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ORIGINAL ARTICLE

Generation and characterization of a scFv against recombinant coat protein of the geminivirus tomato leaf curl New Delhi virus

Adel M. Zakri · Angelika Ziegler · Lesley Torrance · Rainer Fischer · Ulrich Commandeur

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Abstract We report the establishment of a hybridoma cell line secreting the monoclonal antibody (mAb) HAV, which recognizes the coat (AV1) protein of tomato leaf curl New Delhi virus (ToLCNDV), a begomovirus. The cell line was obtained following immunization of mice with purified recombinant AV1 fused to glutathione S-transferase (GST). A single-chain variable fragment (scFv-SAV) was assembled from hybridoma cDNA, but sequence analysis revealed a single nucleotide deletion causing a frame shift that resulted in a 21-residue N-terminal truncation. The missing nucleotide was restored by in vitro site-directed mutagenesis to create scFv-RWAV. The binding properties of mAb HAV and the corresponding scFvs were characterized by western blot, ELISA and surface plasmon resonance spectroscopy. MAb HAV bound to AV1 with nanomolar affinity but reacted neither with the N-terminal region of the protein nor with the GST fusion partner. This suggested that the antibody recognized a linear epitope in a region of the coat protein that is conserved among begomoviruses. Both scFvs retained the antigen specificity of mAb HAV, although the dissociation rate constant of scFv-RWAV was tenfold greater than that of scFv-SAV, showing the importance of restoring the 21 N-terminal amino acids.

A. M. Zakri · R. Fischer · U. Commandeur (✉)
Institute for Molecular Biotechnology (Biology VII),
RWTH Aachen University, Worringerweg 1,
52074 Aachen, Germany
e-mail: commandeur@molbiotech.rwth-aachen.de

A. M. Zakri · A. Ziegler · L. Torrance
Plant Pathology Programme, Scottish Crop Research Institute (SCRI), Invergowrie, Dundee DD2 5DA, UK

R. Fischer
Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Forckenbeckstrasse 6, 52074 Aachen, Germany

Present Address:
A. M. Zakri
Center of Excellence in Biotechnology Research (CEBR),
King Saud University, Riyadh, Saudi Arabia

Introduction

The ectopic expression of recombinant antibodies can be used to prevent virus infections, a strategy known as immunomodulation [3, 33]. The feasibility of immunomodulation has been demonstrated for human viruses [22], but there have been few reports thus far of the same technique applied in plants [24, 25, 29, 36]. The use of immunomodulation to counter geminivirus infections would be particularly valuable, since these viruses cause millions of dollars worth of losses in some of our most important food and commodity crops, including maize, wheat, tomato, pepper, tobacco, bean, cotton, squash, beet and cassava.

The genome of geminiviruses consists of one or two single-stranded DNA (ssDNA) molecules, 2.5–3.0 kb in size. The coat protein encoded by ORF AV1 (or V1) has several functions, including the determination of vector specificity [10, 11], the protection of viral DNA during transmission [2, 7] and self-association to form multimeric assemblies [8, 27]. Although coat proteins from different genera show only limited sequence similarity, it is thought that all members of the family *Geminiviridae* share the overall geminate particle structure [30].

As a first step in developing an immunomodulation strategy against commercially important geminiviruses, we report the production of HAV, a monoclonal antibody (mAb) directed against the coat protein (AV1) of tomato

leaf curl New Delhi virus (ToLCNDV), a begomovirus. We also prepared two single-chain variable fragment (scFv) derivatives of mAb HAV and tested the three antibodies for their binding specificity and affinity. The potential role of these antibodies as part of an immunomodulation strategy is discussed.

Materials and methods

Overexpression of recombinant AV1 fusion proteins in bacteria

The *AV1* gene was amplified from a full-length cDNA clone of ToLCNDV DNA-A, yielding a ~0.8 kb product that was fused upstream of the glutathione S-transferase (GST) gene in vector pGEX5x-3 (Amersham Pharmacia Biotech), which had been digested with *Sal*I and *Not*I. A similar strategy was used to clone *AV1* downstream of the maltose-binding protein (MBP) gene in pMAL-cx2 (New England Biolabs). The N-terminal 54 amino acids of AV1, which contain the nuclear localization signal (NLS) [16, 17, 20, 28], were also prepared as a GST fusion by removing the sequence encoding the C-terminal portion of AV1 in pGEX-AV1. This was achieved by digesting the vector with *Bsr*G1 and *Not*I, generating blunt ends, and subsequent ligation. The GST and MBP fusion proteins were expressed in *Escherichia coli* BL21DE3 (Novagen) and purified by affinity chromatography. Glutathione Sepharose 4B from Amersham Pharmacia Biotech was used for purification of GST fusion proteins, whereas amylose resin from New England Biolabs was used for purification of MBP fusion proteins.

Preparation of mAb HAV

A hybridoma cell line secreting antibodies specific for ToLCNDV coat protein AV1 was established by standard hybridoma technology. Briefly, two female Balb/c mice were immunized with the GST-AV1 fusion protein. Using GST-AV1 and MBP-AV1 fusion proteins as antigens for selection, the antiserum was monitored by ELISA after the third injection to determine antibody titers, and after the sixth immunization, a hybridoma cell line secreting the mAb HAV was isolated. Antibody purification using Protein-A Ceramic HYPERD F BioSepra (Ciphergen Biosystems) were carried out according to the manufacturer's standard protocols.

Amplification and cloning of the mAb HAV variable domains

MAb HAV mRNA was reverse transcribed using oligo(dT) primers and the Superscript first strand synthesis kit (Invitrogen). The heavy and light chain variable regions (V_H and V_L) were amplified by PCR using a set of degenerate primers binding to the first and fourth framework regions of the murine IgG. The products were gel purified using a QIAquick gel extraction kit (Qiagen) and joined together by splice overlap extension (SOE) PCR in a 25-µl reaction mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs, 3 µl amplified V_H, 3 µl amplified V_L, and 1.25 units *Taq* DNA polymerase (reaction mixture RI). The reaction was heated for 10 min at 95°C, and then seven amplification cycles (1 min 95°C, 1.5 min 60°C, 1.5 min 72°C) were carried out, followed by a pause at 60°C while 25 µl of reaction mixture RII was prepared and added (final concentration 1× PCR buffer, 2.5 mM $MgCl_2$, 1 mM dNTPs, 2 µl mV_H forward primers mixture, 2 µl mV_K backward primers mixture, 1.25 units *Taq* DNA polymerase). The annealed V_H and V_L products were amplified using 35 cycles of 1 min 95°C, 1.5 min 61°C, and 1.5 min 72°C, followed by 10 min at 72°C. The amplified scFv was inserted into the *Sfi*I/*Not*I sites of pHENHI [26] (a modified version of pHEN4II [35] containing an N-terminal *pel*B leader peptide that targets the expressed protein to the periplasmic space and a C-terminal His_6 tag for purification by Ni-NTA chromatography). The resulting vector was introduced into *E. coli* XL1-blue cells (Stratagene).

Expression and purification of scFvs by IMAC

The scFvs were expressed in *E. coli* XL1-blue cells (Stratagene), extracted from the bacterial periplasm and purified by IMAC. Single colonies were cultured overnight in 5 ml 2YT medium containing 100 µg/ml ampicillin (2YTA) and 1% (w/v) glucose at 30°C on a shaking platform (200 rpm). The culture was diluted with 50 ml 2YTA containing 0.1% (w/v) glucose and maintained at 30°C for 2 h. The cells were then sedimented by centrifugation at 4,000×*g* for 20 min at 4°C and resuspended in 200 ml 2YTA containing 1 mM IPTG and incubated overnight at 30°C with shaking at 200 rpm. The culture was then centrifuged (4,000×*g*, 20 min, 4°C), and the supernatant (S1) was kept on ice. The pelleted bacteria were resuspended in 10 ml ice-cold PBS buffer containing 1 mM EDTA. The suspension was incubated at 4°C for 15–30 min followed by centrifugation (4,000×*g*, 40 min, 4°C). The supernatant (S2) was mixed with the first supernatant (S1), and the proteins were precipitated by adding solid ammonium sulfate (0.3 g/ml supernatant) and stirring for a few minutes. The precipitated proteins (periplasmic extract) were collected by centrifugation (4,000×*g*, 40 min, 4°C), and the pellet was resuspended in 1–2 ml ice-cold PBS and dialyzed against PBS prior to IMAC affinity purification according to the

manufacturer's instructions. The purified scFvs were freeze-dried and stored at −20°C.

In vitro site-directed mutagenesis

A QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) was used for DNA mutation according to the manufacturer's protocols using the following primers:

5′scFv1 (CAGCTTCTGCAGTCAGGGACTGCACTGGCAAAAC) and
3′scFv1 (GTTTTGCCAGTGCAGTCCCTGACTGCAGAAGCTG)

The sample reaction was prepared as follows: 1× PCR buffer, 5–50 ng dsDNA template, 0.2 mM of each of the dNTPs, 125 ng 5′scFv1 primer, 125 ng 3′scFv1 primer, 2.5 units *PfuUltra* HF DNA polymerase, and up to 50 µl dd H_2O. The amplification was carried out under the following conditions: 30 s 95°C, then 12× [30 s 95°C, 1 min 55°C, 4 min 68°C]. After DNA amplification, 1 µl of the *Dpn*I restriction enzyme (10 U/µl) was added directly to the amplification reaction and gently and thoroughly mixed. The reaction was then incubated at 37°C for 1 h to digest the parental (i.e., non-mutated) supercoiled dsDNA. Two µl was then used for transformation of XL10-Gold competent cells (Stratagene).

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were coated with 10 µg/ml of each antigen at 37°C for 2 h and blocked with 4% (w/v) skimmed milk in 1× PBS. Dilutions of the antibodies in 1× PBS were added to the coated plates and incubated at 37°C for 1 h. After three washes with PBS containing 0.1% Tween-20, bound antibodies were detected with alkaline-phosphatase-conjugated goat anti-mouse (GAM^{AP}) IgG in blocking buffer and *p*-nitrophenyl phosphate (1 mg/ml, Sigma). ELISA plates were incubated at 37°C for 20–60 min, and the A_{405} was determined.

Triple-antibody sandwich (TAS)-ELISA

Purified IgG (DSMZ AS-0588, specific for TYLCV) was diluted in coating buffer, and 100 µl was added to each well of a microtiter plate and incubated at 37°C for 2–4 h. After three washes with PBS containing 0.1% Tween-20, 200 µl of 4% skim milk powder in PBST was added to each well and incubated for 1 h at 37°C. After removal of blocking solution, 100 µl aliquots of the infected plant material were added to duplicate wells and incubated overnight at 4°C. After washing three times with PBS-Tween, 100 µl of the detection mAbs HAV and DSMZ AS 0546/2 (used as a universal TYLCV probe) diluted 1:500 in conjugate buffer was added to each well and incubated at 37°C for 2–4 h. After three washes with PBS-Tween, 100 µl of alkaline-phosphatase-labelled rabbit anti-mouse IgG (RAM-AP) diluted 1:1,000 in conjugate buffer was added to each well and incubated at 37°C for 2 h. After washing three times with PBS-Tween, 100-µl aliquots of freshly prepared substrate were added, the mixture was incubated at 37°C for 20–60 min and the A_{405} was determined.

Surface plasmon resonance (SPR) spectroscopy

Biomolecular interaction analysis was carried out by surface plasmon resonance (SPR) spectroscopy using the BIAlite™ X semiautomated SPR system (BIACORE, Uppsala, Sweden). All injected samples were dialyzed and diluted in HBS buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% (v/v) Surfactant P20) and subjected to brief low-speed centrifugation prior to injection to remove insoluble components. The AV1 ligand was immobilized on a CM3 sensor chip using an amine coupling kit (BIACORE). The immobilization of proteins on the chip was performed at a flow rate of 15 µl/min. The carboxyl groups on the sensor surface were activated by injecting 100 mM EDC/NHS (*N*-ethyl-*N*′-(dimethylaminopropyl)-carbodimide hydrochloride, 400 mM *N*-hydroxyl-succinimide). After each binding experiment, the surface was regenerated with 15–20 µl 1.2 M guanidine–HCl, pH 1.5–2. The data were analyzed using the BIAevaluation (4.1) software (BIACORE).

Results

Production of an mAb against the coat protein (AV1)

The full-size ToLCNDV coat protein (AV1) and a truncated form comprising the N-terminal 54 amino acids

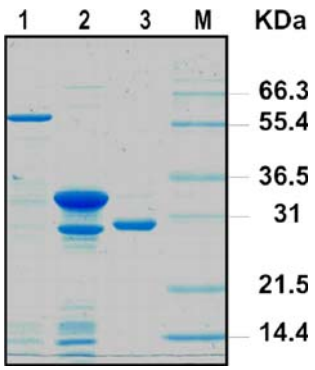

Fig. 1 SDS-PAGE analysis of GST fusion proteins expressed in bacteria and affinity purified. *1* GST-AV1 (~58 kDa), *2* GST-AVNS (~34 kDa), *3* purified GST (~28 kDa), *M* Molecular mass size marker (Mark 12; Invitrogen)

(AVNS) were expressed as GST fusion proteins in bacteria and purified by affinity chromatography (Fig. 1). AV1 was also expressed and purified as a fusion with the maltose-binding protein (MBP) (data not shown). Mice were immunized with GST-AV1. The purified GST-AV1, GST-AVNS, and MBP-AV1 proteins had a tendency to form aggregates and to precipitate. Therefore, after purification, they were immediately frozen till usage. For the Biacore experiments, only the soluble part was used.

Similarly, the ToLCNDV replication initiator protein (AC1) was expressed and purified as GST and MBP fusions following the strategies used for AV1 (data not shown). These preparations served as controls to evaluate the binding specificity of antibodies to GST and MBP fusion proteins, respectively.

Six boosts of GST-AV1 were given at 2-week intervals until a suitable specific antibody titer was achieved. Spleen cells from an immunized mouse were fused with a myeloma cell line to develop a hybridoma clone that secreted a specific antibody against AV1. This resulted in the production and purification of mAb HAV. Indirect ELISA with the purified antibody confirmed that the antibody bound to GST-AV1 and MBP-AV1 but not to GST-AVNS, GST-AC1 or MBP-AC1 (Fig. 2a). In addition, mAb HAV recognized AV1 on a western blot, indicating that the corresponding epitope was linear (Fig. 2b). mAb HAV reacted specifically with leaf extracts from tomato yellow leaf curl virus (TYLCV)- and African cassava mosaic virus (ACMV)-infected plants in TAS-ELISA and western blot assays (Fig. 3a, b, respectively).

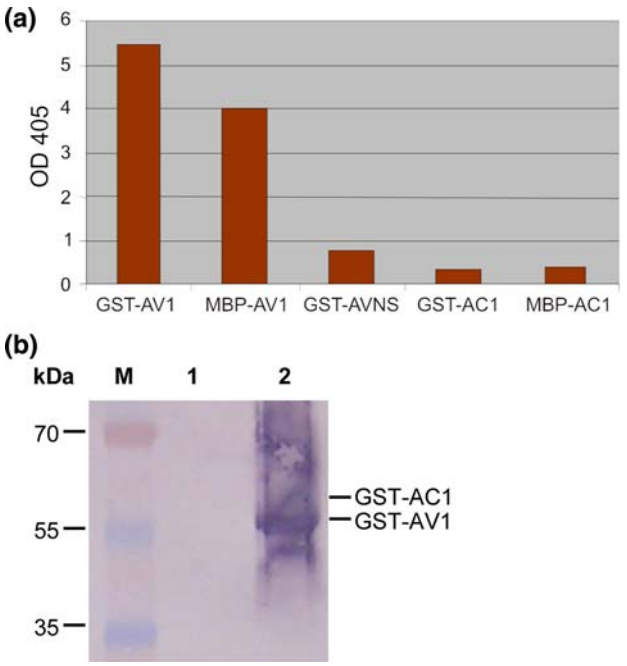

Fig. 2 Analysis of mAb HAV binding activity with recombinant fusion proteins by **a** indirect ELISA and **b** western blot. **a** Purified mAb HAV was incubated on ELISA plates that had been coated with GST-AV1, MBP-AV1, GST-AVNS, GST-AC1 or MBP-AC1 fusion proteins, and bound mAb HAV was revealed by a GAM^{AP} antibody. **b** GST-AC1 (*1*) and GST-AV1 (*2*) were loaded onto an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with diluted mAb HAV (5 µg/ml) for 1 h. Immunodetection was carried out with a GAM^{AP} antibody. *M* pre-stained size marker (Fermentas)

Amplification and cloning of the mAb HAV variable domains

The mAb HAV V_H and V_L genes were amplified separately from cDNA using specific primers, joined together by

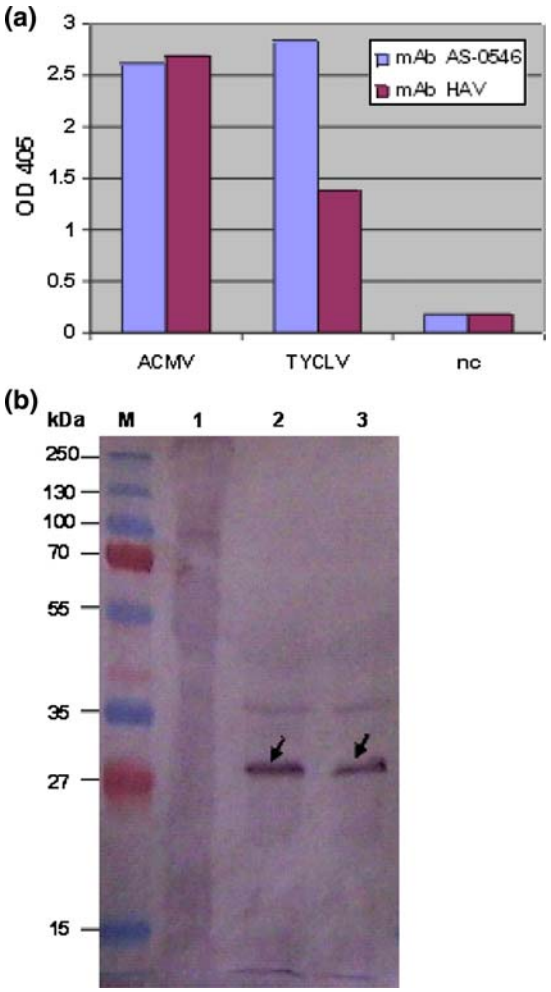

Fig. 3 Analysis of mAb HAV detection ability in infected plant sap by **a** TAS-ELISA and **b** western blot. **a** Infected plant sap was applied to microtitre plates coated with rabbit antiserum AS-0588, and bound mAb HAV was revealed by a RAM^{AP} antibody. *nc* non-infected *Nicotiana benthamiana* as negative control. **b** Leaf extracts from ACMV (*2*) and TYLCV (*3*) infected plants were loaded onto a SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with diluted mAb HAV (5 µg/ml) for 1 h. Immunodetection was carried out with a GAM^{AP} antibody. The position of the coat protein bands are indicated by an *arrow*. *1* non-infected *N. benthamiana*, *M* pre-stained size marker (Fermentas)

SOE-PCR and cloned into the pHENHI vector. Fourteen clones of the transformed XL1-blue cells were picked randomly, and the antibody genes were sequenced. Six clones contained light chains only, and eight clones contained a full-size scFv. The V_H and V_L sequences of all clones were identical, including those containing the light chain only. One of these clones (scFv-SAV) was selected for further analysis. The resulting scFv-SAV was sequenced and expressed in *E. coli*. Western blot analysis of the culture supernatant confirmed the presence of a ~30-kDa band, which had the expected size for a scFv (data not shown). An ELISA was carried out using the culture supernatant, and this showed that secreted scFv-SAV bound specifically, although weakly, to AV1 (data not shown).

Nucleotide sequence analysis of scFv-SAV revealed the presence of a stop codon (TGA) 60 nucleotides downstream of the ATG start codon located within the *Nco*I site. Pairwise alignment of the nucleotide sequence with other antibodies using BLAST revealed a single nucleotide deletion in the scFv-SAV sequence 30 nucleotides downstream of the ATG start codon (adenosine was found in this position in other scFvs). This mutation disrupted the open reading frame and generated a shorter polypeptide from a second ATG codon 27 nucleotides downstream. The expressed scFv had a shorter V_H domain missing the N-terminus but still contained all three complementarity-determining regions (CDRs), which supported weak antigen binding. The missing adenosine in the coding region for the N-terminal part of scFv-SAV was inserted by in vitro site-directed mutagenesis using a QuickChange® II site-directed mutagenesis kit (Stratagene), generating scFv-RWAV.

Both scFv-RWAV and scFv-SAV were expressed in *E. coli* HB2151 as His-tagged proteins and purified by IMAC. The Coomassie-stained gel and western blot (Fig. 4) showed clearly that the molecular mass of scFv-RWAV was slightly higher than that of scFv-SAV, confirming the restoration of the N-terminus.

We carried out an ELISA using purified scFvs, which revealed the high specificity of scFv-RWAV and scFv-SAV against AV1 and a stronger interaction between scFv-RWAV and MBP-AV1 than between scFv-SAV and MBP-AV1 (Fig. 5).

SPR analysis of the AV1 binders

The binding properties of mAb HAV, scFv-SAV and scFv-RWAV to AV1 were characterized further by SPR spectroscopy. The MBP-AV1 antigen was immobilized in flow cell FC2 of the sensor chip, and as a negative control, BSA was immobilized in FC1. For mAb HAV, five different

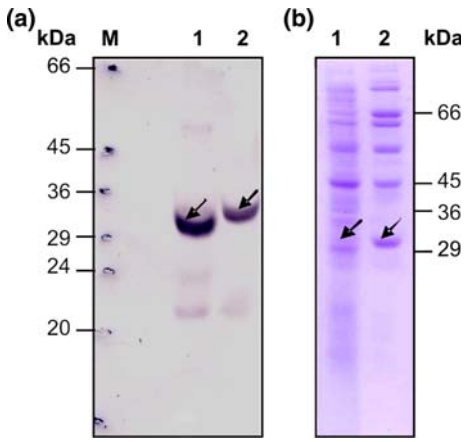

Fig. 4 The scFv-SAV and scFv-RWAV antibodies were expressed in bacteria, purified by IMAC and analyzed by **a** western blot and **b** SDS-PAGE followed by staining with Coomassie brilliant blue. *1* scFv-SAV; *2* scFv-RWAV; *M* molecular mass size markers (Sigma Dalton VII; Sigma). The scFvs were detected in western blots with anti-His and GAM^{AP} antibodies

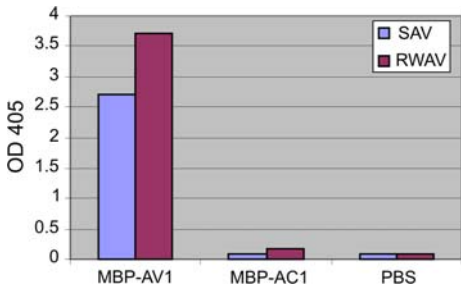

Fig. 5 ELISA reactions of scFv-SAV and scFv-RWAV with AV1. Purified scFvs were applied to microtitre plates coated with MBP-AC1, MBP-AV1 or PBS. Bound scFvs were detected with anti-His and GAM^{AP} antibodies. MBP-AC1 and PBS were used as negative controls

concentrations of antigen were tested (17, 35, 70, 140 and 280 nM), and every concentration was tested three times. The FC2-FC1 data (obtained by subtracting the FC1 control) were analyzed using the BIAevaluation (4.1) software (Fig. 6; Table 1). MAb HAV was shown to have a K_D of 1.23×10^{-9} M with a slow dissociation rate. Similar experiments with equivalent concentrations of scFv-SAV and scFv-RWAV injected over the FC and visual comparison of the sensorgrams (the maximum analyte binding capacity) (Fig. 7) as well as comparison of the values for the dissociation rate constant (kd) (Table 1) showed that scFv-RWAV interacted more strongly with AV1 and had a slower dissociation rate (7.51×10^{-3} s^{-1}) than scFv-SAV (1.35×10^{-2} s^{-1}), indicating that frame correction and restoration of the N-terminus successfully increased the binding properties, albeit not to the value of the parent mAb HAV.

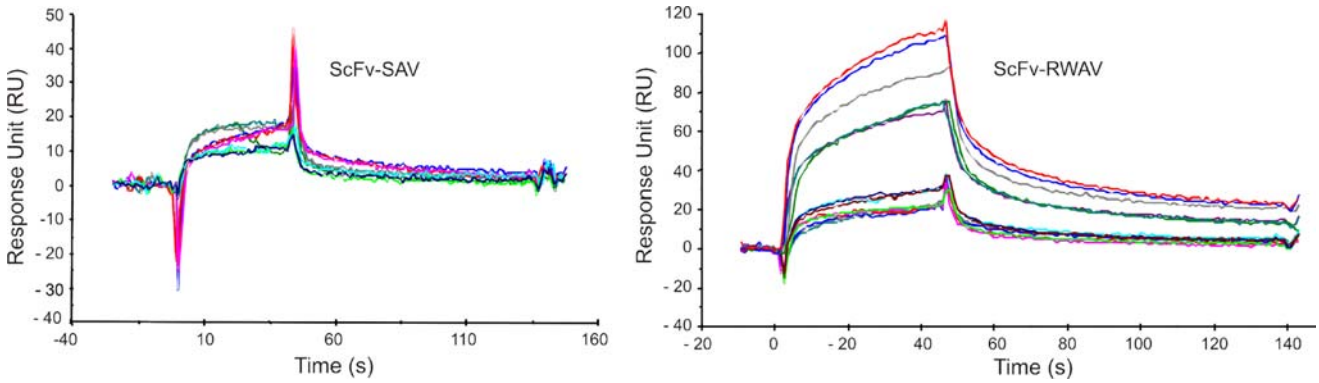

Fig. 6 Sensorgrams (FC2-FC1) of biomolecular interaction between mAb HAV and MBP-AV1. The MBP-AV1 antigen was immobilized on a BIACORE CM3 sensor chip on FC2, and BSA was immobilized on FC1. Increasing concentrations (17, 35, 70, 140, and 280 nM) of mAb HAV were injected over both FCs, and the data were analyzed using the BIAevaluation (4.1) software

Table 1 Kinetic analysis of mAb HAV, scFv-SAV and scFv-RWAV binding to MBP-AV1, showing values for association (ka) and dissociation (kd) rate constants and equilibrium dissociation constant (K_D)

	ka ($M^{-1}s^{-1}$)	kd (s^{-1})	K_D (M)
mAb HAV	1.21×10^5	1.48×10^{-4}	1.23×10^{-9}
scFv-RWAV	–	7.51×10^{-3}	–
scFv-SAV	–	1.35×10^{-2}	–

The Chi square and *T* values (not shown) were within acceptable error ranges

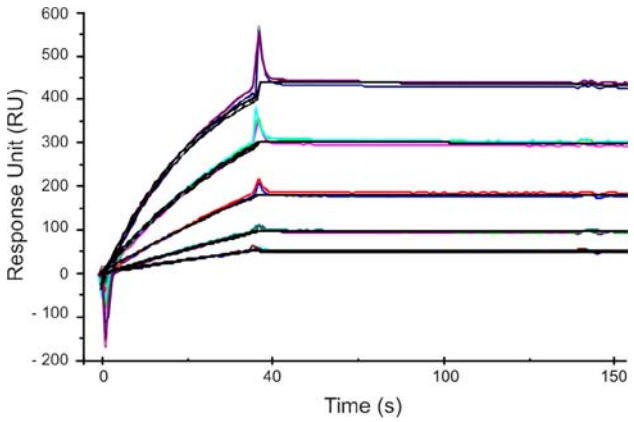

Fig. 7 Sensorgrams (FC2-FC1) of biomolecular interaction between AV1-specific scFvs and MBP-AV1. The MBP-AV1 antigen was immobilized on a BIACORE CM3 sensor chip in FC2, and BSA was immobilized on FC1. Increasing concentrations of scFvs were injected over both FCs, and the data were analyzed using the BIAevaluation (4.1) software

Discussion

The purified mAb HAV showed high specificity and reactivity with AV1 protein preparations in ELISA and western blots, recognizing both MBP-AV1 and GST-AV1 in ELISA and showing no cross-reactivity with fusion proteins containing the unrelated replication initiator protein, MBP-AC1 and GST-AC1. These results exclude interactions between mAb HAV and the linker or any other amino acid residues fused to the AV1 protein. In addition, mAb HAV did not react to fusion protein containing the 54-residue N-terminal portion of AV1, indicating that the epitope lies within the central or C-terminal parts of the protein. Since most of the variability in the amino acid sequences of begomovirus coat proteins is limited to the N-terminal 60–70 amino acid residues, with the remainder of the coat protein sequence being highly conserved [1, 9], it is possible that mAb HAV may react with the coat protein of several begomoviruses. In support of this theory, we found that mAb HAV also reacts with recombinant coat protein preparations from a TYLCV isolate from Iran (unpublished data). This theory was further supported by the TAS_ELISA reactions of mAb HAV with extracts from begomovirus-infected plants, as strong and intermediate reactions between mAb HAV and ACMV- and TYLCV-infected plant sap, respectively, were observed. The reaction of mAb HAV with ACMV was as high as that of mAb AS 0546/2 and lower in the case of TYLCV. A possible explanation for this observation is that mAb HAV was generated against recombinant coat protein of ToLCNDV, whereas mAb AS 0546/2 was raised against TYLCV. Furthermore, the epitope is likely to be linear, since mAb HAV bound to denatured MBP-AV1 fusion protein and to the coat proteins of ACMV and TYLCV in western blots. The high affinity of mAb HAV was further reflected by kinetic data from SPR spectroscopy.

A recombinant scFv was created from cloned mAb HAV DNA. After expression in *E. coli*, the corresponding scFv-SAV was found to be truncated, and this was traced to a single nucleotide deletion, which was corrected by site-

directed mutagenesis to produce the full-size scFv-RWAV. In ELISA, scFv-RWAV reacted more strongly with AV1 than scFv-SAV, but because of the high level of impurities, it was difficult to determine the active concentration of either scFv. Therefore, we used SPR spectroscopy to compare antigen binding, as this can be done independently of the concentration using the BIAevaluation (4.1) software. In many cases, the dissociation rate constant (or off rate) is the crucial parameter controlling the presence of a useful function [4]. With viruses, the capacity of an antibody to neutralize virus infectivity tends to correlate with the antibody off rate rather than the equilibrium constant [23, 31, 32, 34]. SPR showed that scFv-SAV bound weakly even at the highest available concentration, whereas scFv-RWAV dissociated more slowly. The weak binding of scFv-SAV compared to scFv-RWAV probably reflects the missing 21 amino acids from its V_H framework 1 region, which is known to have a high impact on scFv functionality, conformation, stability and yield [5, 12, 14, 18, 21]. Framework 1 of the V_H4-34 gene segment is essential for the interaction between cold agglutinin antibodies and the I antigen [19]. The amino acids at positions H6, H7, and H10 define the distinct conformations of V_H framework 1 [13]. Particularly H6, a buried glutamine or glutamate residue, has a strong effect on antibody functionality [12, 18, 21], and substitutions can have a drastic effect on stability and affinity [6]. Furthermore, Khalifa et al. [15] showed that binding kinetics are affected by framework residues remote from the binding site. In many cases, PCR-induced sequence changes in the framework regions reduced antigen binding, yield and thermodynamic stability, although in some cases the resulting scFvs remained fully functional [5, 12]. The ability of scFv-SAV to bind AV1 despite the missing 21 amino acids is surprising, and no similar example has been reported to date.

Although the affinity of scFv-RWAV for AV1 was higher than scFv-SAV, it was still lower than that of mAb HAV. This can be attributed to the different valences of these molecules in addition to the presence of dimers, multimers and impurities. Even so, the binding affinity was adequate for the detection of AV1 in ELISA. Future work will focus on the expression of scFv-RWAV in plants and the assessment of in vivo binding activity with the aim to produce plants with transgenic resistance to ToLCNDV and possibly other begomoviruses.

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