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Transgenic breeding of anti-*Bombyx mori* L. nuclear polyhedrosis virus silkworm *Bombyx mori*

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Silkworm strains resistant to *Bombyx mori* L. nuclear polyhedrosis virus were obtained through transgenic experiments. *piggy*Bac transposon with an A3 promoter were randomly inserted into the silkworm, driving the enhanced green fluorescent protein (*EGFP*) reporter gene into the silkworm genome. Polymerase chain reaction results verified the insertion of the extraneous *EGFP* gene, and fluorescence microscopy showed that the *EGFP* was expressed in the midgut tissue. The morbidity ratio of the nuclear polyhedrosis decreased from 90% in the original silkworm strain to 66.7% in the transgenic silkworm strain. Compared with the resistance to the *Bombyx mori* L. nuclear polyhedrosis virus in the Qiufeng strain, which is commonly used in the production, there was an increase of 33 centesimal points in the transgenic silkworms. The antivirotic character in the Chunhua×Qiuyue strain, which was bred from a different transgenic family, was about 10 centesimal points higher than that in the Qiufeng×Baiyu, another crossbreed used in production. Our results indicated a good application value of the transposon-inserted mutation in the breeding of anti-BmNPV silkworm strain.

Keywords silkworm; transgenic; *piggy*Bac; BmNPV; antivirotic

Nuclear polyhedrosis is caused by the *Bombyx mori* L. nuclear polyhedrosis virus (BmNPV), a member of the subfamily Eubaculovirinae of the family Baculoviridae [1]. Nuclear polyhedrosis is one of the deadliest diseases that can strike the mulberry silkworm, *Bombyx mori*. The most economical and effective way to prevent the disease is by breeding the antivirotic silkworm strain. Previous genetic development research has shown that resistance to BmNPV is controlled by one major gene and several minor genes belonging to the incomplete patroclinous heredity with the effective gene number of 2.31 [2,3]. There are significant differences among the various silkworm strains, such as between the antivirotic strain and the normal strain, as the latter is three times as susceptible to nuclear polyhedrosis. Our results showed that the resistance to the virus was positively related to the silkworm's cocoon weight, suggesting that the successful breeding of antivirotic silkworm strains may possibly be the economical merit strains.

Rcently, the application of transgenic technology conducted by the injection of *piggy*Bac transposon has become widely used [4]. Previous research has shown that the Nistari strain has high transformation efficiency when injected with the *piggy*Bac transposon [5]. Also, extraneous target genes have been successfully inserted and expressed in the silkworm by *piggy*Bac transformation system [6–8].

Random insertion of a *piggy*Bac transposon into the silkworm genome changes gene expression. Based on this, we screened transgenic silkworm stranis with high resistance to BmNPV.

Materials and Methods

Materials

Nistari, a non-diapause, multivoltine silkworm strain, were bred and raised by Zhejiang University's Laboratory of

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Germplasm Innovation and Molecular Breeding in Silkworm and Bee (Hangzhou, China). The *piggy*Bac transposon (pPIGA3GFP) with A3 promoter and enhanced green fluorescent protein (*EGFP*) reporter gene as well as the helper plasmid (pHA3PIG) were provided by Dr. Toshiki Tamura (National Institute of Sericultural and Entomological Science, Tsukuba, Japan).

Embryo injection and screening of transgenic silkworms

Embryo injection and polymerase chain reaction (PCR) analysis were carried out [5]. In brief, eggs were harvested at the syncytial preblastoderm stage 1–6 h after being laid in at 25 ºC environment. The mixture of vector pPIGA3GFP and helper plasmids (15–20 nl; 1:1, 0.4 μg/μl total DNA concentration) in 0.5 mM phosphate buffer (pH 7.0) containing 5 mM KCl was microinjected into each egg. The embryos were allowed to develop at 25 ºC and 75% humidity. G_0 moths were mated randomly. In G_1 generation, we screened the positive individuals by observing EGFP fluorescence with an Olympus SZX12 stereomicroscope (Tokyo, Japan)

Excitation filter spectral width was set between 460 nm and 490 nm, and emission filter spectral width between 510 nm and 550 nm was used for detection. G_1 positive transgenic individuals were mated and generated G_2 generation.

Total DNA was extracted from the posterior silk glands of G_2 fifth instar larvae that had been frozen in liquid nitrogen and stored at –20 ºC. The obtained DNA (<1 μg) was used for PCR analysis. The target fragment of 1341 bp nucleotide of pPIGA3GFP was amplified using the primer pair 5′-ACGACGGCAACTACAAGACC-3′ and 5′-GCGGAGAATGGGCGGAACT-3′. Amplification was carried out with a 4 min denaturing step at 96 ºC, followed by 35 cycles of 1 min at 96 ºC, 1 min at 60.9 ºC, 1.5 min at 72 ºC and a final extension at 72 ºC for 10 min. Amplified products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Comparing the resistance to BmNPV

The single G_2 batch was reared, and the positive individuals mated to generate G_3 silkworms. We chose 90 silkworms from each family for resistance testing, and others were reared for the strain reserve. Each set of 90 silkworms were divided into three groups at the beginning of the third instar. Three mulberry leaves (4 cm×4 cm) incubated with 200 μl BmNPV (5×10^7/ml) were added to each test group. The silkworms were kept at room temperature (26 ºC –27 ºC). The morbidity ratio was investigated in the forth instar.

Results

Screening positive transgenic silkworm

In total 25,058 eggs were microinjected, and 708 female moths and 869 male moths were obtained from G_0 generation. All of them were fertile and had a percent of 6.29% in the total microinjected eggs. In G_1 larvae, the positive transgenic individuals with *EGFP* were found in 214 broods with a percent of 13.57% to the total living moths (708+869) and 0.854% in the total eggs (25,058). PCR analysis in G_2 larvae showed that the *EGFP* gene had been inserted and expressed in the genome of transgenic silkworms (data not shown). The anatomical images observed under fluorescence indicated that the *EGFP* driven by the A3 promoter, which mainly promotes gene expression in muscle tissue, could be expressed in midgut (**Fig. 1**).

Comparing resistance to BmNPV in transgenic silkworm strains

Ten transgenic families numbered T1–T10 were chosen for antivirotic testing, and the normal Nistari strain was used as control. The transgenic families' resistance to BmNPV was elevated compared with the control's. The morbidity ratio decreased from 90% in the control group and to 66.7%, the lowest ratio, in T1 transgenic group, suggesting that resistance to BmNPV approximately be increased by 23.3 centesimal points (**Table 1**). Comparing the results of the T1, T4, T6, T7 and the most commonly used silkworm strain, Qiufeng, showed that resistance to BmNPV was elevated by approximately 33.4 centesimal points in the T7 silkworm family (**Table 2**).

Hybridized combinations of anti-BmNPV silkworm strains

The T4 and T6 families were crossbred with the strains Feng1 and Qiufeng, two strains used in the production. The T7 family was hybridized with the strains 54A and Baiyu. In the crossbreeding process, the *EGFP* was used as a maker and the strains with high resistance to BmNPV were reserved. At the same time, other economic characters were also analyzed. After five generations of selection, two hybridized combination strains, Chunhua×Qiuyue and Qiuyue×Chunhua, from the transgenic silkworm strain were obtained. The hybridized strains' resistance to BmNPV was compared to that of strains Qiufeng×Baiyu and Baiyu×Qiufeng, which were used in production. The concentrations of BmNPV used for the test were 5×10^6/ml and 5×10^7/ml. Evidence of high

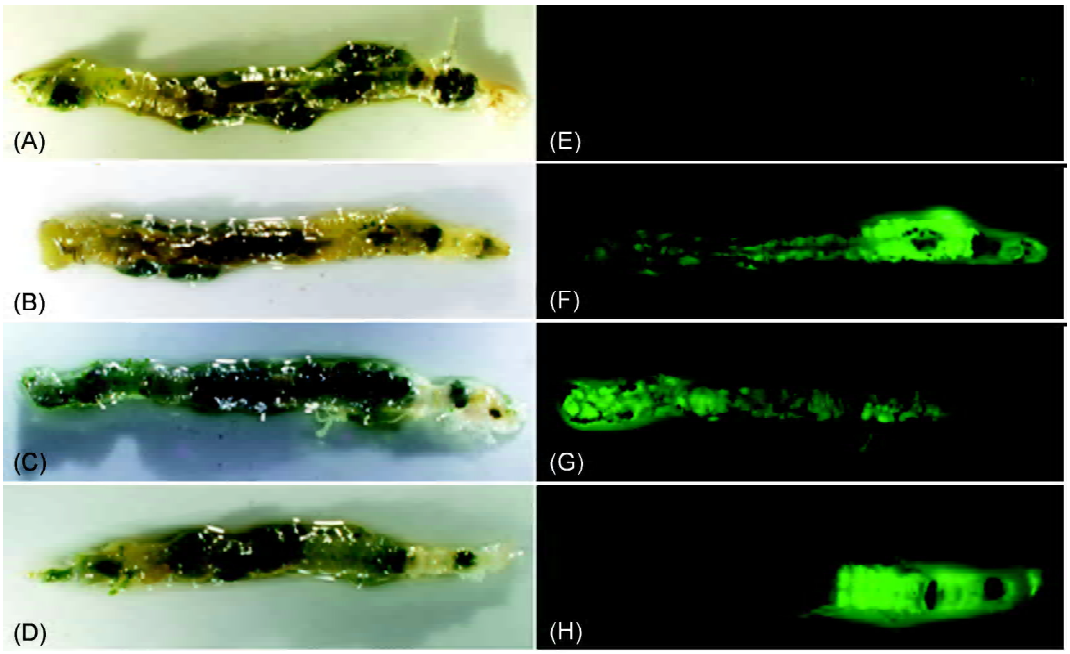

Fig. 1 Enhanced green fluorescent protein gene expression in the midguts of G_3 transgenic silkworms (A) The midgut from non-transgenic silkworm under the bright-field. (B–D) The midguts from transgenic sikworms under the bright field. (E) The midgut from non-transgenic silkworm under the green fluorescence fields. (F–H) The midguts from transgenic sikworms under the green fluorescence fields.

Table 1 Morbidity ratio of different transgenic silkworm families treated with BmNPV (%)

Strain	CK	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
Morbidity ratio	90.0	66.7	80.0	81.1	81.1	81.1	72.2	73.3	87.8	86.7	84.4

Table 2 Morbidity ratio in different transgenic silkworm families and Qiufeng treated with BmNPV (%)

Strain	Qiufeng	T1	T4	T6	T7
Morbidity ratio	96.7	90.0	86.7	71.1	63.3

resistance to BmNPV in the hybridized transgenic silkworm strains became more apparent when treated with higher concentrations of BmNPV. The Chunhua×Qiuyue reciprocal hybrids had a 10.28 centesimal point resistance to BmNPV, which was higher than that of the Qiufeng×Baiyu reciprocal hybrids (**Table 3**).

Discussion

It has been proven that the *piggy*Bac transposon was most probably inserted into the function genome areas when being transformed [9]. Using RNA interference technology, researchers have found that target silence of baculoviral immediate early-1 gene as well as other preventative mechanisms could increase the silkworm resistance to baculovirus [10,11]. The silence of essential viral gene *lef-1* of BmNPV may also induce silkworm resistance to BmNPV [12]. Our results showed that in the transgenic silkworms, A3 promoter in the *piggy*Bac transposon could induce extraneous gene expression in the midgut tissue (**Fig. 1**), and some transgenic silkworm families showed high resistance to BmNPV (**Tables 1–3**). These results excluded the target gene silence of BmNPV related genes that acquire a rather common resistance to BmNPV in several transgenic silkworm strains.

There are two possible reasons for this phenomenon. One is that the transposon may be inserted into a functional locus in the genome that is related to silkworm resistance to BmNPV, thus resulting in the low morbidity ratio in transgenic silkworms as the insertion of transposon

Table 3 Morbidity ratio of hybridized combinations treated with different concentrations of BmNPV (%)

Hybridized combinations	Control (water)	BmNPV	
		5×10^6/ml	5×10^7/ml
Qiufeng×Baiyu	0.00	12.22	26.67
Baiyu×Qiufeng	0.00	3.33	38.33
Average	0.00	7.78	32.50
Chunhua×Qiuyue	0.00	8.89	30.00
Qiuyue×Chunhua	3.33	1.11	14.44
Average	1.67	5.00	22.22

affected the physiology of resistance to BmNPV. The mechanism may be related to the activation of BmNPV receptor in midgut or the reduced binding ability between them. The other possibility relates to the EGFP protein expressed in the midgut as a result of the A3 promoter. Interestingly, no matter where the transposon was inserted into the genome and regardless of the transgenic family, all the resistances to BmNPV increased, indicating that the expression of EGFP in the midgut might be protective to the silkworm (**Tables 1–3**). Previous research also reported that EGFP, RFP (red fluorescent protein) afford some protection against the damaging effects of ultraviolet light in baculoviruse [13]. We assumed that EGFP functioned similarly in this case. Both of the above hypotheses are possible, while the latter holds greater potential. However, these mechanisms still need to be verified by further researches.

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