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Isolation of recombinant antibodies (scFvs) to grapevine virus B

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Abstract

A panel of 15 recombinant single chain antibodies (scFv) specific to grapevine virus B (GVB) were recovered from a human combinatorial scFv antibody library using the phage display technique against purified virus particles. Two selected scFv-encoding genes were expressed in recombinant *Escherichia coli* cells as dimeric antibodies. Successful detection of GVB in tissues of herbaceous hosts and grapevine was obtained in a direct binding assay using dimeric scFvs. This reagent was also shown to substitute efficiently for a GVB polyclonal serum in standard DAS-ELISA test used routinely for diagnosis.

Keywords: scFv; Recombinant antibodies; Grapevine virus B; Phage display; ELISA

1. Introduction

Grapevine virus B (GVB) is the putative agent of the corky bark syndrome of the rugose wood complex, one of the economically most important and widespread virus disease of the grapevine (Martelli, 1999). GVB filamentous particles ca. 800 nm long containing a single stranded positive sense RNA of 7599 nt in size is routinely multiplied in *Nicotiana occidentalis* (Saldarelli et al., 1996). An effective measure for preventing GVB dissemination is represented by production and planting healthy stocks, whose sanitary status is assessed by serological and/or molecular assays. However, the performance of ELISA is impaired by the low titer of standard polyclonal antisera currently available (Boscia et al., 1997).

Recently, the expression in bacteria of recombinant proteins incorporating variable antibody chains, has been described. These "single chain fragment variable" (scFvs) proteins are expressed in fusion with bacteriophage coat proteins and maintain the original antibody binding properties (McCafferty et al., 1990; Hogenboom et al., 1991). Virus specific scFvs can be either obtained from cloned antibody genes derived from selected hybridomas, or selected from libraries containing up to 10^8 different antibody genes (Griffiths et al., 1994). Once selected, scFvs genes could be maintained stably in and expressed from bacterial plasmids, allowing the production of large quantities of synthetic antibodies. Thus this technology has the potential for developing fully recombinant ELISA kit for plant virus diagnosis (Toth et al., 1999; Griep et al., 2000; Uhde et al., 2000).

In the present study, scFvs specific to GVB were isolated from a phage library displaying scFvs and were able to detect GVB in infected tissues of herbaceous hosts and grapevine both in the native and recombinant dimeric form.

2. Materials and methods

2.1. Propagation and purification of GVB

GVB was propagated in *N. occidentalis* plants grown in a climatized greenhouse and purified from a minimum of 100 g of infected plant material according to Boscia et al.

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(1993). The concentration of purified viral preparations was determined spectrophotometrically using a E_{260} (specific extinction coefficient) of 2 (e.g. 1 $O.D._{260\,nm}$ unit = 2 mg/ml). A GVB-infected grapevine, was used as positive control in ELISA.

2.2. Selection of anti-GVB scFv

scFvs were selected from the Human Synthetic VH + VL Library (Griffiths et al., 1994) according to Griep et al. (2000). Briefly, immunotubes (Nunc MaxiSorpTM, Nalge Nunc International, Rochester) were coated with GVB preparations diluted in 50 mM Na_2CO_3, pH 9.8, for 14 h at 4 °C and phages were eluted in 0.2 M glycine-HCl, pH 2.2, and neutralized successively with 1 M Tris–HCl, pH 9.1. Three rounds of selection were carried out using increasing stringency conditions which consisted in reducing progressively the GVB concentration for coating, from 100 to 10 and 1 μg/ml, respectively, and increasing from 10 to 20 the washing steps. Each of the three-phage populations obtained was tested for specificity to GVB by "polyclonal phage-ELISA", whereas individual bacterial clones expressing GVB-reacting phages were selected by "monoclonal phage-ELISA".

2.3. Analysis of selected phages

cDNAs corresponding to the scFv sequences were amplified directly from bacterial colonies and the DNA obtained restricted with the enzyme *MvaI*. PCR amplification was done with primers fdseq1 (5′-GAATTTTCTGTATGAGG-3′) and LMB3 (5′-CAGGAAACAGCTATGAC-3′), and RFLP fragments analyzed in 5% PAGE in TBE buffer (1× TBE: 890 mM Tris, 890 mM boric acid, 25 mM Na_2EDTA, pH 8.3). Phagemid DNA was purified using a Qiagen (Qiagen GmbH, Germany) mini-prep kit and nucleotide sequence determined by automated sequencing (MWG-Biotech AG, Germany).

2.4. Sub-cloning of scFv gene in pTMZ1 CLZIP and production of dimeric scFv

Purified phagemid DNA was digested with NcoI and NotI, and the gel-purified fragment subsequently ligated into a NcoI/NotI digested pCLZIP vector (Kerschenbaumer et al., 1997). The ligation mix was transfected in *E. coli* ER2566 bacteria and plates incubated overnight at 37 °C on LB agar medium containing 100 μg/ml ampicillin. Positive colonies were identified by small-scale expression and restriction analysis of the extracted recombinant pCLZIP vector. Individual transformants were grown at 37 °C in LB medium containing 100 μg/ml ampicillin up to a $O.D._{600\,nm}$ of 2, and stored at −80 °C in 25 μl aliquots in 15% glycerol. For each round of expression an aliquot of frozen bacteria was inoculated in 50 ml LB medium containing 100 μg/ml ampicillin and grown up to a $O.D._{600\,nm}$ of 0.6 at 37 °C. The temperature was decreased to 21 °C and the cells were induced for 14 h with 1 mM IPTG. The cells were then centrifuged at 6000 × *g* for 10 min, resuspended in 1/20 initial volume of 20% sucrose 30 mM Tris–HCl, pH 8, and incubated for 5 min at 0 °C after drop to drop addition of Na_2EDTA, pH 8, to a final concentration of 1 mM. Bacteria were centrifuged and resuspended as above in 5 mM $MgSO_4$ and incubated for 60 min at 0 °C for extraction of fusion proteins from the periplasmic fraction. Supernatant obtained after centrifugation at 8000 × *g* for 20 min, was dialyzed for 24 h against lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8) and the His-tagged scFv protein eluted by IMAC purification, according to the manufacturer's instructions (Qiagen GmbH, Germany). His-tagged scFv proteins were also recovered by IMAC purification from the LB medium after ammonium sulfate precipitation of the total protein contents. Proteins solubilized in lysis buffer were dialyzed and purified as described above. Column eluted fractions with the highest protein contents were identified by SDS-PAGE and Western blot analysis using AP conjugated goat anti-human IGg f(ab′) (Jacksons Scientific, USA) following standard procedures (Sambrook et al., 1989).

2.5. ELISA

GVB particles from purified preparations (2 μg/ml in PBS: 0.8% NaCl, 0.02% KH_2PO_4, 1.15% Na_2HPO_4, 0.02% KCl, pH 7.4), infected *N. occidentalis* or grapevine tissues (1 g tissue/10 ml extraction buffer: 0.1% Tween, 2% PVP in PBS) were trapped overnight at 4 °C directly in ELISA plates. After four washings in PBST (0.1% Tween in PBS) and blocking for 2 h in PBSTM (5% skimmed milk in PBST) at room temperature (RT), particles were detected by polyclonal ("polyclonal phage-ELISA") or monoclonal ("monoclonal phage-ELISA") phage preparations, recombinant scFvCL ("scFvCL-ELISA") and GVB Mab B2 ("direct-ELISA") as described below.

For detection with phage-bearing scFvs, polyclonal populations ("polyclonal phage-ELISA") of each round of panning (10^{10} cfu/well in PBSTM) or supernatant fluid containing monoclonal phages ("monoclonal phage-ELISA") produced according to "Griffin1" protocol (http://www.mrc-cpe.cam.ac.uk/g1p.php), were incubated for 2 h at room temperature. scFv-phage/GVB immunocomplexes were detected by rabbit-anti-M13 antibodies (Sigma Chemical, St Louis, USA) diluted 1:800 in PBSTM and incubated for 2 h at RT, and finally incubated for the same time with anti-rabbit AP conjugated antibodies (Sigma Chemical, St Louis, USA) diluted 1:2000 in conjugated buffer (0.1% Tween, 2% PVP, 0.2% in PBS). Positive reactions were revealed by adding 100 μl of *p*-nitrophenyl phosphate (*p*-NPP) substrate per well and scored by absorbance readings at 405 nm within about 60 min. Each step was followed by four washings in PBST as above.

For detection with scFv–CL fusion proteins ("scFvCL-ELISA"), eluted proteins were diluted 1 to 1 or 1 to 3 in PBSTB (0.1% BSA in PBST) and incubated O/N at 4 °C. Immunocomplexes were detected by incubation for 2 h with

AP conjugated goat anti-human IGg f(ab′) diluted 1:2000 in conjugated buffer and ELISA plates were developed as above.

GVB Mab B2 ("direct-ELISA") was used as control by incubation for 2 h at RT at the dilution of 1:2000 in PBSTB and subsequent incubation with the same conditions with anti-mouse AP conjugated polyclonal serum (Sigma Chemical, St Louis, USA). ELISA plates were developed as above.

GVB detection was also made by indirect DAS-ELISA based on plate trapping by a polyclonal serum and detection by Mab B2 and anti-mouse AP conjugated antibodies as described above.

3. Results

3.1. Selection and characterization of GVB-specific scFvs

Three rounds of panning were carried out against purified GVB particles, decreasing virus concentration and increasing the number of washings, in order to select high affinity binding phages. An increase of reactivity toward GVB was observed by "polyclonal phage-ELISA" with the three-phage populations obtained after each round of panning (Fig. 1). Specificity to GVB of the obtained phage populations, was also demonstrated by the lack of reactivity against purified grapevine virus A (GVA) preparations, used as negative controls.

A total of 56 individual scFv-displaying phages, selected at random from the phage population of the third round of panning, were tested for their reactivity toward purified GVB particles in a "monoclonal phage-ELISA" (data not shown) and their scFv genes were amplified with primers LMB3 and fdseq1. Analysis of the PCR products by gel electrophoresis showed that only 15 phages bearing intact scFv DNA genes of ca. 1000 kb (data not shown) were able to detect GVB either as purified particles or in infected *N. occidentalis* tissues by "monoclonal phage-ELISA" (Fig. 2). These full-length scFvs corresponded to 28% of the total selected phages whereas the remaining phages contained either rearranged genes or no genes at all (data not shown).

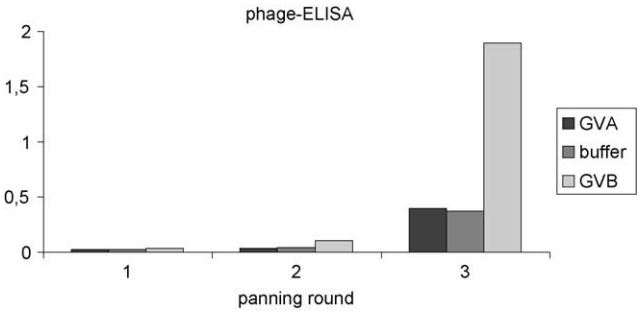

Fig. 1. Monitoring the progress of panning by "polyclonal phage ELISA". Plates were coated with purified GVB particles (0.2 μg/well) and detection was carried out by phage populations originating from each round of panning as described. GVA viral particles were used at the same conditions to demonstrate specificity of binding.

Molecular analysis of these 15 GVB-reacting phages was carried out by RFLP digestion of the amplified scFv genes using the restriction enzyme *MvaI*. Three classes of scFvs genes were found, one represented by clone 27 which gave the highest values in "monoclonal phage-ELISA", was studied further by sequencing. Search for homologies in the database VBASE showed that this scFv 27 originated from the germline segment 3-09 of the VH3 family (data not shown).

3.2. Expression of scFv–CLZIP fusion proteins and ELISA detection

Two highly reacting scFvs, clones 27 and 35, were individually subcloned in pC_LZIP, a construct expressing a scFv in fusion with the constant domain of the mouse light chain (CL), a leucine zipper dimerization domain (ZIP) and a His-tag (Kerschenbaumer et al., 1997), designed to give dimeric scFvs. Bacteria containing recombinant plasmids (pC_LZIP-27 and -35) were tested for protein expression at different temperatures and times of growth. The optimal conditions for expression were 21 °C for 14 h, under which an average yield of scFvCL-ZIP fusion protein of about 1 mg/l of initial liquid culture was obtained (data not shown).

Specific reactivity of scFvCL-ZIP-27 and -35 to GVB was tested repeatedly in "scFvCL-ELISA" using GVB-infected *N. occidentalis* and grapevine and their respective healthy controls using a scFv amount/well ranging between 0.5 and 2.5 μg. The results of a typical assay, reported in Table 1, shows that both scFvCL-ZIP are able to recognize the homologous virus in *N. occidentalis* and grapevine tissues. $O.D._{405\,nm}$ infected/healthy ratios were higher than 2.5, in the case of the scFvCL-ZIP-35, and higher than 3 in the case of the scFvCL-ZIP-27, thus confirming the efficiency of the two monomeric scFvs in "monoclonal phage-ELISA" for detecting GVB.

Dimeric scFvCL-ZIP-27 was also tested in a DAS-ELISA format to coat the plates instead of the polyclonal antiserum to GVB used currently. In a first experiment dimeric scFvCL-ZIP-27 was used at decreasing dilutions to evaluate its coating

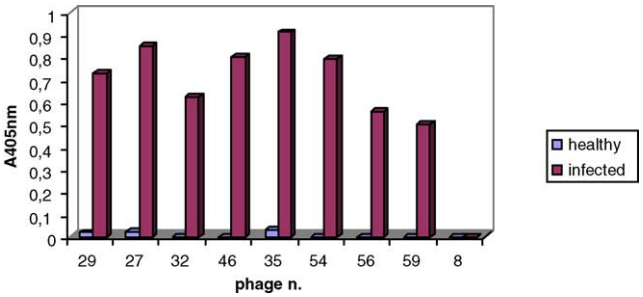

Fig. 2. "Monoclonal phage ELISA" of purified phages randomly selected from the third round of panning. Plates were coated with sap from healthy or GVB-infected *N. occidentalis* plants and detected as described.

Table 1
Detection of GVB by "scFvCL-ELISA" in *N. occidentalis* and grapevine extracts

Detecting antibody[a]	GVB-infected *N. occidentalis*	GVB-infected grapevine	Healthy leaf extract (*N. occidentalis*/grapevine)
$scFvC_L$-27	0.5	0.635	0.05/0.107
$scFvC_L$-35	0.255	0.226	0.065/0.1
Mab-B2	0.530	0.315	0/0.022

[a] Absorbance readings ($A_{600\,nm}$) were recorded after 1 h incubation with the substrate.

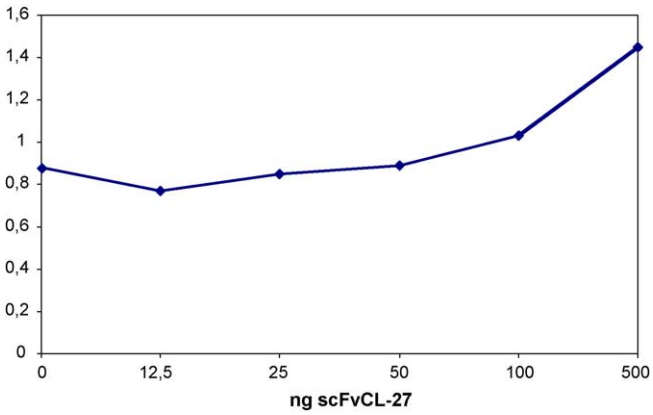

Fig. 3. Detection of GVB by DAS-ELISA in infected *N. occidentalis* tissues. Different amounts of dimeric scFvCL-27 were tested in the coating step whereas detection was done with Mab-B2 and rabbit anti-mouse AP conjugated antibodies.

ability. Absorbance at 405 nm proved to be directly correlated to the amount of the scFvCL-27 used for coating, indicating in 50 ng the minimum amount necessary to saturate the well (Fig. 3).

In a second experiment scFvCL-ZIP-27 was compared in DAS-ELISA with the above antiserum for plate coating. Results demonstrated that scFvCL-ZIP-27 is as efficient as the GVB antiserum in trapping virus particles since $O.D._{405\,nm}$ readings of 1.55 and 1.15 were obtained with the recombinant and traditional antibodies, respectively, using sap of infected *N. occidentalis* plants. Corresponding absorbances of sap from healthy plants were 0.15 and 0.

4. Discussion

Using a phage display system a series of 15 scFv specific for GVB were selected from a human combinatorial scFv antibody library. All 15 scFv-bearing phages were able to recognize the virus adsorbed to ELISA plates from infected tissues of *N. occidentalis*. Specificity of the selected scFvs was demonstrated by their lack of reactivity toward the taxonomically and serologically distantly related GVA.

Careful evaluation of the diversity of selected scFvs by *MvaI* RFLP fingerprinting, showed that they belong to three different classes. The finding that two antibodies, scFv-27 and 35 showed higher O.D. values in "monoclonal phage-ELISA" induced to use them for further studies.

In agreement with previous reports (Kerschenbaumer et al., 1997), it was shown that these two scFvs, dimerized through their expression as a fusion with the amphipatic CLZIP domain, retain and increase their reactivity toward GVB particles adsorbed to ELISA plates. Dimerized scFv-27 was also able to identify the virus extracted from infected grapevine tissues, a substrate in which GVB is very difficult to detect because of its low titer. In addition scFvCL-27, was an effective coating reagent, being able to substitute rabbit serum in a commercial diagnostic DAS-ELISA used for routine detection of GVB in plant material (Agritest S.r.l., Valenzano, Italy). This latter finding makes scFvCL-27 a promising candidate for the development of a complete recombinant assay employing enzyme-tagged-scFv as a secondary detection reagent, as reported in previous work (Griep et al., 2000). Since a rapid reduction of reactivity of scFvCL-27 was observed after purification and storage at −20 °C, optimisation of storage conditions needs to be sought.

This report confirms the ability of phage display technology to select recombinant antibody fragments specific for a plant virus, that, in the case of GVB is of particular importance because of its well-known low immunogenicity. The results allow us to envisage an immediate use for diagnosis through the development of complete recombinant ELISA reagents, and the possible induction of resistance to GVB through the expression of these scFv in transgenic plants.

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