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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