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Expression of $HpaG_{Xooc}$ Protein in *Bacillus subtilis* and its Biological Functions

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$HpaG_{Xooc}$, 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 resistance in plants, and enhancing plant growth. To express and secret the $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ in *B. subtilis*. The ELISA analysis determined the optimum condition for $HpaG_{Xooc}$ expression in *B. subtilis* WBHF. The biological function analysis indicated that the protein $HpaG_{Xooc}$ from *B. subtilis* WBHF elicits hypersensitive response (HR) and enhances the growth of tobacco. The results of RT-PCR analysis revealed that $HpaG_{Xooc}$ induces expression of the pathogenesis-related genes *PR-1a* and *PR-1b* in plant defense response.

Keywords: $HpaG_{Xooc}$, *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 $HrpN_{Ea}$ 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 $HrpN_{Ea}$ 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 $hpa1_{Xooc}$ 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 $HpaG_{Xooc}$ in *E. coli* [16]. Our further comparison analysis has revealed its function similarity to the $HrpN_{Ea}$, 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 $hpa1_{Xooc}$ encoding protein $HpaG_{Xooc}$ hexa-histidine 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 $HpaG_{Xooc}$, respectively.

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The optimum condition for $HpaG_{Xooc}$ expression in *B. subtilis* WBHF was determined through ELISA analysis. Moreover, the roles of this recombinant protein $HpaG_{Xooc}$ from *B. subtilis* 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. *E. coli* DH5α was used as the host for all plasmids; *B. subtilis* 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 *E. coli* and *B. subtilis* 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 $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MnCl_2$, 5 μM $FeCl_3$, 1 μM $ZnCl_2$, and 2 μM thiamine) were used for all *B. subtilis* [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.

DNA Manipulation and Transformation

The isolation and manipulation of recombinant DNA were performed using standard techniques. *E. coli* and *B. subtilis* were transformed as described by Sambrook *et al.* [25] and Spizizen [27]. All enzymes used in this study were purchased from TaKaRa Bio Inc. (Japan). The specific primers used for the PCR are described in Table 2.

Purification of Hexahistidine-tagged $HpaG_{Xooc}$ Protein from *B. subtilis* WBHF

Hexahistidine-tagged $HpaG_{Xooc}$ protein was purified by one chromatographic step using HisTrapHP (GE Healthcare) as described in the protocol of the manufacture. Briefly, the supernatant, which was centrifuged from 200 ml of the cultures in super-rich medium with kanamycin incubated at 37°C for 96 h, was dialyzed overnight against imidazole buffer (20 mM, pH 7.5) and then loaded onto the HisTrapHP column. The target protein was bound to the column and the nonspecific proteins were removed by rinsing with wash buffer (20 mM K_2PO_4, pH 7.4, 500 mM NaCl, and 20 mM imidazole). The proteins were eluted with a gradient of 10–500 mM imidazole. Purified protein $HpaG_{Xooc}$ was aliquoted and stored at −20°C after dialysis against the wash buffer in the absence of imidazole. Protein concentration was measured with a BCA-100 protein quantitative analysis kit (Biocolor Biotech, Shanghai, China) using bovine serum albumin as the standard.

Western Blot Analysis

Western blot analysis was performed as previously described [25]. Briefly, proteins were separated by 12% SDS-PAGE and then electroblotted to a polyvinylidene difluoride (PVDF) membrane (Millipore) and probed with primary antibody 6× His-tag polyclonal antibody, and visualized by enhanced chemiluminescence (ECL) (Genscript Biotechnology Co., Ltd, Nanjing, China) after binding with secondary antibody goat anti-rabbit IgG-HRP. Alternatively, the rabbit $HpaG_{Xooc}$ polyclonal antibody prepared according to the protocol

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Source or reference
Strains		
E. coli DH5α	F⁻ ϕ80d*lacZ* ΔM12 *minirecA*	Stored in this lab
Bacillus subtilis		
168	*trpC2*	Stored in this lab
WB800	*trpC2; nprE nprB aprE epr mpr bpf vpr wprA*; Cm^r, Hyg^r	[36, 38]
OKB105	*pheA1 sfp*$^+$, surfactin producer, JH642 transformed with DNA of ATCC 21332	[18]
WBP5	*trpC2; nprE nprB aprE epr mpr bpf vpr wprA*;WB800 transformed with pMA5; Cm^r, Hyg^r, Km^r, Ap^r	This study
WBHF	*trpC2*; *nprE nprB aprE epr mpr bpf vpr wprA*; *hpa1*$_{Xooc}^+$, producer of $HpaG_{Xooc}$; WB800 transformed with pM43HF; Cm^r, Hyg^r, Km^r, Ap^r	This study
OKBP5	*pheA1 sfp*$^+$, producer of surfactin; OKB105 transformed with pMA5; Km^r, Ap^r	This study
OKBHF	*pheA1 sfp*$^+$, *hpa1*$_{Xooc}^+$, producer of surfactin and $HpaG_{Xooc}$; OKB105 transformed with pM43HF; Km^r, Ap^r	This study
Plasmids		
pMA5	pUB110 derivative; contains the HpaII promoter for the expression of cloned genes; ColE1; *repB*; Km^r, Ap^r	[8]
pPICZHP	pPICZa derivative carrying a 411 bp *hpa1*$_{Xooc}$ gene; Zeo^r	Invitrogen
pMD18-T	T-clone site vector; *lacZ*; Ap^r	TaKaRa
pMA5HF	pMA5 carrying His-tagged *hpa1*$_{Xooc}$; Km^r, Ap^r	This study
pMD18P43SP	pMD18-T carrying a fragment with *ble* gene, promoter P43 and *nprB* gene; Ap^r	This study
pM43HF	The ble-p43-nprB fragment from pMD18P43SP was inserted into the SnaBI and NdeI sites of pMA5HF for the expression of protein $HpaG_{Xooc}$; Km^r, Ap^r	This study

[a]Resistance markers: Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Hyg^r, hygromycin resistance; Zeo^r,Zeocin resistance

Table 2. Oligo DNA primers used in this study.

Name	Sequence of primers (5'-3')[a]
P1	CATATGAACTCTTTGAACACACAATTC (NdeI)
P2	AAGCTTTCAATGATGATGATGATGGTC(HindIII)
P3	GGTTTAAAGGTGGAGATTTGATAGGTGGTATGTTT
P4	CTTGGTCAAGTTGCGCATGTGTACATTCCTCTCTT
P5	AAGAGAGGAATGTACACATGCGCAACTTGACCAAG
P6	CATATGTGCTGCAGCTGAGGCATGTGTT (NdeI)
P7	GGCGTACGTATTTATTAACTTCTCCTAG (SnaBI)
P8	AAACATACCACCTATCAAATCTCCACCTTTAAACC
PR-1a sense primer	GTGTAGAACTTGACCTGGGA
PR-1a anti primer	TTCGCCTCTATAATTACCTGGA
PR-1b sense primer	CATGCCCCAAACTCTCAACAAG
PR-1b anti primer	TAGCACATCCAACACGAACCGA
EF-1α sense primer	AGACCACCAAGTACTACTGCAC
EF-1α anti primer	CCACCAATCTTGTACACATCC

[a]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 $HpaG_{Xooc}$ in the supernatants of *B. subtilis* strains. Firstly, the standard $HpaG_{Xooc}$ purified and the diluted supernatant samples from *B. subtilis* 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 waster at 37°C for 1 h, and washed five times. The primary polyclonal antibody rabbit anti-$HpaG_{Xooc}$ 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 (Amresco) was used as the substrate for developing at 37°C for 30 minutes; the reactions were stopped by the addition of H_2SO_4. The plates were read on a Multiskan Ascent microplate photometer (Thermo, U.S.A.).

Time-Course Analysis of $HpaG_{Xooc}$ Protein Expression

To optimize the $HpaG_{Xooc}$ expression in *B. subtilis* WBHF, a time-course analysis of the level of protein expression was performed. The super-rich medium with kanamycin was used for growth of WBHF, and then the supernatants containing the target protein from 1-ml cultures at various time points were collected by centrifugation for 2–3 minutes. Finally, the $HpaG_{Xooc}$ protein samples were concentrated by precipitation with 5% trichloroacetic acid (TCA) and resolved by SDS-PAGE and analyzed by Western blot and ELISA.

Determining the Stability of the Recombinant Plasmid pM43HF

The structural stability and segregational instability of the recombinant plasmid pM43HF in the presence or absence of kanamycin under selective or nonselective pressures for approximately 140 generations were tested according to the protocol modified on the method described by Bron and Luxen [5]. In the process of the experiments, samples were taken at different time points, diluted to obtain single colonies, and plated on LB agar plates (nonselective 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.

Assaying Bioactivities of the Recombinant Protein $HpaG_{Xooc}$

Inducing hypersensitive response (HR) was carried out by infiltrating the purified recombinant protein $HpaG_{Xooc}$ into the intercellular space of leaves of tobacco (*Nicotiana tabacum* L. 'xanthi'), and HR was observed after 12–24 h of inoculation.

Promoting plant growth with the recombinant protein $HpaG_{Xooc}$ was done according to the following procedure: Sterile tobacco seeds with lower temperature treatment were soaked in 15 μg/ml of $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ from *B. subtilis* 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 *B. subtilis* WBP5 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 $HpaG_{Xooc}$ Protein Expression Vector pM43HF

To construct an overexpressive and secretive vector in *B. subtilis*, the strong promoter P43 and signal peptide element *nprB* were chosen in this study. The P43 promoter is a

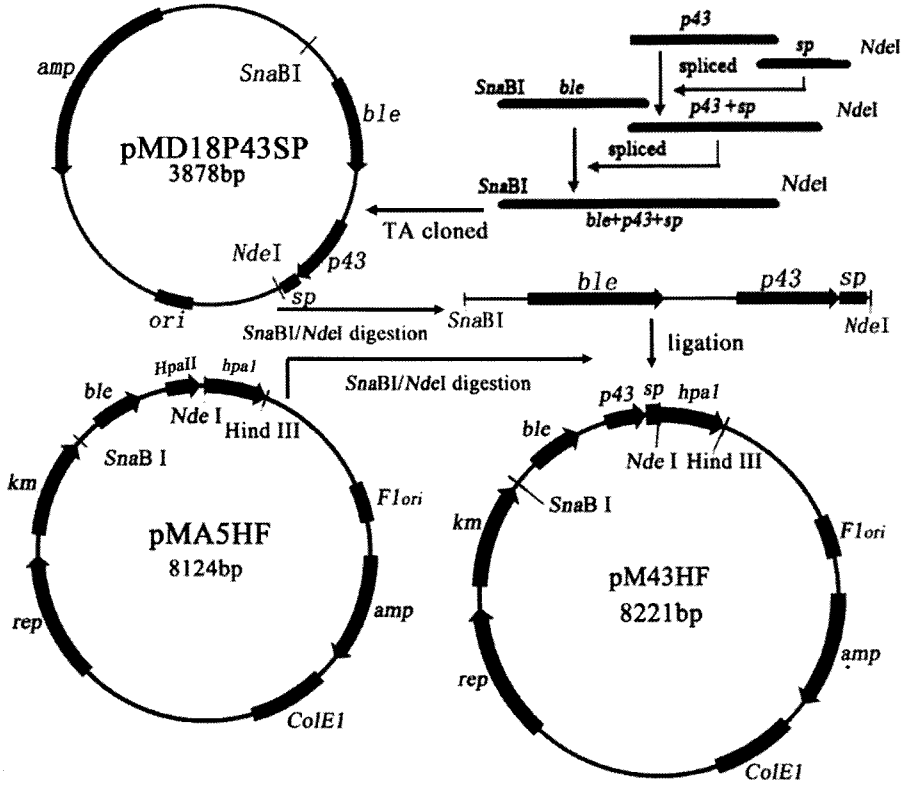

Fig. 1. Construction of HpaG$_{Xooc}$ expression vector pM43HF.
The sequence region encoding HpaG$_{Xooc}$ protein hexa-histidine fused in its C-terminal was cloned into the NdeI and HindIII sites of pMA5, generating pMA5HF; subsequently, the *ble* gene, promoter P43, together with signal peptide gene *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 *nprB* gene, the ble-p43-sp segment form pMD18P43SP was cloned into the SnaBI and NdeI sites of pMA5HF, and the resulting plasmid was designated as pM43HF.

well-characterized overlapping promoter that is functional during both the exponential and stationary growth phases [32]. The signal peptide *nprB* has a typical cleavage site (ASA-A) for signal peptidase I and acts to secrete the neutral protease B in the *B. subtilis* [30].

In the first step, the DNA fragment encoding the HpaG$_{Xooc}$ protein hexa-histidine fused in its C-terminal was amplified by PCR from the plasmid pPICZHP using primers P1 and P2, and cloned into the NdeI and HindIII sites of pMA5 [8], an *E. coli-B. subtilis* shuttle vector, creating pMA5HF. Sequentially, the genomic region coding for promoter P43 was amplified from *B. subtilis* 168 using primers P3 and P4; the signal peptide *nprB* gene-encoding sequence (sp) was amplified from *B. subtilis* 168 using primers P5 with the initial codon ATG for substituting the original TTG and P6 with NdeI site; and the *ble* gene was amplified from pMA5 using primers P7 with SnaBI site and P8. These above three PCR fragments were spliced into the ble-p43-sp segment by overlapping extension, and cloned into the pMD18-T simple by using a TA cloning kit (TaKaRa, Japan), and the resulting plasmid was named pMD18P43SP. To replace the HpaII promoter with P43 promoter and introduce into the *nprB* gene, the ble-p43-sp segment from pMD18P43SP was cloned into the SnaBI and NdeI sites of pMA5HF, and designated as pM43HF (Fig. 1). The entire clone region was confirmed by sequencing (Invitrogen Biotechnology Co., Ltd.).

Expression of Protein HpaG$_{Xooc}$ in *B. subtilis* Strains OKB105 and WB800

Protein HpaG$_{Xooc}$ from rice pathogenic bacterium *Xanthomonas oryzae* pv. *oryzicola*, inducing hypersensitive response and disease resistance, has been expressed *in E. coli* [16, 17]. However, *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$_{Xooc}$ as a new type of effective biological agent for controlling plant diseases and enhancing plant growth, we chose *B. subtilis* OKB105 (a surfactin producer) as host strain for expressing HpaG$_{Xooc}$.

At the beginning, we constructed the plasmid pMA5HF and transformed it into *B. subtilis* OKB105; disappointedly, the results of SDS-PAGE indicated the expression level of protein HpaG$_{Xooc}$ 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 *nprB*, and introduced it into *B.*

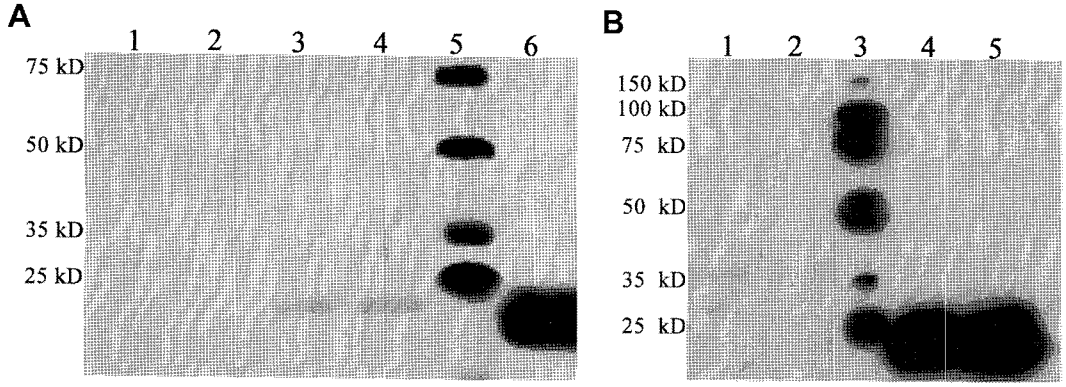

Fig. 2. Western blot analysis of $HpaG_{Xooc}$ in genetically modified strains.
The supernatant of 1 ml of the cultures was concentrated 10-fold to 100 μl using 5% trichloroacetic acid (TCA), and 5 μl aliquots were used in the Western blot analysis. **A**. Detection of $HpaG_{Xooc}$ in *B. subtilis* 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 $HpaG_{Xooc}$ in *B. subtilis* WBHF ($HpaG_{Xooc}$ polyclonal antibody as the primary antibody). Lane 1, WB800; lane 2, WBP5; lane 3, molecular mass markers; lane 4, WBHF; lane 5, purified $HpaG_{Xooc}$.

subtilis OKB105 to construct the genetically modified *B. subtilis* OKBHF. To verify the $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ in the supernatants of the OKBHF cultures through ELISA. The results indicated that the concentration of the $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ protein from 36 to 48 h of incubation suggested that $HpaG_{Xooc}$ was probably unstable and sensitive to some proteases in *B. subtilis* OKBHF.

To avoid degradation of $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$, and the resulting genetically modified strain was assigned as *B. subtilis* WBHF. This Western blot analysis was done with the rabbit $HpaG_{Xooc}$ polyclonal antibody as the primary antibody and goat anti-rabbit IgG-HRP as the 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ Protein in *B. subtilis* WBHF

To optimize the expression level of protein, pM43HF was constructed, and the amount of $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ protein produced in different time points increased from 12 to 84 h of incubation (Fig. 3B). To more precisely measure the amount of $HpaG_{Xooc}$ 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 $hpa1_{Xooc}$ 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 $HpaG_{Xooc}$ increased from 12 to 84 h of incubation, and the highest level of expression of $HpaG_{Xooc}$

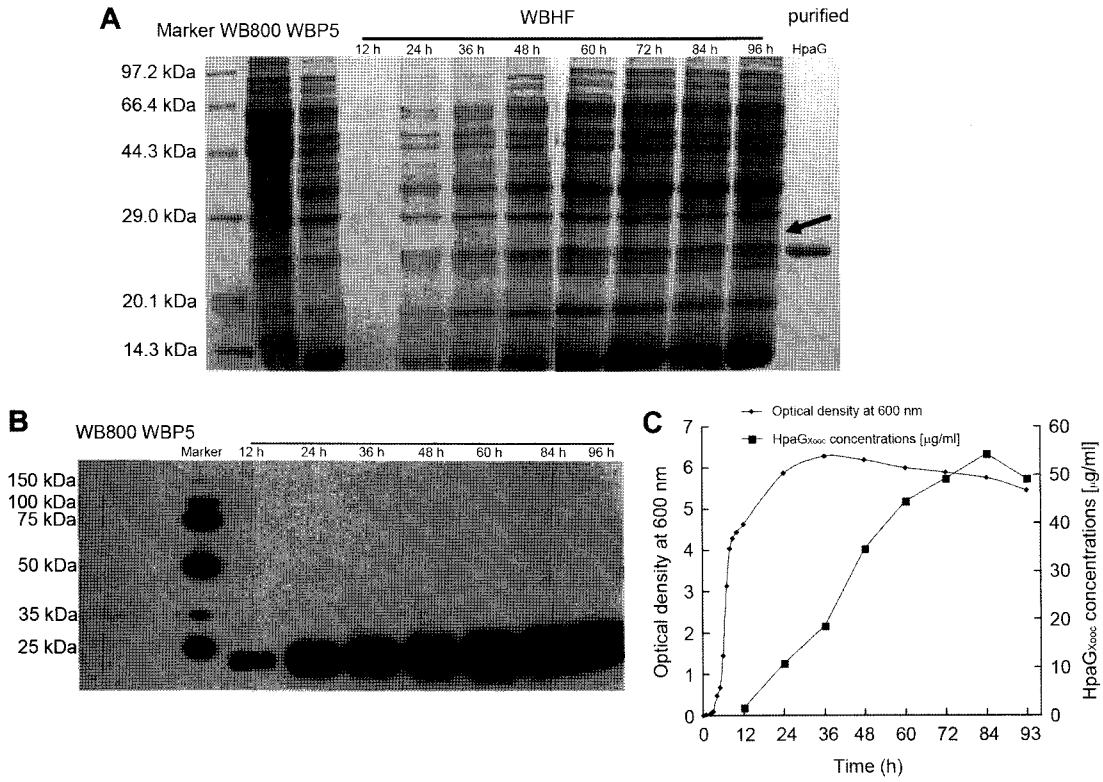

Fig. 3. Time-course analyses of HpaG$_{Xooc}$ protein expression.
A. SDS-PAGE analysis. **B**. Western blot analysis. **C**. Enzyme-linked immunosorbent assay (ELISA) and the growth curve.

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$_{Xooc}$ protein in the so-called optimum medium, and found that this medium was able to improve the expression of HpaG$_{Xooc}$ 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$_{Xooc}$ 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.*

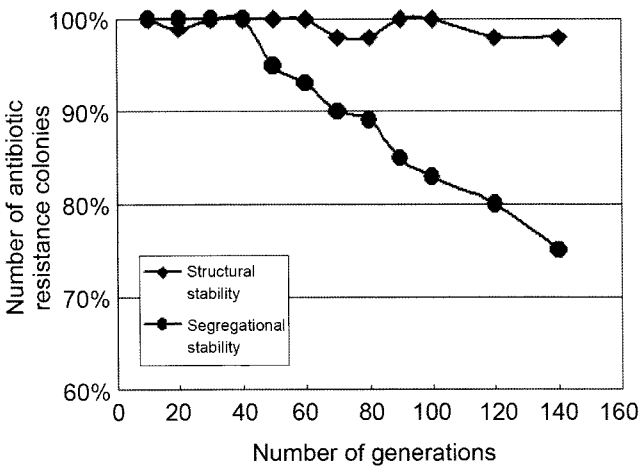

Fig. 4. Structural and segregational stabilities of the recombinant vector pM43HF.

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 indispensable 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.

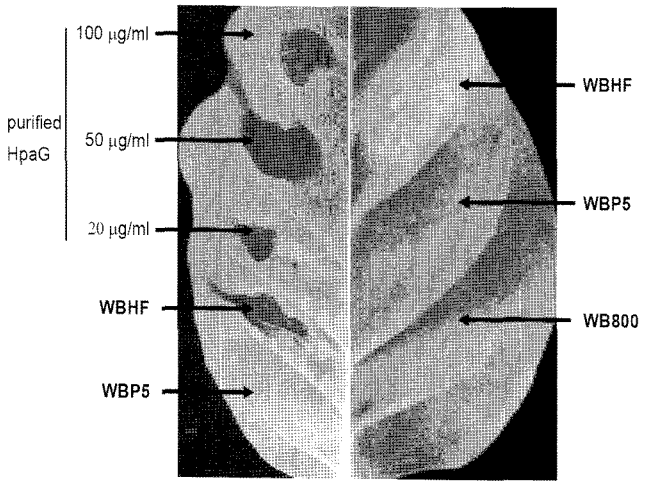

Fig. 5. Hypersensitive response (HR) induced with the recombinant protein $HpaG_{Xooc}$ from *B. subtilis* WBHF.
The symptoms of the tobacco leaves after infiltration with the protein supernatant solutions of WBHF, WB800, and WBP5 as well as the purified $HpaG_{Xooc}$ of WBHF. These symptoms were photographed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

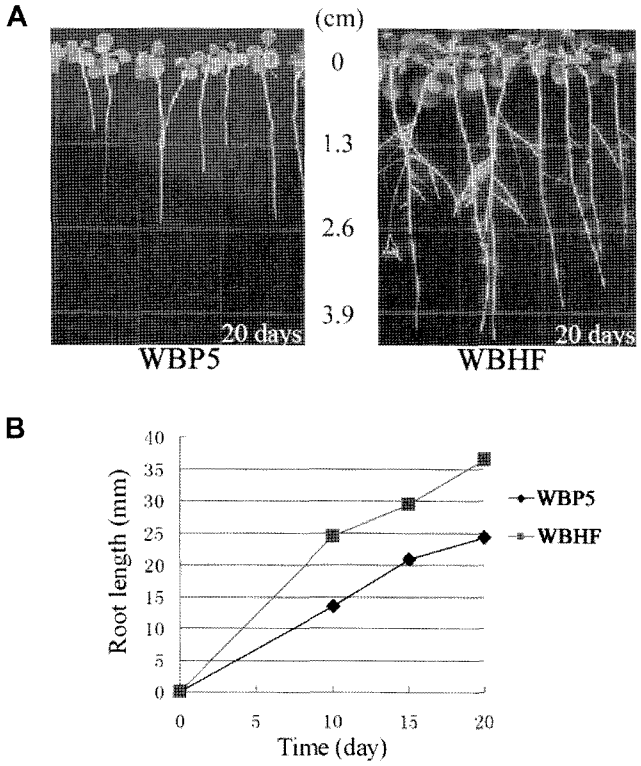

Fig. 6. Effect of $HpaG_{Xooc}$ protein from *B. subtilis* WBHF on the growth of tobacco.
A. Seedlings of tobacco germinated from the soaked seeds with $HpaG_{Xooc}$ from WBHF (right) and the control from WBP5 (left). The length of the square Petri dish used for the growth of tobacco is indicated (middle). **B.** The growth curves of the tobacco root treated with $HpaG_{Xooc}$ and the control as described above. This experiment was repeated three times.

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

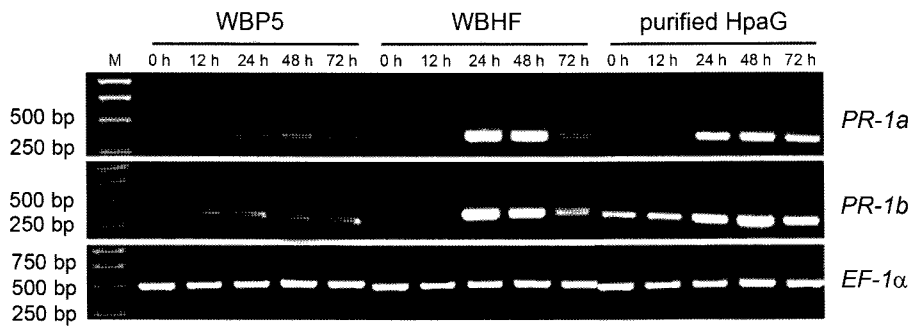

Fig. 7. $HpaG_{Xooc}$ induced the expression of the genes *PR-1a* and *PR-1b*.
The products of the RT-PCR reactions were resolved on an agarose gel and used to examine the expression of *PR-1a*, *PR-1b*, as well as *EF-1α* (standard control) as shown, respectively. The *B. subtilis* strains that produced the protein solutions and the purified protein $HpaG_{Xooc}$ used for treating the tobacco leaves at different time points are indicated above the gel. The DL2000 marker lane (M) is shown with values to the left indicating molecular sizes in nucleotides.

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-1a* is involved in the SA-medium signal pathway [23], and *PR-1b* 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-1a* and *PR-1b* in defense responses [11].

To confirm whether the recombinant protein $HpaG_{Xooc}$ from WBHF induces the plant defense response, the expressions of the *PR-1a* and *PR-1b* 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 $HpaG_{Xooc}$ spraying on the lower leaves of the tobacco seedlings led to an increase in the expression level of the *PR-1a* and *PR-1b* 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 $HpaG_{Xooc}$ protein induces the expression of the relevant genes *PR-1a* and *PR-1b* 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 $HrpN_{Ea}$ of *Erwinia amylovora*. $HpaG_{Xooc}$ 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, $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ from *B. subtilis* WBHF (Figs. 5–7).

$HrpN_{Ea}$, 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 $HrpN_{Ea}$ 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 $HpaG_{Xooc}$, its coding sequence fused

with 6×histidines in its C-terminal was cloned into pMA5 [8], generating pMA5HF. However, when pMA5HF was transformed into *B. subtilis* OKB105 [18], the expression level of $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ in *B. subtilis* OKBHF, though its expression is still lower (Fig. 2A). Because $HpaG_{Xooc}$ is sensitive to proteases, it is likely that most $HpaG_{Xooc}$ is degraded by the proteases in *B. subtilis* OKBHF. To further improve the expression level of $HpaG_{Xooc}$, *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 $HpaG_{Xooc}$ in the WBHF was significantly higher than that in the OKBHF (Fig. 2A).

Although the ELISA showed that the expression level of $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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 $HpaG_{Xooc}$ 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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