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Selection of beet necrotic yellow vein virus specific single-chain Fv antibodies from a semi-synthetic combinatorial antibody library

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Abstract

Methods for the generation of monoclonal antibodies against plant viruses are limited because current hybridoma techniques do not allow efficient exploitation of the immune repertoire. Moreover, the immunization procedures often lead to a bias towards an immunodominant contaminant in the immunogen preparation and not to the plant virus itself. The selection of six different single-chain antibody variable fragments (scFv) against beet necrotic yellow vein virus from a semi-synthetic human combinatorial antibody library showed the feasibility of the phage display system. No bias towards minor contaminants in the purified virus preparation was observed in ELISA, as all the selected scFvs reacted only with beet necrotic yellow vein virus infected plant homogenates. In addition, two of the isolated beet necrotic yellow vein virus-specific scFvs could be produced in *E. coli* as a scFv fusion protein with alkaline phosphatase, and were applied in ELISA as specific ready to use antibody-enzyme conjugates. Because of their specificity, these antibodies have potential to be used as reagents in sensitive diagnostic assays for routine testing for beet necrotic yellow vein virus in sugar beets.

Abbreviations: BNYVV – beet necrotic yellow vein virus; MAbs – Monoclonal Antibodies; PAbs – Polyclonal antibodies; PhAbs – phage-antibodies; scFv – single-chain variable fragment; scFv-AP/S – single-chain variable fragment genetically fused to alkaline phosphatase.

Introduction

Most diagnostic assays for plant viruses utilize polyclonal antibodies (PAbs) or monoclonal antibodies (MAbs). MAbs do have some advantages over PAbs. They are homogeneous and well defined reagents which allow standardization of assays between different laboratories (Regenmortel, 1986; Regenmortel, 1990). Due to the fact that MAbs recognize single epitopes they may give less cross-reactions, discriminate between viral strains (Torrance et al., 1986; Torrance, 1995) and MAbs directed against conserved epitopes may allow grouping of virus isolates for epidemiological and taxonomical studies.

It is difficult to raise MAbs to some plant viruses because minor contaminants in the isolated virus fraction exhibit immunodominance (George and Converse, 1988). This was shown to be the case for beet necrotic yellow vein virus (BNYVV), a furovirus causing rhizomania (Tamada and Baba, 1973). During purification the virus aggregates with host cell constituents (Putz and Kuszala, 1978) and only a few MAbs were obtained reacting specifically with BNYVV, while the majority of the MAbs reacted with healthy plant antigens. It is noteworthy that polyclonal antisera collected at the time of spleen removal had high virus-specific and low anti-healthy plant sap titers. The same phenomenon has been observed in developing MAbs to

cauliflower mosaic virus (George and Converse, 1988) and blueberry red ringspot virus (Hepp and Converse, 1986).

Various methods have been described to reduce the immune response to plant contaminants to enhance the yield of virus-specific MAbs. For instance, the induction of immunological tolerance in neonatal mice (Hsu et al., 1990), the use of immunosuppressive agents like cyclophosphamide (Matthew and Sandrock, 1987), or use of antigen based B-cell selection systems (Parks et al., 1979). The common drawback of these methods is that the conditions have to be established for each virus.

Current hybridoma methods (Köhler and Milstein, 1975) for the generation of MAbs against plant pathogens do not satisfactorily exploit the immune repertoire. While the antibody repertoire is estimated to consist of over 10^8 different antibodies, only a few thousand different hybridoma clones are obtained, of which on average less then 1% produce antigen binding antibodies. Thus, the chance that a useful hybridoma derived MAb will be obtained is low. This diversity and efficiency problem was recently addressed by advances in molecular immunology. Forced cloning of DNA, encoding antibody variable heavy (V_H) and light (V_L) chain domains with RT-PCR (Orlandi et al., 1989; Sastry et al., 1989), allowed amplification of the antibody repertoire encoding sequences. Expression of V_H and V_L domains as a single-chain Fv (scFv) fusion protein (Figure 1) in *E. coli* was made possible by joining them with a flexible linker peptide (Huston et al., 1988). Cloning of a pool of scFv encoding genes, in which V_H and V_L were randomly combined, enabled the generation of large combinatorial antibody libraries which have a diversity comparable to the natural immune repertoire (Huse et al., 1989; Marks et al., 1991). This, in combination with the display of functional antigen binding fragments on the tips of filamentous phage, created a powerful system (Hoogenboom et al., 1991; McCafferty et al., 1990; Clackson et al., 1991) to obtain specific MAbs. The phage display system (Figure 1) allows direct selection of rare specificities from combinatorial antibody libraries through successive rounds of phage growth and selection for antigen binding.

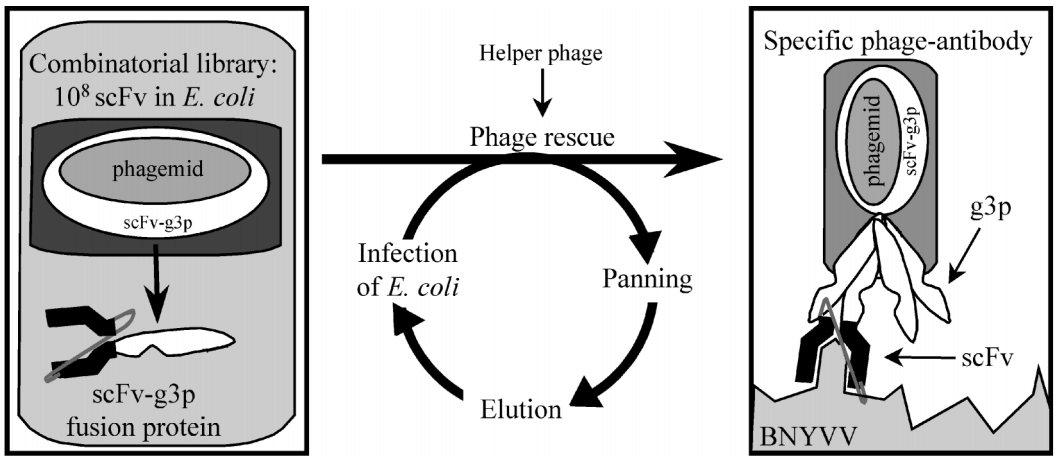

Figure 1. Outline of antibody engineering and selection of antigen-specific antibodies by phage display technology. The minimal requirements of an antibody for antigen recognition are located in the antigen-binding site. The V_H and V_L domains, joined by a linker peptide encoding DNA fragment, are inserted into a phagemid vector as a single-chain Fv segment. In the scFv encoding phagemid an ampicillin resistance gene, an origin of replication for *E. coli* and a packaging signal which is required for phage-assembly are present. Depending on the *E. coli* strain used, the scFvs can be expressed as soluble proteins in HB2151 bacteria, wherein translation halts at an amber stop codon (located between scFv and g3p). Fusion proteins, of scFv with the minor coat protein of filamentous phage Fd, are obtained from TG1 bacteria in which translation proceeds at the amber codon. Selection of BNYVV-specific scFv-antibodies from a combinatorial antibody library can be achieved using phage display. Helper phages, which contain the entire phage genome but lack an efficient packaging signal, are used to 'rescue' phagemids from a combinatorial antibody library. When both helper phage and phagemid are present within the same bacterium, phage-antibodies are assembled which carry scFv-antibodies on their surface and contain the scFv encoding phagemid vector. Consequently, within PhAbs, the genotype and the phenotype are linked. In order to select for antigen specificity, PhAbs, rescued from a combinatorial antibody library, are allowed to bind to immobilized BNYVV (Panning). Washing removes the PhAbs that lack affinity for BNYVV. Bound PhAbs are eluted and the selected PhAbs are applied into sequential rounds of panning until the desired affinity is obtained.

For instance, Bradbury et al. (1993) showed the feasibility of this technique by selecting antigen binding phages from a pool of non-binding phages, even at a ratio of one binding in 10^9 irrelevant phages. In addition, the phage display system may also prove to be a more universal method to obtain specific MAbs, since the antibody specificities are not biased towards immunodominant epitopes.

To study the versatility of the phage display system, we have applied it to BNYVV. This virus was chosen as a model system because it is a classic example of the difficulties outlined in raising specific MAbs. We examined the feasibility of the phage display system in isolating a diverse repertoire of BNYVV-specific antibody fragments from a semi-synthetic combinatorial antibody library (Nissim et al., 1994).

Materials and methods

Bacterial strains. *E. coli* strains used for the isolation of recombinant antibodies, were TG1 (K12, Δ(*lac-pro*), *sup*E, *thi*, *hsd*D5/F′*tra*D36, *pro*A$^+$B$^+$, *lacI*^{q}, *lac*ZΔM15) for selection of specific phage-antibodies and HB2151 (K12, *ara*, Δ*lac-pro*), *thi*/F′/*pro*A$^+$B$^+$, *lacI*^{q}ZΔM15) for expression of soluble scFv-antibody fragments. HB2151 bacteria were grown at 30 °C in 2TY containing 100 μg ml^{-1} ampicillin and 2% (w/v) glucose (Sambrook et al., 1990). When the OD_{600} reached 0.5, the bacteria were pelleted and the supernatant was discarded. The bacteria were resuspended in 2TY + AMP (100 μg/ml), induced with 1 mM IPTG and grown for 18 h at 16 °C.

Purification of beet necrotic yellow vein virions. Beet necrotic yellow vein virus (Dutch isolate) was purified from mechanically inoculated *Chenopodium quinoa* according to a slightly modified protocol from Putz and Kuszala (1978). Infected *C. quinoa* leaves (200 g) were homogenized in a mixture of 400 ml CCl_4 and 800 ml extraction buffer (0.1 M Tris/HCl buffer pH 9 containing 0.14 M NaCl; 5% (v/v) ethanol) and 2% (w/v) polyvinylpyrrolidon. The mixture was stirred for 15 min at room temperature (RT) and then centrifuged for 15 min at 5,000× *g* (GSA rotor, Sorvall). To the collected supernatant, 5% (w/v) PEG-8000 was added and stirred at RT for 15 min and subsequently at 4 °C for 90 min. The virions were pelleted at 16,000× *g* (GSA, Sorvall) for 20 min. After resuspending the pellets in 100 ml extraction buffer and stirring overnight at 4 °C, sucrose was added to 3.3% (w/v) and the mixture was centrifuged at 27,500× *g* for 20 min (SW28, Beckman) to remove large aggregates. Subsequently the virions were pelleted at 110,000× *g* (SW28, Beckman) for 2 h through a 3 cm layer of 20% (w/v) sucrose in extraction buffer. The pellets were resuspended in 50 ml extraction buffer and, after stirring for 30 min at RT, centrifuged for 10 min at 1,600× *g* to remove insoluble precipitates. Half of the supernatant was adjusted to a density of 1.1 g/cm^3, half to 1.3 g/cm^3 with $CsCl_2$. The two halves were layered 1 : 1 and centrifuged at 154,000× *g* (SW41Ti, Beckman) for 18 h. The virions containing band was collected and dialyzed overnight against 0.1 M Tris/HCl buffer pH 9 containing 0.14 M NaCl and then centrifuged for 5 min at 12,000× *g* to remove aggregates. The virion concentration was determined with a Beckman photospectrometer using a formula $[(1552 \times A_{280}) + (-757.3 \times A_{260})]$, described by Warburg and Christian (1942), which gives the protein concentration in μg ml^{-1}. The concentration was adjusted to 1 mg ml^{-1} with 0.1 M Tris/HCl buffer pH 9 containing 0.14 M NaCl and 1 ml aliquots were frozen in liquid nitrogen and stored at −74 °C until use.

Expansion of the antibody library. From a stock of the combinatorial library, which was constructed by Nissim et al. (1994), 50 μl was plated on a 240 × 240 mm minimal plate (Sambrook et al., 1990) to which 1 mM thiamin was added. The bacteria were grown overnight at 30 °C, scraped from the plate and resuspended in 50 ml of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose. A freezer stock (Sambrook et al., 1990) was prepared from 10 ml of these bacteria. One bacterial OD_{600} unit was taken from the remaining 40 ml, the volume adjusted to 10 ml by adding of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose, and the bacteria were grown at 37 °C while shaking (250 rpm). When an OD_{600} of 0.5 was reached, 10^{11} helper phages (M13K07, Pharmacia, Uppsala, Sweden) were added. The 15 ml tube containing the mixture was put in a waterbath without shaking to allow for optimal infection. After 30 min the bacteria were pelleted (2,100× *g*, 10 min) and resuspended in 25 ml 2TY containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin. The bacteria were transferred to a 1 liter Erlenmeyer, with 225 ml of prewarmed (37 °C) 2TY containing 100 μg ml^{-1} ampicillin and 25 μg ml^{-1} kanamycin, and were grown for 18 h at 30 °C while shaking (250 rpm).

Preparation of phage-antibodies for panning (large-scale production). The 250 ml overnight culture was harvested and the bacteria were removed by centrifugation (10,000× *g*, 20 min, GSA, Sorvall). The phages in the supernatant were precipitated by adding 50 ml of 20% (w/v) PEG-6000/2.5 M NaCl and mixed thoroughly for 1 h at 4 °C. The precipitated phages were pelleted (8,000× *g*, 40 min) and resuspended in 20 ml of sterile H_2O. Precipitation was repeated by adding 4 ml of PEG/NaCl and mixed for 20 min at 4 °C. The phages were pelleted (17,000× *g*, 10 min; SS34, Beckman), resuspended in 2.5 ml sterile H_2O and the stock was stored at 4 °C for further use. Usually 5×10^{13} phages were produced, as was established by plating TG1 bacteria on selective ampicillin plates after infection with serial dilutions of the phage suspension.

Panning procedure. Immunosorbent tubes (Maxisorb, Nunc) were coated with 40 μg BNYVV (10 μg ml^{-1} in 50 mM $NaHCO_3$, pH 9.5) for 2 h on a rollerbench at 25 °C and for another 18 h at 4 °C. The tubes were washed 3 times with PBS and blocked with PBM (PBS containing 2% (w/v) skimmed milk powder) for 30 min. Simultaneously, 2 ml of phage-antibodies was mixed with 2 ml of 2 × PBM and preincubated for 30 min. After removing the blocking solution from the tubes, 4 ml of the phage-antibodies containing PBM was added to an antigen-coated tube. Phage-antibodies were allowed to bind to BNYVV for 30 min on a roller bench and for another 90 min without rotation. Free phages were removed by washing the tubes 20 times with 4 ml PBS containing 0.1% (v/v) Tween-20 and for another 20 times with 4 ml PBS to remove the detergent. After washing the tubes were rinsed with 4 ml 0.1 M Tris/HCl pH 8.9 containing 0.14 M NaCl. Bound phages were eluted by adding 1 ml of a 0.5 mg ml^{-1} BNYVV solution in 0.1 m Tris/HCl pH 8.9 containing 0.14 m NaCl to the tube and incubated for 45 min with rotation, followed by a wash with 0.75 ml PBS. The BNYVV-eluted phages and phages in the PBS washing were pooled. One ml aliquot, of the BNYVV-eluted phages, was used to infect 9 ml of *E. coli* TG1 bacteria, for 30 min at 37 °C in a water bath. The infected *E. coli* cells were pelleted (2, 100× *g*, 10 min) and subsequently resuspended in 1 ml of 2TY, containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose. From these suspensions 50 μl aliquots were taken and plated in serial dilutions on 2TY agar plates containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose to establish the number of eluted phages and to make freezer stocks (Sambrook et al., 1990) of TG1 bacteria which were growing in single colonies. The remaining 950 μl was plated separately on 240 × 240 mm minimal plates (Sambrook et al., 1990) to which 1 mM thiamin was added, and grown for 18 h at 30 °C. Further enrichment of BNYVV-specific phage-antibodies was achieved by three additional panning rounds, which were performed according to Figure 1.

Preparation of phage-antibodies for ELISA (medium-scale production). From a streak of the bacterial freezer stocks, prepared during the panning procedure, single colonies were picked and plated on minimal plates (Sambrook et al., 1990) to which 1 mM thiamin was added. The bacteria were grown for 18 h at 30 °C, scraped from the plate and resuspended in 2 ml of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose. One bacterial OD_{600} unit was adjusted to 1 ml by adding of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose, and 2×10^{10} helper phages (M13K07, Pharmacia). The 1.5 ml Eppendorf tube containing the mixture was put in a waterbath for 30 min without shaking to allow for optimal infection at 37 °C. The bacteria were pelleted (8,000× *g*, 10 min) and resuspended in 750 μl 2TY containing 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin. The bacteria were transferred to a 24-well culture plate and grown for 18 h at 30 °C while shaking (100 rpm). The 750 μl overnight culture was harvested and the bacteria were removed by centrifugation (8,000× *g*, 10 min). If necessary, the phages in the supernatant were precipitated by adding 150 μl of 20% (w/v) PEG-6000/2.5 M NaCl and mixing well for 1 h at 4 °C. The precipitated phage-antibodies were pelleted (8,000× *g*, 10 min), resuspended in 250 μl of PBM and stored at 4 °C until further use.

Preparation of phage-antibodies for ELISA (small-scale production). After each panning round, 100 single colonies were picked and inoculated in 100 μl of 2TY containing 100 μg ml^{-1} ampicillin and 1% (w/v) glucose in a well of a 96-well culture plate. To study the effect of the panning also 100 single colonies were picked from the original library before any selection was carried out (Panning round 0). The bacteria were grown shaking (200 rpm) for 18 h at 30 °C. From these overnight cultures 5 μl was taken, inoculated in a new 96-well plate well, containing 100 μl of 2TY with 100 μg ml^{-1} ampicillin and 1%

(w/v) glucose and grown shaking for 1 h at 37 °C. To allow super-infection, 50 µl aliquots of 2TY containing 100 µg ml^{-1} ampicillin, 1% (w/v) glucose and 2.5×10^9 helper phages (M13K07, Pharmacia) were added to each well and incubated for 30 min without shaking and for 1 h with shaking (200 rpm) at 37 °C. The bacteria were pelleted (2, 800× *g*, 10 min) and resuspended in 200 µl 2TY containing 100 µg ml^{-1} ampicillin and 50 µg ml^{-1} kanamycin. The bacteria were grown shaking (200 rpm) at 30 °C for 18 h, removed by centrifugation (2,800× *g*, 10 min) and the phage-antibody containing supernatant was stored at 4 °C until further use.

Phage-ELISA. The phage-ELISA was essentially carried out according to Clark and Adams (1977) and to Tijsen (1985). Briefly, ELISA plates (Labstar, Costar, Cambridge, UK) were coated overnight with 100 µl of polyclonal rabbit-anti-BNYVV antibodies (3 µg IgG ml^{-1} in 50 mM $NaHCO_3$, pH 9.5) at 4 °C. The plates were washed with PBST (PBS containing 0.1% (v/v) Tween-20) and blocked with PBM for 30 min at 37 °C at 200 µl per well. The microtiter plates were washed twice with PBST and incubated for 1 h at RT with 100 µl/well of a *C. quinoa* leaf homogenate (50 mg dry leaf material ml^{-1} in PBM), with or without BNYVV lesions. After each incubation step the microtiter plates were washed four times with PBST. The plates were subsequently incubated for 1 h at RT with phage samples, diluted 1 : 1 in PBM; with polyclonal mouse-anti-M13 antibodies, diluted 1 : 10,000 in PBM; and finally with polyclonal rat-anti-mouse antibodies conjugated to alkaline phosphatase (Jackson Immuno-Research Laboratories, Inc., Westgrove, PA) diluted 1 : 2500 in PBM at 100 µl per well. The ELISA was developed by adding 100 µl *p*-nitrophenylphosphate substrate per well and absorbance readings were made at 405 nm, usually within 60 min. A reaction in phage-ELISA was considered to be positive if the threshold value (mean of the background + three times the standard deviation) was exceeded.

Mva*I-fingerprinting.* Fingerprinting was performed on PCR amplified scFv-DNA using the restriction enzyme *Mva*I. Single colonies were tooth picked and grown for 4 h in 2TY containing 100 µg/µl ampicillin and 1% (w/v) glucose. The bacteria were boiled for 3 min and pelleted at 14,000× *g* for 10 min. From the supernatant 2 µl was added to a 48 µl PCR mix containing 2.5 µM dNTPs; 0.25 U Super Taq DNA polymerase (HT Biotechnology, Cambridge, UK); 10 µM Forward primer (5′-AGG AAA CAG CTA TGA CCA TGA TTA CGC CAA G-3′) and 10 µM Backward primer (5′-GCC CAA TAG GAA CCC ATG TAC CGT AAC ACT G-3′); 2 mM $MgCl_2$ and 50 mM Tris/HCl, pH 8 and 25 cycles (1 min 94 °C; 2 min 72 °C) were performed on a thermal cycler (Perkin Elmer). From the PCR mix, 20 µl was added to 36.5 µl H_2O and 6,5 µl of buffer H (Boehringer, Mannheim, Germany). After mixing 2 µl (1U/µl) *Mva*I (Boehringer) was added and incubated 18 h at 37 °C. The *Mva*I digestion patterns were analyzed on a 3% FMC Metaphor agarose gel (Epicentre Technologies, Madison, USA).

Cloning of scFvs into pDAP2/S. The genes encoding the scFvs, BNY-8 and BNY-10, were PCR amplified using the forward and backward primers, *Hin*dIII and *Not*I digested, gel purified, ligated into *Hin*dIII/*Not*I digested pDAP2/S vector (Kerschbaumer et al., 1997) and transfected to *E. coli* TG1 bacteria. Transformed bacteria were picked and tested for the expression of scFv-alkaline phosphatase fusion-proteins through incubations with *p*-nitrophenylphosphate.

Production of scFv-AP/S fusion-proteins. Cultures were grown and induced as described by Kerschbaumer et al. (1997). Pelleted bacteria (5,000 rpm in a GSA rotor during 20 min at 4 °C) were resuspended in 1/20 volume (referring to the original culture size) of a 50 mM Tris/HCl pH 8 buffer (containing 30% sucrose and 1 mM EDTA). After incubation for 5 min at 0 °C the bacteria were pelleted (10,000 rpm in a GSA rotor during 20 min at 4 °C) and the produced proteins were extracted by an osmotic shock from the periplasm with 1/20 volume (referring to the original culture size) of 5 mM $MgSO_4$ and incubation for 45 min at 0 °C. The bacterial debris was removed by centrifugation.

ELISA. An antigen coated plate (ACP)-ELISA was used to assess the specificity of the recombinant scFv-AP2/S fusion proteins in the osmotic shock fractions according to standard methods, in which the plates were washed four times between each incubation. Briefly, the wells of a 96-well microtiter plate were coated with the appropriate antigen in 0.1 M $NaCO_3$ (pH 9.6) for 2 h at 37 °C. After blocking with PBMT-5% (PBS containing 5% skimmed milk powder and 0.1% Tween-20) at 200 µl/well for 30 min at 37 °C, the wells were incubated with scFv-AP2/S diluted in 100 µl of PBMT-1%

for 1 h at 37 °C. The reaction was visualized by a subsequent incubation with *p*-nitrophenylphosphate.

Results

Selection of BNYVV binding clones from a phage-antibody library. To select BNYVV-specific monoclonal antibodies, scFv expressing phages were rescued from a semi-synthetic human antibody library containing 10^8 different antibody encoding phagemids (Nissim et al., 1994), and subjected to panning (Figure 1). The rescued phages, containing 10^{13} phage-antibodies (PhAbs), were allowed to bind to immobilized BNYVV particles. Bound PhAbs, were eluted by specific elution with competing antigen. To monitor the efficiency of selection during the panning experiments, the phage recovery was measured after each panning round. Determination of the phage recovery (Figure 2A) showed a clear enhancement. Especially in the third round where a strong increase in phage recovery, about 1,000 fold, was found. Because the fourth panning round did not show a further increase in phage recovery, a fifth selection round was not applied.

To verify if any of the selected phage-antibodies were BNYVV-specific, a polyclonal sandwich phage-ELISA was carried out with samples taken from the stocks of phage-antibodies which were prepared by large-scale production for each panning round. In this BNYVV-specific phage-ELISA, the polyclonal PhAbs showed increasing signals for subsequent rounds of panning (data not shown). The same stocks of polyclonal PhAbs were also tested for their reactivity using a phage-ELISA format in which purified BNYVV was coated directly to the plate. Although the signals from the sandwich ELISA were stronger than those obtained from the ACP phage-ELISA (data not shown), both ELISAs showed a similar increase. To ensure that differences in ELISA signals could be contributed to enhancements in specificity and not to degradation of the oldest samples during storage, and to allow the PhAbs obtained from the fourth selection round to be included in the ELISA as well, PhAbs were rescued simultaneously from the freezer stocks of the bacteria. When comparable amounts of PhAbs were tested in ELISA, again an increase in BNYVV binding was observed up to the third panning round (Figure 2B). This seemed to be a superlative level, as the signal showed no further increase after the fourth round of selection.

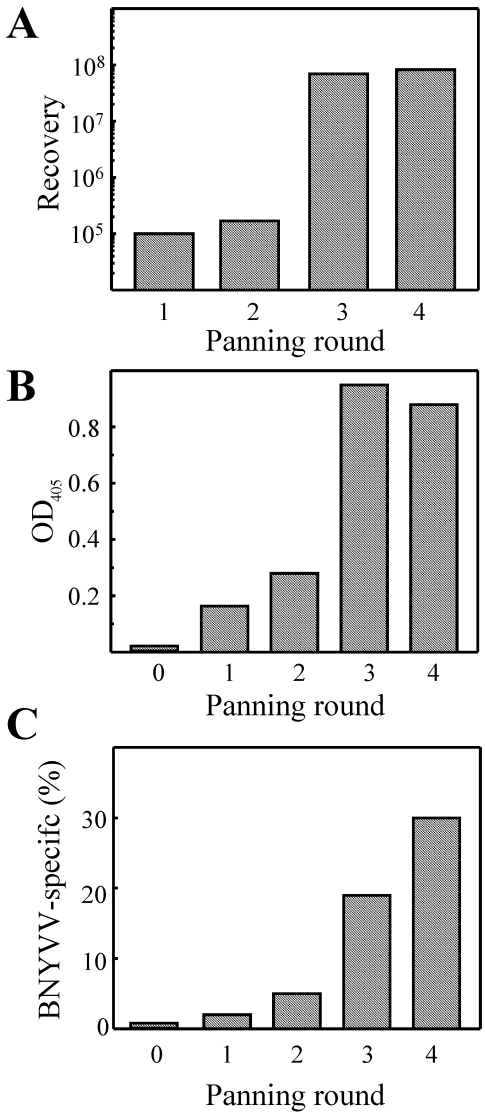

Figure 2. Recovery and specificity of BNYVV-binding phage-antibodies in four sequential rounds of panning. (A) The number of recovered PhAbs was counted and the recovery was plotted for each subsequent panning round. (B) Phage-antibodies were produced (medium-scale production) and comparable amounts were tested in polyclonal phage-ELISA. Phage-antibodies that were rescued from a stock of the antibody library before any selection had been applied served as negative control (panning round 0). (C) In addition the percentage of BNYVV-specific phage-antibody producing clones (small-scale production) was shown by a monoclonal phage-ELISA. Colonies were counted positive in case the derived signal exceeded the mean ($n = 3$) of the negative control pHen (non-scFv expressing phage) plus three times the standard deviation.

Characterization of BNYVV-specific phage-antibodies. The polyclonal character of the phage-ELISA does not give a clear image of the antibody specificity because of different growth rates and expression levels between individual clones. Therefore, 100 single clones were isolated from each panning round. The monoclonal phage-antibodies, produced by these individual clones, were tested separately in a phage-ELISA (Figure 2C) and showed a strong increase in the number of positive clones from 0% before selection to as much as 30% after the fourth round of selection.

To investigate the diversity within the selected monoclonal phage-antibodies, a DNA fingerprint was carried out. The scFv encoding DNAs, of the clones which were positive in the phage-ELISA, were amplified by PCR and digested with the restriction enzyme *Mva*I. When the digestion products were analyzed on an agarose gel (Figure 3A), a high variation was observed within the isolated monoclonal phage-antibodies. A total of six different *Mva*I-patterns was found within the positive clones. These monoclonal PhAbs were designated BNY-1, BNY-2, BNY-3, BNY-5, BNY-8 and BNY-10, of which PhAb BNY-10 had become the most abundant PhAb after three rounds of selection. Up to 90% of the positive colonies possessed the same *Mva*I restriction pattern as PhAb BNY-10. In contrast, after four rounds of selection PhAbs like BNY-2, BNY-3 and BNY-5 had completely disappeared from the antibody pool.

To study the specificity of the selected MAbs towards healthy and BNYVV infected *C. quinoa* plants, one representative colony of each pattern was selected, streaked and after phage-antibody production tested for its reactivity in a phage-ELISA (Figure 3B). Of the isolated PhAbs, five gave high reactions with BNYVV infected plants but no reaction with healthy *C. quinoa* plants in the BNYVV-specific phage-ELISA. The only MAb that failed to give a positive reaction in this ELISA corresponds to the restriction pattern of MAb BNY-2 which was found in a positive colony obtained from the first panning round but nowhere else. However, when the phage-ELISA was repeated with a PEG-purified and concentrated BNY-2 phage-antibody, a positive signal was found for BNYVV infected but not with healthy *C. quinoa* (Figure 3B: BNY-2p).

Although the selected monoclonal phage-antibodies reacted specifically with BNYVV in a phage-ELISA, they failed when they were expressed in *E. coli* strain HB2151 as soluble scFv antibodies. When the HB2151 bacteria were fractionated after induction with

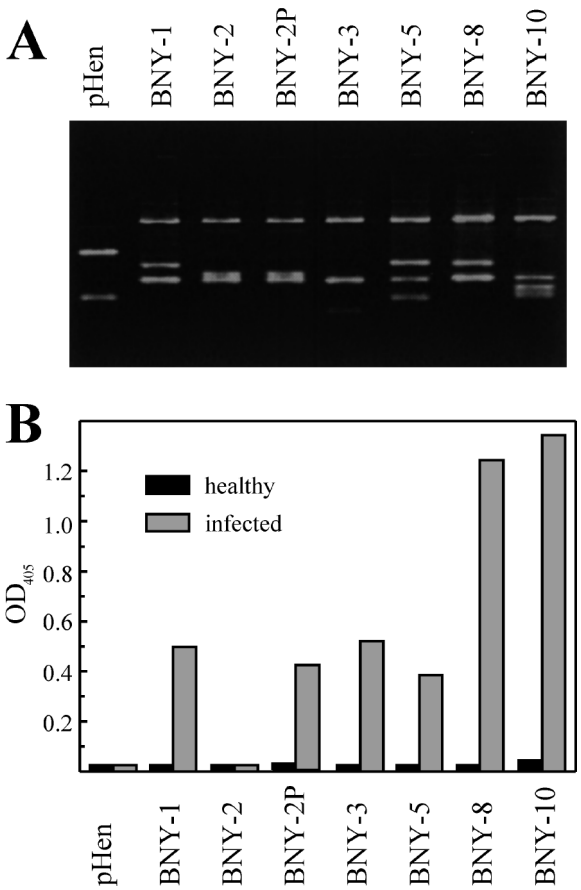

Figure 3. (A) Restriction patterns of BNYVV binding clones which were obtained during selection. PCR-products (1100 Bp) of the DNA encoding the BNYVV-specific antibody fragments were digested with the restriction enzyme *Mva*I. The digested fragments were analyzed on a 3% FMC metaphor agarose gel. (B) The specificity of the isolated PhAbs for BNYVV is shown in a phage-ELISA with homogenates of healthy and BNYVV-infected *C. quinoa* leaf material. Supernatants of infected *E. coli* cultures were tested directly for BNYVV specificity in the phage-ELISA after 1 : 5 dilution in PBM. Rescued (non-scFv expressing) phages, obtained from TG1 bacteria which were transformed with a no scFv containing phagemid (pHen), served as a negative control.

IPTG, and tested by Western blotting for the presence of expressed scFvs, high signals were found within the cytoplasm (data not shown) but not within the periplasm. Apparently the transport of the scFvs to the periplasm is troublesome and the result is formation of inclusion bodies. An improvement was observed when the scFv genes of BNY-8 and BNY-10 were recloned into the expression vector pDAP2/S

(Kerschbaumer et al., 1997) which allowed the scFvs to be expressed as scFv-alkaline phosphatase fusion proteins (scFv-AP/S). After extraction of the scFv-AP/S fusion proteins from the periplasm, specific signals were obtained in ELISA (Figure 4).

Discussion

Six different BNYVV-specific monoclonal phage-antibodies were selected from a semi-synthetic human combinatorial antibody library (Nissim et al., 1994), as was assayed by *Mva*I digestion of PCR amplified scFv encoding DNA. Each of these PhAbs reacts specifically with BNYVV from *C. quinoa* leaf homogenates in a sandwich ELISA. When the phage display technique is compared to the hybridoma technique a number of advantages are observed. First, laboratory animals are no longer required. Second, selection of antibodies from semi-synthetic combinatorial antibody libraries with phage display is much faster and less labor-intensive. Third, there is no bias towards certain minor plant contaminants in the antigen preparation. Finally, the specificity and affinity of the MAbs can be guided in a certain direction through manipulations during selection. For instance, high affinity antibodies can be obtained using low amounts of antigen or the specificity can be enhanced by elution with competing antigen.

Although elution with competing antigen gave a steep rise in BNYVV-binding PhAbs in the described selection procedure (Figure 2C) and finally yielded a high PhAb diversity, it generally has the disadvantage to select for antibodies with high dissociation rates. Another disadvantage is the high amount of antigen that is required for elution: over 2 mg of purified BNYVV was required for four rounds of panning and elution. If the antigen is merely used for coating of the immunosorbent tubes then only 160 μg is needed. However, in cases where non-specific binding of the filamentous phage to the antigen is high, as was found for BNYVV in a pilot study, specific elution might be the only way to enrich for target-specific PhAbs during panning.

When the goal of selection is to obtain a diverse repertoire of antigen-specific antibodies with regard to recognition of different epitopes on the antigen or sets of scFvs recognizing the same epitope, care should be taken not to lose antibody diversity through applying too many rounds of selection. The percentage of positive clones improved with each subsequent round of panning (Figure 3C) and 30% was reached after four

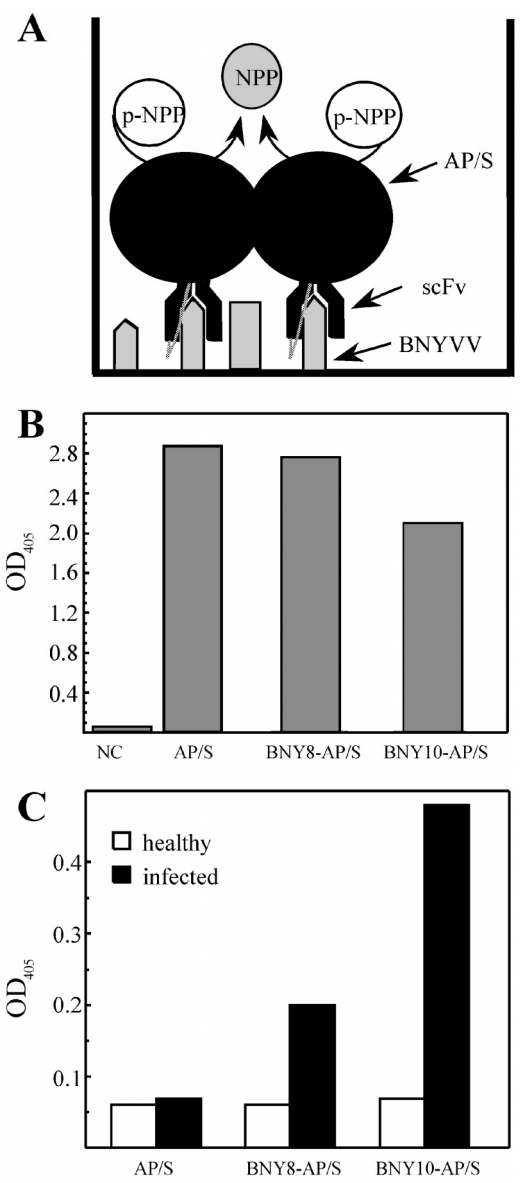

Figure 4. (A) Outline of BNYVV-specific ELISA. The ELISA plate was coated with the target antigen BNYVV. For detection of BNYVV, scFv alkaline phosphatase fusion proteins were used and binding of the bifunctional antibody-enzyme conjugate was visualized with *p*-NPP substrate. (B) Alkaline phosphatase activity in the osmotic shock fractions was measured 10 minutes after the addition of 10 μl osmotic shock fraction to 100 μl of *p*-NPP substrate, where 10 μl of 5mM MgSO4 served as the negative control (NC). (C) ELISA plates were coated either with healthy or BNYVV infected *C. quinoa* in carbonate buffer. After blocking, 100 μl of the alkaline phosphatase, BNY8-AP/S or BNY10-AP/S containing osmotic shock fraction was added (diluted 1 : 1 in PBM) to the wells and compared for binding with BNYVV.

rounds of selection. When more subsequent rounds of selection would have been performed the 100% would probably have been reached. However, a decrease in the diversity was observed after the third round of selection. This indicates that too many rounds of selection results either in antibodies with the highest affinity for the most abundant epitope or in antibodies which are produced at a faster rate and which are not necessarily the most specific.

The inability of *E. coli* to export the selected BNYVV-specific scFvs to the periplasm was observed. Several mutations are known which can enhance the transport of scFvs to the periplasm (Nieba et al., 1996) and it might be worthwhile to introduce these mutations into the BNYVV-specific antibodies. The presence of an *E. coli* protein domain (AP/S) on the C-terminus of the scFv molecule already seemed to increase the stability and the folding efficiency of the soluble scFv. In addition, the dimeric character of alkaline phosphatase may also add an avidity factor to the affinity of the scFv-AP/S fusion protein for the antigen. Moreover, production of MAbs in *E. coli* offers several important features for the standardization of detection assays. Production of scFv-enzyme fusion proteins might give a better reproducibility of the detection of antigens in various immunoassays since the highly variable step of antibody enzyme conjugation is omitted. The recombinant scFv antibodies can be purified via co-expressed affinity tags (Casey et al., 1995; Neri et al., 1995) to yield ultra pure, ready to use alkaline phosphatase conjugated reagents.

It is interesting that all the scFv expressing clones isolated with the phage display procedure are BNYVV-specific and do not react with antigens from the *C. quinoa* homogenate. Phage display rather allows PhAbs to be selected on the presence of a specific epitope than on the basis of immunogenicity. For example, glycoproteins are highly immunogenic. Therefore, the immune response elicited by a virus preparation that is slightly contaminated with a plant glycoprotein will be biased to that particular glycoprotein. The result is a high proportion of host-specific MAbs. If the same virus preparation would be used to select PhAbs from a combinatorial antibody library, specific clones are obtained against the most abundant epitope present; i.e., the virus. This was what was observed for the BNYVV virus preparation, the virus is almost pure but still contains some plant contaminants, yet the PhAbs obtained with this method are BNYVV-specific. It would be worthwhile to evaluate their use in a routinely applied standardized detection assay for BNYVV in sugar beets. They certainly have potential, as they are highly BNYVV-specific.

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