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Epitope identification and *in silico* prediction of the specificity of antibodies binding to the coat proteins of *Potato Virus Y* strains

Hans Keller[1], Rikus Pomp[1], Jaap Bakker[2] and Arjen Schots[1]
[1]*Laboratory of Molecular Recognition and Antibody Technology, Wageningen University;* [2]*Department of Nematology, Wageningen University, Binnenhaven 5, P. O. Box 8123, 6700 ES Wageningen, The Netherlands (Phone: +31317485261; Fax: +31317484254; E-mail: arjen.schots@wur.nl)*

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Abstract

A phage library containing 2.7×10^9 randomly expressed peptides was used to determine the epitopes of three monoclonal antibodies that bind to the coat protein of *potato virus Y*. Construction of the consensus sequences for the peptides obtained after three selection rounds indicated that each antibody recognized a different epitope located within the first 50 N-terminal amino acids of the coat protein. The location of the epitopes was confirmed by heterologous expression of the N-terminal part of the coat protein in *Escherichia coli*, and, subsequently, by performing an immunological test with the three antibodies. The accuracy of the phage library was demonstrated by predicting *in silico* the cross-reactivity of the three antibodies with other potyvirus family members. ELISA and *in silico* predictions revealed the same results in almost every case. The potential of peptide phage libraries to optimize the use of antibodies in plant virology is discussed.

Introduction

The genus *Potyvirus* (family *Potyviridae*) consists of around 200 virus species that account for almost 25% of known plant viruses (Shukla et al., 1994). *Potato virus Y* (PVY) is the type member which can cause significant yield losses in potato (*Solanum tuberosum l.*), pepper (*Capsicum sp.*) and tomato (*Lycopersicon esculentum mill.*). Under natural circumstances aphids transmit PVY in a non-persistent manner, which may lead to severe epidemics. Three PVY strains have been reported and their differentiation is based on the symptoms in test plants and resistance responses. PVY^O (common strain) occurs worldwide and causes severe systemic crinkle symptoms in potato and systemic mottling in tobacco plants. The PVY^N strain (tobacco veinal necrosis strain) induces necrotic symptoms in tobacco and mild symptoms in almost all potato cultivars. Most of the potato cultivars are hypersensitive for the PVY^C strain (stipple streak strain). In tobacco this strain induces systemic mottling. Additionally PVY^{NTN} acts as a group of isolates that is able to cause severe chlorotic mosaic in potato leaves and necrotic ring spots in tubers (Jones, 1990; Le Romancer et al., 1994).

Millions of immunoassays are annually carried out on potato seed tubers in order to prevent uncontrolled spreading of PVY. Enzyme-linked immunosorbent assays (ELISAs) are rapid and reliable, and are based on antibodies directed against the viral coat protein. To ensure reproducibility and comparability of these assays, monoclonal antibodies (MAbs) are gradually replacing the traditionally used polyclonal antibodies. MAbs contribute to the standardization as they bind to only one epitope and because batch-to-batch variation is absent. Subsequently it would be desirable to determine the epitopes recognized

by these MAbs. Knowledge of the molecular structure of the epitopes, especially if they are continuous, provides a tool to develop artificial antigens that can be used as reliable positive controls. At present positive controls are in most cases based on infected plant material, which may result in serious problems. The samples are often unstable (Cerovska and Filigarova, 1995) or difficult to standardize, and are often prohibited by quarantine authorities as they may contribute to an unwanted spread of pathogens. Alternatives for positive controls are therefore urgently needed. Synthetic peptides or small proteins expressed in bacteria containing the antigen moieties are an attractive alternative for infected plant material.

The first step in constructing genes encoding these artificial antigens is to identify the epitope recognized by the antibody that is used in the immunoassay. Revealing the molecular basis of epitopes can be achieved by using a peptide phage library or a solid phase peptide library (PEPSCAN). Phage display has the advantage that the same library can be used for different antigens, whereas for PEPSCAN each time new peptides have to be synthesized. Peptide phage display (Smith, 1985) has successfully been applied to identify epitopes of monoclonal (Cwirla et al., 1990; Smith, 1991; Wobus et al., 2000) and polyclonal antibodies (Folgori et al., 1994), both of continuous and conformational make-up. Remarkably, peptide phage libraries have hardly been applied in plant virology for this purpose (He et al., 1998).

Knowledge of the molecular structure of epitopes provides the possibility to predict *in silico* the cross-reactivity of antibodies. The epitopes can directly be used to screen the databases for identical or similar sequences, either by performing a general search or a specific search among family members. The outcome of the alignment will indicate the specificity of the antibody and will make random testing of related members superfluous.

Our objective was to determine the epitopes of three antibodies binding to the coat protein of PVY^N. Several binding peptides were selected from a twelve amino acid M13 peptide library. The sequences from these peptides were used to construct consensus sequences, which were aligned with the PVY coat protein, thereby revealing the position of the epitopes. In addition, these epitope sequences were used to predict *in silico* the antibody cross-reactivity with other potyviruses.

Materials and methods

Propagation and purification of the PVY virus

Nicotiana rustica plants were inoculated with PVY^C (isolate 509 Zeeuwse Blauwe, 1969, the Netherlands), PVY^N (isolate 602 Bintje, 1963, the Netherlands), PVY^{NTN} (isolate 752 Hermes, Austria) or PVY^O (isolate 702 Bintje, 1957, the Netherlands). Four weeks after inoculation, leaves were harvested. About 100 g of infected leaf materials were crushed in 300 ml ice cold extraction buffer (0.05 M K_2HPO_4, 0.01 M EDTA, 1% sodium sulfite, pH 7.6, 5% ethanol), filtered and centrifuged at 8,000 × *g* for 30 min at 4 °C (Sorval SLA-3000). The supernatant was stirred for one hour at 4 °C in extraction buffer containing 1% Triton X-100 (final concentration). The virus particles were pelleted by centrifugation in a Beckman SW41 (30,000 rpm, 3.5 h at 4 °C.) on a 20% sucrose cushion. The pellet containing the virus was resuspended in PBS and stored at −80 °C.

Monoclonal antibodies

The PVY antibodies Y-2, Y-3,4 and Y-5 were selected and produced according to Boonekamp et al. (1991). Antibodies Y-2 and Y-5 were conjugated to alkaline phosphatase according to the supplier's manual (Boehringer).

Phage display

The PhD-12 phage peptide library (New England Biolabs) containing 2.7×10^9 individually expressed peptides was used to map the epitopes. The PhD-12 phage display peptide library is based on a combinatorial library of random twelve-mer peptides fused to a minor coat protein (pIII) of M13 phage. In the first selection round immunosorbent Maxi-sorb tubes (Nalgene Nunc) were coated with 100 μg ml^{-1} antibody in coating buffer (50 mM carbonate buffer, pH 9.8). In the second and the third round, respectively, 10 and 1 μg ml^{-1} antibody was used for coating. In all selection rounds the phage library was incubated for 1 h at room temperature. Tubes were washed 10 times

with TPBS (PBS containing 0.1% Tween-20) and thereafter 10 times with PBS. The bound phages were eluted with 0.2 M Glycine-HCl (pH 2.2), 1 mg ml^{-1} BSA. After 10 min the eluted phages were transferred to a clean tube and neutralized with 150 µl 1 M Tris-HCl (pH 9.1) and used for amplification.

ELISA

Maxisorp immuno ELISA plates (96 well, Nalgene Nunc) were coated overnight in coating buffer at 4 °C with Y-2, Y-3,4 or Y-5 (1 µg ml^{-1}), blocked for 1 h at room temperature with 5% skimmed milk in PBS containing 0.1% Tween-20. Thereafter the plates were incubated with medium culture containing phages from the third panning round. The culture medium with phages was diluted 1:10 with PBS containing 0.1% Tween-20. After incubation the plates were washed and incubated with 1:2000 anti-fd (Sigma), washed again and incubated with 1:2000 anti-rabbit alkaline phosphatase (Sigma). All incubations were performed for 1 h at 37 °C and the antibodies were diluted in PBS containing 0.1% Tween-10 and 0.1% BSA. To determine the cross-reaction of Y-2, Y-3,4 and Y-5 with other potyvirus family members, ELISA plates were coated with the antibodies (1 µg ml^{-1}) overnight at 4 °C. After blocking, virus infected plant material (1 g leaf material ground in 10 ml PBS) was used for incubation. For detection of the different viruses, Y-2 and Y-5 conjugated with alkaline phosphatase were used.

Production of thio-PVY

RNA was extracted from purified PVY^N and converted into cDNA using an Oligo-dT primer and SuperScriptII reverse transcriptase (Invitrogen). The produced cDNA was used as the template to amplify 165 nucleotides, coding for the first 55 amino acids of the N-terminus of the coat protein. The primers PVYnFor 5'-GGAAATGACACAATCGATGC-3' and PVYnRev 5'-TCTCA TTTTGGACGTGATAGCTT-3' were used in the PCR reaction. The amplified fragment was ligated into the pBAD/Thio-TOPO vector (Invitrogen), electroporated into TOP10 cells (Invitrogen) and sequenced. ER2537 cells (New England Biolabs) were transformed with the plasmid containing the correct insert. One of these clones was grown at 37 °C in shaker-flasks in a total of 1 l of 2xTY containing 100 µg ml^{-1} ampicillin. The cells were induced with 0.02% arabinose (final concentration) at $OD_{600} = 1$ and grown for an additional 4 h. The cells were removed from the culture medium by centrifugation, 20 min 8,000 rpm at 4 °C (Sorval SLA-3000) and resuspended in lysis buffer (50 mM potassium phosphate, pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole). The cells were lysed by sonication at 20% amplitude for 20 s with a pause of 40 s (Vibra Cell) and kept all the time on ice. Cell debris was removed by centrifugation, 30 min 20,000 rpm at 4 °C (Sorval SS-34). The fusion protein (thio-PVY) was purified from the supernatant using a Ni-NTA agarose column (Qiagen) according to the Qiagen Ni-NTA purification protocol.

Results and discussion

Peptide selection and epitope determination

A random peptide M13 phage library was employed to determine the exact amino acid sequence of the epitopes recognized by the antibodies. In three rounds, binding peptides were selected. To favour the conditions for strong binders, the coated antibody concentration was decreased in each next selection round. An ELISA using the amplified phages selected in the first round showed no detectable binding signals. Phages selected in the second round showed little binding that increased significantly for phages selected in the third round (data not shown). Ten individual phage clones selected by each of the three antibodies from the third selection round were picked randomly and tested for their binding capacity (Figure 1). The result showed that all the peptides bound specifically to the antigen binding site and not to other parts of the antibody. The amino acid sequence of the peptides was determined by sequencing the single stranded phage DNA. The peptides selected for each antibody were placed in order of affinity and were aligned. From the alignment the consensus sequence was determined (Figure 2).

Peptides binding to antibody Y-3,4 have in common a sequence containing serine- threonine - x-lysine (ST*x*K), wherein the x stands for any

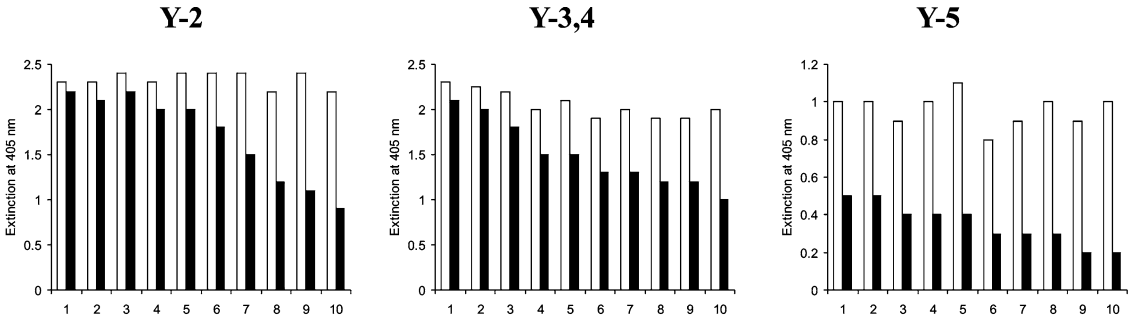

Figure 1. Binding and competition assay of selected peptide phages binding to the antibodies. White bars represent the binding and dark bars represent the competition of the peptide phages with the natural antigen (PVY^N). Phages are placed in order of competition.

Figure 2. Alignment of the sequenced peptides binding to the antibodies Y-3,4, Y-2 or Y-5. The peptides are numbered from 1 to 10 in order of affinity. The peptides with number 1 show the highest affinity. Gray boxed letters represent the residues putatively involved in binding to the antibody. The deduced consensus is presented in bold type under the aligned sequences. The lower case x represents any amino acid and h represents a hydrophobic residue.

amino acid. The peptide showing the weakest binding lacks the serine residue, indicating that this residue consolidates the binding. Since no peptides were found without the threonine-x-lysine motif, we concluded that these residues are absolutely essential for binding. Furthermore, almost every peptide contained a glycine (G) residue, which was located either on one or two positions upstream of the serine. Absence of this glycine residue resulted in weaker binding characteristics as is shown by peptide number 9. These findings resulted in the consensus sequence GGSTSK.

The peptides binding to antibody Y-2 all contained the sequence aspartic acid-x-asparagine (D*x*N). Beside this motif a lysine (K) residue was also present in all of the peptides. However, the location of the residue was not fixed and it was found either two or three amino acid positions upstream of the aspartic acid residue. Remarkably this change in position did not have any influence on the binding strength (see Figure 2). We therefore concluded that the lysine residue might be part of a conformational epitope. Most of the peptides also contained alanine (A), and when present this amino acid followed after the asparagine residue. In two of the peptides this alanine was substituted for valine, which did not have an effect on the binding. In most of the peptides a serine residue (S) was found after the alanine. The consensus sequence constructed from the peptides binding to Y-2 was KxDxNAS.

Arginine (R) was present in all the peptides binding to Y-5 and was therefore used as a starting position to align the peptides. Half of the peptides had a proline (P) preceding the arginine residue. The residue most often found on the third position was leucine and in fewer situations valine or isoleucine, thereby indicating a strong preference of the antibody for a hydrophobic residue. On the fourth position lysine was found in many peptides and on the fifth position half of the peptides contained alanine. On the sixth position again a

hydrophobic residue was found. From these findings we concluded that the consensus sequences for Y-5 is PRLKAh, where h represents a hydrophobic residue.

Location of the epitopes

To obtain the coat protein sequences of the four PVY strains used in our experiments, mRNA was extracted from the purified viruses and converted into cDNA. The region coding for the N-terminal part of coat protein was sequenced. Figure 3 shows the alignment of the coat proteins from the four different strains, and the consensus sequences representing the putative epitopes. The consensus sequence for the epitope of Y-3,4 was present in the N and NTN strains and was found at the amino acid positions 8 to 13. The TxK motif, necessary for binding, was absent in both the PVY^C and PVY^O strains, explaining the specificity of antibody Y-3,4 for the N and NTN strains. The DxN motif responsible for binding antibody Y-2 was found further downstream at positions 33 to 36. The alignment demonstrated that this motif was present in the coat protein of all strains, explaining the cross-reactivity of the antibody. In two of the strains, the alanine following the aspartic acid was substituted for valine. As already shown for the peptides, this substitution did not have an influence on the binding. The lysine in the consensus was not found at the same position in the coat protein. This result, together with the changing position of the lysine in the peptides, makes it plausible that the residue is part of a conformational epitope. The antibody Y-5 recognized all four strains, and in all these strains the sequence PRIKAI was found at positions 45 to 50, matching convincingly with the consensus sequence.

To confirm the *in silico* analyses, the coat protein of PVY was also heterologously expressed. The N-terminal part of the PVY^N coat protein gene coding for the first 55 amino acids was amplified and cloned in frame with the C-terminus of thioredoxin. Protein analysis showed that the fusion protein (thio-PVY) was expressed at high levels in *E. coli* and could be purified using a nickel column. Thio-PVY was used in ELISA to confirm that the epitopes are indeed located in this part of the coat protein. The ELISA plates were coated with the antibodies and incubated with thio-PVY. The captured thio-PVY was detected using either Y-2 or Y-5, both conjugated to alkaline phosphatase. The results, presented in Figure 4, show that the three antibodies recognized specifically thio-PVY and not thioredoxin without fusion.

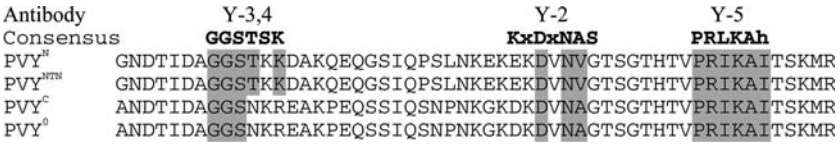

Figure 3. N-terminal parts of the coat proteins of the four PVY strains aligned with the consensus sequence of the binding peptides. The gray blocks mark the residues also present in the consensus sequence.

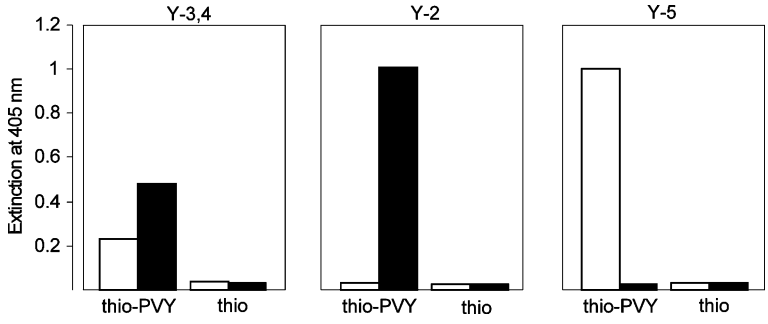

Figure 4. Confirmation of the location of the epitopes using the heterologously expressed fusion protein (thio-PVY). Antibodies Y-3,4, Y-2 or Y-5 were coated and incubated with thio-PVY or with the negative control thioredoxin (thio). The captured thio-PVY was detected with Y-2-AP (white bars) or Y-5-AP (dark bars), which were conjugated with alkaline phosphatase.

From this result it was obvious that although Y-2 and Y-5 gave high signals with thio-PVY, the signal of Y-3,4 was considerably lower. Possibly the translocation of the epitope from the very end of the N-terminus of the coat protein into the middle of the fusion protein had a negative effect on binding. Nevertheless, the signal was still far above the background level, indicating specific binding.

In silico *prediction and experimental validation of cross-reactivity with other potyviruses*

Antibodies raised against PVY show in many cases cross-reactivity with other potyviruses (Joisson et al., 1992; Jordan, 1992). The classical method to determine the specificity of monoclonal antibodies is to test them randomly with other potyviruses. To test the ability to predict the cross-reactivity of antibodies *in silico*, the determined epitopes were aligned with the coat protein sequences of the seven potyviruses and these viruses were tested with the antibodies in an ELISA (see Figure 5). For the epitope of antibody Y-3,4 no sequence match was found and ELISA confirmed that the antibody did not bind to any of these seven potyviruses. The motifs DxNV or DxNA, essential for binding Y-2, were present in five of the seven potyviruses and ELISA showed binding of the antibody whenever these motifs were present. The PRIKAI sequence, the epitope of Y-5, was found in two of the seven potyviruses. Here the ELISA showed, in contrast to the peptides displayed on phage, that the antibody would only bind in those cases were the complete epitope sequence was present. One exception to the PRIKAI sequence was found. In the *lettuce mosaic virus* (LMV), isoleucine was substituted for threonine and this change did not inhibit the antibody binding.

The epitope motifs were also used to search in the NCBI database for identical sequences. For the epitope of Y-3,4 only homology with PVY^{N} and PVY^{NTN} was found, indicating that this antibody recognizes exclusively these strains and none of the other potyviruses stored in the database. The short epitope of Y-2 was found in 23 different family members and the epitope of Y-5 was present in 10 other potyviruses.

The results demonstrate that combining epitope mapping with database mining can be used to predict *in silico* the specificity of antibodies and can trace possible cross-reaction candidates. Unravelling the epitopes of antibodies or raising antibodies against specific regions may be one of the steps to design resistance against pathogens. Tavladoraki et al. (1993) showed that expression of a single chain antibody fragment (scFv) directed against artichoke mottled crinkle virus in tobacco resulted in a reduced incidence of infection and delayed symptom development (Tavladoraki et al., 1993). Voss et al. (1995) showed that also full length antibodies reduce the TMV infectivity in *Nicotiana tabacum* plants. Amino acid sequences in the PVY coat protein that have vital functions, and which are present in all the known virus strains could be ideal targets for designing resistance. In this aspect the N-terminal region of potyvirus coat proteins is particularly interesting; although non-essential for replication it is involved in cell to cell movement (Dolja et al., 1994, 1995). Deletion of 25 amino acids in the N-terminal region of the coat protein of *tobacco etch virus*

Figure 5. Alignment of the N-terminal part of the coat protein of PVY^{N} with the coat protein of seven other potyviruses. The gray blocks indicate the residues involved in binding, and are exclusively shown in those cases where ELISA confirmed binding of the antibody. PVY-Potato Virus Y N-strain. Sequences were obtained from the NCBI database and the accession numbers are placed between brackets. BCMNV-*Bean common mosaic necrosis virus* (U19287), CDV-*Colombian datura virus* (CAD26690), LMV-*Lettuce mosaic virus* (P31999), PRSV-*Papaya ringspot virus* (Q01901), PVA-*Potato virus* A (AAB24779), PVV-*Potato virus* V (S55510), WMV-2-*Watermelon mosaic virus* (AAA48497).

(TEV) resulted in a reduction of cell-to-cell movement in tobacco and completely abolished systemic spreading of the virus (Dolja et al., 1994). Other motifs in the N-terminal region like the DAG amino acid triad, are involved in the transmission by aphids (Atreya et al., 1990, 1991). These properties combined with the fact that most of these regions are hyperimmunogenic (Fernandez-Fernandez et al., 2002) provide excellent opportunities to design antibodies that could inhibit the spread of PVY.

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