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Short communication

Engineering of a single chain variable fragment antibody specific for the *Citrus tristeza virus* and its expression in *Escherichia coli* and *Nicotiana tabacum*

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Abstract

Citrus tristeza virus (CTV) is one of the most destructive citrus virus diseases in the world. The construction of an engineered antibody, EMBL accession number AJ278109, able to specifically recognize its antigen, i.e. the coat protein of CTV, directly on infected plant material without any purification or manipulation of the entire woody plant. The potential uses of this engineered antibody are discussed.

Citrus agricultural products are important worldwide, both as fresh fruit and processed products. The *Citrus tristeza virus* (CTV), belonging to the *Closteroviridae* family, is a serious threat and a limiting factor for a competitive citrus industry in Europe (about 40 million trees lost in Spain since 1957). The action of this citrus pathogen could result in important economical loss involving both production and fruit quality (Wallace, 1956; Bar-Joseph et al., 1979). CTV is semi-persistently transmitted by different aphid species and causes death of sweet orange, mandarin and grapefruit, especially when grafted on sour orange. Different strains of CTV have been isolated and show different infection abilities. The effect of infection depends not only on the strain, but also on the different species involved (insect vectors, grafted plant variety) and on the different combinations of plant and rootstock as well, resulting in different syndromes including stem pitting, quick decline due to phloem necrosis, and tree death (Bar-Joseph et al., 1989; Cambra et al., 1995).

This virus cannot be controlled by chemicals and the morphological diagnosis of symptoms is only possible at an advanced stage of infection, when the epidemic is underway. Quarantine, the use of tolerant species such as rootstocks and/or cultivars, and the production of disease-free plant sources represent the preventive methods available to control this disease.

New techniques and standard methods for early, quick and reliable identification of CTV isolates are needed to check the potential spreading of severe strains into Mediterranean areas. In recent years, several CTV-specific monoclonal antibodies raised against the CTV coat protein have been developed. ELISA procedures have improved CTV detection and provided ways to recognize epitopic variations in the coat protein which are often related to infection severity (Vela et al., 1986; Permar et al., 1990). Recently, it has been shown that engineered antibodies are able to play an important role in diagnosis (Fecker et al., 1996; Toth et al., 1999; Terrada et al., 2000) and in developing transgenic plants (Benvenuto et al., 1991; Le Gall et al., 1998; Tavladoraki et al., 1993). We chose a monoclonal antibody to engineer a single chain antibody (scFv-αCTV) which was expressed in *Escherichia coli* (*E. coli*) and characterized. Furthermore, *Nicotiana tabacum* was selected as a plant expression model

system and tobacco transgenic plants were produced and tested for the presence of the engineered antibody.

Purified scFv-αCTV was tested for specificity in immunodetection analysis for agricultural application.

Construction and characterization of the single chain variable fragment anti-CTV

The mRNA (kindly provided by Prof. M. Cambra) extracted from the hybridoma 3DF1 cell line, specific for the CTV coat protein (Vela et al., 1986, 1988), was used as starting material to obtain a single chain antibody (scFv-αCTV-3DF1) by PCR amplification of the variable regions of the heavy and light immunoglobulin chains. The following primers were used: VL1FOR: 5′d(CCGTTTGATCTCGAGCTTGGTGCC)3′; VH1 FOR2: 5′d(TGAGGAGACGGTGACCGTGGTCCCT TGGCCCCAG)3′; VL1BACK: 5′d(GACATCGAG CTCACTCAGTCTCCA)3′; VH1BACK: 5′d(AGGTS MARCTGCAGSAGTCWGG) (S = C or G; M = A or C; R = A or G; W = A or T) The complete cloning strategy and further details are summarized in Figure 1. The final DNA coding for scFv-αCTV was automatically sequenced to check the assembly in the final vectors and the correct coding regions (EMBL accession number AJ278109). The inferred amino acid sequence belongs to the immunoglobulin class and the product of translation was expected to adopt proper folding and to have biological activity, due to the presence of the amino acids involved in correct folding in the right positions (Chothia et al., 1985; Chothia and Lesk, 1987; Lesk and Tramontano, 1992; Foote and Winter, 1992; Knappik and Plückthun, 1995).

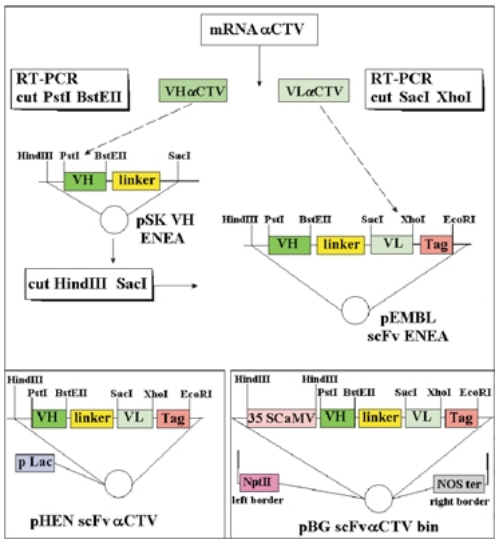

Figure 1. The cloning strategy. The purified mRNA from hybridoma cell line was used as template for PCR amplification by oligonucleotides. The scFv cassette was directly used for insertion of VL, while the insertion of VH was made in two steps into the two plasmids (pSK-VH cassette and pEMBL-scFv cassette). The linker consists of $(G_4S)_3$. The bacterial pHEN-scFv-αCTV and the plant pBG-scFv-αCTV expression vectors are shown including the restriction sites, the promoter regions, the tag-myc sequence and the NOS terminator region.

Expression of ScFv-αCTV in *E. coli*

ScFv-αCTV was expressed in *E. coli* strain *HB2151*. To induce the expression, a bacterial growth up to 0.9 optical density (OD) at 600 nm was diluted 1 : 100 in 2×TY (Amp 100 mg/ml, glucose 0.1%) and then IPTG (isopropyl-b-D-thiogalactopyranoside) 1 mM f.c. was added, followed by 16 h incubation at 25 °C. Two extraction protocols were tested to recover the protein from the *E. coli* system based on a mild buffer (mild osmotic shock in TES (0.2 M TRIS-HCL pH 8.0, 0.5 mM EDTA, 0.5 M sucrose), diluted in water 1 : 4 at 0 °C for 30 min) and a strong buffer (10 mM glycylglycine, 1 mM EDTA, 2 M urea, pH 7.5, at 0° for 60 min). Both these extraction systems only influence the yield of the recovered proteins and not their molecular weight (the strong one worked more than 1000 times better than the mild one), as shown by Western blot analyses carried out using the following procedure. All protein extracts were run in 12% SDS PAGE, according to most general protocols and subsequently transferred and fixed on nitro-cellulose filter to show the protein presence using Ponceau red (by BIORAD) and ECL developing system (by Amersham). The nitrocellulose filter was incubated with blocking solution for 45 min at room temperature, then with monoclonal antibody anti-tag 9E10 1 : 200 for 60 min at room temperature in buffer (TRIS 0.05 M pH 7.5, NaCl 0.15 M, EDTA 5 mM pH 7.5, Triton 0.05%) and gelatine 0.2%, (Evan et al., 1985), and finally with goat anti-mouse IgG Horseradish Peroxidase Conjugate (BioRad) diluted 1 : 3000 for 60 min at room temperature in buffer (TRIS 0.02 M pH 7.5, NaCl 0.15 M, Tween20 0.05%) and 0.2% gelatine. Developing reaction was carried out by ECL system, as above, followed by filter exposure on Kodak film at room temperature for 10 min.

The results are shown in Figure 2, with a band of about 28,000 Da, as expected. Unfortunately the

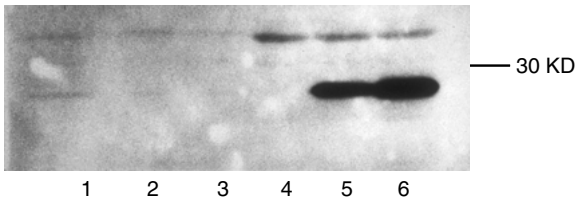

Figure 2. The Western blot. The proteins were detected by 9E10 monoclonal antibody that recognizes the tag-myc peptide. The detectable protein scFv-αCTV (positive band) has a molecular weight of 28,000 Da as expected. Lines 1–3 show 'mild' extracts from three pHEN-scFv-αCTV *E. coli* clones (positive band). Line 4 is the urea extract from one negative *E. coli* clone. Lines 5 and 6 are the urea extracts from two positive pHEN-scFv-αCTV *E. coli* clones.

strong protocol was not useful for testing the functionality of the recovered engineered antibody, due to its denaturing effect and to the inefficacy of renaturating protocols (data not shown). Therefore, the mild protocol was selected to prepare scFv-αCTV for use in immunoprinting analyses for testing its functionality and binding activity.

Immunoprinting test on woody infected plant material

After the expression, the ScFv was characterized and tested for 'high specificity' in immunodetection analysis for agricultural application. These tests were carried out on transverse sections of fresh stems from both healthy and infected (T388, T300 and T397-P CTV isolates) plant materials (not showing manifest symptoms) printed on nitro-cellulose, according to the routine protocol for CTV detection (Garnsey et al., 1993). The filters were incubated with the parental monoclonal antibody (3DF1) and with the scFv-αCTV purified from *E. coli*.

The biological materials were collected from the IVIA (Instituto Valenciano de Investigaciones Agrarias-Valencia-Spain). The T397-P isolate was not revealed by the parental monoclonal antibody. The negative control was performed using an unspecific scFv. Figures 3A, B and D show, in the same order, four lines of transversal section prints (from left to right): healthy tissue, tissue infected by T397-P (undetectable by 3DF1 mAb-αCTV), tissue infected by T388 and tissue infected by T300 (detectable by 3DF1). Figure 3A shows results obtained using scFv-αCTV. As expected, both healthy and T-397-P infected tissues, gave negative signals, whereas T-388 and T-300 infected tissues gave positive signals. Figure 3B shows results obtained using parental mAb-αCTV. The pattern is very similar to the previous one. Figure 3D is a negative control obtained using an unspecific scFv. In this case, all the different tissues did not manifest a substrate precipitation. These results show that scFv-αCTV retains the parental characteristics of affinity for the CTV coat-protein and it is able to show the 'topographic' localization of the virus in phloem tissues.

Expression of ScFV-αCTV in *Nicotiana tabacum* plants

In order to test the expression of this scFv-αCTV in a plant, *N. tabacum* was selected as a model system. The cassette scFv-αCTV from pEMBLscFv-αCTV was cloned into the pBG(dAbs)BIN vector, able to transform leaf disks by *Agrobacterium* infection. The pBG(dAbs)BIN vector contains the restriction sites useful for inserting any engineered antibody (dAb or scFv). The 35S-CaMV promoter drives the expression, the NOS terminator region stabilizes the transcription product and the NPT II gene confers the Kanamycin resistance to select transgenic plants (see Figure 1). pBG-scFv-αCTV-BIN was inserted by electroporation into *Agrobacterium tumefaciens* GV3101 strain. The transformed *Agrobacterium* colonies were used to infect leaf disks from *N. tabacum* grown *in vitro*, following the usual procedures (Maliga et al., 1995). After regeneration, the resulting plants were tested to check the presence of scFv by RT-PCR (Kit OneStep RT-PCR by Takara) and by Western blot.

Tobacco transgenic plants were produced and tested for the presence of the engineered antibody. ScFv-αCTV was detected by RT-PCR in transgenic plants (Figure 4). The protein extracted from the plants, analyzed by Western blot, did not differ from the protein obtained from *E. coli* expression (data not shown). Furthermore, results from structural considerations and chemical characterization of the purified scFv-αCTV indicate that it has an intrinsic stability and a natural propensity to form dimers (data not shown), thus suggesting possibly better performances compared to other similar constructs. In conclusion, this scFv-αCTV is able to be fully expressed in bacteria and in *N. tabacum*. The purified scFv retains its functionality and specificity and may be used to develop a

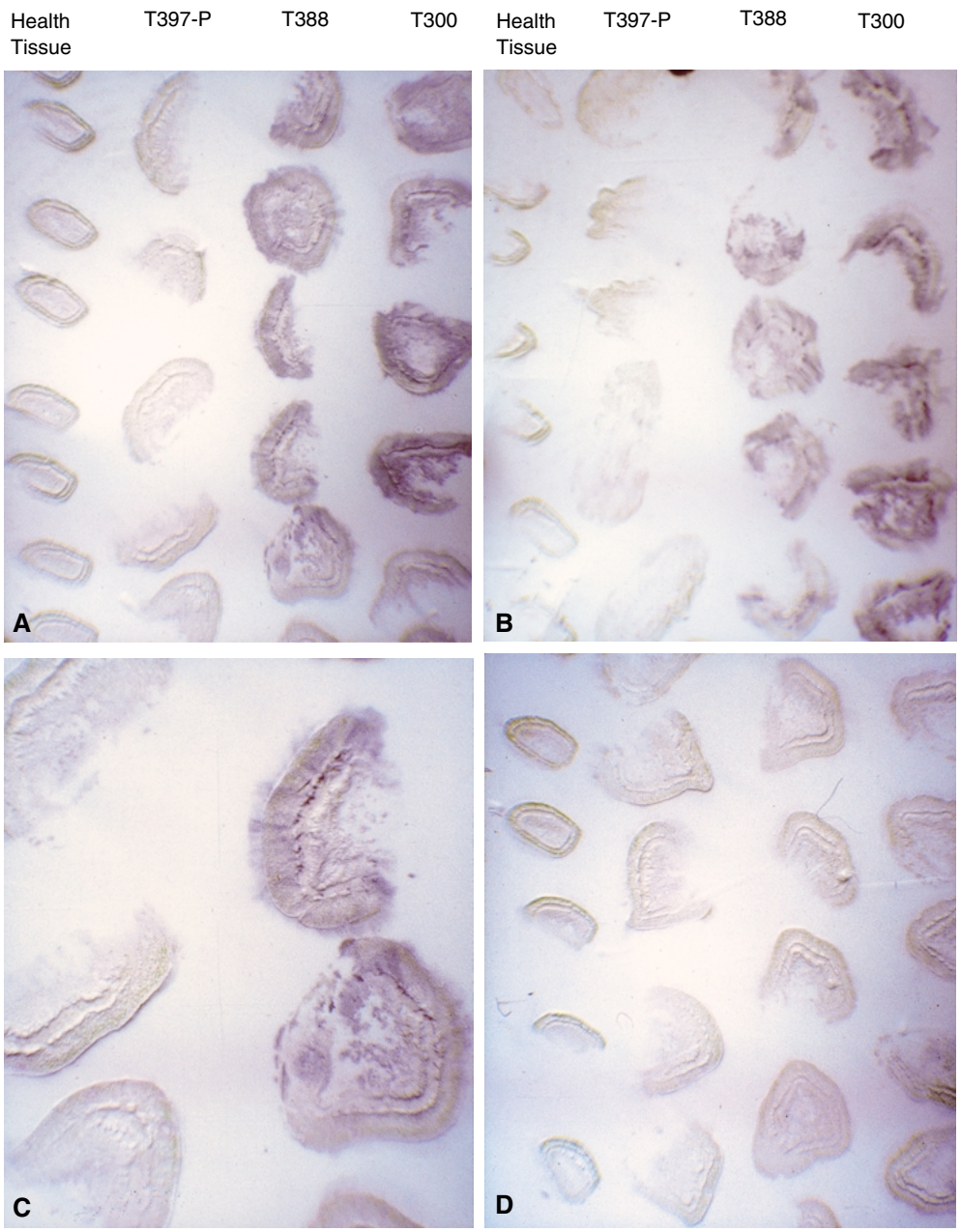

Figure 3. Immunoprints of transversal sections of plant materials. In photos A, B, D, each column of tissue prints (from top to bottom) refers to the healthy tissue, the tissue infected by T397-P, the tissue infected by T388 and the tissue infected by T300 (from left to right). Photo A: Immunoprints incubated with scFv-αCTV; photo B: Immunoprints incubated with mAb αCTV; photo D: Immunoprints incubated with an unspecific scFv (A, B, D were carried out using a light microscopy). In photo 3C is shown a magnified detail of photo A.

diagnostic kit in agriculture. It also represents a promising tool to gain insight into plant-virus interactions and to develop transgenic plants as a new strategy against infection.

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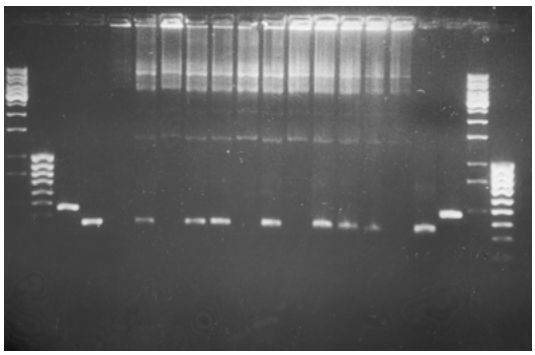

Figure 4. RT-PCR from *N. tabacum* transformed by pBG-scFv-αCTV-BIN. VH chain was amplified using VH1FOR2 and VH1BACK primers. Lines 1 and 19: marker size 100 bp (Takara); lines 2 and 20: marker size 1 kb (Takara); lines 3 and 18: positive controls of RT-PCR kit; lines 4 and 17: positive control pEMBL-ENEA scFv-αCTV; lines 5 and 16: negative control *N. tabacum* wild type; lines 6–15 RT-PCR samples from *N. tabacum* plants transformed by pBG-scFv-αCTV-BIN.

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