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Construction of Plant Expression Constructs Harboring Full-length *Bt* Cry1Ac22 Toxin Gene and Truncated Functional Domains of *Bt* Cry1Ac22 Toxin and Arabidopsis Transformation

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Abstract The full-length *Bt* toxin gene (3 534 bp) and truncated functional domains of *Bt* toxin (1 959 bp) of *cry1Ac22* were amplified by PCR from *Bt* strain W015−1. The PCR products were ligated into plant expression vector pBI121 cutting off the ß−glucuronidase gene to make the constructs of pBI121−Cry1Ac22F and pBI121−Cry1Ac22T. The constructs carrying with *Kanamycin* resistant marker were transferred into T-DNA vector and then validated by restricted enzyme digestion and PCR identification. Arabidopsis transformation with the transfered T-DNA vector were performed during the flowering stage mediated by *Agrobacterium tumufaciens*. Transgenic Arabidopsis seeds with positive *Kanamycin* resistance were harvested in this research that facilitate the understanding of *Bt* toxin functions in plant transgenic breeding.
Keywords *Bt* Cry1Ac22 toxin; *Bt* toxin functional domain; Plant expression construct; *Arabidopsis thaliana*; Genetic transformation

Background

Bt-toxin protein gene is the most commonly used insect-resistant gene, which is widely applied in crops such as transgenetic *Bt* cotton, *Bt* rice and woods like poplar eucalyptus to kill the pests of Lepidoptera, Coleoptera and so on (van Wordragen et al., 1993). *cry1Ac* is a insect disinfestation gene which has been extensively used in transgenetic cotton exhibited high toxicity against Lepidopteran. According to the research, toxicity peptide of insecticidal crystal protein Cry1Ac is composed of three typical structural domains. Structural domain Ⅰ locating in peptide chain at N-terminus, which is an alpha helical bundle, formed by six or seven amphipathic alpha helix enclosing a hydrophobic alpha helix, participated plasmalemmal perforation.

The domain Ⅱ is located in the middle of peptide chain. It is an anti-parallel β−pleated sheet consisted of three Greek key topological structures. And its apical loop participate in the integration of toxin and receptor protein. While the domain Ⅲ at C-terminus is a sandwich structure which consisted of two anti-parallel β−pleated sheets, lining up in jelly roll topological structure, and can prevent protease having over degradation on toxin molecule (Schnepf et al., 1998).

cry1Ac22 gene was cloned from the strain W015−1 isolated from the dissected guts of diapausing larvae of the silkworm (Xie et al., 2010), which can be efficiently expressed in the *E. coli* (Liu et al., 2010) and yeast (Liu et al., 2010). The expressional products have showed high insecticidal activity against larvae of *Plutella xylostella*. The expressed Cry1Ac22 toxicity protein in *E. coli* can be hydrolysed into a molecular weight of about 65 kD protein, which still showed insecticidal activity (Xie et al., 2010). And it is consistent with the former report that the Cry1Ac protein expressed in the *Bacillus thuringiensis* can be

hydrolysed to the 65 kD core protein (Lenin et al., 2001). Therefore, it is essential to carry out the research to express the total length *cry1Ac22* and the functional domain from *Bt* W015−1.

In the experiment, we cloned the full length of *cry1Ac22* gene of 3 534 bp in length and its function domains of 1 959 bp by PCR, and constructed them to plant expression vector pBI121 to make the constructs of pBI121−Cry1Ac22F and pBI121−Cry1Ac22T both carrying with *Kanamycin* selection marker.

Arabidopsis thaliana was transformed during the flowering stage mediated by *Agrobacterium tumufaciens*, lots of transgenic *Arabidopsis thaliana* seeds with positive *Kanamycin* resistance were harvested in this research that facilitate the understanding of *Bt* toxin functions in plant transgenic breeding.

1 Results

1.1 Cloning of the full length *cry1Ac22* gene and its function domain

cry1Ac22 full length gene of 3 534 bp (Figure 1A) and *cry1Ac22* function domain of 1 959 bp (Figure 2A) were amplified by specific primers. Both of the target genes were ligated to pMD18−T vector and positive clones were identified by enzymetic digestion of *Bam*HⅠand *Sal*Ⅰ, respectively (Figure 1B). And then named as pMD18−T-Cry1Ac22F for full length gene (Figure 1C) and pMD18−T-Cry1Ac22T for truncated gene (Figure 2B), respectively. Finally, we sequenced the positive clones to validate the sequence of Cry 1Ac22 by GenBank database.

1.2 Plant expression construct of pBI121−Cry1Ac22F and pBI121−Cry1Ac22F

Full length gene *cry1Ac22* cutting from recombinant

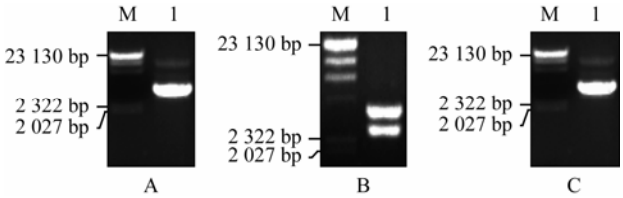

Figure 1 PCR amplification of *cry1Ac22* and digestion of pMD18−T-Cry1Ac22F by restriction enzymes
Note: M: λ DNA/*Hin*dⅢ; A: 1: *cry1Ac22*; B: 1: pMD18−T; C: 1: Digestion of pMD18−T-Cry1Ac22F with *Bam*HⅠand *Sal*Ⅰ

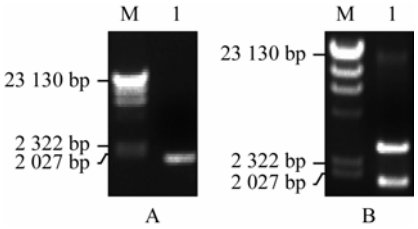

Figure 2 PCR amplification of *cry1Ac22T* and digestion of *cry1Ac22T* with *Bam*HⅠand *Sal*Ⅰ
Note: M: λ DNA/*Hin*dⅢ; A: 1: *cry1Ac22T*; B: 1: Digestion of pMD18−T-Cry1Ac22T with *Bam*HⅠand *Sal*Ⅰ

plasmid pMD18−T-Cry1Ac22 was ligated into plant expression vector pBI121 to make a plant expression construct named as pBI121−Cry1Ac22F, and then was transformed into *Escherichia coli* JM109. While the construct of pBI121−Cry1Ac22T for truncated gene was constructed by the same way. Both of the constructs were validated by digesting with *Bam*HⅠand *Sal*Ⅰ. A 3.5 kb target fragment and a 18 kb vector fragment was cut for pBI121−Cry1Ac22F (Figure 3). Whereas a 1.9 kb target fragment and a 18 kb vector fragment for pBI121−Cry1Ac22T (Figure 4).

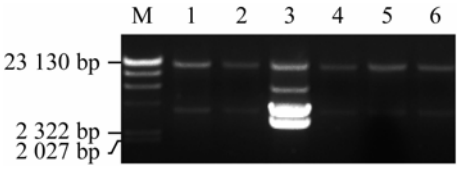

Figure 3 Digestion of pBI121−Cry1Ac22F with *Bam*HⅠand *Sal*Ⅰ
Note: M: λ DNA/*Hin*dⅢ; 1~6: Transformants; 1,2,4,5,6: Positive clones

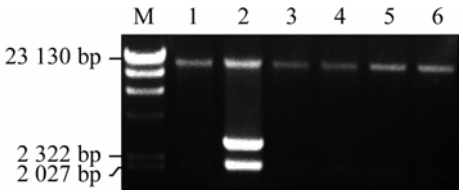

Figure 4 Digestion of pBI121−Cry1Ac22T with *Bam*HⅠand *Sal*Ⅰ
Note: M: λ DNA/*Hin*dⅢ; 1~6: Transformants; 1,3,4,5,6: Positive clones

1.3 Identification of *Agrobacterium* recombinant plasmid by PCR

The constructs were transformed into *Agrobacterium* EHA105, and the plasmids were extracted by using commercial extract kits. The positive clones were amplified and identified by the same specific primers as

for the constructs. A 3.5 kb target band for pBI121−Cry1Ac22F (Figure 5), and a 1.9 kb target band for pBI121−Cry1Ac22T (Figure 6), were amplified that indicated both of them have been transformed into *Agrobacterium* EHA105 successfully.

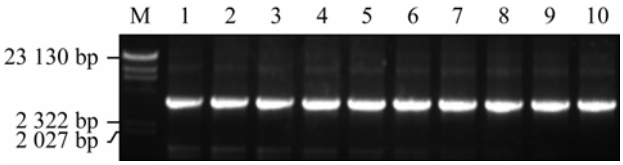

Figure 5 PCR identification of pB1121−Cry1Ac22F
Note: M: λ DNA/*Hin*dⅢ; 1~10: Positive transformants

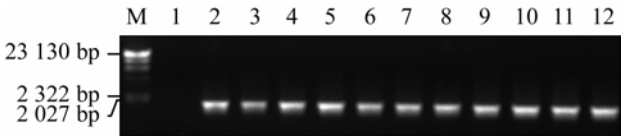

Figure 6 PCR identification of pB1121−Cry1Ac22T
Note: M: λ DNA/*Hin*dⅢ; 1~10: Positive transformants

1.4 Identification of of transgenic *Arabidopsis thaliana*

The *cry1Ac22* full length gene and *cry1Ac22 truncated* gene were transformed into the genome of *Arabidopsis thaliana* plants mediated by *Agrobacterium*, respectively. We identified that the plants of No.3, No.4, No.6, No.7, No.8 and No.10 were exsiting the 3.5 kb *cry1Ac22* full length fragment, (Figure 7).

Whereas for truncated *cry1Ac22T* transformation, we found that *cry1Ac22T* gene has been integrated into *Arabidopsis thaliana* plants with the plants of No.3, No.5, No.6, No.9, No.10 (Figure 8).

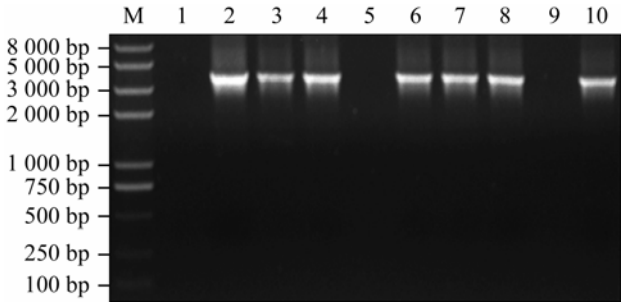

Figure 7 Identification of transgenic *cry1Ac22F* plants
Note: M: DL2000 plus ladder, 1: Negative control (DNA from non-transgenic plants); 2: Positive control (pBI121−Cry1Ac22); 3~10: DNA from putative transgenic plants

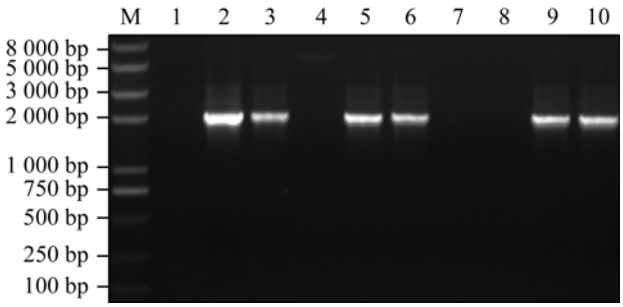

Figure 8 Identification of transgenic *cry1Ac22T* plants
Note: M: DL2000 plus ladder, 1: Negative control (DNA from non-transgenic plants); 2: Positive control (pBI121−Cry1Ac22T); 3~10: DNA from putative transgenic plants

2 Discussions

Bacillus thuringiensis cry1A toxin genes are the most commonly used as insecticidal targeted gene, among them, *cry1Ac* gene was extensively used for developing transgenic *Bt* cotton. There are numbers of reports that the full length *cry1A* genes were transformed into many crops including cotton, rice, potato, corn, canola, soybean, sugarcane, peanut etc. but the amounts of *Bt* toxins expressed in these transgenic plants were considerable low and were not enough toxic dose to the targeted pests (Romeis et al., 2006). These researches indicated that the full length sequence of the *cry1A* gene be not suitable for developing transgenic crops. Therefore, enhancement of *Bt* toxicity in transgenic plants would be essential goals in *Bt* transgenic breeding.

In this research, we designed two kinds of plant expression constructs, pBI121−Cry1Ac22F and pBI121−Cry1Ac22T, and transformed into the model of dicot plant, *Arabidopsis thaliana*. Both of the gene were transformed into Arabidopsis genome. The preliminary studies on the expressed toxin in Arabidopsis plant indicated that the expression of truncated domain be higher than that of full length gene (data not showed). We believed that the *Bt* toxin shoud be well designed for transgenic plants than completely employed from those deposited in GenBank.

3 Materials and Methods

3.1 Materials and reagent

Escherichia coli JM109, pMD18−T vector, pBI121 plant expression vector were deposited in our lab. DNA restriction endonucleases *Sac* Ⅰ, *Bam*HⅠ, DNA Marker, T4 DNA ligase, *Taq* DNA polymerase and

DNA extraction kit were bought from TAKARA company. Antibiotics, biochemistry and molecular biological reagents were bought from Shanghai Bioen gineering Company, Harbin Demei Biology Company etc. Primers were synthesized by Beijing Aoke Biotechnology Company. Other reagents were all domestic analytically purity.

3.2 Extraction of plasmid, purification and preparation of competent cell for *E. coli*

Plasmid DNA of *E. coli* and *Agrobacterium* were extracted by alkali lysis method (Zhang et al., 2000); the competent cells for the *E. coli* were prepared by the $CaCl_2$ method followed by the method of Sambrook et al (1992).

3.3 Primer designed and synthesized

Two pairs of specific primers were designed based on the conserved *cry1Ac22* gene sequence and function domain (Table 1), which were synthesized by the Nanjing Jinsite Biotechnology Company. The restriction enzyme site *Bam*H Ⅰ was added in the forward primer, the *Sal* Ⅰ was added in the reverse primer.

Table 1 PCR primers

Genes	Primers	Sequences (5'−3')
cry1Ac22F	Forward primer	GGATCCATGGATAACAATCCGAACATC
	Reverse primer	GTCGACTGAGTTTGCATGAGACTATTC
cry1Ac22T	Forward primer	GGATCCATGGATAACAATCCGAACATC
	Reverse primer	GTCGACTTACGTAACTAAATTGG

Note: The restriction sites are underlined

3.4 Cloning of *cry1Ac22F* and *cry1Ac22T*

We respectively amplified the *cry1Ac22F* gene and *cry1Ac22T* using the W015−1 genomic DNA as templates with specific primer. PCR procedure is as following: 94℃ for 5 min in advance, 30 cycles of 94℃, 1 min, 54℃, 1 min and 72℃, 30 s, 72℃, 10 min for extension and then 4℃ until moved to use. The PCR products were electrophoretically separated in the agarose gel and then were recovered to ligate to the pMD18−T vector. Two sequencing clones of *cry1Ac22F* and *cry1Ac22T* were completed to be transformed into the competent cell. The positive recombinants were identified by the *Bam*H Ⅰ and *Sal* Ⅰ digestion and then were sequenced.

3.5 Construct of pBI121−Cry1Ac22F and pBI121−Cry1Ac22T

pBI121 vector is a commonly plant expression vector driven by the CaMV35S promoter with kanamycin resistant gene. The recombinant plasmid of pMD18−T-Cry1Ac22F and pBI121−Cry1Ac22T were digested by the restriction enzymes *Bam*H Ⅰ and *Sal* Ⅰ. The digested fragments were ligated into the pBI121 vector that was also digested with the same restriction enzyme, and then were transformed into *E. coli* JM109 and identified by the restriction enzyme. The positive clones of pBI121−Cry1Ac22F and pBI121−Cry1Ac22T were screened by the enzyme digestion. Plant expression constructs of pBI121−Cry1Ac22F and pBI121−Cry1Ac22T were showed as the figure 9 and figure 10.

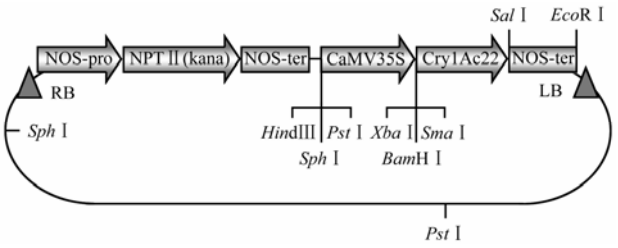

Figure 9 Structure of the construct of pBI121−Cry1Ac22F

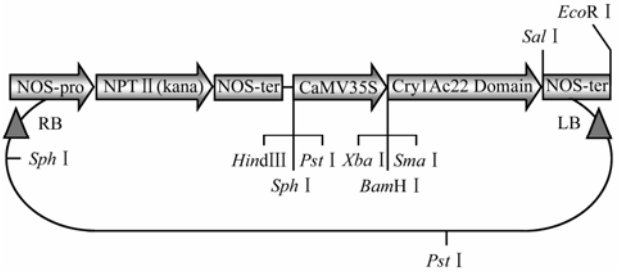

Figure 10 Structure of the construct of pBI121−Cry1Ac22T

3.6 Identification of *Agrobacterium* recombinant constructs by PCR

These two constructed plant expression vectors were transformed into the *Agrobacterium* competent cells. The *Agrobacterium* plasmid DNA was extracted using the alkali split kits, the positive clones were identified by using the primers that were used to constructs of pBI121−Cry1Ac22F and pBI121−Cry1Ac22T in the table 1. PCR procedure is followed as: 94℃, 5 min for pre-denaturation, 30 cycles of 94℃, 1 min, 54℃, 1 min and 72℃, 30 s, finally 72℃, 10 min for extension and then 4℃ for ever. The PCR products were electrophor etically separated in the agarose gel.

3.7 Transformation of Arabidopsis

Arabidopsis (*Arabidopsis thaliana*) was transformed by the approach of inflorescence infiltration, followed as the procedures of Clough with slight modifications (Clough et al., 1998). *Agrobacterium* baterials with constructs of pBI121−Cry1Ac22F and pBI121−Cry1A c22T respectively were inoculated in 10 mL LB liquid medium, and shaked incubation at 28℃ over a night, and then added the incubating liquid into 500 mL LB liquid medium on the ratio of 1% bacteria. While the density of *Agrobacterium* reached OD_{600}=1.0~1.2 at 28℃, the strains were collected by centrifuged with 3 000 r/min at 4℃ for 15 min , After removing the supernatant liquid and the precipitate was dissolved into medium with 1/2 MS with 5% (w/v) Sucrose and 0.044 umol/L Benzylamine purine to suspend *Agrobacterium* and then make its final density reach 5 μL/L by adding surfactant SilwetL−77.

The inflorescence of *Arabidopsis thaliana* was immersed into the infiltration utensils filled with 500 mL suspensions by soaking in *Agrobacterium* liquid medium for 10~15 min and then continuing to culture in darkness for 2 days. Transformed plants were cultivated in the normal growth conditions.

3.8 Validation of positive transgenic Arabidopsis by kanamycin selection and PCR detection

Kanamycin resistant selection and PCR detection were carried out according to the experimental methods of Clough et al (1998). Putative transgenic Arabidopsis seeds were soaked by *Agrobacterium*, treated by the 95% ethanol and 3.0% Sodium hypochlorite containing 0.05% Tween and then rinsed by 4~5 times in the sterile water. Steriled seeds were suspended in 2 mL 0.1% steriled agarose and seeded into 9 mm dish in diameter, 4 mg seeds per dish which contained 1/2×MS with 50 μg/mL kanamycin (KM) and 0.8% agorose. Culture conditions were as follows:

Firstly, these dishes were treated at 4℃ 2 days, then treated at 24℃ for 23 h light and 1 h darkness cultured 7~10 days. Finally, the status of plantlet colour and root developmental condition was easily judged whether the plantlets harbored resistant gene.

The resistant plantlets were transplanted into culture pan. The transgenic Arabidopsis genomic DNA were extracted from the Arabidopsis young leaves carrying the kanamycin resistant gene by using modified CTAB method. Then employing non-transgenic Arabidopsis genomic DNA as negative control, pBI121−Cry1Ac22F and pBI121−Cry1Ac22T plasmid as positive control, we used transgenomic DNA as template to identify the target genes by using specific primers for *cry1Ac22F* and *cry1Ac22F*, the expected amplified products were 3 534 bp and 1 959 bp respectively. PCR procedure was as follows: 94℃ 5 min pre-denaturation, 30 cycles of 94℃ 1 min, 54 ℃ 1 min and 72℃ 30 s, final cycle , 72℃ 10 min for extension and then 4℃ for ever. The amplified products were separated by agarose gel electrophoresis.

Author Contributions

ZL is the person who designed and conducted this experiment; ZL and YZ finished the data analysis and paper preparation. SL conducted experimental design and result analysis; XF is the PI of this project, involving in project design, data analysis, paper modification. All authors had read and consented the final text.

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