SELECTION OF PHAGE-DISPLAY PEPTIDES THAT BIND SPECIFICALLY TO THE OUTER COAT PROTEIN OF RICE BLACK STREAKED DWARF VIRUS

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Summary. – Several peptides that could bind specifically to the outer coat protein encoded by the S10 gene of Rice black streaked virus (RBSDV) were isolated from a phage-display random 12-mer peptide library. The sequence analysis showed that the amino acid motif (K)K**(*)P, the asterisk denoting any amino acid, might be the core sequence by which the peptides bind to the target protein. The peptide 1 that had a high affinity to RBSDV outer coat protein was synthesized by a chemical method and its fusion protein with glutathione-S-transferase (GST) was produced in an *Escherichia coli* expression system. The dot and Western blot analyses indicated that RBSDV could be detected with a high sensitivity in crude extracts of diseased plant leaves using a purified GST fusion protein. The circular dichroism (CD) spectroscopy revealed that the synthesized binding peptide but not a non-binding peptide could bring about a marked change in the conformation of outer coat RBSDV protein. Since the protein functions only when it has correct conformation, the peptides binding specifically to it could possibly disturb the function of the virus outer coat protein and might be used to block the transmission pathway of the virus. Summing up, as these peptides showed a high specificity and sensitivity and diagnostic potential for RBSDV, they may represent the basis of a novel strategy for development of resistance to RBSDV.

Key words: phage-display peptide; outer coat protein; Rice black streaked dwarf virus

Introduction

RBSDV (species *Rice black streaked dwarf virus*, genus *Fijivirus*, family *Reoviridae*) is transmitted by a brown planthopper, *Laodelphax striatellus* Fallen, and infects cereal crops causing severe damage in China and eastern Asia (Bai *et al.*, 2001; Fang *et al.*, 2001; Zhang *et al.*, 2001a). The virus particle has two coats, outer and inner (core) that encapsidate 10 segments of double stranded genomic RNA (S1-S10). So far, all of RBSDV genome segments have been sequenced (Zhang *et al.*, 2001b). The sequence analysis showed that S1-S6, S8 and S10 have a single large open reading frame (ORF), while S7 and S9 have two non-overlapping ORFs. Western blot analysis indicated that S8 and S10 encode the core and outer capsid proteins, respectively, and S7 and S9 encode non-structural proteins (Isogai *et al.*, 1998).

Virus coat proteins play major roles in virus replication, packaging and recognition of the insect vector and plant host. In viral RNA replication they may be involved in the regulation of positive strand RNA synthesis either directly or as a consequence of encapsidation of the newly synthesized RNA (Houwing and Jaspars, 1986). It has also been demonstrated that the Cucumber mosaic virus (CMV)

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Abbreviations: CD = circular dichroism; CMV = Cucumber mosaic virus; ELISA = enzyme-linked immunosorbent assay; GRHV = Grass carp hemorrhage virus; GST = glutathione-S-transferase; HPLC = high performance liquid chromatography; HRP = horseradish peroxidase; MAb = monoclonal antibody; ORF = open reading frame; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RBSDV = Rice black streaked dwarf virus; RSV = Rice stripe virus; SP = special protein; TBST = 50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, and 1% Tween 20 (v/v)

coat protein plays an important role in aphid transmission in which its primary or higher structure is solely responsible for the interaction of the virions with the inside of the aphid stylet (Chen and Francki, 1990; Gera *et al.*, 1979). Changing a few amino acid residues could result in dramatic changes in transmission of the virus by different aphid vectors (Perry *et al.*, 1998). The CMV coat protein has also been shown to function as a host range determinant (Ryu *et al.*, 1998). Various functions of the CMV coat protein are dependent on highly conserved domains within its primary structure which could provide targets in a novel strategy for development of resistance to CMV (Gough *et al.*, 1999).

Phage-display random libraries have been employed to isolate peptide ligands that specifically bind to wide range of target "ligates" and to map epitopes of monoclonal antibodies (MAbs) (Boots *et al.*, 1997; Stern *et al.*, 1997; Young *et al.*, 1997). Some reports have shown that some phage-display peptides could interfere with protein-protein interactions (Bottger *et al.*, 1996, 1997; Liang *et al.*, 1997). Dyson and Murray (1995) have isolated phage-display peptides that could bind to the core antigen of hepatitis B virus and interfere with the assembly of virus particles. Wang *et al.* (2000) have selected a specific peptide from a nona-peptide library which could *in vitro* inhibit the replication of Grass carp hemorrhage virus (GRHV). Gough *et al.* (1999) have obtained phage-display peptides that could bind to the CMV coat protein. Moreover, dot blot analysis showed that a fusion peptide expressed by *E. coli* could be used as a specific and highly sensitive tool in detection of CMV (Gough *et al.*, 1999). Several phage-display peptides specifically binding to a special protein (SP) of Rice stripe virus (RSV) and able to change its conformation *in vitro* have been selected (Zhang *et al.*, 2002).

In this report, the peptides that could bind with high specificity to the outer coat protein of RBSDV were selected from a phage-display peptide random library. This result might form the basis of a novel strategy for development of resistance to RBSDV.

Materials and Methods

Target protein and phage-display random peptide library. Preparation and purification of the outer coat protein of RBSDV were performed according to the methods described by Bai *et al.* (2002). The fusion protein expression vector pGEX-S10 containing RBSDV S10 and pGEX-3x (Amersham Pharmacia Biotech) was constructed. pGEX-S10 was used for transformation of *E. coli* BL21. When the transformed cells reached the mid-log phase, 1mmol/l IPTG was added to induce the protein expression. After 6 hrs the cells were harvested by centrifugation, resuspended in the phosphate-buffered saline (PBS) and lysed by sonication on ice. The supernatant collected after centrifugation representing crude total proteins of *E.coli* was used for purification of the GST-S10 fusion protein by a glutathione-Sepharose 4B column chromatography (Amersham Pharmacia Biotech).

Library bio-panning. Immunotubes were coated overnight at 4°C with the GST-S10 fusion protein or GST only (100 μg/ml in 100 mmol/l $NaHCO_3$, pH 8.6). First, the library was bio-panned for 5 x 1 hr at room temperature using the immunotubes coated with GST in order to get rid of the phages that specifically bound to GST. Then the bio-panned library was used to select the phages that could specifically bind to the outer coat RBSDV protein. Further bio-panning steps were performed according to the recommendations of the manufacturer. The PH.D.-12™ Phage Display Peptide Library Kit was purchased from New England Biolabs.

Enzyme-linked immunosorbent assay (ELISA) was performed in 96-well plates coated overnight at 4°C with 200 μl of 100 μg/ml GST-S10 per well. Five wells coated with GST only served as a control. The coated plates were blocked with 3% (w/v) non-fat dried milk for 2 hrs at 4°C, washed with the TBST (50 mmol/l Tris-HCl pH 7.5, 150 mmol/l NaCl, and 1% Tween 20(v/v)). The blocked phages were added to the wells and left to interact with the target protein for 1hr at room temperature. After 6-fold washing with TBST, the wells were filled with a horseradish peroxidase (HRP)-conjugated M13 antibody (the combinatorial library of random peptide 12-mers fused to a minor coat protein of M13 phage) for 1 hr at room temperature with agitation and then rinsed with TBST again 6 times. ABTS (200 μl, Sigma) and H_2O_2 were added to each well and incubated at room temperature for 10 mins in the dark. At last, A_{490} was read in a micro-plate reader.

Nucleotide sequencing. Thirty-eight phages that gave the highest ELISA absorbance values were selected and their DNAs were prepared and sequenced by a commercial company (Genecore, Shanghai, P.R. China).

Expression and purification of the peptide-GST fusion proteins. The expression vector was constructed by PCR. The primers were designed according to the phage DNA sequence and a part of the vector pGEX-3x sequence. The primer sequences were as follows: (a) 5′-AGG AAG AAG ACT CGT CCT AGT CGG CCG GAA TTC ATC GTG ACT GAC TG-3′and (b) 5′-ACG AGT CTT CTT CCT CCG CGC ATG GGG GAT CCC ACG AAC TTC-3′. The PCR was performed as follows: 94°C for 5 mins; 94°C for 1 min, 55°C for 1min, 72°C for 5 mins, altogether 30 cycles. The PCR products after purification by use of the Gel Extraction Kit (Huashun, China) was used to transform *E. coli.* DH5α competent cells. Plasmid containing the gene encoding the peptide 1-GST fusion protein was extracted and identified by DNA sequencing. The methods used for the expression and purification of the peptide-GST fusion proteins were the same as those for GST-S10.

Chemical synthesis of the phage peptide 1 was carried out by a commercial company (GL Biochem, China). Its sequence was N-Tyr-Ser-His-Ser-His-Ala-Arg-Arg-Lys-Lys-Thr-Arg-Pro-Ser-Arg-Pro-Gly-Gly-Gly-Ser-C. The peptide was purified by high-performance liquid chromatography (HPLC) by the same company as above and its purity was 78.63%

Dot blot and Western blot analyses. Crude extracts from diseased plants were spotted onto a nitrocellulose membrane (NC, for dot blotting) or were blotted onto a NC membrane after electrophoresis in 12.5% polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE, for Western blotting). The blots were blocked with a 1% blocking buffer (Roche) for 1 hr at room

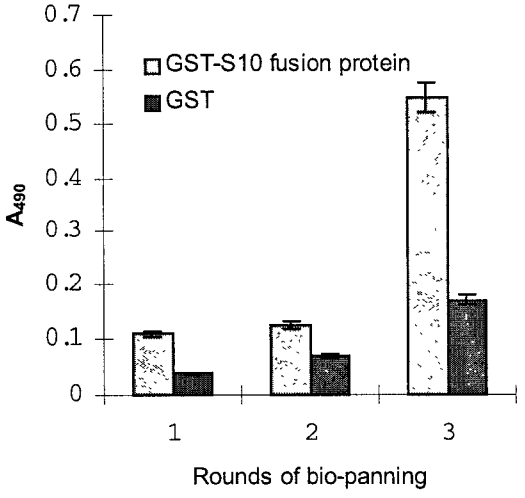

Fig. 1

Enrichment of phages after three rounds of bio-panning as determined by ELISA

temperature and incubated with GST-peptide (1 μg/ml in PBS) and then with a polyclonal GST antiserum (Amersham Pharmacia Biotech) in a 0.5% blocking buffer for 1 hr at room temperature. Finally the blots were washed with TBST (0.1% Tween 20) for 3 x 10 mins and incubated in a goat anti-rabbit immunoglobulin-alkaline phosphatase conjugate for 1 hr. After washing with TBST for 3 x 10 mins the blots were incubated in BICP/NBT as substrate for 10 mins in the dark.

Target protein conformation analysis. The conformation of the outer coat RBSDV protein was analyzed by CD spectroscopy using a JASCO715 apparatus with the following parameters: the cell length of 0.1 nm, the band width of 1.0 nm, the resolution of 0.2 nm, the sensitivity of 20 mdeg, the response of 0.25 sec, the speed of 200 nm/min, and the accumulation of 5.

Results

Enrichment of phages

A phage-display random 12-peptide library was used to screen the peptides that could bind specifically to the outer coat RBSDV protein of. Three rounds of bio-panning against RBSDV target protein immobilized on the immunotube wall were carried out. Using ELISA, the degree of enrichment of polyclonal phages was measured after each round of bio-panning. After 3 rounds of bio-panning, the phages bound specifically to the outer coat RBSDV protein were enriched significantly (Fig. 1), while the phages bound to GST were just slightly enriched.

Nucleotide sequences analysis

In order to obtain peptides with high affinity to the outer coat RBSDV protein, the affinities between the monoclonal phage-display peptides and the outer coat RBSDV protein were measured. A hundred and ninety-two clones eluted in the round three were isolated and assayed by ELISA. Thirty-eight clones, which gave the strongest signals in monoclonal ELISA, were selected for further characterization by nucleotide sequencing (Table 1). The sequences obtained represented 21 different peptides. Eleven out of 38 peptides were the peptide 1; this

Table 1. Nucleotide sequences of peptide genes and amino acid sequences of peptides

Peptide No.	Nucleotide sequences of peptide genes	Amino acid sequences of peptides	Frequency
1	CATGCGCGGAGGAAGAAGACTCGTCCTAGTCGGCCG	HARRKKTRPSRP	11
2	GCGGCGCCTCGTTTGAAGCATCCTCCGAAGTCGATT	AAPRLKHPPKSI	6
3	GTTCAGACTAAGATTAAGACTAAGCATAAGAAGCCT	VQTKIKTKHKKP	2
4	ACTCTGCCTAAGAAGCGTAAGAATCCTCCTCGTGGT	TLPKKRKNPPRG	2
5	CTGAATCCTACTAATACGCTGTGGCCGACGGCGCAG	LNPTNTLWPTAQ	1
6	AATTTTAAGCCGCCTATTAAGACTCCTCCTTCGCAG	NFKPPIKTPPSQ	1
7	ATTGGTAGGACGAAGAAGGATCATGAGCTGGGTCGT	IGRTKKDHELGR	1
8	CGTCATAAGAAGCGGAAGCCGCCTCAGAGGTTGGCG	RHKKRKPPQRLA	1
9	ATGCTGAATATGCCGGTGCGTCCTGCTTTGGTTCCT	MLNMPVRPALVP	1
10	GGTACTCATATGTCGNCGGTGATGAGTGGTCTGCGT	GTHMS*VMSGLR	1
11	GGTCCGAATGTTGTTCCGAGTATTCCGGATAATCCG	GPNVVPSIPDNP	1
12	GGTCAGTCGTTGCCTTTGTCTTTGGCTCTTTCTGCG	GQSLPLSLALSA	1
13	ACTGCGCATCTGATTAGTACTCCTGTGTATCCTCTT	TAHLISTPVYPL	1
14	AATTCGCTGGAGAGGACTAATACTTGGTATAGGTCG	NSLERTNTWYRS	1
15	GCTCATGGGCCTACGTAGAGGCCGTGGCCGACGCCT	AHGPTQRPWPTPG	1
16	GTTACGCTGAAGCAGCTTACGCCTTCTCCTTATCCT	VTLKQLTPSPYP	1
17	ACTAAGAAGAAGAATAAGAATCGTCGTGATGATGCG	TKKKNKNRRDDA	1
18	AGGCCGACTAAGAAGAAGTTTCCGCAGTCTCAGCGT	RPTKKKFPQSQR	1
19	CCTTTTTCTACGGCTTATTTTCCTTATTCTGTTTCT	PFSTAYFPYSVS	1
20	CAGATGCCTCGGCCTCCTGCTTGGACTCCTCCATAT	QMPRPPAWTPPY	1
21	CAGGCTGCTTGTGAGCAGGTTCTTCCGACTGGTCGT	QAACEQVLPTGR	1

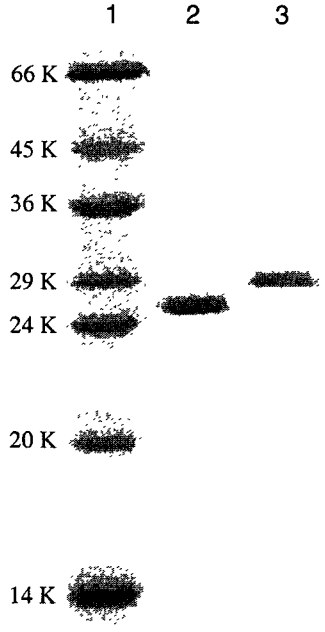

Fig. 2

Expression and purification of the peptide 1-GST fusion protein

The expression was carried out in *E. coli* BL21. The purification was carried out by glutathione-Sepharose 4B column chromatography. Protein size marker (lane 1), GST (lane 2), and the peptide 1-GST fusion protein (lane 3).

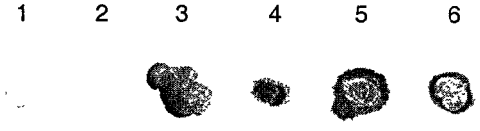

Fig. 3

Detection of RBSDV in crude extracts of diseased plants by dot blot analysis using the peptide 1-GST fusion protein as a probe

Crude extracts from healthy rice leaves (1 and 2), GST control (3), RBSDV control extract (4), a crude extract from diseased rice leaves (5), and a crude extract from diseased wheat leaves (6).

finding indicated that the peptide 1 had the highest frequency. The frequency of the peptide 2 occupied the second place. The amino acid sequence analysis showed that 65.8% of the peptides shared the common motif (K)K**(*)P, the asterisk denoting any amino acid,, that indicated that this motif might be the core sequence by which the peptides bound to the target protein.

Expression and purification of peptide-GST fusion proteins

The vector pGEX-3x containing the gene encoding the peptide 1-GST fusion protein was used for transformation of *E. coli* BL21. After the cell culture reached the mid-log

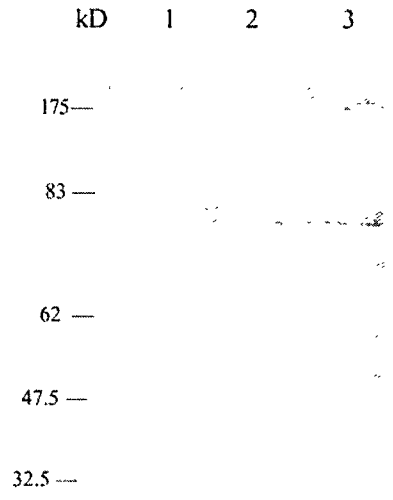

Fig. 4

Detection of RBSDV in crude extracts of diseased plants by Western blot analysis using the peptide 1-GST fusion protein as a probe

Crude extract from healthy rice leaves (lane 1), crude extract of diseased wheat leaves (lane 2), and crude extract of diseased rice leaves (lane 3).

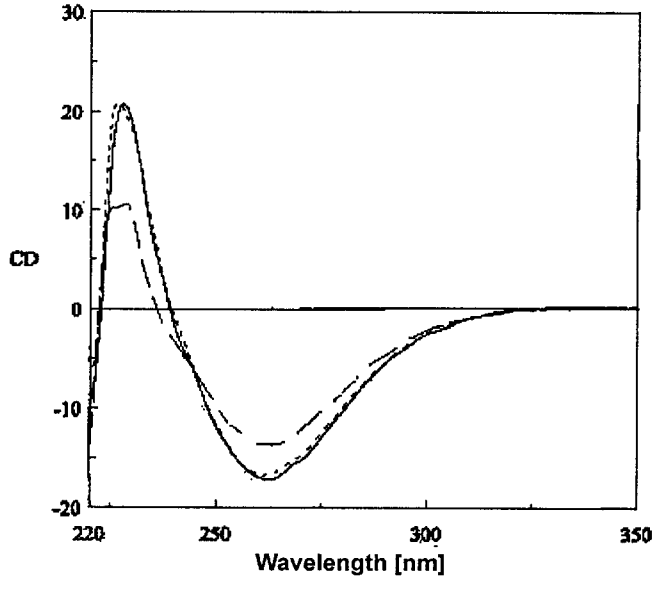

Fig. 5

CD spectroscopy of outer coat RBSDV protein

No peptide added (continuous line), a non-bilding peptide added (dotted line), and the peptide 1 added (broken line).

phase at 37°C, IPTG was added to induce the protein expression. The fusion protein was purified from the crude cell extract by glutathione-Sepharose 4B column chromato-

graphy. The relative molecular mass (M_r) of the purified protein was about 29 K, which was fully in accord with the expected size (Fig. 2). Thus the peptide 1 gene was successfully expressed as the peptide 1-GST fusion protein in *E. coli*.

Dot blot and Western blot analyses

In order to prove that the selected peptide could be used to detect RBSDV in crude extracts of diseased rice or wheat, dot blot and Western blot analyses were performed (Figs. 3 and 4). For dot blot analysis 15 µl of a RBSDV crude extract was spotted directly onto a NC membrane. The results revealed that RBSDV could be detected though its amount in the diseased plants was very low (Fig. 3). Western blot results showed that the peptide-GST fusion protein did not cross-react with intrinsic proteins of the plants since there were no bands in the healthy plant samples. However, two proteins were detected in the diseased plant samples, and they both were similar in size to those of the RBSDV particle. The reason that there were more than one band might be a degradation of the outer coat RBSDV protein (Fig. 4).

Conformation analysis of the target protein

In order to investigate whether the selected peptide could change the conformation of the outer coat RBSDV protein due to binding, the CD spectroscopy was performed. The results showed that the conformation of the fusion protein consisting of the outer coat RBSDV protein and GST changed significantly after adding the peptide 1 at the final concentration of 0.67 mmol/l (Fig. 5). However, there was no conformation change when a non-binding peptide, which could only bind to the SP of RSV was added (Fig. 5).

Discussion

The phage-display technology allows the isolation of peptides with specific binding to target proteins from a vast library of random peptides. In this study, we isolated the peptides that could bind to the outer coat RBSDV protein after three rounds of bio-panning. The nucleotide and amino acid sequence analyses showed that the motif (K)K**(*)P might be the core sequence by which the peptides bind to the target protein. However, some other peptides not containing this motif also had high ELISA absorbance values. This could be caused by the presence of different binding sites for the target protein. The selection of the peptide 1 for expression as a GST fusion protein in *E. coli* as well as for chemical synthesis was based on its highest ELISA absorbance value and frequency in the phage sequencing analysis. The dot blot and Western blot analyses showed that RBSDV could be detected with high sensitivity in crude extracts of diseased leaves of rice and wheat. These results also indicated that the peptide could bind to a native as well as denatured target protein. Since the fusion protein of the peptide could be expressed to high levels in *E. coli* and the expressed protein could be purified easily, the peptides that specifically bound to outer coat RBSDV protein could be used as a cheap and sensitive diagnostic tool.

The isolation of peptides that specifically bind to virus proteins aimed at development of a novel disease resistance strategy. Peptides that bind to replicase proteins, movement proteins or coat proteins have been isolated and fused to carrier proteins for expression in plants or insects. The binding of these fusion proteins of peptides *in vivo* could compromise virus function and lead to tolerance/resistance (Gough *et al.*, 1999). So far, some studies have confirmed that plants could acquire virus resistance by expressing antibodies to fragments of recombinant viral coat protein (Tavladoraki *et al.*, 1993; Zimmerman *et al.*, 1998). In this study, the selected peptide changed the conformation of the outer coat RBSDV protein significantly *in vitro*. Because the protein needs for its function an optimal conformation, a change of its conformation leads to disturbance or block of its normal function. In addition, earlier studies have demonstrated that the conformation of some proteins changes due to the binding of peptides (Chang *et al.*, 1997; Inooka *et al.*, 2001; Mensseb *et al.*, 1999).

Thus we can assume that the functions of the outer coat RBSDV protein might be blocked or disturbed by the peptide tested in this study, since the peptide changed its conformation. Summing up, we not only demonstrated that the selected phage-display peptide could be used as a sensitive diagnostic protein, but it may also represent the basis of a novel RBSDV transgenic resistance strategy. However, further study is needed to prove whether the peptide binding specifically to the outer coat RBSDV protein can block or disturb the virus replication or transmission provided the peptide is expressed in the plants or planthoppers.

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