Expression of the fusolin gene of *Choristoneura fumiferana* entomopoxvirus in the baculovirus insect cell system

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The fusolin (*fus*) gene of *Choristoneura fumiferana* entomopoxvirus was cloned and expressed in insect cells via the baculovirus expression vector system using the *polh* promoter. A *Pst I*/*EcoRI* restriction enzyme fragment (1115 bp) which includes the *fus* gene was cloned into pBlueBac4.5. This engineered plasmid and linearized DNA of *Autographa californica* nuclear polyhedrosis virus (*AcNPV*) were cotransfected into *Spodoptera frugiperda* cells. Selection and purification of high level recombinant baculovirus were performed by polymerase chain reaction and plaque assay. Expression of the fusolin protein (approximately 38 kDa polypeptide) in *S. frugiperda* cells was determined by SDS-polyacrylamide gel electrophoresis.

*Key words: entomopoxvirus, *fus* gene, baculovirus, expression.*

**Introduction**

Entomopoxviruses (EPVs) are insect viruses that can infect various species belonging to members of the orders Lepidoptera, Coleoptera, Diptera and Orthoptera. EPVs have occlusion bodies, also called spheroids, which look like those of baculovirus (ERLANDSON, 1991). The spheroid of many EPVs contains the virions and another spindle-like structure, which seems to be composed of single protein subunit called fusolin (DALL et al., 1993), a protein toxic for insects. The fusolin protein, product of *fus* gene, is of agricultural importance. Because of the replication of a subfamily of entomopoxviruses (Chordopoxvirinae, poxvirus of vertebrates) in vertebrates, the entomopoxviruses are not used as biological control agent to kill harmful insects in the nature (ARIF, 1995). On the other hand, killing of harmful insects using baculoviruses is harmless since they do not infect vertebrates (LUCkow & SUMMERS, 1988, LUCkow, 1991).

In previous studies, it was shown by western blot analysis that anti-fusolin antibodies reacted positively with a protein of approximately 98 kDa (DALL et al., 1993). On the other hand, sequence analysis indicated that the cleaved mature protein has a predicted molecular mass of 37.730 kDa (YUEN et al., 1990; DALL et al., 1993). The discrepancy between the predicted molecular mass of...
fusolin and reduced mobility in polyacrylamide gel is still unresolved (ARIF, 1995). In order to help to understand this discrepancy, the initial step is to express the fus gene in a heterologous expression system.

Baculovirus is routinely used as a vector for high level expression of foreign genes in insect cell cultures. Polyhedrin (product 28 kDa) gene promoter is a strong promoter of very late gene (expresses between 20 and 72h p.i.) and is not essential for virus replication in cultured insect cells. It is exploited to drive the expression of foreign genes (KING & POSSE, 1992; O’REILLY et al., 1992). It is very important that recombinant baculoviruses and their products, which are near authentic and often biologically active, are used in biological control against harmful insects in agriculture. Since they are natural agents, they do not affect the ecological equilibrium dramatically (DEMIRBAĞ et al., 1998).

In order to generate a recombinant baculovirus with no potential hazard for use in agriculture as biological control agent and to confirm the molecular size of the cloned fus gene product, we aimed to express the fus gene of *Choristoneura fumiferana* entomopoxvirus in a baculovirus expression system.

**Material and methods**

**Insect cell culture and viruses**

The *Spodoptera frugiperda* (Sf-9) cell-line maintained in disposable tissue culture flasks (Corning), in TNM-FH medium supplemented with 10% fetal bovine serum was used (HINK, 1970). Linearized Bac-N-Blue DNA (BstU1-digested DNA of *Autographa californica* nucleo polyhedrovirus, Invitrogen) was utilized for transfection. Wild-type *Autographa californica* NPV (AcNPV) was used as a control.

**Plasmid vectors and bacterial strain**

pTZD1 (a gift from Dr. Just V. LAK, Wageningen Agricultural Univ., Wageningen, The Netherlands) was used as source of fus gene. The plasmid pBlueBac4.5 used to transfer the fus gene into AcNPV and Bac-N-Blue DNA used to generate expression vector were purchased from Invitrogen (USA). The *E. coli* JM101 strain was also used to transform pTZD1 and transfer vectors.

**Cloning the fus gene fragment into baculovirus transfer vector**

pTZD1 transformed into JM101 strain of *E. coli*, and transformed *E. coli* were grown on Luria Bertani (Difco) agar containing 100 μg/mL ampicillin using standard procedures (SAMBROOK et al., 1989). Plasmid DNA was isolated and digested by *PstI/EcoRI*. The restriction fragment (1115 bp) including complete fus gene (1071 bp) and additional nucleotides was purified from 1% agarose gel using Geneclean II (Bio 101) DNA isolation Kit. The purified fragment was cloned into *PstI/EcoRI* digested pBlueBac4.5 and transferred into *E. coli*. One of the recombinant plasmids carrying the effective size of fus gene fragment was designated as pAcRN1 (recombinant transfer vector).

**Infection and transfection of insect cells**

*S. frugiperda* cells were co-transfected by pAcRN1 and linearized DNA of AcNPV (Bac-N-Blue DNA) (O’REILLY et al., 1992). Recombination was performed between Bac-N-Blue DNA and pAcRN1. Thus, fus gene was inserted into Bac-N-Blue DNA by homologous recombination. Recombinant plaques were recognized by color development upon addition of X-gal (50 mg/mL) and by the absence of polyhedra in these cells. The putative recombinants were plaque-purified four times to reach genetic homogeneity (KING & POSSE, 1992; O’REILLY et al., 1992). Recombinant AcNPVs (AcNPV/RN1) were produced again in Sf-9 cells, viral titer (pfu/mL) was calculated by plaque assay and stored at 4°C until needed.

**Polymerase chain reaction (PCR)**

Recombinant AcNPVs (AcNPV/RN1) forms blue plaques, were produced in Sf-9 cells and viral DNA was isolated from extracellular progeny viruses. The fus gene was amplified by using 5’-TTTACTGGTTTGTAA-CAGTTTTTG-3’ and 5’-CAACACCGACGACAGATC-CAG-3’ primers which have been designed for flanking regions of *polh* (total 435 bases). PCR assay was performed in a 50 μL aliquot of reaction mixture containing 1 μg of viral DNA, 5 μL of 10X PCR buffer, 0.25 μM of each deoxynucleoside, 100 ng forward and 100 ng reverse primer, 0.3 μL of 50 mM MgCl₂, 1.5 units Taq DNA polymerase and sterile water to a final reaction volume of 50 μL. The cycle condition consisted of an initial 2 min denaturation at 94°C followed by 30 cycles of 1 min of denaturation at 94°C, 2 min of annealing at 55°C, 3 min of extension at 72°C and a final 7 min of extension at 72°C (Hybaid). Ten microliters PCR amplified samples were analysed in an agarose gel stained with ethidium bromide and visualised under UV transillumination.

**Expression of recombinant protein**

*S. frugiperda* cells in a 6-well disposable plate (1 x 10⁶ cells/well, Corning) were infected with a multiplicity of infection of 10 plaque-forming units (pfu) per cell of wild type and recombinant virus (AcNPV/RN1) (BNONIA et al., 1993). Uninfected (as control) and wild-type infected cells were harvested at 48 h p.i., however, recombinant virus-infected cells were harvested at 24, 48 and 72 h p.i., and lysed by a detergent lysis method (INVITROGEN, 1996). Protein extraction procedures were as described by O’REILLY et al., (1992). Proteins were electrophoresed in 10% SDS-PAGE at 30 mA according to LAEMMLI (1970) with a 20 μL of sample volume, and stained with silver nitrate as described by SAMBROOK et al. (1989).
Results

Construction of the transfer vector and expression vector

In this study, we expressed *fus* gene of entomopoxvirus in a *polh*-based *Autographa californica* nuclear polyhedrosis virus (AcNPV, Baculovirus) expression vector system. The first step of the engineering of a recombinant baculovirus was the construction of baculovirus transfer vector in which the coding sequence of the *polh* gene was replaced by the coding sequence of the *fus* gene of *Choristoneura fumiferana* entomopoxvirus (CfEPV, Entomopoxvirinae). The transfer vector pBlueBac4.5 contains, in addition to a unique cloning site downstream from the polyhedrine promoter, a gene cassette having lacZ, which is expressed under the control of pETL. This promoter is constitutively expressed in infected insect cells and drives the expression of the marker gene. pTZD1 contains the complete *fus* gene starting one base pair upstream of the ATG codon and ending 10 nucleotides downstream of the stop codon. A 1115 bp *PstI/EcoRI* fragment of pTZD1 which includes the *fus* gene, was inserted into pBlueBac4.5 to generate baculovirus transfer vector pAcRN1 (Fig. 1).

Since the genome of AcNPV is too large to insert the *fus* gene directly, insertion of foreign gene into AcNPV is performed only by ho-

![Diagram](image)

**Fig. 1.** Construction of the transfer vector pAcRN1. The *fus* gene fragment was excised from pTZD1 with *PstI/EcoRI* endonuclease and cloned into the transfer vector, pBlueBac4.5, linearized with *PstI/EcoRI*. The recombinant transfer vector pAcRN1 contains the *polh* promoter (*P_{PH})*, a bacterial β-galactosidase selection marker (*lacZ)*, the baculovirus early-to-late promoter (*P_{ETL})*, the *polh*-gene-flanking sequences for homologous recombination (ORFs) and the *fus* gene (dotted box).
mologous recombination. *S. frugiperda* cells were co-transfected with pAcRN1 and linearized DNA of wild type AcNPV, the Bac-N-Blue DNA, by “lipofection” (King & Posse, 1992; O'Reilly et al., 1992) helping the entrance of pBlueBac4.5 and Bac-N-Blue DNA into *S. frugiperda* cells. Recombinant viruses (AcNPV/RN1, Fig. 2) were distinguished from wild type virus in a plaque assay (Summers & Smith, 1987) by the absence of polyhedra and by their blue coloration with the addition of X-gal (50 mg/mL). Twelve “blue” plaques were purified to genetic homogeneity. The inserted *fus* gene and flanking region of *polh* (1550 bp) was amplified by PCR and was analysed in a 1% agarose gel (Fig. 3). However, the PCR amplification of the control showed a 839 bp fragment. The restriction pattern of these viruses was analysed and all the recombinant viruses appeared to have inserted *fus* gene fragment in the correct orientation as expected (data not shown). One clone was selected for further analysis.

Fig. 2. The construction of baculovirus expression vector, AcNPV/RN1, based upon the *polh*-replacement vector pAcRN1. After a co-transfection of transfer vector pAcRN1 and linearized baculovirus DNA, Bac-N-Blue DNA into *S. frugiperda* cells, the *fus* gene is introduced into the *polh* locus. The arrows indicate the direction of transcription.
Expression of fus gene

*S. frugiperda* cells, infected with wild type AcNPV, were harvested at 48 h p.i. and cells infected with recombinant virus (AcNPV/RN1) were harvested at 24, 48 and 72 h p.i., and analysed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In cells infected with AcNPV/RN1 polyhedrin (28 kDa) was absent, instead a protein of approximately 38 kDa was observed (Fig. 4).

Discussion

Baculoviruses are capable of expressing foreign genes under the control of the major late *polh* and *p10* promoters. The *polh* gene is dispensable for virus replication and the expression of a foreign gene is based on the allelic replacement of the *polh* gene (King & Possee, 1992; O’Reilly et al., 1992). Recombinants are usually recognized by their inclusion body negative appearance. Using a novel transfer vector pBlueBac4.5, the screening for recombinants containing the gene of interest was facilitated by the co-expression of X-gal as a marker. All analysed blue recombinant viruses displaying the identified DNA patterns had a correct insertion of the *fus* gene. The fact that these recombinants were easily retrieved suggests that the 38 kDa fusulin protein produced within *S. frugiperda* cells is not toxic intracellularly.

*S. frugiperda* cells infected with recombinant virus (AcNPV/RN1) were analysed by light microscopy (data not shown). The results of this investigation indicate the absence of polyhedra in cells infected with recombinant virus. Since the recombinant virus lacks the coding sequence of *polh* gene, the production of polyhedra is not expected. On the other hand, we observed the polyhedral inclusion bodies in *S. frugiperda* cells which were infected by the wild type AcNPV.

A 1550 bp fragment including the *fus* gene was amplified by PCR and analysed in a 1% agarose gel (Fig. 3). Fusolin protein was expressed in *S. frugiperda* cells at the expected size of 38 kDa at relatively high level. The protein was visible in SDS-PAGE by silver nitrate stain (Fig. 4) and it is identified as the fusolin. This result also confirms
the earlier studies (Dall et al., 1993; Yuen et al., 1990) indicating that fus gene encodes an approximately 38 kDa polypeptide. Previous studies demonstrated that anti-fusolin serum reacted positively with 98 kDa protein (Dall et al., 1993). Although presence of a potential glycosylation site was detected in the protein, chemical methods capable of detecting glycan moieties did not give positive results. We think that the fusolin is not glycosylated in baculovirus expression vector system. Therefore, an approximately 38 kDa protein was detected.

The results show that fus gene is expressed in baculovirus expression vector system, and the expressed fusolin polypeptide is about 38 kDa. Further studies will be performed to isolate, characterize and test the biological activity of this protein.

Acknowledgements

This study was supported by Karadeniz Technical University Research Foundation (KTU 96.111.004.7.).

References


Received November 25, 2000
Accepted September 3, 2001