SELECTION AND EXPRESSION OF PEPTIDES WHICH CAN CHANGE THE CONFORMATION OF P20 PROTEIN OF RICE STRIPE VIRUS

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Summary. – Phages with high affinity to the P20 protein of rice stripe virus (RSV) were enriched from phage-displayed random 12-mer peptide library after three rounds of phage display screening. Nine different peptides from the enriched library were selected by enzyme-linked immunosorbent assay (ELISA). The P20 protein from raw extracts of rice leaves infected with RSV could be detected by those 9 peptides displayed on the phage, which suggested that a peptide could be an effective tool for diagnosis of RSV in rice and planthopper. Circular dichroism (CD) spectra of P20 fusion proteins with the binding phages and non-binding phages showed that the conformation of P20 protein was changed after binding to each of the 9 selected 12-mer peptides, which suggested that these peptides might disrupt the function of the P20 protein. Thereafter, those peptides might be used to develop plant resistance and disrupt virus transmission. Three of the 12-mer peptide genes were fused with the glutathione-S-transferase (GST) gene in the vector pGEX 3X. The fusion proteins were obtained from an *Escherichia coli* expression system and purified. The fusion proteins might have a potential to develop a plant peptide-based resistance to its pathogens and virus diagnosis. It also provided a tool (i) to confirm the inhibition of the function of P20 protein by the fusion peptides *in vivo*, and (ii) to detect the function of P20 protein and the interaction between the virus and its vector.

Key words: peptide; phage display; P20 protein; protein conformation; rice stripe virus

Introduction

Rice stripe disease, caused by RSV, brings great losses to rice yields (Toriyama, 1995). RSV is a single-stranded segmented RNA virus (the species *Rice stripe virus,* the genus *Tenuivirus* (van Regenmortel *et al.,* 2000)). It is transmitted among rice by planthopper (Ramirez and Haenni, 1994). There are seven genes in four segments of RNA genome (Ishikawa *et al.*, 1989). A single, large open reading frame (ORF) is within the negative-sense RNA1, which encodes an RNA-dependent RNA polymerase (RDRP) (Falk and Tsai, 1998). Furthermore, each of ambisense RNA 2, 3, and 4 contains two ORFs that are located near their 5'terminus (Kakutani *et al.*, 1990, 1991; Takahashi *et al.*, 1993). Two proteins translated from RNA3 and RNA4 are major non-capsid proteins (NCP). The NCP from RNA4, P20 (M_r of about 20 K), is frequently found by immunodetection in the cell nucleus, cytoplasm and vacuoles of infected rice and planthopper tissue but not in the RSV virion (Qu *et al.*, 1997, 1999). This suggests that P20 is not a movement protein of RSV. It is probably a protein essential for virus propagation in the host and/or virus transmission, but its precise function is still unclear (Qu *et al.*, 1999).

Conventional approaches to control of RSV infection include insecticides or isolation of virus-tolerant rice cultivars. Recently some strategies involving the production of transgenic plants have been employed (Hayakawa *et al.*, 1992). These strategies are based on the expression of

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Abbreviations: BSA = bovine serum albumin; CD = circular dichroism; ELISA = enzyme-linked immunosorbent assay; cRNA = complementary RNA; GST = glutathione-S-transferase; ORF = open reading frame; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RSV = rice stripe virus

a viral protein in plants leading to either disease resistance or delay of symptoms. The coat protein (CP) gene of RSV was introduced into two *Japonica* varieties of rice by electroporation of protoplasts. The resultant transgenic plants expressed CP at high levels and exhibited a significant level of resistance to virus infection (Hayakawa *et al.*, 1992).

Different from all strategies mentioned above, ours is to control the propagation of RSV in planthopper so that the transmission of RSV can be terminated by *Wolbalchia,* a kind of bacterial symbiont infecting planthoppers, which has a procreation superiority in population (Werren, 1997). As we know, peptides are widely used to disrupt protein function (Brian *et al.*, 1998). They can also be used for the detection and diagnosis of viral infections of plants too (Gough *et al.*, 1999). (i) Microinjection of candidate peptides able to bind target proteins into *Wolbalchia* or (ii) transformation of *Wolbalchia* or the planthopper with the genes encoding these peptides can stop or weaken the transmission of RSV.

Phage display is the most efficient and widely used method to screen ScFv antibodies (Griffiths and Duncan, 1998) and peptides or ligands that bind specifically to a wide range of target proteins (antigens) and to map epitopes of monoclonal antibodies (Rodi and Makowski, 1999; Sidhu *et al.*, 2000). It can also be used for analysis of protein-protein interactions (Rodi *et al.*, 1999). In this study, the P20 protein was selected to be a target protein due to its important role in RSV propagation. Several peptides that could bind to the P20 protein were obtained using a phage-displayed library. These peptides might be further used to inhibit RSV transmission.

Materials and Methods

Purification of GST and the GST-P20 fusion protein was done according to Qu *et al.* (1999). The *E. coli* cells containing the plasmids with GST or the GST-P20 genes (Qu *et al.*, 1999) were cultured overnight and then the cultures were diluted to grow to log phase in the 2×YT medium (17 g Bacto tryptone, 10 g Bacto yeast extract, and 5 g NaCl per liter of medium). Expression of the GST or the GST-P20 fusion protein was induced with 0.1 mmol/l IPTG. The cells were collected after 6 hrs and resuspended in phosphate-buffered saline (PBS). The cells were sonicated and centrifuged and the resulting supernatant was used for purification of GST or the GST-P20 fusion protein by chromatography on a glutathione-Sepharose 4B column. The column was washed thoroughly with PBS and the proteins of concern were eluted with a glutathione-elution buffer (10 mmol/l reduced glutathione and 50 mmol/l Tris-HCl pH 8.0).

Phage display sreening. Immunoplates were coated with GST or the GST-P20 fusion protein (100 μg/ml) at 4°C overnight. The coated plates were blocked with bovine serum albumin (BSA, 5 mg/ml) in 0.1 mol/l $NaHCO_3$ pH 8.6. After the coated plates were washed 3 times with a Tris-buffered saline (TBS, 50 mmol/l Tris-HCl pH 7.5 and 150 mmol/l NaCl), a phage library (Ph.D.-12™, New England Biolabs) was added to the plates coated with GST and incubated at room temperature for 1 hr. The procedure to subtract phages binding GST was repeated 5 times. The subtracted library was added to the plates coated with the GST-P20 fusion protein and incubated at room temperature for 1 hr with gentle shaking. Then the plates were washed 5 times with TBS containing 0.1% (v/v) Tween 20. Each well was eluted with 0.2 mol/l glycine-HCl pH 2.2 at 37°C for 8 mins and neutralized with 1 mol/l Tris-HCl pH 9.1. The eluted phages were added to a log phase *E. coli* ER2738 culture in LB medium and grown at 37°C for 4 hrs. The phages were precipitated with 20%(w/v) polyethylene glycol 8000 in 150 mmol/l NaCl and used in another round of phage display screening. The phage display screening was repeated three times with increasing concentration of Tween 20 (the first round with 0.1% (v/v) Tween 20 and the others with 0.5% Tween 20 (v/v) separately). For each round of phage display screening, the eluted and amplified phage was titrated separately.

ELISA of isolated phages. Following the final round of panning and titration, plaques were selected randomly and amplified by infecting *E. coli* cells and shaking for 4 hrs at 37°C. The phages were added to the plates coated with the P20 fusion protein and incubated at 37°C for 1 hr. The plates were washed 6 times with PBS containing 0.5% (v/v) Tween 20. A diluted horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia, 1:10 000 dilution in PBS containing 2.5% skim milk and 0.5% Tween 20) was added to each well and the plates were incubated at 37°C for 1 hr. The plates were washed 6 times with PBS containing 0.5% Tween 20, and a substrate (ABTS) in 0.09% H_2O_2 was added to each well. A_{490} was monitored at regular intervals for each clone. Wells containing no antigen or GST served as negative controls.

Nucleotide sequencing. DNA fragments to be sequenced were purified according to Wilson (1993). The DNA fragments encoding the P20-binding peptides from the selected phages were sequences in an ABI 377 automatic DNA sequencer using the sequencing primer 5'-CCCTCATAGTTAGCGTAACG-3'.

CD spectroscopy. The CD spectra were obtained with a 0.1 cm pathlength cell over a wavelength range from 250 nm to 190 nm, at an interval of 0.2 nm using a JASCO 715 spectropolarimeter. For each sample, five spectra and baselines were collected and averaged, and their SD values were calculated at all the wavelengths used. A P20 sample contained 0.15 mg/ml fusion protein in H_2O. Phages (10^{12} PFU) in TBS were added to each sample and incubated at room temperature for 15 mins. Secondary structures were determined using a normalized constrained least squares algorithm (Wallace and Teeters, 1987).

Detection of P20 protein in diseased rice plants with phage-displayed peptides. The ground rice leaves infected with RSV were suspended in PBS. After a low speed centrifugation the supernatant containing total raw protein from the diseased rice plant was collected. The plate wells were coated with the raw protein and the ELISA was performed as described above. Wells containing no antigen or a raw protein from healthy rice served as negative controls.

Expression of selected peptides fused with GST. Primers were designed to contain the multiple coding site of the expression vector pGEX 3X and a partial segment of selected peptides (Table 1).

Table 1. Primers used in cloning peptide genes in the expression vector

Peptide	Primer sequence
No.1	F: 5'-TGGATTTGGACGGATGGGCAGTGGTGTGAATT CATCGTGACTGACTG-3'
	R: 5'-ATCCGTCCAAATCCAACACACACTGGGGATCCCACGAA CTTCCACACACATGGGGATCCCACGACCTTC-3'
No. 2	F: 5'-TGGCTTCATCCGTCTCCGTGGGTGACTGAATT CATCGTGACTGACTG-3'
	R: 5'-AGACGGATGAAGCCAAAACGGATAGGGGATCCCACGAA CTTCAAACGGATAGGGGATCCCACGACCTTC-3'
No. 3	F: 5'-TGAGGAGGTTTGGTTTCCATTTACGGAATT CATCGTGACTGACTG-3'
	R: 5'-AAACCAAACCTCCTCATTCCAAGACCAGGGGATCC CACGAACTTCGGGGATCCCACGACCTTC-3'

F = forward. R = reverse.

Table 2. Phage output/input ratios of each round of phage display screening

Round No.	Phage input (PFU)	Eluted phage (PFU/μl)	Phage output (PFU)	Phage output/input ratio
1	4×10^{10}	44	5.06×10^{4}	1.27×10^{-6}
2	1.9×10^{12}	400	4.6×10^{5}	1.31×10^{-7}
3	1.1×10^{12}	11400	2.42×10^{7}	1.19×10^{-5}

A PCR was performed (94°C for 1 min, 55°C for 1min, and 72°C for 5 mins, 30 cycles) to obtain a pGEX 3X vector containing a fragment of selected peptide. PCR products were used for transformation of *E. coli* strain BL21. The transformants with a peptide gene inserted into the multiple cloning site of pGEX 3X vector correctly were identified by sequencing. A GST-peptide fusion protein was purified according to the protocol described above.

Results

Enrichment of high affinity phages by phage display screening

The number of eluted phages and the output/input ratio for each round of phage display screening are shown in Table 2. Because the concentration of Tween 20 increased from 0.1% to 0.5%, not only the phages bound unspecifically to P20 were eluted, but also the phages with low affinity to P20 were washed out. This was the reason why the output/input ratio of the second round of screening was smaller than that of the first round. Under the same washing conditions, the input/output ratio of the third round of screening showed great increase compared to that of the second round. The results showed that the enrichment of high affinity phages by the phage display screening was very effective.

The amplified phages from each round of screening were used to assess their ability to bind P20 by ELISA. After 3 rounds of screening the phages displayed a greatly increased affinity to the GST-P20 fusion protein and a little increased affinity to GST, indicating that enrichment of the peptides binding specifically to P20 had occurred (Fig. 1).

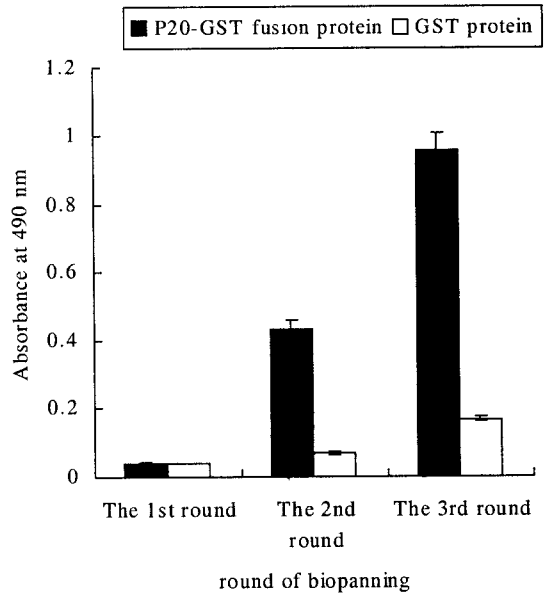

Fig. 1
The binding affinity of the screened phages to P20 and GST for each round of phage display screening as detected by ELISA

Three rounds of screening were carried out. P20 fusion protein was immobilized on ELISA plate and detected with 10^{10} PFU of the amplified phage. A_{490} was measured 15 mins after incubation with the substrate. Abscissa: rounds of screening.

Selection of peptides with affinity to P20

To assess the binding of phage peptides to P20, 144 plaques from eluates of the third round of screening were

Table 3. Nucleotide and deduced amino acid sequences of selected peptides

Peptide No.	Nucleotide sequence	Amino acid sequence
1	AGTGTGTGTTGGATTTGGACGGATGGGCAGTGGTGT	SVCWIWTDGQWC
2	TATCCGTTTTGGCTTCATCCGTCTCCGTGGGTGACT	YPFWLHPSPWVT
3	TGGTCTTGGAATGAGGAGGTTTGGTTTCCATTTACG	WSWNEEVWFPFT
4	ATGTGTTCTCATTGTCATGTGGCTCTTTCTGGGCCG	MCSHCHVALSGP
5	CATTCGCAGCATGTTTCTCTTGTTTCGTGGCATTGG	HSQHVSLVSWHW
6	GCGTAGTCGTTGACTCTGTGGCATCATTTTAGTATG	AQSLTLWHHFSM
7	TCTTCTTGGTTGATGCATATGGATGGGCGTGCTATT	SSWLMHMDGRAI
8	CAGGGTCCGAATGAGGGTTTGCTGCCGTTTTGGACT	QGPNEGLLPFWT
9	GTTCCGGCTAAGCGTGATATGACTTATTTTTCTACT	VPAKRDMTYFST

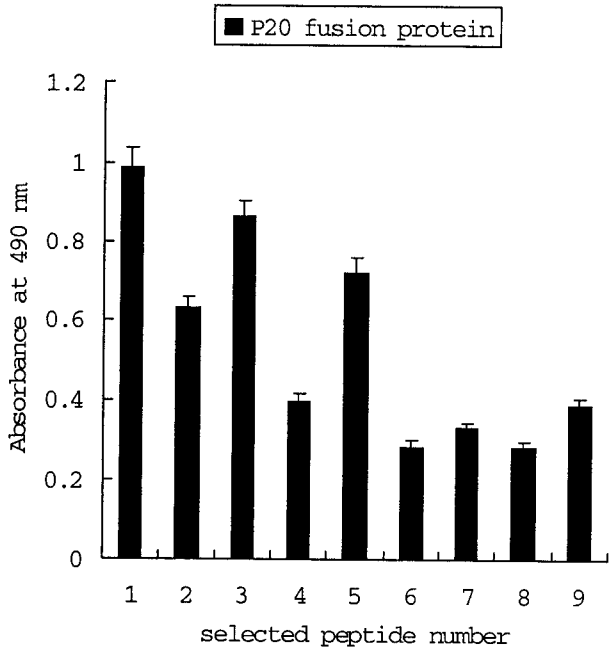

Fig. 2
The binding affinity of 9 selected peptides to the P20 fusion protein as detected by ELISA

randomly selected, amplified and assayed by ELISA. Thirty-two plaques which had definite affinity to the P20 fusion protein and had no affinity to GST were obtained and sequenced (Table 3). According to the sequences of DNA fragments encoding P20-binding peptides from selected phages 9 totally different peptides were obtained. Among those 32 clones, 21 clones belonged to peptide 1 and 4 clones belonged to peptide 2. No functional domains were found in the 9 peptides. The common motif WxWxxxxW was detected in peptides 1 and 3, and the motif PFW was found in peptides 2 and 8. The ELISA A_{490} of the 9 selected peptides ranged from 0.28 to 0.988, and peptides 1, 3 and 5 showed the strongest signals (Fig. 2).

Forty plaques from the third round of phage display screening were selected randomly for nucleotide sequencing. The latter revealed 19 different deduced amino acid sequences. Among them, the sequence of peptide 2 was repeated 17 times, the sequence of peptide 8 was repeated 4 times, and the sequence of peptide 1 was repeated 3 times. These results contradicted the data mentioned above and might be caused by low titers of amplified phages leading to weak ELISA signals. Also it might be caused by a non-random selection of plaques. No consensus motif(s) or protein domain(s) were found among the 19 peptides.

CD analysis

Interactions of selected peptides with P20 were further characterized by CD spectroscopy. The results demonstrated that P20 underwent a substantial conformational change upon binding to all selected peptides (Fig. 3). The spectra of fusion proteins with and without the control peptide (X-6) which had no affinity to P20 were almost the same, but the spectra of P20 with the 9 selected peptides differed significantly from those with the control peptide (Fig. 3). These differences were much greater than the standard deviations of these measurements. The interactions between the selected peptides and P20 caused the changes in shape and peak position of the curve (Fig. 3). The spectra of the P20 fusion protein with the control peptide and peptide 1 at 193 nm, 208 nm and 221 nm differed over 20%. The secondary structures of the control peptide and the selected peptides were investigated and significant differences were found (Table 4).

Comparison of the spectra of P20 combined with each of the 9 peptides demonstrated that the peptides 1 and 4 led to greatest differences while the remaining 7 peptides behaved similarly. This result suggested the 7 peptides bound to the same site of P20 caused the same conformational change of P20, while peptides 1 and 4 bound to another two sites of P20. The function of a protein is closely related to its conformation. The protein function is altered or even lost if its structure is changed. When the selected peptides bound to P20 its conformation changed significantly and its function

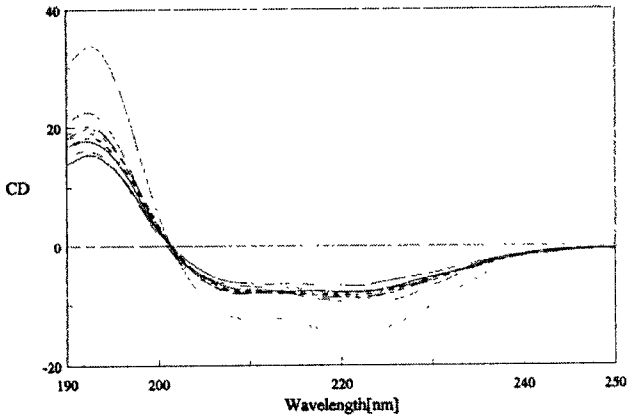

Fig. 3
CD spectra of P20 with the selected peptides

The non-binding peptide (negative control, the uppermost curve).

Table 4. Secondary structure of P20 as influenced by selected peptides

Peptide No.[a]	α-helix (%)	β-sheet (%)	Turn (%)	Random (%)
X-6 (control)	64.1	0.0	4.2	31.6
1	67.7	0.0	0.0	32.3
2	54.9	14.3	4.4	26.4
3	58.5	13.5	0.7	27.3
4	68.4	2.5	1.0	28.1
5	58.1	7.0	6.7	28.2
6	58.9	11.6	2.8	26.8
7	63.4	2.7	5.6	28.3
8	56.0	12.7	2.3	28.9
9	54.5	3.3	8.6	33.6

[a]The peptide added to P20.

might be influenced either. This assumption suggests that the selected peptides might inhibit the function of P20 and could be candidate inhibitors of replication and/or transmission of RSV.

Detection of RSV with selected phage-displayed peptides

The selected phage-displayed peptides were used to assay their binding affinity to a raw protein extracted from rice leaves infected with RSV. These phages gave significantly stronger ELISA signals than those obtained with a raw protein extracted from healthy rice leaves or with a peptide with no affinity to P20 (both ELISA A_{490} values were lower than 0.05, Fig. 4). These results indicated that the phage-displayed peptides could be used to diagnose RSV in rice.

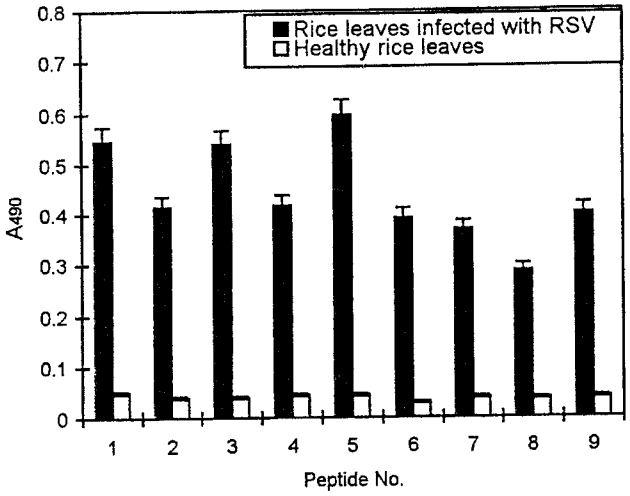

Fig. 4
Detection of RSV with the selected phage-displayed peptides

A_{490} of negative controls (wells coated with a protein from healthy rice leaves and phages displaying a non-binding peptide) were below 0.05.

Discussion

The purpose of screening peptides that could bind to the P20 protein from the phage display library is to isolate candidate inhibitors of replication and transmission of RSV. It was confirmed that most selected peptides bound to P20 at the sites that coincided with natural ligand-binding sites, and consequently acted as antagonists or agonists of natural protein-protein interactions (Brian *et al.*, 1998). Natural binding sites possessed features that predisposed the selected peptides for ligand binding. Delano *et al.* (2000) have isolated peptides that bound to the constant fragment of immunoglobulin G (IgG-Fc) and have found that a consensus binding site that interacted with at least four natural proteins was also the preferred site for peptide binding. This result suggested that peptides could be inhibitors of protein functions because a lot of binding peptides is likely to target biologically relevant sites. Furthermore, binding peptides inhibit protein interactions and disrupt protein functions *in vitro* (Brian *et al.*, 1998). Thus it was possible to select peptides binding to a viral core protein and interrupting replication and transmission of RSV.

Eckert *et al.* (1999) synthesized a D-peptide according to the sequence of a selected peptide from phage display library for D-gp41 of HIV-1. The D-peptide could bind to

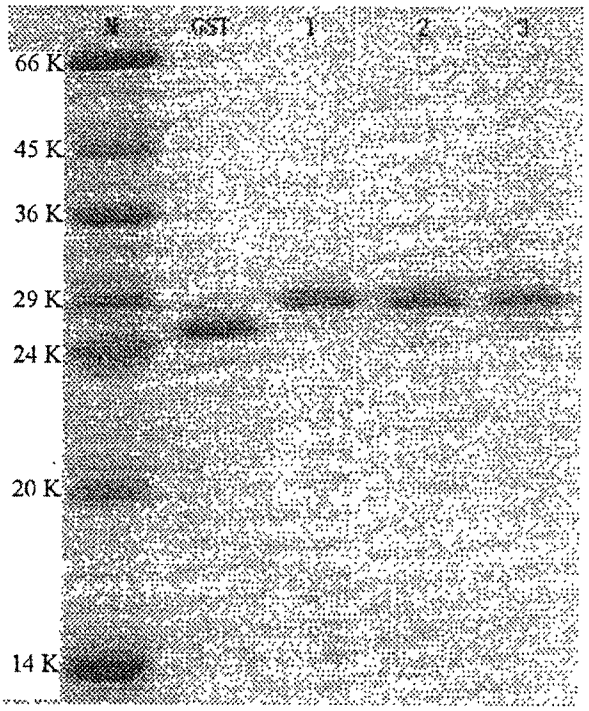

Fig. 5
SDS-PAGE of purified fusion proteins

Protein size marker (lane M), GST (lane GST), and the fusion proteins containing GST and peptides 1, 2, and 3, respectively (lanes 1, 2, and 3).

gp41 and so to inhibit HIV-1 binding to the cell membrane receptor and entering into the cell *in vitro* and *in vivo* (Eckert *et al.*, 1999). These results confirmed that binding peptides could be inhibitors of a viral protein function.

Based on the abovementioned results and the particularity of RSV transmission, the screening of peptides binding to P20 was chosen as a strategy to find an inhibitor disrupting replication and/or transmission of RSV. Because there were no efficient methods of transformation of the planthopper and of detection of small amount of virus variants in the planthopper, the detection of changes in the P20 protein conformation was chosen as a marker of disruption of the P20 function. The CD spectra of P20 with the selected binding peptides and with the non-binding peptide showed that the P20 conformation was significantly changed by the binding peptides but remained unchanged by the non-binding peptide.

Since the function of a protein depends on its structure/conformation and an alteration of its structure/conformation may alter also its function, the selected peptides under study possibly altered or even disrupted the function of P20. The selected binding peptides could be candidate inhibitors of replication and/or transmission of RSV.

The success of selecting binding peptides highlighted the potential of this technique for developing peptide-based resistance to plant pathogens. The peptide genes could be transferred into the rice plant, the planthopper vector or *Wolbachia*, and the products of these genes could bind to and inactivate RSV in the host. In addition, the peptides could be used to detect accurately RSV in rice and the planthopper in surveillance of the disease development, thus avoiding a cross-reaction caused by a polyclonal antiserum and difficulties in preparing a polyclonal antiserum. The expression of the peptide-GST fusion proteins provided a tool to confirm the inhibition of the P20 protein by the selected peptides *in vivo* and to analyze the function of P20 and interaction between the virus and the vector.

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References

Brian KK, Alexei VK, Robin H (1998): From peptides to drugs via phage display. *Drug Discover Today* **8**, 370–378.

Delano WL, Ultsch MH, de Vos AM, Wells JA (2000): Convergent solutions to binding at a protein-protein interface. *Science* **287**, 1279–1283.

Eckert DM, Malashkevich VN, Hong LH, Carr PA, Kim PS (1999): Inhibiting HIV-1 Entry: Discovery of D-peptide inhibitors that target the gp41 coiled-coil pocket. *Cell* **99**, 103–115.

Falk BW, Tsai JH (1998): Biology and molecular biology of viruses in the genus *Tenuivirus*. *Annu. Rev. Phytopathol.* **36**, 139–163.

Gingery RE, Nault LR, Bradfute OE (1981): Maize stripe virus: characteristics of a new virus class. *Virology* **182**, 99–108.

Gough KC, Cockburn W, Whitelam GC (1999): Selection of phage-display peptides that bind to cucumber mosaic virus coat protein. *J. Virol. Methods.* **79**, 169–180.

Griffiths AD, Duncan AR (1998): Strategies for selection of antibodies by phage display. *Curr. Opin. Biotechnol.* **9**, 102–108.

Hayakawa T, Zhu Y, Itoh K, Shimamoto K, Toriyama S (1992): Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus. *Proc. Natl. Acad. Sci. USA* **89**, 9865–9869.

Ishikawa K, Omura T, Hibino H (1989): Morphological characteristics of rice stripe virus. *J. Gen. Virol.* **70**, 3465–3468.

Kakutani T, Hayano Y, Hayashi T, Minobe Y (1990): Ambisense segment 4 of rice stripe virus: possible evolutionary relationship with phleboviruses and uukuviruses (Bunyaviridae). *J. Gen. Virol.* **71**, 1427–1432.

Kakutani T, Hayano Y, Hayashi T, Minobe Y (1991): Ambisense segment 3 of rice stripe virus: the first case of a virus containing tow ambisense segments. *J. Gen. Virol.* **72**, 465–468.

Qu Z, Liang D, Harper G, Hull R (1997): Comparison of sequences of RNAs 3 and 4 of rice stripe virus from China with those of Japanese isolates. *Virus Genes* **15**, 99–103.

Qu Z, Shen D, Xu Y (1999): Western blotting of RSV gene products in rice and insects. *Chin. J. Genet.* **26**, 331–337.

Ramirez BC, Haenni AL (1994): Molecular biology of *tenuiviruses*, a remarkable group of plant viruses. *J. Gen. Virol.* **75**, 467–475.

Rodi DJ, Makowski L (1999): Phage-display technology: finding a needle in a vast molecular haystack. *Curr. Opin. Biotechnol.* **10**, 87–93.

Rodi DJ, Janes RW, Sanganee HJ, Holton RA, Wallace BA, Makowski L (1999): Screening of a library of phage-displayed peptides identifies human Bcl-2 as a Taxol-binding protein. *J. Mol. Biol.* **285**, 197–203.

Sidhu SS, Lowman HB, Cunningham BC, Wells JA (2000): Phage display for selection of novel binding peptides. *Methods Enzymol.* **328**, 333–363.

Takahashi M, Toriyama S, Hamamatsu C, Ishihama A (1993): Nucleotide sequence and possible ambisense coding strategy of rice stripe virus RNA segment 2. *J. Gen. Virol.* **74**, 769–773.

Toriyama S (1995): Viruses and molecular biology of *Tenuivirus*. In Singh RP, Siongh US, Kohmoto K (Ed.): *Pathogenesis and Host Specificity in Plant Diseases.* Vol.3, *Viruses and Viroids.* Pergamon, Oxford, pp. 211–223.

van Regenmortel MHV, Fauquet CM, Bishop DHL (2000): *Virus Taxonomy. Classification and Nomenclature of Viruses. Seventh Report of the International Committee on Taxonomy of Viruses.* Academic Press, San Diego-San Francisco-New York-Boston-London-Sydney-Tokyo.

Wallace BA, Teeters CL (1987): Differential absorption flattening optical effects are significant in the circular dichroism spectra of large membrane fragments. *Biochemistry* **26**, 65–70.

Werren JH (1997): Biology of Wolbachia. *Annu. Rev. Entomol.* **42**, 587–609.

Wilson RK (1993): High-throughput purification of M13 templates for DNA sequencing. *Biotechniques* **15**, 414–422.