is an expression system with increased versatility for producing foreign gene products on a large scale, since silkworms can be easily and cost-effectively cultured. Moreover, compared to BmNPV, recombinant AcNPV is less toxic to the host culture for the loss of some viral elements,

Table 1. Test of pest-killing activity of rHa-Bar and wt-HaNPV to 3rd instar larvae[a]

Virus PIBs/g[b]	2×10^5		2×10^4		2×10^3		2×10^2		2×10	
Type of virus[c]	r	wt	r	wt	r	wt	r	wt	r	wt
Lethal rate%[d]	91.5	86.3	83.7	70.8	66.9	47.4	41.2	31.3	27.8	20.6
Adjused lethal rate %[e]	94.3	88.9	86.3	72.9	68.9	48.8	42.5	32.3	28.7	21.3

[a]Number of 3rd instar larvae was 120 per group.
[b]PIB represents polyhedrin inclusion bodies, concentrations were determined by counting inclusion bodies under microscope.
[c]In the second line "r" represents the rHa-Bar and "wt" represents the wt-HaNPV.
[d]Lethal rateThe Lethal rate was obtained using conventional statistics.
[e]Adjusted lethal rate The adjusted lethal rate was calculated after eliminating dead with no symptoms of virus infection.

which have stricter species demands.

Therefore, disorders decreased to the lowest level, desirably sustaining mass production for foreign genes. In this work, a novel Bac-to-Bac system named Hanpvid, containing the HaNPV genome was developed *via* homological recombination between HaNPV and plasmid pTS containing the transposon/ shuttle cassette. The recombinant Hanpvid can be easily constructed in bacteria and provides a simple and programmed approach for obtaining recombinant HaNPV, and also presents more opportunities to screen out highly effective toxic genes for application as a baculovirus pesticide. There are some similarities between the Hanpvid system and the host range expanded recombinant Bonpvid system. However, since the Hanpvid system conserves the entire HaNPV genome, except for *polh*, which is mainly expressed in the very late phase and could be regained by insertion of a heterologous *polh*, it is appropriate and applicable to utilize Hanpvid as a special pesticide to control *Heliothis armigera*. The expression of foreign *bar* would greatly improve the toxicity of recombinant virus, and the original viral genome is intact, therefore the toxicity from wild type virus is substantially preserved.

Earlier research work regarding *barnase* and *barstar* mainly focused on the external folding of the protein-protein structure (Maugen *et al.*, 1982). Due to lethal activity to the host system, the application of *barnase* is always limited to a comparatively small range. More recently, some researchers have focused on the use of *barstar* to counteract *barnase* (Deyev *et al.*, 1998; Kuvshinov *et al.*, 2001; Jagannath *et al.*, 2001). Leuchtenberger (Leuchtenberger *et al.*, 2001) constructed a stable mammalian cell line expressing *barnase* driven by a promoter, which has a minimal basal activity in the uninduced state. The construction of the transformed cell line AM1-NB described in this work, in which *barstar* can be expressed stably should be a novel approach for the application of *barnase*.

bst driven by the IE1 promoter of AcNPV was integrated into the chromosome of cotton bollworm cells Hz-AM1, and *bar* driven by a strong polyhedron gene promoter of AcNPV was inserted into the Hanpvid genome. Pie1, an immediate early promoter active during both early and late phases of virus infection, is less effective than the polyhedron gene promoter, which is active only in the very late phase of virus infection (Maruniak *et al.*, 1981). When rHa-Bar derived from Hanpvid infects AM1-NB cells, the expression of *bst* driven by IE1 gene promoter inhibits the activity of *barnase*, which results in the normal proliferation of recombinant rHa-Bar in both early and late phases. However, in the very late phase, the expression of *bar* driven by *polh* gene promoter, which is more effective than Pie1, will be higher than that of *Barstar*, suggesting that the recombinant virus rHa-Bar has residual toxicity for transformed AM1-NB cells. For our purposes, two equally effective promoters driving *bar* and *bst* are much more desirable.

Because various pests cause serious damage to numerous crops annually and the highly lethal *barnase* leads to the death of *Heliothis armigera*, which is more resistant to traditional chemical insecticides (Cuyno *et al.*, 2001), the technique for AM1-NB construction and *barnase* utilization may be applicable to small scale insecticide production.

However, the production cost of a *bst* transgenic AM1-NB cell line would be prohibitive and could not sustain large-scale insecticide production. Presently, the incubation of baculovirus in host pests is still the major method for producing biopesticides. Unfortunately, compared to other baculoviruses and genetically engineered baculoviruses, the virion yield of rHa-Bar is relatively low due to its lethality to *Heliothis armigera*. We attempted to construct other recombinant Hanpvid containing *bst* but no *polh*, to rescue the replication of rHa-Bar when *Heliothis armigera* larvae are infected by rHa-Bar together with other recombinant Hanpvid carrying only *bst*. Since rHa-Bar regained the polyhedrosis phenotype, it is easy to isolate recombinant Hanpvid containing only *bst* from rHa-Bar, by for example sucrose gradient centrifugation. Even if a mixture containing rHa-Bar and *bst*-containing Hanpvid is utilized as an insecticide, there would be mainly rHa-Bar remaining to exert its biofunction if spilled in the field, since the latter lacks the polyhedrosis phenotype and can only infect larvae through oral administration, with difficulty.

NPV is more suitable than BV for pesticide production because it is more stable in the field and can penetrate the mid-gut tissues of pests after oral infection (Zhu *et al.*, 1998a). In the present study, the additional insertion of *polh* from AcNPV into the Hanpvid genome allowed the recombinant virus rHa-Bar to regain its polyhedrosis phenotype, because polyhedron protein was expressed, which greatly improved the toxicity of and demonstrated marked advantages of rHa-Bar as a biopesticide (Mishra *et al.*, 1998).

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