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Improved detection of *Beet necrotic yellow vein virus* in a DAS ELISA by means of antibody single chain fragments (scFv) which were selected to protease-stable epitopes from phage display libraries

Brief Report

K. Uhde[1,*], **R. J. Kerschbaumer**[2], **R. Koenig**[1], **S. Hirschl**[2], **O. Lemaire**[3], **N. Boonham**[4], **W. Roake**[5], and **G. Himmler**[2]

[1]Biologische Bundesanstalt für Land -und Forstwirtschaft, Institut für Pflanzenvirologie, Mikrobiologie und biologische Sicherheit, Braunschweig, Germany
[2]Institut für angewandte Mikrobiologie, Universität für Bodenkultur, Wien, Austria
[3]Institut National de la Recherche Agronomique, Unite de Recherche Vigne et Vin, Equipe Vection, Colmar, France
[4]Central Science Laboratory, Sand Hutton, U.K.
[5]Cambridge Antibody Technology, U.K.

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Summary. The detection of *Beet necrotic yellow vein virus* (BNYVV) in stored sugar beets by means of monoclonal antibodies or antibody single chain fragments (scFv) often poses problems, because the immunodominant C-terminal epitope of the viral coat protein is readily lost due to proteolysis. Clones which produce scFv specific for protease-stable BNYVV epitopes were selected from two naive phage display libraries. Fusion proteins of the scFv with a human IgG kappa chain (expressed from the newly designed vector pCL) or with alkaline phosphatase, respectively, allow the ELISA detection of BNYVV even in stored sugar beets with a sensitivity which was comparable or often higher than that achieved with polyclonal antibodies.

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Beet necrotic yellow vein virus (BNYVV) is the causal agent of sugar beet rhizomania, one of the economically most important virus diseases in Europe. In the past two decades the yield losses due to this disease have been alleviated by

*Present address: RWTH-Aachen, Institut für Biologie I, Aachen, Germany.

growing partially resistant sugar beet varieties. The breeding of such varieties was based on the selection of genotypes for which ELISA had revealed a reduced rate of virus multiplication and/or translocation. Because the production of coating and detecting reagents for ELISA would be much cheaper in bacterial cultures than in animals or in hybridoma cell cultures, we have previously cloned the coding sequences for the heavy and light chains of the anti-BNYVV monoclonal antibody 14CG6 [2] into the vector pCOCK in which the two sequences are joined by a linker sequence [4]. We thus obtained the coding sequence for a BNYVV-specific antibody single chain fragment (scFv). Insertion of this sequence into the vectors pZIP1 and pDAP2/S allowed the bacterial expression of a bivalent miniantibody and an alkaline phosphatase-scFv fusion protein with an increased specific enzyme activity, respectively. These fusion proteins were used as coating and detecting reagents, respectively, in a double antibody sandwich ELISA [7].

The procedure for obtaining the coding sequences for scFv from hybridoma cell lines, however, is laborious and only a small portion of the clones obtained from the hybridoma cell line 14CG6 in pCOCK enabled the expression of functionally reactive BNYVV-specific scFv. With several other hybridoma cell lines, we failed to obtain clones expressing BNYVV-specific scFv altogether ([4]; L. Fecker and R. Koenig unpubl). The hybridoma cell line 14CG6, from which we had obtained the BNYVV coat protein-specific scFv, has two disadvantages. The monoclonal antibody (MAb) which it produces is specific for the C-terminal epitope 4 on the viral coat protein. This epitope is readily cleaved off by proteases, e.g. in beets which have been stored for a prolonged time [10]. In addition, the binding capacity of the scFv 14CG6-derived miniantibody for BNYVV is inhibited by high concentrations of plant material.

A promising alternative for obtaining coding sequences for scFv is the use of large naive phage display libraries. This approach has the additional advantage of circumventing the use of immunized animals. Vaughan et al. [13] and Sheets et al. [11] have described libraries which contain 1.4×10^{10} and 6.7×10^{9} different phage clones, respectively; each clone expresses an unique scFv fused to the phage gene3 product. A panning procedure, in which phages expressing scFv reactive with a given antigen, e.g. a virus, are trapped by the immobilised antigen, allows the selection of scFv together with the gene encoding them [14]. By modulating the selection conditions a variety of scFv adapted to specific needs can be obtained. In this paper we describe the use of the two phage display libraries mentioned above for selecting scFv which allow the detection of BNYVV infections even in samples in which the virus, due to prolonged storage, has lost the C-terminal epitope of its coat protein and in which the concentration of plant proteins is especially high. To obtain improved coating reagents a new vector pCL was designed which allowed the expression of the scFv as fusion proteins with the constant domain of the IgG kappa chain. For the high yield production of the scFv-alkaline phosphatase fusion protein the newly described vector pDAP2/S^{tet} [8] was employed.

The selection of scFv-producing phage clones from the Sheets and the Vaughan libraries was done in a panning procedure similar to that described by Harper

Table 1. Selection protocols for beet necrotic yellow vein virus (BNYVV)-specific scFv

A from the Sheets library

Round	BNYVV[a]	Eluant	Phage added[b]	Phage output
1	50	TEA[c]	1×10^{12}	4.0×10^{4}
2	17	TEA[c]/Trypsin[d]	$1 \times 10^{13}/1 \times 10^{13}$	$7.4 \times 10^{6}/4.5 \times 10^{5}$
3	10	TEA/Trypsin	$1 \times 10^{13}/1 \times 10^{13}$	$1.0 \times 10^{8}/2.4 \times 10^{7}$
4	6.5	TEA/Trypsin	$1 \times 10^{13}/1 \times 10^{13}$	$6.2 \times 10^{7}/1.4 \times 10^{7}$

B from the Vaughan library

Round	BNYVV[a]	Eluant	Phage added[b]	Phage output
1	50	TEA[c]	1×10^{12}	4.5×10^{4}
2	17	TEA[c]/Trypsin[d]	$1 \times 10^{13}/1 \times 10^{13}$	$8.4 \times 10^{4}/2.2 \times 10^{5}$
3	10	TEA/Trypsin	$1 \times 10^{13}/1 \times 10^{13}$	$9.3 \times 10^{6}/1.8 \times 10^{5}$
4	6.5	TEA/Trypsin	$1 \times 10^{13}/1 \times 10^{13}$	$8.4 \times 10^{8}/2.8 \times 10^{9}$

[a] μg/ml, in ELISA coating buffer [1]
[b] Plaque forming units/ml
[c] 100 mM triethylamine, pH 12
[d] 1 mg trypsin/ml PBS

et al. [5] (Table 1). An aliquot of the output of the fourth panning round of each library was plated on TYE-agar and for a total of 1100 individual colonies (550 for each library) 1 ml mini-expression cultures in 2 × YT medium were checked in a MAb 9E10-mediated ELISA [4] for the production of scFv reacting with BNYVV in infected leaves of *Chenopodium quinoa* and in stored sugar beet. The virus was trapped by polyclonal antibodies (PAb) and the binding of the scFv to the trapped virus particles was detected by MAb 9E10 which specifically reacts with the Myc tag on the scFv [3]. The coding sequences of scFv giving ELISA readings at least as high as the BNYVV PAb-alkaline phosphatase conjugates were excised from the original phagemid vectors by digestion with SfiI and NotI and were subcloned into the newly designed expression vectors pCL and pDAP2/S^{tet} [8]. The vector pCL was generated by replacing the ecphoA1 gene of the vector pDAP2 [6] by the coding sequence of the human C kappa domain together with a hexahistidine tag. Bacterial expression and purification by means of immobilized metal affinity chromatography of scFv-fusion proteins was achieved essentially as described previously [7]. The coding sequences for scFv were analysed by a commercial company (Seqlab, Göttingen) using the primers pHEN seq (5′ CTA TGC GGC CCC ATT CA-3′) and LMB3 (5′ CAG GAA ACA GCT ATG AC 3′) for sequencing.

In order to obtain scFv specific for protease-stable epitopes on BNYVV particles, we have studied the reactivity of 25 different BNYVV preparations with MAb 14CG6 and with PAb, respectively, in an ELISA in which these antibodies

were used as detecting reagents and in which the virus particles were trapped to the plates by means of PAb. One virus preparation reacted only with the PAb, but failed to react with MAb 14CG6 indicating that it had lost its normally immunodominant C-terminus. This preparation was used for the selection of scFv from the two above-mentioned phage display libraries. The supernatants of 30 out of 1100 mini-expression cultures yielded ELISA readings in a MAb 9E10-mediated ELISA which were at least as high as those produced by PAb with sap from stored BNYVV-infected sugar beets. The selection of scFv by means of ELISA proved to be advantageous as compared to the previously used dot blot procedure [4], because several clones which gave a positive result in the latter procedure failed to react in various ELISA procedures. On the other hand all the clones selected in a MAb 9E10-mediated ELISA produced scFv which were suitable for ELISA also when expressed as kappa chain or alkaline phosphatase fusion proteins.

In order to obtain efficient coating reagents, the coding sequences of 12 scFv giving the highest ELISA readings were excised from the original phagemid vectors by digestion with SfiI and NotI and were subcloned into the newly designed expression vector pCL. This vector allows the expression of the scFv as fusion proteins with the IgG kappa chain. The latter mediates a more efficient binding to plastic than the leucine zipper in the previously used miniantibodies [7] and the expression rate with this vector is higher (results not shown). The trapping efficiency on ELISA plates of the kappa chain fusion proteins of different library-selected scFv for BNYVV, diluted either in standard ELISA washing buffer [1] containing 2% ovalbumin or in the same buffer containing 10% (w/v) ground sugar beet root material, was compared with that achieved with polyclonal antibodies and the MAb 14CG6-derived miniantibodies described previously [7] (Fig. 1). With most of the scFv fusion proteins and the MAb 14CG6-derived

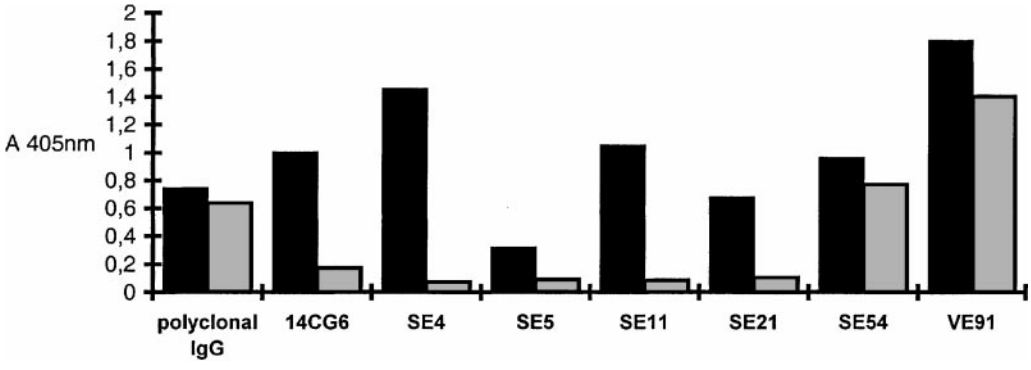

Fig. 1. Comparison of the trapping efficiencies for BNYVV of polyclonal antibodies, the MAb 14CG6-derived scFv miniantibody [7] and of the kappa chain fusion proteins of six different scFv selected from two naive phage display libraries. BNYVV was diluted either in ELISA washing buffer [1] supplemented with 2% ovalbumin (■) or in the same buffer containing in addition 10% (w/v) ground sugar beet root material (■). The trapping of the virus was detected by means of alkaline phosphatase-labelled polyclonal antibodies

miniantibodies the binding of BNYVV was strongly inhibited by the beet extract. However, with polyclonal antibodies and the kappa chain fusion proteins of scFv SE 54 (from the Sheets library) and VE 91 (from the Vaughan library) there was only a weak inhibition by plant sap (Fig. 1).

In order to obtain efficient detecting reagents, the coding sequences for ten different scFv were subcloned into the expression vector pDAP2/S^{tet} [8], a tetracycline resistant version of the pDAP2/S vector [7]. The pDAP2/S^{tet} expression product of scFv SR19 (from the Sheets library) was identified to give the best results under routine testing conditions.

With the combination of the scFv VE 91 kappa chain fusion protein at 10 μg/ml as coating reagent and the scFv SR19 alkaline phosphatase fusion protein at 1 μg/ml as detecting reagent, BNYVV was detected reliably even in sugar beet samples which had been stored or contained plant proteins at a high concentration. Addition of 1% non-fat skimmed milk powder to the diluted detection reagents eliminated background reactions almost completely. The sensitivity of virus detection varied somewhat with different scFv preparations. ELISA readings in a total of about 300 tests done in our laboratories in Germany, France, UK and Austria were comparable to those obtained with the PAb or MAb used routinely; depending on the scFv preparation used they were sometimes up to 30 to 150% higher (Fig. 2). In comparison with MAbs, the scFv gave much lower background readings, resulting therefore in much higher signal to noise ratios (results not shown). The detection limit for the virus was about 1 ng/ml or 100 pg in the 100 μl samples used for testing. A similar value has previously been recorded with PAb [9]. The scFv we have checked failed to detect denatured BNYVV coat protein in Western blotting indicating that they are specific for discontinuous epitopes. From the supernatants of 300 ml shaker flask cultures up to 5 mg/l scFv fusion proteins were obtained. Comparison with known coding sequences for antibodies [12] revealed that the heavy chain coding sequence of SR19 belongs to the V_H3 family and originates from the germline V_H gene segment DP-38, while

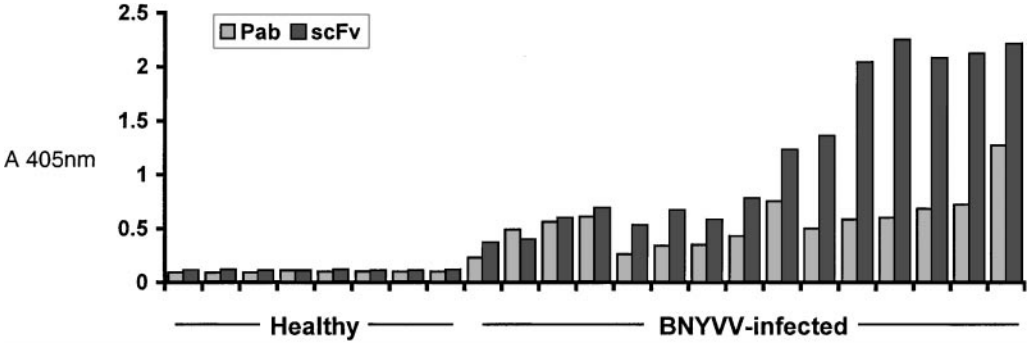

Fig. 2. Detection of BNYVV in sugar beets by means of a conventional ELISA using polyclonal antibodies and a fully recombinant ELISA in which the scFv VE91-kappa chain fusion protein was used for coating and the scFv SR19-alkaline phosphatase fusion protein for detection

the light chain sequence uses a V segment of the VKI subgroup and originates from the germline segment DPK9. The heavy chain coding sequence of VE91 also belongs to the V_H3 family, but originates from the germline V_H gene segment DP-47, while the light chain sequence uses a V segment of the VKI subgroup and originates from the germline segment DPK3.

The results described here together with those reported previously (e.g. [4]) indicate, that it is easier to obtain highly reactive BNYVV-specific scFv from large phage display libraries than by subcloning from hybridoma cell lines derived from immunized mice. Depending on the state of the virus used for panning and on the screening procedure, scFv may be selected which are optimal for a specific application, e.g. for the detection of virus which has lost its immuno-dominant C-terminal epitope or for use in a particular test. For the selection of scFv to be used in ELISA, for instance, the screening should be done by ELISA rather than by a dot blot procedure. Since scFv, like MAbs, may be highly specific for a given epitope, the use of mixtures of scFv may be preferable for the routine detection of viruses.

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Authors' address: Dr. K. Uhde, RWTH-Aachen, Institut für Biologie I, Worringerweg 1, D-52074 Aachen, Germany.

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