

*Full Length Research Paper*

# Genetic analysis of baculovirus resistance in lepidopteran model insect *Bombyx mori* L.

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In order to clarify the resistant mechanism of BmNPV in silkworm, and from negative to prove agricultural pest inheritance of virus resistance, in this study, we used the highly resistant strain NB and susceptible strain 306 as the material through the method of classical genetics experiment, and proved that the baculovirus resistance in silkworm is controlled by a pair of autosomal dominant major gene. At the same time, we used random amplification of polymorphic DNA (RAPD) random primers to screen a molecular marker which are in high linkage with the resistant trait. Validity of the molecular marker was proved in BC<sub>1</sub>, F<sub>2</sub> populations, which further demonstrated that the baculovirus resistance in silkworm is controlled by a pair of autosomal dominant major gene. This can provide an effective research basis for the emergence of baculovirus resistance in pest and its resistant mechanism.

**Key words:** *Bombyx mori*, molecular markers, genetic analysis, biological control, baculovirus resistance.

## INTRODUCTION

Each year, the agricultural economic losses caused by the pest are difficult to estimate. Currently, there are two main methods of chemical control and biological control. Chemical pesticides are likely to cause environmental pollution and damage the ecological balance, so now; it is increasingly popular in the biological control method to reduce agricultural pest damage on the agricultural economy. Concern about the long-term sustainability of agriculture is driving interest in biological control programs to manage pest insect populations. Baculoviruses and *Bacillus thuringiensis* (Bt) are among the most important potential microbial control agents and are sometimes called biopesticides.

The *Helicoverpa armigera* nucleopolyhedrovirus (HaNPV) (Cherry et al., 2000) and the *Cydia pomonella* granulovirus (CpGV) (Huber, 1974) were used worldwide for biological control. The same as Bt gene, baculovirus was considered to be robust against development of insect resistance. In 2005, the first codling moth populations with up to 1000-fold that reduced

susceptibility to CpGV were reported in Germany and France, and then about 35 codling moth orchard populations resistant to CpGV products were observed in several European countries (Asser-Kaiser et al, 2011; Radtke et al., 2011). Through the classical hybridization analysis of genetic experiment, Asser-Kaiser et al. (2007) proved that the rapid emergence of baculovirus resistance in codling moth was due to dominant, sex-linked inheritance, but the identification, cloning, and specific regulatory mechanism of this resistant gene is still unclear.

Sericulture originated in China and spread around the world. There are more than 1000 varieties and strains in China, which has a wide range of characteristics. The silkworm, *B. mori* is an important economic insect and lepidopteran model insect. Unfortunately, the silkworm is particularly susceptible to virus diseases, especially due to *B. mori* nucleopolyhedrovirus (BmNPV), which result in great loss in sericulture. In order to clarify the resistant mechanism of BmNPV in silkworm, and from negative to prove agricultural pest inheritance of resistance to other baculovirus, firstly we carried out the identification of baculovirus resistance on Chinese silkworm resources. Through our investigation to the NPV resistance of 340 species, from the National Center for Silkworm Genetic

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**Table 1.** The segregation of BC<sub>1</sub> progeny after virus administration.

BC <sub>1</sub> population	S <sup>a</sup>	D <sup>a</sup>	Exp <sup>b</sup>	Act <sup>c</sup>	χ <sup>2</sup> <sup>d</sup>	P <sub>0.01,0.05</sub>
(NB×306)×306	206	239	1:1	0.86	1.227	3.84~6.63 <sup>e</sup>
(NB×306)×306	220	227	1:1	0.97	0.055	3.84~6.63 <sup>e</sup>

<sup>a</sup>The letter 'S' and 'D' represent the number of survival individuals and the number of dead individuals in BC<sub>1</sub> progeny, respectively. <sup>b</sup>The value of expectant. <sup>c</sup>The actual value. <sup>d</sup>The value of χ<sup>2</sup> test. <sup>e</sup>95% confidence interval when p < 0.05.

Resources Preservation of China, we found a Chinese local variety with high ability to resist NPV, named NB, whose median lethal concentration was 1000 times higher than the susceptible strain 306 (Chen et al. 1991). The result of real-time qPCR revealed that the relative copy numbers of BmNPV differed most dramatically (10<sup>4</sup> to 10<sup>5</sup> fold) at 72 h pi in the two *B. mori* strains (Qin, 2005). Several silkworm genes associated with resistance to the NPV have been discovered, such as RFP (Hayashiya et al., 1976), Lipases-1 (Nakazawa et al., 2003), gloverin-1,2,3,4 (Bao, 2010), serine protease (Tsuneishi et al., 2004), NADPH oxidoreductase (Selot et al., 2007) and so on.

Subtractive hybridization (Bao, 2008; Bao, 2010) and the research of proteome level have obtained several differential expression genes and proteins (Liu et al., 2008), but so far there is no baculovirus resistant gene cloned in insect. In recent years, Japanese scholars isolated the *nsd-2* gene resistant to the *B. mori* densovirus type 2 (BmDENV-2), and proved that the virus resistance is caused by a 6 kb deletion in the ORF of a gene encoding a 12-pass transmembrane protein, a member of an amino acid transporter family, and expressed only in midgut (Katsuhiko et al., 2008). This result provides an important idea for cloning the baculovirus resistant gene in *B. mori*. In order to clone the baculovirus resistant gene, in this paper we used RAPD random primers screen, a molecular marker which is highly linked with the resistant trait (Yao et al., 2003). Validity of the molecular marker was proved in BC<sub>1</sub>, F<sub>2</sub> populations, which further demonstrated that the baculovirus resistance in silkworm is controlled by a pair of autosomal dominant major gene.

## MATERIALS AND METHODS

### Silkworm strains

Through our investigation to the NPV resistance of 340 species in the National Center for Silkworm Genetic Resources Preservation of China, we found a local variety with high ability to resist NPV, whose median lethal concentration is 1000 times higher than sensitive species. After eight generation of system segregation and filter of high virus concentration, we obtained a high resistance specie, named NB (RR). The sensitive specie was named 306 (rr). A single-pair cross between a female (NB) and male (306) produced the F<sub>1</sub> offspring. For linkage analysis, BC<sub>1</sub> progeny from the cross (NB × 306) × 306 were used, and F<sub>2</sub> progeny from the cross (NB × 306) × (NB × 306) were used.

### Viral inoculation

Newly moulted second instar healthy larvae from BC<sub>1</sub> progeny and F<sub>2</sub> progeny were starved for 6 h, and then inoculated individually through ingestion of a 3 cm<sup>2</sup> piece of mulberry leaf coated with 10 ul suspension Obs (ODV and BV) of concentration of 4.8 × 10<sup>8</sup> NPV polyhedra/ml. After 48 h feeding, we started to survey the segregation of each batch from BC<sub>1</sub> population and F<sub>2</sub> population, and remained the material for the following DNA extraction.

### Selection and validation of molecular marker

Genome DNA was extracted by the method of Bender et al. (1983). Molecular marker of NPV resistance (R<sub>736</sub>) was selected, referred to the SCAR sequence (AY380833) published on the GenBank, and then a pair of specific primer (anti-NPV-F: 5'-GCTACGACCCAGACCTGTACTC-3'; anti-NPV-R: 5'-CGTGCCACGTAATGTAACA-3') was synthesized.

## RESULTS

### Genetic analysis of nucleopolyhedrovirus resistance in the BC<sub>1</sub> progeny from the cross (NB × 306) × 306

According to the result of segregation statistics in the BC<sub>1</sub> population after virus administration, we concluded that the resistant gene in the individual of BC<sub>1</sub> progeny came from the parent NB. The segregation ratio of the statistical results were in line with Mendelian segregation ratio of 1:1 (Table 1), which suggested that the resistant trait in NB is controlled by a pair of dominant gene.

### Genetic analysis of nucleopolyhedrovirus resistance in the F<sub>2</sub> progeny from the cross (NB × 306) × (NB × 306)

From the statistics result of F<sub>2</sub> population after virus administration (Table 2), we can see that the groups of basic statistical results were consistent with the silkworm segregation ratio of 3:1. It further demonstrated that the resistance in NB is controlled by a pair of dominant gene.

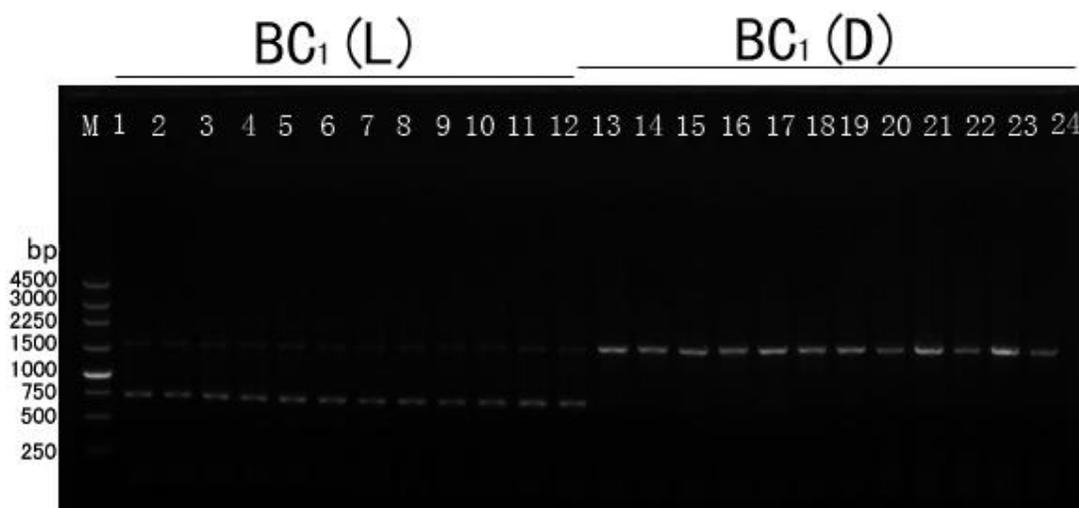
### Genetic analysis of NPV resistant molecular marker in silkworm

Experiment of virus inoculum showed that silkworm resistance to NPV was controlled by a pair of dominant gene. It was further verified by using molecular marker in

**Table 2.** The segregation of F<sub>2</sub> progeny after virus administration.

F <sub>2</sub> population	S <sup>a</sup>	D <sup>a</sup>	Exp <sup>b</sup>	Act <sup>c</sup>	χ <sup>2</sup> <sup>d</sup>	P <sub>0.01,0.05</sub> <sup>e</sup>
(NB×306) F <sub>2</sub>	315	134	3:1	2.35	2.710	3.84–6.63 <sup>e</sup>
(NB×306) F <sub>2</sub>	587	201	3:1	2.92	0.054	3.84–6.63 <sup>e</sup>
(NB×306) F <sub>2</sub>	403	117	3:1	3.44	0.897	3.84–6.63 <sup>e</sup>

<sup>a</sup>The letter 'S' and 'D' represent the number of survival individuals and the number of dead individuals in F<sub>2</sub> progeny, respectively. <sup>b</sup>The value of expectant. <sup>c</sup>The actual value. <sup>d</sup>The value of χ<sup>2</sup> test. <sup>e</sup>95% confidence interval when P<0.05.



**Figure 1.** Amplification result of primer anti-NPV-F and anti-NPV-R against the populations of BC<sub>1</sub> progeny. The letter 'L' and 'D' represent the live individual and death individual, respectively. Lane M, DNA marker; lane 1 to 12, the live silkworm individuals in BC<sub>1</sub> generation after virus administration; lane 13 to 24, the death silkworm individuals in BC<sub>1</sub> generation after virus administration.

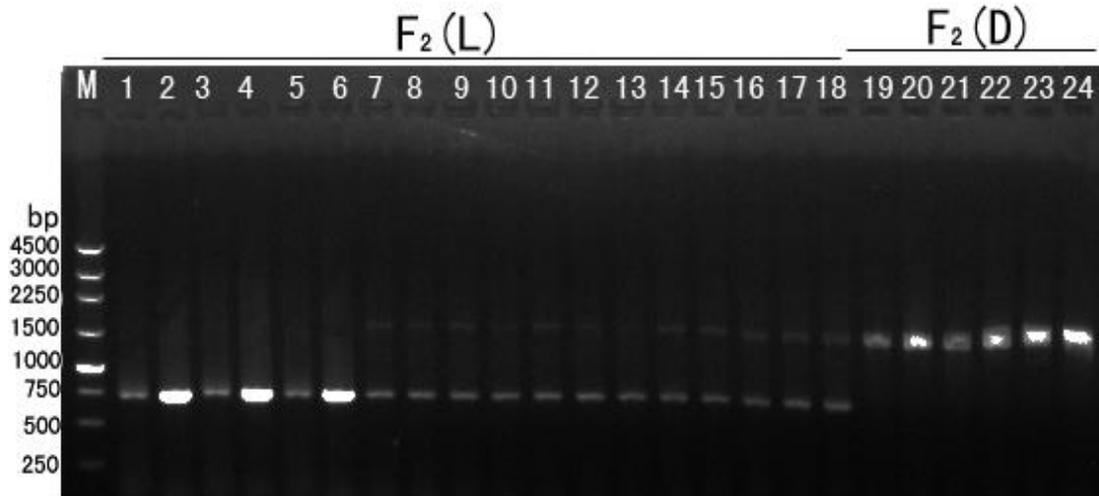
the BC<sub>1</sub> population and F<sub>2</sub> population. Sampling 400 individuals in BC<sub>1</sub> generation were detected, in which about 200 individuals survived after virus administration. At the same time, sampling 400 individuals in F<sub>2</sub> generation were detected, in which about 300 individuals survived after virus administration. The PCR result of molecular marker is shown in Figures 1 and 2, from which we can see that molecular marker segregation ratio in the BC<sub>1</sub> and F<sub>2</sub> population was in line with the segregation ratio after virus inoculation (1:1 and 3:1). This also showed that the NB variety resistant to NPV should be controlled by a dominant major gene from the other side.

## DISCUSSION

Biological control of agricultural pest is one of the most environmentally sustainable development strategy. The use of transgenic Bt crops can effectively protect crops from pest attack, which can reduce the large-scale use of chemical pesticides (Jin et al., 2003). Baculovirus and its transformation products can have the same effective

function (Moscardi, 1999) but the biological control is faced with the ineffective shortcoming which is caused by the increased pest resistance. Insects have evolved many different ways to defend themselves against pathogens (Narayan, 2004), and studies have shown that many agricultural pests have involved the developmental resistance to baculovirus infection with decreasing susceptibilities at older larval stages (Teakle et al., 1986). However, the relevant research about this mechanism of resistance to the virus is still at the initial stage. The silkworm, *B. mori*, is an important economic insect and lepidopteran model insect. In recent years, the result of whole-genome shotgun sequencing in silkworm was reported by Southwest Agriculture University and Genome Research Department of Japan (Xia et al., 2004; Mita et al., 2004), and the BmNPV's sequencing result has been completed, which provide a powerful tool for the research of reciprocal evolution between host and virus as well as the inheritance of resistance mechanism.

Some studies showed that baculovirus was considered to be robust against development of pest resistance, and the resistance was not stable without selection pressure (Huber, 1974; Fuxa, 1993). Our preliminary experiments



**Figure 2.** Amplification result of primer anti-NPV-F and anti-NPV-R against the populations of  $F_2$  progeny. The letter 'L' and 'D' represent the live individual and death individual, respectively. Lane M, DNA marker; lane 1 to 18, the live silkworm individuals in  $F_2$  generation after virus administration; lane 19 to 24, the death silkworm individuals in  $F_2$  generation after virus administration.

of BmNPV resistance identification in silkworm showed that there was only a highly resistant strain found in 344 varieties and strains, so, from the perspective of the resistance evolution, this should be an accidental event and the result of gene mutation. This is the only resistant strain obtained after eight generations of system separation and purification, and now there is little infection caused by BmNPV. This study further proved that the resistance trait in NB was controlled by a dominant major gene. On the contrary, the susceptible strain in our lab is still susceptible to BmNPV after several generations of virus inoculation. This result suggests that the resistance was an event of mutation, and the natural selection and artificial selection only remove the susceptible individuals or groups to retain the resistant individuals. Therefore, baculovirus were considered to be robust against development of insect resistance (Moscardi, 1999; Cory, 2003).

Whether the gender differences of baculovirus resistance gene existed in insect, German scholar proved that codling moth exist resistant differences to baculovirus, and the resistant gene was located on the sex chromosome, through investigation of Mendelian genetics experiment (Asser-Kaiser et al. 2007). Silkworm is the larva of the domesticated silk moth and is easy to distinguish between male and female individuals. Our long-term experiments of silkworm resistance identification showed that the resistant gene in silkworm should be located on the autosome not the sex chromosome, on which there are only micro-effect modifier genes. The result is different from the conclusion of the German scholar. We speculate that there may be two reasons; one is that although the codling moth and silkworm are the same for Lepidoptera,

but their species and genome size are quite different as well as the gene distribution; the other is that the CpGV and BmNPV are baculovirus, and their genome has many similarities, but they belong to the two different genus of baculovirus, in which the CpGV is granulosis virus and its infection is between cells by using an ancient F membrane protein, while the BmNPV is nucleopolyhedrovirus and its infection was mediated by a membrane protein GP64, encoded by itself (Wang et al., 2002). Until now, the two resistant gene have still not been identified, so there is a debate about whether the two resistant genes are the same or not, and this poses the need for further research. At the same time, we also look forward to exposing the insect baculovirus resistance mechanism and genetic mechanism of resistance.

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