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Screening for peptides binding on *Phytophthora capsici* extracts by phage display

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ABSTRACT

Phytophthora blight of pepper is a completely destructive plant disease caused by the oomycete pathogen *Phytophthora capsici* Leonian. *Phytophthora* disease is responsible for major losses in pepper production and the pathogen can survive in soil in the absence of the host plant for many years. Currently, there are no early diagnostic reagents available that specifically target *P. capsici*. Therefore, diagnostic tools that can detect *Phytophthora* are required. In the present study, we screened for *P. capsici*-binding peptides using M13 phage display. After five rounds of biopanning, we identified *P. capsici*-binding peptides from a random peptide library that showed high binding affinity and specificity toward *P. capsici* in the picomolar range. These peptides can be used to develop novel diagnostic probes or potent inhibitors with diverse polyvalencies.

1. Introduction

Phytophthora capsici is a soil-borne fungal pathogen that significantly hampers agricultural production of certain species of peppers and tomatoes (Biles et al., 1992). *P. capsici* has an extensive host range, including but not limited to eggplant, cucumber, watermelon, pumpkin, squash, cocoa, macadamia plants, and peppers (Zhiwei et al., 2006). *Phytophthora* blight on peppers can manifest as spotting and wilting of the roots, stems, leaves, and fruit (Biles et al., 1993). The pathogen can survive in unfavorable soil conditions by forming thick-walled oospores, whereas dissemination and infection are accomplished through the production of motile zoospores from the oospores. The zoospores swim via water present in the soil and are drawn to exudates, which are released by the roots of the potential host plant (Morris et al., 1998). After the zoospores have adhered to the root surface, they encyst and produce a germ tube precisely oriented so that it grows into adjacent host plant tissue. The progression from zoospores to germlings is triggered by environmental signals, some of which are produced by the plant root. Receptors on the surfaces of the zoospores, cysts, and germ tubes detect the environmental signals that trigger or orient each developmental event.

Because of its highly destructive effect on infected plants, strategies to detect *P. capsici* and prevent infection by this pathogen have been extensively investigated. Although chemical treatments for the control of *Phytophthora* blight are effective, the resulting phytotoxicity and chemical residues pose risks to human health and the environment. Thus, alternative methods to control *Phytophthora* blight disease are required. Although many different biocontrol strains have shown some potential for the management of *P. capsici*, strains which can effectively control *Phytophthora* blight of pepper have yet to be found.

In an effort to identify target-specific peptides that bind to *P. capsici* and can thus serve as novel potent inhibitors or diagnostic probes with diverse polyvalencies, we used phage display.

Phage display of randomized peptide libraries has become one of the most powerful technologies for selecting and engineering peptides that target specific molecules. Since it was first reported (Cwirla et al., 1990; Scott and Smith, 1990), phage display technology has become a standard method for selecting peptides with affinities for small molecules (Saggio and Laufer, 1993), receptors (Balass et al., 1993), viruses (Gough et al., 1999), and whole cells (Fong et al., 1994). This technique is used extensively in various pharmaceutical biotechnologies (Sidhu, 2000). Highly diverse libraries can be constructed by fusing degenerate DNA fragments to coat protein genes. Furthermore, library members with particular functions can be isolated when they are encapsulated into the phage particles that display the sequences of selected polypeptides on their surfaces. The sequences of library members with desired binding specificities can be isolated by binding to an immobilized receptor *in vitro* (Kim et al., 2006).

In this study, we identified diverse peptides that bound to *P. capsici* extracts with a high affinity (picomolar range) from an M13 phage peptide library. In addition, these peptides showed little cross-reactivity toward similar *Phytophthora* strains.

2. Materials and methods

2.1. Materials

All *Phytophthora* strains used in this study, including *P. capsici*, were provided by the Korean Agricultural Culture Collection (KACC). The fungi culture media, V8 vegetable juice agar, was purchased from

Abbreviations: *P. capsici*, *Phytophthora capsici* Leonian from pepper (*Capsicum annuum*); *P. capsici*-protein, total cell lysate of *P. capsici* containing soluble protein; PAGE, polyacrylamide gel electrophoresis; ELISA, Enzyme-Linked Immuno-Sorbent Assay.

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Table 1
Isolates of *Phytophthora* used in this study.

Species	Isolate	Source	Characteristics	KACC No.
Phytophthora capsici	P109	Crown of *Capsicum annuum* (red pepper)	*Phytophthora* blight	40483
Phytophthora drechsleri	P9615	*Lycopersicon esculentum* (tomato)	*Phytophthora* root rot	40190
Phytophthora cactorum	Pb36	*Malus pumila var. dulcissima* (apple)	*Phytophthora* stem rot	40166
Phytophthora cactorum	P9781	Fruit of *Prunus persica var. vulgaris* (Peach)	*Phytophthora* fruit rot	40175
Phytophthora cinnamomi	P9815	*Fragaria X ananassa* (strawberry)	*Phytophthora crown rot*	40183
Phytophthora cinnamomi	P9796	*Larix leptolepis* (larch)	Root rot, blight	40182

KACC: Korea Agricultural Culture Collection.

Campbell Soup. The M13 peptide library screening kit (Ph.D. Phage Display Peptide Library kit) was obtained from New England Biolabs. The other chemicals used here, including lysozyme, were purchased from Sigma.

2.2. Preparation of total cell lysates from P. capsici

To screen *P. capsici* (KACC No. 40483 in Table 1) binding peptides, extracts were conducted in several regions of Milyang and Goryeong (Korea). Isolates were collected from pepper plants showing root, stem, leaf, and fruit blight. Preparation of total cell extracts from *P. capsici* was performed as described elsewhere (Jasmine et al., 2003). Briefly, cell pellets were harvested after sonication and dissolved in lysis buffer (20 mM Tris–HCl pH 8.0, 1 mM EDTA, 2% SDS, 10 mM DTT, 5 mM β-ME, 1% Triton X-100) and soluble extracts were collected. Collected pellets were incubated in a resuspension buffer (50 mM Tris–HCl pH 8.0, 1 mM PMSF, 2 mM EDTA, 100 mM DTT, 8 M Urea). The soluble extracts were collected and total proteins were analyzed by 12% SDS–PAGE (polyacrylamide gel electrophoresis).

2.3. Peptide library screening

The Ph.D.-12 Phage Display Peptide Library with 2.7×10^9 peptide variants was used for biopanning. *P. capsici* extract was immobilized onto a polystyrene plate (SPL) via hydrophobic interactions, and wells were then covered with 5% skim-milk (5% skim-milk, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. The wells were washed three times with wash buffer A (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) and used for biopanning. A specified amount of phage was added to the wells and incubated for 1 h at room temperature with gentle shaking. Wells were washed 10 times with buffer A and bound phages were eluted in a chemical elution buffer B (0.2 M glycine-HCl pH 2.2, 1 mg/ml BSA). The eluates were then neutralized with buffer C (1 M Tris–HCl pH 9.1). In subsequent rounds of biopanning (from 2nd to 5th rounds), the concentration of detergent or salt in wash buffer A was increased as follows: 0.3% Tween-20 (second round), 0.5% Tween-20 (third round) and 0.5% Tween-20 plus 500 mM NaCl (fourth and fifth rounds). At the final round, bound phages were eluted by addition of 10 M excess of *P. capsici* extract. Eluted phages at each round were infected into *E. coli* ER2738 cells for amplifcation. After being propagated for 5 h, the bacterial cells were removed by centrifugation at 10,000 rpm for 15 min. The phages were partially purified by precipitation of the culture supernatant with polyethylene glycol (20% (w/v) polyethylene glycol-8000 and 2.5 M NaCl) according to the manufacturer's protocol. After centrifugation at 10,000 rpm for 30 min, phage pellets were resuspended in TBS buffer (50 mM Tris–HCl pH 7.5 and 150 mM NaCl) and used for the next round of biopanning.

2.4. DNA sequencing

The diversity of *P. capsici*-specific peptides was assessed by sequencing the region of the phage genome that codes for the inserted 12-mer peptide and translating to the amino acid sequence. Single-stranded DNA from phages was recovered as follows. An overnight culture of *E. coli* ER2738 was diluted 1:100 in LB (Luria-Bertani) medium and aliquoted into 1-ml volumes, into which an individual phage displaying a *P. capsici*-binding peptide was added. Cells were incubated at 37 °C with shaking (200 rpm) for 5 h and then the supernatant containing the phages was harvested by centrifugation. Two hundred microliters of PEG/NaCl (20% (w/v) polyethylene glycol-8000 and 2.5 M NaCl) solution was added to precipitate the phages, and the resulting pellet was suspended in iodide buffer (containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 4 M NaI) followed by alcohol precipitation. Single-stranded phage DNA was recovered and dissolved in dH_2O. The amino acid sequence of the peptides was deduced from the genetic code information supplied by New England Biolabs.

2.5. Estimation of binding affinities on ELISA

Phages displaying *P. capsici*-binding peptides were prepared and their concentration was determined by measurement of plaque forming numbers per unit volume. To estimate the binding affinities of peptides, *P. capsici* extracts (0.5 μg/well) were placed in individual wells to which the indicated concentrations of phages harboring binding peptide were added and incubated for 30 min at room temperature. The wells were then washed ten times with TBST buffer and probed with an anti-M13 antibody (mouse monoclonal antibody, 1:5000 in TBST, 0.2 μg/ml, Amersham Bioscience) for 1 h at room temperature. After washing with TBST buffer five times, anti-mouse IgG-HRP conjugate was added and incubated for 1 h, followed by a color-developing reaction with TMB substrate solution (3, 3′, 5, 5′-tetramethylbenzidine (TMB)/H_2O_2, Chemicon). After 15 min, the reaction was terminated by the addition of 1 M H_2SO_4 and the optical densities were measured with a Biotrak multi-well plate reader (Amersham Bioscience) at 450 nm.

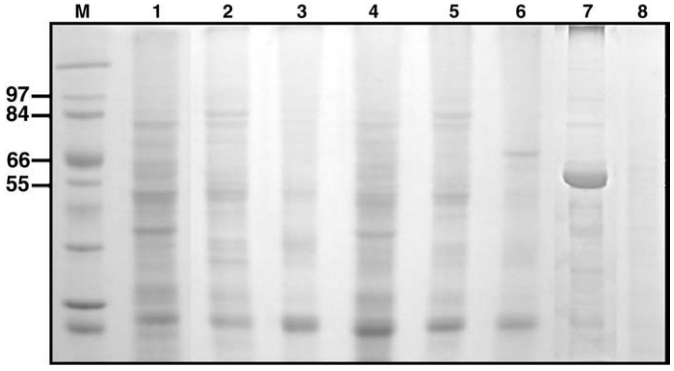

Fig. 1. PAGE analysis of total cell extracts of *P. capsici* and similar *Phytophthora* strains. The extraction process for several similar *Phytophthora* strains and the host plant (pepper) is as described in Materials and methods. The resulting supernatants, containing the soluble target proteins (lanes 1–7), were collected and resolved on 12% SDS–PAGE. M, molecular weight marker; 1, 40483 (*P. capsici*, crown of *Capsicum annuum*, red pepper); 2, 40190 (*Phytophthora drechsleri*, *Lycopersicon esculentum*, tomato); 3, 40166 (*Phytophthora cactorum*, *Malus pumila* var. *dulcissima*, apple); 4, 40175 (*P. cactorum*, fruit of *Prunus persica* var. *vulgaris*, peach); 5, 40183 (*Phytophthora cinnamomi*, *Fragaria X ananassa*, strawberry); 6, 40182 (*P. cinnamomi*, *Larix leptolepis*, larch); 7, extracted protein of normal pepper leaf; 8, inclusion body for the insoluble fraction of 40483, which was dissolved in 8 M urea.

3. Results

3.1. Extraction of total cell lysates form of P. capsici, similar Phytophthora strains and host plant

To screen for *P.capsici*-binding peptides, we first extracted total soluble target proteins from plant cells using liquid nitrogen. Cell pellets were harvested after sonication and dissolved in lysis buffer (20 mM Tris–HCl pH8.0, 1 mM EDTA, 2% SDS, 10 mM DTT, 5 mM β-ME, 1% Triton X-100). For cell lysis, the resuspension was incubated with lysozyme (0.5 mg/ml) and 1 mM PMSF. After 30 min incubation, the cell pellets were removed by centrifugation at 14,000 rpm for 15 min and the supernatant collected. Any remaining particles were removed by filtration (0.45 μm syringe filter). A Bradford assay was used to determine the concentration of recovered protein (595 nm). The soluble extracts were collected and the total protein was analyzed by 12% SDS–PAGE (Fig. 1).

3.2. Peptide library screening

To screen for peptides that specifically bound to *P. capsici* extract, we used a M13 phage display library. We selected for phages displaying *P. capsici*-specific peptides using the two-step screening strategy shown in Fig. 2. We first immobilized *P. capsici* extracts onto a polystyrene plate (SPL) and added the M13 phage library displaying random 12-residue peptides fused to the N-terminus of the gpIII protein. After incubation, we washed the wells with increasing stringency and then eluted the phages by addition of 0.2 M glycine-HCl pH 2.2. At the final biopanning round, soluble *P. capsici* extract was added and adsorbed phages were recovered. After five cycles of biopanning, we selected 50 plaques and determined the DNA sequences of the displayed peptides. Based on the amino acid sequences deduced by the DNA sequencing, the phages could be categorized into five groups according to their amino acid sequence similarities (Table 2).

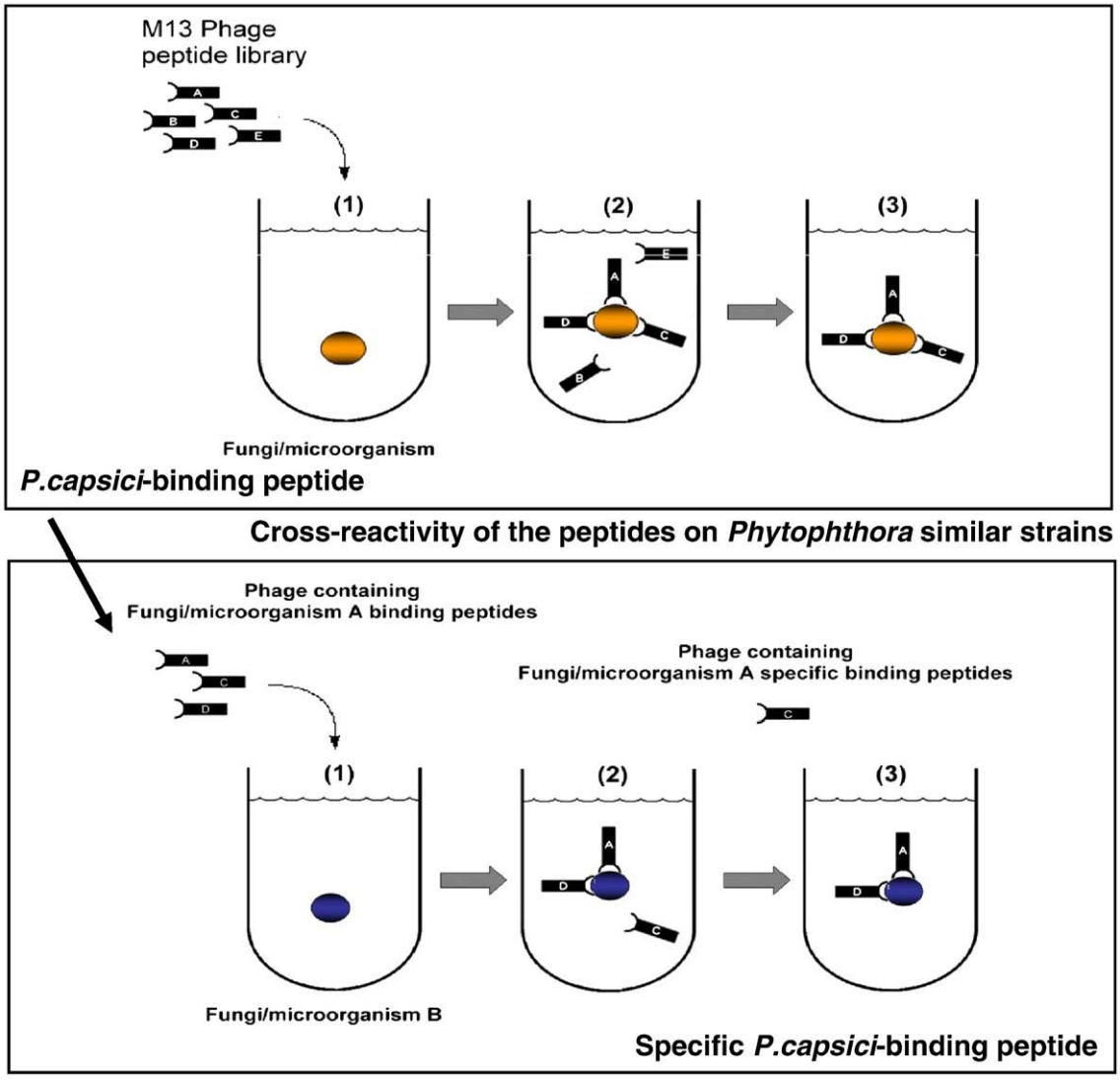

Fig. 2. Schematic diagram of the phage display screen for *P. capsici*-binding peptides. We designed a two-step screening strategy to identify peptides with high affinity and specificity for *P. capsici* extract. First, we screened a family of phage displaying peptides binding *P. capsici* with high affinity. *P. capsici* extract was prepared and placed onto a plate (polystyrene, SPL) and the phage library was added. After five rounds of biopanning, the phages displaying high affinity *P. capsici*-binding peptides were collected and analyzed to determine the amino acid sequences corresponding to the peptides. In the second screening step, we isolated *P. capsici*-specific peptides by a cross-reactivity test. Extracts of several similar *P. capsici* strains and the host pepper plant were prepared and immobilized on a plate, to which phages harboring *P. capsici*-binding peptides from the first screening step were added. After binding, the supernatant containing phages that did not bind to either similar *P. capsici* strains or host plant extracts were harvested and analyzed.

Table 2
P. capsici-binding peptides displayed on M13 phages.

Number	Occurrences[a]	Peptide sequences[b]	Binding affinities[c] (K_d^{app}, pM)
P1	20	**SVSVGMLPSPRP**	24.07 ± 3.40
P2	7	**TIQHQNPPHYAV**	2.76 ± 0.20
P3	8	**GSFASLTNPRVL**	15.13 ± 1.36
P4	13	**SSWILSPYHWGR**	34.59 ± 8.20
P5	2	**SNPHTDNHWPGR**	16.18 ± 1.59

[a] After five rounds of biopanning, 50 plaques were selected and the sequences of the displayed peptides fused to the M13 minor coat protein gpIII were analyzed. Each phage was classified according to the deduced amino acid sequence of the peptide using the BioEdit program. The number of phages in the same group is shown.

[b] Amino acid sequences of peptides were deduced from genetic code information provided by the manufacturer as the library encodes all 20 amino acids with only 32 codons. The third (wobble) position of the codon is G or T. This codon strategy increases the relative frequency of residues coded by a single codon and removes 2 (TAA and TAG) of the 3 stop codons. The amber stop codon TAG is suppressed by glutamine (Q). Each amino acid is shown as a single-letter representation.

[c] Binding affinity was determined as described in Materials and methods. Total cell lysates of *P. capsici* (0.5 μg) were immobilized onto individual wells of a polystyrene plate in random orientation and each phage displaying a peptide was added in a concentration-dependent manner. The amount of bound phage on *P. capsici* extract was estimated by an anti-M13 antibody, which specifically recognizes the major coat protein (gpVIII) of M13 phage. The K_d^{app} value (apparent dissociation constant) was obtained from a binding saturation curve fitted from three independent experiments.

3.3. Estimation of binding affinities

The selected phages displaying *P. capsici*-binding peptides were further characterized to estimate their binding affinities for *P. capsici*. The phages were first amplified and purified. The concentration of each phage was determined by titering serial dilutions of the purified phage solution. To determine binding affinities, an enzyme-linked immunosorbent assay was performed. *P. capsici* extracts were immobilized onto a plate and then each phage was added in a concentration-dependent manner. After 1 h of incubation at room temperature, the wells were extensively washed 10 times with W1 containing 0.5% Tween-20 and 0.5 M NaCl followed by addition of anti-M13 antibody (0.2 μg/ml in TBST), which specifically recognizes the M13 coat protein product of gene VIII (gp8). After a color development reaction with HRP conjugate, the optical density was measured at 450 nm. As shown in Fig. 3, all of the phages showed comparable binding affinities for *P. capsici* in the picomolar (10^{-12}) range. The binding affinity of each peptide displayed on an M13 phage is summarized in Table 2.

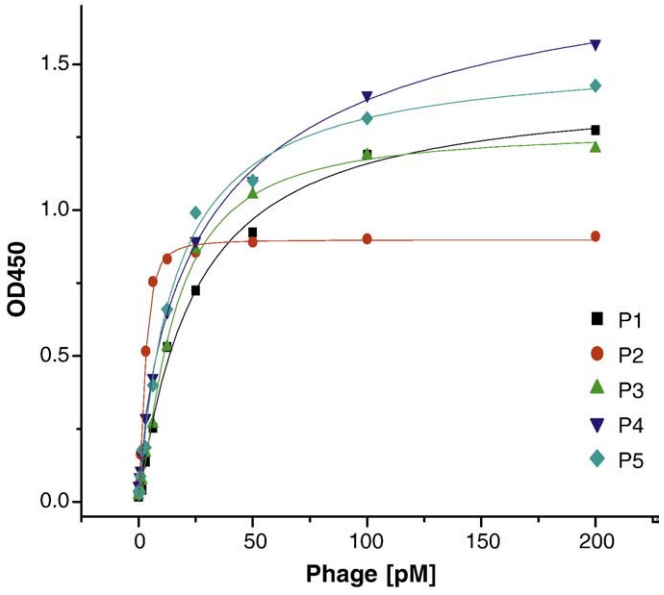

Fig. 3. Determination of binding affinities. The binding affinity of each phage displaying the indicated peptide for *P. capsici* extract was estimated by measuring the binding constant of the phage as described in Materials and methods. The concentration of amplified phage solution was calculated as follows: Concentration = (the total number of phage in solution/6.02×10^{23})/suspension volume. The total number of phages was determined by counting the number of plaques of each phage from titer plates. The data is representative of one of three independent experiments and the binding affinity, which was measured as an apparent K_d, was obtained from non-linear fitting of the saturation binding curve.

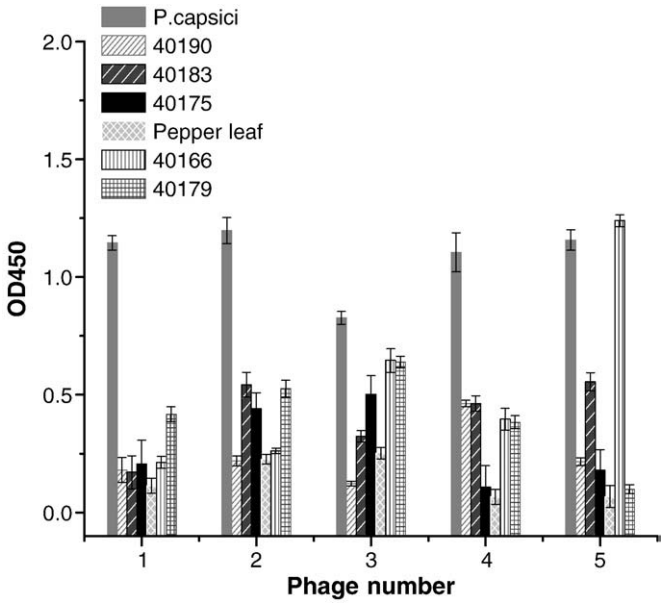

Fig. 4. Cross-reactivity of the *P. capsici*-binding peptides with similar *Phytophthora* strains. The binding specificity of the phages for similar *Phytophthora* strains was determined as illustrated in Fig. 2. The binding of individual phages to similar *Phytophthora* strains was estimated by ELISA using an anti-M13 antibody. The representative data are the average of two independent experiments. Total cell extracts of *P. capsici* and similar *Phytophthora* strains for experiments were obtained as described in Fig. 1 (lanes 2–7).

3.4. Determination of cross-reactivity with Phytophthora similar strains

Next, we determined the cross-reactivity of our peptide selections with several other similar *Phytophthora* strains. Similar *Phytophthora* strains used for cross-reactivity tests are listed in Table 1. We first obtained total soluble protein as described in Materials and methods. After extraction, the *P. capsici*, similar *Phytophthora* strains, and pepper extracts were immobilized onto a polystyrene plate (SPL) and then selected phages (P1–P5) were added. Binding was determined by ELISA as described above. As shown in Fig. 4, phages P1, P2, and P4 showed high specificity for *P. capsici*, whereas P3 and P5 bound to some of the similar *Phytophthora* strains with comparable affinities. Furthermore, it is worth noting that none of the peptides identified in this study recognized the target plant (pepper), suggesting that the peptides are highly specific to the pathogen *Phytophthora*. These results indicate that these peptides are potential novel diagnostic probes.

4. Discussion

Phytophthora blight has caused dramatic sudden pepper death in Europe, North America and in particular, South Korea (Julien et al., 2007). Within the past few years, South Korea has experienced a steady increase in the incidence of root, fruit, and crown rot on peppers caused by *P. capsici* (Chung et al., 2008). Currently, there are few therapeutic agents available to specifically target *P. capsici*. Historically, *Phytophthora* infection has been controlled biocidally by fumigation with methyl bromide or treatment with metalaxyl (Bishop-Hurley et al., 2002). However, this strategy has significant disadvantages, including the risk of occupational exposure, as well as the concomitant loss of microbial diversity and soil tilth. An alternative, environmentally benign strategy

involves the genetic engineering of plants to be less susceptible to infection. Therefore, the isolation of biomaterial peptides is an attractive approach for plant protection and therapeutics.

Peptides have several advantages over antimicrobial molecules for therapeutic and diagnostic purposes. Because the peptides are 10–50 amino acids in length, they can interact with their protein targets in a highly specific manner, more so than small molecules. Furthermore, the biological efficacy of peptides is an advantage when considering these molecules as diagnostic probes for the early detection and treatment of infected plants. Toward this goal, it is essential to identify peptides with diverse binding capabilities and high specific binding affinities for target molecules, which can increase the effectiveness of the diagnostic probes.

In this study, using M13 phage display, we found novel peptides that bind to *P. capsici* extracts. Phage display was first developed with the *E. coli*-specific bacteriophage M13 and was widely used to identify peptides that interact with specific targets (Smith, 1985). The M13 phage particle consists of a single-stranded DNA core surrounded by five different coat proteins. The particle body is covered by several thousand copies of the major coat protein, gpVIII. One end of the particle is capped by five copies of minor coat proteins gpIII and gpVI, while the other end is capped by five copies of two other minor coat proteins, gpVII and gpIX (Marvin, 1998). The peptide library used in this study consisted of DNA fragments fused to the gpIII minor coat protein of M13 phage. We screened for *P.capsici*-specific peptides from this library with a complexity of 2.7×10^9 complexity, and we successfully isolated five representative peptides with high affinity (picomolar range) and specificity (Table 2). The *P. capsici* binding-peptides may be recognizing targets that are found in many *Phytophthora* strains. Moreover, the *Phytophthora* pathogen infects plants, so it is possible that the targets recognized by the peptides are plant-based.

Therefore, the selected phages displaying *P.capsici*-binding peptides were further characterized to estimate their binding affinities for *P. capsici* extract. We determined the cross-reactivities of the identified peptides (P1–5) for several similar *Phytophthora* and host plant (pepper) extracts. Fig. 4 showed that phages carrying these peptides had little reactivity toward similar *Phytophthora* strains or host plant extracts. More importantly, three peptides (P1, P2, and P4) showed highly specific binding on *P. capsici*. These three peptides did not cross-react with any of the similar *Phytophthora* strains or the host plant.

The high specificity and affinity of the peptides indicate that they are potential novel diagnostic probes. The chemical and physical stability of peptides makes them particularly suitable for to the clinical and engineering fields. Our study establishes a foundation upon which novel potent diagnostic probes or inhibitors with increased polyvalencies can be constructed to combat *Phytophthora* blight.

Acknowledgments

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