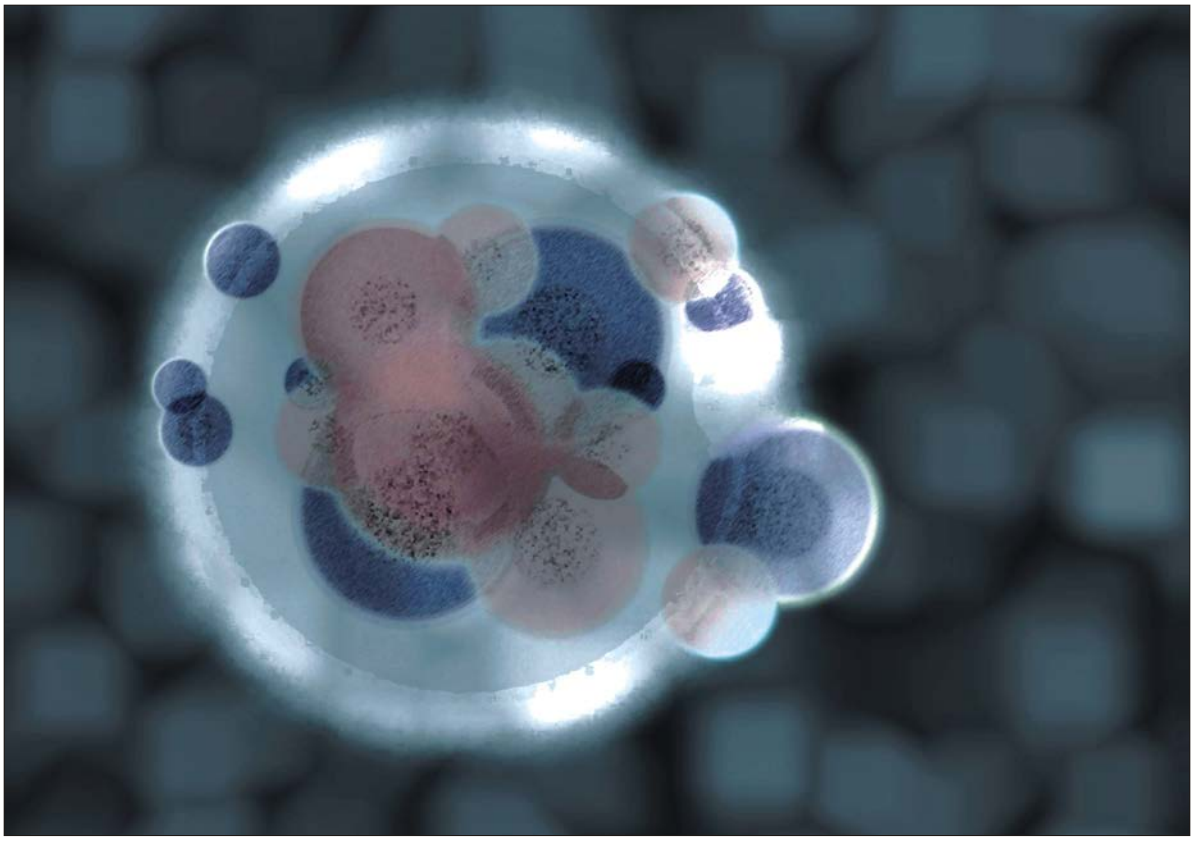

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Biopanning for *Banana streak virus* Binding Peptide by Phage Display Peptide Library

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Abstract: *Banana streak virus* (BSV) is a plant virus that can potentially cause serious problems for banana cultivation, compounded by difficulties in properly diagnosing the infection and identifying the virus itself. In this study, we identified a peptide that can bind to BSV and potentially be developed as a cost effective detection reagent. To select a binding peptide against BSV, biopanning by way of utilising the phage display peptide library was the method used. Three rounds of biopanning successfully yielded a peptide, VVVGSLVVARLR., that bound selectively to BSV.

Key words: *Banana streak virus*, biopanning, peptide, phage display

INTRODUCTION

Banana streak virus (BSV) was first described by Lockhart (1986) in *Musa* sp. as the agent that causes chlorotic leaf streak and necrosis. The virus belongs to the family *Caulimoviridae*, genus *Badnavirus*. BSV has baciliform-shaped particles averaging 30×150 nm in size and a circular dsDNA genome of 7.4 kb (Harper and Hull, 1998). The virus is spread by mealybugs, vegetative propagation and through seeds. Serious problems are encountered in banana cultivation as a result of BSV infection, restricting plant breeding and movement of germplasm and adversely affecting fruit yield and quality (Daniells *et al*., 2001; Strange and Scott, 2005). Severity of banana streak disease is variable, possibly depending on environmental conditions, host and virus genotypes, or stress from other sources (Geering *et al*., 2001; Geering *et al*., 2005; Agindotan *et al*., 2006). It was suggested that BSV infection may have a crucial effect on banana bunch size and fruit quality when floral initiation and early bunch development coincide with a period of increased virus synthesis (Lockhart and Jones, 1999).

Several factors arising from the nature of banana streak disease and the causal agent itself complicate the diagnosis of BSV in banana (Geeing *et al*., 2000; Fargette *et al*., 2006). The sporadic nature of symptom expression throughout the year means diagnosis based on foliar symptom is unreliable (Jones and Lockhart, 1993). Under certain conditions, infected plants may not even show symptoms, or the symptoms may be distinct and plants derived from *in vitro* multiplication usually lack symptom expression. Biological indexing using indicator plants would not work either, because BSV infects only *Musa* sp. and *Ensete* sp. and is not transmissible to these species by mechanical inoculation.

Phage display refers to a powerful selection technique that is used to study peptides or proteins with biologically active binding properties. This technique is usually carried out with a phage display library made up of many recombinant phages, each displaying a different peptide or protein. The peptide or protein is expressed as a fusion with coat protein on the surface of filamentous bacteriophage. The genetic information that encodes the displayed protein is physically linked to its product via the displaying particle (Smith *et al*., 1998; Hoogenboom, 2002). Display libraries with a repertoire of billions of unique displayed proteins are produced by cloning large numbers of DNA sequences into the phage. Proteins or peptides that interact with the desired target such as antibodies, enzymes, DNA-binding proteins and cell-surface receptors can then be synthesized and selected with phage display (Benhar, 2001; Villani *et al*., 2005). A technique named biopanning is then used to screen for the desired epitope. Biopanning involves affinity screening with a phage library against immobilized proteins of interest, specifically bound phages that are recovered and amplified by infection into *Escherichia coli* host cells (He *et al*., 1998; Katakura *et al*., 1998; Giordano *et al*., 2001). Overall, the objective of this study is to select the binding peptide against BSV using the biopanning approach and determine the mimic paratope against the BSV coat protein at the same time. Integrity

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and information of the desired binding peptide could then be further determined through ELISA and sequencing.

MATERIALS AND METHODS

All the methods used in this section were described in kit manufacturer instructions (Ph.D-12™ Phage Display Peptide Library Kit, New England BioLabs®, USA).

Biopanning: A coating solution of 100 μg mL^{-1} of BSV (Agdia #C488) in 0.1 M $NaHCO_3$ (pH 8.6) was prepared and 150 μL of this coating material was dispensed into each well of a microtiter plate which was then incubated overnight at 4°C in a humidified container. The following day, *E. coli* ER2738 was inoculated into 20 mL of Luria-Bertani Broth (LB) in an Erlenmeyer flask and incubated at 37°C with vigorous shaking. Simultaneously, the coating solution in the wells of the microtiter plate was poured away and the wells filled with blocking buffer [10% v/v skim milk in Phosphate-Buffered Saline (PBS) (0.8% w/v NaCl, 0.02% w/v KCl, 0.144% w/v Na_2HPO_4, 0.024% w/v KH_2PO_4)] and incubated at 4°C for 1 h. The blocking solution was then discarded and the wells were washed 6 times with phosphate-buffered saline with tween 20 (PBS-T) (PBS, 0.1% (v/v) Tween-20). Ten microliter of the phage library (4×10^{11} pfu) in 100 μL of PBS-T was dispensed into each coated well and rocked gently for 30 min at room temperature. Following that, any nonbinding phage was discarded. The wells were washed 10 times with PBS-T and bound phage was eluted with 100 μL of trypsin, followed by incubation at 37°C for 30 min. The elute was then pipetted into a microfuge tube and stored at 4°C overnight for amplification the following day. A small amount (about 1 μL) of the elute was used for phage titering. Elute containing the selected phage was enriched by inoculating 20 mL of *E. coli* ER 2738 culture and then incubated at 37°C with vigorous shaking for 4 h. The culture was then centrifuged at 13,000 rpm at 4°C for 20 min. The supernatant was transferred to a fresh microfuge tube, to which a 1/6 volume of PEG/NaCl was added. The desired phage was allowed to precipitate at 4°C overnight. The PEG precipitate was then centrifuged at 13,000 rpm at 4°C for 15 min. Supernatant was discarded and the pellet was dissolved in 1 mL of PBS. This suspension was centrifuged at 13,000 rpm for 5 min to pellet residual cells and the supernatant was then re-precipitated with 1/6 volume of PEG/NaCl and incubated on ice for 60 min, followed by centrifugation again at 13,000 rpm for 10 min. After the supernatant was discarded, the pellet containing selected phage was suspended in 200 μL was the amplified elute.

Phage titration: The amplified elute was titered on LB/IPTG/Xgal plates. Phage titers corresponding to blue plaques formed were determined and this was used to obtain an input volume corresponding to at least $1\text{-}2\times10^{11}$ pfu. Then a second round of biopanning was carried out using the first round of amplified elute as the input phage. Amplified elute from the second round then became the input phage for a third round of biopanning.

For phage titration, a single colony of *E. coli*, ER 2738 was inoculated into 10 mL of LB and incubated with shaking at 220 rpm until mid-log phase (OD_{600} = 0.5). Three milliliter of 45°C equilibrated agarose top was dispensed into sterile culture tubes. Ten-fold serial dilutions of phage in LB were prepared. Once the culture reached mid-log phase, 200 μL of the culture was dispensed into microfuge tubes for phage dilution. Ten microliter of each dilution was then aliquoted into separate tubes, mixed and incubated at room temperature for 5 min. One at a time, infected cells were transferred to a culture tube containing agarose warmed to 45°C and immediately poured onto a pre-warmed LB/IPTG/Xgal plate. The agarose top was spread evenly by tilting the plates. After cooling for 5 min, the plates were incubated overnight at 37°C. The following day, plaques on the plates were counted and converted into plaque forming units (pfu) per 10 μL.

Elisa assay for BSV binding: Two wells of a microtiter plate were coated overnight at 4°C with 100 μL of 100 μg mL^{-1} BSV in 0.1 M $NaHCO_3$ (pH 8.6) and 100 μg mL^{-1} Bovine Serum Albumin (BSA) (as negative control) in 0.1 M $NaHCO_3$ (pH 8.6), respectively. On the following day, excess target solution was shaken out and each well was filled with blocking buffer for incubation at 4°C for 1 h. After the blocking buffer was shaken out and the plate washed 6 times with PBST, phages were added to wells that were now coated with BSV and BSA. Incubation was done for 1 h at 4°C. After that, the wells were washed with PBST and 200 μL of diluted HRP-conjugated anti-M13 antibody (1:5000) (Pharmacia) was then added to each well for incubation at room temperature for 1 h. The plate was washed with PBS-T and horseradish peroxidase (HRP) substrate solution (0.02% (w/v) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 50 mM sodium citrate, 30% (v/v) H_2O_2) was added to each well and incubated at room temperature for 10 min. Optical Density (OD) readings were obtained using an ELISA reader (Multiskan MCC/340P) set at 415 nm.

Phagemid DNA extraction and sequencing: After third round of biopaning, 50 μL of phage and 100 μL of the *E. coli* ER2738 were mixed together in a 10 mL LB broth. The cultures were incubated at 37°C, with shaking at

180 rpm for 4 h. Ten-fold serial dilutions of the phage in LB were prepared. Then, 50 µL of the diluted phage was spread onto a LB/IPTG/Xgal plate and incubated at 37°C overnight. Two colonies were then randomly selected from the plate and inoculated into separate 10 mL LB medium with 100 µL of ER2738 added. These cultures were incubated at 37°C for 4 h with shaking at 180 rpm. After that, the cultures were centrifuged at 6,000 rpm for 15 min and the supernatant was discarded. The alkaline lysis method was used for plasmid extraction (Sambrook *et al.*, 1989). Pellets obtained containing phagemid DNA were then dissolved in 50 µL of dH_2O. Finally, the two phagemids isolated from the phage pool were sequenced using the -96 gIII sequencing primer: 5'-CCCTCATAGTTAGCGTAACG-3'.

RESULTS

Biopanning: Figure 1 shows a plate with blue plaques which were recombinant phage from the phage library that carried the *lacZα* gene. No white colony was observed in this plate indicating that there was no contaminant phage on the plate. Inconsistency in the number of plaques formed from the first round to the third round of biopanning did not prevent subsequent rounds of biopanning after phage amplification (Table 1).

Assaying selected peptides for target binding by ELISA: Before carrying out the ELISA, amplified phages were examined on LB/IPTG/Xgal plates to ensure that they were still viable (Fig. 2). A lawn of blue plaque on the plate indicated that the phages were viable. The OD_{260} reading of the ELISA assay in wells coated with BSV was 0.4142, which was approximately 4 fold higher compared to the negative control (BSA). This shows the input phage had successfully bound to BSV. The readings are shown in Table 2.

Fig. 1: Blue plaques on a LB/IPTG/Xgal plate. Recombinant phage from the phage library that carried the *lacZα* gene are shown as blue plaques on this plate. No white plaque is seen on the plate, an indication that no contaminant phage (wild type) from the environment had grown on the plate

Fig. 2: A lawn of blue plaques on LB/IPTG/Xgal plate. An indicates that the amplified phages

Table 1: Plaques formed during biopanning process

Biopanning round	Unamplified phage (pfu) (output phage after panning process)	Amplified phage (pfu) as input phage for next round of biopanning
First	2.6×10^{6}	2.60×10^{12}
Second	1.3×10^{2}	1.48×10^{13}
Third	7.5×10^{5}	--

Input phage for the first round of biopanning was 5×10^{11} pfu. The values above were based on phage titration on LB/IPTG/Xgal plates. Overall, the number of input phage was higher than the number of output phage, which showed that only a group of phage was selected after each round of biopanning process

Table 2: ELISA assay with BSV

Well	Sample	OD_{260}
1	BSV	0.4142
2	Bovine Serum Albumin (BSA)	0.0997

The OD_{260} reading above shows the affinity of the input phage displayed putative BSV binding peptide against BSV. This is a single replicate and the OD reading is approximately 4 fold higher in wells coated with BSV compared to the negative control

Sequencing: Similar DNA sequences were obtained for the two randomly selected phagemid. The DNA sequence and deduced amino sequence of the peptide that potentially bound to BSV are shown in bold in Fig. 3. Overall, the result shows that the majority of the binding peptide or mimic paratope consisted of the amino acid, valine (Val).

5′-.....TTA TTC GCA ATT CCT TTA GTG GTA CCT TTC TAT TCT CAC TCT
Leu Phe Ala Ile Pro Leu Val Val Pro Phe Tyr Ser His Ser

Start of mature 12-mer peptide-gIII fusion

GTC GTC GTA GGA TCC TTC GTG GTT GCA CGA CTA CGT CCA CCT CCA
Val Val Val Gly Ser Leu Val Val Ala Arg Leu Arg Gly Gly Gly

TCG GCC GAA ACT GTT GAA AGT TGT TTA GCA AAA TCC CAT ACA GAA.....3'
Ser Ala Glu Thr Val Glu Ser Cys Leu Ala Lys Ser His Thr Glu

Fig. 3: Sequencing results of the clone bound to BSV. Two randomly selected clones after the third round biopanning were sequenced and the above results were obtained. Sequence in bold is the peptide that potentially bound to BSV

DISCUSSION

Plantains and bananas (*Musa* sp.) are important but poorly investigated crops that serve as an important source of income and food for millions of people in developing countries. However, an increasing number of new pests and diseases that infect bananas have been identified. BSV is a major virus that infects bananas and affects their yield and quality. Generally, BSV isolates can be detected reliably by Immunosorbent Electron Microscopy (ISEM) or by triple antibody sandwich ELISA (TAS-ELISA) using polyclonal antisera (Ndowora and Lockhart, 1999) and anti-BSV antibody is the main component used in ELISA assay. Therefore in this study, we attempted to identify a peptide that interacts with BSV using a phage display system, which can potentially be developed into binding ligand used for BSV detection.

The phage-displayed combinatorial peptide library was used in this study as it was a potentially rewarding method for rapidly and efficiently mapping protein-protein interactions. Screening a library displaying a billion different peptides by affinity selection required less effort, time and resources (Kay *et al*., 2001). This technique had been extensively used in studies for epitope mapping and vaccine development (Cortese *et al*., 1995; Manoutcharian *et al*., 2001) as well as for the identification of mimotope of non-peptide ligands (Katz *et al*., 1995; D'Mello and Howard, 2001). In general, two types of phage display libraries have been used for epitope identification; Random Peptide Libraries (RPLs) and Gene or Genome Fragment Libraries (GFLs). The random peptide library was used in this study because of its universal nature. The same RPL could be used for mapping epitopes recognized by different antibodies and for isolating mimotopes of proteins or carbohydrates and has a very wide range for application, whereas GFLs are gene-or genome-specific, which means a new GFL would have to be constructed for each individual application (Wang and Yu, 2004).

The principle of a general panning strategy is simple and easy to follow but the expected outcome of any panning experiment needs careful planning (Wang and Yu, 2004). Hence, three rounds of biopanning were carried out in this study to enrich the specifically bound phages. It is not recommended to carry out more than four rounds of panning so as to maintain the sequence diversity among the binding phage. Additional rounds of panning would most likely select for phage that grow, infect or elute better rather than the phage that binds to their targets (Kay *et al*., 2001). However, several modifications could be done to minimize growth competition effect. These include using the colony or plaque lift method for direct screening of a large number of first round binders (Dente *et al*., 1994); reducing rounds of amplification and carrying out statistical analysis on a large number of sequences (Rodi *et al*., 1999); replacing liquid medium with solid media (on a plate) for amplification, growing individual clones separately and then pooling them together for subsequent panning (McConnell *et al*., 1995). Amplification that was done at the interval of each round of panning could increase the final yield of clones and prevent possible loss of rare clones by eliminating the cumulative effect of two or more rounds of panning (Parmley and Smith, 1988). The target phage yield after three rounds of panning in this study was quite high. In addition, further analysis on individual clones could be attempted by a combination of high-detergent washes and intermediate amplification (Parmley and Smith, 1988).

Overall, after three rounds of the biopanning process, a positive ELISA result (OD_{415} = 0.4142) was detected when our target BSV was tested with the phage pool. The majority of the phage in the phage pool after the third round of biopanning was expected to carry similar binding peptides. Hence, two phagemids were randomly isolated from the amplified phage and sequenced. Again, the DNA sequence and deduced amino acids of the binding peptide were obtained as expected.

CONCLUSION

The experiment showed the utility of phage peptide-display libraries as a method for isolating a BSV-binding peptide. This peptide can be used for further evaluation for its sensitivity and specificity as a diagnostic reagent.

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