Phytopathol. Mediterr. (2002) 41, 55–62

Bacterial expressed coat protein: development of a single antiserum for routine detection of *Citrus tristeza virus*

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Summary. *Citrus tristeza virus* (CTV) causes one of the most important citrus diseases world-wide and has recently been detected in Portugal. Early diagnosis based on immunoenzymatic techniques requires significant amounts of reagents. This research describes the isolation of the coat protein gene of CTV, its expression in *Escherichia coli* as a fusion protein containing an N-terminal $(His)_6$ region and its use to raise rabbit polyclonal antibodies. These antibodies could be used at dilutions higher than 1/80,000 at the detection stage in indirect ELISA tests and were also efficient for trapping the virus in ELISA and Immunocapture RT-PCR. These characteristics allowed the production of diagnostic kits based solely on this source of antibodies. A detection spectrum and sensitivity similar to that of a commercial polyclonal antibody kit was achieved.

Key words: recombinant protein, ELISA, diagnosis.

Introduction

Citrus tristeza virus (CTV, genus *Closterovirus*, family *Closteroviridae*) is the largest member of its taxonomic family. Viral particles, 2000 nm in length and 10–12 nm in diameter, are present in the phloem of infected plants. A single stranded positive sense RNA molecule approximately 20,000 nucleotides long constitutes its genome (Bar-Joseph *et al.*, 1985). This nucleic acid molecule is encapsidated by the coat protein (CP), which has a molecular mass of 25 kDa (Sekiya *et al.*, 1991). Another protein with a molecular mass 27 kDa (p27) (Febres *et al.*, 1996) is also associated with CTV virions (Febres *et al.*, 1996). Development of disease symptoms is dependent on the combination of virus strains and hosts. CTV infection has led to the decline and death of millions of citrus trees mostly grafted on sour orange rootstocks (Bar-Joseph *et al.*, 1985). Decline may be in certain cases quick but slow decline for months or years is most frequent. Strains responsible for quick decline or stem pitting symptoms on sweet orange are usually designated as severe strains. Those that do not cause the mentioned symptoms are referred to as mild strains.

As the control of the disease is based on early CTV diagnosis, efforts must be made to develop efficient and rapid diagnostic methods. Large-scale diagnosis based on ELISA and immunoblotting tests requires large amounts of antibodies. Monoclonal antibodies may be more sensitive and allow, as in the case of MCA13 for example (Per-

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mar *et al*., 1990), discrimination between severe and mild strains from Florida. However, the risk of the appearance of strains that lack an epitope previously thought to be conserved is real (Kano *et al*., 1991) and may give rise to false negatives when monoclonal antibodies are used. Thus, polyclonal antibodies or mixtures of monoclonal antibodies seem more appropriate for broad-spectrum detection of the virus.

Raising polyclonal antibodies requires large amounts of purified virions to be used in immunization procedures. In addition, the antigen may not maintain its characteristics along successive purifications. The use of CTV coat protein expressed in and purified from an *Escherichia coli* system to raise polyclonal antibodies overcomes these limitations, and avoids the production of antibodies against host proteins that may be present in the viral preparations (Nikolaeva *et al*., 1996). Production of antisera against bacterially expressed CTV coat protein (rCP-CTV) has been previously described (Manjunath *et al*., 1993, Nikolaeva *et al*., 1995, Bar-Joseph *et al*., 1997). However these antisera could not be used as the sole source of antibodies to detect CTV in ELISA tests, due to their low efficiency for trapping the virus. To overcome this problem an alternative but complex immunisation scheme combining the use of rCP-CTV and partially purified virus particles was described by Bar-Joseph *et al*. (1997). In this paper we describe the production of immunoreagents to detect a broad spectrum of CTV strains by ELISA, based solely on polyclonal antibodies against rCP-CTV.

Materials and methods

Virus isolates

The CTV isolates (see Table 1) used in this work were obtained from Reunion Island, Spain and Portugal (mainland and the Island of Madeira) and Morocco (Zemzami *et al*., 1999). The virus isolates were propagated by grafting infecting material onto sweet orange (*Citrus sinensis* cv. Madame Vinous) and Mexican lime (*Citrus aurantifolia*) plants. These were maintained in an insect proof greenhouse which was kept under 30°C and 35°C in winter and summer seasons respectively. Mild and severe symptoms were observed due to infection with different strains within this collec-

Table 1. ELISA values of samples from diverse origins maintained in collection or obtained in the field, tested with the biotinylated kit produced from the As 19-21 antiserum.

Origin	A_{405}	Symptoms[c]
Positive samples		
Portugal - Madeira Island	1.38 (1.81)[a]	SP
	2.02 (1.62)[a]	SP
	2.30 (2.12)[a]	SP
	1.86[b]	
	1.76[b]	
	1.65[b]	
	0.42[b]	
	0.41[b]	
Portugal - Algarve	1.05 (0.88)[a]	M
	1.77 (1.49)[a]	M
	1.58 (1.45)[a]	M
	1.28	M
	1.95	M
Portugal - Setúbal	1.26 (1.27)[a]	M
	1.33 (0.79)[a]	M
	0.62	M
	1.15	M
	1.53	M
Reunion Island	1.24	SP
	1.40	SP
	1.10	SP
	0.86	SP
	1.42	SP
	1.00	SP
Morocco	1.68	M
	0.85	M
	1.65	SP
	1.32	SP
	0.98	M
Negative samples		
Portugal - Island of Madeira	0.01[b]	
	0.04[b]	
	0.02[b]	
	0.03[b]	
	0.03[b]	
	0.03[b]	
	0.02[b]	
	0.01[b]	
	0.02[b]	
	0.05[b]	
	0.03[b]	

[a] Bracketed values indicate the results obtained with a commercial kit (Bioreba).
[b] Field samples. The readings of the negative controls ranged from 0.1 to 0.2 and were previously subtracted. The status of the negative samples was confirmed by IC/RT-PCR.
[c] M (mild), typical symptoms observable on Mexican Lime. SP (stem-pitting), stem pitting in branches of Madam Vinous sweet orange.

tion (Table 1). Additional uncharacterised isolates were collected in the field.

Isolation, amplification and cloning of the coat protein gene

The CTV virions of the mild isolate of Spanish origin CTV-19 were directly isolated from an infected plant by immunocapture and the coat protein gene amplified as described by Nolasco *et al.* (1993). The immunocapture was carried out using IgG from a commercial CTV detection kit (Bioreba, Reinach, Switzerland). The primers used to amplify the whole CP gene by PCR were based on the Genbank accession M76485 and had the following sequences: CTV10 (reverse primer): 5'-ATCAACGTGTGTTGAATTTCC-3' and CTV1 (forward primer): 5'- ATGGACGACGAAACAAAGAA-3'. The PCR products were TA-cloned in the pCRII vector (TA cloning system – Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. For expressing purposes the insert was transferred to the pTrcHisA vector (Invitrogen) and sequenced to verify the correct in frame insertion, according to standard procedures (Sambrook *et al.,* 1989). The pTrcHisA-19.21 construction was used to transform TOP 10 *E. coli* cells (Invitrogen).

Expression and purification of recombinant coat protein

For the preparative scale production of the recombinant protein a 60 ml *E. coli* culture in SOB medium (2% Bacto-tryptone, 0.5% Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl) medium containing ampicillin was grown at 37°C and induced with 1 mM isopropyl-thio-β-D-galactoside (IPTG) when bacterial culture density reached OD_{600} = 0.6. The cells were harvested at 5 h after induction, pelleted and resuspended in 12 ml of guanidinium lysis buffer (6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.8), ultra sonicated on ice and the insoluble debris eliminated by centrifugation at 13,200 g for 5 min. The fusion protein was purified from the lysate by metal affinity chromatography in Ni^+ ProBond columns (Invitrogen) according to the manufacturer's instructions. Partially purified fusion protein was dialysed against 10 mM Tris (pH 8.0) containing 0.1% Triton X-100, quantified by the Bradford method (Bradford, 1976) and analysed by denaturing SDS-PAGE (15%) on Tris-glycine (pH 8.3) buffer and Western blotting according to standard methods (Laemmli, 1970; Ausubel *et al.*, 1990). The final purification of the fusion protein was achieved by cutting the band from a preparative SDS-PAGE (15%) gel.

Immunization, production and conjugation of polyclonal antibodies

Two rabbits were immunized with the purified fusion protein (crushed polyacrilamide gel band containing the protein) by a specialised firm (Eurogentec, Saraing, Belgium). The immunization scheme consisted of antigen injections on days zero, 14, 28 and 56, with 35 μg of protein per injection. Bleedings were done at days 38 (2 ml), 66 (22 ml) and 80 (50 ml). The antiserum obtained from the last bleeding of rabbit B (designated AS 19-21) was purified by affinity chromatography on a protein A-sepharose column. Part of the IgG thus obtained was biotinylated by incubation with N-hydroxysuccinimidobiotine (5:1, w:w) at 25°C for 2 hours followed by dialysis against PBS (pH 7.4).

Immunoenzymatic-assay procedures

Standard ELISA procedures were used. Plant extracts were obtained as previously described (Bar-Joseph *et al.*, 1979). All the assays were conducted in microtiter plates (ref. 650161 Greiner-Labortechnik GmbH, Frickenhausen, Germany) using 100 μl per well of reagents. Absorbance readings of samples were recorded at 405 nm (A_{405}) in a Titerteck Multiscan reader without previous subtraction of blanks. The A_{405} values presented in the results are the mean of two replicates or three replicates in the case of the negative controls. The mean A_{405} value of the negative controls was subtracted from the mean value of the samples before plotting the data. The positive/negative threshold was fixed as 6,965 times the standard deviation (corresponding to a 99% confidence level in a one-tailed T distribution). Indirect double antibody sandwich ELISA (IDAS-ELISA) was used to test the detecting ability of antisera and purified IgG. Thus microtiter plates were coated with a mixture of CTV specific monoclonal antibodies (3DF1+3CA5) obtained from Ingenasa (Valencia, Spain) diluted 1:1000 and the antibodies from AS 19-21 were detected with an alkaline phosphatase anti-rabbit IgG conjugate (Sigma A-3687, Sigma Aldrich, St.

Louis, USA) diluted 1:10,000. The trapping ability of the purified IgG in the coating step was also assayed by IDAS-ELISA. In this case plate coating was done with IgG from AS 19-21 at (1 μg ml^{-1}) and detection was achieved by monoclonal antibodies (3DF1+3CA5) diluted 1:10,000, followed by an anti-mouse IgG alkaline phosphatase conjugate diluted 1:10,000. The biotinylated kits prepared with IgG from AS 19-21 were used in the following conditions: plate coating carried out for 3h at 37°C with 1 μg / ml of AS 19-21 IgG, followed by overnight incubation with viral antigens at 4°C, incubation with biotinylated-IgG diluted 1:1,000 for 3 h at 37°C, incubation for 1 h at 37°C with ExtrAvidin alkaline phosphatase conjugate (Sigma E-2636) diluted 1:8,000. Comparisons were done with a commercial kit to detect CTV from Bioreba following the instructions enclosed, but using volumes of 100 μl of reagents per well. Immunodot blot and immunotissue print protocols were based on Garnsey *et al.* (1993) using nitrocellulose membranes (45 μm) which had been previously moistened with PBS. Blocking was done with 5% low fat milk solution. Detection was done with crude AS 19-21 antiserum (1:10000 dilution). Detection of immobilised AS 19-21 antibodies was carried out by goat IgG raised against anti-rabbit IgG conjugated with alkaline phosphatase (1:10,000 dilution). A pre-made solution of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP-NBT, Roche 1681451Basel, Switzerland) was used as phosphatase substrate.

Results

Expression and purification of the recombinant CTV coat protein

In preliminary experiments no novel peptide band of the correct size (about 28 KDa as expected for the fusion of the CTV-CP plus the leader sequence of the expression vector) could be detected by SDS-PAGE analysis in the bacterial culture medium or in the soluble fraction after induction of expression of the pTrcHisA-19.21. However, analysis of the insoluble fraction showed a new band of the correct size, which became stronger with increasing growth times. The specificity of this band was demonstrated by Western blot analysis with 3DF1+3CA5 monoclonal antibodies. As the intensity of this specific band was weak, alternative conditions of induction (0.3 mM IPTG) and bacterial growth (24°C) were assayed but no better results were obtained (Fig. 1). Preparative expression of the recombinant CTV coat protein was then done under the conditions described in the Materials and methods. The protein yield after affinity chromatography was 9.6 mg per litre of cell culture. However SDS-PAGE analysis of the affinity chromatography eluate still showed the presence of additional contaminant proteins of higher molecular mass, thus reducing the specific rCP-CTV yield. The rCP-CTV could be identified in this preparation by western blot analysis with 3DF1+3CA5 monoclonal antibodies, migrating as an isolated band (Fig. 1).

Performance of the antisera as detecting antibodies

Indirect ELISA assays were done to study the performance of the antisera and purified IgG as detecting antibodies (Fig. 2). Crude antisera from both rabbits (final bleeding) demonstrated a very good detecting ability of the virus in extracts of infected plants. An antisera dilution of 1:80,000 still produced a difference of about 1.5 absorbancy units between infected and healthy samples. Purified IgG at the same dilution (from a 1 mg ml^{-1} stock) also showed positive results but the absorbancy was very close to the discriminating positive/negative threshold. Similar results were obtained with a few CTV isolates. In comparative assays the IgG from the Bioreba kit could not detect the virus at dilutions higher than 1/40,000.

A parallel assay was done with the rCP-CTV (1 μg ml^{-1}) purified according to the Materials and methods instead of infected plant sap. In this case, the antigen could barely be detected by the crude antisera. Purified IgG from our serum or from Bioreba also failed to detect it (data not shown).

The ability to detect membrane bound CTV virions was tested by immunotissue printing and dot blot tests (Fig. 3). There was a clear distinction between imprints of healthy and of infected samples. These assays allowed the detection of CTV isolates from Reunion Island, Portugal (mainland), Spain and the Island of Madeira.

Performance as trapping antibodies

The performance of the AS 19-21 IgG for trapping the antigen in the coating step was assayed

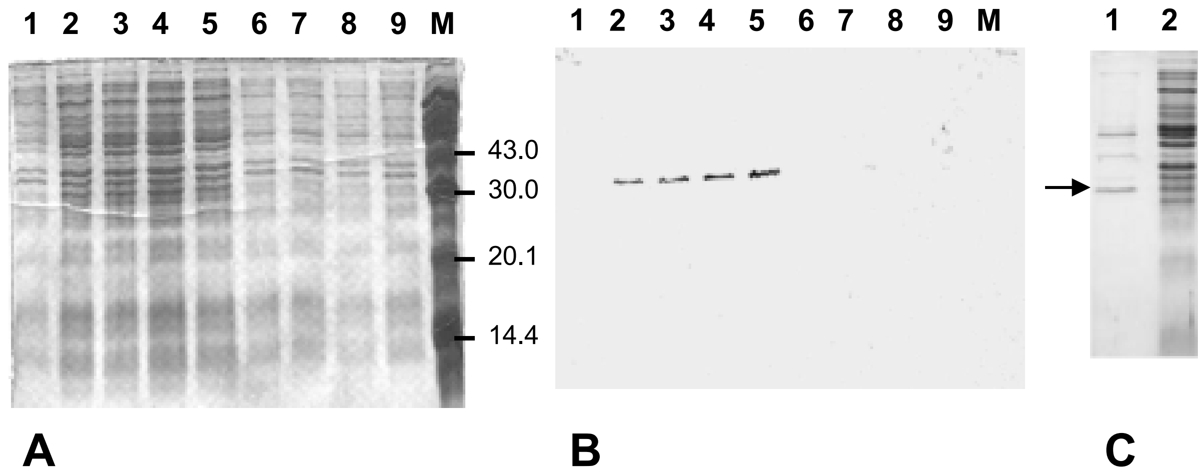

Fig. 1. Analysis by SDS-PAGE (15%) and Western blot of cellular extracts of *Escherichia coli* cultures harbouring the expression vector pTrcHisA 19-21. Panel A) SDS PAGE (15%) analysis of cellular extracts of cultures grown at 37°C (lanes 1 to 5) and 24°C (lanes 6 to 9). Lane 1, time 0, expression not induced. Lanes 2 and 3, respectively, 3 h and 5 h after induction with 0.3 mM IPTG. Lanes 4 and 5, respectively, 3 h and 5 h after induction with 1 mM IPTG. Lanes 6 to 9, same time and concentration of IPTG as in lanes 2 to 5. M, molecular marker, LMW (Pharmacia LKB Biotechnology, Sweden) with indicated molecular mass in KDa. Coomassie blue staining. Panel B) Western blot analysis of a twin gel of panel A. Panel C) SDS PAGE (15%) analysis of purified (lane 1) and unpurified (lane 2) rCP-CTV. The arrow indicates the position of the band that was used as immunogen.

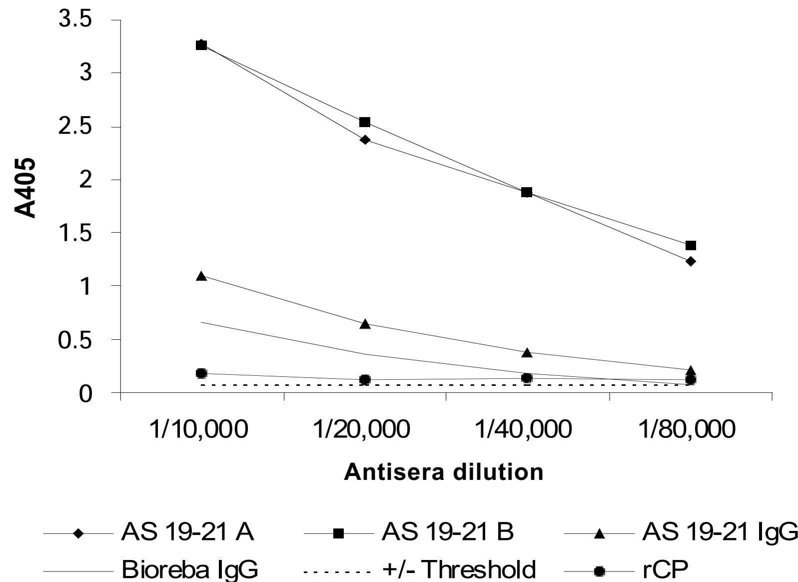

Fig. 2. Detecting ability of antisera and IgG produced against bacterially expressed CP in IDAS-ELISA. AS 19-21 A and B - crude antisera from two rabbits A and B tested against infected (isolate CTV 19) plant sap. AS 19-21 IgG - immunoglobulin purified from AS 19-21 B, tested against infected (isolate CTV 19) plant sap. Bioreba IgG - immunoglobulin from a commercial kit, tested against infected (isolate CTV 19) plant sap. rCP - antisera AS 19-21 A, tested against the purified recombinant protein (1 μg ml^{-1}). The dilutions at which the antisera were assayed are indicated. Absorbancy readings were done after 30 minutes of incubation with the enzyme substrate. The values obtained with the negative controls ranged from 0.09 to 0.17 and were subtracted.

by IDAS-ELISA using extract of plants infected with a Spanish CTV isolate. A parallel assay was conducted with IgG from Bioreba. Both sources of IgG gave similar values of absorbancies (differences of infected and healthy controls were around 1 unit after 45 min. of incubation with the substrate). The ability of the immunoglobulin to immunocapture diverse CTV isolates in IC/RT-PCR was also assayed in comparison with direct adsorption of virions on uncoated tubes. A clear increase in intensity of the band was obtained when the tubes were previously coated with the AS 19-21 IgG (data not shown).

Use of AS 19-21 IgG in DAS ELISA kits

In view of its good ability for trapping and detecting the virus, AS 19-21 IgG was used as the sole source of antibodies to produce a diagnostic kit. After optimisation, the kit was assayed with selected isolates from diverse origins (Table 1). The performance of this kit was equivalent to an ELISA kit of commercial origin (Bioreba), and in addition it also detected a broad spectrum of CTV isolates. These characteristics have permitted its use as part of a CTV eradication programme currently being conducted in Portugal.

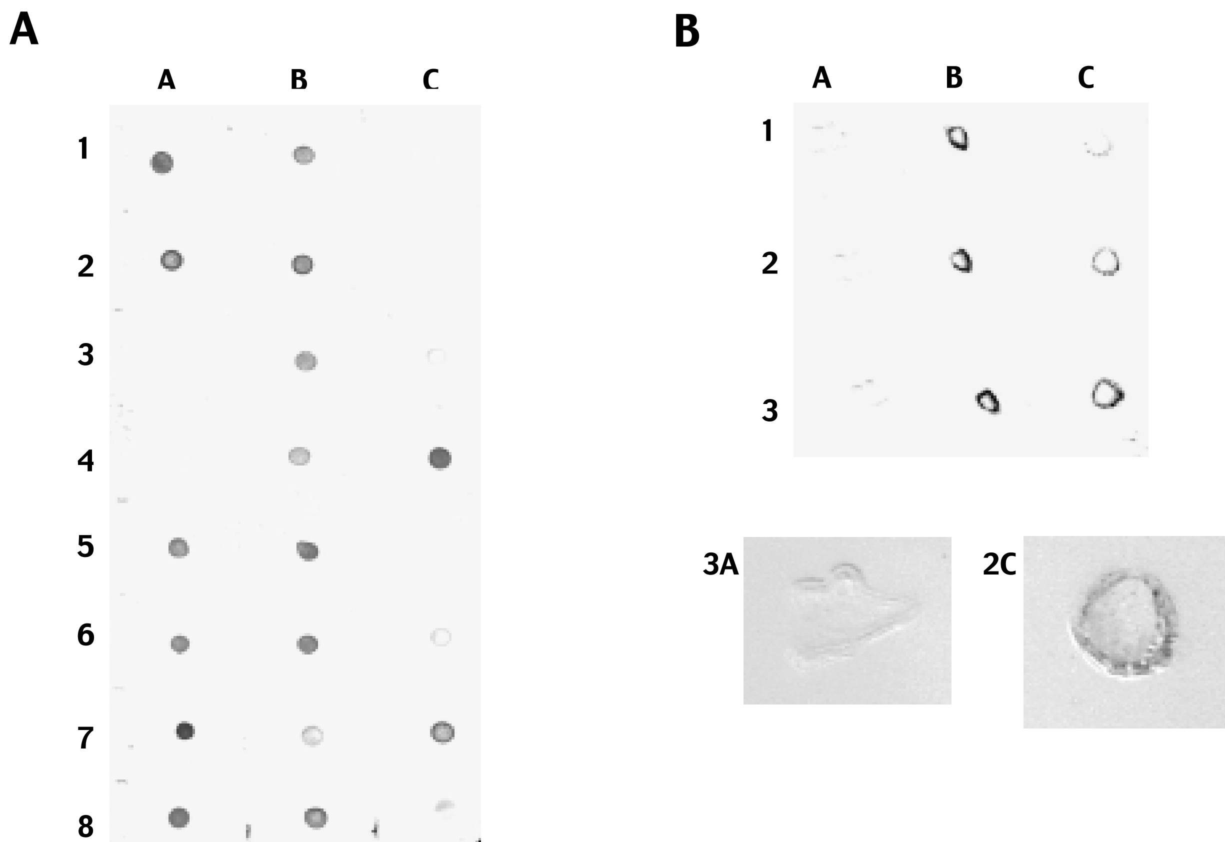

Fig. 3. Detection of membrane bound virions. Panel A: immunodot assay with diverse field samples from Reunion Island; negative controls are in column C, rows 1 and 2. Panel B: immunotissue prints of sections of healthy (column A) and infected (columns B and C) sweet orange stems. Amplified imprints of samples 3A and 2C show the difference between a weak positive reaction (2C) and a negative reaction (3A) in which too much pressure was exerted during the imprint procedure.

Discussion

The purpose of this work was to produce immunoreagents for an ELISA kit that could be used for routine diagnosis of CTV in Portugal. The major source of the disease is still the infected budwood illegally introduced from Spain, but there is now the risk of introduction of severe strains from the Island of Madeira (Nolasco *et al*., 1998). The heterogeneity of CTV strains makes it useful to have broad-spectrum antibodies available that could detect exotic strains. The disadvantage of generating antigen by expressing the coat protein gene is the risk of expressing a clone corresponding to a rare variant of the virus. On the other hand, this strategy offers additional possibilities of selecting (or even trimming) the amino acid sequence.

The yield of the expressed recombinant CP in our system was very low (less than 10 mg l^{-1}) as compared to 100 mg or 280 mg per litre obtained by Nikolaeva *et al*. (1995) and Manjunath *et al*., (1993) respectively, for a fusion protein containing the CP of CTV, although these authors used different systems for expressing the recombinant protein. Attempts to improve the yield by using a sub-optimal temperature for the culture growth were unsuccessful. However in the present study a higher yield proved to be unnecessary. In fact, the recombinant protein that was obtained from a 60 ml culture was sufficient for the immunization of two rabbits.

The performance of the AS 19-21 antibodies at the detecting stage of IDAS-ELISA was good as assessed in comparative tests conducted with polyclonal antibodies from a commercial kit (Bioreba). Good performance was also obtained when used as detecting antibodies in antigen-membrane bound techniques, with isolates from diverse origins. This is in accordance with the results from other authors (Manjunath *et al*., 1993; Nikolaeva *et al.,* 1995, 1996; Pappu *et al*., 1995), who have shown that the antibodies produced against an *E. coli* expressed recombinant CTV-CP perform well as detecting antibodies. However, it is generally accepted that this kind of approach is not adequate to produce good trapping antibodies. This would rule out the possibility of producing diagnostic kits based solely on a single antibody source. The reasons for the usually poor trapping ability of such antibodies are not fully understood. It has been suggested that antibodies produced against recombinant proteins recognise sequential epitopes rather than conformational ones. This would be due to the incorrect folding of the bacterial expressed protein. Bar-Joseph *et al*. (1997) were able to overcome this drawback by using an immunization scheme in which the primary injection was carried out with the recombinant protein and the booster injection with a partially purified preparation of CTV. However our results clearly showed that our antibodies could successfully be used at the trapping stage of ELISA or IC/RT-PCR experiments. In addition, there are reports (Vaira *et al*., 1996) with other viruses showing that antibodies against expressed proteins may have good ability to trap the viral antigens. These characteristics enabled us to prepare an efficient diagnostic kit that is based solely on a single antiserum produced against the expressed protein. These reagents are now routinely used for CTV screening in Portugal.

It is intriguing however that the same protein preparation that was used as immunogen could be barely detected in IDAS-ELISA using the homologous antibodies. The reasons for this were not further investigated. It was suggested that the fusion protein, following purification and with the effect of time and storage, could acquire a different conformation, hiding the reacting epitopes. These are most probably sequential, since the immunisation was done with a denaturing SDS-PAGE purified protein band. Such a conformation is perhaps more stable than the native conformation of the viral CP, which would allow a partial denaturation exposing sequential epitopes. In western blot analysis the rCP-CTV may be detected because it is also denatured. From a practical point of view, this characteristic prevents the use of the rCP-CTV as a positive control for ELISA, which could be important in countries where CTV has not yet been detected. In these cases lyophilised bark from twigs of infected plants could be used.

Since the expression plasmids can be stored for long periods, the production of bacterial expressed viral proteins is a good strategy to provide uniform immunogens for antibody production as it avoids laborious virus purification methods and the need for expensive equipment.

Acknowledgements

This work was supported by the grant PRAXIS XXI 3/3.2/Hort/2159-95.

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Accepted for publication: February 28, 2002