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Research Article

Development and molecular analyses of Xanthomonas pthA specific scFv recombinant monoclonal antibodies

Hamideh Raeisi[1], Mohammad Reza Safarnejad[2*], Seyed Mehdi Alavi[3], Naser Farrokhi[4*], Seyed Ali Elahinia[1], Hossein Safarpour[5] and Farshid Sharifian[3]

1. Department of Plant Protection, Faculty of Agricultural Sciences, Guilan University, Rasht, Iran.
2. Department of Plant Viruses, Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization of Iran (AREEO), Tehran, Iran.
3. Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
4. Department of Cell & Molecular Biology, Faculty of Life Sciences & Biotechnology, Shahid Beheshti University G. C, Tehran, Iran.
5. Cellular and Molecular Research Center, Birjand University of Medical Sciences, Birjand, Iran.

Abstract: The *Xanthomonas citri* pv. *citri* (*Xcc*) is causal agent of bacterial citrus canker which is major disease of citrus throughout the world. The pthA bacterial effector protein is presented within the infected plants and indispensable of canker. The scFv antibodies are valuable tools for diagnosis and suppression of pathogens within plants. The present article describes developing and characterization of specific recombinant monoclonal scFv antibodies against pthA effector protein. For this aim, the gene encoding pthA protein was heterologously expressed in *Escherichia coli* and used for screening of Tomlinson phage display antibody library to pinpoint specific single chain variable fragment (scFv). In each round of panning, the affinity of phage towards pthA was checked by enzyme linked immunosorbent assay (ELISA). The data was indicative of about 50% of the monoclonal phages to be reactive strongly against pthA protein. Among the positive clones, 5 samples (A12, B8, C1, H8 and G8) were capable of detecting *Xcc*-infected plant samples and recombinant pthA protein. Restriction fragment length polymorphism showed similar banding pattern for all 5 scFvs as renamed to pthA-scFG8. HB2151 *E. coli* cells were infected by the phage bearing pthA-scFG8, and the expression of the peptide was induced by IPTG to produce a 30 kDa recombinant molecule. I-TASSER was used for homology modeling of both scFv and pthA and docking was carried out by Hex program. The latter demonstrated binding energy of −784 kcal/mol in scFv-pthA.

Keywords: Biopanning, citrus bacterial canker, phage display, single chain fragment variable

Introduction

Management of plant diseases with drastic effects on both quality and quantity of the produce comes in many forms and shapes. Developing recombinant antibodies by the virtue of phage display has promised both plant pathologists and plant growers a rather efficient and rapid diagnostic tactic (Hust *et al*., 2002; Safarnejad *et al*., 2008; Yang *et al*., 2013; Yuan *et al*., 2015), next to providing opportunities to circumvent such devastating effects (Tavladoraki *et al*., 1993; Safarnejad *et al*., 2009; Cervera *te al*., 2010; Hemmer *et al*., 2018). The latter mainly comes

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* **Corresponding authors**, e-mail: mrsafarnejad@yahoo.com, n_farrokhi@sbu.ac.ir
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with the introduction of the genes encoding single chain variable fragment (ScFv) to the host plant genomes via transgenesis. The gene encoding a recombinant antibody is made up of a variable heavy and light chains connected with a short polypeptide linker. The recombinant antibody would be capable of binding to varieties of target proteins such as phytoplasma surface protein (Yuan *et al*., 2016), viral coat proteins or other enzymes involved in virus proliferation (Gil *et al*., 2011), fungal hydrolytic enzymes that are active in pathogenesis (Cheng *et al*., 2015; Peschen *et al*., 2016), and bacterial effector proteins.

The effector protein of *Xanthomonas citri* subsp. *citri* (*Xcc*), the causative agent of Citrus canker, is pthA that leads to hypersensitive response (HR) in resistant hosts and pathogenicity in susceptible genotypes (Gottig *et al*., 2010; Roeschlin *et al*., 2017) and non-host plants (Mysore and Ryu, 2004). Thus, it is being considered as the major determinant of *Xcc* pathogenicity (Brunings and Gabriel, 2003; Al-Saadi *et al*., 2007). The complete nucleotide sequence of pthA has 17.5 copies of 102-bp repeats, tandem repeats of 34 amino acids, three nuclear localization signals (NLSs) to direct the pthA into the host nucleus (Swarup *et al*., 1992; Domingues *et al*., 2010; Pereira *et al*., 2014), and C-terminal transcriptome activation conserved domain. The protein belongs to AvrBs3/pthA family also known as transcription activator-like (TAL) effectors that is being translocated to the host plant via a type III effector protein (Römer *et al*., 2007). The tandem repeats of pthA amino acids are proposed to be involved in protein-protein and protein-DNA interactions (Boch *et al*., 2009; Moscou and Bogdanove, 2009). This section apparently defines both pathogenicity and avirulence (Yang and Gabriel, 1995; Kay and Bonas, 2009). It was shown that the pthA interacts with α-importins (Szurek *et al*., 2001; Domingues *et al*., 2010), cyclophilin, thioredoxin and ubiquitin conjugating enzyme (Domingues *et al*., 2010, 2012), high mobility group (HMG), and poly(A)-binding proteins (De Souza *et al*., 2012). Overall, it has been proposed that TAL effectors such as pthA functioning to affect mRNA processing and translation (De Souza *et al*., 2012), activate host transcription by inhibiting peptidyl-prolyl cis-trans isomerase activity of cyclophilin through binding to its regulatory C-terminus domain (Domingues *et al*., 2012), and targeting the promoters of some genes such as *lateral organ boundary* (Hu *et al*., 2014; Jia *et al*., 2016; Zhang *et al*., 2017), 2-oxo-glutarate/FE(II)-dependent dioxygenase (DIOX) and *CCNBS* genes (Pereira *et al*., 2014). Given that, the pthA is a specific protein of the pathogen, it could be a good candidate to develop a recombinant antibody for serological test or production of resistant plants to *Xcc*.

Xcc causes severe damages to many Citrus species, affecting quality loss via forming yellow chlorotic rings on fruits and other parts, and finally leads to significant yield reduction (Gottwald *et al*., 2002; Li *et al*., 2012). Therefore, developing any means that help to reduce the use of chemical bactericides and antibiotics would be beneficial for human health, off-target organisms, and the environment.

Here and for the first time, we have developed a scFv antibody against pthA via screening a human combinatorial phage peptide library, followed by bioassay of the heterologously expressed antibody in *E. coli* to further corroborate on its specificity towards *Xcc*. The results were complemented with molecular docking of the scFv antibody within the pthA.

Materials and Methods

Production of pthA recombinant protein

A partial sequence of pthA (606 bp) from the COOH-terminal of the native sequence with the highest antigenicity index was cloned in pET28a with NH_2-terminally 6× His tag (Mokhtari *et al*., 2015). Protein expression was induced in *E. coli* Rosetta strain (DE3) by 1 mM IPTG for 16 h and purified under native condition by immobilized metal ion affinity chromatography (Qiagen, Hilden, Germany) following the manufacturer's manual. Purified protein was separated on 12% SDS-PAGE (Supplementary Fig. 1) and blotted onto a membrane to be treated by commercial anti-His tag (Abcam, UK) (Surendran *et al*., 2015).

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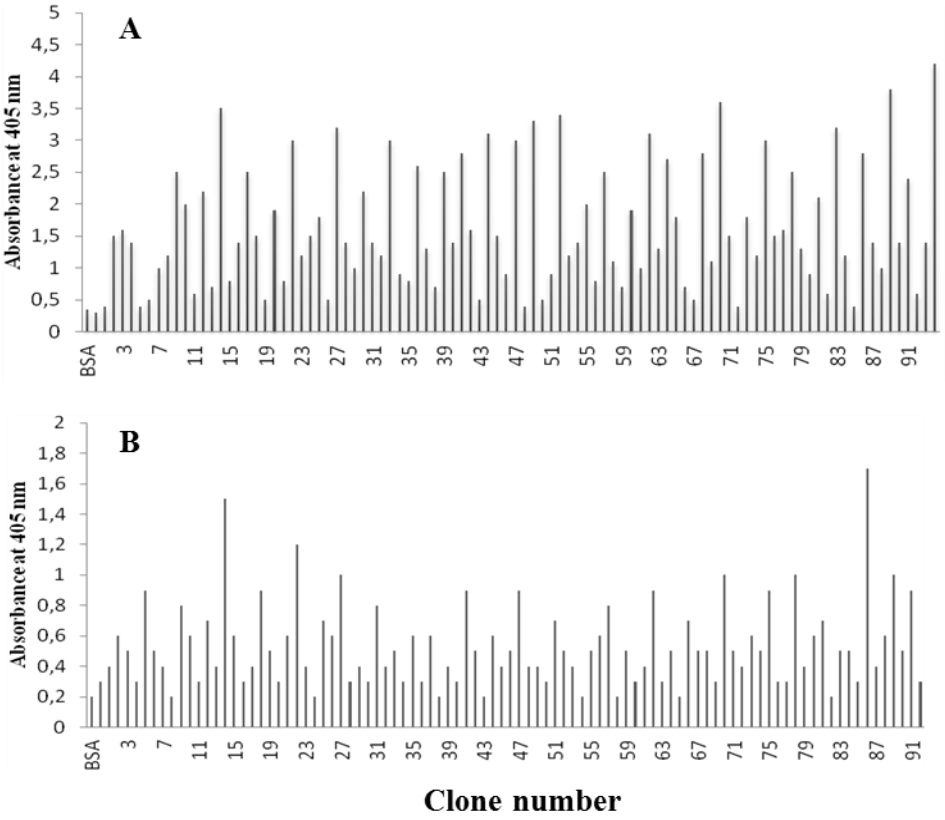

Figure 1 Screening of selected clones from Tomlinson I libraries in monoclonal phage ELISA. Binding activity of selected monoclonal phages (A) and soluble scFv antibodies (B) to pthA protein were assayed via indirect ELISA.

Phage panning and screening

Tomlinson I naïve scFv phage libraries (Source Bioscience, UK) were used for selection of specific monoclonal antibody against pthA protein of *Xcc*. Preparation of phage library was performed using a TG1 library culture upon infection with M13K07 helper phage and the titer was determined. Phage particles were rescued from infected bacteria using M13KO7 helper phage. Purified phage were used in the next round of biopanning. Finally, three rounds of panning were carried out (Raeisi *et al*., 2018).

Identification of specific recombinant phage to pthA

Screening was carried out by phage-ELISA. *E. coli* TG1 cells were infected with 100 µl of eluted phage and plated on TYE agar containing 1% (w/v) glucose and 100 µg/ml ampicillin. Bacterial colonies (94) were arbitrarily selected and were cultured in a microtiter plate. Bacterial cells were grown in 2 × YT (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH = 7.2) containing 100 µg/ml ampicillin and 1% (w/v) glucose at 37 °C. Helper phage suspension was added (~10^9 cfu) at OD_{600} = 0.4. For each phage clone, one well was coated with 10 ug/ml pthA and blocked with 3% BSA in 1 × PBS and another uncoated well blocked as a control. Phages were added to the coated plates and incubated at 37 °C for 2 h and washed three times in 0.1% PBST. The reaction between bound phages and antigen (pthA) was detected with anti-M13 antibodies conjugated to horseradish peroxidase (Abcam, UK) for 1 h at 37 °C in the presence of 2, 2-azino-di-3-ethylbenz-thiazoline sulfonate (ABST) (Fermentase, Vilnius, Lithuania). The absorbance was recorded at 405 nm in an ELISA reader (Tecan, Switzerland).

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Production of soluble scFv antibodies

To induce scFv production, HB2151 *E. coli* cells were infected with produced monoclonal phage supernatant. Bacterial cells were grown in 2 × YT containing 100 µg/ml ampicillin and 0.1% (w/v) glucose at 37 °C. The induction was initiated at OD_{600} = 0.7 - 0.8 with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 16 h at 30 °C. The cells were isolated by centrifugation (2000 × g / 10 min / 4 °C) and supernatants were used for subsequent assays. For each analyzed scFv fragment, a well was coated with 10 ug/ml pthA in PBS and blocked with 3% BSA in PBS whereas an uncoated well was blocked as a control. After three times of washing with 0.1% PBST, the prepared cell culture supernatants were added to the coated plates, the plates were incubated at 37 °C for 2 h, and washed three times in 0.1% PBST. The anti-c-myc tag antibody (Abcam, USA) was incubated in each well for 2 h at 37 °C and washed three times in 0.1% PBST. The reactions between antibodies and antigens were detected by adding horseradish peroxidase-conjugated goat anti-mouse IgG (Abcam, UK) for 1 h at 37 °C. After final wash with PBST, the color reaction was initiated by adding ABST as substrate for a period of 30 - 60 min at 22 °C. The absorbance was recorded at 405 nm in an ELISA reader. Reacting clones shown in ELISA were used selectively for western blot.

To evaluate the specificity of the produced antibody against *Xcc*, indirect ELISA against the plant protein extracts from healthy and infected lime was performed. Extraction of plant protein was carried out in 1:3 (w/v) extraction buffer (1× PBS, pH = 7.5, 5 mM EDTA, 5 mM β-mercaptoethanol or 2% (v/v) polyvinyl pyrrolidone in PBS). Wells of the microplate were coated by healthy and infected plant extracts and purified recombinant pthA protein as the positive control and stored at 4 °C for 16 h. The pthA-scFv antibodies were added and incubated for 2 h at 37 °C. Reaction between antibodies and antigens were detected by adding anti-*c-myc* tag antibody at 37 °C for 2 h that followed by goat anti-mouse IgG conjugated with horseradish peroxidase for 1 h at 37 °C. The substrate (ABST) was added for a period of 30 - 60 min at 22 °C. The absorbance was read at 405 nm in an ELISA reader. The samples were considered as positive where the mean ELISA (A_{405} nm) value of sample was at least twice greater than that of the negative control.

Western blot analysis of pthA proteins using scFv antibodies

Purified pthA and bovine serum albumin (BSA) were separated on SDS-PAGE. Proteins were transferred to Millipore polyvinylidene difluoride (PVDF) membrane (Sigma-Alderic, Germany) according to the instructions by the manufacturer. After blocking with PBS buffer containing 5% powdered skimmed milk (w/v), the membrane was incubated with scFv proteins at 4 °C for 16 h. The scFv proteins were detected by anti-c-myc monoclonal antibody, followed by anti-mouse IgG conjugated to alkaline phosphatase (Sigma, USA). The target proteins were finally revealed by adding substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma, Deisenhofen, Germany).

Endonuclease digestion of scFv and Sequence analysis

ELISA positive clones were PCR amplified using pHEN (sense: 5'-GCCGCTGGATTGTTATTAC CTCT-3' and antisense: 5'-AGAGAGAGATAGT TTGTAGAGA-3') primers to amplify a 950 bp fragment of scFv antibody coding sequence. Thermal cycling was carried out with an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C: 1 min, 54 °C: 1 min, 72 °C: 1 min, and a final extension step at 72 °C for 10 min. The amplicons were digested with *Bst*NI (Fermentas, Lithuania) and analyzed on 2% (w/v) agarose gel electrophoresis.

pHEN primers used for sequence analysis and sequence alignments with the human germline gene were done using IMGT/V-QUEST (http://www.imgt.org).

Production and purification of scFvs

The *E. coli* HB2151 was cultured in 2 × YT at 37 °C. At OD_{600} = 0.7 - 0.8 was induced with 1 mM IPTG for 16 h at 30 °C. The cells were

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harvested by centrifugation (5000 × g / 20 min / 4 °C). The pellet was incubated with 0.05 volume of 50 mM Tris-HCl buffer containing 30% sucrose and 1 mM EDTA (pH = 8) for 5 min at 0 °C. Extraction of scFvs was carried out using 0.05 volume of 5 mM $MgSO_4$ for 45 min at 0 °C. scFvs were purified via immobilized metal ion affinity chromatography (Qiagen, Hilden, Germany). Purified protein was separated on SDS-PAGE (Surendran *et al*., 2015).

Homology modeling and molecular docking

I-TASSER server service (https://zhanglab .ccmb.med.umich.edu/I-TASSER/) was used to generate 3D models of recombinant pthA and scFvG8. The models were refined by ModRefiner (http://zhanglab.ccmb.med.umich. edu/ModRefiner/). HEX (version 6.0, http://hex.loria.fr/) was used to investigate the mode of interaction between the recombinant pthA and scFvG8 refined models. The possible interactions of the structural models were analyzed and visualized using Pymol software version 1.5.0.1 (http://pymol.findmysoft.com). The final model was selected based on the largest cluster size and minimal local energy.

Results

Screening of the library by affinity selection

Tomlinson I scFv library was used for 3 rounds of biopanning with 10^{13} cfu of phages per round. The panning process was performed by coating of an immunotube with the recombinant pthA protein. After each round of panning, the ability of eluted phages to detect antigen were tested by indirect ELISA. Eluted phages with affinity towards pthA were increased in following rounds of panning, while BSA remained unchanged (Supplementary Fig. 2). Binding of monoclonal phages to recombinant pthA protein was about 50% (Fig. 1A).

Isolation of pthA-binding scFv fragments

Eluted monoclonal phages were used to infect HB2151. Binding activities of the phages were checked against pthA by ELISA that demonstrated 25% positive reaction. ELISA values at 405 nm were at least twice greater than that of the negative control (Fig. 1B). Among the positive clones, 5 samples, A12, B8, C1, H8 and G8, had the highest signals. Two clones C8 and D5 with smaller absorption were considered as negative control.

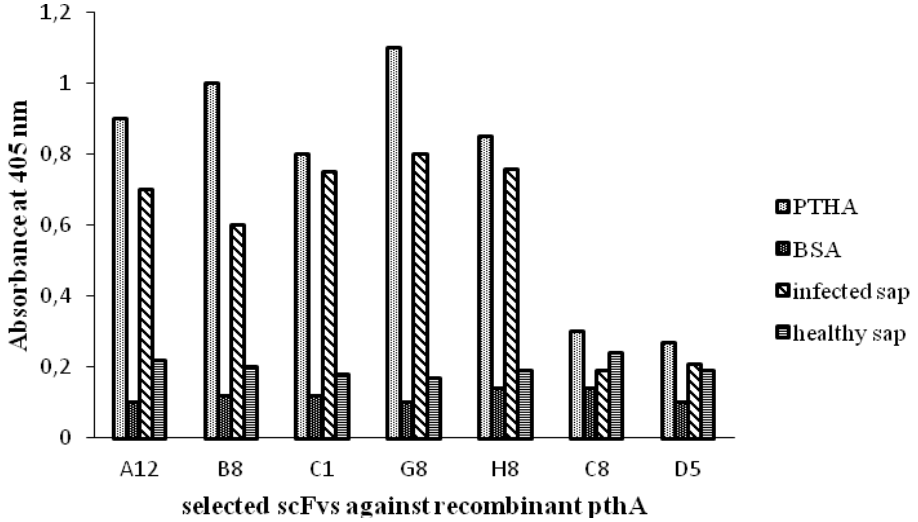

Figure 2 Detection of *Xcc*-infected plant samples via indirect ELISA with scFvs antibodies (G8, B8, A12, C8 and D5) developed against pthA determined by absorbance at 405 nm. Bovine serum albumin (BSA), purified recombinant pthA, proteins from infected and healthy lime plants were used as the samples. C8 and D5 were used as the negative control.

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Detection of *Xcc*-infected plant samples with pthA-specific antibody was checked using indirect ELISA. The results demonstrated that the 5 antibodies strongly reacted with the pthA in infected samples. However, no significant reaction was detected in extracts from the healthy plants as negative control (Fig. 2).

Immunoblot analysis

Western analysis was carried out to assess the specificity of the scFvs antibody against pthA. The results demonstrated that all 5 scFvs strongly reacted with recombinant pthA and revealed a single protein band at the expected size of 30 kDa; indicating a specific recognition (Supplementary Fig. 3).

Characterization and purification of monoclonal pthA-binding scFv

The variations within the scFv encoding sequences were revealed using fingerprint analysis. The restriction enzyme analysis was performed using *Bst*NI. The results showed a similar restriction pattern for all scFv fragments (data not shown). The isolated plasmids from positive clones were used for sequence analysis using specific primers for pHEN vector. The results indicated that all the positive clones selected in monoclonal ELISA contained the same sequence, and therefore named pthA-scFvG8.

The pthA-scFvG8 was purified by IMAC and separated on SDS-PAGE. Proteins were transferred to PVDF and revealed that the molecular weight of scFv is ~30 kDa (Supplementary Fig. 4).

Sequencing analysis of scFv sequences

Plasmid extraction carried out from each colony with positive result in ELISA experiment and sequence analyses were performed by pHEN specific primers. Sequences were translated and aligned together and with the available sequences in IMGT database (http://www.imgt.org). The amino acid sequences of scFvG8 with the complementarity determining regions (CDRs) and framework regions (FRs) are shown in Fig. 3.

Homology model building and molecular docking

The estimated accuracy of the modeled pthA and scFvG8 are shown in Supplementary Table 1. The structure with lesser energy was chosen for further analysis (Fig. 4A). Quality evaluation of the modeled structure of scFvG8 was performed using Ramachandran plot; 91.7 of the residues were placed into the combination of favored and allowed categories (Fig. 4B). These results showed that the scFv model was suitable for the molecular docking study.

Figure 3 Deduced amino acid sequence of scFvG8 antibody. VH and VL domains are linked with a linker; the CDRs of the scFv, His-tag and c-Myc tag sections are indicated.

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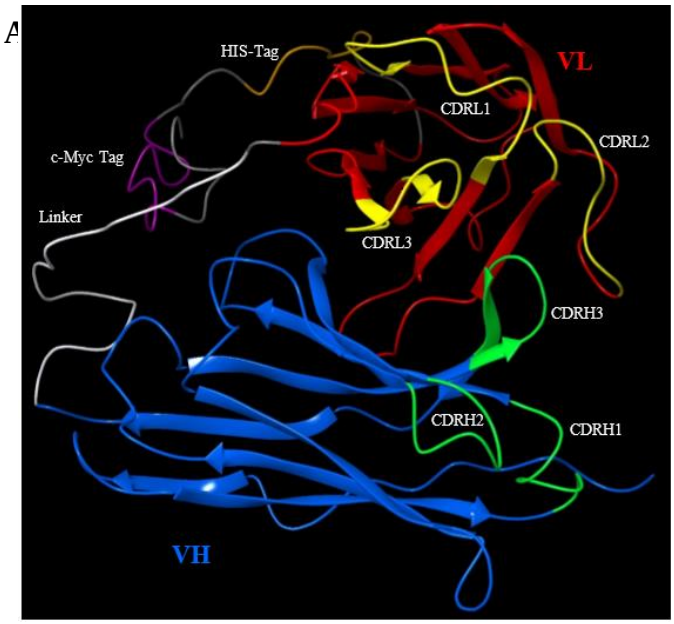

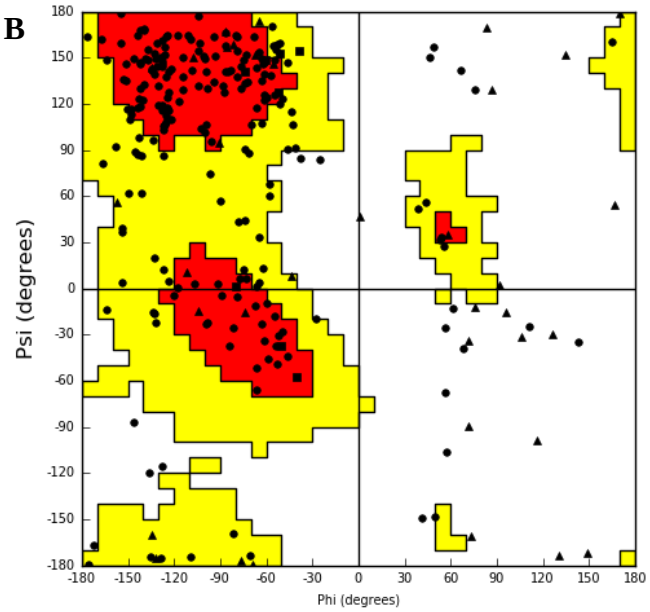

Figure 4 Three-dimensional homology model of scFv. (A) Three dimensional model of scFvG8. (B) Ramachandran plot of the scFvG8 model.

Table 1 Estimated accuracy of the pthA and anti-pthA scFvG8 models.

Protein Name	C-Score	TM-Score	RMSD (Å)
anti-pthA scFv	0.13	0.73 ± 0.11	5.7 ± 3.6
pthA	-3.06	0.37 ± 0.13	13.0 ± 4.2

Hex was used to visually evaluate the interaction of the scFvG8 with pthA that allows highly efficient modeling of full peptide flexibility and significant flexibility of a protein receptor. The pthA and BSA (as negative control, PDB: 4f5s) 3D models were

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docked onto scFvG8 model. In the case of pthA-scFvG8, the suggested model with the lowest energy docking (among 30 models) was selected (Fig. 5A). According to the docking outputs, the binding energy of scFvG8 was estimated −784 kcal/mol when bounded to pthA. This is approximately 2-fold stronger than that of BSA-scFvG8 (−419 kcal/mol). These results are in agreement with the ELISA experiments.

Fig. 5B provides details of the interaction between the two partners, which reveal the binding pthA protein to the gap between VL and VH domains of scFv. Furthermore, the hydrogen bond interaction analyses revealed that the complex was stabilized by 11 van der Waals (Ala 103, Tyr 166, Ala 59, Tyr 183, Ala 55, Tyr 52, Asp 101, Ala 55, Ser 225, Tyr 166, Cys 102) and two intermolecular hydrogen bonds (Ala 103 and Ser 56) (Fig. 5B, Supplementary Table 2).

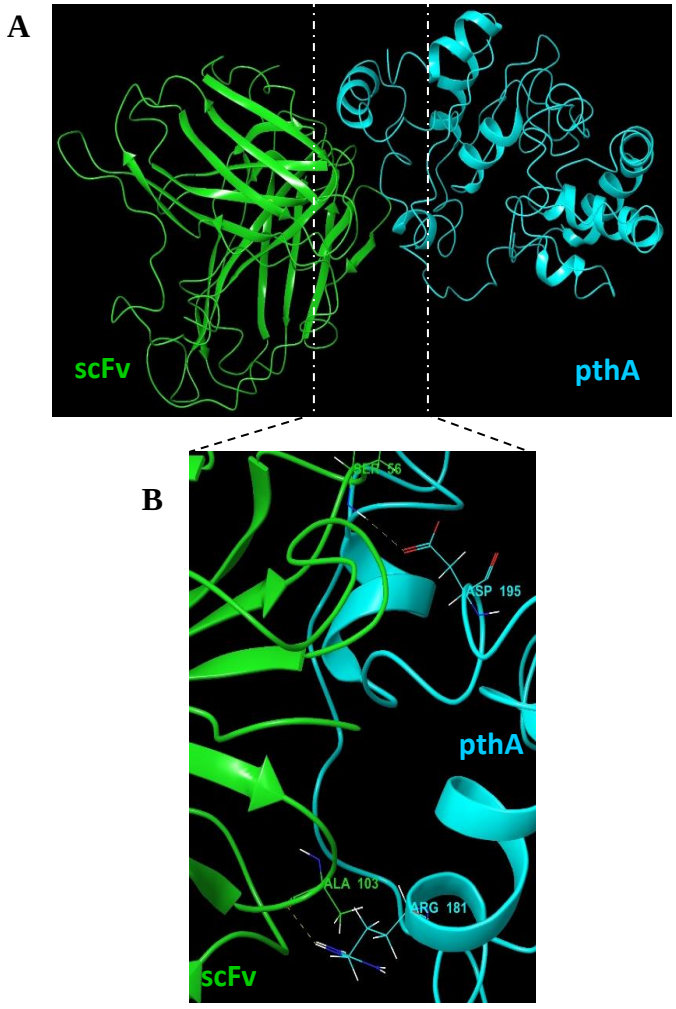

Figure 5 Three-dimensional binding of scFvgG8 to pthA. (A) 3D binding poses of pthA (blue) and scFv (green). (B) Intermolecular interaction analyses of the scFv with pthA. The hydrogen bonds involved in the interactions with the scFv are shown.

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Table 2 Residues of the pthA bound to the residues of the anti- pthA scFv. Amino acid residues involved in the hydrogen bonds with the scFv are presented.

Anti pthA amino acid	pthA amino acid
103: Ala	181: Arg
56: Ser	195: Asp

Discussion

The effector protein, pthA, through type III secretion system (T3SS) are being injected into the host cell cytoplasm, that later is transported into the nucleus with the help of importins α and β to cause disease (Dalio *et al.*, 2017; Gochez *et al.*, 2018). Therefore, if pthA as a prompt candidate could be suppressed, then the disease onset is expected to be hampered as we have seen in our study (Cervera *et al*., 2010). Here, we prepared a monoclonal antibody via phage display technology against pthA recombinant protein. The use of phage-display technology allows the isolation of peptides such as Fab and scFv with specific binding characteristics from a library of random short amino acid sequences (McCafferty *et al.*, 1990). A scFv contains six specific zones, which are knowns as complementary determining regions (CDR): CDR L1-L3, and CDR H1-H3 (Elgert, 2009). It is also known that all six CDRs of the antibody may interact with the antigen (Kunik *et al.*, 2012).

Here, Tomlinson I naïve scFv phage libraries were applied for selection of specific monoclonal antibody against pthA protein of *Xcc*. The results of three rounds of biopanning of library demonstrated a very strong enrichment for isolated phage against pthA. RFLP showed a similar restriction pattern for all scFv fragments, and therefore the monoclonal antibody of all 5 samples were renamed to pthA-scFG8.

The IMGT/V-QUEST analysis of V_H and V_L chains of scFvG8 antibody are shown in Figure 3. A total of six CDRs (three in each chain) are identified. I-TASSER server service (Zhang, 2008) was used to generate 3D models of recombinant pthA and scFvG8. The accuracy of the 3D models of recombinant pthA and scFvG8 was evaluated by the Ramachandran plot analysis method, which showed 91.7 of the residues were placed into the combination of favored and allowed categories, demonstrating that the bond lengths, bond angles, and dihedral angles of the entire molecule are reasonable. Furthermore, docking results of scFv-pthA showed that the binding domain was mainly formed by two intermolecular hydrogen bonds.

The study demonstrates for the first time that phage display can be used to generate antibody fragments that specifically recognize *Xcc*-infected plant. This research could be the basis for the development of plantibodies against *Xcc*.

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تولید و بررسی مولکولی آنتی‌بادی منوکلونال نوترکیب scFv اختصاصی pthA باکتری Xanthomonas

حمیده رئیسی[1]، محمدرضا صفرنژاد[2*]، سیدمهدی علوی[3]، ناصر فرخی[4*]، سیدعلی الهی‌نیا[1]، حسین صفرپور[5] و فرشید شریفیان[3]

۱- گروه گیاه‌پزشکی، دانشکده علوم کشاورزی، دانشگاه گیلان، رشت، ایران.
۲- بخش ویروس‌شناسی گیاهی، مؤسسه تحقیقات گیاه‌پزشکی کشور، سازمان تحقیقات آموزش و ترویج کشاورزی ایران، تهران، ایران.
۳- بخش بیوتکنولوژی گیاهی، پژوهشگاه ملی مهندسی ژنتیک و زیست فناوری، تهران، ایران.
۴- گروه علوم و زیست فناوری گیاهی، دانشکده علوم و فناوری زیستی، دانشگاه شهید بهشتی، تهران، ایران.
۵- مرکز تحقیقات سلولی و مولکولی، دانشگاه علوم پزشکی بیرجند، بیرجند، ایران.
پست الکترونیکی نویسندگان مسئول مکاتبه: mrsafarnejad@yahoo.com, n_farrokhi@sbu.ac.ir
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چکیده: بیماری شانکر باکتریایی مرکبات توسط باکتری *Xanthomonas citri* subsp. *citri* (*Xcc*) ایجاد می‌شود از جمله مهم‌ترین بیماری‌های مرکبات در دنیا می‌باشد. پروتئین pthA به‌عنوان پروتئین افکتور، نقش اساسی در فرایند بیماری‌زایی باکتری در گیاه دارد و لذا غیرفعال‌سازی آن با استفاده از آنتی‌بادی‌های نوترکیب می‌تواند منجر به ایجاد مقاومت علیه بیماری شود. در این پژوهش تولید آنتی‌بادی نوترکیب scFv علیه پروتئین pthA باکتری *Xcc* مورد بررسی قرار گرفته است. برای این‌منظور، ابتدا ژن رمزگذاری‌کننده پروتئین pthA به‌صورت نوترکیب در میزبان باکتریایی *Escherichia coli* بیان شد و به‌عنوان آنتی‌ژن برای غربالگری کتابخانه‌های نمایش فاژی Tomlinson و جداسازی قطعات ژنی نواحی متغیر آنتی‌بادی اختصاصی (scFv) استفاده گردید. در هر دور از غربالگری، اختصاصیت فاژهای حاصله علیه pthA توسط آزمون الیزا (ELISA) مورد بررسی قرار گرفت. نتایج حاصله نشان‌دهنده قابلیت اتصال حدود ٪۵۰ از فاژهای مونوکلونال به پروتئین pthA می‌باشد. از بین فاژهای مونوکلونال جداسازی شده، پنج نمونه (A12، B8، C1، G8 و H8) قادر به ردیابی گیاهان آلوده به *Xcc* و پروتئین نوترکیب pthA بودند. بررسی پلی‌مورفیسم طول قطعات آنتی‌بادی به‌وسیله آنزیم برشی، الگوی باندی مشابه برای هر پنج scFv نشان داد. تولید آنتی‌بادی در سویه HB2151 باکتری *E. coli* توسط فاژمید حاوی ژن pthA-scFG8 صورت پذیرفت. بیان ژن در باکتری توسط IPTG القا شده و پروتئین نوترکیب با وزن ملکولی در حدود ۳۰ کیلو دالتون تولید گردید. پیش‌بینی ساختار سه‌بُعدی scFv و pthA با روش مدل‌سازی همسانی (Homology modeling) در I-TASSER صورت پذیرفت و داکینگ بین scFv و pthA توسط برنامه Hex انجام شد که نتایج آن نشان‌دهنده انرژی اتصال ۷۸۴ kcal/mol بین scFv-pthA بود.

واژگان کلیدی: غربالگری، شانکر باکتریایی مرکبات، نمایش فاژی، قطعات ژنی نواحی متغیر آنتی‌بادی

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