PeBL1, a novel protein elicitor from *Brevibacillus laterosporus* strain A60, activates the defense responses and systemic resistance in *Nicotiana benthamiana*

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ABSTRACT
In our study, we report the identification, characterization, and gene cloning of a novel protein elicitor (PeBL1) secreted from *Brevibacillus laterosporus* strain A60. Through a purification process consisting of ion exchange chromatography and HPLC, we isolated a protein that was identified by ESI-QTOF-MS/MS. The 351-bp PeBL1 gene produces a 12833 Da protein with 116 amino acids that contains a 30-residue signal peptide. The PeBL1 protein was expressed in *Escherichia coli*. The recombinant protein can induce a typical hypersensitive response (HR) and systemic resistance in *Nicotiana benthamiana* as the endogenous protein. PeBL1-treated *N. benthamiana* exhibited a strong resistance to the infection of TMV-GFP and *P. syringae* pv. *tabaci* compared to control *N. benthamiana*. In addition, PeBL1 triggered a cascade of events that resulted in defense responses in plants, including reactive oxygen species (ROS) production, extracellular medium alkalization, phenolic compounds deposition and several defense-related genes. Real-time quantitative PCR analysis indicated that the known defense-related genes *PR-1*, *PR-5*, *PDF1.2*, *NPR1* and *PAL* were up-regulated to varying degrees by PeBL1. This research not only provides insights into the mechanism by which beneficial bacteria activate plant systemic resistance but also sheds new light on a novel strategy for biocontrol using strain A60.
INTRODUCTION

In nature, plants live in complicated surroundings with various beneficial microbes and potential plant pathogens. In order to prevent infection by pathogens, plants have evolved defense mechanisms leading to a basic innate immunity (1, 2). Additionally, beneficial bacteria can generate a protective action which indirectly makes plant resist the infection of further pathogens through the elicitation of plant defense system (3). This defensive capacity is systemic, for example, root treatment with beneficial bacteria could extend to aboveground plant parts, triggering resistance in the whole plant. Resistance responses triggered by nonpathogens is so-called induced systemic resistance (ISR), which can efficient resist a broad spectrum of pathogens, including bacteria, fungi, viruses, nematodes, and insects (4-6). ISR is phenotypically similar to the well-studied systemic acquired resistance (SAR) motivated by an incompatible pathogen (7). Among these ISR-inducing bacteria, most are plant growth-promoting bacteria (PGPB), which are related to many plant species and generally present in a variety of environments (8). The most well-studied class of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the rhizosphere (9).

ISR has been documented in lots of plant species, for example, Arabidopsis thaliana, tomatoes, beans, cucumbers, radishes and tobacco (2, 6). Globally, ISR has been considered as a three-step procedure that contains perception of the elicitor, signal transduction, and defense-related gene expression triggering the resistance in the whole...
plant. ISR-associated signal transduction mechanisms have been demonstrated, even if comparatively less well understood than SAR. The salicylic acid (SA)-, jasmonic acid (JA)-, and ethylene (ET)-dependent pathways are major players in the regulation of signaling networks that are involved in induced defense responses (10-12). SA is an important signaling molecule involved in the induction of systemic resistance and local defense reactions (13). Several pathogenesis-related (PR) genes, such as PR-1a, PR-1b, PR-5, are generally used as markers of SA-dependent defenses. JA, a signaling molecule, is involved in many different aspects of plant biology including defense and development (14). ET regulates several processes in plants and has been implicated in defense responses. Normally, ISR is mediated by a signaling pathway in which JA and ET play key roles (6, 15). However, Saskia et al. demonstrated that activation of an SA-dependent pathway is a feature of ISR-inducing biocontrol bacteria (16). Maurhofer et al. reported that ISR induced by *P. fluorescens* strain CHA0 in tobacco is related to PR protein accumulation, suggesting that ISR and SAR share similar mechanisms. Thus, the defense mechanisms of ISR must be further studied (17).

The plant resistance system is the condition of enhanced defensive capacity. Plant defense responses triggered by elicitors of biotic and nonbiotic origins are a part of the plant resistance and play important roles in the signal exchange between the plant and the microbe. The elicitors, derived from various organisms including bacteria, fungi, viruses and oomycetes, have different chemical natures and include proteins, glycoproteins,
peptides, lipids and oligosaccharides (18-20). For example, Harpins are multifunctional protein elicitors produced by Gram-negative plant pathogenic bacteria (21). The fungal elicitors Hrip1, PevD1, and MoHrip1 from *Verticillium dahliae*, *Alternaria tenuissima*, and *Magnaporthe oryzae*, respectively, have been applied to plants to improve their pathogen resistance (22-24). However, a few reports have focused on elicitors that were isolated from biocontrol bacteria. For example, Dimethyl disulfide (DMDS), an elicitor produced by an ISR-eliciting *B. cereus* strain, can suppress plant fungal diseases and play a crucial role in ISR by *B. cereus* C1L (25). Massetolide A produced by *Pseudomonas fluorescens* SS101 is involved in ISR-eliciting defensive capacity in tomato against *Phytophthora infestans* (26). Surfactins and fengycins produced by *Bacillus subtilis* S499 can also act as elicitors of ISR (5). In contrast to the many researches performed with PAMPs used as models for early defense-related events, very little information is available about the perception mechanisms of ISR-specific protein elicitors (27).

In general, a defense reaction triggered by elicitors can be divided into two stages. The first stage occurs minutes after using an elicitor and contains ion fluxes across the cell membrane, extracellular medium alkalization and ROS. In the plant defense reaction, ROS are considered to play an important role in the elicitor signal transduction system and be also associated with the hypersensitive response (28), as a marker of the plant defense reaction (29, 30). ROS have been demonstrated to be sufficient for the induction of plant secondary metabolite accumulation and are required in the plant defense reaction.
The second stage occurs hours after elicitor ingestion and involves the activation of defense-related genes correlated with cytoderm reinforcement, the synthesis of phytoalexins, the accumulation of PR proteins and induction of defense compounds, such as phenolic compounds, callose and PAL (phenylalanineammonia lyase) (33).

_Brevibacillus laterosporus_, a gram-positive, aerobic spore-forming bacterium previously classified as _Bacillus laterosporus_, can produce diverse metabolites with antifungal activity, which can control the infection of plant pathogens as biocontrol agents (34). We have previously screened a novel strain A60 that was isolated from the soil of mango plants in Changjiang, Hainan province, China (E108°46.029′N19°15.635′), which was identified as _B. laterosporus_ by phenotypic characterization and 16S rRNA sequencing (35). In addition to antimicrobial activity, strain A60 also exhibited the induction of systemic resistance in numerous types of plants, such as wheat, pepper and Chinese cabbage. The control efficiencies against _Phytophthora capsici_ and _Peronospora parasitica_ in pepper and Chinese cabbage that were treated with strain A60 Aqua (5×10⁹ Cfu/mL) were 81.6% and 73.7%, respectively, after 10 days. In particular, the yield of Chinese cabbages after treatment with A60 Aqua (5×10⁹ Cfu/mL) increased by 13.2% compared to the wild type. Based on the excellent effect, microbial fertilizer of _B. laterosporus_ strain A60 Aqua has been registered (No. 2014-2058) and has achieved large-scale production in the factory of Henan province, with an annual output of 5000 tons. The application area has increased to three millions of acres. In previous studies, a
novel antimicrobial peptide BL-A60 with a molecular mass of 1602.0469 Da was isolated and purified from strain A60 (35). However, the metabolites involved in the activation of systemic resistance by strain A60 have not been completely studied.

In this study, we report the purification and characterization of the novel protein elicitor PeBL1 from *B. laterosporus* strain A60. PeBL1 activated certain early plant defense responses and the systemic resistance in *N. benthamiana* against infection by TMV-GFP and *P. syringae* pv. *tabaci*. Our research helps to elucidate the mechanisms of *N. benthamiana* systemic resistance triggered by the PeBL1 and provides a novel strategy for using *B. laterosporus* strain A60 to control plant disease.

**MATERIALS AND METHODS**

**Bacteria and plant cultivation**

The strain A60 was preserved at the China General Microbiological Culture Collection Center (CGMCC No: 5694) and maintained on Luria-Bertani medium (LB: 10 g tryptone, 5 g yeast extract, 10 g NaCl per 1 L distilled water) at 37 °C in the dark. *N. benthamiana* seeds were germinated on 1/2 Murashige and Skoog (MS) medium in a growth chamber that was maintained at 25°C with 12 h of light and 12 h of darkness. Following germination, the seedlings were transferred to an autoclaved soil mix containing 1:3 (v/v) high nutrient soil and vermiculite in 8×7.5×7.5-cm pots. One plant per pot was kept in the growth chamber at 25°C with 50% humidity and 16 h of light.

**Establishment of *N.benthamiana* cell culture**
Tobacco (N. benthamiana) seeds were soaked in 75% ethanol for 45 sec and in 10% sodium hypochlorite for 10 min, followed by three washes with sterilized water. The sterilized N. benthamiana seeds were cultivated for callus in MS medium. The callus were cut into small pieces after 15 days and suspended in liquid MS medium at pH 5.0 supplemented with inositol (100 mg/ml), 0.2% KH$_2$PO$_4$, 3% sucrose, 2,4-dichlorophenoxyacetic acid (0.2 mg/ml) and HCl (1 mg/ml) under shaking at 130 rpm at 25°C in the dark. Subcultures were inoculated with 4 mL of 5 day-old stock suspensions (36).

Isolation and detection of crude protein

B. laterosporus strain A60 was cultured in 3000 ml LB medium with shaking at 180 rpm for 48 h at 37°C, and the supernatant was collected after centrifugation (4700×g, 15 min, 4°C). Solid ammonium sulfate was added to the supernatant to achieve 80% relative saturation (w/v) at 4°C overnight. The precipitate was harvested by centrifugation (12,000×g, 20 min, 4°C), redissolved in 200 ml buffer A (25 mM MES–NaOH, pH 6.2) and dialyzed against buffer A for 48 h. Before filtering the crude protein with a 0.22 μm filter (Millipore, SuZhou, China), the insoluble debris was removed from the dialysate by centrifugation (12,000 × g, 10 min, 4°C). A portion of the crude protein (50 μl) was tested for elicitor activity (HR-inducing activity).

Purification of protein

Further purification was performed using the ÄKTA Explorer protein purification System
The crude protein was loaded onto an ion exchange chromatography HiTrap™ SP FF column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with elution buffer (25 mM MES–NaOH, pH 6.2). The bound proteins were eluted with a linear gradient of increasing NaCl in elution buffer at a flow rate of 2 ml/min. All fractions were collected and injected to a desalting column (GE Healthcare, Uppsala, Sweden) for elicitor activity analysis. The purified protein was monitored for elicitor activity. The pooled active fraction after desalting was purified through High-Performance Liquid Chromatography (HPLC) on a C18 reverse-phase column injected onto a Zorbax Eclipse XDB-C18 reverse-phase column (150 × 4.6 mm, 5 μm, Agilent) equilibrated with 5% ACN (acetonitrile)/2 mM NH₄FA/0.1%FA/water. The pooled active fraction was eluted with chromatography-grade ACN using a linear gradient of increasing from 20% (v/v) to 100% (v/v) over 30 min at a flow rate of 0.2 ml/min. All of the peaks were automatically collected by the Fraction Collectors (Agilent). Each peak was freeze-dried, redissolved in ultrapure water (Milli-Q, US), and tested for elicitor activity. The fraction with elicitor activity was chromatographed again to ensure its purity, and the molecular weight was determined via Tricine-SDS-PAGE.

**Mass spectrometry analysis and gene identification**

The protein sample was isolated on a Tricine-SDS-PAGE gel and digested overnight using MS-grade Trypsin Gold (Promega, Madison, WI, USA). The digested peptides
were reacted with succinimidyl-2-morpholine acetate (SMA) in order to analysis by MS/MS. The purified peptides were sprayed into quadrupole time-of-flight (Q-TOF) mass spectrometer (MicrO TOF-QII™, Bruker Daltonics K.K., Tokyo, Japan) with an electrospray ionization (ESI) ion source. The MS/MS data were automatically analyzed by the MASCOT search engine (Matrix Science, London; http://www.matrixscience.com), using the following parameters: Database, NCBIInr; taxonomy, *B. laterosporus*; enzyme, trypsin; Type of search, MS/MS Ion Search. The peptide and fragment mass tolerance were set at 0.1 Da. Proteins with probability based MOWSE scores exceeding the threshold (p<0.05) were definitely identified.

The genomic DNA was extracted from *B. laterosporus* strain A60 using an E.Z.N.A.™ bacterial DNA Kit (Omega Bio-tek, Norcross, GA, USA). A pair of gene-specific primers was designed to amplify the *PeBL1* gene (GenBank accession no. KM668059) sequence deduced from the mass spectrometry analysis and the Mascot database search. The primer sequences were designed as follows: forward primer, 5’-ATGAAAAAAGCTGTCTCAAC-3’ and reverse primer, 5’-TTAGTAGGGAACAGTTATATT-3’. The PCR product was cloned into the pMD 18-T vector (TaKaRa, Dalian, China) and verified by DNA sequencing (Beijing Genomics Institution, Beijing, China).

**Expression in *E.coli* and purification of recombinant protein**

The *PeBL1* gene, without its predicted signal peptide, was inserted into the
pET30-TEV/LIC vector (Novagen, Darmstadt, Germany). Then, the recombinant plasmid was transformed into the *E. coli* BL21 DE3 (TransGen Biotech, Beijing, China). The primers, including a fragment of the pET30-TEV/LIC vector and the 5’ and 3’ ends of the *PeBL1* gene, were designed as follows: forward primer, 5’-TACTTCCAATCCAATGCCACACCAGCCAAACACTC-3’, and reverse primer 5’-TTATCCACTTCCAATGCTATTAGTAGGGAAACAGTTATATTC-3’. The DNA was isolated by electrophoresis and observed by staining with Gold View (SBS Genetech, Beijing, China) and using Trans 2K DNA marker (TransGen Biotech, Beijing, China). The PCR product was cloned into the vector using ligation-independent cloning (37), and verified by DNA sequencing. To express the recombinant *PeBL1* protein, the bacteria were first grown at 37°C for 4 h, and the recombinant protein was subsequently induced by the addition of 0.2 mM isopropyl b-D-1-thiogalactopyranoside (Sigma, St. Louis, MO, USA) to the media at 16°C for 14-16 h. The acquired cells were disrupted two times with an ultrasonic disruptor for pooling the supernatant. The mainly purification procedure of recombinant *PeBL1* as follows: affinity chromatography with a His-Trap HP column (GE Healthcare, Waukesha, WI, USA), and a HiTrap desalting column (GE Healthcare, Waukesha, WI, USA). The purified protein was tested for elicitor activity and detected by Tricine-SDS-PAGE. Protein markers (Thermo Scientific, USA) were used to evaluate the apparent molecular mass of the purified recombinant proteins.
Characteristics of the PeBL1 protein

The HR-inducing activity of PeBL1 was evaluated in N. benthamiana leaves. The N. benthamiana leaves was injected with samples (50 μl) or a control prepared by using a syringe to cover areas of 1-2 cm². The HR symptom was examined after 24 hours according to the previously described method (38). The amplification of the tobacco HR marker gene, HSR203, was performed with reverse transcription-polymerase chain reaction (RT-PCR) with the following primers: HSR203 (forward, 5′-TGTACTACACTGTCTACACGC-3’; reverse, 5′-GATAAAAGCTATGTCCCACCTCC-3’) and EF1α (forward, 5′-AGACCACAAAGTACTACTGAC-3’; reverse, 5′-CCACCAATCTGTACATCC-3’) as a positive control.

In order to ascertain the influence of pH on elicitor activity, the pH of the elicitor was adjusted to 4, 6, 8 or 11 with NaOH or HCl, incubated overnight, dialyzed, and then used in the elicitor bioassay.

In order to determine elicitor heat stability, four aliquots of purified protein were incubated at 4, 25, 50 and 75°C for 15 min, and subsequently the elicitor activity of the treated proteins was tested.

Detection of hydrogen peroxide production and alkalinization of the extracellular medium

The histological localization of hydrogen peroxide production in N. benthamiana leaves
was determined as previously described (39). Briefly, PeBL1 (5 μM) or Tris-HCl (negative control) was injected into 8-week-old leaves. Subsequently, the leaves were isolated after 24 h of treatment and soaked in 3,3′-diaminobenzidine (DAB)-HCl (1 mg/mL, pH 3.8) solution. After incubation for 8 h in the dark, the treated leaves were placed in 95% ethanol at 65°C to remove chlorophyll and observed under an Olympus Stereomicroscope SZX9 (Olympus America, Inc., Melville, NY, USA). ROS production in tobacco cell suspensions was quantified by chemiluminescence using luminol (40). In brief, 250 μl cell were incubated with 300 μl of buffer (pH 5.75) containing 10 mM HEPES, 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄ for 1 h at 26°C. Then PeBL1 (10 μM) and luminol (0.3 mM) were mixed into buffer and rotated in a shaker. The chemiluminescence was expressed as nanomoles of H₂O₂ per gram fresh weight of tobacco cells using a standard calibration curve and monitored by the GloMax-96 Luminometer (Promega, Madison, WI, USA).

The alkalinization of extracellular medium was performed in the tobacco cell suspensions (41). The test was conducted simultaneously in 3x10 mL flasks (test, negative control and positive control), each of which contained 1 g fresh weight (f.wt) of cells per 5 mL of cell suspension. Tobacco cells were pre-equilibrated with an orbital shaker for approximately 45 min at 26°C until a steady pH value (5.0-5.2) was achieved and then treated with PeBL1 (10 μM). The pH was observed for 90 min after PeBL1 addition. Flg22 (1 μM) and buffer were added as a positive control and a negative control, respectively. The
changes in the pH of the suspension mediums were monitored using a pH meter (Sartorius Stedim, Germany).

**Detection of phenolic compound accumulation in tobacco cell culture**

For the measurement of phenolic compound accumulation in tobacco cells, 300 μl of tobacco cell suspension was examined after incubation with 50 μl (10 μM) PeBL1 in the dark at 26°C for 108 h under epifluorescence, Zeiss Axiovert 100 M inverted microscope equipped with a confocal laser scanner (Zeiss LSM 510, Oberkochen, Germany).

Tris–HCl buffer (pH 7.3) was used as a negative control.

**Bioassay for PeBL1-induced disease resistance in Nicotiana benthamiana**

We used the TMV-GFP, a recombinant virus in which jellyfish green fluorescent protein (GFP) gene was extended into the coat protein (CP) ORF of native TMV. The GFP was visualized by using a 100-W long-wave UV lamp (Black Ray model B 100AP; UVP, Upland, USA). The recombination did not influence the infection and movements of virus in *N. benthamiana* (42, 43). Three leaves each from six plants were injected at one spot per leaf with 10 μM PeBL1 or Tris–HCl buffer as a negative control. Three days later, the upper three non-treated (systemic) leaves were inoculated with TMV-GFP. The concentration of TMV-GFP solution was 0.5 g diseased leaves in 10 ml ultrapure water (Milli-Q, US). The signs and diameter of TMV lesions in each leaf were analyzed at the two, four, and six day post-inoculation (dpi) as previously described (44, 45). For the diameter of the lesions, 10 randomly lesions were measured for each plant. Three
replications were performed. The inhibition of TMV lesion was calculated using the formula below.

\[
\text{Inhibition} \% = \left\{ \frac{\text{(number(size)of lesions on control leaves - number(size)of lesions on elicitor-treated leaves)}}{\text{number(size)of lesions on control leaves}} \right\} \times 100\%
\]

To assay whether PeBL1 can induce systemic resistance and diminish disease signs in *N. benthamiana*, we next included a phytopathogenic bacteria (*P. syringae pv. tabaci*) in our experiments. *P. syringae pv. tabaci* were cultivated at 28°C in King’s B media (46) for 24 h and harvested with centrifugation, then washed three times and resuspended in sterile distilled water at an OD600 nm = 0.6 (1×10^7 CFU/ml). The method that *N. benthamiana* plants were treated with PeBL1 and buffer was just as described in the assay of TMV resistance. After three days, upper three non-treated leaves were inoculated with *P. syringae pv. tabaci* by soaked for 45 s. Signs were assessed 4 days after challenge with *P. syringae pv. tabaci*. Leaves were detached and sterilized to remove epiphytic bacterial populations. Three samples were collected from each leaf with a sterilized hole punch and ground with a pestle in 100 µl sterile water. The sample suspensions were vortexed completely and serially diluted to 10^-3. The bacteria in the dilution were inoculated on a King’s B Kan^25 plate and grown for 2 d. The number of the colonies on each plate was counted. The area of each sample was approximately 0.1963 cm^2 and CFU/cm^2 was calculated by multiplying by the dilution factor.

Analysis of the expression of defense-related genes induced by PeBL1 using
real-time quantitative PCR (RT-qPCR)

To study the mechanisms of the defense responses induced by PeBL1 in *N. benthamiana* plants, *N. benthamiana* plants that were infiltrated with PeBL1 or buffer on three leaves were assayed for the induction of several defense-related genes. A small fragment was collected from the upper leaves at the indicated times and rapidly frozen in liquid nitrogen. The fragments were placed in RNase-free tubes and frozen at -80 °C until use. Control plants were infiltrated with buffer. Total RNA was extracted with the EasyPure™ Plant RNA Kit (TransGen Biotech, Beijing, China). The cDNA was generated using the TransScript™ All-in-one SuperMix for qPCR Kit (TransGen Biotech, Beijing, China), and the concentrations of the cDNAs were adjusted to be equal. RT-qPCR was performed to determine the relative expression levels of several defense-related genes and conducted using the TransStart Green qPCR SuperMix UDG (TransGen Biotech, Beijing, China). The specific genes were designed from the coding sequences of each gene using Beacon Designer 8.12 (Table 1). The PCR was processed on an IQ-5 Real-Time System (Bio-Rad, USA) under the following program: 50°C for 2 min, then 94°C for 10 min. followed 43 cycles of 94°C for 5 s, and 60°C for 30 s. A melting curve was established from 65 to 95°C. Three technical replicates were amplified for each sample, including negative controls. The *EF-1α* (elongation factor 1α) gene was used as a reference gene for normalization. The quantification of the relative changes in gene transcript level was performed using the comparative 2-ΔΔCt method (47). The mean deviation was
calculated from the standard deviation (SD) in the ΔΔCt value using the formula $2^{(\Delta\Delta Ct \pm SD)}$.

**Protein assay**

All protein concentrations were measured using the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA).

**Statistical analysis**

All of the experiments and data presented in this paper were repeated at least three times. The data are presented as the mean ± the standard deviation and significant differences between the treatments and the controls were determined by analysis of variance using SAS.

**RESULTS**

**Isolation and purification of the elicitor protein**

Dialyzed crude protein from *B. laterosporus* was shown the HR activity. To purify the active fractions, we performed cation exchange chromatography on the crude protein using a HiTrap™ SP FF column, which produced one unadsorbed fraction and three adsorbed fractions (A, B and C). All fractions were injected into *N. benthamiana* leaves to check for elicitor activity (data not shown), and the active fraction of peak B (Fig. 1a) was further purified. Using agilent HPLC with a Zorbax Eclipse XDB-C18 reverse-phase column, five main peaks (B1, B2, B3, B4 and B5) were obtained (Fig. 1b). Peak B5 was demonstrated to have strong activity and underwent chromatography again under the
same conditions. We found that fraction B5 was a single peak (Fig. 1c) and showed a
single band on Tricine-SDS-PAGE with a relative apparent molecular weight of 12 kDa
(Fig. 1d). We specified the protein as PeBL1.

**Characterization of the PeBL1 protein**

*N. benthamiana* leaves infiltrated by the purified PeBL1 were induced an apparent
necrotic zone in the infiltration area at 24 -32 h post-infiltration (Fig. 2A), while there
was no necrosis on the leaves that were infiltrated with buffer. The early symptoms in the
infiltrated area are clearly transparent approximately 10-14 h after infiltration (data not
shown). PeBL1 also induced the expression of the *HSR203* gene (Fig. 2B) in *N.
*benthamiana* leaves, which is regard as the HR-specific gene (48).

PeBL1 was stable at 4°C, 25°C and retained its elicitor activity. However, at 50°C and
75°C, PeBL1 was thermally denatured with a loss of HR activity (data not shown).

We also found that PeBL1 only showed HR activity within an appropriate pH range. After
incubation for 12 h at 4°C in a series of pH solutions (4, 6, 8 or 11), only the PeBL1 at pH
6 or 8 could show HR activity (data not shown).

The Chou & Fasman Secondary Structure Prediction Server (CFSSP) analysis of the
secondary structure of PeBL1 indicates that the percentages of residues of helices, sheets
and turns are 50.0%, 63.8% and 12.9%, respectively. PeBL1 has very low identity to any
known protein structure and function, and PeBL1 also lacks any conserved domains in
the sequence, indicating that PeBL1 is a novel protein.
Mass spectrometry analysis and cloning of PeBL1

The protein sample was excised from the Tricine-SDS-PAGE for LC/MS analysis of in-gel digested protein for amino acid sequence determination of PeBL1. The results of the mass spectroscopy analysis were searched and performed by Mascot. Based on the Mascot search results, we obtained the best matching protein that had the highest score (score: 194), which contained two different reliable peptides: (a) $^{45}$TSNETWNLGSHIR$^{57}$ (score: 67) and (b) $^{88}$FTAVPNAGYVYK$^{102}$ (score: 47). MOWSE scores greater than 25 were significant for these search results. The protein (GenBank accession no. ERM19151.1) was from B. laterosporus PE36. Using the sequence of PE36 strain, we designed the PCR primers to clone the PeBL1 gene.

The PeBL1 gene (GenBank accession no. KM668059), whose full-length was 351 bp, encoding a protein of 116 amino acids with a theoretical molecular weight of 12833 Da, was amplified from the B. laterosporus A60 genomic DNA. The SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) was used for Signal peptide analysis of the PeBL1 gene sequence, revealing that the protein contained an 30-residue signal peptide, indicating that PeBL1 was a secreted protein.

Expression and purification of recombinant protein

The sequence encoding residues 31–116, without the signal peptide, was cloned into the pET30-TEV/LIC vector downstream of a 6xHis tag. After amplifying the sequence, the PCR product was cloned into the vector using. Subsequently, the recombinant expression
vector was transformed into *E. coli* BL21 DE3 cells. The prokaryotically expressed His<sub>6</sub>-PeBL1 was soluble in *E. coli*, and subsequently was purified over a His-Trap HP column (Fig. 3a), and a HiTrap desalting column (Fig. 3b). The purified recombinant protein was characterized by a single band with 12 kDa on Tricine-SDS-PAGE (Fig. 3c). The purified recombinant protein can also induce a typical HR in *N. benthamiana* (data not shown).

**Induction of ROS production and alkalinization of the extracellular medium by PeBL1**

A burst in oxidative metabolism that leads to the accumulation of superoxide (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub> is considered a significant early event in the plant defense system (49). The PeBL1 elicitor could induce H<sub>2</sub>O<sub>2</sub> accumulation in *N. benthamiana* leaves. Hydrogen peroxide polymerized by 3, 3’-diaminobenzidine (DAB), which forms a dark red–brown precipitate, was detected, and the sites of H<sub>2</sub>O<sub>2</sub> accumulation were obviously observed microscopically in the stomata and veins of *N. benthamiana* leaves (Fig. 4A). ROS production induced by the PeBL1 was determined in tobacco cell suspension by chemiluminescence and compared to a Tris-HCl buffer treatment negative control and an flg22 (1 μM) treatment positive control (Fig. 4B). PeBL1 treatment caused a rapid increase in hydrogen peroxide, which reached a maximum at about 20 min, followed by a gradual decrease to a level similar to that of the negative control, just a little less than the well-known elicitor flg22.
The alkalinization of the extracellular medium, another early key event that occurs after challenging tobacco cell suspension with PeBL1 and is thought to result from elicitor-induced ion fluxes caused by PeBL1, was also analyzed. The alkalinization of the tobacco culture medium following PeBL1 treatment was assessed by determining the pH of the cell medium, which significantly increased from 5.2 to 5.8 within 25 min in comparison to the negative control. Then, the pH slowly decreased to the initial pH value at 90 min. The positive control Flg22 induced a slightly higher and quicker maximal extracellular pH shift than PeBL1 (Fig. 4C).

**Accumulations of phenolic compounds**

Phenolic compounds, secondary metabolism molecular components including precursors for antifungal compounds, cytoderm reinforcement and SA, help to control plant diseases. Under UV excitation, tobacco cells can present fluorescence attributable to the accumulation and oxidation of phenolic compounds, such as scopoletin, scopolin and ferulic acid (50, 51). After a 108 h incubation with PeBL1, phenolic compounds accumulated in the tobacco cells and were observed in blue under fluorescence microscopy (Fig. 5b). In contrast, accumulation of phenolic compounds was not detected in control tobacco cells that were treated with Tris-HCl buffer (Fig. 5d).

**PeBL1-induced disease resistance in N. benthamiana**

The numbers and diameters of TMV lesions in systemic leaves of PeBL1-treated plants were obviously lower than in control plants (Table 2). The greatest reductions in both the
numbers and the diameter of TMV lesions were approximately 43%, at 4 days post inoculation (dpi) (Fig. 6A).

We also found that PeBL1 can enhance systemic resistance against *P. syringae pv. tabaci* in *N. benthamiana*. In the PeBL1-pretreated plants, the occurrence of disease signs was delayed and the bacterial population had decreased by 30% at 4 dpi (Fig. 6B).

**Expression of defense-related genes induced by PeBL1 in *N. benthamiana***

To further study the mechanism of PeBL1-induced plant systemic resistance, we assayed the expression levels of PR proteins of families of the *PR-1a* and *PR-5* (thraumatlin-like proteins) gene, which are markers of the SA-dependent defense pathway. The expression levels of the two genes were both significantly up-regulated within five days in the PeBL1-pretreated *N. benthamiana* in relation to untreated plants. The maximum level of the *PR-1a* gene increased by 30-fold at 4 days post-treatment (dpt) (Fig. 7A). The expression of the *PR-5* gene continuously increased over five days and achieved a maximum increase of 8-fold at 5 dpt (Fig. 7B). The expression levels of *PR-1a* and *PR5* were also up-regulated during the majority of growth stages for buffer-infiltrated plants, however, with an apparently lower intensity than observed for the PeBL1-treated plants (Fig. 7A and B). To analyze the JA/ET-dependent pathways, we examined the expression of the plant defensin1.2 (*PDF1.2*) gene (52). The expression level of *PDF1.2* was not influenced by the negative control, but was 5-fold greater in PeBL1-treated *N. benthamiana* (Fig. 7C). The non-expressor of pathogenesis-related 1 (*NPRI*) gene is
important for activating PR gene expression and is regarded as a key regulator of the
crosstalk between SA- and JA-dependent defense pathways (53). The measurement of
transcript accumulation for the NPR1 gene indicated a slight increase in expression
between 2 and 5 dpt compared to untreated plants, while the expression level did not
increase in buffer-infiltrated controls during the same time period (Fig. 7D). The
transcription level of the defense-related enzyme, PAL, was also determined. The
expression level of PAL increased 3-fold at 2 dpt and then began to decrease, while PAL
was only up-regulated by 1.5-fold at 2dpt in the buffer-infiltrated controls (Fig. 7E).

DISCUSSION

The B. laterosporus strain A60 has shown a well-known ability to act as a biocontrol
agent and induce systemic resistance in plants and produce various antagonistic factors
such as a parasporal crystal, an extracellular protease, a lipopeptide antibiotic and a
pseudopeptide (35, 54-56). However, the molecular mechanisms involved in the
activation of systemic resistance by strain A60 have not yet been completely elucidated.
In this study, we report a novel secreted protein elicitor of 12.833 kDa (PeBL1) from the
culture supernatant of B. laterosporus that elicits systemic resistance in N. benthamiana.
The PeBL1 protein contains a secretory signal sequence and 116 amino acid residues. The
amino acid sequence of PeBL1 does not exhibit homology or identity to other reported
protein elicitors, indicating that PeBL1 is a novel elicitor. We also successfully cloned the
PeBL1 gene and purified the prokaryotically expressed recombinant protein. Both the
recombinant and native PeBL1 protein can induce HR in *N. benthamiana* leaves. A concentration of 2.5 μM PeBL1 protein was sufficient to induce HR within 24 h, and the induced HR did not increase the size of infiltrated zone, even when high concentrations of the protein were applied, suggesting that HR induced by PeBL1 was typical.

Furthermore, the HSR203 gene, which is considered an HR-specific gene (48), was expressed in PeBL1-infiltrated *N. benthamiana* leaves. In general, HR is a part of the plant innate immunity and induces a signaling cascade that activates plant defense responses, consequently, leading to systemic resistance (57).

We examined several defense-related early events involved in plant-elicitor interactions.

One of the early events during the HR process is the production of ROS in an oxidative burst. Numerous reports have demonstrated that ROS play a key role in the whole plant defense system and often appear in host or non-host plants after treatment with an elicitor (28). ROS regulate multiple cellular functions in plants, including alterations of redox status that directly affect specific plant transcription factors and regulate antimicrobial activity (58). In this study, we detected H$_2$O$_2$ in *N. benthamiana* leaves via histological imaging and ROS production in tobacco suspension cells induced by PeBL1. In comparison with a known elicitor, flg22, used as a positive control, PeBL1-treated and flg22-treated tobacco suspension cells showed similar ROS production patterns, indicating that PeBL1 performs similarly to this well-known elicitor. ROS act as signaling molecules and interact with various molecules including calcium ions, NO
(Nitric Oxide), lipids and mitogen-activated protein kinase (MAP kinase), which are general regulatory elements (59, 60). These signaling molecules may be involved in physiological phenomena and can regulate several processes in interconnected branch pathways and defense signaling pathways (61, 62). However, the involvement of these signaling molecules in PeBL1-regulated signal transduction is still indistinct, and further study is required. As observed for other elicitors, PeBL1 induces the extracellular medium alkalization of tobacco cell suspension, indicating that it might be involved in the restoration of the ion influx and pH gradient between the cytosol and the apoplast. All of the data indicate that PeBL1 is a real elicitor and the key player for inducing defense responses in N. benthamiana.

To clarify the downstream signaling pathways of defense responses induced by PeBL1 in N. benthamiana, we investigated the behavior of the PR-1a, PR-5, PDF1.2 and NPR1 genes using RT-qPCR. Considering that the plant defense responses can also be elicited by the damage associated with infiltration, we analyzed the buffer-infiltrated plants as negative controls. We found that the relative expression levels of these defense-related genes were differentially up-regulated after infiltration with PeBL1 and higher than those caused by damage at certain stages (Fig.7). The coordinate expression of SA-responsive PR genes, JA/ET-responsive PDF1.2 gene and the signaling regulatory gene NPR1 may indicate that PeBL1 elicits systemic resistance using a complex signaling network, which most likely includes SA and JA/ET-dependent pathways. However, the exact signaling
The phenylpropanoid biosynthetic pathway is known to be involved in plant defense system because the antimicrobial compounds produced by this pathway, such as lignin, phytoalexin and phenolic compounds, act as plant barriers to pathogens (50). Especially phenolic compounds play key roles in interactions between plants and soil microorganisms and are involved in preformed plant defenses (63). The first enzyme in the phenylpropanoid biosynthetic pathway is PAL, which provides precursors for the formation of antimicrobial compounds (64). In this study, we found that \textit{PAL} gene was up-regulated (using RT-qPCR) and that phenolic compounds were produced after PeBL1 treatment (using fluorescence microscopy), indicating that PeBL1 can induce the accumulation of antimicrobial compounds involved in defense responses.

To determine if the activation of the defense system in \textit{N. benthamiana} can impart resistance to phytopathogens, experiments were performed by inoculating \textit{N. benthamiana} with a TMV-GFP recombinant virus and virulent \textit{P. syringae pv. tabaci}. The numbers and diameters of TMV lesions in systemic leaves of PeBL1-treated plants were obviously lower than in control plants (Table 2). PeBL1 may potentially transfer TMV resistance via the accumulation of antiviral compounds and creation of a physical barrier, which suppress virus replication or movement, respectively, in \textit{N. benthamiana}. In addition, the disease signs of the wildfire pathogen \textit{P. syringae pv. tabaci} were alleviated and the bacterial population was significantly decreased after PeBL1 treatment.
potentially because the pretreatment of *N. benthamiana* with PeBL1-influences the perception and invasion of the pathogen. In conclusion, the defense systems activated by PeBL1 in *N. benthamiana* are effective against a broad spectrum of pathogens, including viruses and pathogenic bacteria.

In summary, we have reported the purification, characterization and gene cloning of a secreted protein elicitor PeBL1 from *B. laterosporus* strain A60. Both the recombinant and native PeBL1 protein can induce a typical HR and activate defense-related early events and antimicrobial compounds production in *N. benthamiana*, indicating that PeBL1 is an excellent candidate as a plant defense activation agent to challenge infections with pathogens. PeBL1 could be effectively utilized in bio-pesticides and transgenic crops to reduce the application of chemicals to food plants with benefits to human health. Furthermore we have provided a foundation for expanding the understanding of the signaling transduction mechanism of plant defense responses by PeBL1. Our researches not only give insights into molecular mechanisms and biological functions of PeBL1 in the activation of plant systemic resistance but also strongly support the potential applications of *B. laterosporus* strain A60 in biocontrol and sustainable agriculture.

**ACKNOWLEDGMENTS**

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Figure 1 The purification of PeBL1 from *B. laterosporus*. a. The crude protein was loaded on a HiTrap™ SP FF 5 ml column at a flow rate of 2 ml/min. Three peaks (A,B,C) were collected, and the target protein was included in peak B. b. Chromatography of peak B using a C18 reversed-phase column. The concentration of the ACN in the eluted solvent was raised from 20% (v/v) to 100% (v/v) over 30 min using a linear gradient at a flow rate of 0.2 ml/min. Five main peaks (B1, B2, B3, B4 and B5) were obtained and the peak B5 showed HR activity. c. We chromatographed peak B5 again under the same conditions and a single peak was observed. d. Tricine-SDS-PAGE analysis of the peak B5, PeBL1, showing a single band with Coomassie Brilliant Blue R-250 staining. (B5: PeBL1, M: protein molecular weight marker)

Figure 2 The hypersensitive response induced by PeBL1 in *N. benthamiana*. A. The response was observed at 24 h post-injection: the right side of leaf was treated with elicitor and the other side was treated with Tris–HCl buffer as a negative control. B. Total RNA prepared from *N. benthamiana* leaves treated with buffer (−) or PeBL1 (+) was used as a template in RT-PCR assays. The expression of the *HSR203* gene was induced by PeBL1.
Figure 3 Purification of recombinant PeBL1. 

a. Total *E.coli* expressed proteins were purified by a His-Trap HP column. The peak P2 which mainly includes recombinant PeBL1 was eluted with elution buffer (25 mM Tris, 200 mM NaCl, 500 mM Imidazole, pH 8.0). 

b. The peak P2 was loaded on a HiTrap™ desalting column at a flow rate of 5 ml/min. The purified and desalted recombinant protein (peak D1) was isolated with saline ion (peak D2). 

c. The purified and desalted 6×His-tagged PeBL1 (D1) protein showed a single band with a molecular mass of 12 kDa on Tricine-SDS-PAGE. *M* protein molecular weight marker; *P2* the peak P2.

Figure 4 ROS burst and extracellular medium alkalinization in tobacco following PeBL1 treatment. 

A. Microscopic observation of H$_2$O$_2$ accumulation in *N. benthamiana* leaves. 

a PeBL1-treated leaves; b buffer-treated leaves. H$_2$O$_2$ accumulation (as indicated by diaminobenzidine staining) appeared in the veins and stomata of elicitor-treated leaves, but not in buffer-treated leaves. Scale bar=50 µm. 

B. ROS formation in tobacco cell culture following elicitor treatment, Flg22 treatment, and buffer treatment was detected in 96-well plates by chemiluminescence. ROS formation in both the PeBL1-treated and Flg22-treated cell cultures reached a maximum at approximately 20 min and declined thereafter to the level of the negative control. 

C. The kinetics of the extracellular medium alkalinization induced by PeBL1 (10 µM) in tobacco cell suspension. A distinct pH
increase in the elicitor-treated cell culture was monitored for the first 5 minutes, and the pH reached the maximum level at the 25 minutes. As positive control Flg22 induced a slightly higher and quicker maximal extracellular pH shift than PeBL1. Each data point represents three replicates. The error bars represent ±SD of the mean.

Figure 5 Phenolic compound deposition in tobacco cells following PeBL1 treatment. Tobacco cells treated with PeBL1 (a) and elicitor buffer (c) under bright field observation; tobacco cells treated with PeBL1 (b) and elicitor buffer (d) under fluorescence microscopy (using a 365 nm excitation filter and a 445 to 450 nm longwave pass filter). Scale bar=20 µm.

Figure 6 Systemic resistance induced by PeBL1 against the infection of TMV-GFP and P. syringae pv. tabaci in N. benthamiana. A. The numbers and diameters of TMV-GFP lesions (green fluorescence spot) in systemic leaves of protein-treated plants were significantly lower than in control plants. GFP pictures were taken under UV illumination. a. The upper PeBL1-treated leaves were imaged at 4 dpi. b. The upper buffer-treated leaves were imaged at 4 dpi. B. Against P. syringae pv. tabaci. The Cfu/cm² of buffer-treated N. benthamiana was compared with the cfu/cm² of PeBL1-treated N. benthamiana. The latter significantly reduced the infection of P. syringae pv. tabaci, with an inhibition ratio of 30% at 4 dpi. The results are mean values
from three independent experiments ±SD. The statistical analyses were performed using Student’s t-test. The asterisks indicate a significant difference between the treated and control samples. (**P < 0.01)

Figure 7 Expression levels of *N. benthamiana* defense-related genes relative to the control.

*N. benthamiana* leaves were infiltrated with 10 μM PeBL1 or buffer. At the indicated times, a small fragment was taken from the upper leaves and the amounts of mRNA of five defense-related genes were measured by RT-qPCR. a, b, c, d and e show the relative expression levels of *PR-1a*, *PR-5*, *PDF1.2*, *NPR1* and *PAL* genes, respectively. The samples were normalized against *EF-1α*. The expression levels are represented as fold change in relation to the untreated control. The results are mean values from three independent experiments ±SD. The statistical analyses were performed using Student’s *t*-test. The asterisks indicate a significant difference between the PeBL1-treated samples and buffer-infiltrated controls at the same point (*P < 0.05, **P < 0.01, ***P < 0.001).
TABLE 1. Primers used for real-time qPCR of defense-related and internal control genes.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>PR-1a</td>
<td>5’-CCCTGACATTCTCAAGTGTAAT-3’</td>
<td>5’-CCATTTGTACACCTGGAACCCTAGC-3’</td>
</tr>
<tr>
<td>PR-5</td>
<td>5’-CCGAGTTGATGAGACTGGAG-3’</td>
<td>5’-CCTCTGATGGGTGATTAGTGCA-3’</td>
</tr>
<tr>
<td>PDF1.2</td>
<td>5’-GGAATGGGCAAATCCATGCG-3’</td>
<td>5’-ATCCTTGGTCAGACAAACG-3’</td>
</tr>
<tr>
<td>NPR1</td>
<td>5’-ACATCGAGGAAGCAGTAG-3’</td>
<td>5’-GTGGCGAGGACTAGTCAAC-3’</td>
</tr>
<tr>
<td>PAL</td>
<td>5’-GTTATGCTCTTAGAAGTGCC-3’</td>
<td>5’-CCGTTGATGCTTATGTTTA-3’</td>
</tr>
<tr>
<td>EF-1a</td>
<td>5’-TGTGATGTTTTTTGGTGTCCTTTA-3’</td>
<td>5’-TCAAAAGAAATGCAGACAGACTCA-3’</td>
</tr>
</tbody>
</table>
**TABLE 2.** PeBL1 induces a significant resistance against TMV in tobacco

<table>
<thead>
<tr>
<th>Time after TMV inoculated</th>
<th>Number of lesions</th>
<th>Diameter of lesions (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PeBL1</td>
</tr>
<tr>
<td>2d</td>
<td>14.33±4.15</td>
<td>9.61±3.22</td>
</tr>
<tr>
<td>4d</td>
<td>37.17±9.22</td>
<td>21.22±5.57</td>
</tr>
<tr>
<td>6d</td>
<td>58.39±16.56</td>
<td>44.28±10.74</td>
</tr>
</tbody>
</table>

Data is representative of three replicates and nine plants for each replicate. Values are means±standard deviation. Values with the same letter in the same column are not statistically different at 5% significance level.