MOLECULAR ANALYSIS OF A CHINESE *CITRUS TRISTEZA VIRUS* ISOLATE SHOWING ANOMALOUS SEROLOGICAL REACTIONS

C.X. Wang[1,2], N. Hong[1,2,3], G.P. Wang[1,2,3], J.K. Zhang[2] and L. Hui[1,2]

[1] *The Key Laboratory of Plant Pathology of Hubei Province, Huazhong Agricultural University, Wuhan 430070, P.R. China*
[2] *National Indoor Conservation Center of Virus-free Gemplasms of Fruit Crops, Huazhong Agricultural University, Wuhan 430070, P.R. China*
[3] *College of Plant Science and Technology, Huazhong Agricultural University, Wuhan 430070, P.R. China*

SUMMARY

A Chinese isolate of *Citrus tristeza virus* (CTV), denoted HB1, was not recognized by a polyclonal antiserum (PAb-I) raised to the Italian CTV isolate 0002 and ten monoclonal antibodies to the Chinese isolate S4 (MAbs-S4). However, it reacted with a polyclonal antiserum (PAb-L5) raised to the Chinese isolate L5 in both ELISA and Western blots. To understand this unusual serological behaviour, the CP gene sequence of HB1 and other CTV isolates was determined. The results showed that HB1 was closely related to L5 at the nucleotide and amino acid levels. However, three amino acid residues in the HB1 CP differed from those of L5, which were conserved in all other isolates sequenced, including CTV-0002 and S4. This suggested that the failure of PAb-I and MAbs-S4 to recognise HB1 could depend on these molecular changes. Prediction of Antigenic Indexes and Surface Probability in four isolates (HB1, S4, L5 and 0002) with the Peptidestructure and Plotstructure softwares, showed that the HB1 tracing contained two small peaks at aa 84 and 190 that did not appear in any of the other profiles, but no apparent difference in aa 98. However, HB1 and L5 showed a similar small peak at the 5' end which did not occur in the S4 and CTV-0002.

Key words: *Citrus tristeza virus*, CP gene, sequencing, Western blot, serology.

INTRODUCTION

Citrus tristeza virus (CTV), a member of the genus *Closterovirus*, family *Closteroviridae*, is the casual agent of a destructive disease of citrus (Bar-Joseph *et al.*, 1989). The virus is transmitted by several aphid species and dispersed to new citrus areas mostly by infected propagating material (Roistacher and Bar-Joseph, 1987). The biological characteristics of several CTV strains have been described. Some induce very mild symptoms or are essentially symptomless, even in the most sensitive citrus species, whereas others induce quick decline and death of plants grafted on sour orange rootstocks (*Citrus aurantium*). Other strains cause stem-pitting, stunting, low yield and poor fruit quality of grapefruit and sweet orange, regardless of the rootstock (Bar-Joseph *et al.*, 1989), or cause seedling yellows (Garnsey *et al.*, 1987).

CTV virions are flexuous, about 2000 × 11 nm in size, and encapsidate a single-stranded, positive sense genomic RNA *ca*. 20 kb in size, comprising 12 open reading frames (ORFs) that code for up to 19 protein products (Karasev *et al.*, 1995; Mawassi *et al.*, 1996; Yang *et al.*, 1999; Vives *et al.*, 1999). Two proteins 25 and 27 kDa in size, encoded by ORF 7 and 8, respectively (Sekiya *et al.*, 1991; Febres *et al.*, 1996), are the major coat protein (p25, CP), that coats about 95% of the particle length and the minor coat protein (p27, CPm) that coats only one end of the particle (Sekiya *et al.*, 1991; Febres *et al.*, 1996).

In recent years, CTV isolates have been differentiated using specific antibodies, and the molecular constitution of epitopes studied. Thus, Permar *et al.* (1990) produced the monoclonal antibody MCA-13 that could discriminate severe from mild isolates occurring in Florida. Nikolaeva *et al.* (1998) reported that stem pitting isolates could be differentiated from non-stem pitting isolates in sweet orange in Florida and California by I-DAS-ELISA using a polyclonal antibody raised against a bacterially-expressed CTV CP for trapping and the monoclonal antibody 3E10 for detection, or using two polyclonal antisera to recombinant CTV CP. Pappu *et al.* (1993, 1995), using site-directed mutagenesis of selected amino acid residues, showed that the epitopes for MAbs 3DF1 and MCA-13 are linear and close to amino acid (aa) residues 2 and 124, respectively. Nikolaeva *et al.* (1996) screened 30 CTV-specific MAbs and assigned them to five groups based on epitope specificity.

Our group has raised a polyclonal antiserum and monoclonal antibodies to two CTV isolates (Wang *et al.*, 2006) and initiated a serological survey of Chinese CTV strains, in the course of which an isolate from pummelo (HB1) was identified, that differed serologically from all previously studied isolates. In an attempt to understand the molecular mechanisms underlying

Corresponding author: G.P. Wang
Fax: +86.27.87287735
E-mail: gpwang@mail.hzau.edu.cn

such diverse serological behaviour, the CP gene sequences of HB1 and other CTV isolates were determined and compared.

MATERIALS AND METHODS

Virus isolates and plant materials. Three CTV isolates, i.e. HB1 from pummelo, and S4 and L5 from sweet orange collected from Hubei province, were transmitted by grafting and maintained in seedlings of Mexican lime (*Citrus aurantifolia*) and sweet orange (*Citrus sinensis*). All isolates induced chlorosis, flecking in the leaves and stem pitting in the branches of both hosts. Five previously sequenced CTV isolates, HN-K (AY953982), JX-1-1 (AY953983), JX-2-7 (AY953980), ML-12 (EF028325) and ML-13 (EF028326) collected from the Chinese provinces of Hunan and Jiangxi were also maintained in Mexican lime (Zhang *et al.,* 2006). Six other isolates from sweet orange and two from hybrid citrus trees on trifoliate orange (*Poncirus trifoliata*) rootstocks, were recovered from citrus groves in the Wuhan area (Hubei province). Young shoots were collected from the quadrant of the trees and mixed together to prepare extracts for ELISA and RT-PCR.

Serological reagents and ELISA. The reagents used were: (i) the polyclonal antiserum PAb-I raised to purified particles of the Italian isolate CTV-0002; (ii) the polyclonal antiserum PAb-L5 and a family of monoclonal antibodies (MAbs-S4) generated with purified particles of Chinese isolates L5 and S4, respectively (Wang *et al.*, 2006). Goat anti-mouse/rabbit immnoglobulins (Ig-Gs) conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HRP) were from Promega (Madison, WI, USA). Two indirect ELISAs protocols were used to detect CTV infections, i.e. protein A sandwich (PAS)-ELISA according to Hong *et al.* (1992) with PAb-I and PAb-L5, and triple antibody sandwich (TAS)-ELISA, basically as described by Vela *et al.* (1986). PAb-L5 and a cocktail of ten Mabs generated from S4 were used as coating and detection antibodies, respectively, then the HRP-conjugated goat anti-mouse IgG was added, and 3,5'-tetramethylbenzidine (TMB) was used as substrate. Crude extracts were prepared by grinding 0.1 g petioles in 1 ml 0.01 M phosphate buffered saline with 0.05% Tween-20 (PBST) (pH 7.2, containing 2% polyvinyl-pyrrolidone and 0.1% BSA) and the extracts were centrifuged for 10 min at 5,000 rpm. Healthy Mexican lime was used as negative control. The threshold of optical density at 450 nm (OD_{450}) for positive reaction was fixed at twice the average value of the negative control. All tests were duplicated.

RNA extraction and PCR. Total RNA from CTV-infected and healthy citrus shoots was extracted using TRI-zol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Virus-specific primers targeting the two ends of the CP gene were designed based on the published nucleotide sequence of CTV isolate T36 (Karasev *et al.*, 1995). The sense primer was CP1 5' ATGGACGACGAAACAAAGA3', and the anti-sense primer was CP2 5'TCAACGTGTGTTGAATTTCC3'. The isolated RNA (1µg) was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) at 42°C for 1 h. The CP gene was amplified using *Taq* DNA polymerase (TaKaRa, Dalian, China) under the following conditions: 94°C, 3 min (1 cycle); 94°C, 30 sec denaturation; 53-56°C, 30 sec annealing and 72°C, 45 sec extention (30 cycles) followed by an extention step at 72°C, 5 min. PCR products were analyzed in 1.2% agarose/EB gels in Tris-acetate-EDTA buffer.

Cloning and sequencing of CTV CP gene. The nucleotide sequence of the CP gene of HB1, S4 and L5 isolates were determined. Amplified products were purified by a gel recovered kit (TaKaRa, Dalian, China) and ligated into the vector pMD 18-T (TaKaRa, Dalian, China). Plasmids containing the inserted CP gene (pMD-CP) were grown in *E. coli* DH5α cells and purified by the alkaline lysis method (Sambrook and Russell, 2001). At least three independent clones of each isolate were sequenced using the ABI Prism Big Dye Terminator cycle sequencing protocols.

Expression of fusion proteins and Western blot analysis. The recombinant plasmids containing the CP gene (pMD-CP) were digested with *BamH* I and *Hind* III and the purified target DNA fragments were ligated into the expression vector pET-28b(+) (Clontech, Mountain View, CA, USA). The constructed plasmids (pET-28b-CP) were transformed into *E. coli* BL-21 (DE3) plysS. Fusion proteins in E. *coli* were induced with 0.5 mM isopropylthio-β-D-galactoside (IPTG) at 37°C and harvested after 4-8 h. Cells were centrifuged and resuspended in lysis buffer (125 mM Tris-HCl, pH 6.8, containing 4% SDS, 20% glycerin, 10 mM 2-mercapthoethanol). The expressed proteins were separated in 12% SDS-PAGE and visualized by staining with a 0.25% Coomassie Blue G250 solution. The expressed proteins and the native proteins from purified virus products were subjected to Western blot analysis. Proteins were separated by SDS-PAGE and transferred onto Hybond™-P membranes (Amersham Biosciences, Little Chalfont, UK). The membranes were blocked in 0.5% BSA-PBST, incubated subsequently in different antibodies and AP-conjugated goat anti-rabbit/mouse IgG, and visualized with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Sequence analysis and construction of phylogenetic tree. The nucleotide sequences of CP genes from differ-

ent isolates or deduced amino acid sequences were aligned using the Clustal W of the MegAlign procedure supplement within the DNASTAR package (version 7.0) and GeneDoc (version 2.6, 2002). The phylogenetic relationships of HB1 with S4, L5, and five other Chinese isolates, and 12 sequenced isolates from the GenBank database were determined using the Bootstrap Neighbor-Joining method in MEGA 3.1 software (http://www.megasoftware.net). The 12 isolates from GenBank included two mild isolates, T385 (from Spain, Y18420) and T30 (from Florida, AF260651), seven severe isolates, T36 (from Florida, U16304), CTV-0002 (from Italy, AJ518841), VT (from Israel, U56902), SY568 (from California, AF001623), NUagA (from Japan, AB046398), 13C (from Portugal, AF184113), Cheju (from South Korea, AF249279) and Bangalore (from India, AF501867), and isolates *CTV* (from Mexico, DQ272579) and Qaha (from Egypt, AY340974) the severity of which is unknown.

The CP sequences of HB1, S4, and L5 were deposited in the GenBank database and assigned the accession numbers EF028324, EF063109, and EF016126, respectively.

The prediction of Antigenic Indexes and Surface Probability of four isolates (HB1, L5, S4 and CTV-0002) was investigated with the Peptidestructure and Plotstructure softwares from Wisconsin Package Version 9.1, Genetic Computer Group, Madison, WI, USA.

RESULTS AND DISCUSSION

Serological characterization of CTV-infected samples. Samples from of 16 citrus accessions were tested for CTV by PAS- and TAS-ELISA with the polyclonal antisera and monoclonal antibodies previously mentioned. Results showed that, except for HB1, all plants tested yielded the same results regardless of the ELISA protocol used. By contrast, HB1 reacted positively with PAb-L5 in PAS-ELISA but not with PAb-I in PAS-ELISA and the cocktail of MAbs-S4 in TAS-ELISA (Table 1). These results were confirmed by repeated tests with samples from the same trees collected in different seasons (data not shown).

Amplification of the CP gene. When accessions HB1, A4, Y1, and A6 that showed different reactions in ELISA were assayed by RT-PCR, products with the expected size (672 bp) were amplified from HB1, A4, and Y1, but not from A6 and or from healthy Mexican lime (Fig. 1). This was taken as an indication that accession A6 was not infected by CTV. Annealing temperature was important for the successful amplification of the CP gene from sample HB1. Initially, 56°C was used for primer annealing, but no amplification products were obtained (Fig. 1, lane 3). When annealing temperature

was decreased to 53°C, amplification products with the same size as those from A4 and Y1 were obtained in 1.2% agarose/EB gel, but the amount of PCR products from HB1 was apparently lower than that from A4 and Y1 (Fig. 1, lane 4).

Table 1. Serological detection of CTV from different samples by three ELISA protocols with antibodies from different sources.

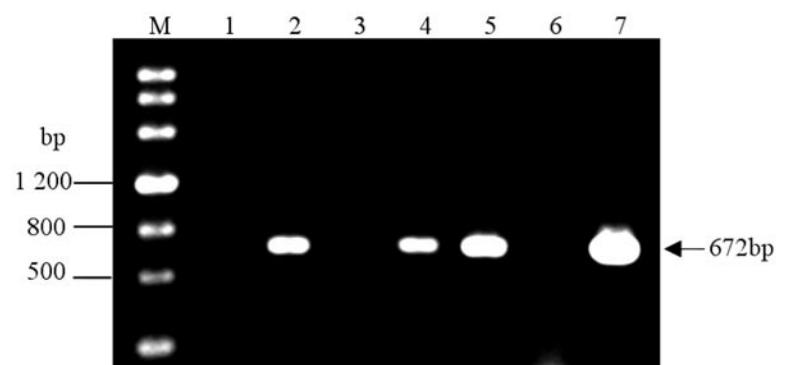| CTV isolates	PAS-ELISA (PAb-I)[a]	PAS-ELISA (PAb-L5)	TAS-ELISA (MAbs to S4)
P1	+	+	+
P3	-	-	-
GL2	+	+	+
Y0	-	-	-
Y1	+	+	+
L4	+	+	+
A4	+	+	+
A6	-	-	-
HB1	**-**	**+**	**-**
L5	+	+	+
S4	+	+	+
HN-K	+	+	+
JX-1-1	+	+	+
JX-2-7	+	+	+
ML-12	+	+	+
ML-13	+	+	+

[a] + positive reaction; - negative reaction.

Fig. 1. Detection of CTV in different samples by RT-PCR. M: DNA marker (Tiangen, Beijing, China); lane 1: healthy Mexican lime; lane 2: L5; lane 3: HB1 (annealing temperature 56°C); lane 4: HB1 (annealing temperature 53°C); lane 5: A4; lane 6: A6; lane 7: Y1.

Expression of CP gene and Western blot analysis of expressed proteins. The amplified fragment from HB1 CP was cloned, inserted into the vector pET-28b (+) and successfully expressed in *E. coli* BL-21 (DE3) pLys S cells under the induction of IPTG. The molecular mass of the expressed CTV-specific fusion proteins was about 29 kDa. The amount of recombinant proteins did not show a significant difference within inducing periods from 4 h to 8 h (Fig. 2). When 2% glucose was added to the *E.coli* BL-21 cultures during protein induction, the yields of recombinant CP increased significantly (data not shown). Western blot analysis with PAb-I, PAb-L5 and MAbs-S4 showed that both recombinant and native CP of purified HB1 produced clearcut signals with PAb-L5 (Fig. 3, lane A2 and A4) but

not with PAb-I and MAbs-S4 (Fig. 3, lane B3 and C3). However, native proteins from purified L5 reacted positively with all antibodies tested (Fig. 3, lane A3, B4 and C4). No signal was produced by extracts from healthy Mexican lime (Fig. 3, lane A5, B5 and C5). Results from Western blot analysis also showed that native CP of purified isolate L5 migrated faster than that of HB1 (Fig. 3, lane A3 and A4).

Sequence analysis. Results from sequence comparisons of CPs showed that most CTV isolates from China, including HB1 and L5, were severe strains with the conserved phenylalanine residue at position 124, whereas ML-13 had isoleucine at position 122, and the substitution of phenylalanine for tyrosine which occurs in the majority of mild isolates (Pappu *et al.*, 1993). Furthermore, HB1 had identities ranging from 90.4% to 99.0% at the nucleotide level, and from 94.2% to 98.7% at the amino acid level with the other isolates sequenced. Identity at both nucleotide and amino acid levels among HB1, L5, the Californian severe stem pitting isolate SY568 (Yang *et al.*, 1999), and Japan seedling yellows isolate NUagA (Suastika *et al.*, 2001) was higher than 97%, confirming the response of indexing. The phylogenetic tree constructed with amino acid sequences of 20 different CTV isolates (Fig. 4) showed that, in agreement with serological reactions HB1, and L5 were very closely related, and clustered away from isolates CTV-0002 and S4.

When the predicted amino acid sequence of HB1 CP was compared with that of CTV-0002 and of L5, S4, HN-K, JX-1-1, JX-2-7, ML-12, and ML-13, all of which had reacted with PAb-I and MAbs-S4 in ELISA, it was found that HB1 had a consistent change in three amino acid residues at positions 84 (S→L), 98 (G→D) and 190 (G→C) (Fig. 5) that differentiated it from all the above isolates (Fig. 5) as well as from other sequenced CTV isolates from GenBank (not shown). This suggested that
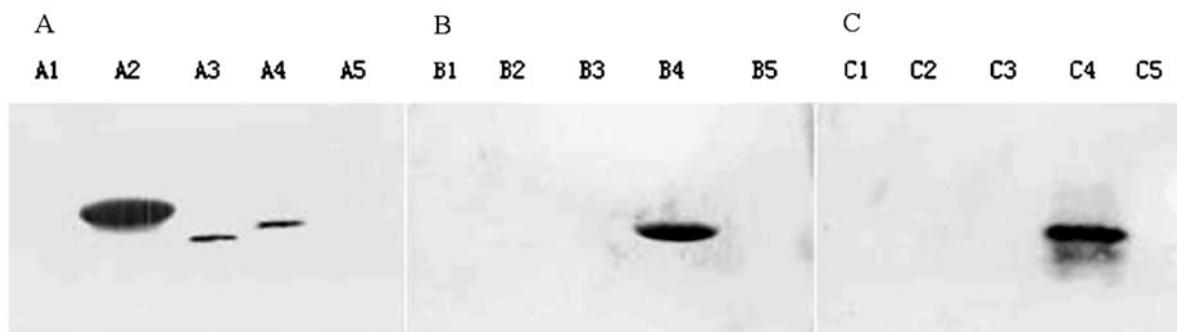

Fig. 2. SDS-PAGE analysis of the recombinant protein expressed in *E. coli* BL-21. M: protein molecular weight markers (Tiangen, Beijing, China). Lane 1: total proteins from *E. coli* cells transformed with pET-28b and induced by IPTG for 6 h. Lane 2: total proteins from non-induced *E. coli* cells transformed with pET-28b-CP. Lanes 3, 4 and 5: total protein from *E. coli* cells transformed with pET-28b-CP and induced by ITPG for 4 h, 6 h and 8 h respectively. Arrow indicates the proteins expressed in *E. coli* cells carrying pET-28b-CP.

Fig. 3. Western blot analysis of the expressed HB1 fusion protein and the native proteins from purified isolates HB1 and L5, using antibody PAb-L5 (A), PAb-I (B) and MAbs-S4 (C). Lanes A1, B1, and C1: total proteins from non-induced cells transformed with pET-28b-CP. Lanes A2, B2, and C2: total proteins from cells carrying pET-28b-CP induced by ITPG for 4 h. Lanes A3, B4, and C4: native proteins from purified L5; Lanes A4, B3, C3: native proteins from purified HB1. Lanes A5, B5, and C5: extracts from healthy Mexican lime.
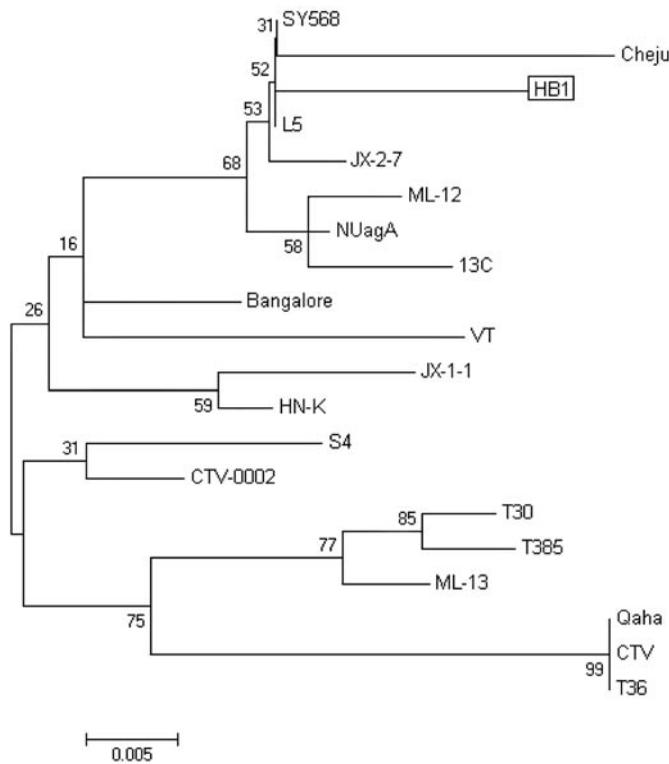
Fig. 4. Phylogenetic analysis of 20 CTV isolates based on CP predicted amino acid sequences using Neighbor-Joining analysis. The values at the nodes indicate the number of times out of 1000 trees that this grouping occurs after bootstrapping the data. The scale bar represents a distance of 0.005 substitutions per site. Accession numbers of the sequences used to generate the tree are given in the text.

the changes observed in these three positions could be involved in the failure of PAb-I and MAbs-S4 to recognise HB1. In fact, among the eight CTV isolates mentioned above, a total of sixteen amino acid sites were different, although these differences did not affect recognition of any of these isolates by PAb-L5, PAb-I, and MAbs-S4.

Comparison of HB1, L5, S4 and CTV-0002 profiles generated by Peptidestructure software disclosed that the HB1 tracing had two small peaks at the level of aa 84 and 190 which were absent in the other profiles, but were apparently identical to these at the level of aa 98 (Fig. 6). This was taken to indicate that the recorded diversity of these three amino acids between HB1 and S4/CTV-0002 is unlikely to account for the observed serological difference. However, HB1 and L5 showed a similar small peak, which did not occur in both either CTV-0002 or S4, at the 5' end of the sequence, a region characterized by a high probability to be surface-exposed (Fig. 6) and was shown to be strongly immunogenic (Pappu *et al.*, 1995). This may be the reason for the different reaction of HB1 with respect to Pab-L5 (positive, presence of the peak) and to PAb-I and S4 (negative, absence of the peak).

Notwithstanding the alleged serological variability of CTV (Permar *et al.*, 1991; Nikolaeva *et al.*, 1998; Albiach-Marti *et al.*, 2000; Wang *et al.*, 2006), commercial antisera used for surveys and eradication programmes have so far proven able to detect the wide array of known viral isolates. The unexpected finding that PAb-
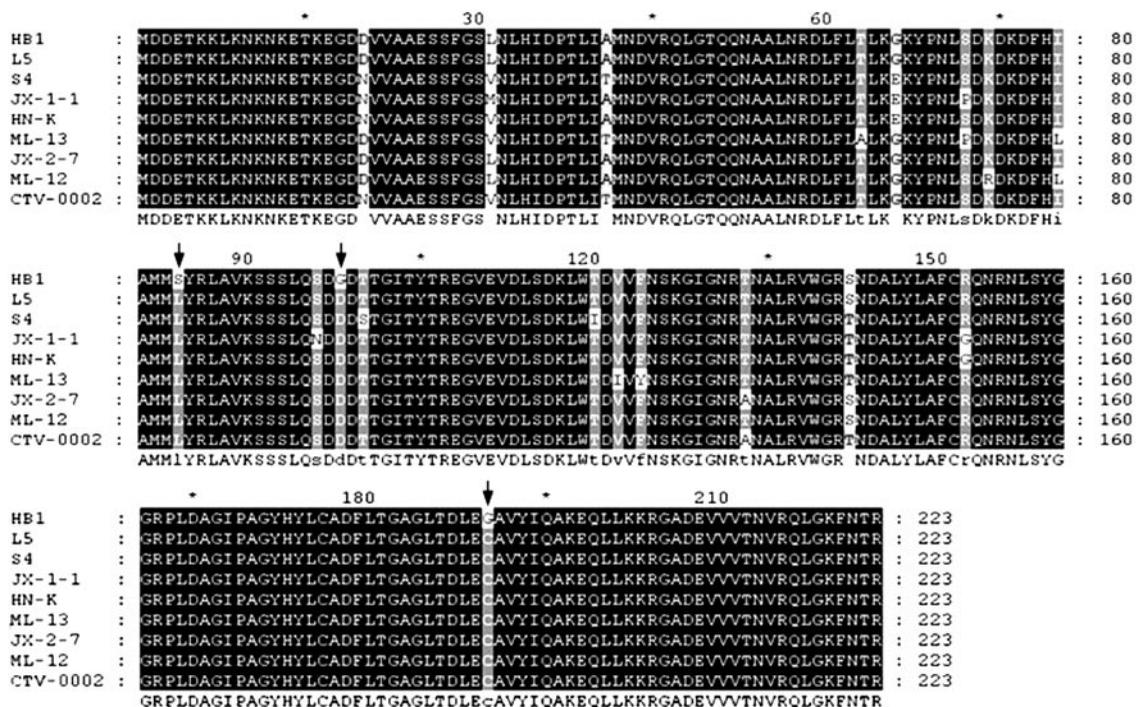

Fig. 5. Alignments of the predicted CP amino acid sequences of eight CTV isolates from China and Italy (CTV-0002). Arrows show positions of amino acids changed in HB1 and conserved in all other isolates.
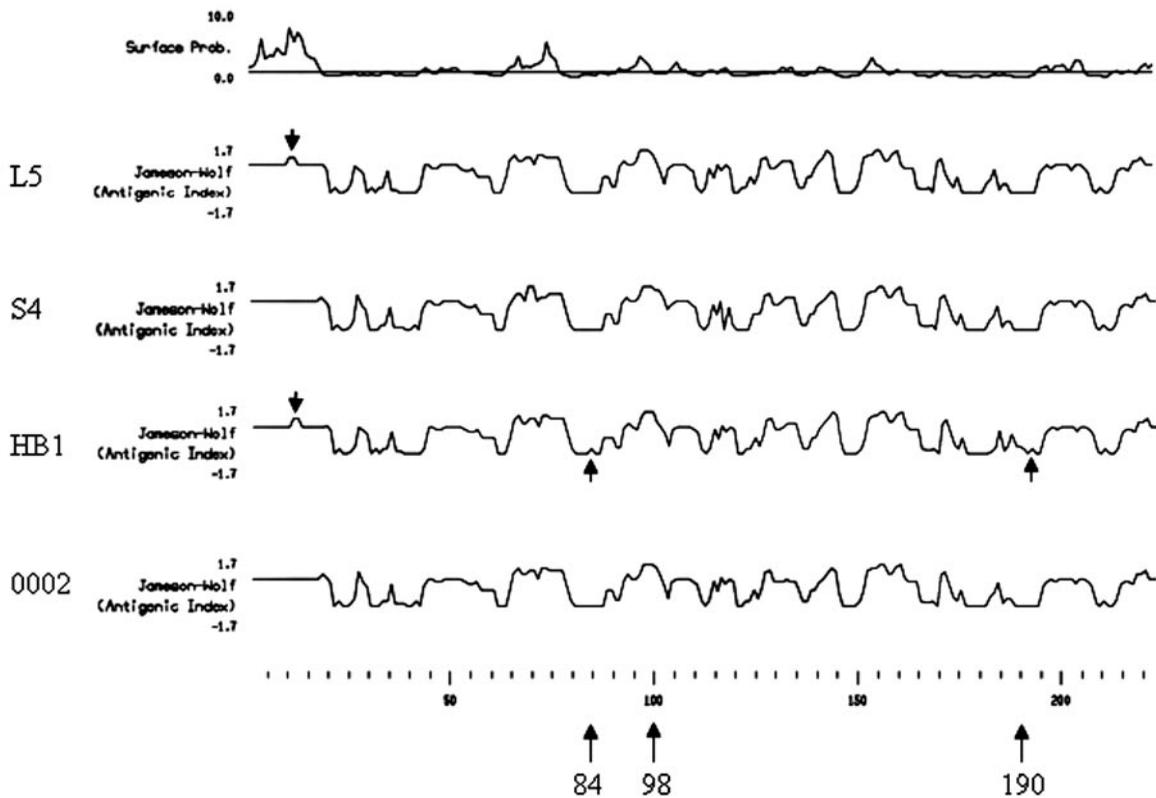
Fig. 6. Prediction of Antigenic Indexes and Surface Probability of isolates HB1, L5, S4 and 0002 with the Peptidestructure and Plotstructure softwares. Arrows indicate peaks that differentiate the four isolates.

1, thought to be a broad-spectrum antiserum, did not recognise at least one viral strain (HB1) is interesting, as yet unexplained, and requires further study.

REFERENCES

Albiach-Marti M.R., Guerri J., Cambra M., Garnsey S.M., Moreno P., 2000. Differentiation of citrus tristeza virus isolates by serological analysis of p25 coat protein peptide maps. *Journal of Virological Methods* **88**: 27-37.

Bar-Joseph M., Marcus R., Lee R.F., 1989. The continuous challenge of citrus tristeza virus control. *Annual Review of Phytopathology* **27**: 291-316.

Febres V.J., Ashoulin L., Mawassi M., Frank A., Bar-Joseph M., Manjunath K.L., Lee R.F., Niblett C.L., 1996. The p27 protein is present at one end of citrus tristeza virus particles. *Phytopathology* **86**: 1331-1335.

Garnsey S.M., Gumpf D.J., Roistacher C.N., Civerolo E., Lee R.F., Yokomi R.K., Bar-Joseph M., 1987. Toward a standard evaluation of the biological properties of citrus tristeza virus. *Phytophylactica* **19**: 151-147.

Hong N., Liu F.C., Wang G.P., Wang X.F., 1992. The detection of apple chlorotic leaf spot virus and apple stem grooving virus by protein A sandwich ELISA. *China Fruits* **1**: 44-47.

Karasev A.V., Boyko V.P., Gowda S., Nikolaeva O.V., Hilf M.E., Koonin E., Niblett C.L., Cline K., Gumpf D.J., Lee R.F., Garnsey S.M., Dawson W.O., 1995. Complete sequence of the citrus tristeza virus RNA genome. *Virology* **208**: 511-520.

Mawassi M., Mietkiewska E., Gofman R., Yang G., Bar-Joseph M., 1996. Unusual sequence relationships between two isolates of citrus tristeza virus. *Journal of General Virology* **77**: 2359-2364.

Nikolaeva O.V., Karasev A.V., Powell C.A., Gumpf D.F., Garnsey S.M., Lee R.F., 1996. Mapping of epitopes for citrus

tristeza virus-specific monoclonal antibodies using bacterially expressed coat protein fragments. *Phytopathology* **86**: 974-979.

Nikolaeva O.V., Karasev A.V., 1998. Serological differentiation of the citrus tristeza virus isolates causing stem pitting in sweet orange. *Plant Disease* **82**: 1276-1280.

Pappu H.R., Pappu S.S., Manjunath K.L., Lee R.F., Niblett C. L., 1993. Molecular characterization of a structural epitope that is largely conserved among severe isolates of a plant virus. *Proceedings of the National Academy of Sciences USA* **90**: 3641-3644.

Pappu H.R., Pappu S.S., Kano T., Koizumi M., Cambra M., Moreno P., Su H.J., Garnsey S. M., Lee R.F., Niblett C.L., 1995. Mutagenic analysis and localization of a highly conserved epitope near the amino terminal end of the citrus tristeza closterovirus capsid protein. *Phytopathology* **85**: 1311-1315.

Permar T.A., Garnsey S.M., Gumpf D.J., Lee R.F., 1990. A monoclonal antibody that discriminates strains of citrus tristeza virus. *Phytopathology* **80**: 224-228.

Roistacher C.N., Bar-Joseph M., 1987. Aphid transmission of citrus tristeza virus. *Phytophylactica* **19**: 163-167.

Sambrook J., Russell D. W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, USA.

Sekiya M. E., Lawrence S. D., McCaffery M., Clike K., 1991. Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristeza virus. *Journal of General Virology* **72**: 1010-1020.

Suastika G., Natsuaki T., Terui H., Kano T., Ieki H., Okuda S., 2001. Nucleotide sequence of citrus tristeza virus seedling yellow isolate. *Journal of General Plant Pathology* **67**: 73-77.

Vela C., Cambra M., Cortes E., Moreno P., Miguet J. G., Perez de San Roman C., Sanz A., 1986. Production and characterization of monoclonal antibodies specific for citrus tristeza virus and their use for diagnosis. *Journal of General Virology* **67**: 91-96.

Vives M.C., Rubio L., Lopez C., Navas-Castillo J., Albiach-Moreno P., 1999. The complete genome sequence of the major component of a mild citrus tristeza virus isolate. *Journal of General Virology* **80**: 811-816.

Wang C.X., Wang G.P., Hong N., Jiang B., Liu H., Wu K.W., 2006. Production of polyclonal and monoclonal antibodies against citrus tristeza virus and their efficiency for the detection of the virus. *Chinese Journal of Biotechnology* **22**: 629-634.

Yang Z.N., Mathews D.H., Doods J.A., Mirkov T.E., 1999. Molecular characterization of an isolate of citrus tristeza virus that causes severe symptoms in sweet orange. *Virus Genes* **19**: 131-142.

Zhang J.K., Hong N., Wang G.P., 2006. Analysis of sequence variability in CP gene of citrus tristeza virus. *Journal of Agricultural Biotechnology* **14**: 259-264.