Expression of HpaG\textsubscript{Xooc} Protein in Bacillus subtilis and its Biological Functions

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HpaG\textsubscript{Xooc}, from rice pathogenic bacterium Xanthomonas oryzae pv. oryzicola, is a member of the harpin group of proteins, eliciting hypersensitive cell death in non-host plants, inducing disease and insect resistant in plants, and enhancing plant growth. To express and secret the HpaG\textsubscript{Xooc} protein in Bacillus subtilis, we constructed a recombinant expression vector pM43HF with stronger promoter P43 and signal peptide element nprB. The SDS-PAGE and Western blot analysis demonstrated the expression of the protein HpaG\textsubscript{Xooc} in B. subtilis. The ELISA analysis determined the optimum condition for HpaG\textsubscript{Xooc} expression in B. subtilis WBHF. The biological function analysis indicated that the protein HpaG\textsubscript{Xooc} from B. subtilis WBHF elicits hypersensitive response (HR) and enhances the growth of tobacco. The results of RT-PCR analysis revealed that HpaG\textsubscript{Xooc} induces expression of the pathogenesis-related genes PR-1a and PR-1b in plant defense response.

Keywords: HpaG\textsubscript{Xooc}, Bacillus subtilis, biological function

The harpin protein group, which is secreted by many plant pathogenic bacteria during infection, elicits multiple plant responses, resulting in multiple beneficial effects on crop improvement [1]. Wei and his colleagues [33] first found and identified protein HrpN\textsubscript{Ea} produced in nature by Erwinia amylovora. This protein elicits the hypersensitive response (HR) and enhances plant photosynthetic and nutrient uptake abilities, resulting in enhanced growth and fruit/flower production. EDEN Bioscience has further studied this protein and developed the commercial product Messenger® as the first commercial application of harpin protein. Moreover, the HrpN\textsubscript{Ea} protein was industrially produced in genetically engineered strain Escherichia coli [4].

Bacillus subtilis has been studied extensively for over 50 years. It is the optimum model for extensive researches in the analysis of medical, environmental, and industrial important bacteria owing to its amazing capacity to produce antibiotics, insecticides and enzymes. It has been reported that B. subtilis can produce more than 60 different types of antibiotics as well as some compounds that act as plant growth promoters [24]. For example, B. subtilis synthesizes lipopeptides through the nonribosomal peptide synthetases pathway [29, 37]. Among these lipopeptides, surfactin shows antimicrobial, antiviral, and antitumor activities [2, 14, 18, 22, 31].

Over the past decades, because of the overuse of chemical pesticides as well as various deleterious environmental consequences, the exploration of natural biological products for management of crop diseases and improvement of crop productivity has been accumulating [9]. Some wild-type B. subtilis strains are potential biocontrol agents and plant growth promoters [2, 20, 24, 26]. In addition, B. subtilis has been explored as a host for the production of various heterogeneous secretory proteins because of its excellent properties [6, 10, 35]. Recently, the construction of pHCMC expression vectors (exhibiting full structural stability) and B. subtilis strains WB800 (an eight protease null mutant) provided us a new avenue for the stable and high efficient expression of heterogeneous proteins in B. subtilis [19, 36, 38].

More recently, our group cloned the gene hpa1\textsubscript{Xooc} from rice pathogenic bacteria Xanthomonas oryzae pv. oryzicola (Xooc) that causes causing bacterial leaf streak of rice (Oryza sativa) for which control measures are limited, and expressed and purified its product HpaG\textsubscript{Xooc} in E. coli [16]. Our further comparison analysis has revealed its function similarity to the HrpN\textsubscript{Ea}, despite their sequences being completely different [17, 33]. In this study, to construct genetically modified B. subtilis strains for producing higher efficient, broad-spectrum, and stable biological control agents, the gene hpa1\textsubscript{Xooc} encoding protein HpaG\textsubscript{Xooc} hexahistidine fused in its C-terminal was cloned into an expression vector under the control of the strong promoter P43 and the signal peptide element nprB in B. subtilis [32, 38], and the resulting plasmid pM43HF was introduced into the host strains B. subtilis OKB105 (a surfactin producer) [18] and B. subtilis WB800 (protease null mutant) [36, 38] for expressing and secreting HpaG\textsubscript{Xooc}, respectively.

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The optimum condition for HpaG<sub>Xooc</sub> expression in <i>B. subtilis</i> WBHF was determined through ELISA analysis. Moreover, the roles of this recombinant protein HpaG<sub>Xooc</sub> from <i>B. subtilis</i> in enhancing plant root growth and inducing hypersensitive response and plant defense response were also analyzed.

**Materials and Methods**

**Bacterial Strains, Plasmids, and Growth Conditions**

The bacterial strains and plasmids used in this study are described in Table 1. <i>E. coli</i> DH5α was used as the host for all plasmids; <i>B. subtilis</i> WB800 and OKB105 were the hosts for plasmid pM43HF. The various new plasmids and strains constructed in this study are described in the text.

Luria-Broth (LB) was used for the growth of <i>E. coli</i> and <i>B. subtilis</i> strains. The super-rich medium, which consists of 2.5% yeast extract, 1.5% tryptone, and 0.3% potassium phosphate, and the optimum medium (super-rich medium supplemented with 1% xylose, 0.1% glucose, 2 mM MgCl<sub>2</sub>, 0.7 mM CaCl<sub>2</sub>, 50 µM MnCl<sub>2</sub>, 5 µM FeCl<sub>3</sub>, 1 µM ZnCl<sub>2</sub>, and 2 µM thiamine) were used for all <i>B. subtilis</i> [13, 34]. When required, antibiotics were added to the following final concentrations: ampicillin (Ap), 100 µg/ml; chloramphenicol (Cm), 5 µg/ml; kanamycin (Km), 50 µg/ml; hygromycin (Hyg), 50 µg/ml.

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>Source or reference</th>
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<tr>
<td><strong>Strains</strong></td>
<td></td>
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</tr>
<tr>
<td>E. coli DH5α</td>
<td>F&lt;sup&gt;+&lt;/sup&gt;φ80&lt;del&gt;lacZ Δm12 minirecA</td>
<td>Stored in this lab</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>168 trpC2</td>
<td>Stored in this lab</td>
</tr>
<tr>
<td>WB800</td>
<td>trpC2; nprE nprB aprE aprB mpr bpf vpr wprA; Cm&lt;sup&gt;r&lt;/sup&gt;, Hyg&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[36, 38]</td>
</tr>
<tr>
<td>OKB105</td>
<td>pheA&lt;sup&gt;+&lt;/sup&gt; sfr&lt;sup&gt;+&lt;/sup&gt;, surfactin producer, J1642 transformed with DNA of ATCC 21332</td>
<td>[18]</td>
</tr>
<tr>
<td>WBP5</td>
<td>trpC2; nprE nprB aprE aprB mpr bpf vpr wprA; WB800 transformed with pMA5; Cm&lt;sup&gt;r&lt;/sup&gt;, Hyg&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>WBHF</td>
<td>trpC2; nprE nprB aprE aprB mpr bpf vpr wprA; hpa1&lt;sup&gt;Xooc&lt;/sup&gt;&lt;sup&gt;r&lt;/sup&gt;, producer of HpaG&lt;sub&gt;Xooc&lt;/sub&gt;; WB800 transformed with pM43HF; Cm&lt;sup&gt;r&lt;/sup&gt;, Hyg&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>OKBP5</td>
<td>pheA&lt;sup&gt;+&lt;/sup&gt; sfr&lt;sup&gt;+&lt;/sup&gt;, producer of surfactin; OKB105 transformed with pMA5; Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>OKBHF</td>
<td>pheA&lt;sup&gt;+&lt;/sup&gt; sfr&lt;sup&gt;+&lt;/sup&gt;, hpa1&lt;sup&gt;Xooc&lt;/sup&gt;&lt;sup&gt;r&lt;/sup&gt;, producer of surfactin and HpaG&lt;sub&gt;Xooc&lt;/sub&gt;; OKB105 transformed with pM43HF; Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pMA5</td>
<td>pUB110 derivative; contains the HpaII promoter for the expression of cloned genes; ColE1; repB; Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[8]</td>
</tr>
<tr>
<td>pPICZHP</td>
<td>pPICZa derivative carrying a 411 bp hpa1&lt;sub&gt;Xooc&lt;/sub&gt; gene; Zeo&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pMD18-T</td>
<td>T-clone site vector; lacZ; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>TaKaRa</td>
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<tr>
<td>pMA5HF</td>
<td>pMA5 carrying His-tagged hpa1&lt;sub&gt;Xooc&lt;/sub&gt;; Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pMD18P43SP</td>
<td>pMD18-T carrying a fragment with ble gene, promoter P43 and nprB gene; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pM43HF</td>
<td>The ble-p43-nprB fragment from pMD18P43SP was inserted into the SmaBl and Ndel sites of pMA5HF for the expression of protein HpaG&lt;sub&gt;Xooc&lt;/sub&gt;; Km&lt;sup&gt;r&lt;/sup&gt;, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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*Resistance markers: Ap<sup>r</sup>, ampicillin resistance; Km<sup>r</sup>, kanamycin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Hyg<sup>r</sup>, hygromycin resistance; Zeo<sup>r</sup>, Zeocin resistance.*
Table 2. Oligo DNA primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence of primers (5'-3')</th>
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<tr>
<td>P1</td>
<td>CATATGAACTTTTGAACACACAATTC (NdeI)</td>
</tr>
<tr>
<td>P2</td>
<td>AAGCTTTCAATGATGTAGATGTAGTGTC (HindIII)</td>
</tr>
<tr>
<td>P3</td>
<td>GGTGTAAAGTTGGAATTAGTGATGTAGTGTTT</td>
</tr>
<tr>
<td>P4</td>
<td>CTTGGTGCAATGCTGCTGTCATCCTCCTCCTT</td>
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<td>P5</td>
<td>AAGAGAGAATGACATGCGGAATTAGTGTTT</td>
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<tr>
<td>P6</td>
<td>CATATGTCGTGCAGCTGAGGCATGTGTT (NdeI)</td>
</tr>
<tr>
<td>P7</td>
<td>GGGCGTACGTAACATTTATATACTTCTCCTAG (SmaBI)</td>
</tr>
<tr>
<td>P8</td>
<td>AACATACCCACCTATCAAACTTCCACACCTTTAAACC</td>
</tr>
<tr>
<td>PR-1a sense primer</td>
<td>GTGTGAAACTTGGACCTGGGA</td>
</tr>
<tr>
<td>PR-1a anti primer</td>
<td>TTCGCCCTCTATATACATTCCGGA</td>
</tr>
<tr>
<td>PR-1b sense primer</td>
<td>CATGCCAAAACACCTCACAACAG</td>
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<tr>
<td>PR-1b anti primer</td>
<td>TAGCACATCACCACCAACAGACCA</td>
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<tr>
<td>EF-1a sense primer</td>
<td>AGACCACAAAGTACTAAGCAC</td>
</tr>
<tr>
<td>EF-1a anti primer</td>
<td>CCACAACTCTGGACACATCC</td>
</tr>
</tbody>
</table>

*Restriction sites in primers are underlined.

as described by Li et al. [15] and diaminobenzidine (DAB) (Beijing Biosynthesis Biotechnology Co., Ltd, China) was also applied to this analysis.

ELISA

Enzyme-linked immunosorbent assays (ELISAs) were carried out for measuring the amount of HpaG<sub>xooe</sub> in the supernatants of <i>B. subtilis</i> strains. Firstly, the standard HpaG<sub>xooe</sub> purified and the diluted supernatant samples from <i>B. subtilis</i> WBHF were loaded onto ELISA 96-well plates, coated with plastic wrap, and incubated at 4°C overnight. After washing with washing buffer (0.5% Tween 20 in PBS), the plates were then blocked with 2% nonfat dry milk in double-distilled water at 37°C. PBS), the plates were then blocked with 2% nonfat dry milk in double-distilled water at 37°C for 1 h, and washed five times. The primary polyclonal antibody rabbit anti-HpaG<sub>xooe</sub> immunoglobulin G (1/5,000) was added and incubated in the 96-well plates at 37°C for 1 h; the plates were washed six times followed by the addition of the secondary antibody goat anti-rabbit IgG-HRP (1/10,000) to each well, incubated at 37°C for 1 h. Finally, after washing seven times, 3,3',5,5'-tetramethylbenzidine (TMB) in hydrogen peroxide (Armesco) was used as the substrate for developing at 37°C for 30 minutes; the reactions were stopped by the addition of H<sub>2</sub>SO<sub>4</sub>. The plates were read on a Multiskan Ascent microplate photometer (Thermo, U.S.A.).

Time-Course Analysis of HpaG<sub>xooe</sub> Protein Expression

To optimize the HpaG<sub>xooe</sub> expression in <i>B. subtilis</i> WBHF, a time-course analysis of the level of protein expression was performed. The super-rich medium with kanamycin was used for growth of <i>B. subtilis</i> WBHF and the upper leaves were collected at different time points, diluted to obtain single colonies, and plated on LB agar plates (selective media) to determine the number of viable cells, and on kanamycin-containing LB plates (selective media) to determine the number of plasmid-containing cells.

As a result of the experiments, samples were taken at different time points, diluted to obtain single colonies, and plated on LB agar plates (selective media) to determine the total number of viable cells, and on kanamycin-containing LB plates (selective media) to determine the number of plasmid-containing cells.

Assayng Bioactivities of the Recombinant Protein HpaG<sub>xooe</sub>

Inducing hypersensitive response (HR) was carried out by infiltrating the purified recombinant protein HpaG<sub>xooe</sub> into the intercellular space of leaves of tobacco (<i>Nicotiana tabacum</i> L. ‘xanthi’), and HR was observed after 12–24 h of inoculation.

Promoting plant growth with the recombinant protein HpaG<sub>xooe</sub> was done according to the following procedure: Sterile tobacco seeds with lower temperature treatment were soaked in 15 µg/ml of HpaG<sub>xooe</sub> solution for 6 h, and then planted on agar medium, and the length of root was measured after 10 days.

Analysis of PR-1a and PR-1b Genes Expression by RT-PCR

The lower leaves of the tobacco seedlings sprayed with 15 µg/ml of the recombinant protein HpaG<sub>xooe</sub> from <i>B. subtilis</i> WBHF and the upper leaves were collected at different time points for isolating the total RNA, using Trizol reagent according to the instructions from the manufacturer (Invitrogen Biotechnology Co., Ltd.). Inactive proteins (15 µg/ml) from <i>B. subtilis</i> WB5 was used as the control. The detection of gene expression was carried out according to the methods described elsewhere [11, 21]. The primers for the targeted genes (PR-1a and PR-1b) as well as the control gene (EF-1α) are listed in Table 2. The RT-PCR products were resolved on agarose gel to determine the expression level of the target gene.

RESULTS

Construction of HpaG<sub>xooe</sub> Protein Expression Vector pM43HF

To construct an overexpressive and secretive vector in <i>B. subtilis</i>, the strong promoter P43 and signal peptide element nprB were chosen in this study. The P43 promoter is a...
Expression of HpaG\textsubscript{Xoo}\textsubscript{e} Protein in Bacillus subtilis

The sequence region encoding HpaG\textsubscript{Xoo}\textsubscript{e} protein hexa-histidine fused in its C-terminal was cloned into the Ndel and HindIII sites of pMA5, generating pMA5HF; subsequently, the ble gene, promoter P43, together with signal peptide gene \textit{nprB} (sp) were spliced into the ble-p43-sp segment by overlapping extension, and inserted into the pMD18-T simple by TA cloning, creating pMD18P43SP; finally, to replace the HpaII promoter with the P43 promoter and introduce into \textit{nprB} gene, the ble-p43-sp segment form pMD18P43SP was cloned into the SnaBI and Ndel sites of pMA5HF, and the resulting plasmid was designated as pM43HF.

Expression of Protein HpaG\textsubscript{Xoo}\textsubscript{e} in \textit{B. subtilis} Strains OKB105 and WB800

Protein HpaG\textsubscript{Xoo}\textsubscript{e} from rice pathogenic bacterium \textit{Xanthomonas oryzae} pv. \textit{oryzicola}, inducing hypersensitive response and disease resistance, has been expressed in \textit{E. coli} [16, 17]. However, \textit{B. subtilis} has been regarded as an attractive host for the secretion of heterologous proteins. In this study, to produce the mixture of surfactin and protein HpaG\textsubscript{Xoo}\textsubscript{e} as a new type of effective biological agent for controlling plant diseases and enhancing plant growth, we chose \textit{B. subtilis} OKB105 (a surfactin producer) as host strain for expressing HpaG\textsubscript{Xoo}\textsubscript{e}.

At the beginning, we constructed the plasmid pMA5HF and transformed it into \textit{B. subtilis} OKB105; disappointingly, the results of SDS-PAGE indicated the expression level of protein HpaG\textsubscript{Xoo}\textsubscript{e} under the control of the HpaII promoter is extremely low (data not shown). Thus, we constructed the expression vector pM43HF using the stronger promoter P43 and signal peptide element \textit{nprB}, and introduced it into \textit{B.
**Fig. 2.** Western blot analysis of HpaG<sub>Xooc</sub> in genetically modified strains.

The supernatant of 1 ml of the cultures was concentrated 10-fold to 100 µl using 5% trichloroacetic acid (TCA), and 5 µl aliquots were used in the Western blot analysis. A. Detection of HpaG<sub>Xooc</sub> in *B. subtilis* OKBHF (6× His-tagged polyclonal antibody as the primary antibody). Lane 1, OKB105; lane 2, OKBP5; lane 3, OKBHF (24 h); lane 4, OKBHF (36 h); lane 5, molecular mass marker (6× His-tagged proteins); lane 6, WBHF (24 h). B. Detection of HpaG<sub>Xooc</sub> in *B. subtilis* WBHF (HpaG<sub>Xooc</sub> polyclonal antibody as the primary antibody). Lane 1, WB800; lane 2, WBP5; lane 3, molecular mass markers; lane 4, WBHF; lane 5, purified HpaG<sub>Xooc</sub>.

*B. subtilis* OKB105 was transformed with pM43HF for stably expressing foreign protein HpaG<sub>Xooc</sub>, and the resulting genetically modified strain was assigned as *B. subtilis* OKBHF. To verify the HpaG<sub>Xooc</sub> expression in *B. subtilis*, SDS-PAGE and Western blot analysis were performed. The purified protein samples from the wild-type *B. subtilis* OKB105, *B. subtilis* OKBP5 (OKB105 harboring plasmid pMA5 alone), and *B. subtilis* OKBHF were separated by 12% denaturing SDS-PAGE alongside 6× His-tagged protein standard as marker (Genscript Biotechnology Co., Ltd, Nanjing, China), and electroblotted into the membrane, and then probed with 6× His-tagged polyclonal antibody followed by the addition of the secondary antibody goat anti-rabbit IgG-HRP. The bands were detected by enhanced chemiluminescence (ECL). The results of the Western blot analysis are shown in Fig. 2A, lanes 1–4. An intense band representing HpaG<sub>Xooc</sub> at around 24 kDa was detected in *B. subtilis* OKBHF after 24 h and 36 h of incubation, respectively. We further precisely measured the amount of the HpaG<sub>Xooc</sub> in the supernatants of the OKBHF cultures through ELISA. The results indicated that the concentration of the HpaG<sub>Xooc</sub> in *B. subtilis* OKBHF was about 0.85 µg/ml at 36 h and 0.2 µg/ml at 48 h (data not shown). These results demonstrated that HpaG<sub>Xooc</sub> protein was capable of expressing and secreting into the supernatant under the control of the P43 promoter and *nprB* gene in *B. subtilis* OKBHF. This decrease in amount of HpaG<sub>Xooc</sub> protein from 36 to 48 h of incubation suggested that HpaG<sub>Xooc</sub> was probably unstable and sensitive to some proteases in *B. subtilis* OKBHF.

To avoid degradation of HpaG<sub>Xooc</sub> protein, *B. subtilis* WB800, an eight protease-deficient strain and excellent host for the expression of foreign proteins [36, 38], was also transformed with pM43HF for stably expressing foreign protein HpaG<sub>Xooc</sub>, and the resulting genetically modified strain was assigned as *B. subtilis* WBHF. This Western blot analysis was done with the rabbit HpaG<sub>Xooc</sub> polyclonal antibody as the primary antibody and goat anti-rabbit IgG-HRP as the secondary antibody, and developed with diaminobenzidine (DAB). The results of the Western blot analysis confirmed that the His-tagged proteins from *B. subtilis* WBHF is the HpaG<sub>Xooc</sub> protein (Fig. 2A, lane 6; Fig. 2B, lanes 4 and 5), and the amount of this protein was more drastically increased in *B. subtilis* WBHF than that in *B. subtilis* OKBHF cultures at 24 h of incubation (Fig. 2A, lanes 3 and 6).

**Optimum Conditions for Expression of HpaG<sub>Xooc</sub> Protein in *B. subtilis* WBHF**

To optimize the expression level of protein, pM43HF was constructed, and the amount of HpaG<sub>Xooc</sub> was measured at different time intervals of growth on super-rich medium by time-course analysis. The supernatants containing the target protein from 1 ml cultures at various time points were collected by centrifugation for 2–3 minutes, and then concentrated by precipitation with 5% trichloroacetic acid (TCA), and resolved by SDS-PAGE and analyzed by Western blot.

The SDS-PAGE analysis revealed that the foreign protein HpaG<sub>Xooc</sub> secreted by *B. subtilis* WBHF was a specific band at around 24 kDa, which could be obviously observed after 12 h (Fig. 3A). The results of the Western blot analysis also showed that the amount of HpaG<sub>Xooc</sub> protein produced in different time points increased from 12 to 84 h of incubation (Fig. 3B). To more precisely measure the amount of HpaG<sub>Xooc</sub> protein in WBHF, ELISA was done at the different time points. The results of the ELISA and the growth curve for *B. subtilis* WBHF are showed in Fig. 3C. The results showed that *hpol<sub>Xooc</sub>* gene, under the control of the P43 promoter, was continuously expressed throughout the exponential growth phase and into the late stationary phase, since the P43 promoter is a well-characterized overlapping promoter that is functional during this period [32]. The concentration of HpaG<sub>Xooc</sub> increased from 12 to 84 h of incubation, and the highest level of expression of HpaG<sub>Xooc</sub>
protein reached up to 54 µg/ml at 84 h, and then was reduced to 49 µg/ml at 96 h.

The medium for growth of cultures is also a limiting factor for expression of protein. The medium with different components of carbon sources and supplemented with trace amounts of metal ions probably affects the expression level of recombinant protein [13, 34]. Consequently, we measured the expression of HpaG\textsubscript{Xooc} protein in the so-called optimum medium, and found that this medium was able to improve the expression of HpaG\textsubscript{Xooc} protein, increasing 1.5 times at 36 h (18.5 µg/ml in the super-rich medium; 27.3 µg/ml in the optimum medium); however, its effect was not significant. Owing to some influence of the metal ions in this optimum medium in the purification and biological analysis, we still chose the super-rich medium as the source for expressing HpaG\textsubscript{Xooc} protein.

**Stability of the Recombinant Plasmid pM43HF**

To some extent, the expression level of a foreign protein in a bacterium is dependent on the stability of the recombinant plasmid in this organism. It is essential to study the structural and segregational stabilities of the recombinant plasmid pM43HF in *B. subtilis* WBHF in the presence or absence of kanamycin under selective or nonselective pressures. This study was performed according to the procedure described in the Material and Method section. The results of this study disclosed that the recombinant plasmid pM43HF was extremely stable under selective pressure, with 98% colonies containing plasmid pM43HF after 140 generations on the LB plates with kanamycin. Moreover, even in the absence of kanamycin, there were still 83% colonies with plasmid pM43HF after 100 generations of incubation (Fig. 4). Taken together, the recombinant plasmid pM43HF is very stable in the *B. subtilis* WBHF strain.
subtilis WB800 before 100 generations, suggesting the genetically modified B. subtilis WB800 might keep the higher level producing HpaG_{Xooc} protein for 100 generations even in the absence of the expensive antibiotic kanamycin. However, after 100 generations, pM43HF is segregationally unstable in B. subtilis.

**Roles of HpaG_{Xooc} Protein from B. subtilis WBHF in Inducing Hypersensitive Response (HR) and Enhancing the Growth of Non-Host Tobacco**

Our colleagues have previously manifested the biological functions of HpaG_{Xooc} protein expressed in E. coli BL21 in inducing hypersensitive response (HR) and disease resistance in tobacco [16]. However, it is indispensible to investigate the biological roles of the recombinant His-tagged HpaG_{Xooc} as a heterogeneous protein secreted by B. subtilis WB800.

Reportedly, 20 µg/ml of Hrp protein solution is a sufficient dose to induce HR on tobacco leaves [11]. Thus, 20 µg/ml HpaG_{Xooc} in the total protein from the supernatants of B. subtilis WBHF and the control strains WB800 or WBP5 was respectively infiltrated into the space of leaves of tobacco to induce HR. The results of HR observation after 24 h of inoculation showed that the HR spots were noticeable on those sites of the tobacco leaf treated with the purified HpaG_{Xooc} and the supernatants containing HpaG_{Xooc} from WBHF. Nevertheless, HR spots were invisible on the other sites of the same tobacco leaf treated with those supernatants containing the same concentrations of proteins from WB800 or WBP5 in the absence of HpaG_{Xooc} (Fig. 5). The results of this study defined the same role of the recombinant His-tagged HpaG_{Xooc} from both B. subtilis WB800 and E. coli BL21 in inducing HR.

**Fig. 5.** Hypersensitive response (HR) induced with the recombinant protein HpaG_{Xooc} from B. subtilis WBHF. The symptoms of the tobacco leaves after infiltration with the protein supernatant solutions of WBHF, WB800, and WBP5 as well as the purified HpaG_{Xooc} of WBHF. These symptoms were photographed after 24 h of treatment.

It has been reported that 15 µg/ml of harpin protein solution was sufficient in promoting the germination of tobacco seed and the growth of tobacco root [11]. In an attempt to test the role of HpaG_{Xooc} protein from B. subtilis WBHF, the surface-sterile tobacco seeds with lower temperature treatment were soaked in 15 µg/ml HpaG_{Xooc} protein solutions from B. subtilis WBHF or inactive proteins solutions from B. subtilis WBP5 for 6 h, respectively, and then planted on the agar medium in the square Petri dish. The root length of the tobacco seedlings was measured at the 10th, 15th, and 20th days. The effect was evident in 15 days to 20 days and increased with time (Fig. 6B).

The results of statistics analysis (t test) indicated that HpaG_{Xooc} protein of B. subtilis WBHF had significant effect on the growth of tobacco root (Fig. 6).

**HpaG_{Xooc} Protein Induces the Expression of the Pathogenesis-related Genes PR-1a and PR-1b in Plant Defense Response**

In plant defenses, the salicylic acid (SA)-medium signal pathway, ethylene (ET)-medium signal pathway, as well as jasmonate (JA)-medium signal pathway are three major plant
defenses signal pathways, playing roles in the induction of plant growth enhancement (PGE) and insect resistance (IR) [12, 23]. Many genes are involved in these defense signal pathways. Among these effectors, the PR-1α is involved in the SA-medium signal pathway [23], and PR-1β is associated with the ET signal pathway [7, 28]. The harpin protein acting as an elicitor suppresses the JA signaling, activates the ET and SA pathways, and induces the expression of the pathogenesis-related genes PR-1α and PR-1β in defense responses [11].

To confirm whether the recombinant protein HpaG\textsubscript{Xoo} from WBHF induces the plant defense response, the expressions of the PR-1α and PR-1β genes were analyzed in tobacco treated with proteins from WBP5 and WBHF strains by RT-PCR. Expression of the gene EF-1α, which encodes eukaryotic translation elongation factor 1-alpha in all eukaryotic cells for mRNA translation, was used as an internal control. Expression of the gene EF-1α was constitutive in all cases. The HpaG\textsubscript{Xoo} spraying on the lower leaves of the tobacco seedlings led to an increase in the expression level of the PR-1α and PR-1β in the upper leaves, highly obvious in the period of 24 to 48 h since being treated. However, the expression levels of these two genes were constant in the upper leaves of the tobacco seedlings treated with the control from WBP5 (Fig. 7). Taken together, these results were consistent with the previous reports [11], suggesting that the HpaG\textsubscript{Xoo} protein induces the expression of the relevant genes PR-1α and PR-1β through activating the SA and ET signal pathways.

**DISCUSSION**

HpaG proteins, produced by the genus *Xanthomonas*, contain two to four copies of the glycine-rich motif characteristic of harpin proteins, and cysteine absent in other harpins such as HrpN\textsubscript{Ya} of *Erwinia amylovora*. HpaG\textsubscript{Xoo} of *X. oryzae* pv. *oryzicola* contains two glycine-rich motifs and one cysteine residue [17]. Although there are some differences in the sequence and component of amino acids, HpaG\textsubscript{Xoo} exhibits similar biological functions as other harpins in inducing hypersensitive cell death and defense responses as well as enhancing plant growth [16]. The biological function analysis in this study further verified these roles of the protein HpaG\textsubscript{Xoo} from *B. subtilis* WBHF (Figs. 5–7).

HrpN\textsubscript{Xa}, namely the commercial product Messenger®, is industrially produced in *E. coli*, and secreted into the periplasm of *E. coli* [4, 33]. In the process of HrpN\textsubscript{Xa} purification, the outer membrane crackdown of host bacterial *E. coli* strain causes additional procedure and cost. However, some serious problems including low expression rates, formation of inclusion bodies, improper protein folding, and toxicity can occur during the process of heterologous gene expression and purification in *E. coli*. On the other hand, compared with *E. coli*, the Gram-positive bacterium *Bacillus subtilis* offers an efficient secretion apparatus that guides the expressed protein directly into the culture supernatant [3]. Because of its nonpathogenic nature and high secretion capacity and the existence of a great deal of fermentation technology, *B. subtilis* has long been used in industry for the production and secretion of proteins [6, 10, 35]. In addition, some *B. subtilis* strains themselves were reported to be effective for the biocontrol of multiple plant diseases and enhancing plant growth. So far, four *B. subtilis* strains, GB03, MBI600, QST713, and FZB24, have been registered in the EPA (U.S. Environmental Protection Agency) as commercial biocontrol products [2, 20, 24, 26]. Therefore, constructing a genetically engineered *B. subtilis* strain capable of directly secreting protein into extracellular cultures is becoming more attractive.

In this study, we constructed the recombinant expression vector pM43HF for expressing and secreting harpin protein in *B. subtilis*, for the first time. The recombinant plasmid vector is a key component in the protein expression system. We chose pMA5, an *E. coli*-*B. subtilis* shuttle vector, as the starter, carrying the essential replication regions and HpaII promoter of plasmid pUB110. To facilitate purification and identification of protein HpaG\textsubscript{Xoo}, its coding sequence fused
with 6×histidines in its C-terminal was cloned into pMAS [8], generating pMA5HF. However, when pMA5HF was transformed into B. subtilis OKB105 [18], the expression level of HpaG$_{Xoo}$ was extraordinarily lower under the weak HpaII promoter (data not shown). Thus, to replace the weaker HpaII promoter, the strong promoter P43 and signal peptide nprB were introduced into pMA5HF, constructing HpaG$_{Xoo}$ expression and secretion vector pM43HF (Fig. 1).

B. subtilis OKB105, a surfactin producer, which shows antimicrobial activities, is a potential biocontrol agent for plant diseases [2, 14, 18, 22, 31]. To produce the mixture of HpaG$_{Xoo}$ and surfactin as a new type of broad-spectrum biocontrol agent for plant diseases, the expression vector pM43HF was transformed into B. subtilis OKB105 to fabricate genetically modified strain B. subtilis OKBHF. The results of SDS-PAGE and Western blot analysis demonstrated that the expression of HpaG$_{Xoo}$ in B. subtilis OKBHF, though its expression is still lower (Fig. 2A). Because HpaG$_{Xoo}$ is sensitive to proteases, it is likely that most HpaG$_{Xoo}$ is degraded by the proteases in B. subtilis OKBHF. To further improve the expression level of HpaG$_{Xoo}$, B. subtilis WB800, an eight protease-deficient strain and excellent host for the expression of foreign proteins, was also transformed with pM43HF to construct another genetically modified strain, B. subtilis WBHF. The results of SDS-PAGE and Western blot analysis revealed that the expression level of HpaG$_{Xoo}$ in the WBHF was significantly higher than that in the OKBHF (Fig. 2A).

Although the ELISA showed that the expression level of HpaG$_{Xoo}$ in the B. subtilis WBHF in super-rich medium reached up to 54 µg/ml at 84 h (Fig. 3), it is possible to improve the yield through further optimization. In fact, the so-called optimum medium for production of human interleukin-3 had no significant effect on the HpaG$_{Xoo}$ expression [34]. However, the medium recipe should be optimized based on individual product. In addition, temperature and oxygen should also be considered as important limiting factors. It is required to further optimize conditions for the highest expression. A genome-based survey of the secretome of B. subtilis has shown that a total of at least 27 proteases are present in the membrane, cell wall, and culture medium [30]. It is likely that the recombinant protein HpaG$_{Xoo}$ is still degraded at a certain degree in B. subtilis WB800, which is just an eight proteases-deficient mutant.

To some extent, the expression level of foreign protein in bacteria is dependent on the stability of recombinant plasmid in this organism. The replication region of pM43HF originates from the so-called rolling-circle-type repB of pUB110, which might lead to segregational instability [5]. Our results showed that plasmid pM43HF is fully stable under selective conditions, and becomes unstable under the nonselective condition, especially after 100 generations (Fig. 4). This segregational instability affects the continuing expression of HpaG$_{Xoo}$, and finally fails to secrete this protein in B. subtilis. The use of antibiotics to keep the stability of the recombinant plasmid for the continuing expression is not commercially viable because of their high cost. Hence, more studies on improvement on stability of the recombinant plasmid pM43HF are indispensable.

Taken together, the results of this study indicate the stronger promoter P43 has significant regulatory roles in enhancing yield of protein HpaG$_{Xoo}$ and the signal peptide nprB functions in extracellularly secreting the protein. However, structural instability of the recombinant plasmid and degradation of the recombinant protein secreted into the medium have long been two problems in B. subtilis as protein factory [35]. Constructing a more efficient and stable expression vector based on pM43HF, and developing new B. subtilis strains with more proteases deficiency and stronger antimicrobial natural products as hosts for producing new biological control agents are in progress.

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References


