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Detection of Tobacco mosaic virus with antibody mimics based on M13 phages

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Detection of *Tobacco mosaic virus* with antibody mimics based on M13 phages

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1. Introduction, Knowledge, Objectives

The production of pathogen-free products is of vital importance for consumer protection and hence for the product safety of horticultural products. Due to the robustness of serological methods, their relatively simple handling and suitability for routine virus testing, these methods will also have an important role in future. However, the procedures are based on finite resources, because the antibodies used for the test have to be produced and consistently validated. Therefore “antibody mimics” were developed which detect (similar to an antigen-antibody-reaction) the presence of a pathogen. In this work, phage-based molecules were used to detect *Tobacco mosaic virus* (TMV). The phage display technique was first developed in 1985 (Smith) and subsequently improved e.g. by Marks *et al*. (1992), Paschke (2006) and Koide *et al*. (2012). These so called “phage libraries” were afterwards used to identify specific protein-protein interactions with target molecules. In these libraries millions of phages, which present unique fusion proteins on their surface, are screened against target molecules e.g. streptavidin to find tightly binding single phages. Depending on the type of library, unique proteins are fused to minor or major coat proteins of the phages. In this study, the minor coat protein (P3) of a M13-phage was used, which displays 12 additional amino acids located close to the N-terminus. After the selection of appropriate phages, these can be tested and compared in standard ELISAs.
The aim of this study was the detection of TMV with antibody mimics specific to this virus. On the one hand TMV is an important virus infecting many horticultural crops on the other hand it can be easily propagated and purified for the intended study.

2. Material and Methods

TMV (strain OhioV; Koerbelin *et al*. (2012)) was mechanically inoculated in *Nicotiana benthamiana* plants. Three weeks after the inoculation TMV was purified according to Dijkstra and Jaeger (1998). A screening procedure against purified TMV for the presence of phages binding tightly to TMV was carried out with the Ph.D.™-12 phage library (New England Biolabs). According to the manufacturer the library contains 10^9 independent clones. These represent approximately 1 millionth of the 4.1 * 10^{15} theoretical possible 12-mer sequences.
TMV particles were suspended in a coating solution (0.1 M $NaHCO_3$, pH 8.6) and added to the wells of a microtiter plate. After overnight incubation the solution was discarded from the wells, which were subsequently blocked with blocking solution (0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml bovine serum albumin (BSA) in H_2O) for at least 1 hour at 4 °C. After 6

washing steps with TBST (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) + (0.1 % [v/v] Tween-20) the library was diluted with 100 µl TBST and loaded in the TMV coated wells followed by an incubation step of 60 minutes. Non-binding phages were discarded and wells were washed 10 times with TBST. The remaining bound phages were eluted with 100 µl of elution buffer (200 mM Glycine-HCl (pH 2.2), 1 mg/ml BSA) and amplified by adding the eluate to *E. coli* cells (Strain: ER2738). The infected bacterial cells were incubated for 4.5 hours at 37 °C with vigorous shacking. Bacterial cells were centrifuged for 10 minutes at 12000 g (4 °C). The supernatant was collected and phages were precipitated by adding 1/6 volume of 20 % polyethylene glycol 8000 (PEG)/ 2.5 M NaCl. After an overnight incubation at 4 °C the PEG precipitate was collected by centrifugation at 12000 g for 15 minutes at 4 °C. The pellet was further processed according to the manufacturer's instructions before the phages were suspended in 200 µl of TBS (TBST without Tween-20). 100 µl were used in additional rounds of biopanning (see Figure 1).

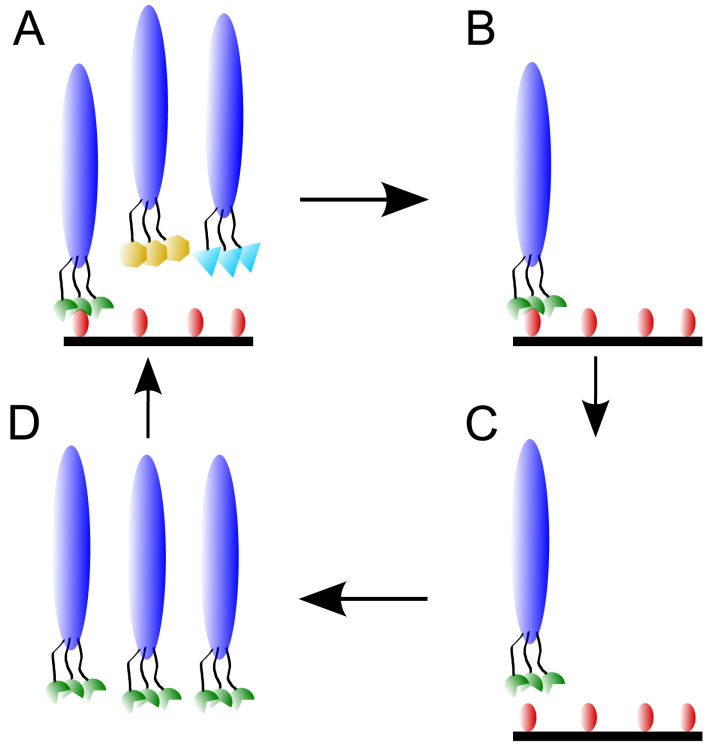

Figure 1: Schematic overview of typical biopanning procedure. A: Incubation of phage library with an immobilized target molecule; B: Washing and removal of unbound phage; C: Elution of phage(s) bound to the target molecules; D: Amplification of eluted phage(s) for subsequent rounds of biopanning.

After 3 rounds of biopanning individual phage clones were selected and propagated. The 12 amino acid extension of the P3 gene was determined by sequencing of the phage DNA. In addition, single phages were tested in an individual plate trapped „phage ELISA", where the TMV target particles were coated to microtiter plates (overnight) and blocked with blocking solution (2 % milk powder (w/v) for 2 h). Phages mixed with blocking solution were added and incubated for 1.5 h. Non-binding phages were removed by washing. For

final detection of bound phages a *horseradish peroxidase* (HRP)/Anti-M13 Monoclonal Conjugate antibody (GE Healthcare) (incubation: 1 h) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) as substrate solution in 0.05 M citric acid (pH 4.0) combined with 0.051 % hydrogen peroxide was used. The colour reaction was quantified with a BioRad microplate reader at 405 nm.

3. Results

After optimization steps (e.g. selection of a suitable microtiter plate, data not shown), three panning rounds were conducted with purified TMV. Nine phages were selected and sequenced. Five phages with sequences similar to those suspected to bind to a plastic surface and one phage containing an incomplete sequence were excluded from further tests. The three remaining phages with identical amino acids in the variable region (DWAQLTQRWYLR) were mixed and used in a phage ELISA as shown in Figure 2. A clear difference between the phage mixture (phage clones 1, 3 and 5) specifically binding to purified TMV and non-binding wild type M13 phage was found. Measuring the absorption 5 minutes after adding the substrate revealed readings tenfold higher in case of specific binding phages compared with the wild type M13 phage or with the control reactions without TMV as a target.

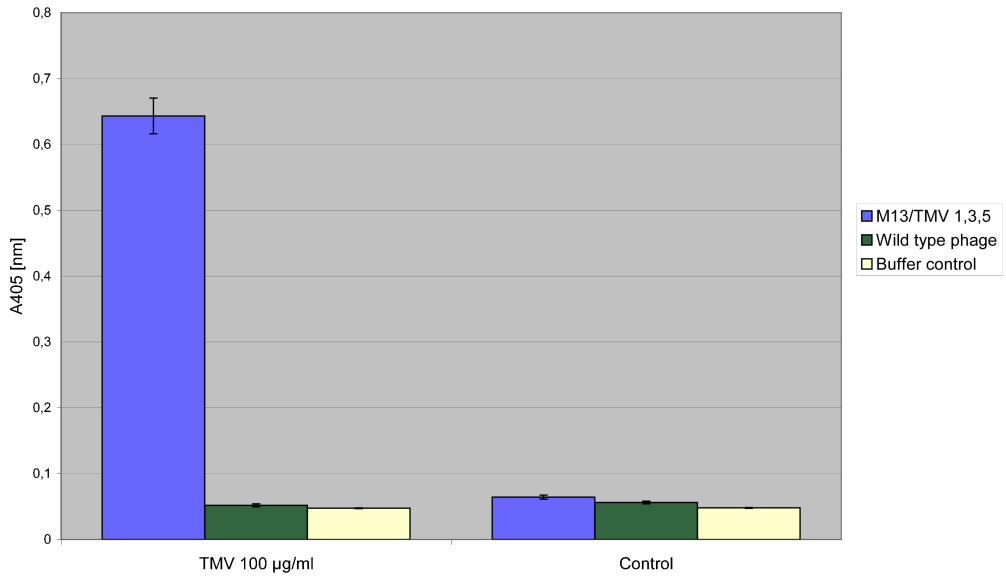

Figure 2: Phage ELISA against purified TMV and without TMV (Control). Phage clones 1, 3 and 5 possessing identical 12 amino acids in the variable region were mixed and used for the experiment. As a negative control, wild type phage and wells without TMV target were used. Readings were taken 5 min after adding substrate to the wells (n=3); bars show standard deviation.

4. Discussion

It is shown that a screening procedure with phages against purified virus particles leads to selection of phages specifically reacting with TMV in phage-ELISA experiments, revealing high readings within a few minutes. All positively tested phages revealed a stretch of identical amino acids in the variable region, which is thought to be involved in the specific binding to the target protein. Wild-type M13 phages without the specific amino acid stretch failed to detect TMV.
So far, single-chain variable fragments (scFvs) fused to M13 phages have been shown to detect plant viruses, e.g. *Tomato leaf curl New Delhi virus* (Zakri *et al*. 2010). Generally phages derived from a peptide library can be an interesting alternative compared to common scFv libraries for virus detection as shown here for TMV or for *Banana streak virus* (Heng *et al*. 2007).
Additional steps are necessary to determine the sensitivity and specificity of these phages. For this purpose it must be demonstrated that phage based antibody mimics are also able to detect different TMV strains from various host plants preferably out of crude plant saps. Furthermore it will be tested, if closely related viruses are also detected.

5. Conclusions

This study gives a first indication that antibody mimics selected from a Ph.D.™-12 phage library (New England Biolabs) against TMV could be a valuable tool to supplement and improve ELISAs for the detection of plant viruses.

6. Literature

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