Fully “Recombinant Enzyme-Linked Immunosorbent Assays” Using Genetically Engineered Single-Chain Antibody Fusion Proteins for Detection of *Citrus tristeza virus*

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ABSTRACT

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Recombinant single-chain variable fragment antibodies (scFv) that bind specifically to *Citrus tristeza virus* (CTV), which cause the most detrimental viral disease in the citrus industry worldwide, were obtained from the hybridoma cell lines 3DF1 and 3CA5. These scFv were geneti-

cally fused with dimerization domains as well as with alkaline phosphatase, respectively, and diagnostic reagents were produced by expressing these fusion proteins in bacterial cultures. The engineered antibodies were successfully used for CTV diagnosis in plants by tissue print enzyme-linked immunosorbent assay (ELISA) and double antibody sandwich-ELISA. The fully recombinant ELISAs were as specific and sensitive as conventional ELISAs performed with the parental monoclonal antibodies, showing the usefulness of recombinant antibodies for routine detection of a virus in woody plants for the first time.

Tristeza is one of the most destructive diseases of citrus worldwide (4). It is caused by *Citrus tristeza virus* (CTV), which is naturally spread by aphids in a semipersistent manner and is also easily transmitted by grafting (3). Since 1957 the disease has caused the decline and death of close to 40 million trees grafted on sour orange rootstocks in Spain (12) and several million trees in other citrus growing areas in the Americas, Australia, and South Africa (4). CTV is a monopartite virus with flexuous-filamentous particles of approximately $11 \times 2,000$ nm (3). It has a plus sense single-stranded RNA genome of approximately 19,296 nucleotides (25) encapsidated in the coat protein (25 kDa). The virus has numerous isolates differing in biological (symptoms and aphid transmissibility) and serological properties (1,8,9,39) and genome sequence (25,46).

The control of this economically important virus is achieved by preventive methods such as quarantine, use of disease free budwood and CTV-tolerant rootstocks. Eradication programs and the use of cross-protection strategies with mild isolates can also contribute to the control of the disease (40). All these control strategies require a large number of detection tests usually performed by enzyme-linked immunosorbent assays (ELISA) with polyclonal or monoclonal antibodies (MCA) (2,13,17). The best results are obtained by specific MCAs (17,45) produced by hybridoma technology (29). In spite of the high serological variability of the virus, a mixture of two MCAs (3DF1 and 3CA5) are able to detect all CTV isolates tested (10). These antibodies, patented in 1984 (No. 536923RPI), are well characterized (10,33,44,45) and

are considered an international reference for CTV diagnosis. In addition, the 3DF1 epitope has been identified (35). With these commercially available MCAs (INGENASA, Madrid, Spain), approximately 2 million samples have been analyzed since 1990. For these reasons we selected both MCAs to be subcloned from hybridoma cell lines and expressed in *Escherichia coli* as single-chain variable fragment antibody (scFv) fusion proteins to be used in a number of ELISA variants for CTV detection.

The scFv technology (5,23) allows the cloning of variable (V) genes from pre-existing MCA cell lines, linking them with a flexible peptide as a single chain Fv (scFv). These constructs can be expressed in bacteria (19,36,37) as soluble proteins or fused with the capsid proteins of filamentous phages (47). In addition, antibody genes can be expressed fused with other proteins such as alkaline phosphatase (AP) (15,28) or with amphipathic helices (27,34,37). The expression of these antigen binding proteins in bacterial cultures provides standardized diagnostic reagents that are theoretically able to replace conventional monoclonal or polyclonal antibodies and conjugates, providing significant advantages in time and cost. However, their applications for diagnostic purposes are scarce. In plant pathology, serological detection is widely used, but although some recombinant constructs have been produced (6,20,21,27,32,42,43,48) only few of them have been applied for routine ELISA tests.

The goal of this work was to produce engineered scFv fusion proteins for detection of CTV and to evaluate their ability to detect the virus under routine assay conditions in comparison with conventional MCAs. To our knowledge, this is the first report that homologous recombinant antibodies are as efficient and useful as conventional MCAs for routine detection of a virus in woody plant materials. The success of the developed engineered reagents in binding and detecting CTV in extracts and in tissue blots opens new possibilities for easy and fast production of detecting reagents.

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MATERIALS AND METHODS

VH and VL amplification. Purification of mRNA from 3CA5 and 3DF1 CTV-specific hybridoma cell lines (stored at INGENASA) was performed with a micro mRNA purification kit (Quick-Prep, Amersham Pharmacia Biotech, Uppsala, Sweden). The cDNA was obtained by reverse transcription of this mRNA with the specific primers CH1FOR (an equimolar mixture of MOCG1-2FOR: 5' CTC AAT TTT CTT GTC CAC CTT GGT GC 3'; MOCG3FOR: 5' CTC GAT TCT CTT GAT CAA CTC AGT CT 3'; and MOCMFOR: 5' TGG AAT GGG CAC ATG CAG ATC TCT 3') and CKFOR (5' CTC ATT CCT GTT GAA GCT CTT GAC 3') (H. Hoogenboom, unpublished data). VH and VL regions of both antibodies were amplified by polymerase chain reaction with cDNAs as templates with primers VH1backSfi (5' C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC SAG GTS MAR CTG CAG SAG TCW GG 3'), VH1for2LiAsc (5' ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3'), Vk4forNot (an equimolar mixture of JK1NOT10: 5' GAG TCA TTC TGC GGC CGC CCG TTT GAT TTC CAG CTT GGT GCC 3'; JK2NOT10: 5' GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAG CTT GGT CCC 3'; JK4NOT10: 5' GAG TCA TTC TGC GGC CGC CCG TTT TAT TTC CAA CTT TGT CCC 3'; and JK5NOT10: 5' GAG TCA TTC TGC GGC CGC CCG TTT CAG CTC CAG CTT GGT CCC 3'), and Vk2backLiAsc (5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GAG CTC ACC CAG TCT CCA 3'). These primers are based on the primers previously reported (14), which were modified to introduce the *AseI* site and the linker sequence (16). Alternatively VH and VL regions from MCA 3DF1 were amplified with primers VH1FOR2 (5' TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CCA 3'), VK1FOR (5' CCG TTT GAT CTC GAG CTT GGT GCC 3'), VK1BACK (5' GAC ATC GAG CTC ACT CAG TCT CCA 3'), and VH1BACK (5' AG GTS MAR CTG CAG SAG TCW GG 3') (14).

Cloning in pDAP2, pDAP2/S, pZIP, and pCLZIP vectors. Each VH sequence was amplified with VH1backSfi and Vh1forLiAsc and cloned into *SfiI*- and *AseI*-digested pDAP2 (28). Transformation of *E. coli* TG1, plasmid purification, and selection of recombinants were performed by standard protocols (41). The plasmid DNA of 10 clones harboring the respective VH region were pooled and restricted with *AseI* and *NotI*. The corresponding VL sequence, amplified with Vk4forNot and Vk2backLiAsc, was inserted and *E. coli* TG1 were transformed with these constructs. Individual clones of the resultant pDAP2-3DF1scFv and pDAP2-3CA5scFv constructs were expressed in a 20-ml scale for screening as described (27), and the supernatants were checked for scFv-AP fusions with binding activity by double-antibody sandwich (DAS)-ELISA (described below).

TABLE 1. Comparison of double-antibody sandwich, enzyme-linked immunosorbent assay (ELISA) optical density at 405 nm (OD₄₀₅) values obtained with extracts of sweet orange cv. Pineapple noninfected and infected with *Citrus tristeza virus* (CTV) isolates T-388, T-397-P, and T-302^a

Isolate	OD ₄₀₅ ^b			
	3DF1scFv-AP/S	3DF1	3CA5scFv-AP/S	3CA5
T-388	1.062 ± 0.035	0.892 ± 0.004	0.792 ± 0.018	0.876 ± 0.034
T-397-P	0.053 ± 0.005	0.056 ± 0.002	0.501 ± 0.027	0.606 ± 0.049
T-302	1.188 ± 0.004	1.031 ± 0.004	0.814 ± 0.025	0.901 ± 0.007
Control	0.043 ± 0.001	0.072 ± 0.004	0.072 ± 0.005	0.046 ± 0.004

^a The method was performed by coating microplates with a mixture of monoclonal antibodies (MCAs) 3DF1 and 3CA5 and with 3DF1scFv-AP/S or 3CA5scFv-AP/S as recombinant conjugates or alkaline phosphatase-conjugated MCAs 3DF1 or 3CA5. The isolate T-397-P, defective in the 3DF1 epitope (9), was not detected by 3DF1 MCA or 3DF1scFv but was detected by 3CA5 MCA and 3CA5scFv.

^b Average of four ELISA wells containing 100 µl per well after 1 h substrate incubation.

The 3CA5scFv and 3DF1scFv with CTV binding properties were subcloned from pDAP2 into *SfiI/NotI*-digested pDAP2/S and pZIP1 vectors (26). 3CA5scFv was also inserted in vector pCLZIP (26). Transformation of *E. coli* TG1 was done as described (41).

Cloning in pEMBL-ENE and pHEN-ENE vectors. The pEMBL-ENE contains a scFv cassette *HindIII/EcoRI* consisting of the VH region (inserted into *PstI* and *BstEII* restriction sites), the linker sequence, the VL region (inserted into *SacI* and *XhoI* restriction sites), and finally the *c-myc* tag sequence. The VH cDNA derived from MCA 3DF1 was amplified with VH1FOR2 and VH1BACK and was *PstI/BstEII*-inserted into the plasmid. In a second cloning step, the VL-gene, amplified with VK1FOR and VK1BACK, was inserted between the *SacI* and *XhoI* restriction sites of pEMBL-ENE containing the VH gene. After sequence analysis, the 3DF1scFv gene was excised from pEMBL-ENE and was swapped into pHEN vector (22) for prokaryotic expression. The pHEN vector was used to transform *E. coli* HB2151.

Sequence. Both scFv fragments were sequenced with an aflatoxin biosynthesis inhibitor (PRISM DNA Sequencer 377, Perkin-Elmer, Langen, Germany) or by CEINGE Service, Naples University.

Expression, purification, and storage of the recombinant diagnostic reagents. The expression products of the pDAP2/S constructs (3DF1scFv-AP/S and 3CA5scFv-AP/S), of the pZIP1 constructs (3DF1scFv-ZIP and 3CA5scFv-ZIP), and of the pCLZIP construct (3CA5scFv-CLZIP) were obtained by incubating the respective *E. coli* TG1 clones at 16°C for 72 h. Proteins (except 3CA5scFv-AP/S) were purified from cultured supernatant by immobilized metal affinity chromatography as described (27). The molecular weight of the purified proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The 3CA5scFv-AP/S fusion protein was concentrated from the supernatant by ammonium sulfate precipitation, redissolved in Luria-Bertani (LB) medium, and dialyzed against LB medium diluted 1:1 with phosphate-buffered saline (PBS). HB2151 bacteria containing the pHEN-3DF1scFv plasmid were induced for expression by incubating them with isopropyl-β-D-thiogalactopyranoside (1 mM) at 25°C for 16 h. Several periplasmic extraction methods were tested: (i) protein borate extraction, after induction, bacterial pellet was resuspended in 1:10 volume of 200 mM sodium borate buffer (pH 8.0) containing 160 mM NaCl, incubated on ice for 1 h, and centrifuged 15 min at 12,000 × g; (ii) protein mild extraction: bacterial pellet was treated with a mild osmotic shock in 0.2 M Tris-HCl (pH 8.0), 0.5 mM EDTA, and 0.5 M Sucrose, diluted in water 1:4, incubated at 0°C for 30 min, centrifuged at 5,000 × g for 10 min, and centrifuged again at 48,000 × g for 15 min; and (iii) protein urea extraction (38). In each case, protein extracts were collected in the final supernatant and analyzed by SDS-PAGE and western blot. The membranes were incubated with MCA antitag 9E10 for detection of scFv. Na-Azide (0.1%) was added to the purified proteins and to concentrated 3CA5scFv-AP/S and stored in aliquots at 4°C or at -20°C, mixed with an equal volume of glycerol.

Tissue print ELISA. Immunoprinting or direct tissue print immunoassay (30) was performed as described for CTV detection (7,11,18). Nitrocellulose membranes (HA45, Millipore Corp., Bedford, MA) were used to imprint fresh sections of healthy and CTV-infected plant materials. The following species were used as healthy controls: sweet orange (*Citrus aurantifolia*) cvs. Pineapple and Mexican lime (*C. aurantium*). The infected plant material had been previously inoculated with a number of CTV isolates from the collection maintained at Instituto Valenciano de Investigaciones Agrarias (IVIA). The selected isolates differed in aggressiveness: T-388 (very aggressive), T-397 and T-397-P (intermediate), and T-302 (mild). The isolate T-397-P, defective in 3DF1 epitope (9), was not recognized by 3DF1 MCA. The printed membranes were blocked by incubation in 1% bovine serum albumin in distilled water for 1 h. Tissue print direct ELISA was performed by

incubation of the membranes for 3 h at room temperature, in parallel assays, with 3CA5scFv-AP/S, 3DF1scFv-AP/S, or with the calf intestinal AP-labeled parent MCA. Tissue print indirect ELISA was also carried out by incubating the printed membranes with 3DF1scFv for 2 h. After washing, membranes were sequentially incubated with MCA 9E10 and with AP-conjugated goat anti-mouse immunoglobulins (Sigma Aldrich, Madrid). In both tissue print ELISAs, after washing, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) were used as substrate. The processed membranes were washed in water, dried, and examined under a binocular microscope at 5 to 20 $\times$ magnification.

DAS-ELISA. ELISA microplates (PolySorp, Nalge Nunc International, Rochester, NY) were coated by incubating for 4 h at 37°C with polyclonal immunoglobulins from antiserum CTV/MN2-IVIA (2 μ g/ml), specific monoclonal antibodies 3DF1 and 3CA5 (1 μ g/ml), or recombinant fragments 3DF1scFv-ZIP, 3CA5scFv-ZIP, or 3CA5scFv-CLZIP (10 μ g/ml), each diluted in 0.1 N sodium carbonate buffer (pH 9.6). The plates were washed and incubated overnight at 4°C with extracts from infected citrus plants. Extracts were prepared by grinding the plant material previously inoculated with CTV isolates T-388, T-397-P, and T-302 (described previously) in PBS-0.2% diethyldithiocarbamate or in a more complex buffer (PBS, 2% ovalbumin, 2% polyvinylpyrrolidone, 10 mM EDTA, and 0.2% Tween 20 at pH 7.2) at a ratio of 1:20 (wt/vol). For detection of the immobilized antigen, 3DF1scFv-AP/S or 3CA5scFv-AP/S (diluted in PBS) were added to the microplate wells and incubated for 3 h at 37°C. As a positive control, AP-conjugated MCAs 3DF1 and 3CA5 (0.1 μ g/ml) were used. After washing, the plates were incubated at room temperature with 1 μ g/ml of *p*-nitrophenyl phosphate in substrate buffer (10% diethanolamine in distilled water, pH 9.8). Optical density (OD) at 405 nm was measured in a photometer (MultiSkan, Titertek, Huntsville, AL) zeroed with an empty plate, after 30, 60, 90, and 120 min of incubation.

For statistical analysis of the different DAS-ELISA formats performed, a one-way analysis of variance using ELISA format as factor was carried out for each isolate $\times$ antibody combination. A LSD test was used for mean comparison.

RESULTS

Synthesis of scFv fragments able to react against CTV. Two different approaches were chosen to obtain functional scFv after subcloning from hybridoma cells. In our first strategy, the VH and VL regions of MCA 3DF1 were sequentially inserted into pEMBL-ENE. The resulting scFv-cassette was subcloned into pHEN1 and was expressed as a fusion with the *c-myc* tag. Clones that expressed 3DF1scFv with CTV specificity were identified by a MCA 9E10-mediated ELISA. SDS and western blot analyses suggested that the urea extraction method had 1,000-fold better yield than the mild extraction method for recovering 3DF1scFv protein. However, these conditions were not useful for recovering functional scFv and a subsequent refolding process had to be performed. Hence, the mild extraction method was used to prepare active 3DF1-scFv for use in tissue print ELISA.

Alternatively, the cDNA of VH and VL of MCA 3DF1 were amplified with primers VH1for2LiAsc and Vk2backLiAsc, respectively, each providing the coding sequence of half of the linker sequence. The VH and VL regions were sequentially cloned into pDAP2, thus joining the two linker halves via the *AscI* restriction site and also linking the scFv fusion to the coding region of *E. coli* wild-type AP (schematic representation of the scFv fusions obtained is shown in Figure 1). The presence of functional scFv gene sequences was confirmed by expression of several colonies, and examination of the scFv-AP fusion protein secreted into the supernatant for CTV binding activity by DAS-ELISA. The latter approach requires fewer cloning steps and also is easier

for testing the scFv-AP fusion proteins. We therefore used this method for subcloning the scFv region from hybridoma cell line 3CA5. For each subcloned MCA, 3DF1 and 3CA5, several clones expressing a highly specific scFv-AP fusion protein were obtained.

The sequences of both scFv fragments, 3DF1scFv, and 3CA5scFv were determined (GenBank Accession No. AF162709 and AF162710, respectively), and compared with the sequences available in the Kabat (1987) database (24). A comparison with inferred amino acid sequences confirmed that the expected amino acids were present in the predicted positions.

Recombinant conjugates. 3DF1scFv and 3CA5scFv were subcloned into vector pDAP2/S (27) to fuse the scFv to an improved *E. coli* alkaline phosphatase (AP/S). In this construct the *ecphoA1* gene was mutated to give a fusion protein with a 35-fold improved specific activity (31). After expression and purification of the AP/S-fused scFv proteins, their efficiency as detection reagents was determined by DAS-ELISA and compared with the efficiency of conventional MCAs. Purified 3DF1scFv-AP/S was able to detect different CTV isolates in infected plants with the same specificity and sensitivity as the parental antibody (Table 1). In contrast, 3CA5scFv-AP/S only identified CTV-infected plants prior to its purification; enzymatic and binding activity was lost after purification. The supernatant of 3CA5scFv-AP/S was concentrated by ammonium sulfate precipitation, and the fusion protein stabilized by redissolving in LB medium and dialysis. This process yielded a recombinant conjugate that was nearly as specific and sensitive as the parental MCA 3CA5 (Table 1). The same results were obtained when both scFv-AP/S fusions were tested by tissue print direct ELISA for detection of CTV (described below). Both recombinant conjugates, purified 3DF1scFv-AP/S and nonpurified, but concentrated, and 3CA5scFv-AP/S were stable after storage at 4°C (after addition of Na Azide) or at -20°C in 50% glycerol for 15 months (Table 2).

Coating reagents. 3DF1scFv and 3CA5scFv were subcloned from pDAP2 into the vector pZIP1 (27) allowing expression of scFv fragments dimerized by a leucine zipper domain (mini-antibodies) (34; Fig. 1). Both scFv fusions (3DF1scFv-ZIP and 3CA5scFv-ZIP) could be purified and immobilized on microplate wells and were able to trap CTV from a semipurified virus preparation. However, when the fused proteins were tested for trapping CTV coat protein (CP) in presence of plant material, reactivity of 3CA5scFv-ZIP was poor. This poor reaction was not observed for 3DF1scFv-ZIP. To circumvent this problem with 3CA5scFv-ZIP, the scFv3CA5 gene was subcloned into another vector, pCLZIP (26), and expressed as a mini-antibody enlarged by a CL-domain positioned between the scFv and the leucine zipper. This new fusion protein (3CA5scFv-CLZIP) was able to trap CTV antigens in the presence of plant material. The trapping of CTV after application of plant extracts on wells coated with 3DF1scFv-ZIP and

TABLE 2. Optical density at 405 nm (OD₄₀₅) values of *Citrus tristeza virus* isolates obtained by enzyme-linked immunosorbent assays (ELISA) with single-chain variable antibody fragments (scFv) fusion proteins freshly prepared, stored at 4°C, and at -20°C for 15 months

Coating reagent ^a	Isolate	Fresh	4°C	-20°C
3DF1scFv-AP/S	T-388	1.824 $\pm$ 0.087	1.690 $\pm$ 0.063	1.784 $\pm$ 0.073
	T-302	1.591 $\pm$ 0.102	1.643 $\pm$ 0.074	1.453 $\pm$ 0.067
3CA5scFv-AP/S	T-388	1.225 $\pm$ 0.046	1.065 $\pm$ 0.089	1.116 $\pm$ 0.058
	T-302	0.998 $\pm$ 0.035	1.103 $\pm$ 0.066	0.932 $\pm$ 0.074
3DF1scFv-ZIP	T-388	1.430 $\pm$ 0.044	NA	1.706 $\pm$ 0.021
	T-302	0.974 $\pm$ 0.056	NA	1.001 $\pm$ 0.045
3CA5scFv-CLZIP	T-388	0.985 $\pm$ 0.032	NA	0.914 $\pm$ 0.033
	T-302	0.704 $\pm$ 0.054	NA	0.756 $\pm$ 0.046

^a ELISA performed by coating with a mixture of 3DF1 and 3CA5 monoclonal antibodies (MCAs) and using the different scFv-AP/S fusions separately as detecting antibodies or by coating with 3DF1scFv-ZIP or 3CA5scFv-CLZIP with a mixture of MCAs 3DF1 and 3CA5 AP conjugated for detection. NA = not analyzed.

Fig. 1. Schematic representation of the single-chain variable antibody fragment (scFv) fusion proteins produced. ScFv-APS = scFv fused to mutated alkaline phosphatase; ScFv-ZIP = scFv fused to the hinge region and ZIP domain for dimerization; and ScFv-C_L-ZIP = scFv fused with the constant region of a human kappa light chain, the hinge region, and the ZIP domain.

3CA5scFv-CLZIP was further optimized when the infected plant extracts were prepared with a “complex” extraction buffer. Both 3DF1scFv-ZIP and 3CA5scFv-CLZIP reacted successfully after storage at -20°C in 50% glycerol for 15 months (Table 2).

Routine CTV detection by recombinant antibody fragments.

The AP/S-fused fragments, 3DF1scFv-AP/S and 3CA5scFv-AP/S, were used to detect CTV CP by tissue print direct ELISA, and the results compared with those from a commercial CTV detection kit (Plant Print Diagnostics SL, Valencia, Spain) using the conventional MCAs 3DF1 and 3CA5. Figure 2 shows the results obtained with some CTV-infected and healthy sweet orange plants. The developed prints show a similar intensity and number of stained areas in the vascular region of sections of sweet orange shoots when using MCA 3DF1 or the recombinant 3DF1scFv-AP/S. The recombinant 3CA5scFv-AP/S was slightly less reactive than the MCA 3CA5. The reaction of a mixture of both scFv-AP/S recombinants was similar to that obtained with the commercially available kit by both CTV-specific MCAs in mixture. In addition, the scFv-AP/S had the same specificity as the parental MCAs. The CTV isolate T-397-P was not recognized by 3DF1 immunoglobulin (Ig)G2B or by 3DF1scFv-AP/S, giving only background signal as with the healthy controls. However, this particular CTV strain was detected by 3CA5 (IgG2B) as well as by 3CA5scFv-AP/S.

The results of a comparison of DAS-ELISA tests performed with MCAs or scFv from the same antibodies are shown in Figure

3. The $\text{OD}_{405\text{nm}}$ values obtained with the recombinant ELISA performed with 3DF1scFv-ZIP for coating and 3DF1scFv-AP/S for detecting were not significantly different to those obtained with conventional DAS-ELISA by 3DF1 conjugated with AP. The recombinant ELISA using both 3CA5-scFv fusion proteins gave lower values than conventional DAS-ELISA using 3CA5 conjugated with AP. However, the $\text{OD}_{405\text{nm}}$ values obtained with fully recombinant DAS-ELISA with a mixture of 3DF1scFv-ZIP and 3CA5scFv-CLZIP for coating and 3DF1scFv-AP/S and 3CA5scFv-AP/S as detecting reagents, were statistically similar to those obtained by DAS-ELISA with a mixture of the 3DF1 and 3CA5 MCAs for coating and the same MCAs conjugated with AP for detection. Figure 3 also illustrates the high trapping efficiency of 3DF1scFv-ZIP, 3CA5scFv-CLZIP, or the mixture of both constructs, compared with those obtained by the corresponding MCAs. No ELISA reaction was obtained with either the 3DF1 MCA or the 3DF1scFv constructs when they were assayed against CTV isolate T-397-P, maintaining the specificity of the parental 3DF1 MCA for the epitope recognition. The $\text{OD}_{405\text{nm}}$ values obtained for this particular CTV isolate did not differ from those given by healthy controls. In addition, the $\text{OD}_{405\text{nm}}$ values of the healthy control were similar for all the ELISA combinations assayed indicating a very low background.

To confirm that the fully recombinant ELISA with two specificities (3DF1 and 3CA5) was able to react with a broad spectrum

Fig. 2. Tissue print enzyme-linked immunosorbent assay (ELISA) of healthy and *Citrus tristeza virus* (CTV)-infected sweet orange cv. Pineapple shoots. The *Citrus* shoots correspond to different CTV isolates: T-388 aggressive (top left), T-300 common (top right), T-397-P common (bottom left), T-302 mild (bottom right), and healthy control (center). Prints were tested by different CTV specific reagents: **A**, MCA 3DF1 conjugated with AP; **B**, 3DF1scFv-AP/S; **C**, MCA 3CA5 AP conjugated; **D**, 3CA5scFv-AP/S; **E**, a commercial mixture of AP conjugated MCAs 3DF1 and 3CA5; and **F**, a mixture of 3DF1scFv-AP/S and 3CA5scFv-AP/S.

Fig. 3. Comparison of OD_{405nm} values from three different double-antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) methods: conventional commercially available, semirecombinant, and complete recombinant. The commercial DAS-ELISA was performed by coating microplates with monoclonal antibodies (MCAs) 3DF1, 3CA5, or with a mixture of both, and using alkaline phosphatase or biotin conjugates. The semirecombinant DAS-ELISA was performed by coating microplates with both conventional MCAs alone or in mixture, and using 3DF1scFv-AP/S, 3CA5scFv-AP/S or a mixture of both as detecting antibodies. The fully recombinant DAS-ELISA was performed by coating microplates with 3DF1scFv-ZIP, 3CA5scFv-CLZIP, or with a mixture of both, and with 3DF1scFv-AP/S, 3CA5scFv-AP/S, or a mixture of both as conjugates. These methods were assayed against extracts from healthy sweet orange cv. Pineapple and plants infected with CTV isolates T-388, T-397-P and T-302. Bar codes indicate the reagent used for coating (C) and the reagent used as conjugate (D). Bar and extensions represent the average $\pm$ SE of four replicates. Within the same isolate and the same antibody bars with the same letter are not significantly different at $P = 0.05$ according to the LSD test.

of CTV isolates, 23 characterized CTV isolates from the IVIA collection and CTV-infected and noninfected field samples from different citrus cultivars were tested in a large screening test. The assays were performed by tissue print direct ELISA with a mixture of 3DF1scFv-AP/S and 3CA5scFv-AP/S. The results were compared with those obtained from testing the same plant material with the CTV detection kit, which uses a mixture of AP-conjugated MCAs 3DF1 and 3CA5. Samples were also analyzed by DAS-ELISA using a mixture of 3DF1scFv-ZIP and 3CA5scFv-CLZIP for coating ELISA microplates and 3DF1scFv-AP/S mixed with 3CA5scFv-AP/S as detecting reagents (fully recombinant ELISA). The results were compared with those obtained by DAS-ELISA with 3DF1 and 3CA5 MCAs for coating and biotin or AP-conjugated MCAs for detection. The results (healthy or infected) were identical, independent of the kind of immunological assay applied (tissue print or DAS-ELISA) as well as of the kind of reagents used (recombinant or conventional) (data not shown). This screening experiment showed that the recombinant reagents could completely replace the conventional kits.

DISCUSSION

Production of recombinant antibodies from hybridoma cell lines secreting specific MCAs is well documented. In this work, scFv-fusion proteins have been produced and serve as diagnostic reagents specific for CTV with a similar efficiency as their parental MCAs 3DF1 and 3CA5. In plant pathology, scFv fusions have been used for diagnosis of plant viruses (20,43) but their efficiency have never been compared with the parental MCAs. Vector pDAP2/S was used to fuse 3DF1scFv and 3CA5scFv to a modified *E. coli*-AP with a 35-fold improved specific activity compared with wild-type *E. coli*-AP. These fusion proteins proved to be efficient recombinant conjugates that were easily produced and facilitate subsequent handling in serological tests. The use of secondary antibodies that can induce nonspecific reactions as well as conventional conjugate preparation by chemical coupling can be avoided. The scFv-AP/S fusions can be used directly in serological tests like tissue print ELISA, a rapid and easily performed technique. The use of 3DF1scFv-AP/S and 3CA5scFv-AP/S in mixture allows large scale testing by tissue print ELISA in a simple and economic way without compromising the sensitivity offered by the parental MCAs. The scFv-AP/S fusion proteins are also excellent substitutes for conjugates in the DAS-ELISA test format.

In order to design a DAS-ELISA based completely on recombinant antibody fragments, bivalent mini-antibodies specific to CTV were constructed, which have the potential to serve as recombinant coating reagents. The mini-antibody 3DF1scFv-ZIP is an excellent coating reagent for ELISA plates. In order to achieve the same trapping efficiency in the case of the 3CA5scFv, the respective mini-antibody had to be enlarged by a light chain constant domain. For unknown reasons, the mini-antibody 3CA5scFv-ZIP was partially inhibited by the plant extract when used as coating reagent. Similar inhibition problems have been described (43). Nevertheless, in our work inhibition could be avoided when the 3CA5-CLZIP is used for trapping the antigen. The four constructs, 3DF1scFv-AP/S, 3CA5scFv-AP/S, 3DF1scFv-ZIP, and 3CA5scFv-CLZIP, allow the performance of a fully recombinant DAS-ELISA for the detection of CTV-isolates, without the need of monoclonal or polyclonal antibodies.

Statistical analysis of the data represented in Figure 3 showed that the ability of 3DF1 MCAs or their recombinant constructs to react against CTV isolates using different ELISA formats was similar. In contrast, the efficiency of the ELISAs performed with 3CA5scFv fusions was slightly lower than that achieved with conventional 3CA5 ELISAs. When both 3DF1scFv and 3CA5scFv fusion proteins were used in mixture it was as sensitive as the conventional AP DAS-ELISA using both MCAs. However, the

highest OD values were obtained by conventional biotin/streptavidin ELISA, due to the amplification effect of this system.

In contrast with data previously reported (43), the scFv fusion proteins produced against CTV were very stable and retained activity during storage for long periods, increasing their practical value for diagnosis.

Both serological techniques, tissue print ELISA, and DAS-ELISA, performed with recombinant antibodies were successfully tested for routine detection of CTV in different laboratories, by previously characterized isolates as well as field-infected trees. The excellent performance of the recombinant CTV-specific antibodies obtained may obviate the need for conventional MCA production from hybridoma cells. The ability of the scFvs in their binding against all the CTV isolates tested suggests that these particular constructs may be effective in inducing resistance in transgenic citrus plants.

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