EplT4 proteinaceous elicitor produced in *Pichia pastoris* has a protective effect against *Cercosporidium sofinum* infections of soybean leaves

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Abstract

A cDNA library was constructed from the mycelium of *Trichoderma asperellum* T4 and a highly expressed gene fragment named EplT4 was found. In order to find a more efficient and cost-effective way of obtaining EplT4, this study attempted to produce EplT4 by using a *Pichia pastoris* (*P. pastoris*) expression system. The gene encoding EplT4, with an additional 6-His tag at the C-terminus, was cloned into the yeast vector pPIC9K and expressed in the *P. pastoris* strain GS115 to obtaining more protein for the further research. Transformants of *P. pastoris* were selected by PCR analysis and the ability to secrete high levels of the EplT4 protein was determined. The optimal conditions for induction were assayed using the shake flask method and an enzyme-linked immunosorbent assay (ELISA). The yield of purified EplT4 was approximately 20 mg/L by nickel affinity chromatography and gel-filtration chromatography (GFC). Western blot and MALDI-TOF-MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer) analysis revealed that the recombinant EplT4 was expressed in both its monomers and dimers. Soybean leaves treated with the EplT4 monomer demonstrated the induction of glucanase, chitinase III-A, cysteine proteinase inhibitor and peroxidase genes. Early cellular events in plant defense response were also observed after incubation with EplT4. Soybean leaves protected by EplT4 against the pathogen *Cercosporidium sofinum* (Hara) indicated that EplT4 produced in *P. pastoris* was biologically active, and would be potentially useful for improving food security.

Keywords EplT4 elicitor; *Trichoderma asperellum*; *Pichia pastoris*; Soybean; Induced resistance.

1 **Introduction**

2 *Trichoderma* spp. fungi have long been recognized as biocontrol agents of plant
3 diseases and are widely used as commercial biofungicides for the control of soil-
4 borne and foliar pathogens [1]. Regular use of *Trichoderma* spp. has provided an
5 economic advantage for food security because of the reduced need to use pesticides.

6 As *Trichoderma* spp. grow and proliferate, they have evolved numerous
7 mechanisms for attacking other fungi. The principal mechanisms for biocontrol have
8 been assumed to be those primarily acting upon pathogens including mycoparasitism,
9 antibiosis, and competition for resources and space [2, 3]. Some *Trichoderma* spp. are
10 able to colonize the intercellular spaces of plant roots up to the cortex layer. Root
11 colonization by *Trichoderma* spp. is frequently associated with the induction of both
12 local and systemic resistance to pathogen attacks [4-6].

13 Although many reports exist on elicitors produced by beneficial rhizobacteria,
14 since their discovery nearly 30 years ago [7], elicitors involved in systemic resistance
15 triggered by *Trichoderma* spp. are not well-studied. Induced resistance has been
16 reported for *Trichoderma harzianum* T39 strain on soybeans [8], a T203 strain on
17 cucumbers [5], and *Trichoderma virens* on cotton and maize [9, 10]. Cellulose and
18 xylanase from *Trichoderma* spp. can elicit resistance in plants independently of their
19 degradation activity [11]. The 18-mer peptaibols from *T. virens* can elicit cucumber
20 defense responses [12]. The dimerization of elicitors from *T. virens* (which secretes
21 the proteinaceous hydrophobin-like elicitor Sm1) and *T. atroviride* (eliciting plant
22 response-like protein, Epl1) has previously been investigated. Both Seidl and Vargas
23 have proposed that aggregation of the elicitors might control the *Trichoderma*-plant
24 interaction and block the activation of induced systemic resistance in plants [13, 14].

1 *T. asperellum* is an effective biocontrol agent against many pathogenic plant
2 species. A directional cDNA library from the mycelium of *T. asperellum* T4 strain
3 fungi was constructed [15] and a highly expressed gene fragment named EplT4 that is
4 homologous to Epl1 protein in *T. atroviride* was found. As Epl1 protein in *T.*
5 *atroviride* is a plant defense response elicitor, which signifies that EplT4 may have
6 the similar function. Given, that high redundancy of a specific cDNA sequence is
7 usually associated with high expression levels, this indicates that it may play an
8 important role in biocontrol. According to sequence analyses, EplT4 belongs to the
9 cerato-platanin (CP) 3-protein family, which consists mainly of proteins produced by
10 fungi that are associated with toxicity and infection processes [16]. The peptides
11 revealed a high similarity to cerato-ulmin, a phytotoxic protein involved in Dutch Elm
12 disease that belongs to the hydrophobin family. Hydrophobins are hydrophobic
13 proteins secreted by many pathogenic fungi. These proteins have the ability to self-
14 assemble into a rodlet structure which is involved in growth and/or developmental
15 processes [17].

16 Purification of native Sm1 protein from *T. virens* results in low protein yield
17 (approximately 150 µg/L) [16] which limits its wider application to small-scale assays
18 (10 µg EplT4 was needed in each trial). Similar results (110 µg/L) were obtained from
19 purification of *T. asperellum* T4 (unpublished data). In order to explore the role
20 played by EplT4, for which significant amounts of proteins were needed, this study
21 aimed to produce EplT4 by recombinant methods. *Escherichia coli*, the most common
22 heterologous protein expression system, does not correctly process many polypeptides
23 [18, 19]. The recombinant CP has been expressed in *E. coli* and the correct product
24 was obtained only after a refolding step and at low yields [17]. The *Pichia pastoris*
25 heterologous gene expression system, which has appropriate post-translational

1 modifications for protein functionality, has been used exclusively to produce high
2 levels of a variety of intracellular and extracellular proteins of interest [20]. *P.*
3 *pastoris* is a methotrophic yeast which requires neither complex growth medium nor
4 culture conditions, is genetically relatively easy to manipulate, and has a eukaryotic
5 protein synthesis pathway. Simple purification of secreted recombinant proteins is
6 possible due to the relatively low levels of native secreted proteins [21, 22].
7 Therefore, the aim of this study was to express EplT4 in *P. pastoris* and demonstrate
8 its elicitation properties in soybean.

9 **Materials and methods**

10 **Materials**

11

12 Pfu DNA polymerase was obtained from Promega (Madison, WI, USA). Restriction
13 endonucleases, T4 DNA ligase, and protein molecular markers were purchased from
14 Fermentas (China). Yeast nitrogen base (YNB) was purchased from Invitrogen. His-
15 bind column was purchased from Novagen (Madison, WI, USA). Superdex 200 was
16 purchased from G.E. Healthcare (USA). The statistical software package SPSS 17.0
17 (IBM, Armonk, NY, USA) was used for statistical analysis. All other chemicals used
18 were analytical grade reagents unless otherwise stated.

19

20 **Strains and Plant Varieties**

21

22 Biocontrol agent *T. asperellum* strain T4 was obtained from the China General
23 Microbiological Culture Collection Center (number CGMCC3.14975). *P. pastoris*
24 expression vector pPIC9K and *P. pastoris* host strains GS115 (his-mut+) were
25 obtained from Invitrogen. *E. coli* Top10F⁺ was used for propagation of plasmids

1 (Invitrogen). *Cercosporidium sofinum* (Hara) strain 15 (which causes frog eye leaf
2 spot disease on soybeans) and soybean (*Glycine max*) variety Suinong 10 were
3 provided by the Heilongjiang Academy of Agricultural Sciences, China. The EplT4
4 protein sequence was aligned with other CP proteins available in the GenBank
5 database using the ClustalX (version 1.8) multiple sequence alignment program. The
6 alignment results were manipulated using GeneDoc software (version 2.12).
7 Phylogenetic analysis was performed with the protein sequences aligned by ClustalW
8 using the neighbor-joining algorithm. A phylogenetic tree was generated, and a
9 distance matrix was produced, using the MEGA (version 3.1) program.

10

11 Construction of *P. pastoris* Expression Plasmid of EplT4

12

13 Primer design was based on the nucleic acid sequence of *EplT4* from published
14 databases (Accession No. HM572232), and the primers P1 and P2 (Table 1) were
15 designed for PCR amplification from cDNA including the completed coding DNA
16 sequence (CDS). The signal peptides were analyzed by Signal P 3.0 server
17 (<http://www.cbs.dtu.dk/services/SignalP>). The native signal peptides were removed
18 and an additional sequence encoding a 6-His tag was fused at the 3' end of the EplT4
19 gene for easy purification and detection of the gene product. The amplified EplT4
20 gene was purified using the UNIQ-10 Column Collection Tube Kit and digested with
21 *EcoR* I and *Not* I, and ligated with similarly digested pPIC9K. The ligated product
22 was transformed in *E. coli* Top10F' cells and the positive colonies were selected by
23 colony PCR. Positive transformants were further confirmed by sequencing, using the
24 dideoxy chain-termination method [23]. This strategy placed EplT4 (without its native

1 N-terminal signal sequence) under the AOX promoter of pPIC9K vector and used the
2 *S. cerevisiae* signal peptide for secretion out of the cells.

3

4 Transformation and Screening for EplT4-Productive Clones

5

6 *Sal* I was used to linearize 5-10 µg of pPIC9K-EplT4 plasmid DNA, and the linear
7 DNA was then transformed into *P. pastoris* GS115 cells with an electroporator
8 (Eppendorf: conditions: 1.1 kV). The electroporated cells were immediately
9 suspended in 1.0 mL of ice-cold 1.0 M sorbitol and spread on MD plates (1.34% yeast
10 nitrogen base containing ammonium sulfate and lacking amino acids, $4 \times 10^{-5}\%$
11 biotin, 2% dextrose, and 1.5% agar). The His⁺ transformants resistant to increasing
12 concentrations of G418 were screened according to the manufacturer's protocol
13 (Invitrogen). The “boiling-freezing-boiling” method was used to prepare the *P.*
14 *pastoris* genomic DNA [24]. His⁺ transformants were analyzed by PCR using the α-
15 factor primer and 3'-AOX1 primer (Table 1) to investigate whether the EplT4 gene
16 had integrated into the *P. pastoris* genome.

17 For screening EplT4-productive clones, several single colonies of *P.pastoris*
18 were inoculated into 50 mL of buffered glycerol-complex medium (BMGY; 1% yeast
19 extract, 2% peptone, 100 mM of potassium phosphate, 1.34% YNB, $4 \times 10^{-5}\%$ biotin,
20 and 1% glycerol, pH 6.0) in 250 mL flasks at 28°C, with shaking at 220 rpm until an
21 OD₆₀₀ value of 6 was attained. The cell pellets were harvested and resuspended in 100
22 mL of BMMY (1% yeast extract, 2% tryptone, 1.34% YNB, $4 \times 10^{-5}\%$ biotin, 0.5%
23 methanol (v/v) in 100 mM potassium phosphate, pH 6.0) in 1 L flask with shaking at
24 220 rpm at 28°C. The resuspended culture was induced for 96 h by addition of 100%
25 methanol to a final concentration of 0.5% (v/v) every 24 h. The supernatant was

collected by centrifugation at $10,000 \times g$ at 4 °C for 10 min and analyzed by Western blot. The primary antibody and secondary antibody were mouse anti His-tag and goat anti mouse IgG, respectively.

Optimization of Conditions for Cell Growth and EplT4 Expression

To define the optimal conditions for EplT4 expression, different factors that could affect the host cell growth and EplT4 yield were tested, including the time following induction (24, 48, 72, and 96 h), pH values (4.0, 5.0, 6.0, and 7.0), and final concentrations of methanol (0.5%, 1.0%, and 2.0%). The EplT4 yield was determined by an enzyme-linked immunosorbent assay (ELISA). Antibodies used were as described above.

For high-level production of EplT4, the highest G418-resistant *P. pastoris* clone was selected as seed, while the high-cell density fermentation was conducted in a 19 L fermenter (NLF, Bioengineering AG, Switzerland) as previously described [25]. Glycerol batch fermentation (high-cell density fermentation) was performed according to the Pichia Fermentation Process Guidelines (Version B, 053002). When the initial glycerol was depleted as indicated by a dissolved oxygen spike, glycerol fed-batch fermentation was initiated by starting the fed-batch medium at a rate of $18.4 \text{ mL h}^{-1} \text{ L}^{-1}$ of initial medium volume. In the methanol fed-batch phase, temperature, pH and DO (dissolved oxygen concentration) were monitored and maintained at set points of 30°C, pH 5.0 and 30-40%, respectively. The pH was adjusted by the addition of ammonium hydroxide as needed. The growth curve of recombinant *P. pastoris* was determined by weighing the wet weights of the cells from fermentation

1 mixture taken at 24 h intervals. In addition, Western blot was employed to assess the
2 expression of EplT4 secreted into the culture medium.

3

4 Purification and Characterization of the Secreted Recombinant EplT4

5

6 The entire culture medium obtained from the high-cell density fermentation was
7 harvested by centrifugation at $8,000 \times g$ for 15 min at 4°C. The resultant supernatants
8 were precipitated overnight with 80% ammonium sulfate. The resulting precipitate
9 was then centrifuged at $12,000 \times g$ for 20 min, collected and dissolved in binding
10 buffer of a His-bind column. The protein dissolved in binding buffer was passed
11 through a 0.45 μm membrane filter and applied to the His-bind column equilibrated
12 with binding buffer (NaCl 0.5M; Tris 20mM; Imidazole 5mM, and pH 7.9). After
13 washing with washing buffer (NaCl 0.5M; Tris 20mM; Imidazole 60 mM, and pH
14 7.9), the recombinant protein was eluted with elution buffer (NaCl 0.5M; Tris 20mM;
15 Imidazole 1M, and pH 7.9). The fractions containing EplT4 were pooled, dialyzed
16 against 10 mM NH_4HCO_3 and concentrated to 5 mL. They were then purified further
17 by gel-filtration chromatography (GFC) through a HiPrep 26/60 Sephacryl S-100 HR
18 (16×60 cm; G.E. Healthcare) pre-equilibrated with Tris/NaCl buffer (Tris 50mM;
19 NaCl 100mM, pH 7.0). The column was then eluted with 50 mM Tris and 100 mM
20 NaCl at a flow rate of 1.0 mL/min. The fractions that showed maximum absorbance at
21 280 nm were isolated. The purity of protein was assessed by a 15% SDS-PAGE
22 followed by Coomassie blue staining. The fractions containing recombinant EplT4, as
23 determined by SDS-PAGE, were pooled, dialyzed (10 mM NH_4HCO_3) and
24 lyophilized for further experiments.

Both the bands of EplT4 monomer and its dimer candidate after separation on SDS-PAGE were excised manually and subjected to in-gel trypsin digestion [26]. The digestion products were extracted and prepared for MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometer) analysis. The peptide mass fingerprint was analyzed in a MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometer).

Recombinant EplT4 Activity Tests

The production of H₂O₂ in two week-old soybean unifoliate leaves was examined 12 h after application of either EplT4 monomer (10 µg) or sterile water (negative control). Drops (10 µg) of EplT4 or sterile water were placed on the slightly punctured surface of the leaves. The leaves were then infiltrated with nitro 3,3'-diaminobenzidine (DAB, Sigma), incubated overnight, fixed and cleared in alcoholic lacto-phenol solution [27]. A red-brown precipitate was observed using an Olympus microscope BX-51 (Olympus America Inc.) at ten-fold magnification.

To test for autofluorescence, 10 µg of EplT4 monomer, 50 nmol of 2,6-dichloroisonicotinic acid (INA, Sigma), or sterile water was applied to soybean leaves as described above. After 12 h of incubation, the induction of autofluorescence in leaves was observed. Treated leaves were cleared in alcoholic lacto-phenol solution, incubated in 70% glycerol, and mounted on slides [27]. Microscopy was performed using an Olympus BX-51 fluorescent microscope at twenty-fold magnification (excitation wavelengths from 470 to 490 nm, emission wavelengths from 510 to 550 nm, and a dichroic mirror at 505 nm).

C. sofinum (Hara) Resistance Assay of Soybean Leaves

C. sofinum (Hara) strain 15 was grown for 10 days on PDA at 28°C under constant light. Conidiospores were scraped from plates, filtered through gauze, and washed three times in distilled water, followed by centrifugation for 5 min at $10,000 \times g$. Conidiospores were counted using a hemacytometer, spore suspension was adjusted to 1×10^4 conidia/mL and Tween 20 was added to a final concentration of 0.005%. Fourteen day-old soybean unifoliate leaves were elicited applying EplT4 (10 µg) to the cut petiole and salicylic acid (SA, Sigma) at 30 nmol/leaf was used as positive control. After 12 h of incubation at 27°C, 50 µL of *C. sofinum* (Hara) conidiospores was placed on the surface of each leaf. The seedlings were incubated in a growth chamber at 27°C, with a 14 h photoperiod and 60% humidity for two weeks. Leaves were photographed with a digital camera (Nikon). Lesion percentage was determined using Image Pro Plus 6.0 software. Each treatment contained five repetitions and the experiment was repeated twice. Data were analyzed by ANOVA and Fisher's PLSD test ($P < 0.05$).

Expression Analysis of Soybean Defense-Related Genes

Real-time quantitative PCR (qPCR) was used to evaluate gene expression in soybean leaves after 12 h of incubation of EplT4 monomer (50 µg) or sterile water (negative control). The leaves were harvested and frozen immediately in liquid nitrogen. Total RNA was extracted using Trizol reagent according to the manufacturer's protocols. Total RNA (5 µg) from each sample was reverse-transcribed into cDNA in the presence of oligo(dT) primer. The synthesized cDNA was used as a template for

qPCR. Six pathogenesis-related genes involved in plant defense pathways were selected for expression analysis. Actin 11 was used as an internal reference to normalize the amount of total RNA present in each reaction. All genes and primers used in this study are presented in Table 1. The expression levels of the genes were calculated from the threshold cycle according to the $2^{-\Delta\Delta CT}$ method [28].

Results

Cloning and Expression of EplT4 in *P. pastoris*

The EplT4 from *T. asperellum* T4 is a 138 aa protein with a predicted molecular mass of 14.5 kDa (containing signal peptide), and an isoelectric point (pI) of 6.68 (Supplement Fig. 1a). A putative signal peptide of 18 aa with a cleavage site between alanine-18 and aspartic acid-19 at its N-terminal and four conserved cysteine residues located in conserved amino acid positions were identified in EplT4 protein (Supplement Fig. 1a). Sequence alignment and phylogenetic tree analysis with homologues from other fungi revealed that EplT4 shows a high level of similarity to the fungal proteins of *T. atroviridis* and *T. virens* (Supplement Fig. 1b).

The recombinant pPIC9K-EplT4 vector was constructed by inserting complete EplT4 CDS (excluding the secretion tag and with an additional 6-His tag at the 3' end) into the *EcoR* I and *Not* I sites in the pPIC9K vector (Supplement Fig. 2). The resulting vector was transformed into *P. pastoris* strain GS115, and approximately 300 transformants were obtained on MD plates. Further screening of the transformants with different concentrations of G-418 yielded five colonies on 0.3 mg/mL G-418. After a preliminary screening, one transformant that contained the appropriate size of the EplT4 gene insert, and also produced an extracellular protein

1 in the crude filtrate as analyzed by Western blot (Fig. 1), was selected. The yeast cells
2 harboring an empty pPIC9K vector did not yield the PCR product or the extracellular
3 protein (Fig. 1a lane 2, 1b, lane 4).

4 5 High-Cell-Density Fermentation to Produce Recombinant EplT4

6
7 The optimum time to induce EplT4 expression is shown (Fig. 2a). A significant
8 increase in the amount of recombinant protein was observed with increasing induction
9 time, peaking at 48 h. Therefore, EplT4 was collected at 48 h after induction. As
10 shown in Fig. 2b, a pH of 5 was found to be optimal. Because methanol is both the
11 inducer and the sole carbon source of *P. pastoris*, its concentration was fundamental
12 in influencing EplT4 production. As revealed in Fig. 2c, EplT4 yield was similar
13 between concentrations of 0.5% and 2.0%. However, both cell growth and EplT4
14 yield declined at a methanol concentration of 5.0% (data not shown). As a result the
15 concentration of methanol in the culture medium was maintained at 1%.

16 To obtain enough recombinant EplT4 for the evaluation of its bioactivity, high-
17 cell density fermentation of the selected clone was conducted in a 19 L fermenter with
18 5 L initial volume. After fed-batch fermentation, the wet weight of the cells reached
19 200 ± 25 g/L (40 h post-seeds inoculation), and the induction medium was gradually
20 added. As shown in Supplement Fig. 3, two protein bands were observed in Western
21 blot analysis of the fermentation supernatant at different induction times. Because the
22 optimum time to induce EplT4 expression was found to be 48 h, the supernatant at 48
23 h was collected for purification. The yield of EplT4 from the fermentor was 50 mg/L
24 which was 1.56 times more than the yield from the shaker flask (32 mg/L).

Purification and Characterization of Recombinant EplT4

Due to the presence of the 6-His tag, the secreted EplT4 was purified by nickel affinity chromatography, resulting in two primary bands (Fig. 3b, lane 2). The purification of EplT4 by gel filtration chromatography on Sephacryl S-100 HR is displayed in Fig. 3a. Fractions corresponding to EplT4 monomer were identified spectrophotometrically at OD₂₈₀ (Fig. 3b, lane 3). This was later confirmed by Western blot using the anti-His-tag antibody. In contrast to the calculated molecular weight of 13.4 kDa (without signal peptide and adding 6-His tag), the recombinant protein migrated as a band with an apparent molecular mass of approximately 17.0 kDa. The final yield of EplT4 monomer obtained from 1 L of basal salts medium induced by 1% methanol was approximately 20 mg/L using Bradford assays [29]. To investigate whether EplT4 could self-associate into higher order to assemble a homodimer, similar to Epl1 and Sm1, the monomer and dimer were subjected to in-gel tryptic digestion. The peptide mass fingerprint of both isoforms revealed similar patterns, with the peptides at m/z 1414.8, 1718.4, 2304.9 and 2505.2 existing in both isoforms (Fig. 4).

EplT4 Induces Plant Defense Reactions in Soybean

EplT4 belongs to the CP protein family, which is usually involved in phyto-pathological phenomena and immunological reactions. The production of hydrogen peroxide and accumulation of phenolic compounds that usually associate with autofluorescence are the early response signals in elicitor recognition [30, 31]. The ability of recombinant EplT4 to elicit these responses was tested and the production of

1 hydrogen peroxide in soybean leaves after exposure to EplT4 was examined.
2 Application of EplT4 (10 µg) resulted in the production of hydrogen peroxide around
3 the puncture. A brownish-red precipitate generated by polymerization of the hydrogen
4 peroxide with nitro 3,3'-diaminobenzidine in the treated tissues was observed (Fig. 5).
5 The induction of autofluorescence was tested in soybean unifoliate leaves. An
6 insignificant response was found in sterile water-treated leaves (negative control),
7 which was probably due to the wounding of the leaf during the treatment application.
8 High levels of autofluorescence were apparent after treatment with 10 µg of EplT4
9 monomer or 50 nmol of INA (SA analog) used as the positive control (Fig. 6).

10

11 EplT4 Induces Resistance Against a Soybean Pathogen

12

13 In order to assess the disease resistance of soybeans, the leaves pretreated with EplT4
14 were inoculated with *C. sofinum* (Hara) strain 15 for 12 h. SA and sterile water were
15 used as positive and negative control, respectively. Control plants treated with water
16 began showing symptoms in the form of frog-eye spots within seven days. Disease
17 assessment was conducted after fourteen days following the inoculation as shown in
18 Fig. 7. In contrast to frog-eye spots observed in water-treated leaves, disease protection
19 was evident in leaves treated with EplT4 and SA, since almost no lesion development
20 could be observed in those plants (Fig. 7, a). The mean lesion area exhibiting
21 symptoms was significantly higher in control plants (5.17 %) than in plants treated
22 with EplT4 (0.75 %) or SA (0.42 %) (Fig. 7, b). The results confirmed that purified
23 EplT4 was able to induce resistance to *C. sofinum* (Hara) in soybeans.

24

25 EplT4 Induces the Expression of Plant Defense Genes

1
2 The expression of these pathogenesis-related protein genes in leaves treated with
3 EplT4 was examined. Two week-old soybean seedlings grown in a gnotobiotic
4 system were used. PR-proteins have been classified into several groups based on their
5 amino acid sequences and biochemical functions. The expression of the PR-proteins
6 either singly or in combination appears to improve plant resistance. PR-protein genes
7 from different families were selected. Gene expression 12 h after application of EplT4
8 monomer to soybean leaves was shown in Fig. 8. Application of EplT4 resulted in up-
9 regulation of glucanase (GLU), chitinase III-A (Chi III-A), cysteine proteinase
10 inhibitor (CPI) and peroxidase gene expression compared to the control (treatment
11 with sterile water). Chitinase I (Chi I) and *Thaumatin*-like protein (TLP) were
12 constitutively expressed in the leaves and showed no response to the EplT4 treatment.
13 In summary, the treatment of plant leaves with EplT4 resulted in inducing some
14 defense-related genes in soybean variety Suinong 10.

15 16 **Discussion**

17 *P. pastoris* has been developed as an excellent host for heterologous protein
18 expression owing to its high expression levels, efficient secretion, proper protein
19 folding and the potential for a very high-cell density fermentation [32]. The
20 expression levels of the EplT4 protein in *T. asperellum* T4 was low, which is a
21 limitation for large-scale production. In this study, the gene encoding EplT4 into the
22 pPIC9K vector was subcloned and the recombinant protein in *P. pastoris* expressed.
23 After induction by methanol, EplT4 protein was secreted into the culture supernatant.
24 The optimal conditions for EplT4 expression in shake flask include induction for 48
25 h, and a culture pH of 5.0 (Fig. 2a, b). Because the shake flask is an open system and

1 the methanol inducer could easily evaporate, the protein yield did not change when
2 different amounts of the inducer were added (Fig. 2c). To obtain sufficient
3 recombinant EplT4, high-cell density fermentation was conducted in a fermenter.
4 EplT4 was purified to homogeneity by nickel affinity chromatography and gel-
5 filtration chromatography. Approximately 20 mg of purified target protein could be
6 obtained from 1 L of culture. Previous work focusing on a related protein, Sm1 from
7 *T. virens* has produced a higher yield than this protein (55 mg) due to less dimers
8 being formed [33]. The monomer of EplT4 showed significant elicitor activity to
9 soybean Suinong 10 against *C. sofinum* (Hara) strain 15.

10 The molecular weight of recombinant EplT4 was approximately 17.0 kDa, which
11 was larger than the predicted value (13.4 kDa). The reasons for the higher molecular
12 mass of EplT4 may be due to post-translational modification by glycosylation. Since
13 the α -factor signal sequence for secretion was fused to the N-terminus sequence of
14 EplT4, an N-terminal extension can result from the inefficient cleavage due to the
15 occlusion of the cleavage site on the secreted protein from the peptidase [34]. A
16 similar phenomenon was observed with expression of the other protein from the CP
17 protein family in *P.pastoris* [17, 33]. The recombinant EplT4 produced by *P. pastoris*
18 could self-assemble into a dimer (Fig. 3, b), as confirmed by Western blot
19 (Supplement 3) and also protein fingerprinting with MALDI-TOF MS (Fig. 4). The
20 dimer was insoluble in a boiling solution containing 10 g of sodium dodecyl sulfate
21 (SDS) in 1 L of 0.1 M sodium phosphate buffer (pH 7.0). This property is similar to
22 that of hydrophobic proteins, which have the ability to self-assemble [35]. Sm1 and
23 Epl1 have different abilities to induce plant defense responses due to a small number
24 of amino-acid differences that determine dimerization [14]. EplT4 is more similar to
25 Epl1 than Sm1 (Supplement figure 1, b) in that it may cause EplT4 forming dimers

1 more easily. In addition, the results of this study have showed that EplT4 can
2 assemble into its dimer at a relatively low level when in low concentration (Fig. 1b).
3 In contrast, the dimer became the main component when high levels of protein were
4 generated by high-cell-density fermentation (Fig. 3b Lane 1 and Supplement 3). The
5 dimer of EplT4 did not show any elicitor activity to soybean (data not shown).
6 However, these aggregates require further investigation to clarify their mechanisms.

7 It is known that accumulation of reactive oxygen species is one of the early
8 cellular events in plant defense responses. EplT4 induced production of H₂O₂ in
9 soybean leaves (Fig. 5) without causing hypersensitive response-associated necrotic
10 lesions (Fig. 6a). It has been reported that accumulation of reactive oxygen species is
11 not necessarily associated with hypersensitive response [36, 37]. This is different to
12 what has been observed for CP-proteins from *Ceratocystis fimbriata* [17],
13 *Magnaporthe grisea* [38] and *Botrytis cinerea* [39]. The proposal is that these
14 pathogens would actively stimulate hypersensitive response in plants with the purpose
15 of generating dead tissue to grow. EplT4 induced accumulation of phenolic
16 compounds when applied to soybean leaves. Accumulation of phenolic compounds
17 usually associated with autofluorescence is an example of another type of plant
18 response to pathogens. A prominent autofluorescence response was observed
19 indicating callose was generated around the treated region [40].

20 The protection by purified EplT4 of soybean leaves against *C. sofinum* (Hara), a
21 common and destructive pathogen of soybeans in China, was investigated. The results
22 of this study showed that EplT4 was able to protect soybean leaves from infection by
23 *C. sofinum* (Hara). The disease in EplT4-pretreated or SA- pretreated leaves was
24 strongly reduced and lesions were minimal. Because humidity is necessary for *C.*
25 *sofinum* (Hara) to infect soybeans, the seedlings were cultured in a growth chamber

1 with 60% humidity. We found that soybean variety Suinong 10, which is normally
2 susceptible to *C. sofinum* (Hara) strain 15, was not diseased when grown below 10%
3 humidity.

4 Due to the fact that PR proteins are usually used as markers of plant responses to
5 pathogens or their elicitors, six PR proteins belonging to different families were
6 chosen. In this study, GLU, chi III-A, cysteine proteinase inhibitor and peroxidase
7 genes were highly induced in response to EplT4 (Fig. 8). Interestingly, chi III-A did
8 not show a response to EplT4, whereas chi I indicated that the two genes may play
9 different roles in plant-pathogen interactions [41, 42]. Thaumatin-like protein gene
10 expression did not increase after being induced for 12 h. It has been reported that
11 thaumatin-like protein gene expression in the stem and leaf was maintained at a
12 constant level until 24 h when it was induced under stress [43].

13 In summary, this study has revealed that a methylotrophic yeast-inducible system
14 is suitable for the high-level expression of the proteinaceous elicitor EplT4, as the
15 yield increased by approximately 180-fold. Although some additional residues were
16 imported into the recombinant EplT4 using alpha factor as signal peptide and 6-His-
17 tag for protein purification, the activity of EplT4 was not affected. EplT4 dimer
18 formation is concentration dependent; the higher level of dimer is due to higher
19 expression levels in the fermentor compared to the shaker flask cultures. The property
20 that EplT4 is able to induce plant immune response without necrosis makes it possible
21 for further applications. Overall, this efficient, cost-effective expression system may
22 open new methods for the possible practical applications of EplT4 in high-level plant
23 growth production systems, thereby improving food security.

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Figures Legends

Fig. 1 Confirmation of *Pichia* transformant by colony PCR and Western blot. (a) Agarose gel of EplT4 amplified by PCR using α -factor primer and 3' AOX1 primer. Lane M molecular marker (DNA ladder 2 kb, Takara); lane 1 positive transformant GS115/pPIC9K-EplT4; lane 2 *Pichia* transformed with empty vector GS115/pPIC9K. (b) Western blot of extracellular production of recombinant EplT4 in transformants induced by 1% methanol after 24 h and 48 h incubation. Lane 4 negative control GS115/pPIC9K (48 h); lanes 5 and 6 GS115/pPIC9K-EplT4 (12 h and 48 h, respectively). Arrow indicates the position of EplT4

Fig. 2 Optimal conditions of EplT4 expression in *P. pastoris*. (a) Expression level of EplT4 in *P. pastoris* over four days incubation with induction by methanol. (b) Expression level of EplT4 at different pH. (c) Expression level of EplT4 induced by different concentrations of methanol

Fig. 3 Purification of EplT4 from *P. pastoris* by affinity chromatography and gel filtration chromatography. (a) GFC separated *P.pastoris* into the EplT4 monomer and dimer. (b) The major bands of EplT4 proteins were analyzed by SDS-PAGE followed by Coomassie blue staining. Lane M marker; lane 1 supernatant from medium; lane 2 protein obtained by affinity chromatography; lane 3 EplT4 monomer protein obtained by GFC

Fig. 4 Comparison of the MS polypeptide fingerprint of the EplT4 dimer (a) and monomer (b) after tryptic digestion. Both isoforms were digested in gel, and the polypeptides were analyzed by MALDI-TOF MS

Fig. 5 H₂O₂ accumulation in soybeans. (a) Soybean leaves were treated with sterile H₂O (negative control) and (b) treated with EplT4 (10 μ g). Treated tissues were harvested 12 h after treatment, and excised leaves were infiltrated with a solution of 3,3'-diaminobenzidine (DAB). In the presence of H₂O₂, DAB polymerized, forming a dark red-brown precipitate. Microscopy was performed using Olympus microscope BX-51 (Olympus America Inc.) at ten-fold magnification. Arrow indicates the application area

Fig. 6 Induction of autofluorescence in soybean leaves. (a) Bright-field image and (b) Fluorescence of soybean leaves. Droplets of sterile H₂O (negative control), EplT4 (10 μ g), or INA (50 nmol, positive control) were placed on the upper surface of slightly punctured leaves. The presence of autofluorescence in treated cotyledons was assessed after 12 h of incubation. Micrographs are centered on the treated region surrounding the application area (indicated by arrows). Microscopy was performed using Olympus BX-51 fluorescent microscope with excitation from 470 to 490 nm, emission from 510 to 550 nm, and 20-fold magnification

Fig. 7 Effect of EplT4 application on fungal disease protection. Soybean leaves were inoculated with *C. sofinum* 12 h after applying a 50- μ l droplet of sterile H₂O (negative control), EplT4 (10 μ g/leaf), or salicylic acid (SA; 30 nmol/leaf, positive control) to the cut petiole. (a) The mean lesion areas caused by each of the three treatments were evaluated and photographed 10 days post-inoculation. (b) The graph illustrates the levels of disease protection observed in each treatment. Each bar represents the mean $\pm$ standard error of 10 plants. Columns with letter in common did not differ significantly according to Fisher's protected least significant difference test at a significance level of $P < 0.05$

Fig. 8 Expression analysis of soybean defense-related genes. GLU (*Beta*-1,3-glucanase), Chi I (chitinase I), Chi III-A (chitinase III-A), TLP (*Thaumatococcus*-like protein), CPI (cysteine proteinase inhibitor). The relative expression levels are equal to their expression after incubation with EplT4 divided by their expression level after being treated with sterile H₂O

Supplemental Figures

Supplemental Fig. 1 Alignment and phylogenetic tree analysis of EplT4. (a) Alignment of EplT4 with other known fungal proteins. Fungal proteins used for alignment were from *Trichoderma asperellum* T4 (ADP09352), *Trichoderma virens* (Q1KHY4) and *Trichoderma atroviridis* (DQ464903). Signal peptides at N-terminal are indicated by a line below the sequences. Four conserved cysteines are indicated by filled triangles. (b) Phylogenetic tree analysis of EplT4 with other proteins from various fungal species. Phylogenetic tree was constructed using CLASTALW program in MEGA software. The fungal proteins used were from *Trichoderma asperellum* T4 (ADP09352), *Trichoderma virens* (Q1KHY4), *Trichoderma atroviridis* (DQ464903), *Ceratocystis platani*(ABM63513), *Auricularia delicata* (EJD54220), *Fomitiporia mediterranea* (EJD01882), *Ceratocystis fimbriata* (ABM63507), *Colletotrichum higginsianum* (CCF39177), *Aspergillus oryzae* (Q2UF42), *Emericella nidulans* (Q5AZK7), *Gibberella zeae* (Q5PSV7), *Gibberella pulicaris* (Q5PSV6) and *Leptosphaeria maculans*(Q8J0U4)

Supplemental Fig. 2 Schematic description of the construct pPIC9K-EplT4. (a) Sequence of EplT4 gene cloned into pPIC9K. (b) Amplified EplT4 cDNA. Lane M molecular marker (DL2000); lane 1 EplT4 cDNA

Supplemental Fig. 3 Western blot of recombinant EplT4 from high-cell-density fermentation. Lanes 1-5 supernatant from medium at 0 h, 24 h, 48 h, 72 h and 96 h, respectively

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1 **Table 1.** Primers used in this study

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Gene/primer name	GenBank No.	Family of PR protein	Primers (5'-3')
<i>Actin 11</i>	BW652479	internal references	ATTTTGACTGAGCGTGGTTATTCC GCTGGTCCTGGCTGTCTCC
<i>Thaumatococcus-like protein</i>	AB116251	PR-2	AATGGCCGTCACGAAAAGC TGAAGTTCGCTGCCTGAGCTA
<i>Chitinase I</i>	AF202731	PR-3	ATTTGGGTGGTGTGGTGACA GTGTTGAGGGTGTGTCAGATTG
<i>Chitinase III-A</i>	AB006748	PR-8	GAAGCTACTCCCTCAGCTCAGC CCTAATGGCCCTGATCCAGTT
<i>Beta-1,3-glucanase</i>	U08405	PR-5	CCTCCCAATGATGGCGTTTT GCCCCATTGCTCACTAGGAA
<i>Cysteine proteinase inhibitor</i>	D31700	PR-6	CCAACCTGGATGGCAATCTG GCTGGATAGTCTTGATCGCATGAT
<i>Peroxidase</i>	L78163	PR-9	CAGGTGGTCATACGTTTGGAAGA AGGGTTTCCAGTGTTGCTGAA
P1			CGGAATTCGATACCGTCTCGTACG ATACCGGCT
P2			ATAAGAATGCGGCCGCTTAATGAT GGTGATGGTGATGGAGGCCGAGT TGCTCACAGC
α -factor primer			TACTATTGCCAGCATTGCTGC
3'-AOX1 primer			GCAAATGGCATTCTGACATCC

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