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Full Length Research Paper

Preparation of scFv against HrpA of *Pseudomonas syringae* pv. tomato DC3000

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Hrp pilus is a pathogenic bacterial surface appendage which serves as a tool for the transformation of bacterial effecter proteins into the plant cell. Hrp pilus is assembled by HrpA protein. In this study, a HrpA protein was successfully expressed, purified and a phage library single-chain fragment variable (scFv) was constructed. After six rounds of bio-panning, a specific scFv against HrpA protein named scFv-FE5 was screened by phage display. The results of enzyme-linked immunosorbent assay (ELISA) and western blotting analysis showed that scFv-FE5 could recognize HrpA protein specifically. Current work demonstrates the preparation of the antibody anti-HrpA scFv-FE5 using phage display technology, a very useful tool for generation of a specific scFv to a certain antigen.

Key words: HrpA, expression, scFv, phage display, bio-panning.

INTRODUCTION

Pseudomonas syringae pv. tomato DC3000, a Gram-negative bacteria causing the defense-associated hyper-sensitive response (HR) in plant (Fouts et al., 2003), is one of the major plant pathogenic bacteria that elicits plant disease through the translocation of effecter proteins into plant cells (Alfano et al., 2000). *Hrp* genes are present in almost all Gram-negative pathogenic bacteria, encoding hairpin which is the main component of type III secretion system (Hrp system) (Fu et al., 2006). Many gram-negative phytopathogenic bacteria use type III secretion system to inject type III effector proteins into plant cells to promote pathogenicity (Block et al., 2010). In *P. syringae,* few virulence effectors are translocated into plant cells via type III secretion system encoded by *hrp* genes (Oh et al., 2007). Hence, *hrp* genes may play an important role in pathogenic bacteria colonization and plant defense response induction.

Hrp pilus is formed on the surface of *P. syringae,* which is required for pathogenicity and allergic reactions, and normal function of Hrp type III secretion system depends on Hrp pilus. It is speculated that Hrp pilus on pathogenic bacteria grows through the plant cell wall. Bacterial cell proteins and other virulence factors are secreted through the Hrp pilus, and absorbed by endocytosis of plant cell. The major subunit of the Hrp plius of *P. syringae* pv. tomato DC3000 is the HrpA protein, an important component of the type III secretion system (Deng et al., 1998; He et al., 2003). Hence, HrpA has been suggested as a promising target for prevention and control of diseases.

Single-chain fragment variable (scFv) based on phage display is a versatile technology to generate single-chain antibody for interested antigen (Wang et al., 2006), and provides a useful tool that allows the selection of single-chain antibody that is specific for certain antigen. The technique of scFv has been developed for recognizing molecular target of cancer (Sakai et al., 2010; Zhang et al., 2010), and has the power to mimic the feature of immune diversity and selection, and to synthesize and express unlimited quantities of antibodies. By the tool, we

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are able to characterize the binding property of scFv and investigate the potential use of scFv as a diagnostic tool or therapeutic agent (Eisenhardt et al., 2007; Doppalapudia et al., 2010).

To better understand the role of HrpA in the type III secretion system of *P. syringae* pv. tomato DC3000, we successfully amplified *hrpA* gene and expressed the HrpA protein. A phage antibody library was constructed, and the specific scFv clones against HrpA were screened by bio-panning. Finally, a phage clone named scFv-FE5 was successfully obtained by showing the strongest positive signal in reaction to recognize HrpA protein.

MATERIALS AND METHODS

Bacterial strains and chemicals

P. syringae pv. tomato DC3000 was donated by Prof Zonghua Wang (Fujian Agriculture and Forsetry Universtiy, China). Plasmid pET28a(+) were purchased from Novagen. *E. coli* BL21 (DE3), plasmid pCANTAB-5E, *E. coli* TG1 and HB2151, M13K07 helper phage was stored in our laboratory. DNA restriction enzymes, RNA isolation kits and reverse transcription kits were purchased from Promega. Taq DNA polymerase and T4 DNA ligase were purchased from Takara (Dalian, China). HRP-labeled goat anti-mouse IgG was from Boster Biological Technology Co. (Wuhan, China).

Expression and purification of HrpA protein

To construct the expression vectors pET28a(+)-*hrpA*, the genomic DNA of *P. syringae* pv. tomato DC3000 was extracted by the CTAB DNA Isolation Technique (Current Protocols in Molecular Biology, 2001). The *hrpA* gene was amplified by PCR with the forward primer P1 (AGAAGAATTC ATGGTCGCATTTGCAGGAT, *EcoR* I) and reverse primer P2 (CGGTCTCGAGTTAGTAACTGA TACCTTTAGCGT, *Xho* I). For cloning the *hrpA* gene into pET28a(+) vectors, restriction enzymatic sites, *EcoR* I and *Xho* I, were designed into the primers. Before protein expression, the sequenced plasmid was transformed into *E. coli* BL21 by electroporation, and a single positive colony from the selection plate was inoculated in 5 mL LB liquid media containing 100 μg/mL ampicillin. The culture was incubated overnight with shaking at 37°C, and then transferred to a larger-scale LB media (500 μL culture was transferred into 50 mL fresh LB). Expression of the target protein was induced by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) when OD_{600} of the culture reached 0.8. Cells were harvested by centrifugation after grown for an additional 6 h at 28°C. Then, target protein was purified by Ni^{2+} affinity chromatography.

Immunization and total RNA extraction

Four female Balb/c mice were immunized with the purified HrpA protein by subcutaneous injection. The mixture of HrpA and the same volume of complete Freund's adjuvant was used to immunize mice for the first time, and the later injections were taken every 10 days for further three times with the mixture of HrpA and the same amount of incomplete Freund's adjuvant. The anti-HrpA antibody titer was determined by indirectly ELISA. Once high serum titers were obtained, animals were sacrificed at the 5th day after the last immunization. Total mRNA was extracted from the isolated spleens by Trizol method (Promega Biotech).

Construction of a phage-displayed anti-HrpA scFv library

The first cDNA was amplified with the oligo dT15 primer using the above extracted mRNA as template. The phage-displayed anti-HrpA scFv library was prepared using a recombinant phage antibody system (RPAS) according to the protocols supplied by the manufacturer. Briefly, the amplified V_H and V_L genes were assembled into a scFv gene using a linker sequence. The assembly was re-amplified to incorporate flanking *Sfi* I and *Not* I cloning sites at its 5'- and 3'-ends, respectively. The amplified products were purified, digested with *Sfi* I and *Not* I, and cloned into the phagemid pCANTAB-5E containing E-tag sequence in frame. The recombinant phagemid was then transformed into competent *E. coli* TG1 cells by electroporation, and the transformed cells were spread onto the SOB-AG plates to calculate the capacity of the library.

Bio-panning of phage display library

A 96-well microtiter plate was coated with the purified 10 μg HrpA/ml (100 μl/well) at 4°C overnight, and the plates were washed 3 times with PBS and blocked with 4% PBSM (PBS containing 4% fat free milk) for 2 h at 37°C. Then, the prepared phages were added to the reaction wells and incubated for 2 h at 37°C. Unbound phages were removed by washing 10 times with PBST (PBS containing 0.05% Tween) and 10 times with PBS. Antigen-bound phages were eluted by adding 100 μL of 1.0 M triethylamine for 10 min and then neutralized with 100 μL of 1.0 M Tris-HCl (pH 7.4). The eluted phages were used to infect the *E. coli* TG1 in logarithmic growth phase and plated on SOB-AG medium to determine the titer (Wang et al., 2006).

Screening specific binding clones by phage-ELISA

The picked phage clones were cultured and infected with the M13KO7 for preparation of phage scFv. ELISA was used to determine the binding activity of individual clone. First, the HrpA coated plates were washed and then blocked with 4% PBSM at room temperature for 1 h. Phages scFv derived from individual clone were added into the reaction wells, and incubated at 37°C for 2 h, respectively. The binding activity was detected with HRP conjugated anti-M13 monoclonal antibody by ELISA. Absorbance at 450 nm was measured with a microplate reader.

Soluble expression and extraction of scFv

To obtain the soluble scFv protein, HB2151 was infected with the positive phage, and grown in 2×YT-AG media at 37°C overnight. The culture was then diluted at 1:100 ratio into 100 ml fresh 2×YT media containing 100 μg ampicillin/ml, and continued to grow at 37°C until the OD_{600} reached 0.8. IPTG (final concentration is 1 mM) was added to induce the expression of scFv, and the cultures were further incubated overnight at 28°C. After expression, the cell culture pellet was resuspended in 10 ml ice-cold 1× Tris-HCl, EDTA, sucrose (TES) buffer. After centrifugation, the culture pellet was resuspended by adding ice-cold 1/5×TES buffer, and incubated on ice for 30 min. The contents was transferred to a microcentrifuge tube, and centrifuged at full speed at 4°C for 10 min. The supernatant was carefully collected and analyzed for the presence of soluble scFv by ELISA and SDS-PAGE.
(Tris-HCl, EDTA, sucrose

ScFv specificity analysis

Specificity analysis of the anti-HrpA scFv was carried out by phage ELISA according to Wang et al. (2012). Associated antigens HrpA,

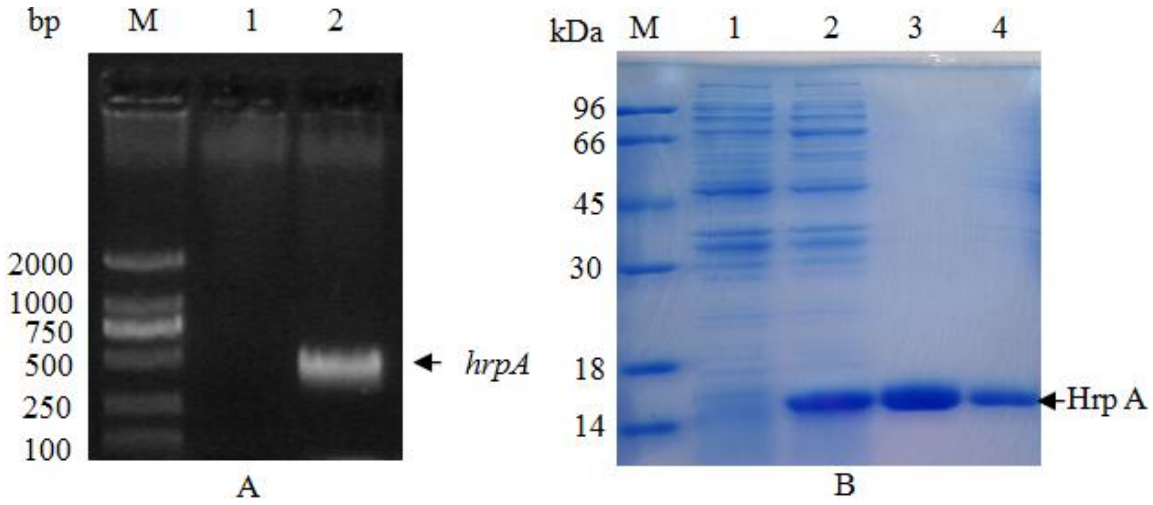

Figure 1. Amplification of *hrpA* gene and expression of HrpA protein. **A.** The amplified fragment of *hrp A* gene. Lane M, DL-2000 Marker; Lane 1, negative control; Lane 2, the amplified fragment of *hrp A* gene. **B.** SDS-PAGE analysis of expression and purification of HrpA protein. Lane M, Mid-Range Protein Molecular Weight Markers; Lane 1, control (induced *E. coli* BL21 containing empty vector pET28a(+); Lane 2, induced *E. coli* BL21 containing recombinant plasmid pET28a(+)-*hrpA*; Lane3-4, the purified HrpA protein by Ni^{2+}-NTA.

HrpJ, HrpZ, 6His, BSA, KLH, and OVA were coated on 96-well plates. After blocking with 4% PBSM, secreted recombinant phage scFv were added to the reaction wells. The specificity of the scFv clone was detected with the HRP conjugated anti-M13 antibody. The enzyme reaction was then performed with TMB as a substrate.

Western blotting and far western blotting

Far western blotting was used to analysis the binding ability of the selected scFv to HrpA. Antigen HrpA with different concentration (10, 20, 40, 80 µg/ml) and BSA (control) were loaded onto PVDF membrane for overnight at 4°C. After blocking and washing, extracted soluble scFv was added for 2 h at room temperature. After washing, the membrane was subsequently incubated with anti-M13 antibody and AP-conjugated goat anti-mouse IgG antibody.

Signals were visualized with substrate BCIP/NBT. To further identify the specificity between the selected positive scFv and HrpA antigen, western blot was performed. The total protein of *P. syringae* pv. tomato DC3000 and the purified HrpA antigen protein was transferred from a SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was treated with soluble scFv according to standard protocol. The last step was the same as that given above.

RESULTS

Expression and purification of Hrp A

Genomic DNA of *P. syringae* pv. tomato DC3000 was isolated and used as the PCR template. The intact DNA fragment of *hrpA* was 342 bp, and a PCR product about 342 bp shown in Figure 1A was amplified. PCR product was cloned into TA vector and sequenced. The result shows that it was identical to *hrpA* gene. The PCR product was digested with restriction enzmyes *EcoR* I and *Xho* I, and was ligated to digested plasmid pET28a(+) to form the recombinant plasmids named pET28a-*hrpA*. To express the target protein effectively, the *E. coli* BL21 containing recombinant plasmid pET28a(+)-*hrpA* was induced by IPTG. The size of fusion protein expressed by pET28a(+)-*hrpA* was about 14 kDa. The results in Figure 1B (Lane 2) show that HrpA was expressed correctly, and the purified target protein was successfully obtained (Figure 1B) (Lanes 3 and 4).

Immunization and extraction of total RNA

To obtain the specific anti-HrpA antibody and higher serum titer, the purified target protein HrpA was used for immunization for four Balb/c mice by subcutaneous inject-tion. The mixture of HrpA and complete Freund's adju-vant was used for immunization for the first time, and the later injections were administered with the mixture of HrpA and incomplete Freund's adjuvant for a further three times. Blood were collected from animals and used for serum titer detection by ELISA. The detection results showed that all four mice had a relatively high titer (Figure 2) and all of them fully met the requirements. Total RNA was extracted from the spleen cells of Balb/c mice that had a higher anti-HrpA serum titer.

Construction of phage-displayed anti-HrpA scFv library

Total RNA was used to generate the cDNA by reverse transcription reaction. The cDNAs were then used as

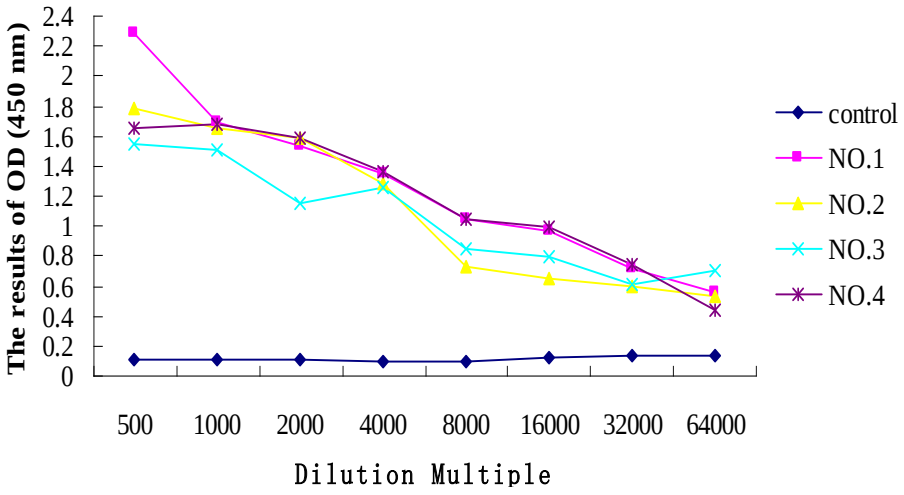

Figure 2. The result of titer assay of serum. The serum from the HrpA immunized mice were used for titer detection by ELISA. No. 1, 2, 3, 4 represent the four immunized mice with high titer respectively, while control represent non-immunized mice as negative control.

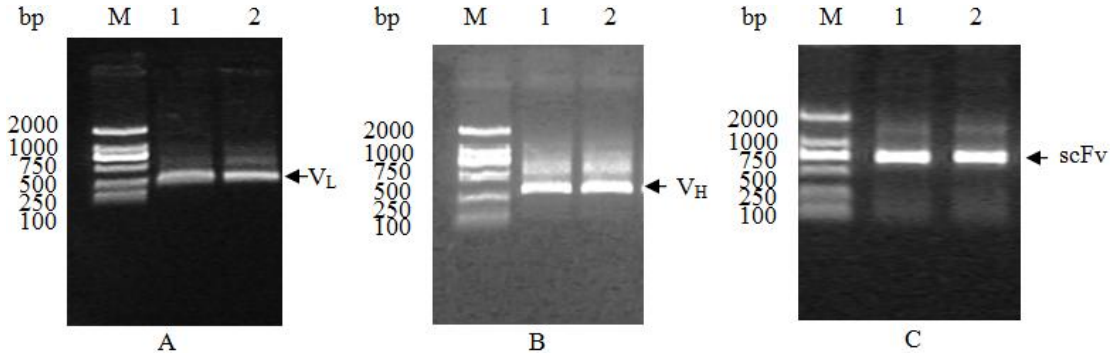

Figure 3. The amplified fragments of V_L, V_H and scFv genes. **A.** The amplified fragment of V_L gene. Lane M: DL-2000 Marker; Lane 1-2: the amplified fragment of V_L gene. **B.** The amplified fragment of V_H gene. Lane M, DL-2000 Marker; Lane 1-2, the amplified fragment of V_H gene. **C.** The amplified fragment of scFv. Lane M, DL-2000 Marker; Lane 1-2, the assemble fragment of scFv.

template to amplify the V_H (340 bp) and V_L (325 bp) genes. As shown in Figure 3, the V_H and V_L genes were amplified successfully (Figure 3A and B). Besides, a scFv DNA fragments with an expected length of 750 bp was obtained (Figure 3C) by overlap PCR with the V_H and V_L genes as template. The phage displayed scFv library was constructed with a transformed rate of 5.6×10^7 (CFU/ml). Fifty (50) clones were randomly selected from the plates for plasmid extraction and analysis, and the result showed that the recombinant rate was 85%. So the size of the phage displayed scFv library was 4.76×10^7(CFU/ml).

Bio-panning and selection of specific HrpA-binding scFv clones

The input and output of the library during bio-panning are shown in Figure 4A, and about 5×10^7 CFU/ml phage clones were checked (input) in each panning round. The elution phage clones (output) were very low at the first two rounds, but the output was kept in a stable level of approximately 4×10^6 CFU/ml after the third round. Lastly, 100 clones from the third round to the six rounds were randomly selected to test the binding ability to HrpA. Finally, 5 scFv clones showing relatively stronger binding ability to HrpA protein were isolated from the library (Figure 4B), and the phage clone with the strongest positive signal designated scFv-FE5 (Figure 4B) was chosen for further study.

Soluble expression and far western blotting

E. coli TG1 was a suppressor strain, allowing the expres-

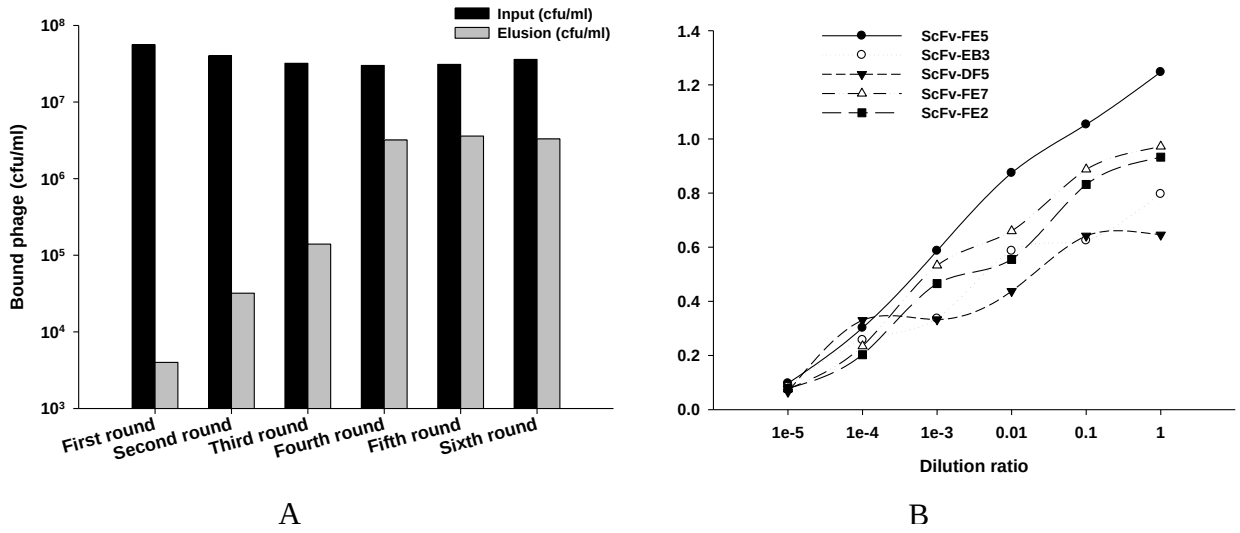

Figure 4. Biopanning and selection of specific HrpA-binding scFv clones. **A.** Biopanning result of the phage-scFv library. The number of input phages in each round were about 5×10^7 CFU/ml. After three rounds biopanning, the number of elution phages was observed 4×10^6 CFU/ml constantly. **B.** ELISA analysis of the binding activity of 5 different anti-HrpA scFv clones. The graph showed the relationship between OD450 value and HrpA concentration.

sion of a fusion protein of the pIII and the scFv. In contrast, *E. coli* strain HB2151 was a non-suppressor strain, so it was used for production of soluble scFv. Soluble expressed scFv was extracted from periplasm, and were evaluated by 14% SDS-PAGE. The protein was detected by staining with Coomassie Brilliant Blue R-250. A 29 kDa protein band was found, corresponding to scFv (Figure 5A). The binding ability of scFv-FE5 to HrpA was firstly analyzed by far western blot. HrpA antigens with different concentrations were loaded onto the PVDF member (BSA was used as control). After the extracted scFv was added, the enzyme-labeled antibody and the substrate was used to develop color. The result shows that the scFv-FE5 could bind to HrpA specifically, but not bind to BSA (Figure 5B).

Specificity analysis of scFv and western blotting

To further identity the specificity of scFv-FE5, some related antigen HrpA, HrpJ, HrpZ, 6His, BSA, KLH, and OVA were used to detect the specificity of scFv-FE5 by indirectly ELISA. As seen in Figure 6A, the scFv-FE5 antibody had stronger signal when detected in ELISA for HrpA antigen compared to other antigens. The result demonstrates that the selected scFv-FE5 could specifically recognize HrpA antigen with no cross-reaction to other related antigen proteins. Besides, the binding activity of the soluble scFv with HrpA was further evaluated by western blotting. As shown in Figure 6B, the scFv-FE5 could recognize the HrpA from *P. syringae* pv. tomato DC3000 and the purified HrpA at the same time, the size of HrpA matched the detected band on the position of 14 kDa. The result demonstrated that the selected scFv-FE5 could recognize HrpA specifically.

DISCUSSION

The type III system (hrp T3SS) in plant pathogens is capable of delivering various effectors into the interior of host cells (Deng et al., 1999). The Hrp pilus has been identified previously from preparations of bacteria recovered from agar or liquid culture (Roine et al., 1997). A major problem with both methods is the fragmentation of the delicate pili and detachment from bacterial cells before examination, and only a low level of constitutive HrpA was expressed in bacteria grown in rich media prior to transfer to inducing conditions (Brown et al., 2001). As it is not easy to get large scale of pure HrpA pilus, we cloned *hrpA* gene of *P. syringae* pv. tomato DC3000 from its genome. The DNA fragment encoding the HrpA was cloned into expression vector pET28a(+); target HrpA was successfully expressed in *E. coli*. The recombinant protein was expressed with a 6His-tag at C-terminal to facilitate the purification of the desired protein. It was showed that expressed HrpA could be easily prepared in large scale, and used for further research.

To gain scFv against HrpA, female Balb/c mice were immunized with purified HrpA, and cDNA was prepared from spleen mRNA of the immunized mice. With a linker molecule, scFv was assembled through joining the amplified V_H and V_L genes together by overlap PCR (Heng et al., 2003). To obtain a large size of phage scFv library, the scFv gene was cloned into pCANTAB-5E vector, and the recombinant vector was transformed into *E. coli*

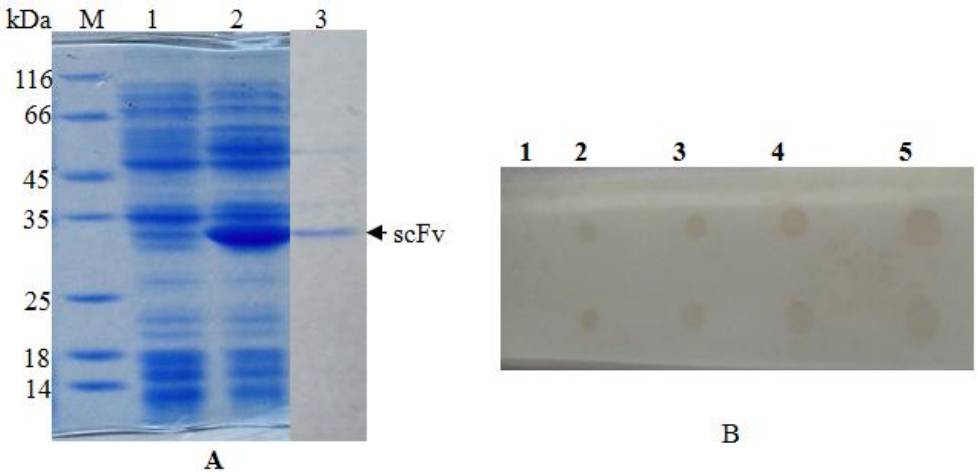

Figure 5. Soluble expression and far western blotting. **A.** SDS-PAGE analysis of soluble expressed scFv. Lane M: protein marker; Lane 1, induced *E. coli* HB2151 containing empty plasmid pCANTAB-5E; Lane 2, induced *E. coli* HB2151 containing recombinant plasmid pCANTAB5E-*scFv*; Lane 3, extracted periplasmic protein containing soluble *scFv.* **B.** Detection of scFv binding ability by far western blot. Antigen HrpA with different concentration and BSA (control) were loaded on to PVDF membrane, and extracted soluble scFv was added. Lane 1, BSA as control (25 ng); Lane 2-5, purified HrpA protein with 25, 50, 100, and 200 ng, respectively.

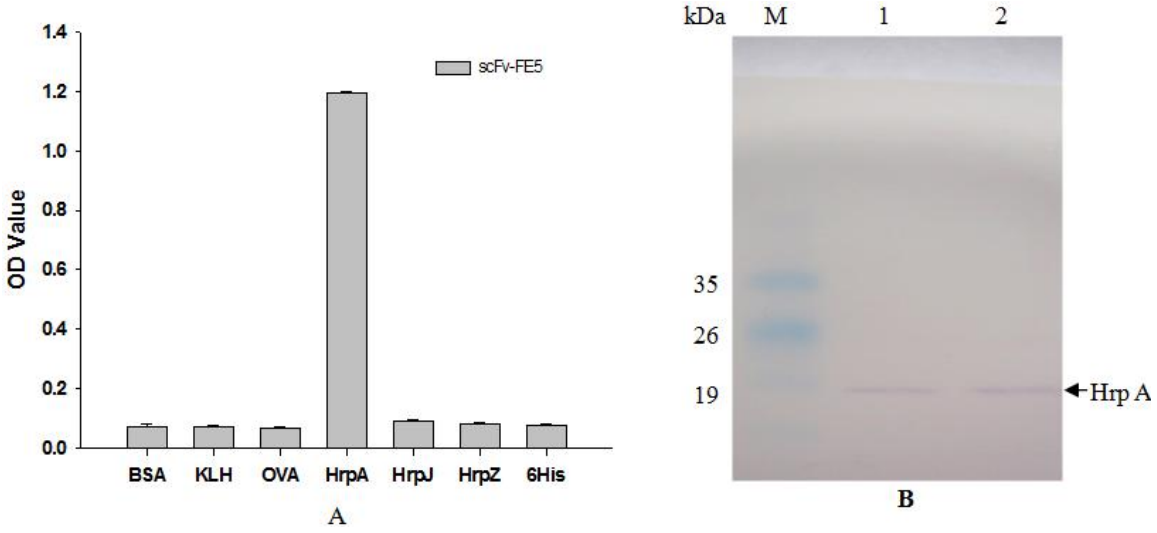

Figure 6. Specificity analysis of scFv and western blotting**. A.** scFv-FE5 specifically binds the HrpA antigen. Associated antigen HrpA, HrpJ, HrpZ, 6His, BSA, KLH, and OVA were coated on 96-well plates in 5 µg /ml (100 µl/well, triplicate), the soluble scFv-FE5 were added to the reaction wells and incubated for 2 h at 37°C. Specificity of the scFv-FE5 clone was determined using an anti-M13 HRP-conjugated antibody. **B.** Western blotting. The total protein of *P. syringae* pv. tomato DC3000 and the purified HrpA antigen protein was transferred from a SDS-PAGE gel onto a polyvinylidene difluoride (PVDF) membrane, and the membrane was treated with soluble scFv-FE5 using standard protocol. The last step were the same to far western blotting. Lane M: pre-stained protein marker; Lane 1: the total protein of *P. syringae* pv. tomato DC3000; Lane1-2: the purified HrpA antigen protein.

TG1 by electronic transformation. The size of the library depends on the transformation efficiency, which is the major limitation for phage display technology (Azzazy et al., 2002; Singh et al., 2010). By using electroporation, the size of the phage displayed antibodies library was 4.76×10^{7} (CFU/ml), which met the needs for scFv bio-

panning.

After six rounds panning, one phage clone which gave the strongest positive signal designated scFv-FE5 was selected. The result of soluble expression of scFv-FE5 indicated that the size of the scFv-FE5 is about 29 KDa. Further western blot analysis showed that the scFv could specifically recognize the Hrp A. All these results show that anti-HrpA scFv was successfully prepared, and the scFv could be used as a tool to study the function of the HrpA in the type III system in plant pathogens in further research. Shahryari et al. (2013) have developed a single-chain variable fragment antibody (scFvIMP6) against the immunodominant membrane protein (IMP) of witches' broom phytoplasma and expressed it in different plant cell compartments. They found that scFvIMP6 binds with high activity and can be used for the detection of Ca. Phytoplasma aurantifolia and is also a suitable candidate for stable expression in lime trees to suppress witches' broom of lime (Shahryari et al., 2013).

The anti-HrpA scFv-FE5 obtained in the study by phage display technology was a very useful tool to develop a kit to detect HrpA antigen, or to be used as a therapy for plant defense-associated hypersensitive response caused by bacteria type III secretion system.

ACKNOWLEDGEMENTS

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