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# Cloning and Characterisation of Nanobodies against the Coat Protein of *Zucchini Yellow Mosaic Virus*

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**Abstract**

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*Zucchini yellow mosaic virus* (ZYMV), in the family *Potyviridae*, causes an economically important disease. Antibodies are valuable reagents for diagnostic assays to rapidly detect viral infection. Here, we report the isolation of camel-derived variable domains of the heavy chain antibody (VHH, also called nanobodies) directed against the coat protein (CP) of ZYMV. Several nanobodies that specifically recognise ZYMV-CP were identified. The isolated nanobodies showed binding not only to recombinant ZYMV-CP but also to native ZYMV, indicating that these nanobodies can be used in diagnostic tools to detect viral infections.

**Keywords**: nanobodies; VHH; naïve library; phage display

*Zucchini yellow mosaic virus* (ZYMV) is a member of the genus *Potyvirus* in the family *Potyviridae* (Shukla & Ward 1989). ZYMV can infect many species and cultivars from the family Cucurbitaceae, and it is the most commonly detected virus in cucurbit species in Saudi Arabia (Al-Saleh 1994). ZYMV infection may result in a complete yield loss when it occurs early in the season (Blua & Perring 1989; Simmons *et al*. 2013), and no recovery phenotype has been reported following infection (Tobias *et al.* 2012). In addition, this disease limits the cultivation of cucurbit crops in certain areas (Al-Shahwan *et al*. 1995; Grafton-Cardwell 1996; Desbiez *et al*. 2002; Gal-On 2007).

Full-size antibodies (Dietzgen & Sander 1982) and their recombinant forms (Ziegler *et al*. 1995; Harper *et al*. 1997; Ziegler & Torrance 2002; Orecchia *et al*. 2008; Zakri *et al*. 2010) have a long history serving as efficient tools for early detection of plant viruses and controlling viral diseases (Nickel *et al*. 2008; Cervera *et al*. 2010). Compared to conventional antibodies, camelids and sharks possess a unique class of antibodies called heavy chain antibodies (HCAB) that are devoid of a light chain (Hamers-Casterman *et al*. 1993; Flajnik *et al*. 2011). Hence, the entire paratope, i.e. the antigen-binding functional region, is found within the heavy chain variable domain. Because of their small size (12–14 kDa), these domains have been termed "nanobodies". They are encoded by a single open reading frame, possess desirable physical, chemical, and molecular properties such as high expression levels, solubility, strong affinities, and thermal stability (Wesolowski *et al*. 2009; Muyldermans 2013), which make them attractive tools for many medical and agronomical applications, including drug discovery, pathogen

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diagnosis, and plant protection (Muyldermans & Lauwereys 1999; Verheesen *et al*. 2003; Cortez-Retamozo *et al*. 2004; Revets *et al*. 2005; Monegal *et al*. 2009; Abbady *et al*. 2011; Flajnik *et al*. 2011; Stijlemans *et al*. 2011; Hassanzadeh-Ghassabeh *et al*. 2013; Muyldermans 2013). In particular, nanobodies have been shown to be less dependent on cellular chaperones because they do not require the assembly of two polypeptide chains and are more tolerant to reducing environments. The latter is of importance because plant viruses are generally located within the cytosol, and antiviral nanobodies have to co-localise to interfere with disassembly, cell-to-cell movement, and replication (Lucas 2006; Laliberte & Sanfacon 2010; Ueki *et al*. 2010; Abbady *et al*. 2011; Harries & Ding 2011; Niehl & Heinlein 2011; Schoelz *et al*. 2011; Tilsner & Oparka 2012; Heinlein 2015). Promising results from the expression of nanobodies in plants have been reported (Winichayakul *et al*. 2009; Teh & Kavanagh 2010; De Buck *et al*. 2013), and transient expression of nanobodies against *Broad bean mottle virus* neutralised the virus infection (Ghannam *et al*. 2015).

Here, we report the isolation of nanobodies against the recombinant ZYMV-CP by phage display panning of a naïve nanobody library. The isolated nanobodies can specifically recognise not only the recombinant protein that was used for panning but also native ZYMV virions. Some of these nanobodies, VP3 in particular, have the potential to be used for early detection of the virus, e.g., for developing lateral flow or biosensor assays, or for genetic engineering of viral resistance.

## MATERIAL AND METHODS

The ZYMV-CP coding sequence was PCR amplified using ZYMV-SA-1 (Al-Saleh *et al*. 2014), cloned into the pIX3.0 vector (Qiagen, Hilden, Germany), expressed in *Escherichia coli* and purified by ion metal affinity chromatography (IMAC) using $Ni^{2+}$-NTA agarose according to the manufacturer's instructions (Qiagen, Germany). The protocol to generate ZYMV-CP-specific nanobodies was based on (Vincke *et al*. 2012) with few modifications. The antigen (purified ZYMV-CP) was immobilised by direct coating onto the surfaces of immunotubes at a concentration of 50 µg/ml. After panning rounds, the resulting libraries were screened against ZYMV-CP by ELISA using mouse anti-HA monoclonal antibody (Sigma-Aldrich, Steinheim, Germany) as the primary antibody, and HRP-conjugated goat anti-mouse (Sigma-Aldrich, Germany) antibody was used as the secondary antibody according to the manufacturer's instructions. Wells were considered positive for ZYMV-CP and nanobody interaction if the absorbance at 450 nm in the antigen-coated well was at least twice that of the PBS-coated well (negative control).

After selecting the positive ZYMV-CP-specific clones, nanobodies were tested again by ELISA using three antigens: 1–10 µg/ml of the recombinant ZYMV-CP, 100 ng/ml of purified ZYMV particles, and 1/25 diluted extracts from infected leaves, while extracts from healthy leaves were used as a negative control. The ZYMV particles, kindly provided by Dr Omar Al-Sogood (King Saud University), were purified from mechanically infected zucchini plants. For this, squash plants showing mosaic symptoms were collected from the central region of Saudi Arabia. Samples were tested and characterized using DAS-ELISA kit from Agdia Inc. (Elkhart, USA) and one of the positive samples was used to infect zucchini seedlings. All antigens were dialyzed against PBS (pH 7.2) before coating plates and continuing the assay as described above. To prepare the leaf extract, 1 g ZYMV-infected leaves were ground in 4 ml potassium phosphate buffer (pH 7.2) and filtrated. The ELISA experiment was repeated 3 times.

The nanobodies were expressed in *E. coli* WK6 cells (Zell & Fritz 1987), extracted from the bacterial periplasm, and purified following protocols described by Vincke *et al*. (2012) using a Ni-NTA Spin Kit (Qiagen, Germany) according to the manufacturer's protocol. Purification was confirmed by SDS-PAGE analysis with Coomassie staining and western blotting using monoclonal anti-poly-histidine antibody (Sigma-Aldrich, Germany) and alkaline phosphatase-conjugated goat-anti-mouse (GAM) for detection.

The surface plasmon resonance (SPR) analyses were performed on a Biacore T200 instrument from GE HealthCare at 25°C using a CM5 sensor chip and HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% v/v Polysorbate 20, pH 7.4) as a running buffer. Purified His-tagged ZYNV-CP was concentrated by ultrafiltration using a 0.5-ml spin column with a 10-kDa MWCO. At the same time, the buffer was exchanged for 10 mM sodium acetate (pH 4.5). The final retentate was diluted 1 : 10 into 10 mM sodium acetate (pH 4.5), and the efficiency of pre-concentration was examined on a blank surface. Immobilization was then performed using an amine-coupling kit, with a flow rate of 10 µl/min and contact time of 420 s for all steps. Briefly, the

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surface was activated by injecting a mixture of equal parts 400 mM EDC and 100 mM NHS, followed by coupling of the ligand and deactivation with 1 M ethanolamine (pH 8.5) and resulting in immobilisation at a level of 2 200 RU. A reference flow cell was generated by activating and deactivating the CM5 matrix under the same conditions. Kinetic analyses were performed at 25°C and a flow rate of 15 µl/min using serial dilutions of the nanobody VP3 (31.25, 62.5, 125, 250, 500, 1 000, 2 000, and 4 000 nM). The association and dissociation phases were 150 and 300 s, respectively. The surface was regenerated with a 60 s injection of 10 mM glycine (pH 2.1). The double-referenced data was plotted using Biacore T200 evaluation software and fitted to a conformational change model. The association and dissociation phases were fitted separately using BIAevaluation software.

## RESULTS

ZYMV-CP-specific nanobodies were isolated by panning the newly built-up variable domains of the heavy chain antibody (VHH) naïve library (unpublished data) against the purified ZYMV-CP. After enrichment by four panning rounds, randomly selected clones were expressed in *E. coli* TG1 cells, and the periplasmic extract was used in ELISA with directly coated ZYMV-CP. Several nanobodies (VP1–VP9) that specifically recognized and bound ZYMV-CP were identified. The selected nanobodies exhibited reactivity against ZYMV-CP that was nearly 10 times higher than that with the negative control. In addition to ZYMV-CP, the clones expressing nanobodies VP1–VP9 showed clear binding to purified viral particles of ZYMV and extracts from ZYMV-infected zucchini leaves (Figure 1A). Although a higher background was observed for coated extracts from healthy leaves, most likely due to cross-reactivity of the secondary antibody against plant proteins, the reactivity against extracts from infected leaves was significantly higher and in agreement with the reactivity observed against purified ZYMV as well as ZYMV-CP. These results clearly show that the selected nanobodies recognise the native viral particles.

Similar results were obtained from a dot blot assay; all nanobodies were bound to both preparations of the virus, purified and in plant extracts (Figure 1B). Additionally, all nanobodies except VP2 showed weak reactivity against the ZYMV-CP by western blot (data not shown).

The selected nanobodies were expressed in *E. coli* WK6 cells and purified using $Ni^{2+}$-NTA agarose. Expression and purification were confirmed by Coomassie-stained SDS-PAGE and western blot analyses (Figure 2). The results revealed a highly pure single band of the expected size for His-tagged nanobodies for each of the different nanobodies. The yield for the purified nanobodies ranged from 6 mg/l to 10 mg/l of the expression culture. Western blot analysis using anti-His tag antibody also showed that all nanobodies were intact or only minor amounts of smaller bands were detected (data not shown).

The purified nanobodies were used to study their binding by surface plasmon resonance. ZYMV-CP was immobilised onto a Biacore CM5 sensor chip by standard 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling (data not shown). Then, an initial screening of all available nanobodies was performed by injecting the nanobodies without intermittent regeneration steps, because it was not known what conditions the

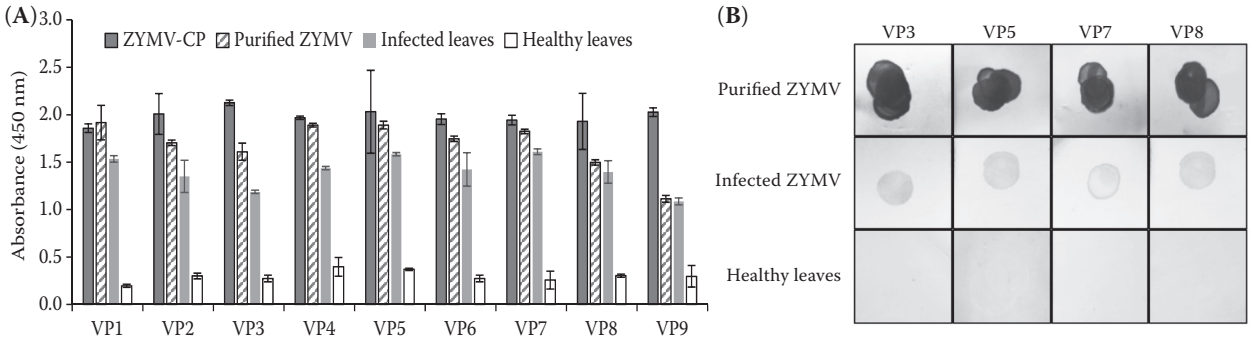


Figure 1. Binding analysis of the isolated nanobodies: (**A**) ELISA showing the binding of the selected nanobodies to recombinant ZYMV-CP (500 ng/ml), purified ZYMV particles (100 ng/ml), and ZYMV-infected zucchini leaves; (**B**) Dot blot analysis showing the binding of nanobodies VP3, VP5, VP7, and VP8 to purified ZYMV particles and extracts from infected zucchini leaves. Healthy leaves were used as a negative control in both assays

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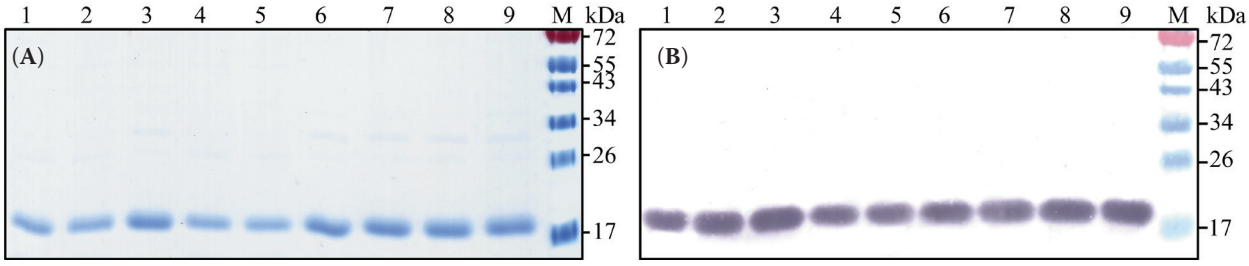


Figure 2. Analysis of the purified nanobodies by SDS-PAGE and western blot: (**A**) Nanobodies separated by SDS-PAGE were visualised by Coomassie staining; (**B**) Western blots were probed with monoclonal anti-poly-histidine antibody and $GAM^{AP}$ and stained with NBT/BCIP substrate

M – pre-stained molecular size marker; 1–9 – Nb VP1–9

immobilised ZYMV-CP would tolerate (i.e., unfolding, aggregation). With the exception of VP3, the nanobodies showed no or minor binding responses only. Several nanobodies that were generated against other antigens were included as negative controls and, as expected, they did not show binding to immobilised ZYMV-CP. Out of the nanobodies that were selected based on their reaction with ZYMV-CP, only VP3 showed significant binding in the SPR experiments. In the next step, regeneration conditions for VP3 binding to immobilised ZYMV-CP were established (data not shown), and a kinetic binding analysis was performed (Figure 3). Notably, the determined maximal response $R_{max}$ indicates that only a small fraction of the immobilised ZYMV-CP was active. The baseline was stable and did not show a significant increase or decrease, and binding to the reference flow cell was low (data not shown). The resulting binding curves were not well described by a simple 1 : 1 binding model, and the association and dissociation clearly exhibited two components. The dissociation phase fit a biphasic model well ($\chi^2$ = 0.009), comprising fast ($k_{diss}$ = 0.087 $s^{-1}$) and slow components ($k_{diss}$ = 0.014 $s^{-1}$). Fitting the association phase to a simple 1 : 1 interaction using the fast component of the dissociation phase resulted in a preliminary estimate ($\chi^2$ = 11.3) of the association rate constant ($k_{ass}$ = 1.2 × $10^4$ M/s), suggesting an overall affinity in the range of 1.2–7.3 µM. In summary, the affinity to the EDC/NHS coupled ZYMV-CP was rather low, and bound VP3 dissociated quickly, which was probably related to modifications introduced during coupling or denaturation as a part of regeneration. Nevertheless, the SPR analysis demonstrated that VP3 binds to ZYMV-CP, therefore corroborating the results from previous experiments, i.e., reactivity in ELISA and dot blot analysis against purified ZYMV and extracts from ZYMV-infected leaves.

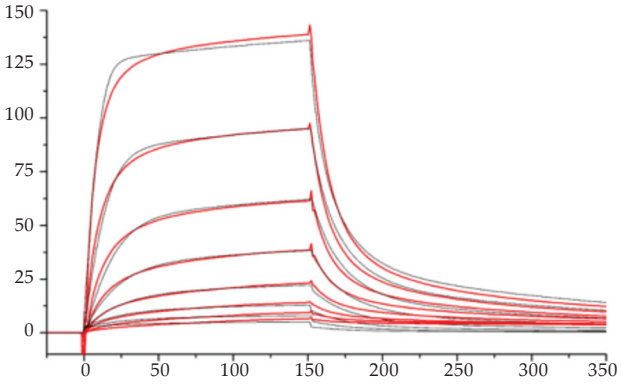


Figure 3. VP3 binding to ZYMV-CP immobilised on a CM5 sensor chip via amine coupling. Experiments were conducted at 25°C using HBS-EP as a running buffer. Different concentrations (31.25–4000 nM) of the VP3 nanobody were injected for 150 s, followed by a dissociation phase of 300 s. The double-referenced sensorgrams (red lines) were fitted to a conformational change model (black lines)

## DISCUSSION

We report here the isolation and characterization of nanobodies against ZYMV-CP from a naïve library. The ZYMV-CP coding gene was amplified and cloned from a local Saudi isolate of ZYMV (Al-Saleh *et al.* 2014) and then overexpressed in *E. coli* and purified via IMAC. The recombinant ZYMV-CP was used as an antigen because of the difficulties in obtaining enough purified viral particles to perform the panning and screening. We also reasoned that the use of recombinant ZYMV-CP might increase our chances of isolating nanobodies that recognised folding and assembly intermediates of the ZYMV presented, e.g., cryptotopes not accessible in intact virions.

With the advancement of large naïve and synthetic antibody libraries and the existence of powerful

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techniques such as phage display, antibodies can be efficiently generated against virtually any antigen. Additionally, isolation, cloning, and recombinant expression are now a routine procedure; therefore, one can quickly and efficiently react to new strains and emerging resistance. In addition, because nanobodies are encoded by single open reading frames, several nanobodies, i.e., resistance genes, can be combined ("trait stacking") to generate broad and durable resistance (Magnus *et al*. 2016; Head *et al*. 2017). Stacking can be achieved by crossing lines that harbour individual nanobody genes but also by co-transforming two or more nanobodies or by engineering fusion proteins comprising multiple nanobodies (multibodies).

Using a VHH naïve library as the nanobody source allowed us to save time and costs, without animal immunization, blood withdrawal, and library construction. Additionally, camel immunisation requires a considerable amount of soluble, properly folded, immunogenic, and proteinaceous antigens (Vincke *et al*. 2012; Hassanzadeh-Ghassabeh *et al*. 2013), which are difficult to obtain in many cases. The major drawback of naïve libraries is that the antigen-specific nanobodies have not affinity matured during immunization and therefore may require subsequent *in vivo* maturation (Koide *et al*. 2007; Hassanzadeh-Ghassabeh *et al*. 2013).

Four phage display panning rounds against ZYMV-CP were performed, resulting in the isolation of nine specific nanobodies. The ELISA and dot blot results showed that the selected nanobodies recognised the recombinant ZYMV-CP specifically, and, as anticipated, ZYMV virions both *in vitro* (as purified particles) and *in vivo* (ZYMV-infected leaves). In ELISA, the reactivity against the infected leaves was, however, slightly lower, which most likely resulted from the lower concentration of ZYMV or the presence of plant proteins in the coating step. The difference was higher in dot blot assay due to the high amount of the purified ZYMV compared to what was used in ELISA. The variation in the absorbance values of different nanobodies may reflect either the affinity or active concentration of the nanobodies. Interestingly, most nanobodies also recognised the denatured CP in the western blot analysis. The bands, however, were very weak and difficult to document, perhaps indicating that the three-dimensional structure of the epitope was destroyed by SDS and that only part of it was still recognizable by the nanobodies.

In the SPR analysis, only nanobody VP3 showed significant binding, whereas the other nanobodies did not recognise the immobilised ZYMV-CP, further suggesting that the nanobodies bind better to native ZYMV virions. The kinetic binding analysis showed that the binding curves were heterogeneous, i.e., they deviated from simple 1 : 1 binding, because of a conformation change (induced fit) and/or heterogeneity in the immobilised ZYMV-CP. This is not surprising, because plant viral CPs have a strong tendency to assemble into higher order structures, i.e., CP oligomers and virions (Kihara 1985). Furthermore, covalent coupling can easily result in obtaining different fractions of the antigen and heterogeneous binding kinetics. Finally, conformational heterogeneity can be caused by (partial) denaturation and refolding during the regeneration step. The most likely explanation for this is that coupling resulted in modification of the antigen, e.g., inactivation of the epitope and/or different presentation than that of ZYMV-CP coated on the wells of the ELISA plates. Indeed, direct immobilisation of antigens to ELISA plates has previously been shown to result in conformational changes (Chung *et al*. 2008). The impact of immobilisation on the reactivity of the nanobodies should be analysed in future studies using capture assays and site-directed coupling strategies. This will be important when assessing the impact of the selected nanobodies on viral replication *in planta* and deducing the mechanism of action. Moreover, binding to intact virions could be analysed by electron microscopy or nanoparticle tracking studies. Ultimately, the nanobodies would have to be expressed in the cytosol of zucchini plants and tested for their capacity to reduce the initial ZYMV infection, as well as cell-to-cell and long distant movement of ZYMV.

The reactivity and specificity of these nanobodies (specially VP3) against ZYMV, specifically in infected leaves, suggest that testing these nanobodies *in vivo* by transient or stable transgenic expression *in planta* might be worthwhile. In the long run, it would be interesting to isolate additional nanobodies to study the effect of multiple nanobodies combined by gene stacking to engineer durable and flexible strategies for generating resistance against ZYMV and other plant pathogens. We anticipate that the work described here represents an initial step toward engineering ZYMV-resistant plants with nanobodies that interfere with virus infection and vector transmission.

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