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Expression of a *Magnaporthe grisea* Elicitor and Its Biological Function in Activating Resistance in Rice

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Abstract: The expression of a protein elicitor from *Magnaporthe griesea* and its biological function in activating resistance in rice (*Oryza sativa* L) were reported. The gene of elicitor was expressed in *Escherichia coli* cells and produced a His_6-fusion protein with 42 kD apparent molecular weight on SDS-PAGE. The purified protein could induce the resistance to blast disease, with the control efficiency of 46.47% and 36.41% at the 14th day and the 21st day after blast inoculation, respectively. After treatment with the expressed protein, the phenylalanine ammonia-lyase (PAL) and peroxidase (POD) activities were promoted in rice plants, meanwhile, the transcription levels of *STKM*, *FAD*, *PBZ1* and *PR1* genes were increased in rice plants. Moreover, after comparing the profile of total rice leaf proteins on two-dimensional electrophoresis gel, about 14 proteins were found to be increased in expression level after the expressed protein treatment. All the results indicated that the expressed protein could act as an elicitor to trigger the resistance in rice.
Key words: elicitor; *Magnaporthe grisea*; induced resistance; protein expression; blast resistance; rice; biological control

In nature, higher plants could initiate various defense responses when they are attacked by pathogens such as fungi, bacteria and viruses. The key factor is that pathogen-derived elicitors are able to be perceived by plants [1]. The term 'elicitor' refers to the molecules that stimulate any of defense responses in plants [2-4]. Most elicitors seemed to fall into two broad categories: many of them are constitutively present in pathogen cell wall, e.g. glucan, chitin fragments as well as bacterial flagellin; the other plays a role as virulence determinants, e.g. haprin, and products of pathogen avirulence (*AVR*) genes [1].

In our previous study, we had isolated a protein elicitor from *Magnaporthe grisea* north strain 1-24 which has 40 kD apparent molecular weight on 12% SDS-PAGE. When foliar application or seed soaking, the elicitor had dual functions that conferred plants resistance and triggered plant growth [5]. Four peptide fragments from the protein elicitor had been successfully sequenced by LC-ESI-MS/MS *de novo* sequencing. All the obtained amino acid sequences were significantly homologous to partial amino acid sequences of the *M. grisea* hypothetical protein EAA52615. To further study the function of the elicitor, the elicitor gene was cloned from *M. grisea* cDNA and named '*PEMG1*', which is for shortening of Protein Elicitor from *M. grisea*. The *PEMG1* gene was expressed in *Escherichia coli* cells and the produced recombinant protein (His_6-PEMG1p) was used as an elicitor to trigger rice (*Oryza sativa* L.) resistance. In order to study the influence of His_6-PEMG1p on rice protection enzyme system, the activities of phenylalanine ammonia-lyase (PAL) and peroxidase (POD) were monitored at different time points after the His_6-PEMG1p treatment. The transcription levels of *STKM*, *FAD*, *PBZ1* and *PR1* genes were also investigated after the His_6-PEMG1p treatment. This research should provide a foundation for further utilizing the protein to promote rice resistance as an elicitor.

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MATERIALS AND METHODS

Gene cloning and plasmid construction

Based on NCBI search results, the primers were designed to amplify the *PEMG1* gene using *Magnaporthe grisea* north strain 1-24 cDNA as template. The sense primer was L, 5'- CGGAATTC ATGAGCGAGTTGTGTCGAA-3', which contained

an *Eco*R I restriction site, and the antisense primer was M1, 5'-CCGCTCGAGGCTGCTGCCAAGGGA-3', which had an *Xho* I restriction site. The PCR product was purified by Cycle-pure Kit (Omega Biotek) and was double digested by *Eco*R I and *Xho* I. The double digested PCR product was ligated into *Eco*R I/*Xho* I site of pET28a vector (Novagen). The constructed plasmid (pET28a-PEMG1) was then transformed into *E. coli* BL21 (DE3) competent cells. The *PEMG1* gene sequence was verified by DNA sequencing using T7 promoter primer and T7 terminator primer.

Protein expression, Western blotting and His_6-PEMG1p purification

BL21(DE3)/pET28a-PEMG1 *E. coli* cells were grown in Luria-Bertani (LB) broth with 50 μg/mL kanamycin in a shaking incubator at 37℃. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mmol/L to induce the expression of His_6-PEMG1p.

SDS-PAGE (12%) was used to analyze the His_6-PEMG1p expression level. Electrophoresis was performed in a Mini-Protein system (Bio-Rad). After the electrophoresis, one of the gels was stained with Coomassie brilliant blue R250 and another gel was used for Western blotting analysis.

Western blotting was performed to further identify the heterogeneously expressed His_6-PEMG1p in bacterial lysate. Polyclonal antibody was produced from *M. grisea* derived PEMG1p and was purified as described by Burns [6] and Olmsted [7]. For Western blotting, *E. coli* lysate containing His_6-PEMG1p was separated by 12% SDS-PAGE. The proteins in the gel were transferred to PVDF membrane using a Trans-blot SD cell (Bio-rad). The PVDF membrane with transferred protein was blocked by 5% BSA in TBST buffer. Blocked membrane was incubated with the primary antibody and alkaline phosphatase conjugated secondary antibody. The His_6-PEMG1p was visualized by alkaline phosphatase chromogen kit (Abcam).

His_6-PEMG1p was purified from *E. coli* lysate using a MagExtractor His-tag kit (TOYOBO) at the native conditions following the manufactures' manual. The purified His_6-PEMG1p was dialyzed against 10 mmol/L Tris-HCl buffer at pH 7.8 to remove imidazole before bioassay.

Plant and pathogen materials

A Japanese japonica rice Nipponbare, provided by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (CAAS), was grown in a controlled chamber under a cycle of 12 hours light at 30℃ and 12 hours dark at 28℃. *M. grisea* north strain 1-24 was kindly provided by Prof. Zhu Chang-xiong at the Institute of Environment and Sustainable Development in Agriculture, CAAS and was grown at 28℃ in a PDA culture medium.

Induced resistance to rice blast

Three-replication experiments were performed. In each experiment, ten rice seedlings with 6-7 leaves were treated by foliar application of His_6-PEMG1p. For foliar application, rice leaves were sprayed with 10 μg/mL His_6-PEMG1p using a sprayer, ensuring a good coverage of His_6-PEMG1p. Control plants were treated with BSA at 10 μg/mL. Rice plants were inoculated with the suspension of *M. grisea* spores (about 450 000 conidia per mL) containing 0.05% Tween-20 at 36 hours after the foliar application. After inoculation, the plants were moved to dark room at 28℃ and 95% relative humidity for 24 hours and then transferred to the greenhouse and sprayed with water twice a day. Macroscopic symptoms and disease index were evaluated on each plant at the 14^{th} days and 21^{st} days after inoculation as the methods described by Fang [8]. The data analysis was performed using SPSS11.5 software for paired sample *t* test.

Enzyme activity test

Rice plants with 6-7 leaves were sprayed with the His_6-PEMG1p and BSA at the concentration of 10 μg/mL. Middle leaves of seedlings were used for measuring the activities of PAL and POD. PAL activity was assayed as previously described by Ke and Salveit [9]. POD assay was carried out as described by Li [10].

Semi-quantitative RT-PCR

The picked genes and their primer sequences were presented in Table 1. To normalize the relation of gene expressions, β-actin gene was added as an internal standard and loading control. DNAMAN

Table 1. List of gene specific primers.

Gene	GenBank ID	Primer	Expected PCR product (bp)
Actin	AC091532	FW: 5'-CCAGGAAATGGAGACTGCCA -3'	700
		RV: 5'- AGTGAGAACCACAGGTAGCAA-3'	
STKM	NM_185437	FW: 5'- GAGAGTGGTGAAGTGGTTGC -3'	700
		RV: 5'- GGCTGAAAGAAGGGATGCTG -3'	
FAD	D78505	FW: 5'- GAGAGTGGTGAAGTGGTTGC -3'	700
		RV: 5'- GGCTGAAAGAAGGGATGCTG -3	
PBZ1	D38170	FW: 5'- CAGCTCTAGCTAGCTACAGG-3'	700
		RV: 5'- CCACGAGAATTTGGACATTT-3'	
PR1	OSU89895	FW: 5'- GACTACGACTACGCCTCCA-3'	700
		RV: 5'- TTTGGGTTGTCTTTGAGCG-3'	

(Lynnon Biosoft) was used to design primers for amplifying the target gene fragments of about 700 bp.

The experiment was independently repeated six times. Rice plants with 6-7 leaves were sprayed with 10 μg/mL His_6-PEMG1p and BSA, respectively. Middle rice leaves were snap-frozen in liquid nitrogen at 36 hours after foliar application. About 100 mg leaf tissues were ground in liquid nitrogen and vortexed in Trizol reagent (Invitrogen) following the manufacture's manual. The RNA quality was checked by running RNA on agarose gels. The RNA concentration and OD_{260}/OD_{280} ratio were also tested on a spectrophotometer for normalizing the reverse-transcript loading and RNA purity. Total 2.5 μg RNA samples were reverse-transcribed with M-MLV (Invitrogen) in the presence of oligo d(T). PCR reactions were performed by using 1 μL (about 0.125 g cDNA) of reverse-transcribed products as templates. After 35 reaction cycles, PCR products were analyzed with 1% agarose gel electrophoresis and images were captured using a Multigenius system (Syngene). The band intensities were analyzed by the Genesnap software (Syngene). The data analysis was performed using SPSS11.5 for paired sample *t* test.

Two-dimensional electrophoresis methods

Two-dimensional gel electrophoresis was designed for measuring the changes of protein expression patterns between His_6-PEMG1p treatment and control. The experiments were performed essentially according to the method of Görg et al [11]. Rice plants with 6-7 leaves were sprayed with 10 μg/mL His_6-PEMG1p and BSA, respectively. Total proteins were extracted from the middle leaves of rice plants at 36 hours after foliar application. The first separation was carried out on immobilized pH gradients (4/7 Immobilin dry strips of 13 cm) with an Ettan IPGphor II isoelectric focusing system (GE Healthcare). Rehydration buffer contained 5 mol/L ultra pure urea, 2 mol/L thiourea, 2% CHAPS, 2% SB3-10, 0.2% ampholine pH 4-7 and 0.1% bromphenol blue. The secondary separation was performed on a Protean II Xi cell (Bio-rad) according to the manufacture's manual.

RESULTS

PEMG1 gene cloning and pET28a-PEMG1 expression plasmid construction

The *PEMG1* gene was amplified by PCR and showed about 1 kb band on 1% agarose gel. The gene was double digested by *Eco*R I/*Xho* I enzymes and ligated into the *Eco*R I/*Xho* I site of pET28a vector for pET28a-PEMG1 expression plasmid construction (Fig. 1). The correct insertion of *PEMG1* gene was verified by DNA sequencing. According to the sequence, the *PEMG1* gene encoded a protein with calculated molecular weight of 34 kD and the *PEMG1* gene sequence has been deposited in GenBank (Accession number EF062504), which showed 99.9% homology with the theoretical EAA52615 protein gene. The amino acid sequences of four polypeptides obtained by LC-ESI-MS/MS *de novo* sequencing and their corresponding sequences in the *PEMG1* gene was compared in Table 2.

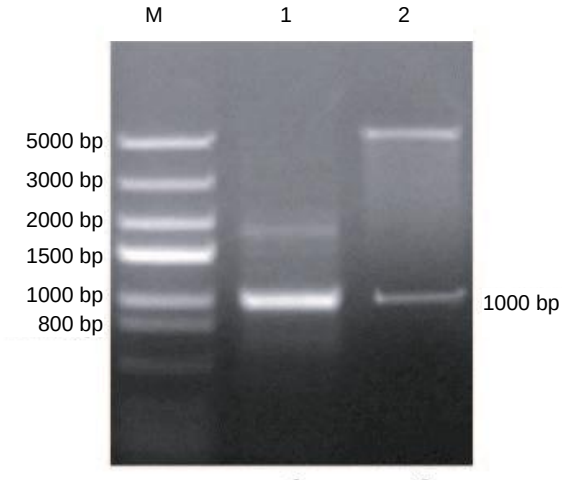

Fig. 1. PCR and *Eco*R I, *Xho* I double digestion of pET28a-PEMG1 plasmid.

Lane 1, PCR amplification of *PEMG1* gene using pET28a-PEMG1 plasmid as template; Lane 2, *Eco*R I and *Xho* I double digestion of pET28a-PEMG1 plasmid.

His_6-PEMG1p protein expression, purification and Western blotting

The His_6-PEMG1p was expressed as a soluble protein in *E. coli* cells. There was a distinguishable extra band in cells harboring pET28a-PEMG1 as shown in Fig. 2-A. The densitometric scanning results revealed that the expression level of His_6-PEMG1p was about 7% of total cellular proteins. The His_6-PEMG1p displayed 42 kD molecular weight on 12% SDS-PAGE and was purified by a MagExtractor His-tag kit (Fig. 2-B) for bioassay. The band was identified to be His_6-PEMG1p by Western blotting (Fig. 2-C).

Table 2. Comparison between obtained amino acid sequences by LC-ESI-MS/MS *de novo* sequencing and *PEMG1* gene predicted amino acid sequences.

Peptide	Obtained (PEMG1p)	Predicted (His_6-PEMG1p)
PEMG1p-1	GNDEEDDTPKAPVK	GNDEEDDTPKAPVK
PEMG1p-2	SFRRDRDDRHAK	SFRRDRDDRHAK
PEMG1p-3	SISYADYLAQQAEKS	SISYADYLAQQAEKK
PEMG1p-4	RFVEQQDRPRG	KFVEQQDRPRG

Induced resistance to rice blast

In the previous study, our findings had demonstrated that foliar application of *M. grisea* derived PEMG1p could confer plant resistance [5]. In this experiment, the biological activity of His_6-PEMG1p on triggering resistance to rice blast was evaluated. Disease indexes at the 14th day and 21st day after blast inoculation were presented in Table 3. The His_6-PEMG1p treated samples exhibited a statistical difference from the control, which showed control efficiency of 46.47% at the 14th day after inoculation and 37.23% at the 21st day after inoculation.

Enzyme activity change by His_6-PEMG1p treatment

Many enzymes involved in plant resistance could be activated by elicitor treatment [12]. To determine the

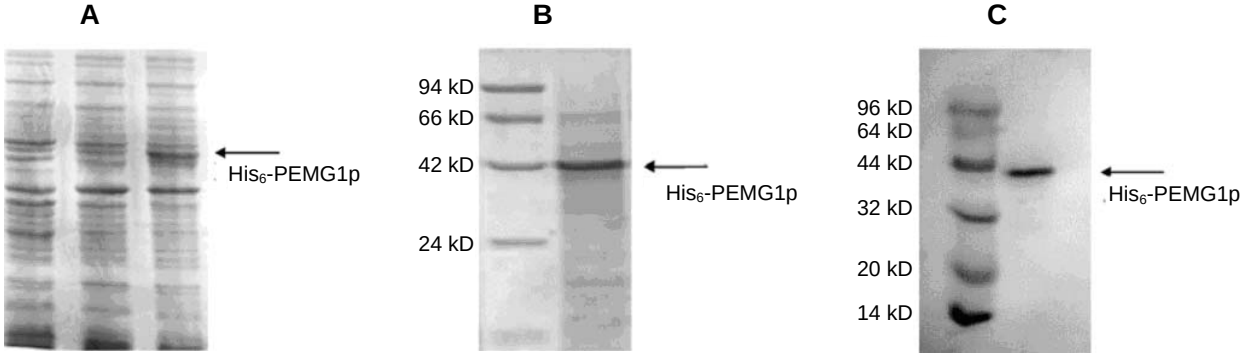

Fig. 2. SDS-PAGE of *E. coli* lysate, purified His_6-PEMG1p and Western blotting of His_6-PEMG1p in *E. coli* lysate.

A. Expressed protein in *E. coli* lysate was separated on 12% SDS-PAGE (Left lane is the pET28a empty plasmid *E. coli* lysate, middle lane is the *E. coli* harboring pET28a-PEMG1 plasmid lysate without adding isopropyl β-D-1-thiogalactopyranoside (IPTG), right lane is the pET28a-PEMG1 vector lysate in the presence of IPTG induction;

B. With silver stain, the purified fraction exhibited a single sharp band on gel (right). The left lane is protein molecular marker, numbers on the left indicate molecular mass in kilo Dalton (kD);

C. Western blotting analysis of expressed His_6-PEMG1p. Left lane is pre-stain protein marker, numbers on the left indicate molecular mass in kilo Dalton (kD). Right lane is His_6-PEMG1p.

Table 3. Disease index and control efficiency of His_6-PEMG1p against rice blast.

Treatment	The 14th day after inoculation		The 21st day after inoculation	
	Disease index	Control efficiency (%)	Disease index	Control efficiency (%)
His_6-PEMG1p	28.53±4.780 a	46.47	38.83±5.120 a	37.23
Control	53.30±4.624 b		61.87±7.203 b	

Values are the means±*SD* of 30 samples; mean values followed by the same letter in the same column are not significantly different at $P \leqslant 0.05$.

Table 4. Comparison between treatment and control in relative signal intensity.

Treatment	*STKM*	*FAD*	*PBZ1*	*PR1*
His_6-PEMG1p	0.780 ± 0.241 a	0.988 ± 0.373 a	0.251 ± 0.176 a	0.321± 0.190 a
Control	0.481 ± 0.173 b	0.621 ± 0.270 b	0.124 ± 0.142 b	0.051± 0.098 a

Values are the means±*SD* of the relative signal intensity ratio of six repeated experiments; Mean values followed by the same letter in the same column are not significantly different at $P \leqslant 0.05$.

ability of His_6-PEMG1p as an elicitor, PAL and POD activities were monitored after treatment of His_6-PEMG1p in rice plants. As shown in Fig. 3, both POD and PAL activities increased after foliar application of His_6-PEMG1p. POD activity increased quickly (4 h after treatment) and reached its maximum level at 8 h after treatment. PAL activity was significantly higher than that of control and reached its maximum level at 9 days after treatment.

Up-regulated transcription of *STKM*, *FAD*, *PBZ1* and *PR1* genes by His_6-PEMG1p treatment

The up-regulation of *STKM*, *FAD*, *PBZ1* and *PR1* genes in rice plants were shown in Fig. 4. The expected PCR products were 700 bp. The constitutive expression of internal control β-actin gene was not significantly altered by His_6-PEMG1p treatment. The relative signal intensity was the ratio of signal intensity between examined genes and β-actin gene (Table 4). The results showed that the expressions of *STKM*, *FAD*, *PBZ1* and *PR1* genes were significantly up-regulated after His_6-PEMG1p treatment.

Difference in protein expression between His_6-PEMG1p treated plants and control

Pre-experiments demonstrated that most rice proteins focused between pH 4 and pH 7 in isoelectric point, and the best resolution was achieved using this pH range. In this experiment, 350 μg of total rice leaf protein was loaded for each electrophoresis. About 450 spots in each gel were stained by Coomassie brilliant blue R250 stain. The protein expression profiles were similar between the samples. The up expression levels of protein were recorded as the arrow indications (Fig. 5). There were 14 spots in different protein expression levels between treated

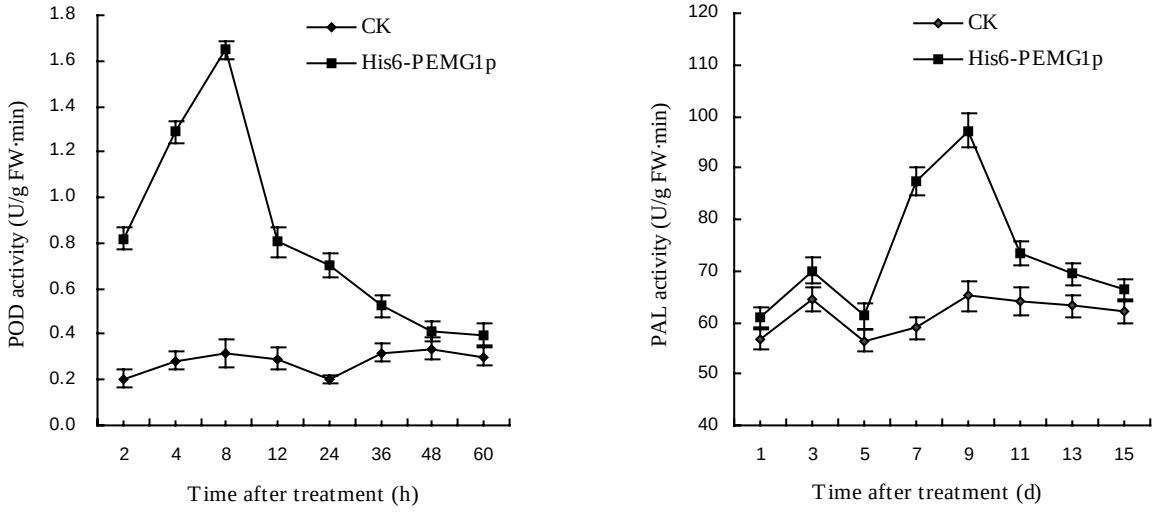

Fig. 3. Enzyme activities of rice plants after His_6-PEMG1p treatment.

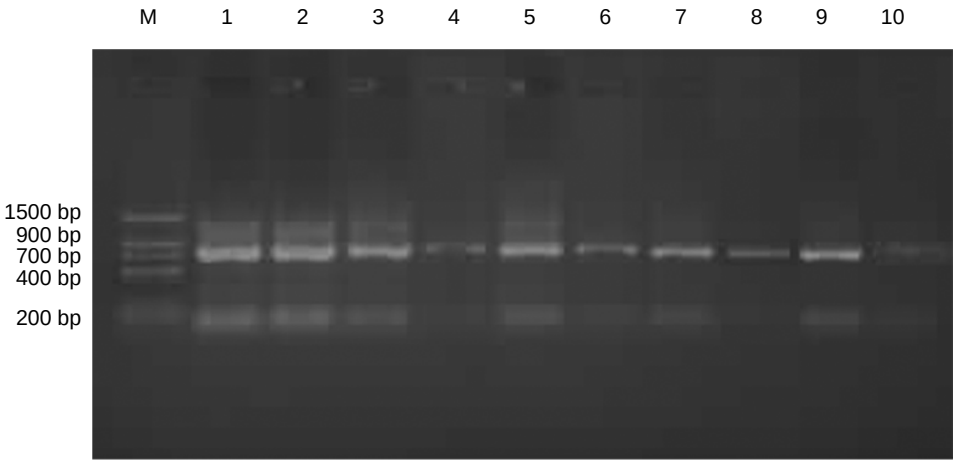

Fig. 4. Semi-quantitative RT-PCR conformation of gene regulation caused by His_6-PEMG1p.

Lanes 1 and 2, β-actin gene RT-PCR (AK059158) (1, Treatment; 2, Control); Lanes 3 and 4, *STKM* gene (NM_185437) RT-PCR (3, Treatment; 4, Control); Lanes 5 and 6, *FAD* gene RT-PCR (D78505) (5, Treatment; 6, Control); Lanes 7 and 8, *PBZ*1 gene (D38170) RT-PCR (7, Treatment; 8, Control); Lanes 9 and 10, *PR1* gene (OSU89895) RT-PCR (9, Treatment; 10, Control).

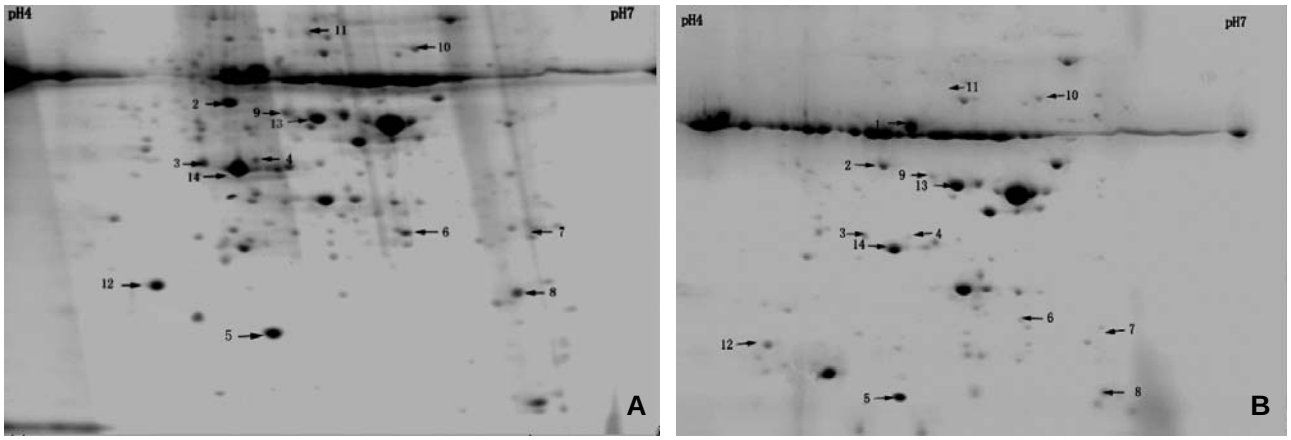

Fig. 5. Two-dimensional electrophoresis profiles of total proteins from rice leaves.

A, Treated with His_6-PEMG1p; B, Control.

plants and the control. The results demonstrated that at least 14 kinds of proteins were activated by His_6-PEMG1p treatment.

DISCUSSION

With the completion of genome sequencing projects for *M. grisea* [13], genome-based protein identification and gene cloning become much easier. In our experiment, the obtained elicitor gene sequence shared 99.9% similarity with that of the hypothetical EAA52615 protein. The 0.1% difference between the obtained gene and the hypothetical protein EAA52615 gene may be due to the different strains of *M. grisea*. To our knowledge, the EAA52615 protein is a hypothetical protein so far, which is deduced from genome sequence. No isolation and no function of the protein were reported yet. In this paper, we expressed the protein in *E. coli* cells and demonstrated that the protein could act as an elicitor on triggering resistance in rice.

Furthermore, the comparison of the PEMG1p protein sequences in NCBI database showed that there were similar sequences in various fungi (only the sequences were published but no physiological function of the deduced proteins was described) such as *Neurospora crassa*, *Chaetomium globosum*, *Aspergillus oryzae*, *Gibberella zeae*. This research

provides the possibility for searching the function of this homology gene in other fungi, and utilizing them as an elicitor for biological control.

The obtained *PEMG1* gene codes a protein with calculated molecular weight about 34 kD. Adding 3 kD molecular weight of His-tag, the theoretical recombinant His_6-PEMG1p molecular weight should be 37 kD. The apparent contradiction between the calculated protein molecular weight and the observed molecular weight may be caused by the protein mobility. This anomalous electrophoretic migration is quite common and has been observed with proteins such as papillomavirus 16E7 protein [14] and *E. coli* AMS protein [15].

The enhancement of rice blast resistance by His_6-PEMG1p would be due to the activation of PAL and POD enzymes and other resistance related genes. PAL is considered as the key enzyme to synthesize lignin and POD, acting as an activator for polymerization and dehydrogenization [16]. Fast promotion of POD and slow increasing of PAL might contribute to deposition of lignin by different metabolic pathways. Deposition of lignin is one of the mechanisms of plant defense, which acts as a physical barrier to arrest the invasion of pathogen [17]. Other defense enzymes may also be responsive to His_6-PEMG1p treatment, though we have not tested here.

The semi-quantitative RT-PCR results showed that the transcription of *STKM*, *FAD*, *PBZ1* and *PR1* genes were increased by the His_6-PEMG1p treatment. Gene *STKM* encodes serine/threonine-protein kinase that catalyzes the phosphorylation of serine or threonine residues on target proteins by using ATP as phosphate donor [18]. The intracellular phosphorylation events control the cell proliferation, chemotaxis, differentiation and stress response [19]. Gene *FAD* encodes ω-3 fatty acid desaturase (ω-3FAD) that acts on membrane lipids to catalyze the formation of trienoic acid. Antisense expression of *ω-3FAD* gene showed enhanced susceptibility against tobacco mosaic virus (TMV) infection [20]. More specifically, one of the strongly elicitor responsive genes encodes the *ω*-3*FAD* and the expression of this gene occurred rapidly around fungal penetration sites. Increasing of *ω-3FAD* expression in plant causes fatty acid metabolism in response to pathogen attack. Activation of *ω-3FAD* may cause the generation of other fatty acid derivatives that either directly act as endogenous signal molecules or indirectly serve as precursors for the synthesis of polymers associated with local suberization of cells or for reinforcement of cell walls thus increase the plant resistance [21]. Genes *PBZ1* and *PR1* encode two kinds of pathogenesis-related protein (PR protein) [21]. Although the functions of PBZ1p and PR1p proteins in disease defense are still unclear, *PBZ1* and *PR1* are valid marker genes for elicitor induced systemic acquired resistance (SAR) [22-24]. In conclusion, the up-regulation of these genes is one of the mechanisms for plant resistance enhancement.

In the present experiment, two-dimensional gel electrophoresis was used to identify interesting proteins in rice plants. The results demonstrated that 14 proteins were increased expression in His_6-PEMG1p treated rice plants. It suggested that His_6-PEMG1p influenced on rice not only at gene transcriptional level but also at protein expressional level. However, what kinds of proteins of this finding are still required to be identified by mass spectrum technology.

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