RESEARCH AND DEVELOPMENT OF BIOENGINEERING PESTICIDE, *Bacillus thuringiensis* WG-001

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ABSTRACT

The biopesticides produced by *Bacillus thuringiensis* have been widely used in the world. On the foundation of exploiting the resource of *Bacillus thuringiensis*, insecticidal crystal protein genes and helper protein genes were cloned, the appropriate recipient strains were screened, different kinds of vectors were constructed, and expression and regulation of insecticidal crystal protein gene were studies. Environmental safe, highly effective genetically modified *Bacillus thuringiensis* strains with stable expression have been constructed. *Bacillus thuringiensis* WG-001 was the first genetically modified pesticide strain which is safety used as a commercial product in China.

KEYWORDS: *Bacillus thuringiensis*; insecticidal crystal protein genes; genetically modified strains; gene expression and regulation

1. INTRODUCTION

Bacillus thuringiensis (*B.t*) can produce insecticidal active protein which has specific insecticidal toxicity that is lethal to insects in their growth metabolism. It can be produced as a biopesticide which can be used in biological pest control. Currently, the research and

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development of *B.t* products are still very popular. Many manufacturers produce a variety of products. The advantages of these products are (1) They are essentially nontoxic to people and pets, and less harmful to the environment. (2) Difference from chemical insecticides, *B.t* insecticides do not kill beneficial insects which includes the natural enemies of insects, as well as beneficial pollinators. (3) The strain can be improved by bioengineering technique to increase the output and effect, and lower the cost. (4) The natural resistance to *B.t* developed by insects is relatively slower because of the diversiform insecticidal crystal protein that *B.t* produces [1]. With the development of *B.t* molecular biology and genetics, the construction of effective engineering strain has become an interesting issue in microbial pesticide research. Even though *B.t* products have become the world most-widely used biopesticide, there are still some disadvantages associated with these products. For example, the highly specific activity of *B.t* insecticides might limit their usage and resistance of pests to the toxin may be developed. Therefore, while exploiting the resource of *B.t*, existing strains need to be modified. The research project is sponsored by the State Key Technology Program, and the genetically modified strain has been constructed using the strategy as follows (Fig. 1).

Construction elements of genetically modified strains

1. Insecticidal crystal protein genes (ICP genes)

The main active component of *B.t* is coden by ICP gene. The ICP genes of different subspecies and strains are different not only on their quantities and existed states, but also on expression levels and insecticidal specificity. Since the first ICP gene from subsp. *kurstaki* [2] was cloned in 1981, a lot of novel ICP genes have been cloned. Until November 20, 2003, 293 genes belonged to 42 classes have been listed. The *cry* genes have been divided to 40 classes and the *cyt* genes have been divided to 2 classes [3].

ICP gene *cry1Ac10* from *B.t* YBT-1520 was cloned in State Key Laboratory of Agricultural Microbiology of Huazhong Agriculture University (HZAU) in 1997. It is the first one that has ever been cloned in China. Since then, 28 ICP genes, such as *cry1Fb3*, *cry2Ab3*, *cry3Aa7* have been cloned in China. Several ICP genes which have activity to *Lepidoptera, Diptera, Coleoptera,* plant and zoo parasitic nematode, and *vip* genes were cloned in HZAU laboratory (Table 1). Compared with *cry1Ac1* to *cry1Ac9,* the difference in

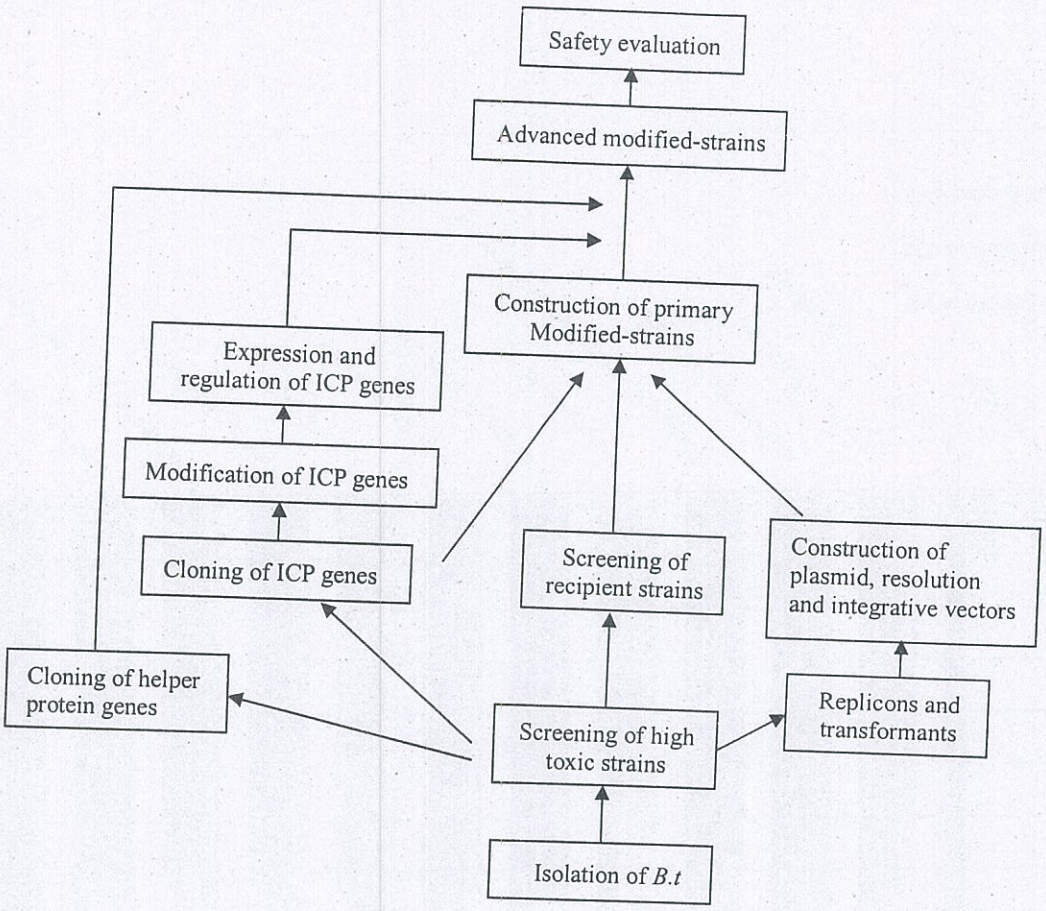

Fig. 1 Construction strategy of genetically modified strains of *Bacillus thuringiensis*

cry1Ac10 is only 4 bp [4]. But the expressed product from *cry1Ac10* indicated higher toxicity to *Helicoverpa armigera* and *Plutella xylostella*. So, *cry1Ac10* is appropriate to be used as a target gene in the construction of modified-strain.

2. Helper protein genes

B.t produces various pesticidal proteins during the stationary phase and the protein usually accumulates in the parent cell in the form of parasporal crystal. However, insecticidal crystal proteins are not always expressed as parasporal crystal. A new kind of PCR cloning technique was developed in HZAU laboratory, which can make the new cloned structural gene correctly pitch the downstream of RBS and promoter. At the same time, it does not bring

Table 1 ICP genes and *vip* genes cloned in HZAU laboratory and their activity

Genes	Pests
cry1Aa10	Lepidoptera
cry1Ac10	Lepidoptera
cry1Ca6	Noctuidae
cry1Ea6	Nematode
cry2Aa5	Lepidoptera Diptera
cry2Aa6	
cry2Aa7	
cry3Aa7	Coleoptera
cry6Aa2	Nematode
cry11Aa	Diptera
cytA	Diptera
vip83	Lepidoptera:*Spodoptera exigua* *Spodoptera litura* *Helicoverpa armigera*

code-shift mutation, whatever enzyme site is appropriate [5]. The helper protein genes *p19*, *p20* and *cytA* from subsp. *israelensis* have been cloned by adopting this technique and studied for their interaction effects. Through the influence on the function of *CytA* protein on cell lysis, it was firstly proved there are biological differences between *P19* and *P20*. Later on it was found that *P19* could be used to enhance the production of *cytA* [6,7,8]. Recombinants having *p19* and *p20* were induced to *B.t* strain which has only *cry1Ab* gene. It was proved that *P19* and *P20* could be used to enhance the production of *cry1Ab* (Fig. 2).

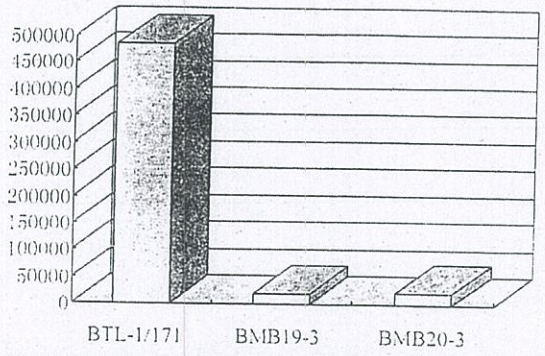

Fig. 2 Enhanced expression of *P20* to *cry1Ab*

The recombinants were then induced to the wild strain YBT-803-1, in order to prove the enhancement to ICPs of wild strain. The process is done for the first time in the world. After that, its enhancement to the production of *cry1Ac* was proved. This enhancement mechanism of *P20* on expression of *cry1* gene was firstly established, which inaugurated a new approach to improve the ICPs genes' expression of modified strain [9].

3. Vectors

A vector is a carrier tool, which can transfer DNA from one cell (a donor) to another cell (a recipient). It plays an important role in construction of engineering strains. Facing the difficulties of the incompatibility of plasmids in construction of modified strain and the unnecessary fragment in gene manipulation, three types of vectors were constructed.

3.1 Plasmid vector:

Three different kinds of replicons were cloned from subsp. *kurstaki* that has high toxicity to Lepidoptera, and subsp. *tenebrionis* that is highly effective for Coleoptera. Then, three new kinds of replicon vectors, which have diverse replication properties, especially from above two subspecies, were constructed. Plasmid replicon ori9741 and small plasmid pBMB2062 from subsp. *kurstaki* YBT-1520 [10,11] were cloned (Fig. 3).

Plasmid replicon ori165 from subsp. *tenebrionis* strain 165 was also cloned. This was the first plasmid replicon cloned from subsp. *tenebrionis* [12]. The coexist of different replication region vectors in a recipient strain could avoid incompatibility and retain stable heredity of plasmid.

3.2 Resolution vector:

Resolution vectors pBMB801 and pBMB1808 [13], which can easily be manipulated in *E.coli*, were constructed using *res* site of Tn4430 (Fig. 4). These resolution vectors, which are similar to endogenetic plasmid of *B.t*, can automatically eliminate those unnecessary foreign fragments such as *E. coli* plasmid fragment, antibiotic gene and reporter gene under the interior resolutionase of *B.t*, and retain the necessary fragment of modified strain at the same time. This kind of modified-strain is the same as natural strain and does not have any safety concern. So, this approach can also be used in construction of other microbial modified-strains.

3.3 Integrative vector:

By using translocation features of Tn4430 and Tn5401, target gene can be transferred to

genome, while transposase does not move. This can improve the stability of target gene. The integrative vectors that were constructed include pBMB14R, pBMB14F, pBMB13R and pBMB13F [14] (Fig. 5).

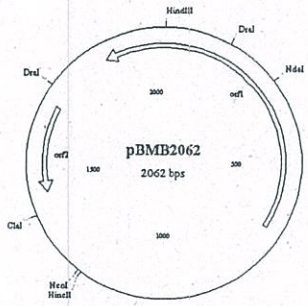

Fig. 3 Restriction enzyme map of plasmid vector pBMB2062

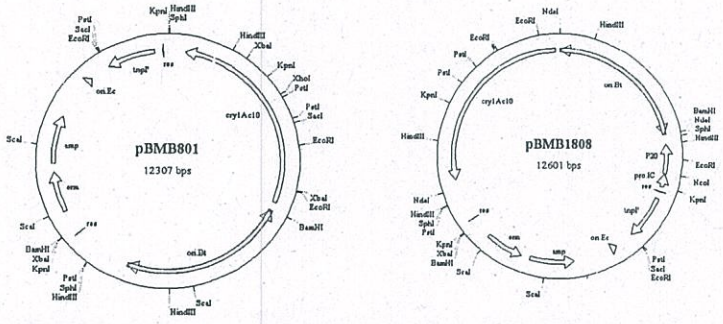

Fig. 4 Restriction enzyme map of resolution vector pBMB801 and pBMB1808

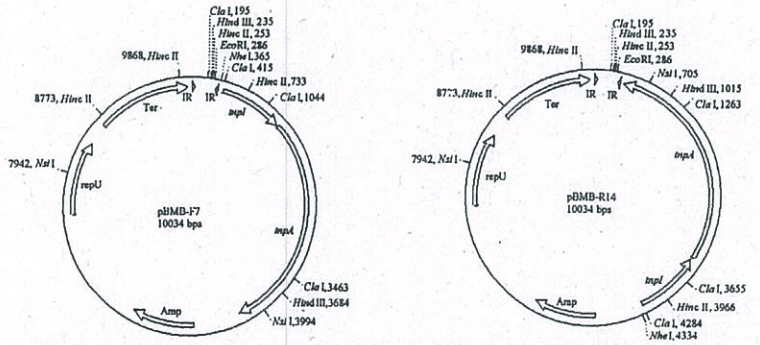

Fig. 5 Plasmid map of integrative vector pBMB-F7 and pBMB-R14

4. Recipient strains

When transferring target ICP gene to host strain, the features of host strain can decide whether or not target gene could be induced in, and also the frequency of transformation.

Therefore, mutant strains which are free of crystal and mutant strains which have neither crystal nor plasmid were screened. Then, naturally high-toxic strains which are appropriate for transformation and expression were screened.

After gradual increased temperature, 9 crystalliferous mutants were screened. By further eliminating plasmid of 4 strains, mutant strain BMB171 (free of plasmid and crystal) was finally obtained. Its transformation frequency is 10^2 higher than international standard host strains with different vectors (Table 2). This laid the foundations for further research of the expression and regulation of ICP genes and of the interactions of different genes [15].

Table 2 Transformation frequency of 4 different alien plamids DNA to different receptors by electroporation

Strain	Transformation frequencies (transformants/μgDNA)			
	pHT3101	pBMB1736	pBTL-1	pHV1249
BMB160	2×10^5	7×10^4	1×10^3	5
BMB168	3×10^5	1×10^4	2×10^3	5
BMB170	4×10^5	3×10^4	2×10^3	40
BMB171	8×10^5	2×10^4	7×10^3	200
YBT-1463	0.1	6	25	0

Table 3 Wild recipients with highly toxic

Strains	Genotypes	Target insects
YBT-1520	*cry1Aa,cry1Ac,cry1Ab,cry2Aa*	**Lepidoptera**: *Helicoverpa armigera*
YBT-1535	*cry1Aa, cry1Ab, cry1Ac, cry2*	**Lepidoptera**: *Plutella xylostella*
YBT-1221	*cry1Aa, cry1Ab, cry1Ac, cry2*	**Lepidoptera**: *Spodoptera exigua*
YBT-0618	*cry3A, ery2*	**Coleoptera**: *Phaedon brassicae*
YBT-917	*cry4A, cry11A*	**Diptera**: *Culex.sp*
YBT-1416	*cry1Aa, cry1C, cry1D, cry2*	**Lepidoptera**: *Spodoptera exigua*
YBT-833	*cry1Ab, cry1A, cry1Aa, cry2*	**Lepidoptera**: *Plutella xylostella*
CT-43	*cry1Ab, cry2Aa, cry2Ab, cry1B*	**Lepidoptera**: *Plutella xylostella* **Diptera**: *Culex.sp* **Coleoptera**:

From more than 1800 *B.t* strains which were isolated by the laboratory at HZAU, some highly toxic wild strains were screened. Those wild strains which have both specific sensitivity to different insect, such as YBT-1520 strain with high toxicity to *Lepidoptera* [16], YBT- 1535, YBT-1221 and YBT-1416 with high toxicity to *Spodoptera exigua* [17], YBT-0618 with high toxicity to Coleopteran insects [18, 19], YBT-917 with high toxicity to Dipteran insects and CT-43 with broad spectra activity to Lepidoptera, Diptera and Coleoptera [20,21], were further screened (Table 3). Their plasmid profiles and gene types, and the activity of single genetic expression product to different specie insects were analyzed. These laid the foundation for independent intellectual property right of the construction of modified-strains. Among the screened-strains, a very special one, YBT-1520, produces the 130 and 65 kDa proteins which have higher toxicity to *P. xylostella* and *H. armigera* than that of HD-1 (Diple- 2X) and NRD-12 strains. It also contains *cry1Aa, cry1Ab* and *cry1Ac* genes and especially, *cry1Ac* has the highest copy number, and is the most appropriate gene to be used as a host strain [22].

Expression and regulation of ICP genes

1. Modification of promoters

Researches on the strong promoter BtI in the *B.t* overlapping promoter BtI-BtII were performed. The results are as follows:

(1) With special deletion by PCR, the fragments of *cry1A* promoter upstream were obtained. The fragments were ligated with a shuttle vector pHT-pSGMU before the BtI promoter. Then, the recom- bined plasmids with deletion of promoter of *cry1A* were transformed into *B.t* strains. The effect of deletion on the expression of *BtI-lacZ* was tested. The results indicated that with deletion in the inverted region and not in bend region, the expression of *BtI-lacZ* in strain 80-21 was identical with full upstream of promoter of *cry1A*. In subsp. *aizawai*, the expression of *BtI-lacZ* with deletion was obviously lower than the wild upstream of *cry1A* promoter (Table 4). The results also suggested the bend region of *cry1A* promoter upstream played an important role in the transcriptional regulation of cry genes [23].

(2) Using different mutants of *cry1A* promoter upstream and testing their effects on expression of *BtI-LacZ* fusion gene, the results indicated that the mutant action in the bend

Table 4 Influence of different mutants of *cry1A* promoter upstream to the expression of *cry-lacZ* fusion gene in different strains

Recombinant strain	Specific β -galactosidase activity
80-21/272wt – *lacZ*	39.4
80-21/272B end – *lacZ*	14.9
80-21/272DM – *lacZ*	23.4
strain 5/272wt – *lacZ*	10.8
strain 5/272B end – *lacZ*	32.1
strain 5/272DM – *lacZ*	18.5

region of *cry1A* promoter upstream caused the decrease in β-gelactosidase activity of *BtI-LacZ* in strain 80-21, which was about 2-2.5 times lower than that with wild *cry1A* promoter upstream. If mutations were in the bend region and in the inverted region at the same time, the increased or decreased margin of *BtI-LacZ* expression would be reduced significantly (Fig. 6). This suggested that different sites of *cry1A* promoter upstream might have different functions in the transcriptional regulation of *cry1A* gene. The regulation would vary in various strains of *B.t* [24].

(3) Study on PDH-E2 binding protein indicated that E2 protein, which would exist mainly in the form of soluble protein, was produced in sporulation phase and its production time and concentration was related to the regulation of *cry1A* upstream [25].

(4) The genes with mutational or deletional upstream of *cry1A* pomoter have different utilization of primary carbon source. This suggested that the expression of *cry* genes was related to the primary carbon sources and the metabolism in cells of *B.t* [26].

(5) In order to investigate the influence of the carbon source on expression of *cry1D-lacZ* in *B.t* with different genetic backgrounds, *cry1D-lacZ* was introduced into different strains and cultured in liquid medium which contained different carbon sources. By analyzing the activity of β-galactosidase, it indicated that *cry1D-lacZ* expressed differently in the same host because of different carbon sources and differences in different hosts utilizing the same carbon sources, which suggested that metabolism path- way of carbon source in HD-133-5 was special. When sodium acetate acted as carbon source, *cry1D-lacZ* could keep high, stable expression in different hosts (Table 5). These results were useful for future fermentation of *B.t* [27, 28].

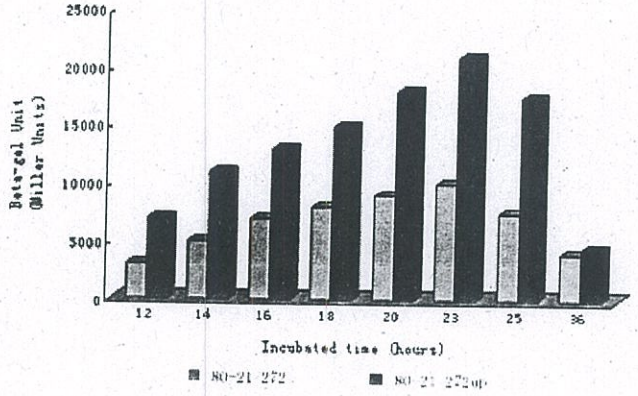

Fig. 6 Influence of *cry1Ab* upstream to the expression of *BtI-lacZ* fusion gene

The sequence analysis of *cry2Ab* suggested that the insertion of *BtII* to upstream of *cry2Ab* resulted in the lack of expression of this gene. Therefore, it is proved that *ORF2*'s positive regulation to *cry2Ab* [29]. These new discoveries provided important academic information for construction of highly expressed modified-strains.

Table 5 Expression activity of different strains with *cry1D-lacZ*

Strains	Activity of β-galactosidase with different carbon sources		
	0.2 % glucose	0.4 % sodium succinate	0.4% sodium acetate
80-21/*cry-1Dwtup-lacZ*	1360	708	1300
5/*cry-1Dwtup-lacZ*	1002	1085	1200
4Q7/*cry-1Dwtup-lacZ*	686	346	810
4Q7/*cry-1DSDup-lacZ*	1789	1140	1800
80-21/*cry-1DSDup-lacZ*	2647	1913	2800
5/*cry-1DSDup-lacZ*	2116	2006	2000

In addition, for convenience of gene's usage, a *BamH*I enzyme site to upstream and a *Hind*III enzyme site to downstream of *cry1Ac10* gene were introduced simultaneously [30]. The promoters of *cry1Ac10, cry1C* and *cry1E* genes, and sporulation-dependent promoters, were replaced with *cry3A* gene promoter, which is a nonsporulation-dependent promoter [31]. The transcription terminator of *cry1C* gene was replaced with that of *cry1Ac10* gene (Fig. 7). By adopting this strategy, if modified-genes were transferred into natural strain with high

toxicity, the expression would not be affected by the native crystal protein genes, which are sporulation-dependent. Then, the recombined strain would highly express crystal proteins, and be expected to have higher toxicity or wider insecticidal spectrum.

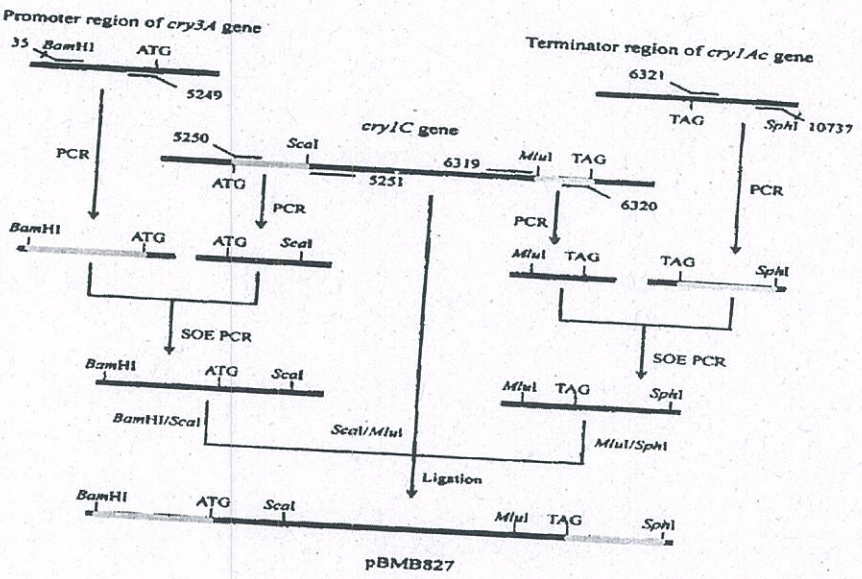

Fig. 7 Alteration of ICP gene *cry1C*

2. Post-transcriptional regulation

Highly effective and expressed modified-strain can be constructed by utilizing the enhanced helper proteins. The laboratory at HZAU introduced the site-specific recombinant plasmid pBMB1808, which contained *P20* helper protein gene and *cry1Ac10* gene, to three wild strains YBT1520, YBT1535 and YBT833, which are highly toxic to *Lepidoptera*, such as *H. armigera* and *P. xylostella*. The expression of 130kDa ICPs of three transformants was more than two times higher than that of host strains. Para- sporal crystals were about 2.5-3 times bigger than those of wild strains. During synthesis, 20kDa helper protein can protect *Cry1Ac* protein from being digested by cell proteinase but it cannot prevent the protoxin digestion after synthesis. Finally, it would improve the expression of *cry1Ac* and the volume of augment crystal, and also increase its insecticidal activity. It was found that 20Kda helper protein can be used in the construction of modified-strains with highly expressed ICPs [32].

Construction of genetically modified-strains

Based on the studies above, several recombined plasmids, which had obvious features, were chosen. They were then induced into different host strains. The following four modified-strains with practical value were constructed.

(1) After transforming the recombined plasmid that contained *cry1Ac10-P20* to the *B.t* mutant strain BMB171, which was free of crystal and plasmid, modified-strain BMB201Ac were obtained. Its ICPs product was 3.5 times more than that produced by the recombined strain BMB304/171, which has only *cry1Ac* gene (Fig. 8) and its insecticidal activity to *H. armigera* of BMB201Ac has 2.5-time improvement [33] (Table 6).

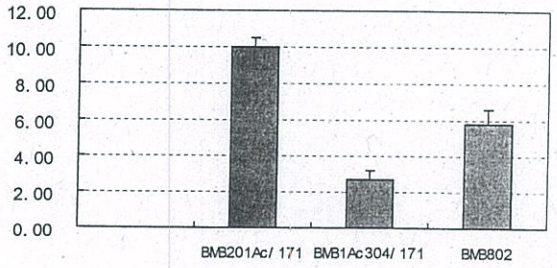

Fig. 8 Effects of 20kDa protein on the expression of *cry1Ac10*

Table 6 Toxicities of *cry1Ac10* transformant against *H. armigera* larvae

Strains	BMB201Ac/171	BMB1Ac304/171	BMB801
LC_{50}	2.632	6.579	4.831

*The results after 72 h infection. ICPM medium was used to cultivate bacteria, LC_{50}: ml/ml

(2) After shortening the plasmid which contained *cry1C*, from 8.5kb to 5.5kb, recombined plasmid pBMBLC-1 was obtained. It was then transformed to wild strain YBT-1535 to obtain modified-strain WG-004, which had 15.6-fold higher toxicity to larva of *Spodoptera exigua*, 140% higher toxicity to *H. armigera*, and 113.64% higher toxicity to *P. xylostella*, compared to natural strain (Table 7) [34].

(3) After transforming the recombined plasmid containing *cry3A* gene to *B.t* wild strain YBT-803-1, wild spectrum modified-strain BMBY-001 was obtained. It indicated high toxicity to *Phyllodecta vulgatissima* larva (LC_{50}0.413 μ l/ml) and to *P. xylostella* (LC_{50}3.319 μ l/ml)

Table 7 Receptor YBT-1535 and its transformant toxicity to three pests

Pests	Strains	Regression equation	Correlative coefficient	LC50 (ml/ml)
Plutella xylostella	YBT-1535	Y=1.390+2.676x	0.92	0.244
	WG-004	Y=1.393+2.835x	0.98	0.180
Helicoverpa armigera	YBT-1535	Y=1.068+1.911x	0.98	1.497
	WG-004	Y=2.600+0.807x	0.97	0.942
Spodoptera exigua	YBT-1535	Y=1.384+0.739x	0.98	78.181
	WG-004	Y=6.870+3.211x	1.00	4.974

Table 8 The toxicity of YBT803-1 and BMBY-001

Pests	Strains	Regression equation	Correlative coefficient	LC50 (ml/ml)
Phyllodecta vulgatissima	YBT803-1	Y=8.8782+1.1461x	0.9870	Not Toxic
	BMBY-001	Y=8.8782+1.1461x	0.9870	0.4132
Plutella xylostella	YBT803-1	Y=9.0422+1.3736x	0.9924	1.1647
	BMBY-001	Y=9.9583+1.8378x	0.9734	3.3189

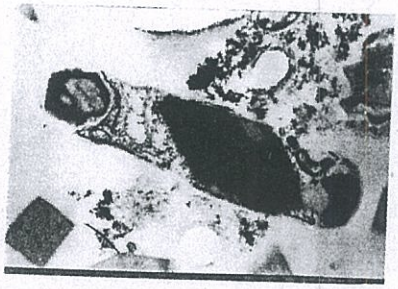

Fig. 9 TEM observation on the spore and crystal in WG-001 (×20,0000)

Table 9 The size of parasporal crystals in YBT1520 and WG-001

Strains	YBT-1520	WG-001
Length	1.85	2.45
Width	0.8	1.06
L/W	2.31:1	2.31:1
V	0.39	0.92

*The values are the average of 30 crystals in each samples; length unit is μ m, volume unit is μ m^3.

Table 8 [35, 36].

(4) After transforming the recombined plasmid pBMB1808 containing *cry1Ac10-P20* to YBT- 1520 strain, which was high toxic to *H. armigera*, the highly effective modified-strain WG-001 was obtained (Fig. 9). Compared with natural strains, its ICPs product was doubled, and parasporal crystal volume was expanded by 2.1-fold (Table 9) [32]. It also indicated

effectiveness in prevention and curing for *H. armigera* and *P. xylostella*.

2. CONCLUSION

In 1998, with the approval of Committee of Safety Evaluation of Agricultural Biogenetic Engineering (CSEABE), the highly effective *B.t* modified-strain WG-001 was tested in Hebei and Guangdong provinces in China. The results indicated that modified-strain WG-001 had no effect on animals, plants and natural enemies of insects, which were inside and around the test area. Based on safety conclusions, environmental release was applied in September, 1999 and commercial production of WG-001 began in 2002. Thus, WG-001 becomes the first genetic modified-strain insecticide that will be registered in China .

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