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EXPRESSION AND PURIFICATION OF *PHYTOPHTHORA INFESTANS* (MONT.) DE. BRAY RXLR EFFECTOR

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Abstract

The RXLR-class effectors play an important role in the pathogenesis of pathogens. They not only can enhance the pathogenicity of the pathogen, but also can be identified as a non-toxic protein to induce cell hypersensitive response (HR) by resistance protein of the host plant. In this study, the gene of RXLR effector was amplified by PCR, then the target gene and expression vector pET-28a were ligated and transformed into BL21(DE3) competent cells to obtain the pET-28a-RXLR recombinant expression plasmid. The protein was induced expressed by IPTG, purified by Ni affinity chromatography and detected by SDS-PAGE. The recombinant expression plasmid pET-28a-RXLR was successfully constructed and expressed in *E. coli* to produce a protein sized 18.4 kDa, consistent with the theoretical value. The high purity RXLR effector was successfully obtained in this study, laying a foundation for its pathogenicity study.

Introduction

Plant-pathogenic oomycetes can cause a variety of diseases in crops, and are especially destructive to economic crops, resulting in enormous economic losses (Kamoun *et al.* 2015). *Phytophthora infestans* is an oomycete that has been studied extensively and is an essential pathogenic factor causing reduced production or no production of potato (Fry *et al.* 2015). The pathogen carries hundreds of effector proteins that are secreted into the apoplast or into the host cells. The effector proteins secreted in the host cells are called cytoplasmic effectors, primarily two classes, Crinkler (CRN) proteins and RXLR proteins (Haas *et al.* 2009, Raffaele *et al.* 2010).

According to gene sequencing and bioinformatics analysis, *P. infestans* has more than 500 RXLR effectors (Haas *et al.* 2009), all including a conserved sequence motif Arg-Xaa-Leu- Arg (RXLR) consensus sequence, in which Xaa represents any amino acid. It is mainly located in gene sparse and repetitive regions, and it evolves faster than genes in dense regions. Therefore, by rapid adaptation RXLR effectors can avoid recognition by R protein (Du *et al.* 2018). Current findings suggested that RXLR effectors can affect autophagy (Dagdas *et al.* 2016), protein degradation and stability (Gilroy *et al.* 2011), kinase/phosphatase signaling (Petra *et al.* 2016), transcription (McLellan *et al.* 2013), RNA binding and small RNA biogenesis (Jing *et al.* 2016), protein secretion (Du *et al.* 2015), endoplasmic reticulum (ER) stress-mediated immunity and Brassinosteroid signaling (Jing *et al.* 2016). RXLR effectors can act both as virulence factors to suppress plant basal immunity, and act as non-toxic factors to activate homologous resistance genes (R genes) in the host. However, the biological activities and molecular mechanisms of most RXLR effectors remain elusive.

In this study, present authors cloned the RXLR gene by prokaryotic expression technique, ligated to the expression vector pET-28a and transformed into BL21 (DE3) competent *Escherichia coli* for protein expression. Then the effector was purified by Ni affinity chromatography and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) detection. It was aimed to obtain the RXLR effector and lay a foundation for subsequent research.

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Materials and Methods

pET-28a prokaryotic vector, BL21(DE3) competent *E. coli*, DNAligase, restriction end nuclease BamHI, restriction end nuclease XhoI, miniprep plasmid kit, agarose gel powder, peptone, yeast extract, glycine, tris-base, and EDTA, etc.

According to the gene sequence from the label-free mass spectrometry sequencing and NCBI database, the primer software was used to design primers containing two restriction sites BamHI and XhoI. The upstream primer was 5'-GACACGGATCCACGCCTACTGACTCTCAGCA-3', and the downstream was 5'-GTGTCAAGCTTTTACGCATAATAGGCGTTGCCCAC-3'. A sufficient amount of target product was amplified by PCR. The PCR protocol was as follows, initial denaturation at 95ºC for 3 min, followed by 22 cycles of denaturation at 95ºC for 22 sec, annealing at 58ºC for 20 sec and extension at 72ºC for 30 sec and then final extension at 72ºC for 5 min. The agarose gel electrophoresis was used to identify the PCR product. The purified fragment was recovered and subjected to restriction enzyme digestion.

The above mentioned PCR product and vector were enzyme digested, respectively. The PCR digestion system contained purified recovered fragments 1 µg, 10 × FD buffer 5 µl, BamHI 1 µl, XhoI 1 µl, and ddH_2O 23 µl. The vector digestion system contained pET-28a 1 µg, 10 × FD Buffer 5 µl, BamHI 1 µl, XhoI 1 µl, and added with ddH_2O to 50 µl. The above systems were placed in a constant temperature water bath at 37°C for 2 hrs. After digestion, the digested vector and fragment were subjected to agarose gel electrophoresis, followed by recovery and purification. Then the concentrations were measured by a spectrophotometer. The purified target DNA fragment and vector were ligated as follows, target DNA fragment 8 µl, digested vector pET-28a 4 µl, 10×T4 DNA ligase buffer 2 µl, T4 DNA ligase 1 µl and added with ddH_2O to final volume of 20 µl, sittingin a PCR machine at 22°C for 1 hr. The ligation solution was transformed to the BL21 (DE3) competent cells. The positive clones were screened for sequencing.

An amount of 1 µl recombinant pET-28a vector was added to 10 µl BL21 (DE3) competent cells, inoculated to an LB agar plate containing 30 µg/ml kanamycin, and cultured overnight in a 37°C incubator. The overnight culture was transferred into 3 litre of LB medium at a ratio of 1 : 100, added with kanamycin to a final concentration of 30 µg/ml, and cultured at 37°C and 220 rpm. When the OD_{600} reached 0.6, the culture was added with IPTG to a final concentration of 0.5mM and induced at 37°C, 220 rpm overnight. The cells were collected by centrifugation at 4,000 rpm for 10 min, and the precipitate was fully resuspended in bacterial lysate. After ultrasonication, it was centrifuged at 12,000 rpm and 4ºC for 20 min. Then the supernatant was collected and subjected to SDS-PAGE.

After ultrasonication, the cells were centrifuged at 12,000 rpm and 4°C for 20 min. The supernatant was collected and purified by Ni affinity chromatography. In brief, an amount of 5 ml Ni-NTA resin was washed and equilibrated with five resin-bed volumes of binding buffer at a flow rate of 5 ml/min. Then the resin was incubated with the sample for 1 hr and then applied to the column and the flow-through was collected. The column was then washed with 20 resin-bed volumes of binding buffer (containing 0.2% Triton X-114) to wash the endotoxin at a flow rate of 5 ml/min, then washed with wash buffer, eluted with elution buffer, with elution collected. The 12% SDS-PAGE was used for purified PXLR protein detection.

Results and Discussion

The virulent RXLR PITG_16275 gene was amplified with designed primers. A single band of about 500 bp was detected by agarose gel electrophoresis, consistent with the theoretical size of 513 bp (Fig. 1). The agarose gel electrophoresis of vector pET-28a after digested by restriction enzymes BamHI and XhoI is shown in Fig. 2.

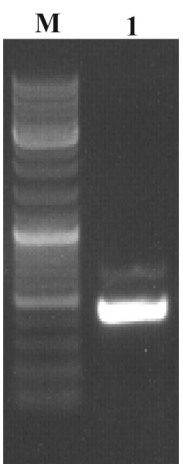

Fig. 1. Agarose gel electrophoresis of the PCR product of the target gene. M: DNA marker; 1: PCR product.

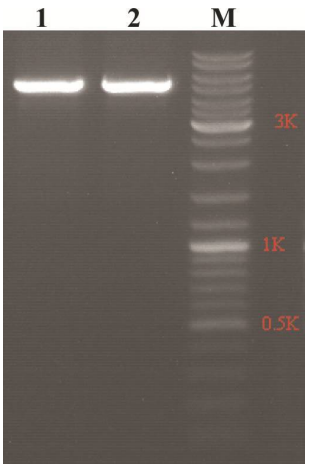

Fig. 2. Agarose gel electrophoresis of vector pET-28a after restriction enzyme digestion. M: DNA Marker; 1 and 2: vector pET-28a after digestion.

The constructed expression plasmid was used to express the target protein in BL21 (DE3) competent cells. After a small amount of induced expression was completed, the cells were collected by centrifugation, and the protein was extracted and subjected to SDS-PAGE. The Coomassie blue staining result showed that the tested induction conditions could be used for subsequent large amount induced expression of target protein, as shown in Fig. 3.

The induction conditions for small expression was used for large induced expression. After induced expression, the cells were disrupted by ultrasonication. A large amount of protein supernatant was prepared and purified by Ni affinity chromatography. The protein purification result is shown in Fig. 4. The target protein molecular weight was approximately 18.4 kD.

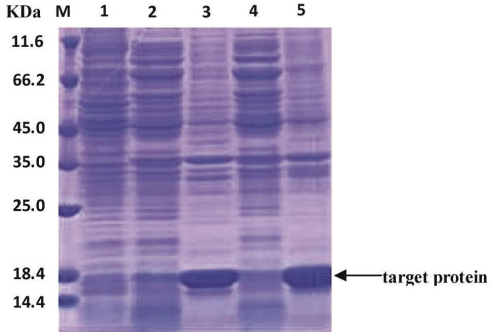

Fig. 3. SDS-PAGE of induced expression of the target protein. M: Protein marker; 1: Total protein before induction; 2: Supernatant of 20°C; 3: Precipitate of 20°C; 4: Supernatant of 37°C; 5: Precipitate of 37°C.

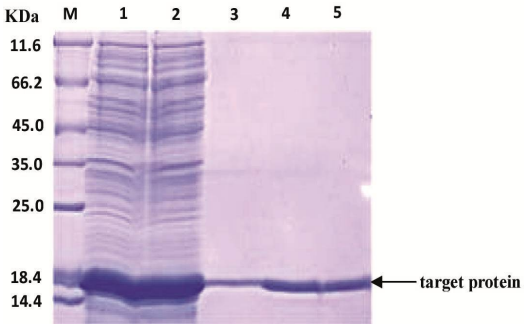

Fig. 4. SDS-PAGE of Ni Sepharose affinity chromatography. M: Protein marker; 1: Loading; 2: Flow-through; 3: 20 mM imidazole elution; 4: 50 mM imidazoleelution; 5: 500 mM imidazoleelution.

After purification, SDS-PAGE and Coomassie blue staining, the final obtained fusion protein was an18.4 kD protein with high purity, indicating successful purification of the target fusion protein (Fig. 5).

P. infestans RXLR effectors are crucial factors causing potato late blight and the RXLR effectors are closely related with its pathogenicity (Saunders *et al.* 2012). In this study, the expression and purification of RXLR effector of *P. infestans* was conducted, providing a basis for revealing the pathway of infection and biological function of *P. infestans.*

Because of its fast growth rate, high expression efficiency, clear genetic background, low cost and short cycle, *E. coli* is the most widely used, most profound studied and most technique matured expression system. The pET series is currently the most commonly used vectors for efficient expression. Therefore, in this study, an *in vitro* prokaryotic expression system was adopted to express the RXLR effector of *P. infestans*. Present experiment demonstrated that the culture condition of 37°C, 220 rpm and a final concentration of 0.5 mM IPTG was most favorable for the induced expression of the target protein.

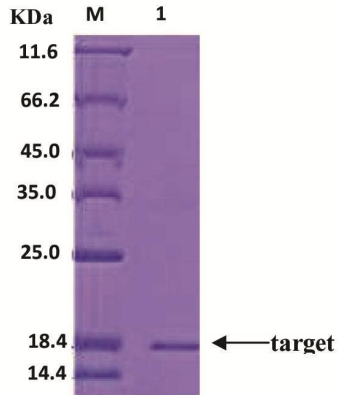

Fig. 5. SDS-PAGE of final pure protein. M: Protein marker and 1: Target protein.

In the future, authors plan to deeply explore the biological functions of RXLR effector of *P. infestans* by pathogenicity assay, subcellular localization, *in vitro* and *in vivo* protein interaction experiments. This study provided a foundation for clarifying the infection mechanism of *P. infestans* and for a better long-term control of potato late blight.

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