Engineering the bacterial endophyte Burkholderia pyrocinia JK-SH007 for the control of lepidoptera larvae by introducing the cry218 genes of Bacillus thuringiensis

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\section*{ABSTRACT}
To expand the usage of endophytes in agriculture and in forestry, the insecticidal gene cry218 of Bacillus thuringiensis was introduced into a poplar bacterial endophyte Burkholderia pyrocinia JK-SH007. The cry218 gene was cloned by polymerase chain reaction (PCR) and was inserted into a PHKT2 expression vector that was introduced into the bacterial endophyte JK-SH007. By using sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE) and western blotting, we confirmed that the engineered bacterial endophyte was successfully constructed, and it harboured insecticidal function after the bioassay in planta. The toxicity of the expressed insecticidal protein was analysed on second instar silkworm. The regression equation showed that the median lethal concentration (LC\textsubscript{50}) of the insecticidal protein was 0.77 (0.57–1.04) g/L at 72 h. The insecticidal bacteria genetically modified in this study have laid the foundation for further exploitation of biocontrol bacteria.

\section*{Introduction}
Bacillus thuringiensis can produce insecticidal Crystal proteins (ICP) with specific virulence on pests which severely damage crops and trees. Thus, B. thuringiensis has been widely used in biological control of pests. Since the 1980s, with the intensive study on the molecular biology and the genetics of B. thuringiensis \cite{1}, constructing recombinant strains with excellent properties had become a direction of developing microbial pesticides all over the world.

By constructing BT engineered bacteria, focus has been laid on a few aspects, like expanding the insecticidal spectrum and extending the effective duration of the toxicity. In 1983, Klier et al. \cite{2} transferred plasmid pBt42-1 carrying an ICP gene into Bacillus subtilis, and then transferred this plasmid to B. thuringiensis. They gained engineered bacteria which were insecticidal to both Ephestia kuehniella and Anopheles stephensi \cite{2}. Later, Rang et al. \cite{3} cloned a CIP gene encoding a 34 kDa crystal protein from B. thuringiensis subsp. thompsoni displaying synergistic activity against the codling moth, Cydia pomonella. The toxicity was tenfold compared to that of the strain BT-OP \cite{3}. In 2013, Pinto et al. \cite{4} cloned and transformed cry1Aa and cry1B genes from B. thuringiensis into Indica rice cultivar IRGA 424 via Agrobacterium tumefaciens. The bioassays with the transformed plants and Spodoptera frugiperda (JE Smith) larvae indicated high rates of mortality to the insect target, with a corrected mortality rate of 94% (cry1Aa) and 84% (cry1B), under laboratory conditions \cite{4}. In a recent study, Yilmaz et al. \cite{5} constructed a plasmid carrying the ICP gene Cry2Aa18 from B. thuringiensis (serotype israelensis), transferred it into the Escherichia coli BL21 (DE3) strain and successfully obtained a high yield of recombinant Cry2Aa18 (6.83 μg/mL). The recombinant protein showed considerable toxicity against last instar larvae of C. piriens with a median lethal concentration (LC\textsubscript{50}) of 630 μg/mL \cite{5}. Through modern biotechnological techniques, the transfer of the cry gene into entophytic bacteria to construct new engineered bacteria could be utilized towards multiple functions involving insecticidal action, disease prevention, nitrogen fixation and increasing production.

Plant endophytes have received much attention in recent years with the discoveries of their biological functions on the host plants, including growth promotion,
antimicrobial properties and long-term colonization of hosts because of the stable living environment in plants. Moreover, entophytic bacteria can multiply constantly. Thus, using entrophic bacteria as the host to extend the half-life of Cry protein and produce ICP in developing biological agents is of great significance [6].

The insecticidal Crystall protein Cry218, whose gene belongs to Cry1ac, has specific insecticidal activity on lepidoptera insects [7]. Combining the functions of a bacterial endophyte together with that of ICP by means of genetic engineering can further strengthen the biocontrol potential of plant endophytes. In this study, the cry218 gene was cloned and inserted into a pHKT2 expression vector [8]. A poplar bacterial endophyte B. pyrrocinia JK-SH007 was used as the ICP-expressing host [9]. The insecticidal bacteria genetically modified in this study were demonstrated to have insecticidal activity on lepidoptera larvae.

Materials and methods

Strains and plasmids

The recipient strain Burkholderia pyrrocinia JK-SH007 was isolated and maintained in the College of Forest Resources and the Environment, Nanjing Forestry University [9]. E. coli strain XL10-Gold was provided by the Department of Bio-engineering, Zhixing College of Hubei University. The selected second instar silkworm, whose phylad belongs to the national professional hybrid seed (Qiu-feng × Baiyu), was provided by Lady Silkworm Company (Hangzhou, Zhejiang, China). The plasmid Cry218-pht304 was a gift from the Biological Laboratory of Huazhong Agricultural University. pHKT2-Cry218 plasmid DNA was kindly provided by Prof. Howard Ceri from Canada. Our group constructed the Cry218 expression plasmid pHKT2-Cry218.

Medium and reagents

The reagents used were as follows: 2× Taqmix (Axy-Prep), DNA molecular weight, loading buffer and other conventional biochemical reagents were from Takara (Dalian, China), the antibiotic trimethoprim (TP) was purchased from Nanjing Boqiao Biotechnology co., LTD (Nanjing, China). The oligonucleotides were synthesized by Sangon Biotech co., LTD (Shanghai, China).

Generation of recombinant plasmids

Two pairs of specific primers (Supplemental Table 1S) were designed respectively according to the DNA sequences of the Cry218-pht304 plasmid and PHKT2 recipient expression vector [10]. These amplified DNA fragments were mixed at a ratio of 1:3 (100 ng vector to 300 ng DNA fragments), and transformed into E. coli XL-10 Gold cells [11]. The recombinant plasmid was DNA sequenced to confirm the construction by Takara (Dalian, China).

Transformation of B. pyrrocinia JK-SH007 competent cells

A single colony of the JK-SH007 strain was picked up and inoculated into 100 mL Super Optimal broth with catabolite repression (SOC) medium and was cultured at 18 °C and 220 r/min until reaching optical density at 600 nm (OD600) of 0.8. The prepared bacteria culture was pre-cooled on ice for 30 min, and then centrifuged at 4 °C/4800 g for 15 min to collect the bacteria precipitate [12]. After adding 30 mL of Wash buffer (10% glycerol, pH 7.0), the bacteria precipitate was vibrated gently, and then was washed again; finally the precipitate was re-suspended in 2 mL of Wash buffer [13]. Aliquots of the prepared competent cells with 100 μL per tube were frozen in liquid nitrogen and stored at −80 °C for further use. The pHKT2-Cry218 plasmid DNA was transformed into Burkholderia pyrrocinia JK-SH007 by electroporation (7500 V/cm, 25 μF, 200 Ω; Bio-Rad Gene Pulser, USA). The positive colonies were confirmed by colony polymerase chain reaction (PCR).

Expression of Cry218 in B. pyrrocinia strain JK-SH007

Protein expression was carried out by culturing bacteria in Luria–Bertani (LB) medium containing trimethoprim (50 μg/mL) at 30 °C until OD600 of 0.8, then rapidly heating up the suspension to 42 °C for 4 h to induce the cells for Cry218 expression. Then the cells were harvested by centrifugation (6000 g, 10 min). Cell pellets were suspended in phosphate buffered saline (PBS; 100 mmol/L, pH 7.8), disrupted by ultrasonication and the cell lysate was subjected to sodium dodecyl sulphate polyacryl amide gel electrophoresis (SDS-PAGE) analysis.

Optimization of recombinant Cry218 production in B. pyrrocinia JK-SH007

B. pyrrocinia JK-SH007(pHKT2-cry218) was used to inoculate 10 mL LB medium containing trimethoprim (50 μg/mL). The cultures were grown to a series of cell densities (OD600 of 0.21, 0.40, 0.61, 0.80, 1.11 and 1.30), and then the cultures were kept at 42 °C in a shaking incubator with vigorous shaking for 4 h. Cells were harvested by centrifugation and subjected to protein quantitation. To
optimize the inducing time, *B. pyrocinia* JK-SH007 (pHKT2-cry218) was used to inoculate 10 mL LB medium containing trimethoprim (50 μg/mL) and the culture was grown until OD_{600} of 0.8. Then the cultures were kept at 42 °C in a shaking incubator with vigorous shaking. Cells were harvested at different time points (4, 8, 12, 16, 20 and 24 h) for protein quantitation.

**SDS-PAGE and measurement of the protein concentration**

Protein samples were separated by 4%–12% (w/v) SDS-PAGE electrophoresis. Target protein bands were detected by Coomassie R-250 staining. Protein concentration was determined with the Bradford Protein Assay and calculated by quantitative densitometry.

**Western blot**

Protein samples were separated on a 4%–12% Tris-glycine gel, and then transferred onto a nitrocellulose membrane. The membrane was soaked in 10 mL TBS-T (20 mmol/L Tris–HCl, 150 mmol/L sodium chloride, 0.05% Tween 20) with 10% (w/v) milk for 1 h at room temperature [14]. Western blot analysis was conducted using primary antibody (Mouse Monoclonal Anti-6 × His IgG antibody, 1:3,000) and a secondary antibody IgG-HRP (horseradish peroxidase-conjugated goat anti-Mouse IgG antibody, 1:5000). The signal was developed using a SuperSignal® West Femto Maximum Sensitivity Substrate, and was recorded by X-ray photography.

**Identification of the heterologous expression protein with mass spectrometry**

After SDS-PAGE, the gel was stained with silver nitrate; gel slices with the target band were excised, digested overnight at 37 °C with trypsin and protein identification was carried out using an Ultraflex II MALDI-TOF/TOF mass spectrometry (MS) (Bruker Daltonic Inc.) in a positive ion reflective mode. The peptide mass spectrometry data were processed with Biotools software, and searched against the National Center for Biotechnology Information (NCBI) non-redundant database through mascot (http://www.matrixscience.com).

**Insecticidal activity detection and statistical analysis**

The genetically engineered bacterial endophyte *B. pyrocinia* JK-SH007 strain was inoculated in LB medium at 30 °C until reaching OD_{600} of 0.8. Then the suspension was rapidly heated up to 42 °C for 12 h. The bacteria were ultrasonically disrupted to release cryproteins (120 w for 15 min at 10 °C) and the samples were then stored at 4 °C. The biomass was determined by the gravimetric method [15]. To evaluate the insecticidal activity of the recombinant strain, fresh mulberry leaves were immersed in recombinant *B. pyrocinia* JK-SH007 lysate with series dilutions (about 0.19, 3.9, 7.9, 15.8 and 31.6 mg total proteins each). The treated leaves were dried in air for 10 min. Twenty-second stage of instar silkworms *Bombyx mori* were added into each dish with treated leaves of 6 concentrations, each of which had 3 repetitions [16]. The silkworms were incubated at 25 °C. The mortality of the silkworms was recorded every 24 h over a period of three days [17]. A corrected mortality ratio formula was chosen to illustrate the results [7]: Corrected mortality rate (%) = (Treatment group mortality – Control group mortality)/(1 – Control group mortality). Five gradient concentrations were tested. The equation of virulence regression and LC_{50} were calculated according to Abbott’s formula [7] and the assessment was made on the basis of dead/alive silkworms to obtain corrected mortality rate values. Additionally, evaluations were made on a proportion scale from 0 (no activity) to 100 (total kill) with an estimated maximum deviation of 5%. LD_{50} was determined by SPSS (version 19.0) based on the principle of probit analysis. To plot the larva mortality curves, bacterial cells were cultured and induced as described in the corrected mortality rate assay section. The freshly prepared *B. pyrocinia* JK-SH007(pHKT2-cry218) or *B. pyrocinia* JK-SH007 cell concentration was adjusted to 1.0 × 10^{13} CFU/L with PBS (100 mmol/L, pH 7.8), and mulberry leaves were coated in these bacterial solutions as described above. The mortality of the larvae was scored every 8 h for 96 h until all the *B. pyrocinia* JK-SH007(pHKT2-cry218) treated insects were dead [18,19].

**Results and discussion**

**Construction of the Cry218 expression vector pHKT2-Cry218**

The Cry218 expression vector was constructed by reverse PCR and *in vivo* homologous recombination. With the plasmid Cry218-pht304 as the template and Cry218F/R as a primer pair, an expected 3.5 kb fragment of the cry218 gene was obtained by the PCR amplification (Figure 1(A)). A 5.3 kb of linear pHKT2 fragment was amplified by PCR with the plasmid pHKT2 as the template and pHKT2/F/R as the primer pair (Figure 1(B)). Both of the DNA fragments were gel recovered, and co-transformed into *E. coli* XL-10 Gold competent cells. The recombinant plasmid DNA was sequenced and the positive recombinant plasmid was designated as pHKT2-
Cry218 (Figure 1(C)). Cry218 ICP expression was controlled by lambda phage PrP1 promoter via heat shock.

Production of recombinant B. pyrrocinia strain JK-SH007

The pHKT2-Cry218 plasmid DNA was introduced into B. pyrrocinia strain JK-SH007 by electroporation. Positive colonies were screened by DNA isolation and PCR screening (Figure 2).

Expression of the Cry218 in B. pyrrocinia strain JK-SH007

The positive colonies were sub-cultured and induced for protein production. A 5 mL culture aliquot was processed for SDS-PAGE and western blotting. The results demonstrated that Cry218 was successfully expressed in this wild-type endophyte bacterial species with an evaluated molecular mass of about 140 kDa (Figure 3). The corresponding gel band slice was further identified by MALDI-TOF MS analysis and was confirmed as Cry218 ICP protein from Bacillus thuringiensis (data not shown).

Optimization the Cry218 expression in B. pyrrocinia strain JK-SH007

To maximize the Cry218 expression in B. pyrrocinia strain JK-SH007, we surveyed the appropriate initial cell density and induction time for high yield of the total Cry218 expression. When the cell density at OD_{600} was 0.8, and the induction time was 12 h, the maximum yield of Cry218 ICP was about 0.25 g/L. Longer induction time and higher cell density would give a persistent decrease in the Cry218 ICP yield (Figure 4).

Bioactivity of the recombinant B. pyrrocinia JK-SH007

The bioassay in planta was carried out to test the toxicity of the engineered recombinant strain to lepidoptera.
pests. The lethal potential of the recombinant strain was determined based on the mortality of the tested larvae, second stage of Bombyx mori instar silkworms. The results showed that 78.3% of the silkworms were dead in 72 h after treatment with 3.16 g/L (about $3.8 \times 10^{13}$ CFU/L) of recombinant bacterial lysate (containing about 0.025 g/L Cry218 ICP; Figure 5). Meanwhile, the JK-SH007 (pHKT2) bacterial lysate was set as a blank control. It was found that the number of deaths in the control group was nearly zero, indicating that the engineered bacterial endophyte B. pyrrocinia JK-SH007 lysate was toxic to the target lepidoptera pest. Furthermore, we performed a corrected mortality rate assay in which the $LC_{50}$ ($P = 0.95$) was calculated to be 0.77 (0.57–1.04) g/L (Table 1). A previous study reported that the $LC_{50}$ value of a pure ICP expressed by engineered bacteria like Cry1ac was in the range of tens of micrograms per millilitre [20]. Thus, the engineered strain B. pyrrocinia JK-SH007(pHKT2-Cry218) we produced has higher efficacy than that in [20].

Finally, we performed the larvae survival assay to test whether the living engineered bacteria possessed insecticidal activity to Lepidoptera larvae. We treated B. mori larvae with a concentration of $1.0 \times 10^{13}$ CFU/L B. pyrrocinia JK-SH007(pHKT2-Cry218) cells. The survival rate showed a decreasing trend; a significant difference ($P < 0.05$) between the treatment and control groups was observed (Figure 6). The median survival time of the group treated with B. pyrrocinia JK-SH007(pHKT2-Cry218) was about 56 h. These results verified the observation that B. pyrrocinia JK-SH007 (pHKT2-cry218) had significant insecticidal activity to B. mori silkworms.

We are very optimistic that the insecticidal activity of this recombinant poplar endophyte as a biopesticide can be further improved, and other ICP genes can also be efficiently co-expressed in the JK-SH007 strain coordinated by genetic manipulation of this expression system to broaden the scope of its application. Moreover, the biological safety and the dynamic changes of engineered bacteria in poplar trees and other plants need to be studied before putting this recombinant strain into

### Table 1. Toxicity of recombinant B. pyrrocinia JK-SH007 to second instar silkworm larvae.

| Insect     | Number of larvae | $LC_{50}$ (95% confidence interval) | Slope ± SEM | Chi-squared |
|------------|------------------|-------------------------------------|-------------|-------------|
| Silkworm   | 320              | 0.77 (0.57–1.04) g/L                | 1.14 ± 0.12 | 3.61        |

Note: Mean values with standard error of the means (±SEM).

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**Figure 4.** Optimization of initial cell density (A) and induction time (B) for recombinant Cry218 in B. pyrrocinia JK-SH007.

**Figure 5.** Corrected death rate of the instar silkworms Bombyx mori under five serial dilutions of recombinant Cry218-007 bacteria lysate.

**Figure 6.** Effect of viable recombinant B. pyrrocinia JK-SH007 bacteria on the survival rate of the instar silkworms Bombyx mori larvae.
the field. Today, in an ever-increasing artificial and natural forest area, the forest pests remain a threat. The constructed strain has great potential for application as a biopesticide, with promising biological defence potential and economic value.

Conclusions

In this study, an ICP gene cry218 from Bacillus thuringiensis was successfully cloned into a Burkholderia pyrocinia expression vector and was expressed in a poplar endophyte Burkholderia pyrocinia strain JK-SH007. After the Cry218 expression conditions were optimised, the bioassay in planta demonstrated that this genetically engineered strain was toxic to Bombyx mori larvae. Thus, this engineered bacterial strain could potentially be considered for mass production by fermentation engineering and application for lepidoptera larvae control. The genetically modified endophyte bacteria in this study laid the foundation for further exploitation of biocontrol bacteria.

Disclosure statement

No potential conflict of interest was reported by the authors.

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