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Generation and characterisation of functional recombinant antibody fragments against RNA replicase NIb from plum pox virus ☆

Olga Esteban,[a,1] Juan Antonio García,[b] María Teresa Gorris,[a] Elvira Domínguez,[b] and Mariano Cambra[a,*]

[a] *Departamento de Protección Vegetal y Biotecnología, Laboratorio de Virología e Inmunología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Apdo, Oficial, 46113 Moncada, Valencia, Spain*
[b] *Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, Campus de la Universidad Autónoma, CSIC, Cantoblanco 28049, Madrid, Spain*

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Abstract

A monoclonal antibody (mAb 2A) able to react against the RNA replicase NIb from plum pox virus (PPV) was obtained and used for generating a specific scFv fragment. The VH and VL coding sequences were cloned and expressed as a fusion scFv protein to alkaline phosphatase. This fusion protein was able to recognise viral NIb in both Western and tissue-print ELISA blots. The affinity and specificity of scFv2A for NIb was similar to that of the parental mAb and the region YLEAFY from PPV-NIb was identified by PEPSCAN assay as the putative epitope. Isolated VH domains from scFv2A were also expressed as fusion to alkaline phosphatase. However, their ability to react against NIb was greatly altered. scFv2A fragments were transiently expressed in the cytosol of *Nicotiana benthamiana* and although they accumulated to low levels, inhibition-ELISA results indicated that they retained antigen-binding activity.

Keywords: Single-chain variable fragment; scFv fusion protein; RNA replicase; Plum pox potyvirus; YLEAFY epitope; Agrobacterium-mediated transient expression

Novel approaches to immunomodulate host–pathogen interactions by expression of antibody genes in plants have emerged over the last few years [1,2]. The feasibility of the immunomodulation approach for engineering plant virus resistance has been shown by different groups with the use of transgenic plants expressing recombinant antibodies (rAbs) directed against structural viral proteins [3–8]. However, the success obtained with these strategies was limited because transgenic plants were only partially protected against infection and effects of the pathogen. The expression of rAbs binding virus replication-associated enzymes, which normally requires a more sophisticated modulation in expression levels to control the replication process, may be a more effective way to interfere with viral infection. Moreover, as most of the infection processes take place in the cytoplasm, rAbs should be targeted to this compartment to facilitate their interference with antigen function. Intracellular expression of rAbs in the cytoplasm has been obtained only with single chain variable fragments (scFvs) because they require only minor post-translational processing. However, the reducing environment of the cytoplasm does not offer always proper folding conditions for scFvs leading in most cases to their misfolding and degradation. Nevertheless, expression of stable and functional antibodies in the cytosol of mammalian and plant cells

☆ *Abbreviations:* scFv, single-chain variable fragment; rAb, recombinant antibody; mAb, monoclonal antibody; MBP, maltose binding protein; ELISA, enzyme-linked immunosorbent assay; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; PBS, phosphatebuffered saline; CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate; BSA, bovine serum albumin; TSP, total soluble protein.

* Corresponding author. Fax: +34-963424001.
E-mail address: mcambra@ivia.es (M. Cambra).
[1] Present address: Department of Chemical and Biomolecular Engineering, University of Illinois, Urbana, IL 61801, USA.

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has been reported [9–14], suggesting that the formation of a disulphide bond in the variable domains of such scFv fragments does not appear to be essential for their function. It is well known that the behaviour of different cytoplasmic scFv fragments is highly variable and their performance under conditions of intracellular expression depends primarily on their amino acid sequence in a way that is as yet unpredictable [9]. Therefore, all plant-produced scFv fragments intended for intracellular immunomodulation should be tested in vivo. Since the generation of stably transformed transgenic plants is time consuming, alternative systems based on two hybrid system [15,16] and plant transient expression like agroinfiltration [17] or use of plant viral vectors [18] have been used to assess scFv gene expression and antigen binding function prior to transformation.

We selected the replicase protein from *Plum pox virus* (PPV) as a model system to investigate the immunomodulation of non-structural viral proteins in plants. This virus is a member of the *Potyvirus* genus that causes a disease with serious agricultural and economic impact on stone-fruit trees [19]. Potyviruses have flexuous filamentous particles containing a single-stranded positive RNA whose expression involves the production of a polyprotein that is processed by three virus-encoded proteases to yield smaller proteins [19]. One of these proteins is the large nuclear inclusion (NIb) protein, which has RNA-dependent RNA polymerase (RdRp) activity [20]. NIb is located mainly in the nucleus of infected plant cells but its replication function presumably takes place in membrane structures in the cytoplasm [21].

In this paper, a murine monoclonal antibody (mAb) recognising a conserved motif in the NIb protein of all PPV isolates and a scFv derived from it were produced and characterised. A fusion scFv protein to alkaline phosphatase was generated and demonstrated to be able to bind to viral native antigen in PPV-infected stone fruit and herbaceous plant material. The activity of VH domains from this scFv fragment was also assessed and the in vivo stability of a leaderless scFv fragment was evaluated by transient expression in *Nicotiana benthamiana*.

Experimental procedures

Overexpression of recombinant NIb protein in Escherichia coli. The cDNA of the complete PPV-NIb coding sequence was amplified by PCR from plasmid pGPPV [22] using the primers NIbGly and NIbC*B (Table 1), which allowed the incorporation of a six glycine-encoding sequence and a TGA termination codon preceding a *Bam*HI restriction site at the 5′ and 3′ ends, respectively, of the amplified fragment. The *Bam*HI-treated PCR fragment was cloned between the *Xmn*I and *Bam*HI restriction sites of the expression vector pMal-c2 (New England Biolabs) giving plasmid pMGNIb. The maltose binding protein-NIb (MBP-NIb) fusion protein encoded by this vector was expressed in *E. coli* JM109 and purified by affinity chromatography on amylose resin following the procedure recommended by New England Biolabs.

Monoclonal antibody production. Hybridoma cells secreting mAbs specific to PPV-NIb protein were produced by standard hybridoma technology [23]. BALB/c mice were intraperitoneally immunised with a solution of 100 μg of recombinant MBP-NIb. Hybridisation, screening for presence of specific antibodies, cell cloning, and mAb isotype determination were performed as previously described [24]. The mAbs were purified from ascitic fluid by affinity chromatography using protein A (Beckman) as recommended by the manufacturer.

Cloning of scFv2A coding sequences. mRNA was extracted from 2.6×10^6 freshly grown hybridoma cells using an mRNA Purification Kit (Pharmacia). VH and VL first-strand cDNAs were synthesised from the mRNA with primers CHFOR (an equimolar mixture of MOCG1-2FOR, MOCG3FOR, and MOCMFOR) and CKFOR (H. Hoogenboom, unpublished data), respectively, and AMV reverse transcriptase (Promega). To make the construct for the scFv fragment, the VH gene was amplified from the cDNA with primers VH1backSfi and VH1for2LiAsc [25] and cloned into the *Sfi*I/*Asc*I-digested pDAP2 vector [26]. For amplification of the VL gene, an equimolecular mixture of oligonucleotides VK5′-1LiAsc, VK5′-2LiAsc, VK5′-3LiAsc, VK5′-4LiAsc, and VK5′-5LiAsc was used as forward primer and the mixture of JK1Not, JK2Not, JK4Not, and JK5Not [25] as reverse primer. The mixture of forward primers is based on primers previously reported [27], which were modified at their 5′ end to introduce the *Asc*I restriction site and the half 3′ of the linker sequence. The VL PCR product was finally cloned into *Asc*I/*Not*I-digested pDAP2VH2A, to produce pDAP2scFv2A, which encodes the anti-NIb scFv (scFv2A) fused to alkaline phosphatase and a C-terminal six-histidine tail.

The *Sfi*I/*Not*I restriction fragment from pDAP2scFv2A containing the anti-NIb scFv coding sequence was further subcloned into the vector SfiI/NotI pDAP2/S tet [28] giving rise to pDAP2/SscFv2A.

The plasmid that produces the scFv2A fused directly to a six-histidine tail was obtained by deleting the alkaline phosphatase gene from pDAP2 by inverse PCR with primers MDAP1 and MDAP2, which contain a *Mlu*I restriction site in their 5′ end. The resulting PCR fragment was digested with *Mlu*I, treated with the methylation-dependent enzyme *Dpn*I to remove the template DNA, gel-purified, and self-ligated to give plasmid pMDAP.

For cloning the VH domain in frame with alkaline phosphatase, the linker region and VL domain of pDAP2scFv2A were deleted by inverse PCR using the primers NVH2A and NpDAP2. These primers were designed in a back-to-back orientation, each containing a *Not*I recognition site in its 5′ end. The resulting PCR fragment was digested with *Not*I and processed as mentioned above, to give pDAP2VH2A. The absence of PCR copying errors was confirmed by automated DNA sequencing.

The sequences of the different oligodeoxynucleotide primers mentioned in this section are listed in Table 1.

Expression and purification of scFv fragments. Fusion proteins scFv2A-his, scFv2A-AP, scFv2A-AP/S, and VH2A-AP were expressed in *E. coli*, extracted from the bacterial periplasm, and purified by IMAC [26]. The scFv fragments were stored at 4 °C in PBS after dialysis against the same buffer. Protein concentrations were determined by the BioRad Protein Assay Kit (Bio-Rad) using bovine serum albumin (BSA) as standard. The purity was checked by SDS–PAGE and Coomassie staining of the gel. Alternatively, the gels were subjected to immunoblotting onto PVDF membranes (0.45 μm, Millipore). Blots were processed using anti-his antibody 3D5 (Invitrogen) as primary antibody and anti-mouse IgG alkaline phosphatase-conjugate (GAM-AP, Sigma) as secondary antibody to detect the recombinant antibody fragments. Blots were developed using BCIP/NBT (Sigma) as substrate.

Affinity and specificity evaluation. The binding affinity of the soluble scFv2A-AP/S fusion protein was determined by competitive ELISA as described in Bosilevac et al. [29]. The ability of this antibody fragment to bind NIb protein was compared with that of its parental mAb.

Table 1
Primer sequences. S = C/G; M = A/C; R = A/G; and W = A/T

Amplification product	Primer name	Primer sequence (5′ → 3′)
PPV-NIb cDNA	NIbGly	GGTGGTGGTGGTGGTGGTTCCAAAACTACACATTGG
	NIbC*B	CGGGATCATTGGTGCACAACAACGTTG
VH first-strand cDNA	MOCG1-2FOR	CTCAATTTTCTTGTCCACCTTGGTGC
	MOCG 3FOR	CTCGATTCTCTTGATCAACTCAGTCT
	MOCMFOR	TGGAATGGGCACATGCAGATCTCT
VL first-strand cDNA	CKFOR	CTCATTCCTGTTGAAGCTCTTGAC
VH domain	VH1backSfi	CATGCCATGACTCGCGGCCCAGCCGGCCATGGCCSAGGTSMARCTGCAGSAGTCWGG
	VH1for2LiAsc	ACCGCCAGAGGCGCGCCCACCTGAACCGCCTCCACCTGAGGAGACGGTGACCGTGGTCCCTTGGCCCC
VL domain	VK5′-1LiAsc	GGTTCAGATGGGCGCGCCTCTGGCGGTGGCGGATCGGAAATTGTKCTCACMCARTCTCC
	VK5′-2LiAsc	GGTTCAGATGGGCGCGCCTCTGGCGGTGGCGGATCGGACATCCAGATGACMCAGWCTMC
	VK5′-3LiAsc	GGTTCAGATGGGCGCGCCTCTGGCGGTGGCGGATCGGATATTGTGATGACMCAGGMT
	VK5′-4LiAsc	GGTTCAGATGGGCGCGCCTCTGGCGGTGGCGGATCGGATGTTGTGATGACCCAAACTCC
	VK5′-5LiAsc	GGTTCAGATGGGCGCGCCTCTGGCGGTGGCGGATCGARYATTGTGATGACCCAGWCTC
	JK1NOT	GAGTCATTCTGCGGCCGCCCGTTTGATTTCCAGCTTGGTGCC
	JK2NOT	GAGTCATTCTGCGGCCGCCCGTTTTATTTCCAGCTTGGTCCC
	JK4NOT	GAGTCATTCTGCGGCCGCCCGTTTTATTTCCAACTTTGTCCC
	JK5NOT	GAGTCATTCTGCGGCCGCCCGTTTCAGCTCCAGCTTGGTCCC
Vector pMDAP	MDAP 1	TATCACGCGTCAGTTTCAGCTCCAGCTTGGTCC
	MDAP 2	TCACACGCGTCACCATCACCATTAAGG
Vector pDAP2VH2A	NVH2A	ATCTGCGGCCGCTGAGGAGACGGTGACCG
	NpDAP2	ACTGGCGGCCGCAGCCCGG
Vector pMGNIb476-518	NIb2	GTAGCTATCGATTGATTGCGGAGACAGCACTGAAG
	NIb3	GTAAGCATCGATGAGTCTGCGCGTCTTTCAGGG

Competitive ELISAs utilised MBP-NIb-coated microtiter wells and the same fusion protein as competitor. MBP-NIb protein, at concentrations of 0.1–25 nM, was incubated with purified mAb (5×10^{-10} M) or bacterial periplasmic fraction containing scFv2A-AP/S (diluted to 8×10^{-11} M in 0.02% PBS–BSA) overnight at room temperature before addition to the ELISA plate wells. Negative controls included unfused MBP and a culture supernatant fraction containing the *Citrus tristeza virus* CP-specific scFv3DF1 [30], not reactive against NIb. Results of competitive ELISAs were read on an ELISA reader (MultisKan Titertek, Labsystems) at 405 nm and plotted as competitor concentration versus percent inhibition of binding.

Serological tests to detect NIb in plant material. The ability of the scFv2A fusion proteins to react against PPV-NIb protein was determined by Western blot and tissue printing-ELISA. Total protein was isolated from healthy and PPV-infected *N. benthamiana* and *Prunus armeniaca* (apricot) seedlings as previously described [31]. Proteins from 20 μl of plant extract were separated on 12% polyacrylamide–SDS gels and transferred to PVDF membranes. After blocking with 3% PBS–BSA, the membranes were incubated with the recombinant antibody (1:16 dilution of the bacterial periplasmic extract in PBS) or with purified Mab 2A (0.25 μg/ml) followed by GAM-AP. Blots were developed with BCIP-NBT substrate.

Prints were prepared by pressing freshly cut non-infected or PPV-infected *N. benthamiana*, apricot, and *P. domestica* (plum) seedlings onto nitrocellulose membranes (Millipore) and processed for tissue-printing ELISA as described in [30].

Identification of the mAb 2A epitope. The parent plasmid used for all deletion mutagenesis reactions was pMGNIb. pMGNIb1-334 and pMGNIb1-430 were obtained by deleting, using standard techniques, a *Hin*dIII (nt 1003–1498 from the sequence coding NIb) or a *Cla*I–*Sal*I (nt 1287 from NIb to 16 nucleotides behind NIb ORF) restriction fragment, respectively. pMGNIb476-518 was generated by inverse PCR using pMGNIb as template and the oligonucleotides NIb2 and NIb3 (Table 1), which include *Cla*I restriction sites, as primers, and self-ligation of the resulting PCR fragment previously treated with *Cla*I and *Dpn*I. The accuracy of the ligation junctions and the absence of PCR-induced mutations were confirmed by automated DNA sequencing. Western blot analysis of the products expressed by these deletion plasmids was carried out as described in Experimental procedures using mAb 2A and anti-MBP polyclonal antibodies (Pabs).

For PEPSCAN analysis [32], a set of 25 13-mer peptides spanning the C-terminal region of NIb (each peptide overlapping with the previous peptide by 12 aa) were synthesised on membrane (Jerini AG, GMBH, Germany). Binding of the anti-NIb mAb, scFv2A-APS, and VH2A-AP to NIb-derived overlapping peptides was tested as described for the Western blot analysis. The PepSpot membrane was developed by reaction with CSPD luminescent substrate (Boehringer) and exposure to X-ray film.

Transient scFv2A expression in N. benthamiana. The plasmid pMDAP-XS was generated from pMDAP by insertion of *Xba*I and *Sal*I restriction sites at the 5′ and 3′ ends of the scFv-his cassette, respectively. The *Xba*I site was introduced by PCR-mediated mutagenesis whereas the *Sal*I site was inserted by cloning a 20-bp oligonucleotide containing the restriction sites *Eco*RI–*Sal*I–*Bam*HI between the *Bam*HI and *Eco*RI sites of pMDAP. The scFv2Ahis-encoding *Xba*I–*Sal*I fragment of pMDAP-XS was ligated into the *Xba*I/*Sal*I-digested 35-pel-scFv[2A10] vector [33] to give pBIscFv2A. This plasmid was transformed into the *Agrobacterium tumefaciens* strain C58(pMP90) carrying the virulence helper plasmid pCH32 [34].

Growth of recombinant *Agrobacterium* was performed as previously described [18]. The cell suspension was incubated at room temperature for at least 1 h before agroinfiltration. A syringe with needle was used to inject the *Agrobacterium* suspension into whole leaves of *N. benthamiana*. Plants were kept for 3 days in a greenhouse before scFv expression analysis.

Accumulation of the scFv2A in *N. benthamiana* was assessed by Western blot as described in Materials and methods and functional antibodies were detected by inhibition-ELISA. For this analysis, MaxiSorp (Nunc) polystyrene microtiter plates were coated overnight at 4 °C with MBP-NIb (10 ng/well) in 50 mM carbonate buffer, pH 9.6. Agroinfiltrated leaf extracts were prepared in PBS, 1% PVP, 2% ovalbumin, and 1 mM PMSF and diluted in 3% BSA. Non-agroinfiltrated leaf extracts were used as negative control. Different dilutions of the extracts were added to the wells and incubated overnight at 4 °C. After washing the plate, periplasmic extract (diluted to 1/40 in PBS) containing scFv2A-APS was added to each well and incubated for 3 h at 37 °C. *p*-Nitrophenol phosphate was used as substrate for development of the plate, which was monitored at 405 nm. Results of ELISA were plotted as dilution of the extract versus percent of binding inhibition.

Results

Production of a monoclonal antibody against NIb protein

To obtain enough immunogen representing the PPV-NIb protein, NIb was overexpressed in *E. coli* JM109 as a fusion to the carboxy-terminus of MBP to facilitate purification of the protein by amylose resin columns. Upon SDS–PAGE, the MBP-NIb protein migrated as a band with the expected mobility (apparent molecular mass of 101 kDa). Mouse immunisation with MBP-NIb yielded mAb-secreting hybridoma clones, from which only one (2A) reacted strongly against MBP-NIb without exhibiting the reaction with MBP protein. This mAb belonged to the IgG1, κ class and reacted with the homologous immunogen on immunoblots (data not shown).

Cloning and expression of anti-NIb 2A recombinant antibodies

Our goal of investigating the immunomodulation of the PPV replicase protein in plant prompted us initially to clone the VH and VL sequences of the mAb 2A from its parental hybridoma cell line. For this purpose, the coding sequences of the VH and VL domains were sequentially cloned into pDAP2 to give the plasmid pDAP2scFv2A, which codes for the scFv2A fused to the *E. coli* wild-type alkaline phosphatase. Confirmation that the correct VL and VH domains were cloned was obtained by automated DNA sequencing of the scFv fragment and comparison with the previously obtained sequences of three independent clones of VL and VH genes. The amino acid sequence of the scFv fragment was deduced from its nucleotide sequence (GenBank Accession No. AJ416563) and compared with murine variable gene sequences in the IMGT database [35]. The VH domain was assigned to the subgroup IGHV1 whereas the V and J segments from the VL domain were included in the subgroups IGKV1 and IGKJ1, respectively.

The scFv2A coding sequence was subcloned into pDAP2/S tet to fuse the scFv to a mutated form of the *E. coli* alkaline phosphatase with enhanced activity

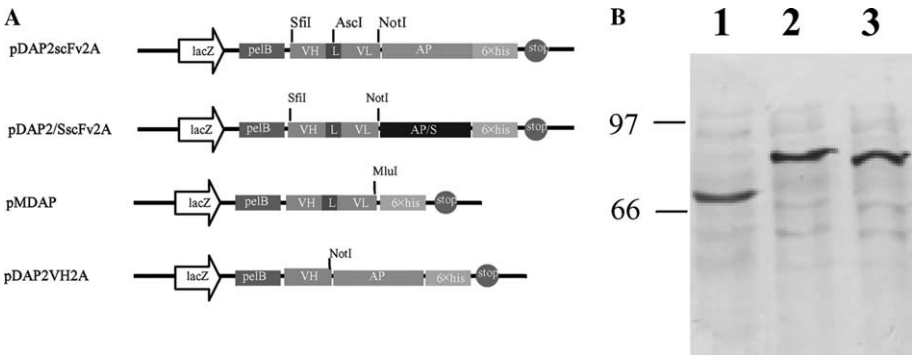

Fig. 1. (A) Schematic diagram/representation of the different antibody fragment constructs generated. (B) Western blot analysis of periplasmic scFv and VH fusion proteins (5 μg) probed with the anti-his mAb 3D5. The M_r values of standards are shown on the left. Lane 1: VH2A-AP; lane 2: scFv2A-AP; and lane 3: scFv2A-AP/S.

(AP/S) [36]. In addition, isolated VH domains from scFv2A were expressed in pDAP2 (Fig. 1A). The scFv and VH fusion proteins were purified from bacterial periplasmic extracts by IMAC. The yield for each fusion protein was variable ranging between 50 and 800 μg/litre of bacterial culture. SDS–PAGE and subsequent immunoblot analysis of IMAC-purified protein fractions (not shown) or bacterial periplasmic extracts (Fig. 1B) revealed a single band of the expected size for each scFv and VH fusion protein.

Affinity and specificity of the anti-NIb 2A antibodies

The binding characteristics of the mAb and scFv2A-AP/S were evaluated through competitive ELISA using MBP-NIb as antigen. Soluble MBP-NIb, but not unfused MBP, competed for binding of scFv2A-AP/S to immobilised MBP-NIb (Fig. 2), demonstrating that scFv2A binds specifically to NIb. To compare the affinity for NIb of the mAb and scFv2A, the ability of MBP-NIb to inhibit the binding of both antibody species to immobilised MBP-NIb was assessed (Fig. 2). The competition curves generated were used to determine the MBP-NIb protein concentration giving 50% inhibition of binding. This value (IC_{50}) can be assumed to be a function of affinity (K_d) and is used to compare antigen binding activities [37]. The control 3DF1scFv-containing periplasmic extracts did not bind MBP-NIb. The IC_{50} of soluble MBP-NIb for scFv and mAb binding to immobilised MBP-NIb was 2.7×10^{-9} and 1.3×10^{-9} M, respectively. Affinities of the isolated 2A VH domains could not be accurately assessed since, although they were able to bind MBP-NIb, their sensitivity in ELISA was much lower than those of scFv and mAb 2A (data not shown).

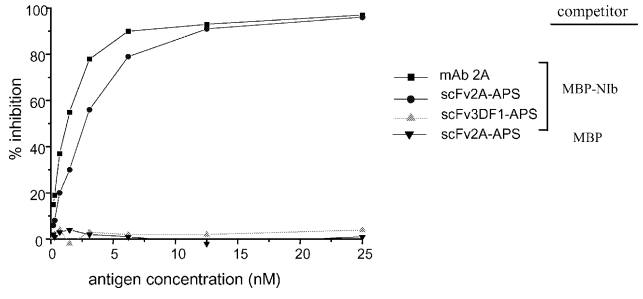

Fig. 2. Competitive MBP-NIb ELISA analysis of scFv2A-APS, mAb2A, and scFv3DF1-APS (mock scFv). Competition with recombinant MBP-NIb bound to microtiter wells was performed with decreasing concentrations of MBP-NIb and MBP (*x* axis). Results are plotted as percent inhibition of binding (*y* axis). Apparent affinity is defined as the reciprocal of the MBP-NIb concentration required to inhibit 50% of maximal binding (IC_{50}).

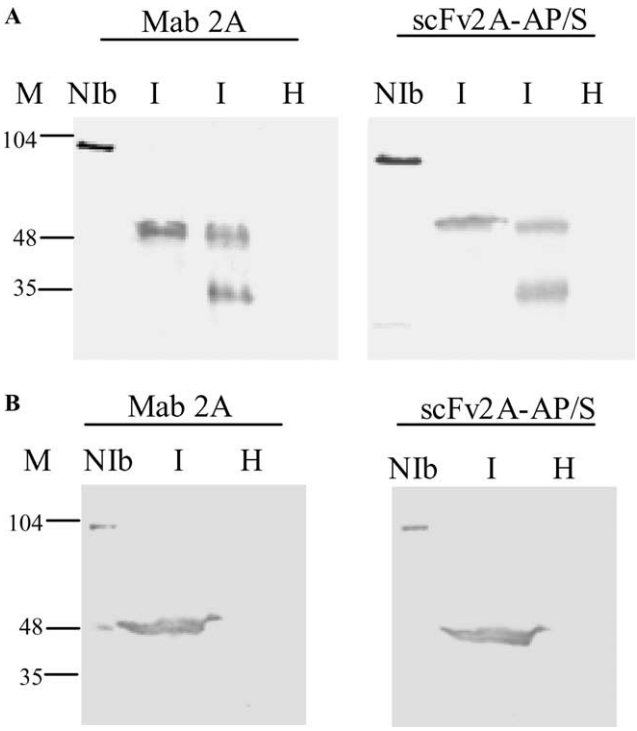

Fig. 3. Immunoblot analysis of 10 μg of total soluble protein from PPV-infected (I) or healthy (H) *N. benthamiana* (A) and *P. armeniaca* (B) plants probed with mAb2A and 16× diluted periplasmic fraction of scFv2A-AP/S. Lane NIb corresponds to recombinant MBP-NIb protein. The M_r values of standards are shown on the left.

Detection of NIb in plant material

Anti-NIb mAb 2A and scFv2A-AP/S fusion proteins were used in Western blot and tissue print-ELISA to detect PPV in virus-infected *N. benthamiana* and apricot. On immunoblot analysis of extracts of the PPV-infected and healthy plants, mAb 2A and scFv2A-AP/S revealed a band specific to the virus-infected plant sample, corresponding to a protein with an apparent molecular mass of ≈59 kDa, the expected size of NIb, and gave no crossreaction with healthy plant proteins (Fig. 3). A minor band corresponding to a ≈40-kDa protein was also observed in some virus-infected plant extracts. This band has not been further characterised, although it probably corresponds to a degradation product of the NIb protein. The same pattern of bands was observed when scFv2A-AP was used (data not shown).

To assess the ability of the 2A antibodies to recognise PPV-NIb protein in the native state, PPV-infected plants were analysed by direct tissue print-ELISA (Fig. 4). All

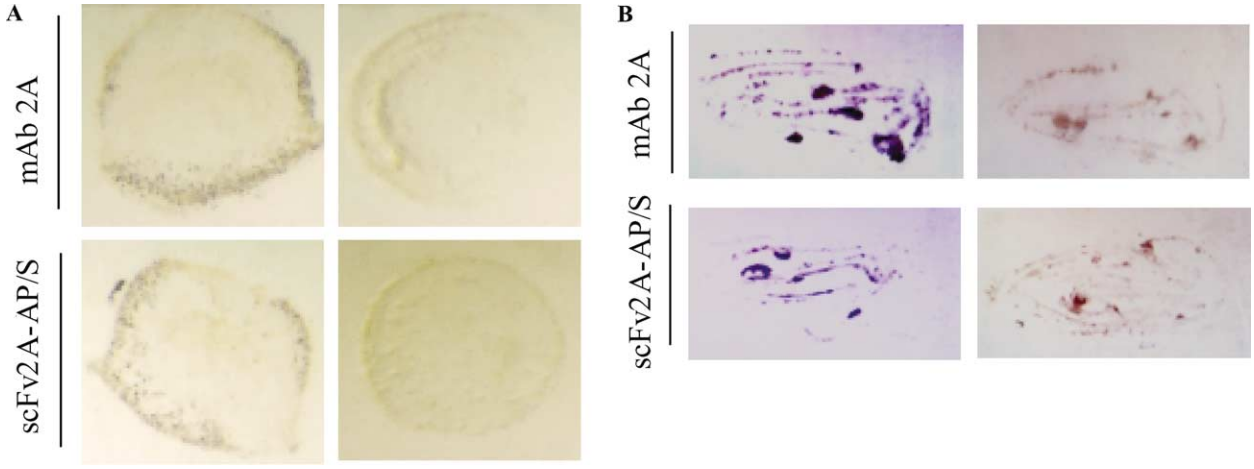

Fig. 4. Localisation of NIb protein in PPV-infected *N. benthamiana* shoots (A) and *P. armeniaca* (B) by tissue print-ELISA with mAb2A and a 16× diluted periplasmic fraction of scFv2A-AP/S. Healthy controls from *N. benthamiana* and *P. armeniaca* are shown on the right of panels A and B, respectively.

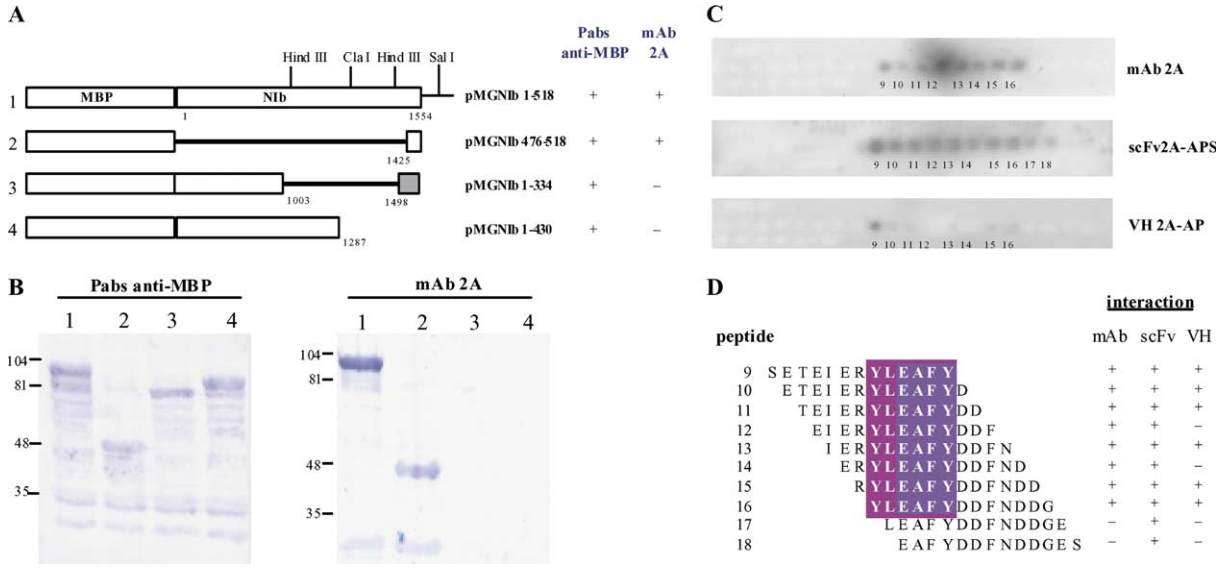

Fig. 5. (A) Schematic representation of deletion forms of the MBP-NIb fusion protein encoded by recombinant plasmids derived from pMGNIb. The structure of full-length MBP-NIb is shown in line 1. The numbers below the diagrams refer to nt positions in the NIb coding sequence. MBP-NIb coding sequences are represented by open boxes. Outframe nt fused to the NIb ORF are represented by a shaded box. Positive and negative reactions in Western blots are indicated by + and − signs, respectively. (B) Western blot analyses of truncated MBP-NIb proteins probed with anti-MBP polyclonal serum (Pabs) (left) and mAb2A (right). Lane 1: pMGNIb1-518; lane 2: pMGNIb476-518; lane 3: pMGNIb1-334; and lane 4: pMGNIb1-430. (C) PEPSCAN analysis of the C-terminal end of NIb using mAb2A, scFv2A-AP/S, and VH2A-AP. A schematic diagram of the overlapping peptides used and their recognition by the different antibody fragments is shown at the bottom.

plant samples positive for Western blot were reactive against scFv and mAb in tissue print-ELISA. The scFv reaction was similar to that obtained with the mAb.

Mapping of the 2A epitope

Initial mapping of the 2A epitope was performed by expressing fusion products of MBP with fragments of the NIb protein using deletion derivatives of pMGNIb (Fig. 5A) and assaying their ability to bind to the corresponding antibody in immunoblots. The three deletion proteins were detected with similar intensities when the immunoblots were probed with anti-MBP polyclonal serum, demonstrating that they accumulated to similar amounts. However, only the product encoded by pMGNIb476-518, which includes the 43 C-terminal amino acids of the NIb protein in frame with MBP, was detected with the mAb 2A (Fig. 5B) or with scFv-2A-AP and scFv2A-AP/S fusion proteins (data not shown). This result indicated that the epitope recognised by both 2A antibodies in NIb was located between amino acids 476 and 517.

To map the 2A epitope more accurately a second approach based on peptide scanning analysis was used. The reaction of mAb 2A with synthetic peptides covering the region of amino acid residues 479–517 revealed the YLEAFY sequence from the NIb protein as the putative epitope (Fig. 5C). This hexapeptide is specific of PPV-NIb, which could explain the lack of reactivity of the 2A antibodies against other potyviruses (data not shown). Strikingly, scFv2A-AP/S was still able to react against peptides lacking Y_{498} or Y_{498} and L_{499} (peptides 17 and 18, Fig. 5C), suggesting that residues Y_{498} and L_{499} do not play an essential role in recognition of NIb, at least in its denatured state.

When the PepSpot membrane was probed with the VH2A-AP fusion protein only a weak reaction against some of the peptides recognised by the mAb and scFv was obtained after a longer incubation time (Fig. 5C).

Transient expression of functional scFv2A in N. benthamiana

Transient expression of scFv2A in *N. benthamiana* leaves was performed by agroinfiltration with *Agrobacterium* cells carrying plasmids pBIscFv2A and pCH32. scFv2A was barely detected in the agroinfiltrated leaves by Western blot analysis (data not shown). However, inhibition ELISA clearly demonstrated the presence of functional scFv2A in the agroinfiltrated tissue. A 1/8 dilution of the agroinfiltrated plant extract inhibited binding of exogenous scFv2A-AP/S to MBP-NIb by up to 32% (Fig. 6). As a negative control, inhibition of NIb binding with non-infiltrated plant extract was only 5%. Using bacterially expressed scFv2A diluted in non-infiltrated plant extract as a standard, scFv2A accumulation in agroinfiltrated tissue was estimated to be approximately 1.3 ng/mg of total soluble protein. Nevertheless, as only the active scFv fraction is determined by this assay, it is likely that the total amount of scFv2A in the plant extract could be higher.

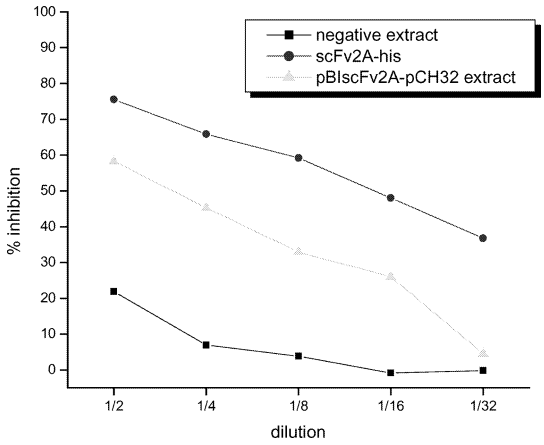

Fig. 6. Inhibition ELISA of scFv2A-AP/S binding to MBP-NIb. Different dilutions of plant extracts agroinfiltrated with C58(pMP90)/pBIscFv2A-pCH32 (triangles) were used to inhibit the binding of bacterially expressed scFv2A-AP/S to MBP-NIb bound to the plate. Non-infiltrated plant extract (squares) and bacterially expressed scFv2A-his diluted in this negative extract (circles) were used as negative and positive controls, respectively. Results are represented as extract dilution (x axis) versus percent inhibition of binding (y axis).

Discussion

We successfully generated a scFv2A fusion protein to alkaline phosphatase that, like its parental mAb, specifically recognised PPV-NIb replicase in vivo (Fig. 4). This fusion protein was able to bind PPV NIb protein extracted from PPV-infected plants without presenting cross-reactivity with other endogenous plant proteins (Fig. 3). Both the parental monoclonal antibody and the scFv fragment exhibited a high affinity, with an estimated IC_{50} close to 10^{-9} M (Fig. 2). The apparent difference in the relative affinities of the scFv and mAb for NIb protein could be attributed to the different valence of these molecules although the existence of dimeric or oligomeric scFv2A fragments in the bacterial periplasmic extract cannot be ruled out.

On the other hand, and contrary to reports demonstrating the prominence of VH domains [38] in determining the protein binding activity from some antibodies, ELISA (not shown) and peptide scanning (PEPSCAN) analysis (Fig. 5C) showed that the affinity of the mAb 2A and its corresponding scFv fragment was not retained by the VH domain alone.

PEPSCAN analysis revealed that the scFv2A recognised a conserved region in the NIb protein of all PPV isolates, not comprised in the NIb sequence of other potyvirus species. The exquisite specificity of this scFv,

together with its ability to recognise native viral NIb, could make it very useful to monitor the spatial and temporal accumulation of PPV-NIb even in plants co-infected with other potyviruses. Furthermore, it has been well established that the viral RNA replication requires a fine balance of different viral and host factors in specific infection stages and subcellular sites [39]. This converts replication-associated enzymes into excellent targets for immunomodulation. Even when total inactivation of the viral antigen was not achieved, alterations in its abundance at a specific phase of the infection cycle could alter the normal course of viral replication. Moreover, preliminary data demonstrating an interference of the mAb 2A on the in vitro RdRp activity of PPV purified replicase complexes encourage the use of these scFv fragments to immunomodulate PPV RNA replication in vivo (V. Escribano, E. Rodriguez-Cerezo, and J.A. García, unpublished results).

Another important question concerns which subcellular compartment should be chosen to target scFvs destined to immodulate. Logically, if interference with the antigen function is desired, scFvs must be targeted to the specific compartment where antigen is located. NIb of several potyviruses, including PPV, accumulate predominantly in the nucleus although their replication probably occurs in a membrane-associated complex in the cytoplasm [21]. The cytosol is, therefore, the compartment of choice to express NIb-binding scFv fragments. However, cytoplasmic antibodies are generally unstable, exhibiting shorter half-lives and low accumulation levels [9]. Stability and functionality is often impossible without disulphide bond formation, but some antibody scaffolds can tolerate the lack of a disulphide bridge and keep their stability and functionality [9]. To assess the activity of cytoplasm-targeted expressed scFv2A fragments, *N. benthamiana* plants were infiltrated with recombinant *Agrobacterium* cells carrying the plasmids pBIscFv2A and pCH32, which express additional copies of virE and virG genes. A signal sequenceless version of the simple scFv2A-his fragment was transiently expressed in *N. benthamiana* and, although this exhibited low accumulation levels, inhibition-ELISA data indicated that it retained the antigen-binding capacity in this compartment (Fig. 6). However, since ELISA was not carried out under reducing conditions, it cannot be ruled out that binding in this assay occurred upon in vitro formation of disulphide bonds. Moreover, recently it has been demonstrated that some scFv antibodies are able to form disulphide bridges in the reducing environment of the cytosol [40], indicating that the redox state of the plant cytosol is not fixed but may change in function of the redox modulation into the plant cell. Thus, even in case in which disulphide bridges determine the functionality and/or stability of the scFv, the result of expression of the scFv in the plant cytosol may be difficult to foresee.

To our knowledge, this is the first report on the expression of a fully functional scFv fragment against a plant viral non-structural protein that is known to play a key role during the plant viral replication. Since the scFv2A binds a conserved motif within the RNA replicase and is accumulated into the plant cytosol, this scFv could be a promising candidate to engineer resistance against PPV and to study the relevance of the YLEAFY domain for the NIb functions in the PPV infection cycle.

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