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Optimization of Fermentation Conditions for EplT4 Production by *Pichia pastoris* Expression System Using Response Surface Methodology

Yun Wang[1], Lei Zhao[1], Hua Cong[1], Chitradee Luprasong [1], Ping Yang[1], Lin Yao[1], Shuang Wang[2], Jin-Xia Fan[3], Xiao-Mei Huang[3] and Qian Yang[1*]

[1]*Department of Life Science and Engineering, Harbin Institute of Technology, Harbin 150001, China*
[2]*Institute of Soil Fertilizer and Environment Resources, Heilongjiang Academy of Agricultural Sciences, China*
[3]*Northeast Agricultural University, Harbin 150030, China*
[*]For correspondence: yangqian84@yahoo.com

Abstract

The design of the optimum conditions for production of EplT4 protein from *Trichoderma asperellum* T4 in *Pichia pastoris* expression system was performed using response surface methodology (RSM). Temperature, pH and Basal Salts were the most important experiment factors selected by Plackett-Burman design. Afterward, the optimal combination of the three factors and the corresponding maximum yield of EplT4 protein were explored using Box-Behnken Design. As shown in the results, the maximum EplT4 protein yield of 38.03 mg/L was predicted when temperature, pH and Basal Salts were 28.5°C, 4.7 and 62.7%, respectively. The maximum yield of 37.70 mg/L EplT4 was obtained under the optimum conditions, which verified the practicability of the optimum method. These results indicated that EplT4 yield was remarkably improved through optimizing the *P. pastoris* expression system using response surface methodology. Furthermore, the obtained EplT4 recombinant protein was proved effective to induce defense responses in soybean. Therefore, this study is meaningful for the application of EplT4 as a promising biofungicide.

Keywords: EplT4 protein; Optimization; Response surface methodology; Defense response

Introduction

Traditional methods used to protect crops from pathogens were mainly dependent on the chemical fungicides. However, the application of chemical fungicide is being replaced by biological control agents, these are basically attributed at least to two reasons, firstly the development of fungicide-resistant strains and secondly the public consideration about the health and environmental impacts of these chemicals (John *et al.*, 2010). In recent years, *Trichoderma* spp., a genus of fungi, has been a focus of research and is extensively used as commercial bio-fungicides for control of soil-borne and foliar plant pathogens (Verma *et al.*, 2007). The previous public reports had demonstrated the modes of action including antibiosis, mycoparasitism, competition and induced plant defense (Howell, 2003). Due to their broader characteristic in terms of production yield and disease control, fungal based biological control agents (BCAs) have gained wide acceptance (Copping and Menn, 2000; Lorito *et al.*, 2010).

Currently, the role of *Trichoderma* spp. is a well-established fact and has become increasingly crucial. By releasing different secondary metabolites and enzymes, these fungi protect crop against many kinds of plant pathogens, including *Phytophthora parasitica*, *Botrytis cinerea* and *Rhizoctonia solani* (Lewis and Lumsden, 2001; Olson and Benson, 2007; Hanada *et al.*, 2009). Epl1, a small protein belonging to Cerato-platanin (CP) family and produced by a few *Trichoderma* spp. has been reported to have an effect on plant pathogenesis and elicitation of plant defense responses (Seidl *et al.*, 2006). Epl1 would be the apparently non-enzymatic protein with an elicitor function. In addition, *Epl1* is an ortholog gene with *sm1* which has been cloned from some *Trichoderma* (*Hypocrea*) species (Buensanteai *et al.*, 2010).

The cellular mechanism of organism producing the protein plays a decisive role on production of an exogenous protein. It is well known that *Pichia pastoris* has many advantages of expressing the proteins for both industrial production and laboratory research, and on the other hand, the fermentation parameters that influence protein productivity and activity can be controlled easily. Compared with mammalian cells, *P. pastoris* is easily to manipulate, needs simple conditions for growth and has the pathway for eukaryotic protein synthesis (Macauley-Patrick *et al.*, 2005). In addition, some characteristics including high-frequency DNA transformation, high levels of protein expression, cloning by functional complementation and eukaryotic protein modifications make *P. pastoris* suitable to foreign protein expression particularly (Cregg *et al.*, 2000).

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Optimization of fermentation parameters is very important to exogenous protein yield (Oskay, 2011). Response Surface Methodology (RSM) as a statistical method is powerful and effective for screening main factors conveniently from the variable to optimize conditions for fermentation (Liu and Wang, 2007; Halim *et al.*, 2009). Meanwhile, interactions between variables can be identified and quantified by this method (Myers *et al.*, 2009). The method was often used to improve the production of secreted proteins in submerged culture. Plackett-Burman (PB) and Box-Behnken Design (BBD) are commonly used experimental designs for RSM. (Reddy *et al.*, 2008; Polak-Berecka *et al.*, 2011).

In this research, the effects of five experimental factors were analyzed using PB design. And then, we optimized the values of the screened variables for EplT4 protein yield by employing *P. pastoris* in submerged fermentation by BBD. Expression analysis of soybean defense-related genes and defense reactions elicited by EplT4 were investigated.

Materials and Methods

Strain and Inoculation Preparation

Biocontrol agent *T. asperellum* strain T4 was deposited (under the number CGMCC3.14975) at China General Microbiological Culture Collection Center. It was isolated from a deep soil sample in Yichun city, China and was cultivated with potato dextrose agar (PDA) at 28°C. *P. pastoris* expression vector pPIC9K and *P. pastoris* host strains GS115 (his_mut+) were from Invitrogen. Soybean (*Glycine max*) variety Kenfeng16 was provided by Heilongjiang Academy of Agricultural Sciences, China.

The Fermentation standard Basal Salts Medium (per liter) contained: Potassium hydroxide 4.13 g, Magnesium sulfate-7H_2O 14.9 g, Calcium sulfate 0.93 g, Potassium sulfate 18.2 g, Glycerol 40.0 g, Phosphoric acid (85%) 26.7 mL and mix together these ingredients, then add water to the final media volume followed by autoclaving. The PTM1 Trace Salts (per liter) contained: Ferrous sulfate-7H_2O 65.0 g, Zinc chloride 20.0 g, Biotin 0.2 g, Cobalt chloride 0.5 g, Boric acid 0.02 g, Sodium molybdate 0.2 g, Manganese sulfate-H_2O 3.0 g, Sodium iodide 0.08 g, Cupric sulfate-5H_2O 6.0 g, Sulfuric Acid 5.0 mL and then add water to a final volume of 1 L. After filter sterilization it was stored at 4^{o}C. The yeast was inoculated in 10 mL Yeast Extract Peptone Dextrose (YPD) in a 100-mL Erlenmeyer flask for seed culture. The cells were cultured at 30°C, 250 rpm for 24 h.

Batch Reactor and Fermentation

EplT4 is produced by *P. Pastoris* in a shake flask culture. The 250-mL flasks with 48 mL of the Basal Salts Medium and 0.2 mL PTM1 Trace Salts were inoculated with 2 mL seed culture. The flasks were incubated at 30°C until the yield of wet cell weight is 20-30 g/L. The culture was divided into several equal parts and centrifuged at 4, 000 g and the harvested cell was used as an inoculum. For optimization studies, the factors effecting fermentation were adjusted according to the experimental design, while the rotational speed was 220 rpm on orbital shaker. As the promoter of pPIC9K is induced by methanol, induction of protein production was achieved by feeding methanol every 12 h. Meanwhile, it was important to fill ammonia into flasks to keep the pH in given values.

ELISA Assay for Protein Yield

The yield of EplT4 protein from supernatant of medium was measured using Enzyme-Linked Immunosorbent Assay (ELISA). For each run triplicate 100 µL aliquots of supernatant was assayed in a 96 microwell plate. Mouse anti His-tag antibody (1:1500 dilution) and goat anti mouse IgG (1:5000 dilution) were used as primary antibody and secondary antibody, respectively. Absorbance at 450 nm was read using a Dynatech microplate reader. A seven-point standard curve using purified EplT4 was included with each plate. The concentration of purified EplT4 was determined by the Bradford method (Bradford, 1976). A blank without protein was subtracted from all other absorbance.

Experiment Design and Statistical Analysis

The PB design, which is an efficient analytical approach for optimization of fermentation condition, was applied to screen factors that had significant influence on the yield of EplT4 protein. It was designed based on the first-order polynomial model:

$$Y=\beta_0+\sum \beta_i X_i \qquad (1)$$

In equation (1) Y represents the response (EplT4 yield), β_0 is the model intercept, X_i is the level of the independent variable and β_i is the linear coefficient. Five factors (temperature, pH, methanol, Basal Salts and Trace Salts) were investigated to identify the key factors significantly influencing the EplT4 production. Based on PB design, all the variables were designed in two levels: +1 for high level and -1 for low level. The curvature and linear effects of the variables were evaluated using center point. Table 1 showed the experimental design containing five factors and Table 2 demonstrates the design matrix. Seven dummy variables were used to calculate the standard error. In this study, five assigned variables in twelve trials were tested including three trials at their center points. Each trial was repeated three times and the mean value obtained was used as the response. If significance of the factor gives a p-value lower than 0.05, the factor was regarded to have significant effect on EplT4 protein yield.

Further optimization was employed by RSM based on BBD(Box and Behnken, 1960). For statistical computing and data analysis, the correlation between the actual and coded values was shown in equation (2):

$$X_i=(A_i-A_0)/\Delta A \qquad (2)$$

Where X_i is the coded value, A_i is the actual value, A_0 is the actual value when the A_i at the center point, and A is the step change of variable. The levels of the variables and the experimental design were shown in Table 3. The matrix includes fifteen trials and each trial was repeated three times and the mean value obtained was used as the response. The second-order polynomial coefficients were calculated and analyzed using the software "Design Expert" (Version 7.1.3) statistical package. The data obtained from statistical analysis was used for evaluating the analysis of variance (ANOVA).

Expression Analysis of Soybean Defense-related Genes Elicited by EplT4

The culture medium obtained from the fermentation was collected by centrifugation at 8,000×g at 4°C for 10 min. EplT4 from supernatants was purified by His-bind column and gel filtration chromatography (GFC). Real-time quantitative polymerase chain reaction (qPCR) was applied to evaluate gene expression in 2-weeks-old soybean leaves after 12 h of incubation of EplT4 (50 μg) or sterilized water (negative control). The leaves were harvested and frozen immediately in liquid nitrogen. The synthesized cDNA from total RNA was used as a template for qPCR. Four pathogenesis-related (PR) protein genes involved in plant defense pathways were selected for expression analysis. The amount of RNA used in each reaction was normalized by internal references gene *actin 11*. All the primers and genes used in this study are presented in Table 4. The gene expression level was calculated from the threshold cycle according to the $2^{-\triangle\triangle CT}$ method.

Recombinant EplT4 Activity Tests

The production of H_2O_2 in two week-old soybean unifoliate leaves was examined 12 h after application of EplT4 (10 μg) or H_2O (negative control). EplT4 or H_2O were placed on the surface of the leaves, which previously had been slightly punctured. Leaves were infiltrated with nitro 3,3′-diaminobenzidine (DAB, Sigma), incubated overnight, fixed and cleared in alcoholic lacto-phenol solution. A red-brown precipitate was observed using an Olympus microscope BX-51 (Olympus America Inc.) at ten-fold magnification (Djonovic *et al.*, 2006).

Results and Discussion

Significant Variables Screening using PB Design

Five variables having effects on EplT4 protein yield were analyzed using PB design. The matrix designed for selecting significant variables affecting EplT4 protein yield and the corresponding responses were demonstrated in Table 2. The significant model terms were screened via F-test for ANOVA (Table 1). The variables with values of Prob>F less than 0.05 were regarded as significant factors and were used for further optimization process.

Table 1: Levels of the variables and statistical analysis of Plackett-Burman design

Code	Variables	Low level (-1)	High level (+1)	Effects (Ex_i)	F-Values	Prob>F
X_1	Temperature	25°C	30°C	0.068	17.02	0.0062[a]
X_2	pH	3	5	-0.048	7.46	0.0341[a]
X_3	Basal Salts[b]	50%	150%	0.046	6.98	0.0384[a]
X_4	Trace Salts[c]	50%	150%	9.334 E-4	6.577 E-3	0.9380
X_5	Methanol	0.2%	2%	0.038	5.12	0.0643

[a]5% significance level

[b]the level of the amount of components Basal Salts (%) compared with standard Basal Slats referred in material

[c]the level of the amount of components Trace Salts (%) compared with PTM1 Trace Salts referred in material

Table 2: Plackett-Burman design matrix for evaluating factors influencing protein yield

Run	X_1	X_2	X_3	X_4	X_5	yield(mg/L)
1	1	-1	1	1	-1	32.32
2	-1	1	1	-1	1	22.06
3	1	-1	-1	-1	1	30.22
4	-1	-1	-1	1	-1	21.45
5	-1	-1	1	-1	1	33.00
6	1	1	-1	1	1	30.43
7	-1	1	-1	1	1	19.78
8	1	1	1	-1	-1	30.10
9	1	1	-1	-1	-1	21.25
10	-1	-1	-1	-1	-1	20.97
11	1	-1	1	1	1	34.07
12	-1	1	1	1	-1	20.12

Dummy variables were not shown in the table

Table 3: Box-Behnken design with three independent variables

Run	Temperature(°C)		pH		Basal Salt (%)		Protein(mg/L)
	X_1	Code X_1	X_2	Code X_2	X_3	Code X_3	Observed[a]
1	30	1	4	0	150	1	17.43
2	30	1	5	1	100	0	31.18
3	28	0	4	0	100	0	35.03
4	25	-1	4	0	50	-1	19.02
5	28	0	5	1	50	-1	36.38
6	25	-1	4	0	150	1	26.24
7	28	0	4	0	100	0	35.77
8	30	1	4	0	50	-1	30.61
9	25	-1	3	-1	100	0	20.80
10	28	0	5	1	150	1	12.82
11	30	1	3	-1	100	0	31.19
12	28	0	3	-1	50	-1	9.03
13	28	0	3	-1	150	1	30.61
14	28	0	4	0	100	0	36.61
15	25	-1	5	1	100	0	29.38

[a]The observed values were the mean values of triplicates

Temperature (0.0062) was identified as the most significant factor, followed by pH of the fermented solution (0.0341) and the contents of Basal Salts (0.0384). The lower values indicate the factors have more significant effects on the EplT4 protein yield. Because the shake flask was used and the methanol inducer could easily evaporate, the protein yield did not change when different amounts of the inducer were added. According to the data, the three variables had

significant effects on protein yield, and RSM design was further employed for optimizing the levels of the three variables. So the three variables: temperature, pH and Basal Salts were selected for further optimization by BBD. Although trace Salts and methanol are not significant variables, they are necessary for the cell growth and inducing protein expression. In each trial 100% of trace salts and 1% of methanol were added.

Optimization of Significant Variables using RSM

A quadratic model consisting of fifteens trials were constructed in this study. The design matrix and the corresponding responses of BBD to evaluate the effects of the variables: temperature, pH and Basal Salts were shown in Table 5. The ANOVA for Response Surface model showed that the model terms, X_1, X_2, X_1X_3, X_2X_3, X_1^2, X_2^2 and X_3^2 were significant ($P < 0.05$). The interactions between temperature and Basal Salts, pH and Basal Salts were significant, respectively. The significance of the model (F-value 42.09) and non-significant lack of fit (F-value for lack of fit 6.86) indicated the model established was a good fit (Table 5). The P-values (0.0003) and lack of fit (0.1299) for the model further verified that the results obtained from the combination of experimental design fit with this model. The coefficient of determination (R^2) and the adjusted coefficient of determination (Adj R^2) were used to measure amount of variation explained. The value of R^2 for equation (3) was 98.70%, indicating that 98.70% of the variability in the response could be explained by the model (The value of R^2 should be within the range of 0-1.0, and Design Experts software identified the fitness of the model with the value approach 1.0). The Adj R^2 value was 96.35% that was adequately advocate for the significance of the model. The coefficients of regression equation were calculated and the value was fitted for a quadratic equations. The responses (Y) are established in the following regression equation:

$$Y = 35.80 + 1.87X_1 + 2.27X_2 - 1.00X_3 - 2.15X_1X_2 - 5.10X_1X_3 - 11.30X_2X_3 - 3.27X_1^2 - 4.38X_2^2 - 9.23X_3^2 \quad (3)$$

In equation (3) Y represents the predicted yield of EplT4, X_1, X_2 and X_3 represent the coded values of temperature, pH and Basal Salts, respectively. The 'adequate precision value' was 19.56, which indicated that the model could be used to navigate the design space because that the values that higher than 4.0 are necessary for a good fit model. As shown in Fig. 1, a positive correlation existed between predicted and experimental values. As the deviation between predicted values and the experimental values was small, it further indicated the good fit of the model.

In order to investigate the optimum levels of the three factors affecting EplT4 protein yield, both response surface curves and contour plots were obtained. The response (EplT4 protein yield) was on the Z-axis against the other two independent variables, while setting trace salts and methanol at the zero levels as shown in Fig. 2-4, which also depicted the interactions between two variables.

Table 4: Primers used in the real-time PCR

Gene Name	GenBank No.	Family of PR protein	Primers (5'-3')
actin 11	BW652479	internal references	ATTTTGACTGAGCGTGGTTAT TCC GCTGGTCCTGGCTGTCTCC
thaumatin-like protein	AB116251	PR-2	AATGGCCGTCACGAAAAGC TGAAGTTCGCTGCCTGAGCTA
chitinase III-A	AB006748	PR-8	GAAGCTACTCCCTCAGCTCAGC CCTAATGGCCCTGATCCAGTT
beta-1,3-glucanase	U08405	PR-5	CCTCCCAATGATGGCGTTTT GCCCCATTGCTCACTAGGAA
cysteine proteinase inhibitor	D31700	PR-6	CCAACCTGGATGGCAATCTG GCTGGATAGTCTTGATCGCAT GAT

Table 5: ANOVA for response surface quadratic model for EplT4 yield

Factors	Statistics				
	Sum of squares	df	Mean square	F-Value	P-Value
Model	1094.75	9	121.64	42.09	0.0003
X_1	28.12	1	28.12	9.73	0.0263
X_2	41.40	1	41.40	14.33	0.0128
X_3	8.00	1	8.00	2.77	0.1570
X_1X_2	18.49	1	18.49	6.40	0.0526
X_1X_3	104.04	1	104.04	36.00	0.0018
X_2X_3	510.76	1	510.76	176.73	< 0.0001
X_1^2	39.60	1	39.60	13.70	0.0140
X_2^2	70.67	1	70.67	24.45	0.0043
X_3^2	314.22	1	314.22	108.73	0.0001
Lack of fit	13.17	3	4.39	6.86	0.1299

R^2 = 99.70%; Adj R^2 = 96.35%

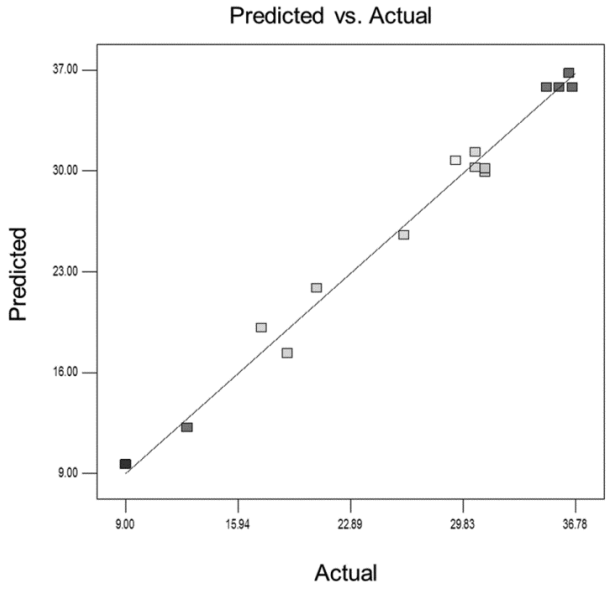

Fig. 1: Parity plot showing the distribution of predicted vs. experimental values of EplT4 protein production

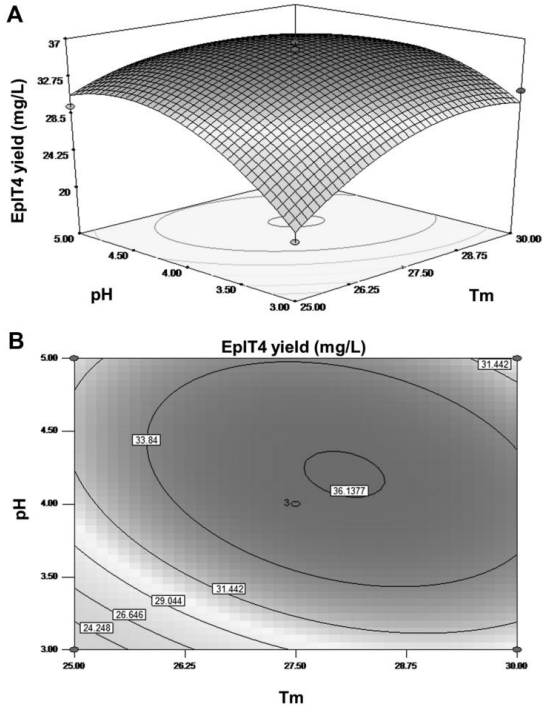

Fig. 2: Response surface curves *A* and contour plots *B* showing the effects of temperature and pH on EplT4 protein production by *P. Pastoris*, with Basal Salts level of zero

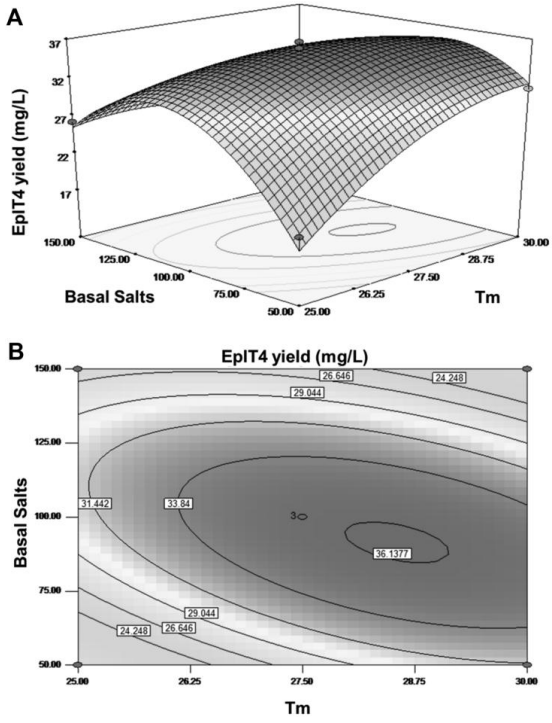

Fig. 3: Response surface curves *A* and contour plots *B* showing the effects of temperature and basal salts on EplT4 protein production by *P. Pastoris*, with pH level of zero

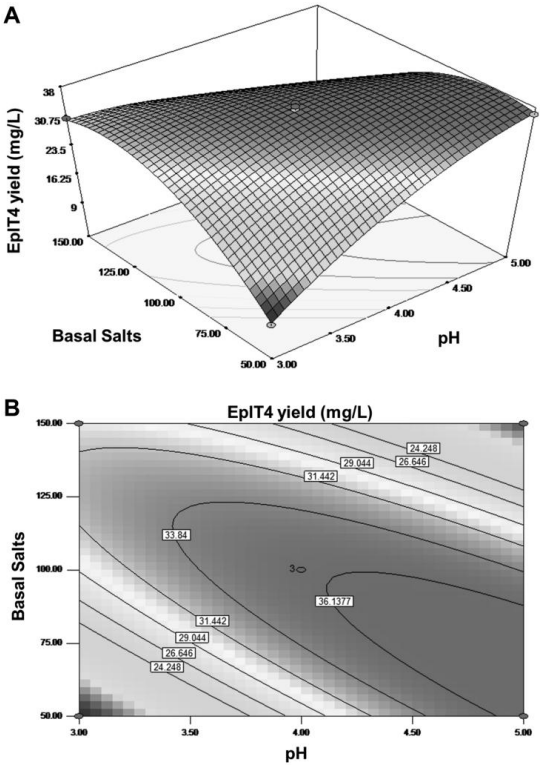

Fig. 4: Response surface curves *A* and contour plots *B* showing the effects of Basal Salts and pH on EplT4 protein production by *P. Pastoris*, with temperature level of zero

It has been reported that the elliptical contour plots were obtained when an evident interaction existed between the independent variables (Xu *et al.*, 2008). As shown in equation (3), the optimum coded values of X1, X2 and X3 were 0.31, 0.80 and -0.65, respectively. Correspondingly, the actual values derived from the model were 28.5°C, pH 4.7 and 62.7% of Basal Salts, respectively. Finally, the maximum proteins yield predicted in the model was 38.03 mg/L.

Validation of the Model

According to the results obtained from BBD, the optimal condition of fermentation was prepared as follows: temperature 28.5°C, pH 4.7, Basal Salts 62.7%, Trace Salts 100%, and Methanol 1%. Under this optimized condition, the maximum EplT4 protein yield was estimated as 38.03 mg per liter culture supernatant and the experimental value observed was 37.70 mg/L in triplicate tests. The result that experimental value was quite close to the predicted value confirmed the validity of the model. The max yield of EplT4 protein was observed within 48 h of induction (date not shown).

EplT4 Induces the Expression of Soybean Defense Genes

In order to investigate the bioactivity of recombinant EplT4,

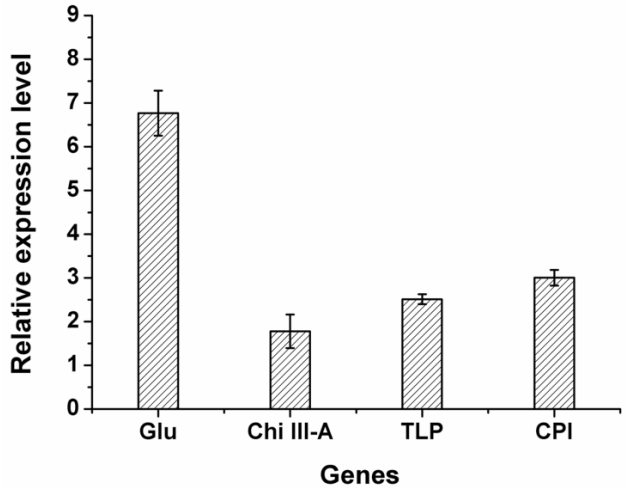

Fig. 5: Expression analysis of soybean defense-related genes. GLU (β-1,3-glucanase), Chi III-A (chitinase III-A), TLP (*Thaumatin*-Like Protein), CPI (cysteine proteinase inhibitor). The relative expression level equal to expression incubation with EplT4 divide by expression treated with water

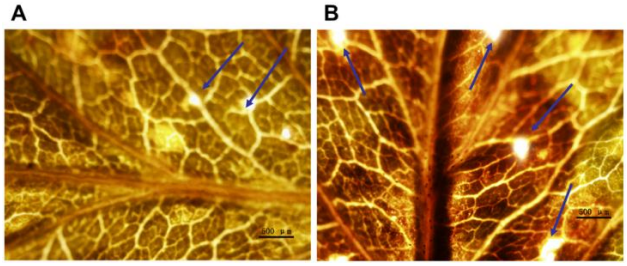

Fig. 6: H_2O_2 accumulation in soybeans. *A* Soybean leaves were treated with H_2O (negative control) and *B* treated with EplT4 (10 μg). Treated tissues were harvested 12 h after treatment, and excised leaves were infiltrated with a solution of 3,3′-diaminobenzidine (DAB). In the presence of H_2O_2, DAB polymerized, forming a dark red-brown precipitate. Micrographs are centered on the treated region surrounding the application area (indicated by arrows).Microscopy was performed using Olympus microscope BX-51 (Olympus America Inc.) at ten-fold magnification

the protein was further applied to soybean. Pathogenesis-related proteins (PR proteins) are usually used as markers of plant response to their elicitors, four PR proteins belonging to different families were chosen. Gene expression 12 h after application of EplT4 to soybean leaves was shown in Fig. 5. Application of EplT4 resulted in up-regulation of Glu, Chi III-A, TLP and CPI genes expression compared to the control (sterile water). The treatment of plant leaves with EplT4 resulted in induction defense related genes in soybean variety Kenfeng 16.

EplT4 Induces Soybean Defense Reactions

EplT4 belongs to the CP protein family, which is usually involved in phyto-pathological phenomena and immunological reactions. The production of hydrogen peroxide in plant is the early response signals in elicitor recognition (Dixon *et al.*, 1994). The production of hydrogen peroxide in soybean leaves after exposure to EplT4 for 12 h was examined. Application of EplT4 (10 μg) resulted in the production of hydrogen peroxide around the puncture. A brownish-red precipitate generated by polymerization of the hydrogen peroxide with nitro 3,3′-diaminobenzidine in the treated tissues was observed (Fig. 6).

The results demonstrated that response surface methodology offer a feasible and efficient approach for optimization of EplT4 production in *P. pastoris*. The obtained EplT4 recombinant protein retained its biological activities and was proved effective to induce defense responses in soybean. The study indicated that recombinant EplT4 produced in *P. pastoris* represent an important resource as potential biofungicide to be used in crops for ecologically safe control of plant pathogens.

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